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Review

Protein Folding Coupled to Disulphide Bond Formation

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Protein folding that is coupled to disulphide bond formation has many experimental advantages. In particular, the kinetic roles and importance of all the disulphide intermediates can be determined, usually unambiguously. This contrasts with other types of protein folding, where the roles of any intermediates detected are usually not established. Nevertheless, there is considerable confusion in the literature about even the best-characterized disulphide folding pathways. This article attempts to set the record straight.

Key words: α-lactalbumin / BPTI / Folding pathways / Kinetic intermediates / Ribonuclease A.

Introduction

Understanding in detail the mechanism of protein folding *in vitro* requires knowing all the conformational states that the protein encounters while it is folding, plus the limiting transition state (Creighton, 1994). The unusual aspect of protein folding is the very large number of conformations accessible to a polypeptide chain of even modest size. Folding generally occurs much more rapidly than expected for encountering the unique native conformation by a random process amongst so many possibilities, even as fast as within milliseconds for small proteins. Nevertheless, unfolded conformations will change on the 10^{-10} s time scale, so a single molecule could sample a very large number of conformations even during folding that is very rapid. Moreover, an experimental sample of unfolded protein contains a large number of molecules, typically 10^{15} to 10^{17}, and each probably has a unique conformation upon initiating folding. Therefore, a very large number of conformations might need to be characterized with a typical experimental sample. Fortunately, the kinetics of refolding are usually observed to be relatively simple and indicate that all the unfolded molecules rapidly equilibrate conformationally (unless they differ in some intrinsically slow isomerization, such as *cis* or *trans* peptide bonds, disulfide bond topologies, etc.) and converge on what can be considered a single, common transition state.

Upon initiating refolding by changing the conditions, proteins are observed to adopt a wide variety of conformations very rapidly, before acquiring the fully folded state. Some remain unfolded, others adopt partly-folded conformations, while many adopt a compact but disordered state that has come to be known as the 'molten globule' (MG) (Kuwajima, 1989; Ptitsyn, 1995). These relatively stable partly-folded and MG conformations are frequently assumed to be crucial for initiating the folding process, so much effort has gone into characterizing them. This is usually difficult experimentally, as kinetic intermediates are present only transiently. Furthermore, they are at best only partly folded, and their consequent conformational heterogeneity makes them inherently difficult to characterize; generally only an average conformation can be determined, and there is even uncertainty about the exact nature of unfolded polypeptide chains under unfolding conditions, which are stable and can be studied at leisure.

Determining the kinetic roles of intermediate species, as to whether they are kinetic intermediates, kinetic traps, or simply the preferred conformational state of the unfolded protein under refolding conditions, is especially difficult with a complex process like protein folding. There is little direct evidence about the kinetic importance of most stable folding intermediates that have been detected (Creighton, 1990, 1994, 1995).

Many of the intrinsic complications and uncertainties about experimental studies of protein folding can be overcome by studying folding that is coupled to formation of disulphide bonds between cysteine residues (Creighton, 1978, 1992, 1994). Using the appropriate methods, the rates of formation and breakage, and the stabilities, of protein disulphide bonds can reflect the role of the protein conformation in bringing each pair of cysteine residues into the appropriate proximity and keeping them there. The most important attributes of this approach are the ability to trap and to characterize all disulphide intermediates and to define their kinetic roles, usually unambiguously. The trapped intermediates are stable, and their conformations can be determined in detail (van Mierlo *et al.*, 1991, 1993, 1994).

It is, of course, necessary that the process of disulphide formation and breakage reflect the conformational folding of the polypeptide chain, and not result from any peculiar chemical properties of the cysteine sulphur atoms or the chemistry involved in making disulphide bonds. The thiol-disulphide exchange reaction (Figure 1) is well-understood (Szajewski and Whitesides, 1980), readily controlled, and easily interpreted. A crucial factor in this reaction is that it is the ionized, thiolate form of the thiol group that is the reactive species. Normal thiol groups of cysteine residues in aqueous environments tend to have pK_a values close to 8.7, but a nonpolar environment or nearby negative charges will increase this, while nearby positive char-

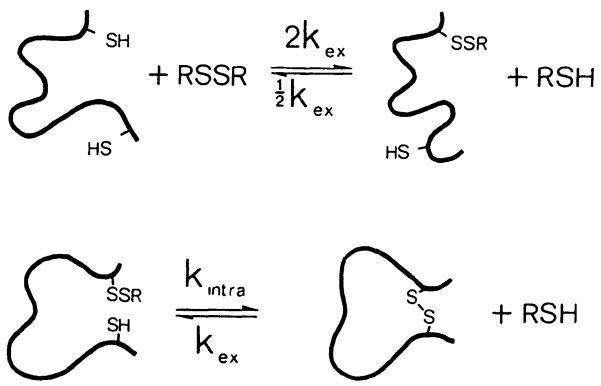

Fig. 1 Formation of an Accessible Protein Disulphide Bond by Thiol-Disulphide Exchange with a Reagent in the Thiol and Disulphide Forms, RSH and RSSR, Respectively.
For a reagent like glutathione, with a thiol group or a disulphide bond chemically like those of proteins, the intrinsic rate constant for the thiol-disulphide exchange reaction between them is k_{ex}. The rate constant most pertinent to protein folding is k_{intra}, which includes the protein conformational transition involved in disulphide formation. If the protein disulphide bond is buried, the reverse rate constant, for its reduction, will be lowered from the indicated value of k_{ex}.

ges decrease the pK_a. The value of the pK_a determines both the ionization of the thiol group and the intrinsic reactivity of the thiolate anion (Szajewski and Whitesides, 1980). Fortunately, there are many techniques for measuring the chemical reactivities of thiol and disulphide groups, and the most appropriate methods used for disulphide formation and breakage yield such information directly in many instances. The pK_a values of thiol groups can be determined relatively easily by monitoring the absorbance at 240 nm of the thiolate anion (Polgár, 1974). Discussions of the relevance of rates of disulphide formation to protein folding should emphasize the rate of the intramolecular step, in which a second cysteine residue of the protein displaces the mixed-disulphide on the first (Figure 1); it is only this step in which protein conformational folding is involved.

Disulphide folding studies are most informative about protein folding if carried out at relatively high pH values where the thiolate anion is present significantly. This minimizes any difference in reactivity arising from differences in pK_a values of the thiol groups. The reactivities with GSSG (Figure 1) of protein thiol groups having different pK_a values are nearly the same at pH 8.7, whereas they will differ accordingly at lower and higher pH values (Darby and Creighton, 1993). For example, a thiol group with a pK_a value that is 1.0 pH unit lower than normal will react 5-fold more rapidly at pH 7.4, but at the normal rate at pH 8.7. A further advantage of high pH is that the thiolate anion is not readily buried, inhibiting the formation of quasi-native conformations that are fully folded, but lacking one or more disulphide bonds and with the unpaired cysteine residues buried (see below). Such quasi-native species can accumulate to high levels with very stable proteins at low pH, and further disulphide formation is not governed by protein folding but by thiol group accessibility and reactivity. Minimizing the occurrence of quasi-native species at high pH tends to keep disulphide formation coupled to protein folding.

Although disulphide folding pathways can be elucidated in detail, and relatively unambiguously, there is a remarkable amount of confusion and controversy about even the best-characterized disulphide folding pathways, those of bovine pancreatic trypsin inhibitor (BPTI), bovine ribonuclease A (RNaseA), and human and bovine α-lactalbumin (αLA). A number of misconceptions are very popular. This article attempts to set the record straight.

Unfolded Proteins Produced Simply by Reducing Disulphide Bonds

The native conformations of small model proteins like BPTI, RNaseA, and αLA unfold upon simply reducing all their disulphide bonds, even under physiological conditions and in the absence of denaturants. Reduced proteins are stable indefinitely in the absence of oxidants, and the unfolded state of the protein can be studied under the same physical conditions as when folding occurs. Folding and disulphide formation are initiated simply by adding small amounts of an oxidant, usually a reagent like the disulphide forms of glutathione or dithiothreitol (Figure 1). Therefore, the same noncovalent interactions will be present in both the unfolded and folded conformations, except for the disulphide bonds. In contrast, the unfolded states of other proteins are stable only under unfolding conditions and are only transient under refolding condition. The reduced proteins are useful stable models of such proteins. Many theories of protein folding would predict that they would generally undergo hydrophobic collapse, adopt substantial secondary structure, or adopt the MG conformation.

The starting point for considering the conformational properties of unfolded proteins must be the idealized random coil. The most realistic definition of a random coil is one where the conformational properties of each residue are independent of those of other residues distant in the primary structure, and where there are no net interactions, other than steric exclusion, between such distant residues. The average conformational properties of this ensemble will depend primarily on the intrinsic conformational preferences of the individual residues, which differ amongst the various amino acids, and on their immediate neighbors in the primary structure. The NMR properties expected of such a random coil have been described (Fiebig *et al.*, 1996); they can correspond closely to the observed properties of small unfolded proteins (Smith *et al.*, 1996).

One of the most thoroughly studied reduced proteins, using a wide variety of techniques, is that of BPTI. Most, but not all, of the evidence indicates that it is very unfolded, approximating a random coil, but with weak interactions between groups close in the sequence. NMR spec-

tra of reduced BPTI, with the thiol groups free, blocked covalently, or replaced by other side chains (Kemmink and Creighton, 1993; Lumb and Kim, 1994; Ferrer *et al*., 1995; Pan *et al*., 1995), are similar to each other and to a set of overlapping peptides representing the entire primary structure (Kemmink and Creighton, 1993). Therefore, only local interactions between groups close in the primary structure can be present. The simplicity of the peptides made it possible to characterize those local interactions that are present (Kemmink and Creighton, 1995). Those most apparent by NMR involved aromatic side-chains interacting with nearby nonpolar groups or a glycine backbone NH group. Each of these interactions was between residues no more than two apart in the sequence, and the same interactions were present in short peptides, some of only four residues. Therefore, the interactions are strictly local in nature, and they could be studied easily. Two in particular could be characterized in detail and quantified (Kemmink and Creighton, 1995); both are weak, being present only about 26% and 64% of the time at 10 °C and having favourable enthalpies of interaction of about −3 kcal/mol. Such weak interactions can be either readily disrupted to adopt alternative conformations or used and strengthened by further folding of the polypeptide chain; examples of both types were observed (Kemmink and Creighton, 1993).

Other studies of reduced BPTI confirmed the presence of these interactions (Lumb and Kim, 1994; Pan *et al*., 1995). A very detailed study of the intact protein (Pan *et al*., 1995) claimed also to detect many further examples of such local interactions, but the possibility that this was due to NMR spin diffusion was not ruled out. The local interactions present in reduced BPTI have been described as 'extensive' (Pan *et al*., 1995) and to indicate the presence of a 'hydrophobic cluster' (Lumb and Kim, 1994) or a 'molten coil' state (Barber *et al*., 1995). In reality, they are isolated, independent and weak interactions between only pairs of residues close in the covalent structure. Other than for a slight tendency to adopt an α-helix near the C-terminus, no evidence for any cooperative interactions or folded structure in reduced BPTI has been presented.

One of the most contentious issues is the overall compactness of the reduced BPTI molecule, with claims that it is not expanded like a random coil but very compact like a MG (Ittah and Haas, 1995) or a 'molten coil' state (Barbar *et al*., 1995). There is considerable evidence that reduced BPTI is not compact, including the absence of NMR interactions between groups distant in the primary structure, the similarity of the chemical shifts of protons in intact reduced BPTI and in short peptides (Kemmink and Creighton, 1993; Pan *et al*., 1995), and its electrophoretic mobility in non-denaturing polyacrylamide gels. The electrophoretic mobility, relative to the same protein in the native conformation, is a sensitive measure of overall molecular dimensions. The decreased mobility of reduced BPTI is very close to that of a very unfolded polypeptide. In contrast, true MG states have compactness and electrophoretic mobilities very close to that of the native conformation (Ewbank and Creighton, 1993a). Moreover, the electrophoretic mobility of reduced BPTI is not affected by urea or by various amino acid replacements (Goldenberg and Zhang, 1993). Only one very slight change was detected (in mutant G12V), and it can be explained by abolishing one of the local interactions noted in reduced BPTI and peptides, of the Tyr10 side-chain with the NH of residue 12, which occurs only when the latter is Gly (Kemmink and Creighton, 1993).

Compact but unfolded protein molecules, especially MGs, tend to bind the dye ANS (1-anilinonaphthalene-8-sulfonate) and to enhance its fluorescence. Weak enhancement of ANS fluorescence by reduced BPTI has been taken to indicate that the protein is a 'molten coil' and not fully unfolded (Ferrer *et al*., 1995). This presumed binding of ANS to reduced BPTI was claimed to have been missed in an earlier study (Darby and Creighton, 1993), but the experimental observations were actually very similar in the two studies. The enhanced fluorescence appeared insignificant in Darby and Creighton (1993) because it was being compared to that by a true MG. Reduced BPTI does apparently bind somewhat more ANS than does native BPTI, but this is only a small fraction of that exhibited by a true MG (Darby and Creighton, 1993). Any unfolded protein will have more exposed nonpolar surface area accessible to ANS than the folded, native conformation, so slightly greater binding under the same conditions does not necessarily mean the presence of a collapsed state.

The experimental evidence that most dramatically suggests a compact conformation for reduced BPTI comes from energy transfer between fluorescent groups introduced into the protein, which indicated distances too short for an unfolded polypeptide chain (Ittah and Haas, 1995). This may, however, result from physical attractions between the two bulky aromatic fluorescent groups that were introduced into the protein for the fluorescence measurements. An attraction between the two groups was indicated by the observation of intermolecular energy transfer between them when they were present at only micromolar concentrations on different reduced BPTI molecules (Ittah and Haas, 1995), concentrations much lower than those where unmodified reduced BPTI aggregates. They are also lower than the millimolar 'effective concentrations' of two groups within the same molecule of reduced BPTI (Darby and Creighton, 1993). Therefore, the attractive interactions between the separate molecules observed at micromolar concentrations will be much greater when they are attached to the same BPTI molecule. In this case, the two fluorescent groups would tend to associate with each other and consequently would report distances shorter than those that would be present in their absence.

Disulfide Formation in Unfolded Polypeptide Chains

In addition to their direct physical properties, the conformational properties of reduced proteins can be further inferred from what disulphide bonds they form initially (Figure 2). A random coil will introduce disulphide bonds between the various cysteine residues not close in the covalent structure in proportion to $n^{-3/2}$ to $n^{-5/2}$, where n is the number of residues between the cysteine residues. Even only small amounts of unstable nonrandom conformation that brings two cysteine residues into proximity to form a disulphide bond will be apparent by a preponderance of disulphide bonds being formed between these two cysteine residues (Figure 2B). For example, a nonrandom conformation that is present in only 1% of the reduced protein molecules, but stabilizes a particular disulphide bond by a modest factor of 10^2, will double the preponderance of that particular disulphide bond over the expected statistical level.

Whatever effect a folded conformation has on the stability of a disulphide bond, the disulphide bond must have the same quantitative effect on the stability of the folded conformation (Figure 2A). Therefore, any nonrandom conformation that stabilizes a particular disulphide bond must be stabilized to the same extent by the presence of that disulfide bond. The stabilizing conformation might be too

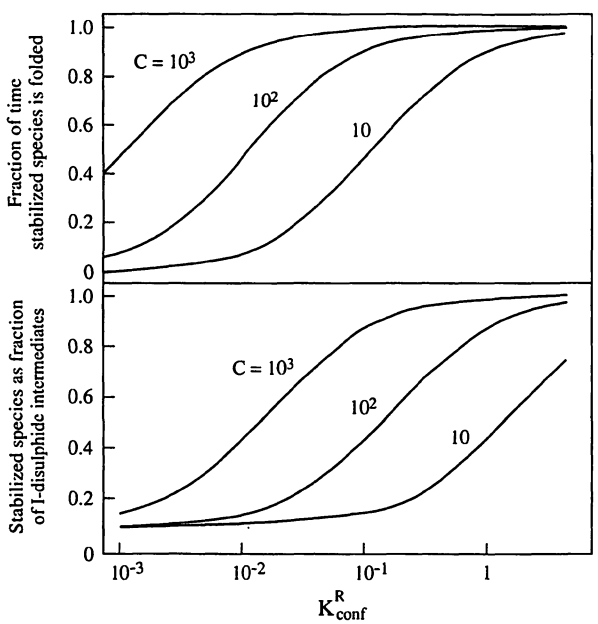

Fig. 2 The Predicted Effects of an Element of Nonrandom Conformation that Stabilizes a Particular Disulphide Bond in a Protein. (A) The stability of the folded conformation in the species with that disulphide bond for various stabilities of the nonrandom conformation in the reduced protein, K^R_{conf}, and for various linkage factors, C, by which the conformation and the disulphide bond stabilize each other. Consider a reduced protein with two or more cysteine residues that is largely unfolded, R_U, but has a tendency to adopt a folded conformation, R_F, that brings two cysteine residues into correct proximity for forming a disulphide bond. The equilibrium constant for this conformational equilibrium is

$$K^R_{conf} = \frac{[R_F]}{[R_U]} \quad (1)$$

The disulphide bond formed will be more stable in R_F than in R_U, by a factor taken to be C. It is a thermodynamic necessity that the presence of the disulphide bond, in the species I, stabilizes the folded conformation by the same factor C:

$$\begin{array}{ccc} R_U & \xleftrightarrow{K_{SS}} & I_U \\ \Big\uparrow\downarrow K^R_{conf} & & \Big\uparrow\downarrow C\,K^R_{conf} \\ R_F & \xleftrightarrow{C\,K_{SS}} & I_F \end{array} \quad (2)$$

Disulphide formation and folding are therefore linked functions, and the two phenomena must have the same quantitative effect on each other. For example, if the folded conformation stabilizes a disulphide bond by a factor of 10^2 ($C = 10^2$) and the folded conformation is present in the reduced protein only 1% of the time ($K^R_{conf} = 10^{-2}$), it will be present 50% of the time in the one-disulphide species I ($K^I_{conf} = 1$).

(B) The effect on the equilibrium concentration of the species I with the stabilized disulphide bond. The particular one-disulphide species I will normally be in equilibrium, by intramolecular disulphide interchange, with all the other one-disulphide species, designated here as $1SS_U$. In the ideal unfolded, random-coil unfolded state, this equilibrium will be statistical in nature and depend simply upon the distances in the primary structure between all the various pairs of cysteine residues:

$$I_U \xleftrightarrow{K_{stat}} 1SS_U \quad (3)$$

On average, the equilibrium constant K_{stat} will have the approximate value of $n-1$, where n is the number of 1-disulphide species possible. The presence of the folded form of I, I_F, as in Equation 2, will increase the total amount of species I:

$$I_F \xleftrightarrow{K^R_{conf}} I_U \xleftrightarrow{K_{stat}} 1SS_U \quad (4)$$

The fraction of one-disulphide molecules present as I will be given by

$$\frac{C\,K^R_{conf} + 1}{K_{stat}} \quad (5)$$

This is depicted here for an average case where the protein has six cysteine residues, $K_{stat} = 14$.

unstable to be populated substantially in the reduced protein, but it will be populated substantially more in the presence of the disulphide bond. The greater the stability of the nonrandom conformation in the absence of the disulphide bond, the greater its stability in the presence of the disulphide bond. Nonrandom conformations in a reduced protein populated sufficiently to make disulphide formation nonrandom must be present in substantial, detectable amounts when the favoured disulphide bond is present. Therefore, it must be possible to detect and to characterize such a conformation.

Reduced BPTI

As expected for a random coil, reduced BPTI forms its first disulphide bonds nearly randomly, and most of the one-disulphide species formed are unfolded (Darby and Creighton, 1993). The first disulphide bonds formed directly are rearranged intramolecularly, however, and the re-

sulting equilibrium mixture is not statistical. The partly-folded (30–51) intermediate (van Mierlo *et al.*, 1993) predominates, because it has a partly-folded conformation and a decreased free energy, but it folds only after the disulphide bond is formed (Darby and Creighton, 1993). Its partly-folded conformation is not expected to be sufficiently stable in the reduced protein to be apparent (Creighton, 1988).

An exception to statistical disulfide formation in reduced BPTI has been reported (Dadlez and Kim, 1995), in that, under some conditions, a disulphide bond is formed most rapidly between Cys 14 and 38; this disulphide bond is also present in native BPTI. This has been claimed to indicate the presence of native-like conformation in the reduced protein (Dadlez and Kim, 1995). If so, the (14–38) intermediate should have been stabilized relative to the other one-disulphide intermediates (Figure 2B), and that nonrandom conformation should have been present in the (14–38) intermediate (Figure 2A). The large magnitude of the kinetic effect in the reduced protein in this case should result in the nonrandom conformation being exceptionally stable in the presence of the disulphide bond. Neither consequence was observed (Dadlez and Kim, 1995), however. The (14–38) intermediate is not more stable than the others; it rearranges very rapidly to the others, and they predominate at equilibrium. Also, an analogue of the intermediate (14–38) was reported to be unfolded under the same conditions (Dadlez and Kim, 1995). [Replacing the other free cysteine residue with α-amino-*n*-butyric acid residues produced a partly-folded structure, but only at very low temperatures (Ferrer *et al.*, 1995; Barbar *et al.*, 1995)].

Rather than nonrandom conformation, the observations indicated that the greater rate of formation of the 14–38 disulphide bond in reduced BPTI is due to an exceptional reactivity of the Cys14 and Cys38 sulphur atoms. This is probably for electrostatic reasons, for the phenomenon occurs primarily at pH 7.3, much less under the more alkaline conditions that are more appropriate for characterizing disulphide folding pathways. Some enhanced reactivity of the Cys14 and 38 thiol groups was detected by chemical modification even at alkaline pH (Creighton, 1992), and it should be enhanced at lower pH values (Darby and Creighton, 1993). It is probably due to the presence of basic residues adjacent to both Cys14 and Cys38 in the primary structure, respectively Lys15 and Arg39. It is known that adjacent basic or acidic residues can alter the ionization and reactivities of cysteine thiol groups and their tendency to make disulphide bonds (Snyder *et al.*, 1983). A subsequent study of the reactivities of the individual cysteine residues in analogues of reduced BPTI and in short peptides confirmed the greater reactivity of the Cys14 and 38 thiol groups and indicated that it was due solely to local interactions that were not affected by urea (Dadlez and Kim, 1996). Likewise, the reactivities of the cysteine thiol groups were quantitatively the same in the peptides and in reduced BPTI, indicating the absence of any stable folded conformation in reduced BPTI affecting them.

Such anomalous thiol reactivity effects should be minimized if disulphide bond formation is to provide information about protein conformation and folding. Their substantial magnitude in Cys14 and Cys38 in the study of Dadlez and Kim (1995, 1996) is undoubtedly due to the use of the relatively low pH of 7.3, where differences due to thiol pK_a are much greater than at higher pH. It was concluded from that study that reduced BPTI is compact, but the only experimental evidence indicating that was a smaller than expected variation in the rate of the intramolecular step in disulphide formation with distance between the cysteine residues in the primary structure. A much greater dependence was observed in the earlier study with five of the fifteen possible pairs of cysteine residues at the more appropriate pH 8.7 (Darby and Creighton, 1993). In any case, studies of disulphide formation in proteins where either or both Cys14 and 38 are blocked or removed indicate that they have no special role in forming the first disulphide bonds, although they do at the two-disulphide stage (Creighton, 1977a; Marks *et al.*, 1987; Goldenberg, 1988; Darby *et al.*, 1995).

Reduced Ribonuclease A

Reduced RNaseA also appears from most of its properties to be very close to a random coil. Nevertheless, it was proposed, on the basis of enzyme activity and immunological measurements, that the native conformation is present in 1.5 to 6% of reduced RNaseA molecules (Garel, 1978; Chavez and Scheraga, 1980). If so, the first disulphide bonds formed in this reduced protein should have been predominantly between native pairs of cysteine residues, and the native conformation should have been greatly stabilized when the native disulphide bonds formed (Figure 2) (Creighton, 1988). Therefore, essentially all the one-disulphide species generated should have had native disulphide pairings and the fully folded native conformation. In reality, all the studies indicate that many different disulphide bonds are formed initially in reduced RNase A and that these intermediates are all unfolded (Creighton, 1979; Xu *et al.*, 1996), as expected if reduced RNase A is a random coil.

The greatest reported exception to random disulphide formation in reduced RNaseA is that between two cysteine residues very close in the primary structure, Cys65 and Cys72, which is present in the equilibrium mixture of one-disulphide species 2.4-fold more than expected (Xu *et al.*, 1996). This comparison was based, however, on simple statistical calculations using solely the number of residues between the cysteines that did not take into account the intrinsic conformational tendencies of the polypeptide chain. The same relative stability of the 65–72 disulphide bond was found in a short peptide of only residues 58 to 72 (Altmann and Scheraga, 1990), indicating that the cause of this stability is strictly local in the polypeptide chain. It was attributed to a favourable backbone geometry and does not result from a nonrandom conformation due to the presence of stabilizing noncova-

lent interactions between groups distant in the covalent structure. The other one-disulphide intermediates appear to be much closer to their expected simple statistical distributions (Creighton, 1979; Xu *et al.*, 1996), indicating that there are probably no noncovalent interactions between groups distant in the covalent structure of reduced RNaseA that affect significantly the relative proximities of any other pairs of the eight cysteine residues. Therefore, all these observations are consistent with reduced RNase A closely approximating a random coil.

The observations with reduced BPTI and RNaseA demonstrate that unfolded proteins under physiological, folding conditions can remain very unfolded and approximate random coils. The nonrandom structure that is apparent in some instances is due to weak, local interactions between groups close in the primary structure. The weakness of these interactions makes them readily broken by productive folding; alternatively, they can be strengthened if they favour further interactions and then ultimately incorporated into the final folded structure (Kemmink and Creighton, 1993). Such local interactions presumably can also influence the average overall conformational properties of the unfolded polypeptide chain, but it otherwise remains unfolded. There is no general hydrophobic collapse or other substantial interactions between residues distant in the primary structure.

The MG Conformation of α-Lactalbumin

Reduced αLA exists as an equilibrium mixture of unfolded and MG conformations (Kuwajima, 1989). The unfolded state of reduced αLA is not as well-characterized as that of reduced BPTI and RNaseA, but its general properties suggest that it is similar (Ewbank and Creighton, 1993b). A detailed study of the homologous hen lysozyme found a situation similar to that in reduced BPTI, approximating a random coil with some of the same types of solely local interactions (Fiebig *et al.*, 1996). The presence of the MG conformation in reduced αLA is apparent from its

(1) relative compactness (Kuwajima, 1989; Ewbank and Creighton, 1993b),
(2) substantial content of secondary structure (Kuwajima, 1989),
(3) significant binding of ANS (Ewbank and Creighton, 1993b), and
(4) the distribution of disulphide bonds it forms (Ewbank and Creighton, 1993a).

The equilibrium between the MG and unfolded conformations is gradually shifted by denaturants to the unfolded state.

Reduced αLA forms very many different first disulphide bonds, mostly non-native. The main effect of its MG conformation is to bring cysteine residues distant in the primary structure into closer proximity than they would be in a fully unfolded conformation (Ewbank and Creighton, 1993a; Creighton and Ewbank, 1994). None of the disulphide bonds, native or non-native, have much effect on the stability of the MG conformation. With three disulphide bonds, non-native disulphide pairings are much more stable in the MG conformation than the native pairings (Ewbank and Creighton, 1991, 1993a). Moreover, the non-native pairings maintain the MG conformation, which cannot have a native-like topology in these instances.

All of the experimental observations indicate quite unambiguously that the MG conformation of αLA is very disordered and without a fixed backbone topology in the absence of native-like constraints, such as the four native disulphide bonds. Nevertheless, the contrary conclusion has been reached in another study because one particular native disulphide bond was found to be more stable than two non-native alternatives (Peng and Kim, 1994; Wu *et al.*, 1995; Peng *et al.*, 1995). Some observations of that study suggest that the conformation stabilizing this particular disulphide bond might be the native conformation rather than the MG (Creighton, 1997). In any case, the slight preference for this one disulphide bond, which is between cysteine residues near the ends of the polypeptide chain, is insignificant when compared to all the other topologies and disulphide bonds that this MG conformation can adopt.

Pathways of Disulphide-Coupled Folding

The pathways of disulphide formation and folding have been elucidated *in vitro* to varying extents for reduced BPTI, RNase A, and αLA (Figure 3). They demonstrate similar general properties, but with variations (Creighton, 1992; Creighton *et al.*, 1995).

Disulphide formation in an unfolded reduced protein is essentially random (when corrected for any differences in reactivities of the cysteine thiol groups), depending primarily upon the distance in the primary structure between the pairs of cysteine residues. The resulting disulphide intermediates will tend to have random disulphide bonds, which can rapidly interchange intramolecularly, and to remain unfolded. Each disulphide bond introduced sequentially into an unfolded protein is increasingly unstable, unless stabilized by the adoption of a folded conformation. An example is RNaseA, which readily forms up to two disulphide bonds randomly, but third disulphide bonds are formed only slowly and are very unstable (Creighton, 1979). The presence of the compact MG conformation primarily increases the probability of forming a disulphide bond between cysteine residues distant in the primary structure, as in reduced αLA (Ewbank and Creighton, 1993a). If the polypeptide chain has sufficient tendency to adopt a fixed conformation that brings two particular cysteine residues into proximity, the disulphide bond between them will be more stable than the other possibilities and the folded conformation will be stabilized (Figure 2); an example is the partly-folded (30–51) intermediate of BPTI (Figure 4) (van Mierlo *et al.*, 1993). Such partly-folded conformations will increase the rate of forming disulphide bonds between cysteine residues brought into proximity (unless that disulphide bond will become buried, see be-

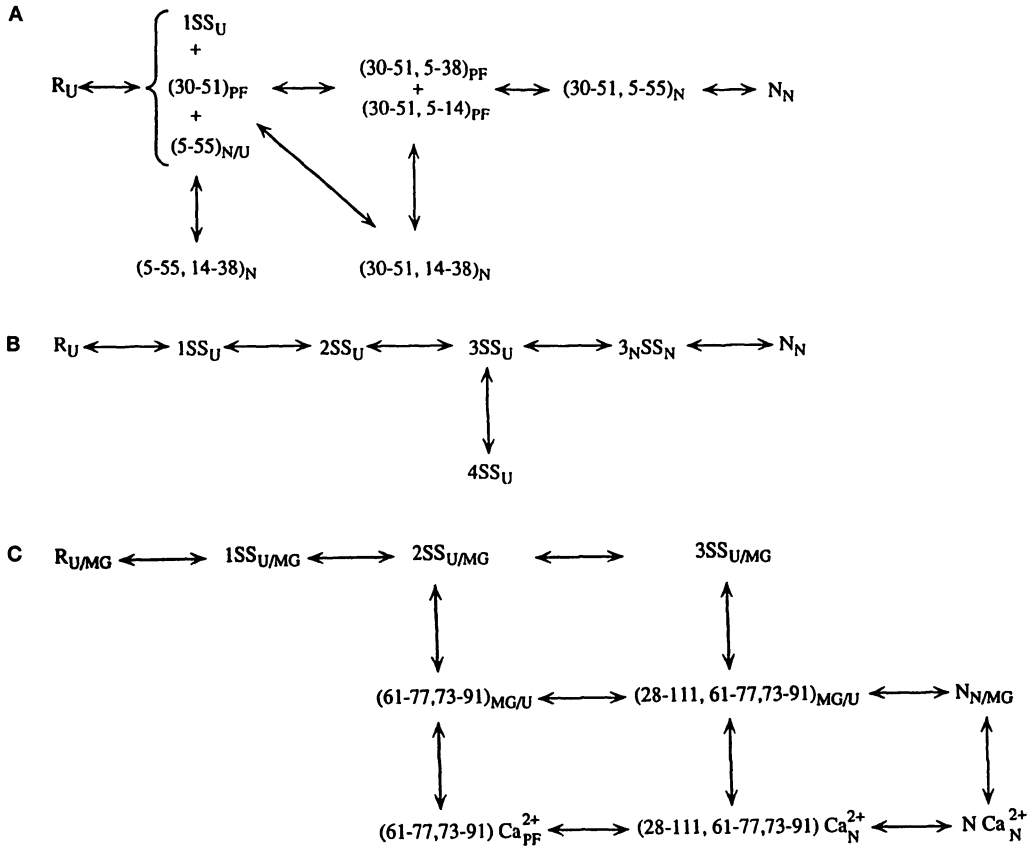

Fig. 3 The Disulphide Folding Pathways of (A) BPTI; (B) RNase A; (C) α-LA.
In each case, R is the fully reduced protein. Intermediates with specific disulphide bonds are indicated by the residue numbers of the cysteine residues paired. A mixture of many isomers with different disulphide bonds (which are usually interconverting by intramolecular disulphide rearrangements when thiol groups are also present) is depicted by the number of disulphide bonds present, e.g. 1SS, 2SS, 3SS and 4SS. Arrows to and from such species can represent the collective formation, breakage or rearrangement of many different disulphide bonds. The designation 3_NSS indicates that all three disulphide bonds are native-like. N is the protein with all the native disulphide bonds: (30–51,14–38,5–55) in BPTI; (26–84,40–95,58–110,65–72) in RNase A; and (6–120,28–111,61–77,73–91) in αLA. The arrows indicate the most important steps, in kinetic terms, and can involve either disulphide formation, breakage, or rearrangement. The predominant conformations of the various species are indicated by the subscripts U (unfolded), MG (molten globule), PF (partly-folded), and N (native). Where two or more conformations are present substantially in equilibrium, there are separated by /. The brackets in (A) indicate that the one-disulphide intermediates included are in equilibrium; the '+' between two more species indicates that they have the same kinetic roles.

low), while inhibiting it when the cysteine residues are kept apart.

Disulphide formation continues in this way, depending upon any change in conformation, until blocked by

(1) the instability of further disulphide bonds,
(2) the acquisition of a quasi-native folded conformation (see below), or
(3) encountering a kinetic block (see below).

In very many cases, the kinetic block is overcome by intramolecular disulphide rearrangements, to reach the fully native conformation with all the disulphide bonds.

The variations on the disulphide-coupled folding pathways (Figure 3) are similar to variations observed in protein folding not involving disulphide formation (Creighton, 1994). Some proteins remain unfolded until undergoing an all-or-nothing folding transition, comparable to RNaseA, which forms initially only random disulphide bonds. In other proteins, the MG conformation is populated during folding, as in the case of α-LA. In others, partly-folded intermediates are detected, as in BPTI (Figure 4).

Quasi-Native Species

In general, a protein disulphide bond generally simply stabilizes a pre-existing protein conformation. The degree of stabilization is determined by

(1) the distance between the cysteine residues in the primary structure (which determines the loss of conformational entropy in forming the disulphide bond and the consequent destabilization of the unfolded state),
(2) the free energy of the disulphide bond itself and the extent to which the folded conformation stabilizes it (Goldenberg and Creighton, 1985), and
(3) any interactions of the thiol groups with the reduced form of the protein (Creighton *et al.*, 1995).

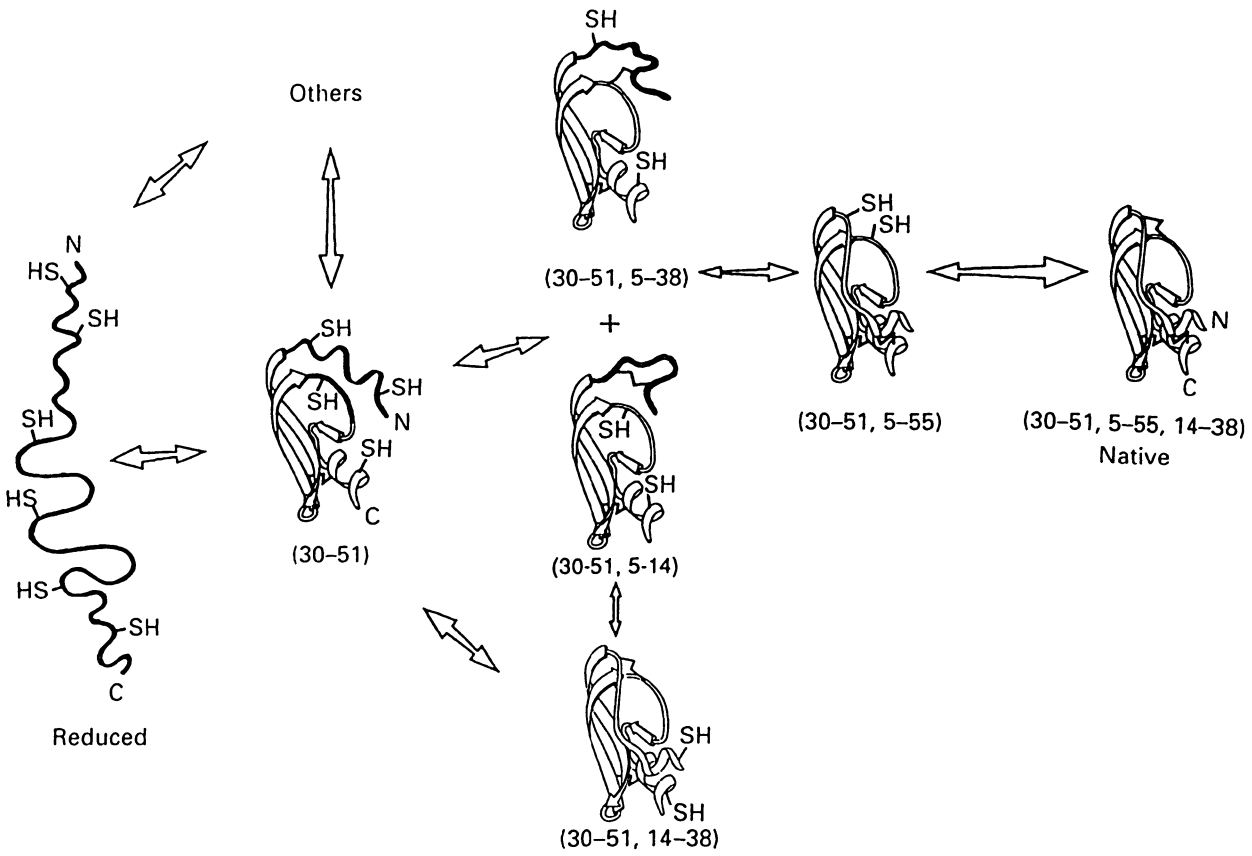

Fig. 4 The Conformational Basis of the Productive Disulphide Folding Pathway of BPTI.
The major disulphide intermediates at pH 8.7 are depicted with their approximate conformations indicated schematically; solid portions of polypeptide backbone are unfolded or very flexible. The non-productive quasi-native species (5–55) and (5–55,14–38) are omitted. The relative rates of the most prominent steps, refering to the value of k_{intra} (Figure 1), are indicated semi-quantitatively by the thickness of the appropriate arrowhead; the wider the arrowhead, the greater the rate in that direction.

If the last factor is especially favourable, even intrinsically stable disulphide bonds can destabilize a folded conformation (Creighton *et al.*, 1995).

In many cases, the native conformation of a protein is sufficiently stable to withstand the loss of one or more stabilizing disulphide bonds. For example, the very same folded conformation is observed, but with varying stabilities, with BPTI lacking any one of its three disulphide bonds, and even in one instance when it is lacking two (van Mierlo *et al.*, 1991, 1993). Less stable homologues of BPTI do not adopt the native-like conformation in the less stable of these species (Kortemme *et al.*, 1996). RNase T1 with just the 6-103 disulphide bond adopts a native conformation (Pace and Creighton, 1986; Mayr *et al.*, 1994). Two such native-like species have been detected with RNaseA, one lacking the 40–95 disulphide bond (Creighton, 1980; Li *et al.*, 1995), the other the 65–72 (Talluri *et al.*, 1994). αLA lacking the 6–120 disulphide bond maintains a native-like conformation if Ca^{2+} is bound (Kuwajima *et al.*, 1990; Ewbank and Creighton, 1993b)

Such species with incomplete disulphide bonds are fully folded, so they are designated as 'quasi-native'. In these species, forming the missing disulphide bonds is not coupled to protein folding. Instead, the rate of disulphide bond formation depends primarily upon the accessibility and reactivity of the protein thiol groups (Figure 1). If the cysteine thiol groups are readily accessible, disulphide bonds can be formed very rapidly because the cysteine residues are in appropriate proximity, as occurs in the (30–51,5–55) intermediate, also designated N^{SH}_{SH}, of BPTI, and such disulphide bonds are generally formed last during disulphide folding (Figure 4). In contrast, cysteine thiol groups that are buried in the interior of the folded conformation and are inaccessible to disulphide reagents (Figure 1) cannot be formed readily. A most extreme example is species (5–55,14–38) of BPTI, where the two remaining thiol groups of Cys30 and Cys51 are buried, inaccessible and unreactive (States *et al.*, 1984; Creighton and Goldenberg, 1984; Eigenbrot *et al.*, 1992). In addition, there is a kinetic block in forming any protein disulphide bond that will be buried after it is formed by the protein conformation (see below), irrespective of whether or not the cysteine thiol groups are reactive.

The kinetic roles of such quasi-native species, in which final disulphide formation is blocked and not linked to folding, is not of immediate relevance to protein folding. For example, there are many unresolved questions about why the quasi-native species of RNaseA lacking the 65–72 disulfide bond can be observed only with very high concentrations of reducing agent and trapped by some un-

usual methods (Talluri *et al.*, 1994; Li *et al.*, 1995), and not by others (Creighton, 1979; Wearne and Creighton, 1988). The discrepancy is usually attributed to the difficulties of trapping by reacting buried residues (Li *et al.*, 1995), but the study of Wearne and Creighton (1988) also used unfolding in urea that should not have been limited by thiol group unreactivity. A more likely explanation is that in the latter study, where the intermediate could not be detected, the rate of generation of this intermediate by reduction with moderate concentrations of thiol reagent was slower than its disappearance, probably by intramolecular disulphide rearrangements; therefore, the intermediate was not populated substantially in this case but was with the very high thiol reagent concentrations. The important conclusion that the rate-limiting step in disulphide breakage and unfolding of RNase A is normally reduction of the first disulphide bond was confirmed by the other study (Li *et al.*, 1995). In any case, the uncoupling between protein folding and disulphide formation in quasi-native species implies that the details of the pathway followed are unlikely to provide information about protein folding. The accumulation of such quasi-native species should be minimized by carrying out studies at alkaline pH, where the cysteine thiol groups tend to be ionized and, therefore, less likely to be buried in a folded conformation.

A more positive aspect of quasi-native conformations is that they demonstrate that disulphide bonds do not determine the folded conformation, as is often mistakenly stated, but only stabilize it. The very same folded conformation is observed in BPTI species lacking any one of the three native disulphide bonds (States *et al.*, 1984; van Mierlo *et al.*, 1991; Eigenbrot *et al.*, 1992).

A reexamination of the BPTI folding pathway found the quasi-native species (5–55), (5–55,14–38) and (30–51,14–38) to predominate and assigned them the most important roles in the folding process (Weissman and Kim, 1991). The especially large quantities of these intermediates that accumulated in that study were due, however, primarily to the use of a low pH, 7.4, where they are more stable. Furthermore, these intermediates accumulate to high concentrations because there is a kinetic block in completing disulphide formation (Creighton, 1977a). The great accumulation of these quasi-native species distracted from the important kinetic intermediates, which occur in lower quantities and more transiently (Darby *et al.*, 1995).

Kinetic Blocks in Disulphide Formation

In virtually all the known disulphide folding pathways (Figure 3), there occurs a kinetic block to the formation of particular disulphide bonds, in that these bonds are formed very much more slowly, if at all, than those preceding; in some cases alternative disulphide bonds are formed more rapidly. In each case, forming the disulphide bond that is blocked would produce a stable folded conformation in which that disulphide bond would be buried and inaccessible. Usually, these are native disulphide bonds. The best-characterized example is the kinetic blockage in forming the 30–51 disulphide bond in the intermediates (5–55) and (5–55,14–38) of BPTI (Figure 4). In both instances, very stable, fully folded native conformations would result.

The reason for these kinetic blockages is apparent from the kinetics of the reverse step, reduction of the buried disulphide bond (Creighton, 1978; Creighton *et al.*, 1995). The kinetics of disulphide formation and breakage in BPTI have been shown to follow microscopic reversibility, in that the ratio of the rate constants for both directions of forming any disulphide bond is the same as the measured equilibrium constant for that step; consequently, the same transition state is encountered in both directions. Reducing a disulphide bond buried in a stable folded conformation by reaction with a thiol reagent (Figure 1) is slow and has a high free energy barrier, because the folded conformation must be perturbed or unfolded for the reagent to gain access to the disulphide bond. Buried disulphide bonds also tend to be the most stable in a folded conformation (Creighton and Goldenberg, 1984). Generally, it is the first step in reducing the disulphide bond that is rate-limiting. Due to microscopic reversibility, the same high-energy transition state is encountered in forming the disulphide bond, and the same step is slow in this reverse direction. Consequently, forming a disulphide bond that will become buried in a stable folded conformation is expected to be slow. This should be a general phenomenon, but it is presently uncertain whether it occurs only when the species lacking the disulphide bond is already at least partly folded (Mendoza *et al.*, 1994; Creighton *et al.*, 1995).

In the disulphide unfolding process, accessible disulphide bonds on the protein surface tend to be the least stable and the most reactive and accessible to the thiol reagent. Consequently, they are the first to be reduced in the native conformation. Likewise, such accessible disulphide bonds also tend to be formed last in disulphide folding. In this case, both forming and breaking disulphide bonds tend to occur most readily by intramolecular disulphide rearrangements, using the thiol groups of the accessible cysteine residues.

These principles are well-illustrated by the BPTI folding pathway (Figure 4) (Creighton, 1977a). The first kinetic block encountered occurs with the very slow formation of the 5–55 disulphide bond in the partly-folded intermediate (30–51). The reason is that the stable folded conformation of (30–51,5–55) would result, in which the 5–55 disulphide bond would be stable and buried and, therefore, reduced only slowly. The very slow rate measured for forming this disulphide bond is that expected from the rate constant for reducing this disulphide bond and the inferred equilibrium constant for the process. The second kinetic block occurs in forming the 5–55 disulphide bond in the quasi-native species (30–51,14–38), which would produce the fully folded native conformation of BPTI with all three disulphide bonds. Again, reducing the 5–55 disulphide bond in native BPTI is very slow, and the very

slow reverse rate of its formation is that expected simply on this basis. The same kinetic block would be expected in forming the 30–51 disulphide bond in (5–55,14–38), but this is obscured by the inaccessibility and unreactivity of the Cys30 and 51 thiol groups in this quasi-native two-disulphide species (States *et al.*, 1984; Creighton and Goldenberg, 1984).

Although these principles have been established only in BPTI, the kinetics of disulphide formation observed with other comparable proteins indicate that they also apply there (Creighton *et al.*, 1995); they would be expected to be universal.

It might be thought that these principles would be specific for disulphide bonds, but it has been pointed out that qualitatively similar, although smaller quantitatively, energetic considerations would be expected with hydrogen bond formation [Figure 9 of Creighton (1978)]. Other factors being equal, forming hydrogen bonds that will be buried in the protein interior should be more rapid when it occurs by hydrogen bond exchange (involving either other hydrogen bonding groups of the protein or solvent water) than by forming hydrogen bonds in isolation. In that regard, it may be pertinent that formation of buried polar interactions has been shown to be a slow step in acquisition of the fully folded state of a protein not involving disulphide bonds (Waldburger *et al.*, 1996).

Kinetic Blockages and Thiol Group Reactivity

In some BPTI intermediates in which there is a kinetic block, such as (5–55,14–38), the cysteine thiol groups are buried and inaccessible (States *et al.*, 1984; Creighton and Goldenberg, 1984). That factor alone would appear sufficient to explain why a disulphide bond cannot be formed readily between them. Probably for that reason, it has become a popular idea that kinetic blocks generally occur because of the folded conformation and because of the thiol groups being inaccessible and nonreactive (Goto and Hamaguchi, 1981; Weissman and Kim, 1991, 1995). Nevertheless, this is not correct, as the experimental data in other instances demonstrate directly and unambiguously (Creighton, 1981).

It is straightforward to determine whether a kinetic block in disulphide formation occurs because the slow step is reaction of the protein thiol groups with the disulphide reagent or the second step of displacement of the resultant mixed-disulfide (Figure 1). If the latter step is slow, the mixed-disulphide intermediate will be generated at a normal rate, but it will not disappear rapidly; consequently, it will accumulate to a substantial extent. If, on the other hand, the initial reaction of the Cys thiol group with the reagent is the slow, rate-determining step, the mixed disulphide will be generated only slowly and will then be rapidly displaced by the second reaction; consequently, the mixed-disulphide will not accumulate substantially, even less than in forming a disulphide bond with no kinetic block. Therefore, the accumulation of substantial levels of the mixed-disulphide species is direct and positive evidence that the reactivity of the cysteine thiol group was not the rate-limiting step (Creighton, 1981).

The experimental observations with the BPTI (30–51) and (30–51,14–38) intermediates are unambiguous and unequivocal. During folding of reduced BPTI with GSSG as disulphide reagent at pH 8.7, where the quasi-native (5–55) is not populated substantially, only two intermediate species are observed to accumulate as -SG mixed-disulphide species: (30–51) with Cys55-SG, and (30–51,14–38) with Cys5-SG and Cys55-SG (Creighton, 1977a). The accumulation of these mixed-disulphide species, and none others, is direct and positive proof that the kinetic blockage of forming the 5–55 disulphide bond in both instances is not due to inaccessibility of the cysteine thiol groups. Instead, it is because the second step of Figure 1 is slow.

It has been argued that the folded conformations of (30–51) and (30–51,14–38) restrict the accessibility of the Cys55 thiol group and that this is the reason for the kinetic block in forming the 5–55 disulphide bond in both instances (Weissman and Kim, 1991, 1995). The folded conformation may cause there to be some decrease in the rate of reaction of Cys55 with disulphide reagents (Figure 1), but this is not the basis of the kinetic block in disulphide formation. The Cys55 thiol group is observed directly to react, by formation of the mixed-disulphide (Creighton, 1977a; Weissman and Kim, 1995). The accumulation of this intermediate demonstrates directly that the second step in forming the disulphide bond is even slower than the first in this case, even though it is solely intramolecular.

Finally, that the second step is rate-limiting in disulphide formation is consistent with the general explanation given above, based on the slow reduction of buried disulphide bonds occurring via the same transition state. The reverse of the second step is normally the slow step in disulphide reduction and would be expected also to be rate-limiting in disulphide formation.

Disulphide Rearrangement Pathways

The BPTI pathway is infamous for normally occurring through intramolecular disulphide rearrangements involving intermediates with non-native disulfide bonds. Weissman and Kim (1991) concluded that the occurrence of specific non-native disulphide bonds was an indication of non-native stabilizing interactions, an unpopular idea amongst protein folders, and attempted to disprove their significance. They found the species with non-native disulphide bonds in somewhat lower quantities than previously, but this was of no significance for their kinetic roles in the pathway. Ironically, those studies confirmed the expected kinetic behaviour of these intermediates and their roles in disulphide rearrangement (Weissman and Kim, 1992). There was, in fact, nothing heretical about the disulphide rearrangement pathway, for

(1) the stabilities of the non-native disulphide bonds were known to indicate that they were not stabilized by any conformation (Creighton, 1977b),
(2) only native-like conformation had been detected in the trapped intermediates (States *et al.*, 1987), and
(3) a pathway involving only native-like nonrandom conformations had been proposed earlier (Creighton, 1990).

In confirmation, the occurrence of the two specific non-native disulphide bonds, 5–14 and 5–38, in the nonnative species (30–51,5–14) and (30–51,5–38), respectively, is now known to occur because of the presence of native-like conformation in the remainder of the partly-folded conformation (van Mierlo *et al.*, 1994).

The disulphide folding pathways of other proteins generally also involve disulphide rearrangements (Creighton, 1992). It is a general phenomenon, because it is the most rapid way of inserting a disulphide bond into a protein when the result will be a folded conformation in which that disulphide bond will become buried.

The disulphide rearrangement pathway of BPTI occurs because of the kinetic block in inserting either the 30–51 or 5–55 disulphide bonds (which are both buried in the native conformation) into species with one other native disulphide bond. The appearance of two native disulphide bonds would cause the fully folded native conformation to become stable, and the 30–51 or 5–55 disulphide bond introduced would become buried. Forming such buried disulphide bonds by the disulphide rearrangement process is more rapid than direct disulphide formation because it is an intramolecular process, whereas forming a disulphide bond by intermolecular reaction with a disulphide reagent is intermolecular (Figure 1). It has long been recognized that the intramolecular transition states for the two processes have very similar free energies, as would be expected if the same conformational transition took place in both (Creighton, 1978); this was subsequently rediscovered by Weissman and Kim (1992).

In some instances of blocks in disulphide formation, alternative disulphide bonds are formed much more rapidly, such as 14–38 in intermediate (5–55) of BPTI (Creighton and Goldenberg, 1984). On the other hand, if no alternatives can be formed rapidly, disulphide formation is slowed dramatically. In some cases, the alternatives are non-native disulphide bonds; these have the potential to rearrange intramolecularly to the native disulphide bond whose formation was blocked, thereby by-passing the kinetic block. This also occurs in the folding pathway of BPTI, in which there are kinetic blocks to forming the 5–55 disulphide bond (Figure 3). The first such block occurs in the partly-folded (30–51) intermediate, which cannot readily form the 5–55 disulphide bond, as it would produce the stable, fully folded intermediate (30–51,5–55). Instead, it can form readily any of the three disulphide bonds possible between Cys5, 14 and 38 (Creighton, 1977a). This is readily explicable on the basis of the partly-folded conformation of this intermediate (van Mierlo *et al.*, 1993, 1994). These three cysteine residues are in flexible or unfolded parts of the polypeptide chain, so there are no restrictions on their forming disulphide bonds. They are not formed because of favourable conformation, but because of the lack of conformation, although the partly-folded conformation does keep them in moderate proximity. The main role of the partly-folded conformation is to restrict disulphide formation with Cys55, even formation of the native 5–55 disulphide bond.

If the 14–38 disulphide bond is formed in (30–51), the quasi-native conformation results, due to the high stability of the native conformation of BPTI (van Mierlo *et al.,* 1991). If the native conformation is less stable, as in some homologues of BPTI, only a somewhat more ordered, but still partly-folded, conformation results (Kortemme *et al.*, 1996). The other two disulphide bonds that can be formed in (30–51), 5–14 and 5–38, are non-native and do not alter substantially the partly-folded conformation (van Mierlo *et al.*, 1994). These two disulphide bonds are still in flexible portions of the chain and can react slowly with the thiol group of Cys55 to rearrange intramolecularly to the native 5–55 disulphide; the stable native-like (30–51, 5–55) results (Figure 4). Direct formation of the 5–55 disulphide bond was blocked kinetically, so the intramolecular disulphide rearrangements appear to have bypassed the kinetic blockage.

Recently, it has been proposed that the disulphide rearrangement pathway of BPTI arises because of the folded conformation of (30–51,14–38) (Weissman and Kim, 1995). This proposal is incorrect, because the rearrangement pathway of BPTI still predominates in the absence of this species. This has been shown using a mutated form of BPTI where (30–51,14–38) is greatly destabilized (Zhang and Goldenberg, 1993) and by covalently blocking (Creighton, 1977a) or replacing (Darby *et al.*, 1995) either Cys14 or Cys38, when the 14–38 disulphide bond and intermediate (30–51,14–38) are not possible. The mistaken proposal arose because intermediate (30–51,14–38) was assigned, solely on the basis of its high accumulation, an incorrect kinetic role as an important intermediate (Weissman and Kim, 1991). Instead, it accumulates to high levels because of the stability of its quasi-native conformation (van Mierlo *et al.*, 1991) and because it is kinetically trapped due to its inability to form readily the missing native disulphide bond (Creighton, 1977a).

Assigning Roles and Importance to Kinetic Intermediates

There is a remarkable degree of confusion about how to interpret the kinetics of protein folding and decide the kinetic importance of any partly-folded species other than unfolded (U) and native (N). Any such structurally intermediate species is generally, in the absence of any experimental evidence to the contrary, assigned a role as a crucial intermediate (I), as in

$$U \longleftrightarrow I \longleftrightarrow N \qquad (1)$$

This kinetic scheme states that all the molecules refold through I and that N would not be formed in the absence of I. It is generally not possible to distinguish experimentally between this kinetic scheme and one where I is an off-pathway species, because U and I are generally in rapid equilibrium. Consequently, the kinetic roles of most intermediate species in protein folding are generally not known.

The disulphide approach to folding has the great advantage that the kinetic roles of all the disulphide species can usually be determined unambiguously. It is straightforward to determine whether a step involves disulphide formation, breakage, or rearrangement, simply by whether the rate is, respectively, proportional to the concentration of the disulphide or thiol reagent, or independent of both (Figure 1). A specific intermediate can be trapped reversibly, purified, and then studied in isolation. Specific cysteine residues can be replaced or blocked, disallowing all species having that residue in a disulphide bond. Finally, each step must involve the formation, breakage, or rearrangement of only a single disulphide bond. The kinetic role of a disulphide intermediate generally remains ambiguous only when that intermediate is in rapid equilibrium with others by intramolecular disulphide exchange.

The BPTI pathway of Figure 4 has been determined unambiguously by such kinetic analysis. Furthermore, it has been shown that this pathway and a single set of rate constants can account for the rates of appearance and disappearance of all the species under the same conditions, but a variety of redox conditions for forming and breaking disulphide bonds (Creighton and Goldenberg, 1984; Darby *et al.*, 1995).

Usually, those partly-folded species in protein folding that are detected in the greatest amounts are considered the most important. However, such species often accumulate to high levels simply because they are kinetically blocked and stable. In general, the least-populated intermediates are those most important kinetically. Rather than its level of accumulation, the kinetic importance of an intermediate should be judged by

(1) what fraction of molecules pass through it during the reaction and
(2) the degree to which the reaction is slowed if that intermediate is not possible.

The non-native two-disulphide intermediates (30–51, 5–38) and (30–51,5–14) of BPTI (Figure 4) fulfill both of these requirements (Darby *et al.*, 1995). Folding through these two specific intermediates accounts quantitatively for the rate of appearance of fully native protein, and complete disulphide formation is blocked in their absence. Consequently, these are the most important kinetic intermediates, even though they are not populated as much as the kinetically-trapped quasi-native species; their kinetic roles had been questioned simply on that basis (Weissman and Kim, 1991). The quasi-native species may predominate, but they fail the second test, in that the rate of BPTI folding is not slowed by their absence.

Although the intermediates (30–51,5–38) and (30–51,5–14) of BPTI are the most important kinetically, their partly-folded conformations serve merely to direct somewhat the disulphide pathway. They probably become unfolded to a substantial degree in the over-all rate-limiting step, just before attaining the fully native conformation. The intramolecular steps in folding are slowed only about 14-fold over-all in the presence of 8 M urea, where all the intermediates are unfolded (Creighton, 1977c, 1978, 1992). The most productive intermediates in folding are too unstable to be detected, which is probably a common feature of protein folding reactions.

The lessons from disulphide-coupled folding are likely to be applicable to protein folding in general.

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Review

Recombinant Allergen-Specific Antibody Fragments: Tools for Diagnosis, Prevention and Therapy of Type I Allergy

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Type I allergy represents a hypersensitivity occurring in almost 20% of the population that is based on the recognition of innocuous airborn antigens (pollen, mite, mould and pet allergens) by specific immunoglobulin E. Allergic symptoms (e.g. allergic rhinitis, conjunctivitis, asthma) are caused by the release of biological mediators from effector-cells after allergen-induced crosslink of receptor-bound IgE. Here we discuss strategies to obtain recombinant allergen-specific antibody fragments (Fabs) from mouse and human cell lines as well as directly from allergic patients lymphocytes via the combinatorial library technology. It is suggested to use recombinant allergen-specific Fabs for the standardization of allergen extracts currently used for diagnosis and treatment, to determine allergen contents in allergen sources and the environment to allow preventive measures and to use allergen-specific Fabs as therapeutic tools to interfere with the allergen-IgE interaction. The latter appears possible because IgE represents the least abundant class of immunoglobulins and there is increasing evidence for a limited diversity among allergens and their B-cell epitopes. Moreover, allergic effector reactions are mostly confined to accessible target organs so that a local application of competing Fabs prior to allergen exposure might represent a feasible therapeutic approach.

Key words: Allergen / Allergen-specific antibody fragments-Fab / Combinatorial libraries / Diagnosis / Prevention / Therapy / Type I allergy/IgE.

Introduction

Type I allergy represents an immunoglobulin-E (IgE)-mediated hypersensitivity reaction towards otherwise harmless antigens (e.g. pollen-, insect-, animal-, and mould proteins). With a prevalence of almost 20%, IgE-mediated diseases represent a health threat of increasing importance in industrialized countries (Miyamoto, 1993). In sensitized patients, innocuous environmental allergens can immediately induce a cascade of allergic reactions by crosslinking of effector-cell bound IgE-antibodies (Segal *et al.*, 1977). Allergic symptoms comprise allergic rhinitis, conjunctivitis, dermatitis, asthma and even anaphylactic shock. Diagnosis of Type I allergy is currently performed by serological measurement of specific IgE-antibody levels and *in vivo* testing (skin prick reactivity) using allergen preparations obtained from various allergen sources. These crude extracts are difficult to standardize regarding their allergen contents. Recent progress in the field of molecular allergen characterization by recombinant DNA technology has brought about two major achievements. First of all, sequence, structural and immunological similarities among the relevant allergens indicate a limited diversity of allergens and their B-cell epitopes. Second, a constantly increasing panel of recombinant allergens has become available for diagnosis and specific therapy of Type I allergy (reviewed in: Scheiner and Kraft, 1995; Valenta and Kraft, 1995). While diagnosis with allergen extracts at best permitted to identify the allergen source against which a patient is sensitized, recombinant allergens allow the precise determination of the patients individual IgE-reactivity profile (allergogram) (Valenta *et al.*, 1991; Valenta *et al.*, 1992; Menz *et al.*, 1996, Pauli *et al.*, 1996). The knowledge about a limited diversity of allergens together with the possibility to determine sensitivity to defined components with the aid of recombinant allergens led us to consider the interaction of allergens and patients IgE antibodies as a possible target for the treatment of Type I allergy. Allergen-specific IgG antibodies present in sera of patients who had received successful immunotherapy are long known as natural competitors of IgE antibodies and therefore represent suitable candidates for specific passive blocking therapy of Type I allergy. In the present contribution we describe strategies to obtain allergen-specific antibodies and recombinant antibody fragments and their use for diagnosis, prevention and therapy of Type I allergy.

Obtaining Allergen-Specific Antibodies and Antibody Fragments (Fabs)

For the production of allergen-specific antibodies and antibody fragments at least three approaches may be pursued. Animals (e.g. mice) can be immunized with allergens to establish hybridomas that secrete monoclonal antibodies. Alternatively it is possible to isolate directly peripheral lymphocytes from allergic patients to establish human allergen-specific cell lines or as a source to construct combinatorial libraries that can be screened for clones with specificity to a given allergen (Figure 1). Allergen-specific antibodies or recombinant antibody fragments (Fabs) may be used for different purposes. They can be applied for the detection and quantifiaction of certain allergens in allergen-sources and extracts that are currently used for diagnosis and therapy, but also for the detection of allergens in the environment and food.

Antibodies represent also valuable research tools to investigate the interaction of allergens and patients IgE antibodies. Those antibodies which compete with the binding of IgE antibodies either bind to/or close to IgE-epitopes or induce conformational changes in the allergen that prevent IgE-recognition. Unexpectedly mouse and human monoclonal antibodies were discovered which increased IgE-binding to allergens, most likely by causing a conformational change that makes additional IgE-epitopes available (Visco *et al.*, 1996; Laffer *et al.*, 1996; Lebecque *et al.*, 1997). While at present there is no evidence that such antibodies may cause disease exacerbation, the beneficial role of antibodies that compete with the allergen-IgE interaction is well established. In order to obtain blocking antibodies, mouse monoclonal antibodies may be tested *in vitro* for their ability to block IgE-binding (Lebecque *et al.*, 1997). If mouse monoclonal antibodies are considered for therapy it will be necessary to modify them by humanization or to produce them as Fabs to reduce their immunogenicity. Human monoclonal antibodies may represent even better candidates for antibody based therapy, however similar as for the mouse antibodies, production via tissue culture methods is rather labor intensive and sets limits as to the number of different antibodies that can be obtained and screened. We therefore suggest to employ combinatorial library technology for the production of recombinant allergen-specific antibody fragments. Using peripheral blood mononuclear cells from allergic individuals it is possible to generate cDNAs coding for heavy chain fragments and light chains by reverse transcription and PCR. Heavy chain and light chain cDNAs can then be combined and co-expressed in bacterial expression systems to represent the complete humoral diversity of the donor (Huse *et al.*, 1989). Clones producing allergen-specific Fabs can then be selected using defined allergens by direct immunoscreening (Huse *et al.*, 1989) or panning (McCafferty *et al.*, 1990; Barbas *et al.*, 1991). In the last decade considerable advances were achieved regarding the expression of functional antibody fragments in *Escherichia coli*. Basically two systems allow the presentation of heavy and light chain variable regions on the surface of filamentous phage. The pCANTAB system (Pharmacia, Uppsala, Sweden) is designed to express single-chain variable fragments (Fvs) of heavy and light chain which are artificially joined with a neutral linker (Winter and Milstein, 1991). In contrast, the pCOMB system co-expresses heavy chain fragments including a portion of their constant region together with the light chain (Barbas *et al.*, 1991). Both systems allow the efficient isolation of antigen-specific scFvs or Fabs by panning of the phage to a given antigen. The advantage of both systems over conventional cell culture technique lies in the fact that both Fabs and scFvs can be produced in *E. coli* (Hoogenboom *et al.*, 1992; Plückthun 1992; Soderlind *et al.*, 1992; Winter *et al.*, 1994; Harrison *et al.*, 1996). The re-

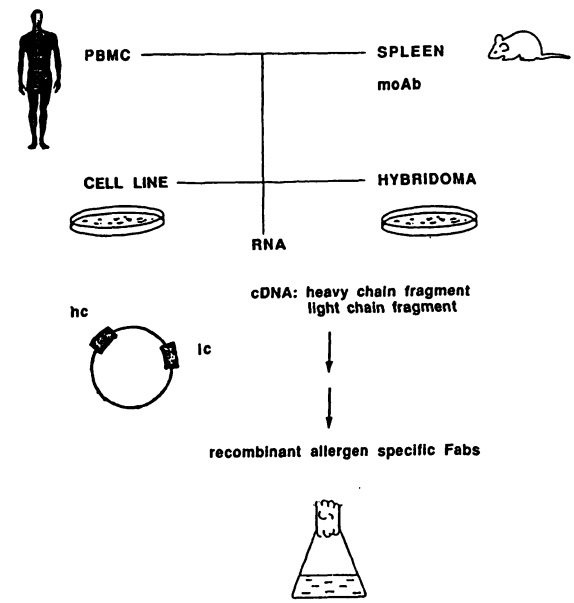

Fig. 1 Obtaining Allergen-Specific Recombinant Fabs. PBMC, peripheral blood mononuclear cells; hc, heavy chain; lc, light chain.

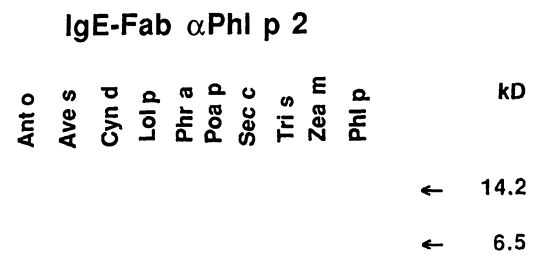

Fig. 2 A Phl p 2-Specific Human IgE Fab Crossreacts with Group 2 Allergens in Pollen from Different Grass Species. Comparable amounts of pollen extracts from ten monocots (Ant o: *Anthoxantum odoratum*, Ave s: *Avena sativa*, Cyn d: *Cynodon dactylon*, Lol p: *Lolium perenne*, Phr a: *Phragmites australis*, Poa p: *Poa pratensis*, Sec c: *Secale cereale*, Tri s: *Triticum sativum*, Zea m: *Zea mays*, Phl p: *Phleum pratense*) were separated by SDS-PAGE, blotted onto nitrocellulose and probed with a human IgE-Fab with specificity for the major timothy grass pollen allergen Phl p 2 (Dolecek *et al.*, 1993).

combinant antibody fragments are small and hence less immunogenic and can be subjected to *in vitro* manipulation (*in vitro* affinity maturation by mutagenesis, shuffling of heavy and light chains or the corresponding variable regions or other manipulations) (Gram *et al.*, 1992; Pack *et al.*, 1995; Low *et al.*, 1996; Hoogenboom, 1997). Since recombinant antibody fragments lack the major part or the complete constant region they will not elicit effector reactions which are mediated via Fc receptors. Using appropriate oligonucleotide primers, recombinant antibody fragments can be produced starting from spleen, cell lines, hybridomas of human or animal origin or by constructing combinatorial libraries directly from patients B-lymphocytes.

Recombinant Allergen-Specific Fabs as Tools to Explore the Allergen-IgE Interaction: Cloning Allergen-Specific IgE

The understanding of the interaction of allergens and patients IgE-antibodies requires structural analysis of the allergen and its IgE-epitopes as well as the characterization of allergen-specific IgE-antibodies (Fedorov *et al.*, 1997). The latter has been a major obstacle because of the extremely low levels of serum IgE, and the low frequency of IgE-secreting B-cells. In order to characterize allergen-specific human IgE antibodies on a molecular level, we have chosen the combinatorial library approach. A combinatorial IgE library was constructed from peripheral blood lymphocytes obtained from a grass pollen allergic patient during the grass pollen season (Steinberger *et al.*, 1996). Using recombinant major timothy grass pollen allergens Phl p 1 (Laffer *et al.*, 1994), Phl p 2 (Dolecek *et al.*, 1993) and Phl p 5 (Vrtala *et al.*, 1993) for panning, phage expressing specific IgE-Fabs could be isolated. cDNAs coding for the IgE heavy chain fragments and their corresponding light chains were sequenced and recombinant soluble IgE

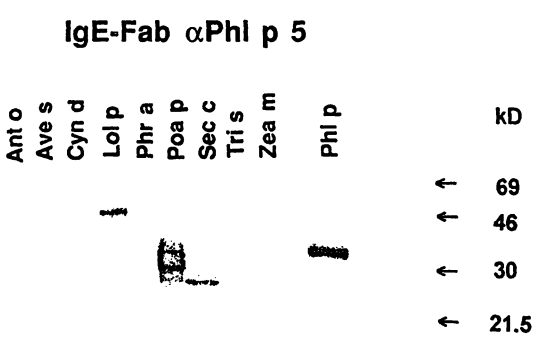

Fig. 3 A Phl p 5-Specific Human IgE Fab (Steinberger *et al.*, 1996) Crossreacts with Group 5 Allergens in Pollen from Different Grass Species.
Comparable amounts of pollen extracts from ten monocots were separated by SDS-PAGE, blotted onto nitrocellulose and probed with a human IgE-Fab with specificity for the major timothy grass pollen allergen Phl p 5 (Vrtala *et al.*, 1993). Abbreviations are as in Figure 2.

Fabs with specificity for the major grass pollen allergens could be expressed and purified up to milligram amounts. As is shown in Figures 2 and 3, human IgE-Fabs with specificity for the major timothy grass pollen allergens Phl p 2 and Phl p 5 crossreact with homologous allergens present in several other grass species, thus explaining allergic symptoms of grass pollen allergic patients after contact with different grass species as being based on immunological crossreactivity. Further molecular and structural analysis of the interaction of IgE-Fabs and their corresponding allergens will provide knowledge regarding their mode of interaction, and help to device specific forms of therapy that are based on the interruption of this contact. Recombinant allergen-specific IgE Fabs lack most parts of their constant region and in particular their Fcε receptor I binding site and therefore are unable to activate allergic effector cells (e.g. mast cells, basophils) to release mediators. As IgE-Fabs contain the variable region needed for allergen-binding they will compete with complete IgE-antibodies for binding to allergens and represent possible therapeutic tools.

Recombinant Allergen-Specific Fabs for the Standardization of Natural Allergen Extracts Used for Diagnosis and Therapy

Diagnosis and specific therapy (specific immunotherapy) of Type I allergy are currently performed with natural allergen extracts. Natural allergen extracts are used for biological (i.e. elicitation of immediate type skin reactions) as well as for serological diagnosis (i.e. detection of IgE-antibodies with specificity for a certain allergen extract) of Type I allergy. The application of increasing doses of allergen extracts by injection (i.e. specific immunotherapy) is known since 1911 as an effective treatment of Type I allergy (Noon, 1911). For both, diagnosis and treatment of Type I allergy, natural extracts are still prepared from the allergen sources and, in addition to allergens, contain a series of other ill defined components. Moreover, extracts prepared from complicated tissues lack certain allergens due to inefficient extraction and degradation. The estimation of the contents of individual allergens in allergen extracts represents therefore a difficult problem that needs to be addressed by standardization procedures. Allergen extracts are currently standardized by biological methods (i.e. by testing their capacity to elicit immediate type skin reactions) and their allergen content is evaluated with antibodies (e.g. serum IgE from allergic patients, polyclonal antisera and monoclonal antibodies raised against defined allergens). Recombinant allergen-specific Fabs will represent extremely useful tools for the standardization of natural allergen extracts. Among the recombinant Fabs, human IgE-Fabs are of particular interest for standardization procedures because they mimick the reactivity of patients serum IgE most closely.

Use of Recombinant Allergen-Specific Fabs for the Measurement of Allergens in Allergen Sources and the Environment; Tools to Prevent Allergen Exposure

Sensitization is believed to occur early in childhood in genetically prone individuals after increased exposure to allergens. The exact measurement of the presence of allergens in our environment (i.e. air, buildings, and food) might therefore represent a relevant first line for allergy prevention. The generation of allergen-specific antibodies and recombinant antibody fragments will allow the precise detection of allergens and their amount in the environment and in various food products. Preventive measures may comprise the elimination of certain allergen sources (e.g. mites), the selection of 'hypoallergenic' plants and crops and the identification of food products with low allergen contents. The latter application may be also of interest regarding the analysis of food which has been modified by genetical engineering.

Allergen-Specific Antibodies and Antibody Fragments for Passive Therapy

There is good reason to expect that allergen-specific Fabs which interfere with the allergen-IgE interaction might be used for therapeutic purposes. Experimental data which indicate a limited diversity of allergens and their B-cell epitopes (reviewed in: Valenta and Kraft, 1995; Valenta *et al.*, 1996) as well as the observation that the IgE-reactivity profile in allergic patients is subjected to little variations encourage to consider passive therapy with blocking antibodies. IgE antibodies represent the least abundant class of immunoglobulins and it should therefore be possible to apply excess doses of competitors. It is further notable that allergic symptoms (allergic rhinits, conjunctivitis, asthma, dermatitis, food intolerance) are mostly confined to certain target organs that are easily accessible and hence allow local therapy instead of systemic application.

Our contention that therapy with blocking allergen-specific Fabs might be a realistic approach is further supported by a body of experimental data on the effectiveness of blocking antibodies. The occurrence of allergen-specific antibodies which did not belong to the 'reaginic' class was reported early (Cooke *et al.*, 1935). It was demonstrated that certain allergen-specific IgG antibodies were able to inhibit anaphylactic reactions (Loveless, 1940; Lichtenstein *et al.*, 1968). Moreover it was speculated that blocking antibodies, in particular those belonging to the IgG_4 subclass might be induced by specific immunotherapy (Aalberse *et al.*, 1983). It was recently shown in a defined experimental system that human monoclonal antibodies with specificity for the major birch pollen allergen, Bet v 1 (Breiteneder *et al.*, 1989), blocked IgE-binding to Bet v 1 and inhibited the Bet v 1-induced histamine release (Visco *et al.*, 1996).

We suggest to use recombinant allergen-specific Fabs, either human IgG or IgE Fabs, for passive (blocking) therapy of Type I allergy. Bacterially expressed Fabs can be modified by standard molecular biological techniques and engineered according to the desired application. To reduce the immunogenicity of the Fabs it is possible to further reduce their size as well as to remove immunogenic parts. Alternatively, mouse monoclonal antibodies can be humanized to reduce their immunogenicity. By *in vitro* maturation, using mutagenesis or CDR rearrangements it is possible to improve the affinity of a recombinant Fab to its antigen. Such a manipulation might even increase the blocking activity of allergen-specific Fabs. Molecular and structural analysis of blocking Fabs might finally provide templates to build synthetic competitors of the allergen-IgE interaction.

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Review

MHC Class II Compartments: Specialized Organelles of the Endocytic Pathway in Antigen Presenting Cells

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Mounting an immune response against foreign, extracellular material requires that this material be internalized by antigen presenting cells, processed to peptide fragments and then displayed on the cell surface for recognition by the T cell receptor on T helper cells. Such peptides, derived from internalized antigens are generally presented to T cells in association with Major Histocompatibility Complex (MHC) class II molecules. Recent work has identified subcompartments of the endosomal/lysosomal system that appear to be important sites for the generation of peptide-MHC class II complexes. These so-called MHC class II compartments receive antigenic fragments from endosomes and lysosomes and load them onto class II molecules. From these compartments, peptide-loaded class II molecules are transported to the plasma membrane, where they can trigger T cells bearing appropriate receptors.

This review summarizes recent work characterizing MHC class II compartments as specialized organelles of the endosomal/lysosomal pathway. Interestingly, MHC class II compartments share many features common to organelles present in a wide variety of specialized cells with quite different functions. Modifying the endocytic pathway to serve the needs of specialized cells may be a common mechanism which allows very different cell types to carry out their disparate functions.

Key words: Antigen presentation / Endocytic pathway / MHC class II molecules / Subcellular organelles.

Introduction

Microorganisms such as viruses, bacteria, and certain fungi are part of our natural environment, but many of these can also lead to infectious diseases. Despite a continuous exposure to these potential pathogens most vertebrates can control an infection because they possess an immune system. In addition to the first-line defense, called 'innate' (inborn) immunity, which is the non-specific engulfment and destruction of microorganisms by phagocytes, the immune system has evolved several more specific ways to eliminate foreign material (Germain, 1994). These mechanisms rely on the ability of an infected cell to signal to the immune system that it harbors an infection. One efficient way infected cells can do this is by displaying on its surface small peptides derived from those infectious agents. These peptides can then be recognized by the T lymphocytes of the immune system, which, upon activation, can mount an immune response resulting in the control and possibly elimination of the infection (Germain, 1994; Townsend and Bodmer, 1989; Townsend *et al.*, 1985). Two subsets of T cells are able to recognize antigenic peptides. Cytotoxic T cells release the content of their cytotoxic granules upon recognition of an antigenic peptide, resulting in rapid destruction of the presenting cell (Kagi *et al.*, 1996). Alternatively, T helper cells, when triggered by a surface displayed peptide, leave the presenting cell intact and assist in the generation of an antibody mediated response (Noelle and Snow, 1992).

How peptides are presented to these two T cell subsets has been worked out in great detail over the past decades. A set of molecules encoded in the major histocompatibility complex or MHC, a genomic region that contains a number of genes involved in immune recognition of an infection (Hansen *et al.*, 1993) were found to play an essential role in this process by the pioneering work of Doherty and Zinkernagel (Zinkernagel and Doherty, 1974 a,b). These molecules come into two forms, the MHC class I and class II molecules; both are transmembrane molecules synthesized on endoplasmic reticulum associated ribosomes and are cotranslationally inserted into the endoplasmic reticulum membrane (Dobberstein and Robinson, 1979; Kvist *et al.*, 1982; Ploegh *et al.*, 1979) (see Figure 1).

The type of immune response and the class of MHC molecules involved appears to be largely dependent on the site of residence of the infectious agent: MHC class I molecules present peptides derived from pathogens that have access to the cytosol (e.g. viruses), whereas MHC class II molecules function in the presentation of peptides derived from antigens that are present exogenously (as is the case for many bacteria) (Jackson and Peterson, 1993; Wolf and Ploegh, 1995).

MHC class I molecules are expressed by all nucleated cells. They associate in the endoplasmic reticulum with another molecule, β-2-microglobulin. Viruses and bacteria that reside in the cytosol are degraded by the cytosolic proteolytic machinery, the proteasome, and the resulting peptides are transported into the endoplasmic reticulum by transporter molecules, the TAP molecules (for trans-

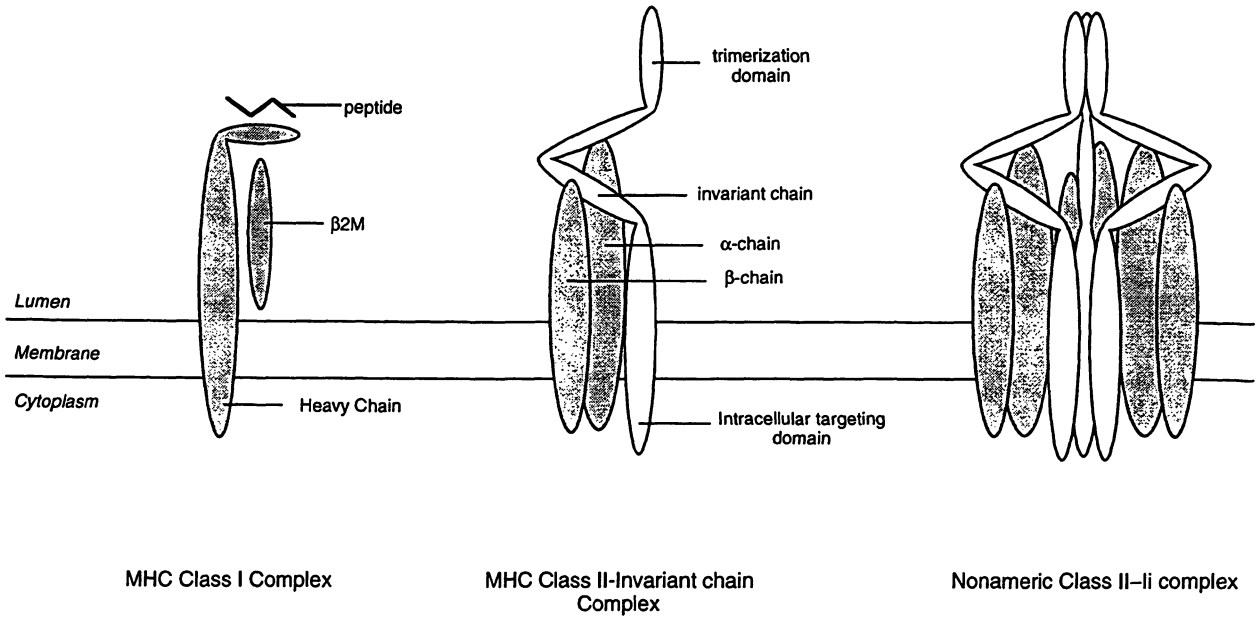

Fig. 1 Model of the MHC Class I and MHC Class II-Invariant Chain I Complexes.
The left panel shows the MHC class I molecule, that in association with β-2 microglobulin can bind antigenic peptides that are imported into the endoplasmic reticulum. The middle panel depicts the MHC class II complex, consisting of an α chain, a β chain and the invariant chain. The right panel shows a model of the nonameric class II/Ii complex that is exported from the endoplasmic reticulum. The MHC class I heavy chain and class II α and β chains have a type I transmembrane topology, i.e. they contain a lumenal N-terminal region and a cytoplasmic C terminal domain. The class II associated invariant chain adopts a type II orientation: it contains a cytoplasmically located N terminus, and a lumenal C-terminal domain. Adapted from Pieters, 1997.

porter associated with antigen processing) (Deverson *et al.*, 1990; Koopmann *et al.*, 1997; Spies *et al.*, 1990). MHC class I molecules become loaded with antigenic peptides in the endoplasmic reticulum, and are then transported via the secretory pathway through the Golgi complex to the cell surface where they can be recognized by a subset of T lymphocytes, the cytotoxic T cells (Townsend *et al.*, 1989). As mentioned, triggering of T cell receptors on cytotoxic T cells usually results in the killing of the antigen presenting cell. Not only viral peptides, but also peptides derived from tumor associated antigens can be presented by MHC class I molecules and are able to trigger cytotoxic T cells (Boon and van der Bruggen, 1996). Therefore, class I restricted presentation is an efficient way to eliminate cells that are detrimental to the organism.

In contrast to MHC class I molecules, class II molecules are expressed by only a subset of cells specialized in the presentation of antigens that are present extracellularly; these include B cells, macrophages and dendritic cells. Expres-sion of MHC class II molecules, however, is inducible in a variety of other cells by interferon gamma, and has recently been shown to be controlled by the MHC class II transactivator protein [CII TA (Pober *et al.*, 1983; Steimle *et al.*, 1993)]. Deposition of peptide-loaded MHC class II mole-cules on the cell surface of antigen presenting cells does not only involve organelles of the secretory pathway, but also of the endocytic pathway.

The endocytic network comprises a heterogeneous set of organelles that function in the uptake of material from the extracellular environment (Mellman, 1996). Therefore, it is also the primary site where extracellular infectious material gains access to the antigen presenting cell. Understanding the mechanisms involved in loading peptides onto class II molecules not only adds to our understanding of the immune system but may also increase our knowledge regarding compartmentalization of eukaryotic cells in general. Recent work has highlighted the involvement of specialized organelles in the process of class II restricted antigen presentation, which is the scope of this review. These organelles, called MHC class II compartments, seem to share several features with other specialized organelles carrying out quite distinct functions.

MHC Class II Molecules in the Biosynthetic Pathway

MHC class II molecules consist of an alpha chain of 35 kDa and a beta chain of 27 kDa molecular weight. These transmembrane molecules display their C termini cytosolically, leaving a large N-terminal region in the lumen of the ER. This N terminal lumenal region is highly polymorphic, and forms the peptide binding site that, after maturation, is displayed at the plasma membrane (Cresswell *et al.*, 1987).

During translation, MHC class II molecules associate with a third transmembrane molecule, the invariant chain [Ii; (Jones *et al.*, 1978; Kvist *et al.*, 1982)]. The expression of Ii is also under the regulation of CIITA, ensuring that all class II molecules form complexes with Ii upon biosynthesis (Chang and Flavell, 1995; Kern *et al.*, 1995). Ii has the

opposite topology as compared to class II molecules, containing a small, N terminal cytoplasmic segment and a relatively large C-terminal region located in the lumen (see Figure 1). Despite the name 'invariant', given because it is not polymorphic, several isoforms of Ii can be coexpressed in most cells (Pieters, 1997; Sant and Miller, 1994; Strubin *et al*., 1986).

The class II/Ii complex consist of one Ii trimer, associated with three MHC class II dimers, a model of which is shown in Figure 1 (Cresswell, 1996; Roche *et al*., 1991). While non-assembled class II and/or Ii molecules are retained and partially degraded in the ER, this nonameric complex is readily transported out of the ER to the Golgi complex (Kvist *et al*., 1982; Pieters *et al*., 1991). Most probably, retention in the ER provides a quality control function to ensure that only properly synthesized and assembled complexes are exported to later stages of the pathway (Arunachalam and Cresswell, 1995; Hurtley and Helenius, 1989; Marks *et al*., 1995; Schaiff *et al*., 1992).

Targeting of MHC Class II/Ii Complexes to the Endocytic Pathway

Peptides presented by MHC class II molecules are usually derived from material or infectious organisms that enter the endocytic pathway of the antigen presenting cells. How MHC class II molecules, being synthesized in the biosynthetic pathway, gain access to this material has been a major question over the past decade. Early work indicated that MHC class II molecules do intersect the endocytic pathway (Blum and Cresswell, 1988; Cresswell, 1985). This was followed by the demonstration that this intersection involves newly synthesized MHC class II molecules, which are sorted at the level of the trans Golgi network from proteins destined for the cell surface (Davidson *et al*., 1991; Neefjes *et al*., 1990).

The MHC class II associated invariant chain appears to play a crucial role at different stages of the biogenesis of the appropriate MHC class II-peptide complexes. First, the C-terminal, lumenal domain of Ii prevents antigenic peptide binding to class II molecules in the endoplasmic reticulum, as the class II molecules should sample the endocytic pathway for peptides (Roche and Cresswell, 1990; Teyton *et al*., 1990). Second, the invariant chain functions in the transport of the class II/Ii complex to endosomes. Site directed mutagenesis showed that the N-terminal, cytoplasmic tail of Ii contains targeting/retention signals for endocytic compartments (Bakke and Dobberstein, 1990; Lotteau *et al*., 1990; Pieters *et al*., 1993). Targeting to the endocytic pathway is mediated by leucine containing stretches of amino acid residues (Pieters *et al*., 1993); deletion or mutation of these leucine containing motifs in the N-terminal cytoplasmic tail abolished endosomal targeting and resulted in the appearance of these Ii mutants at the cell surface.

To analyze the precise pathway followed by the MHC class II/Ii complex, we have been using as a model system the human melanoma cell line Mel JuSo (Johnson *et al*., 1981). This cell line has been a valuable tool for the analysis of class II biology in several aspects. First, these cells express high amounts of MHC class II and invariant chain molecules, as well as other accessory molecules such as HLA-DM that are involved in the actual peptide loading event (cf. below). Second, they can efficiently process and present antigens and third, these cells are well suited for morphological and biochemical analysis (Pieters *et al*., 1991; Tulp *et al*., 1994).

In Mel JuSo cells, as in B lymphoblastoid cells, MHC class II molecules are transported to endosomal organelles following export from the endoplasmic reticulum and transport through the Golgi complex. Upon arrival in the endocytic pathway, degradation of Ii takes place in distinct steps. This degradation, most probably mediated by the endosomal proteases cathepsin S and/or L (Bevec *et al*., 1996; Riese *et al*., 1996), occurs selectively from the lumenal, carboxyterminal region leaving the N-terminal cytoplasmic tail attached to the class II complex (see Figure 2). Interestingly, in B lymphoblastoid cells, Ii proteolysis is much more rapid, and N-terminal proteolytic fragments are detected only when endosomal proteolysis is inhibited (Blum and Cresswell, 1988) while the amount of class II molecules located intracellularly is much smaller in these cells. Given the function of the Ii N-terminal domain as a targeting and retention domain for the MHC class II complex, it is conceivable that the rate of Ii proteolysis and its dissociation from class II molecules dictate transport to, retention in and export from endocytic compartments (Pieters *et al*., 1991).

Specialized Peptide Loading Compartments

The location of MHC class II/Ii complexes in the endocytic pathway has been confirmed by immunocytochemistry in a variety of cell lines (Peters *et al*., 1991; Pieters *et al*., 1991). One puzzling observation in these earlier studies was that the bulk of MHC class II molecules could not be localized to a conventional early endosome, late endosome, or lysosome. Class II containing structures were therefore named 'MIIC's' (Peters *et al*., 1991) or 'multivesicular bodies' (Pieters *et al*., 1991), based on the high concentration of class II molecules or morphological appearance, respectively. The subsequent biochemical isolation and characterization of organelles, part of the endocytic pathway but clearly distinct from conventional endosomes and lysosomes established the class II compartments as separate entities in the endosomal/lysosomal pathway (Amigorena *et al*., 1994; Tulp *et al*., 1994; West *et al*., 1994). Newly synthesized MHC class II molecules are transported to these compartments in association with the invariant chain. Upon arrival in these organelles, the class II associated invariant chain is proteolytically degraded and antigenic peptides, derived from endocytosed proteins, are loaded onto class II molecules. From the MHC class II compartment, peptide loaded class II mole-

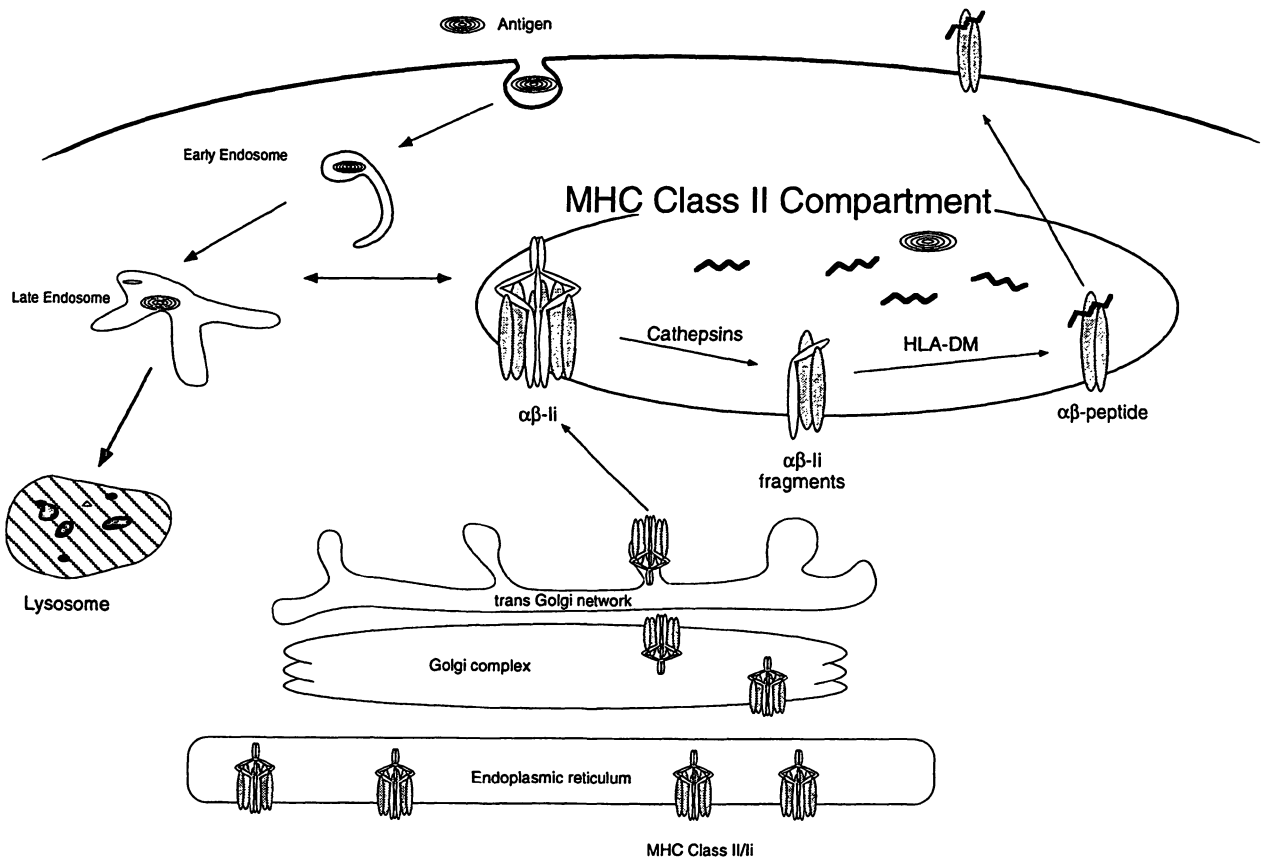

Fig. 2 Intracellular Pathways Followed by MHC Class II/Invariant Chain (Ii) Complexes.
After assembly in the endoplasmic reticulum, the class II/Ii complex is targeted at the trans-Golgi network to MHC class II compartments by virtue of targeting signals that reside in the Ii cytoplasmic tail. Upon arrival in MHC class II compartments, Ii is progressively degraded from its lumenal, C-terminal region through the action of cathepsins. Removal of the Ii C-terminal domains allow MHC class II molecules to acquire antigenic peptides, a process catalyzed by HLA-DM. Antigenic peptides are somehow imported into the MHC class II compartment from the endocytic pathway. Class II molecules loaded with peptides are then exported to the plasma membrane by as yet undefined mechanisms. Modified from (Pieters, 1997).

cules are exported to the plasma membrane for presentation to T helper cells.

A recently discovered molecule, the human leukocyte antigen (HLA)-DM that is involved in the generation of MHC class II peptide complexes, is also located in MHC class II compartments (Nijman *et al.*, 1995; Pierre *et al.*, 1996; Sanderson *et al.*, 1994). HLA-DM was discovered as a 'peptide-loader' when it was observed that mutant antigen presenting cell lines lacking this molecule would perfectly stimulate T cells when supplied with peptides, but could not present peptides when intact antigen was administered (Mellins *et al.*, 1990). This suggested that these mutant cells either had lost the ability to process antigens, or were unable to load intracellularly generated peptides onto MHC class II molecules. Several subsequent studies showed that, both *in vitro* and *in vivo*, HLA-DM is required for loading peptides onto MHC class II molecules (Denzin and Cresswell, 1995; Fung-Leung *et al.*, 1996; Martin *et al.*, 1996; Miyazaki *et al.*, 1996; Sherman *et al.*, 1995; Sloan *et al.*, 1995).

Although HLA-DM molecules have been localized in MHC class II containing compartments, HLA-DM is targeted intracellulary via a tyrosine motif present in its cytoplasmic tail (Lindstedt *et al.*, 1995; Marks *et al.*, 1995). This signal is different from the leucine motif present in the invariant chain cytoplasmic tail responsible for targeting the MHC class II/Ii complex intracellularly, and recent evidence suggests that a distinct machinery may be involved in di-leucine based and tyrosine based targeting (Marks *et al.*, 1996). In accordance with this, we have recently found that in Mel JuSo cells, Ii and DM reside in two physically and functionally distinct MHC class II containing compartments. Furthermore, these two compartments appear to have a different accessibility to endocytosed material; while the class II associated Ii was found to be degraded in an endocytic organelle, HLA-DM dependent peptide loading occurred in a distinct organelle that was not accessible for internalized antigens directly (Ferrari and Pieters, in preparation). The precise mechanisms that regulate the transport of Ii and HLA-DM between these compartments, however, remain to be established.

A general picture describing biogenesis of MHC class II-peptide complexes is depicted in Figure 2. It summarizes work from many different laboratories using a variety of cell lines, including B lymphoblastoid cell lines and melanoma cells.

How does this trafficking scheme look in more physiological situations? Recently, procedures have been developed to obtain large numbers of dendritic cells isolated from peripheral blood (Sallusto and Lanzavecchia, 1994). This opened the possibility to analyze the trafficking pathways of class II molecules in these highly specialized, professional antigen processing and presenting cells (Steinman, 1991). Dendritic cells play an important role in the immune response: in an immature state, they are believed to acquire antigens in peripheral tissues. After migration to the lymphoid organs they can, in a mature state, present these peptides associated with MHC class II to T cells (Cella *et al.*, 1997•; Steinman, 1991). Immature dendritic cells can internalize antigens while most class II molecules reside intracellularly. During maturation, dendritic cells downregulate their endocytic capacity and relocate the MHC class II molecules to the plasma membrane (Cella *et al.*, 1997a, b; Sallusto *et al.*, 1995; Steinman and Swanson, 1995). Thus, the dendritic cell system provides an excellent model to analyze the cell biology of class II restricted antigen presentation.

Morphological studies confirmed the existence of MHC class II compartments in immature dendritic cells, with characteristics very similar to the ones described in B lymphoblastoid cells and melanoma cells (Nijman *et al.*, 1995; Sallusto and Lanzavecchia, 1994, Engering *et al.*, 1997). Surprisingly, after *in vitro* maturation of dendritic cells, MHC class II compartments disappear, concomitant with a shift of the class II molecules from an intracellular site to the plasma membrane (Sallusto and Lanzavecchia, 1994). This suggests that transport of MHC class II molecules to the precise subcellular location in these cells is highly regulated at different stages of maturation. Indeed, several mechanisms seem to operate during DC maturation that redistribute class II molecules among various subcellular compartments (Engering *et al.*, 1997, Cella *et al.*, 1997a).

The Endocytic Pathway as a Host to Specialized Organelles

The MHC class II compartment in antigen presenting cells may be regarded as a specialized endocytic organelle: First, its distribution is restricted to specialized cells that function in the presentation of exogenous antigens. Second, in these cells, it performs a defined function, namely the loading of peptides onto MHC class II molecules. Third, the physical properties as well as the molecular composition of the MHC class II compartment clearly distinguishes it from early and late endosomes.

A number of questions however still remain. How is the MHC class II compartment positioned in the endocytic pathway? What mechanisms control the flow of material to and from these organelles? Is the transport of peptide-loaded class II molecules from the MHC class II compartment to the cell surface regulated? Recent work carried out with dendritic cells suggests that several regulatory mechanisms may be involved in MHC class II restricted presentation. Unfortunately, the further characterization of MHC class II compartments has so far been hampered by the lack of marker molecules other then the class II molecules themselves. However, the MHC class II compartment is, in several aspects, similar to organelles present in at least three other types of specialized cells and may therefore share characteristics with these specialized organelles. These include the glucose transporter-4 (Glut-4) containing vesicles ('Glut-4 compartment') in adipocytes (James and Piper, 1994; Thorens, 1993), cytotoxic granules in T lymphocytes (Griffiths, 1995) and synaptic vesicles in neuronal cells (De Camilli, 1995; Sudhof, 1995).

First, in adipocytes, Glut-4 facilitates the uptake of glucose in an insulin dependent manner. Glut-4 resides in an intracellular, post Golgi compartment and is rapidly transported to the cell surface following stimulation of the cells with insulin (Satoh *et al.*, 1993; Slot *et al.*, 1991). The Glut-4 compartment has characteristics of endosomes but can be isolated as a vesicle population containing only low amounts of endosomal markers such as transferrin receptor and mannose-6-phosphate receptor (Herman *et al.*, 1994; Hudson *et al.*, 1993). Second, cytotoxic granules present in T lymphocytes are secretory granules that are the storage site of a number of lytic proteins (Griffiths and Argon, 1995). Upon activation of T cells, these proteins are released from these organelles, and cause the destruction of the target cell (Griffiths, 1995). Release of the granule content is triggered by engagement of the T cell receptor present at the cell surface (Isaaz *et al.*, 1995). These cytotoxic granules can be considered part of the endosomal/lysosomal pathway, as a number of the resident lytic proteins are targeted there via the mannose-6-phosphate receptor, that is used in most cells to sort lysosomal enzymes from the secretory pathway (Griffiths and Isaaz, 1993). In addition, the pH is mildly acidic in these vesicles, as is also the case for endosomal/lysosomal organelles (Griffiths, 1995). Third, synaptic vesicles, present e.g. in neuronal and neuro-endocrine cells, are vesicular carriers that mediate the secretion of non-peptide neurotransmitters. These organelles originate from endosomes, are acidic and contain membrane proteins that arrive there from the biosynthetic pathway as well as cargo that is imported from the cytoplasm (Clift *et al.*, 1990; Kelly, 1993; Maycox *et al.*, 1992, Schmidt *et al.*, 1997).

The MHC class II compartment may represent a specialization of the endocytic pathway in antigen presenting cells, just as the Glut-4 compartment, cytotoxic granules and synaptic vesicles do in the above described cell types. Interestingly, one determinant involved in intracellular targeting/retention of Glut-4 molecules is a di-leucine motif similar to the intracellular targeting/retention motif of the MHC class II associated invariant chain. The determinants involved in targeting and retaining molecules in cytotoxic granules and synaptic vesicles are not well characterized; at least a subset of these molecules reach these compartments after internalization from the plasma membrane (Griffiths, 1996; Thomas-Reetz and De Camilli,

1994), a pathway that may be utilized by MHC class II/Ii complexes in a number of cases (Pinet *et al.*, 1995; Warmerdam *et al.*, 1996).

How is material from these intracellular storage sites transported to the cell surface? For Glut-4 vesicles, cytotoxic granules and synaptic vesicles a trigger at the cell surface is required for transmitting a signal that results in exocytosis of their content. For Glut-4 vesicles the signal is insulin stimulation; in the case of cytotoxic granules, T cell receptor triggering; and for synaptic vesicles, electrical stimulation causes exocytosis (Isaaz *et al.*, 1995; Satoh *et al.*, 1993; Sudhof, 1995). Whether similar mechanisms are involved in the transfer of class II molecules from the class II compartment to the cell surface is not known.

Conclusion

The pathways followed by MHC class II molecules from their site of synthesis, the endoplasmic reticulum, to the plasma membrane have now well been characterized. In various antigen presenting cells, specialized organelles, the MHC class II compartments, have been described that function in the loading of antigenic peptides onto class II molecules. From these compartments, peptide loaded class II complexes are transported to the cell surface where they can trigger T cell receptors on T helper cells.

These specialized MHC class II compartments are part of the endosomal/lysosomal network and resemble in various aspects other types of specialized organelles that are also part of the endosomal system. Apparently, there is an enormous plasticity within the endosomal network to accommodate specialized functions. As suggested before (Kelly, 1993), all cells may contain a 'housekeeping' endosomal network that performs the basic functions necessary for normal cell growth, such as recycling of LDL/transferrin receptors and trafficking of endocytosed material to lysosomes for degradation. In addition to this basic endocytic system, in specialized cell types endosomal-derived organelles may be present containing proteins involved in carrying out the specific function to which these specialized cells are dedicated. Analysis of the molecular composition of various specialized organelles in disparate cell types might allow identification of common mechanisms that govern traffic of constituents and cargo to and from these different compartments.

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Review

Calcium-Sensing Receptor: Roles in and beyond Systemic Calcium Homeostasis

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The cloning of a Ca_0^{2+}-sensing receptor (CaR) from parathyroid and kidney, coupled with the identification of inherited disorders in Ca_0^{2+}-sensing resulting from inactivating or activating mutations of this CaR, has provided substantial insights into how Ca_0^{2+} is regulated at a systemic level. The CaR plays a central role in mediating, on one hand, the inhibitory effect of Ca_0^{2+} on parathyroid hormone (PTH) secretion and, on the other, the stimulatory action of Ca_0^{2+} on calcitonin secretion, which provide for a sophisticated, bi-directional regulation of the secretion of calciotropic hormones mediated by the same receptor. Indeed Ca_0^{2+} itself can be viewed as a calciotropic 'hormone' acting, along with PTH and calcinotin (CT), on its target tissues involved in mineral ion homeostasis, particularly the kidney. In the kidney, Ca_0^{2+}, acting via the CaR, controls renal handling of divalent mineral ions in the cortical thick ascending limb (CTAL) and perhaps also in the distal convoluted tubule (DCT). In CTAL the CaR not only affects renal calcium handling indirectly by modulating the secretion of PTH but also directly at the level of the tubule, where it is localized on the same basolateral surface of the cells where the PTH receptor is located. Moreover, renal CaRs likely subserve important functions involved in integrating mineral ion metabolism with the homeostasis of water and, perhaps, monovalent cations (e.g., elevating Ca_0^{2+} produces a CaR-mediated inhibition of NaCl reabsorption in the CTAL).

More recent data, however, accumulated since the cloning of the CaR, have suggested additional roles of the receptor in cells uninvolved in mineral ion metabolism. Examples include the brain, intestine and skin, where the receptor may regulate, respectively, the activities of ion channels and probably other neuronal functions, proliferation of the cells of the colonic crypts and differentiation of keratinocytes, presumably in response to local changes in Ca_0^{2+}. It is also possible that the CaR responds to other endogenous agonists, either polyvalent agonists, such as Mg_0^{2+} or organic polycations, including spermine or even protamine. Finally additional studies are needed to determine whether there are additional members of a putative family of structurally-related ion-sensing GPCRs, such as that apparently present in osteoblasts that sense Ca_0^{2+} and, perhaps, in other cell types that recognize additional ions [e.g., Cd_0^{2+} in dermal fibroblasts (Smith *et al.*, 1989)].

Key words: Brain / Keratinocytes / Kidney / Lens epithelium / Parathyroid gland.

Introduction

Calcium ions are known to play a variety of roles in myriad cellular and biochemical processes in essentially all organisms. The involvement of calcium is well recognized in the mechanisms of cellular signaling, secretion, cell division and motility, as well as in muscular contraction, blood clotting, neural transmission, cellular proliferation and differentiation and apoptosis (For review, see Brown, 1991). Therefore, maintenance of a nearly constant level of the extracellular calcium concentration (Ca_0^{2+}) is a high biological priority, which is accomplished by the complex interplay of various organs that include parathyroid gland, thyroidal C-cells, kidney, bone and intestine (for review, see Chattopadhyay *et al.*, 1996b).

Over the years, several lines of evidence suggested the presence of a Ca_0^{2+}-sensing mechanism in parathyroid cells, C-cells and kidney through which Ca_0^{2+} acts as an extracellular first messenger via its own cell surface, receptor-like mechanism (for review, see Brown, 1991). Evidence suggesting the existence of such a Ca_0^{2+}-sensing mechanism in the parathyroid gland came from studies with dispersed bovine parathyroid cells. When subjected to increased levels of Ca_0^{2+}, the cells responded with activation of phospholipase C (PLC) leading to accumulation of inositol 1,4,5-trisphosphate (IP_3) and consequent release of Ca^{2+} from intracellular stores (Nemeth and Scarpa, 1986; Brown *et al.*, 1987). High levels of Ca_0^{2+} also produced a pertussis toxin-sensitive inhibition of agonist-stimulated cAMP accumulation in bovine parathyroid cells (Chen *et al.*, 1989). In addition, these and other studies revealed the capacity of the Ca_0^{2+}-sensing apparatus to recognize other divalent cations (i.e., Ca^{2+}, Mg^{2+}), trivalent cations (e.g., La^{3+} and Gd^{3+}) and even polyvalent cations, such as polylysine, protamine, and neomycin, suggesting that it was a low affinity receptor having rather promis-

cuous polycation-sensing properties (Brown, 1991; Chattopadhyay *et al.*, 1996b). While elevations in Ca_0^{2+} produced a prominent release of Ca^{2+} from its intracellular stores in parathyroid cells, the high Ca_0^{2+}-evoked rise in Ca_i in thyroidal C-cells occurred primarily through opening of voltage-sensitive Ca^{2+} channels, suggesting that these channels might represent an important part of the Ca_0^{2+}-sensing apparatus in the latter cell type (Fried and Tashjian, 1986; Scherubl *et al.*, 1991).

Ca_0^{2+} also exerts several apparently direct actions on diverse cell types within the kidney. Elevations in Ca_0^{2+} inhibit the 1-hydroxylation of 25-hydroxyvitamin D, produce a pertussis toxin-sensitive inhibition of cAMP accumulation in the medullary thick ascending limb (similar to the effect of Ca_0^{2+} on cAMP accumulation observed in parathyroid cells) and inhibit the tubular reabsorption of NaCl, Ca^{2+} and Mg^{2+} in the cortical thick ascending limb (CTAL) (for review, see Brown, 1991). In bone, raising Ca_0^{2+} inhibits osteoclastic bone resorption and increases Ca_i in osteoclasts, a response somewhat reminiscent of that elicited in parathyroid cells by high levels of Ca_0^{2+} (Zaidi *et al.*, 1989).

Cloning and Characterization of G Protein-Coupled CaRs

Cloning of the Bovine Parathyroid CaR

With this background information, Brown *et al.*, employed expression cloning in *Xenopus laevis* oocytes to isolate a full length cDNA encoding the bovine parathyroid calcium-sensing receptor (BoPCaR) (Brown *et al.*, 1993) (Figure 1 shows proposed topology of human CaR). Characterization of BoPCaR revealed that it is a seven membrane-spanning receptor sharing limited homology only with the metabotropic glutamate receptors (mGluRs) among the superfamily of G protein-coupled receptors (GPCR). In the parathyroid gland the receptor is coupled, most likely via G_q/G_{11} (Varrault *et al.*, 1995), to activation of phospholipase C (PLC), presumably through one or more members of the PLCbeta family of the G protein-activated forms of the enzyme. The receptor may also couple to inhibition of adenylate cyclase (Chen *et al.*, 1989), although this later action has not yet been demonstrated unequivocally with the cloned CaR.

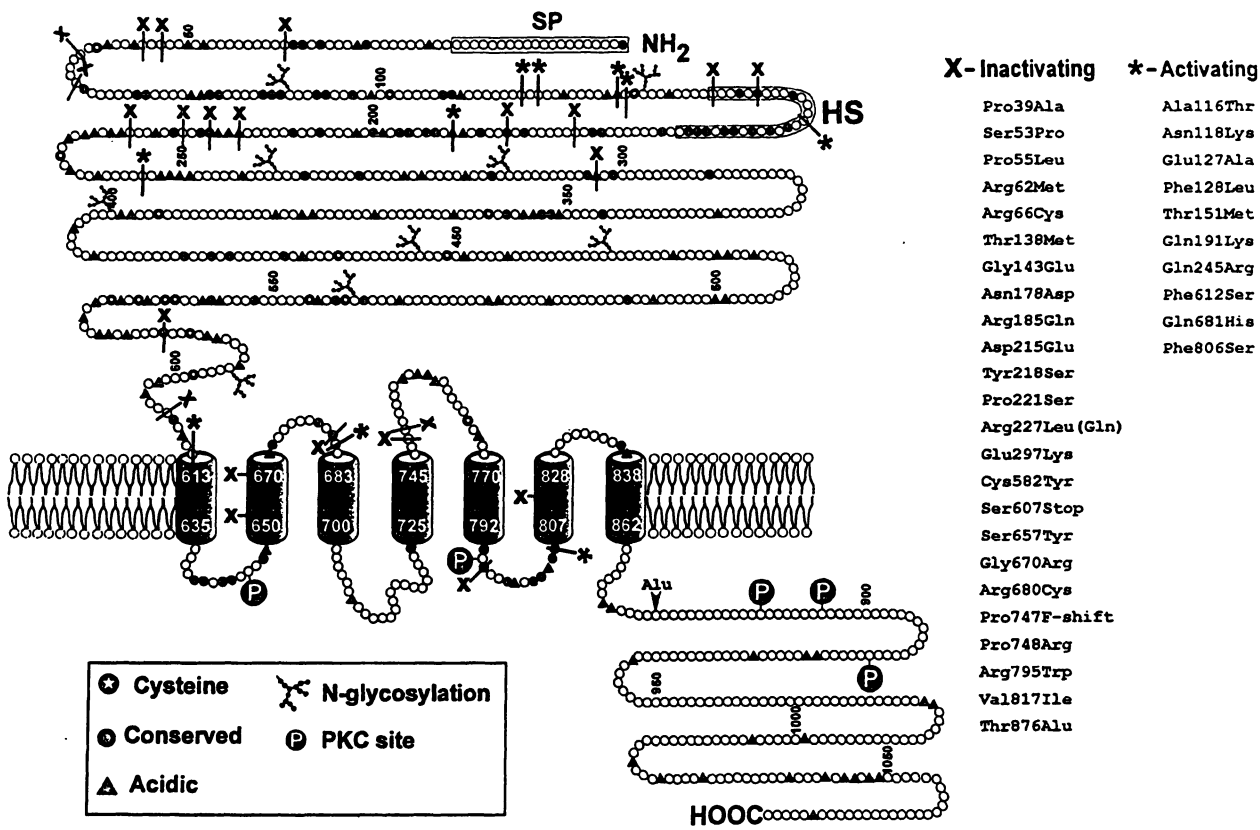

Fig. 1 Schematic Illustration of the Proposed Topology of the Ca_0^{2+}-Sensing Receptor Cloned from Human Parathyroid, Depicting Locations of Activating and Inactivating Mutations.
Abbreviations are as follows: SP = signal peptide; HS = hydrophobic segment. Also shown are positions of missense and nonsense mutations that cause either familial hypocalciuric hypercalcemia (FHH) or autosomal dominant hypocalcemia; the latter are indicated with the three letter amino acid code. The normal amino acid is shown before and the mutant amino acid after the number of the relevant codon. Reproduced with permission from Brown E.M., Bai, M., and Pollak, M. Familial benign hypocalciuric hypercalcemia and other syndromes of altered responsiveness to extracellular calcium. In: Metabolic Bone Diseases, 3rd ed., S.M., Krane and L.V., Avioli, eds. (in press).

Table 1 Selected Features of CaR Cloned from Various Species.

Species	Number of amino acids	Size of protein (kDa)[1]	Putative PKA sites	Putative PKC sites	Putative N-Glycosylation sites	References
Human	1079	130–160	2	5	11	Garrett *et al.*, 1995
Bovine	1085	130–160	2	4	9	Brown *et al.*, 1993
Rat	1079	130–160	2	8	9	Ruat *et al.*, 1995
Rabbit	1070	–	2	6	11	Butters *et al.*, 1997
Chicken	1059	–	2	5	12	Diaz *et al.*, 1997

[1] Most of immunoreactive CaR protein on Western analysis is present as two glycosylated bands with only a small amount of the ~ 120 kDa non-glycosylated CaR.

Cloning of the Ca_0^{2+}-sensing receptor (CaR) was followed almost immediately by the identification of inherited human hyper- or hypocalcemic disorders resulting from loss- or gain-of-function mutations in the CaR, respectively (Pollak *et al.*, 1993; 1994). Mutations reducing the activity of the CaR occur in either a heterozygous form that usually causes a generally asymptomatic, hypercalcemic disorder [familial hypercalcemic hypocalcemia (FHH)] or in a homozygous form that leads to a severe form of hyperparathyroidism in newborn infants [neonatal severe hyperparathyroidism (NSHPT)] (for review see Chattopadhyay *et al.*, 1996). NSHPT can also result, however, from heterozygous inactivating mutations, particularly those that exert a dominant negative effect on the normal allele of the CaR (Bai *et al.*, 1997). FHH is characterized by abnormal parathyroid and renal responsiveness to Ca_0^{2+}. The former produces an elevated set-point for Ca_0^{2+}-regulated PTH release (the level of Ca_0^{2+} half-maximally inhibiting PTH secretion), while the latter causes inappropriately avid renal tubular reabsorption of both Ca^{2+} and Mg^{2+}, thereby producing not only hypercalcemia but also hypermagnesemia in some affected individuals (Chattopadhyay *et al.*, 1996b). Conversely, gain-of-function CaR mutations are associated with an autosomal dominant form of hypocalcemia accompanied by a reduction in the set-point of the parathyroid and an excessive degree of hypercalciuria for any given level of Ca_0^{2+}. The identification of these disorders as well as the development of mice heterozygous or homozygous for targeted inactivation of the CaR gene (Ho *et al.*, 1995) that share many of the biochemical features of FHH and NSHPT, respectively, has provided incontrovertible evidence for the physiological importance of the CaR in mineral ion homeostasis.

Following the cloning of the bovine parathyroid CaR, efforts were made to clone additional organ and species-specific homologs of the receptor and to determine if there were additional CaR isoforms. These screening efforts enabled cloning of additional, highly homologous receptors from organs and tissues involved in calcium homeostasis, such as human parathyroid (Garrett *et al.*, 1995a) and thyroidal C-cells (Garrett *et al.*, 1995b), rat kidney (Riccardi *et al.*, 1995) as well as from rat brain (Ruat *et al.*, 1995) [where the CaR may not play any role in systemic Ca_0^{2+} homeostasis (see below)]. These various CaRs share greater than 90% identity in their amino acid sequences, indicating that they are species and tissue homologs of the same ancestral gene (see Table 1 for a summary of selected features of CaRs cloned from various species to date). Expression of these receptors in *Xenopus laevis* oocytes or in mammalian cells also revealed functional properties very similar to those of BoPCaR, in that they recognize changes in Ca_0^{2+} and Mg_0^{2+} within the physiological, millimolar range (Bai *et al.*, 1997).

Cloning and Characterization of the Rabbit Kidney CaR

Previous studies had shown that the New Zealand white rabbit exhibits a PTH-dependent increase in serum calcium concentration to levels about 30–40% higher than those in humans and most other mammals (Warren *et al.*, 1989). Since rabbits don't suffer any obvious ill effects of their 'hypercalcemia', it was thought that this species might provide a mammalian homolog of FHH, and that there might, therefore, be structural and functional differences between the rabbit CaR and other mammalian CaRs that might explain the altered Ca_0^{2+}-sensing in rabbits. Cloning and functional expression of rabbit CaR from kidney, however, revealed that despite several differences in its amino acid sequence from the human, rat and bovine CaRs, it exhibited no alterations in its affinities for Ca_0^{2+}, Mg_0^{2+} or Gd_0^{3+} (Butters, 1997).

Cloning and Characterization of the Chicken Parathyroid CaR

Another interesting and well-studied animal model in terms of its calcium metabolism is the domestic chicken *(Gallus domesticus)*. Because a laying hen deposits the equivalent of about 10% of its total bodily calcium daily in each egg shell, chickens are remarkable among terrestrial vertebrates in the rapidity with which they turn over bodily calcium stores. During the period of time each day when the egg shell is forming, the ionic Ca^{2+} present in plasma turns over approximately four times every minute. Total plasma calcium in the growing chicken is 10 mg/dl, comparable to that of mammals, but this level doubles during the egg-laying cycle due to the expression of a Ca^{2+}-binding protein, vitellogenin-A, in the serum, while serum ionized Ca^{2+} remains unchanged at 1.2–1.3 mM. These facts

lead us to clone and characterize the cDNA for the chicken CaR from chicken parathyroid gland (Diaz *et al.*, 1997). Functional expression of this CaR in *Xenopus laevis* oocytes revealed agonist-evoked increases in Ca^{2+}-activated Cl^- currents in response to elevated levels of Ca_0^{2+}, Mg_0^{2+} and Gd_0^{3+} that were similar to those of the various mammalian CaRs cloned previously (Diaz *et al.*, 1997). Therefore, a CaR with structural and functional properties very much like those in mammals can apparently provide for the unusually rapid turnover of bodily calcium in the chicken, presumably through adaptations in the effector limb of the mineral ion homeostatic system.

Are there Additional Ion-Sensing Receptors?

Northern blot analysis has revealed the presence of CaR transcripts in a variety of tissues with considerable diversity in the relative abundance and sizes of the different transcripts (Brown *et al.*, 1993; Butters *et al.*, 1997; Diaz *et al.*, 1997; Garrett *et al.*, 1995a, b; Ruat *et al.*, 1995). A compilation of the sizes of the CaR transcripts in various tissues and species is provided in Table 2. Interestingly, more than one transcript is present in every organ of each species studied to date, thereby implying the existence of important biochemical mechanisms involving either differing half lives of the transcripts or alternate promoter usage, issues that have yet to be addressed. The highest levels of expression of the CaR are in tissues known to sense Ca_0^{2+} and to be intimately involved in mineral ion homeostasis, namely parathyroid gland, regions of the kidney involved in divalent cation transport and the thyroidal, calcitonin-secreting C cells.

In the parathyroid gland the CaR likely regulates several aspects of parathyroid functions (for review, see Chattopadhyay *et al.*, 1996b). The inhibition of PTH secretion by elevations in Ca_0^{2+} is severely disrupted in individuals with NSHPT who are homozygous for inactivating CaR mutations as well as in mice homozygous for targeted disruption of the CaR gene (Chattopadhyay *et al.*, 1996b; Ho *et al.*, 1995). Thus the CaR plays a central, non-redundant role in regulating PTH secretion. Recent studies suggest that the CaR may also be involved in the high Ca_0^{2+}-mediated inhibition of PTH gene expression; moreover, the often marked parathyroid cellular hyperplasia present in NSHPT as well as in homozygous CaR 'knockout' mice indicates that the receptor may also directly or indirectly regulate parathyroid cellular proliferation (Chattopadhyay *et al.*, 1996b). Interestingly, the C-cells of the thyroid glands, whose secretion of calcitonin is stimulated rather than inhibited by increases in Ca_0^{2+}, express the same CaR as in parathyroid (Garrett *et al.*, 1995b). High Ca_0^{2+} likewise stimulates hormonal secretion from the CaR-expressing, ACTH-secreting AtT-20 cell line (Emanual *et al.*, 1996). The same receptor, therefore, can apparently mediate either stimulation or inhibition of secretion depending on the cellular context in which it is expressed.

Thus no novel isoforms of the CaR other than the one present in parathyroid and kidney have been isolated to date. Although it is possible that the CaR has been strictly conserved through evolution without undergoing gene duplication, this situation would contrast markedly with that for the mGluRs, where there are at least 8 different mGluR genes, with the different mGluRs varying in the extent to which they couple to various intracellular effector systems (for review, see Nakanishi, 1994). It is also possible, although as yet unproven, that the CaR could be the first identified member of a broader 'cation-sensing' family of cell surface receptors, as hypothesized by some investigators (Quarles *et al.*, 1997). For example, Ca_0^{2+} and other polyvalent cations, such as aluminium Al_0^{3+}, are potent mitogens for osteoblasts and certain osteoblast-derived cell lines, despite the clear absence of the previously cloned CaR as assessed by sensitive techniques to search for its transcripts, such as the use of the reverse transcription-polymerase chain reaction (RT-PCR) (Quarles *et al.*, 1997). Moreover, there may be additional Ca_0^{2+}-sensors structurally distinct from the CaR. A putative Ca_0^{2+}-sensing protein has been cloned from human placenta and rat kidney that is a member of the LDL receptor superfamily (Lundgren *et al.*, 1994). Although antibodies raised against this protein alter the sensing of Ca_0^{2+} by parathyroid cells, the very large protein predicted by the cloned cDNA, which has been called 'megalin', has not been functionally characterized by expression in heterologous systems to prove that it does, in fact, exhibit Ca_0^{2+}-sensing properties (Saito *et al.*, 1994). Finally, osteoclasts are responsive to Ca_0^{2+} as noted above (Zaidi *et al.*, 1989). The Ca_0^{2+}-sensor in the latter cell type has been suggested to be related to the ryanodine receptor, a protein that normally is involved in intracellular Ca_i-induced Ca^{2+} release and has previously been shown to function in the sensing of Ca_0^{2+} (Zaidi *et al.*, 1992). Cloning and expression of the putative osteoclast Ca_0^{2+}-sensor, however, will be necessary to clarify further its structural and functional properties.

Table 2 Sizes of CaR Transcripts in Various Organs of Different Species (in Kb).

Species	Parathyroid	C-cells	Kidney	Intestine	Brain	References
Human	5.6, 4.8, 4.2	5.6	–	–	–	Garrett *et al.*, 1995
Bovine	5.3, 9.5	–	5.3, 2.0	–	5.3	Brown *et al.*, 1993
Rat	–	–	7.5, 4.1	7.5, 4.1, 3.0	7.5, 4.1	Ruat *et al.*, 1995
Rabbit	6.6	–	6.6	3.5	Undetectable	Butters *et al.*, 1997
Chicken	6.2	–	6.2, 1.1	4.2	6.2, 1.1	Diaz *et al.*, 1997

'–' = data not available

Localization and Functions of the CaR in the Kidney

In rat kidney, *in situ* hybridization and RT-PCR performed on isolated nephron segments have localized the CaR to the glomerulus, proximal convoluted and straight tubule, medullary (MTAL) and cortical thick ascending limb (CTAL), distal convoluted tubule (DCT) and cortical (CCD) as well as inner medullary collecting duct (IMCD) (Riccardi *et al.*, 1995, 1996). During renal development in the rat, CaR transcripts and protein are expressed at very low levels prenatally except in large tubules and branching ureteric buds of developing nephrons (Chattopadhyay *et al.*, 1996a). Postnatally, there is a marked increase in CaR expression, especially in the CTAL and to some extent in the CCD. The largest increases in its level of expression correlate with development of the thick limb, which exhibits the highest level of expression of the CaR in the adult kidney as well (Riccardi *et al.*, 1995). In the TAL, the receptor protein is localized predominantly on the basolateral surface of the cell, particularly in the CTAL (Butters *et al.*, 1997), where it probably senses systemic levels of Ca_0^{2+} and Mg_0^{2+} and responds to the changes in the circulating levels of these divalent cations with reciprocal changes in their tubular reabsorption. The decreased and increased responsiveness of these CaRs to Ca_0^{2+} in FHH and autosomal dominant hypocalcemia, respectively, presumably account for the inappropriately low and high urinary excretion of divalent cations in these two disorders (Chattopadhyay *et al.*, 1996b).

Recent studies have shown that the CaR likely mediates the reduced maximal urinary concentrating capacity in hypercalcemic individuals, probably by reducing the activity and/or number of vasopressin-stimulated water channels (e.g., aquaporin-2 channels) resident in the apical plasma membrane of the tubular cells of the IMCD (Sands *et al.*, 1997). A similar action of the CaR during the early postnatal development of the kidney may contribute to the impaired urinary concentration of newborn rats and humans, particularly since the increase in the expression of the CaR postnatally precedes that of the vasopressin receptor in the collecting ducts (Chattopadhyay *et al.*, 1996a). As with the CTAL, alterations in renal water handling in patients with loss- or gain-of-function CaR mutations provide additional indirect evidence for the role of the CaR in regulating renal handling of water. Individuals with FHH concentrate their urine normally despite being hypercalcemic, whereas those with autosomal dominant hypocalcemia may develop impaired urinary concentrating capacity during treatment of their hypocalcemia with calcium and vitamin D, even when their levels of Ca_0^{2+} are within the normal range (Chattopadhyay *et al.*, 1996b). The latter observation, combined with the marked hypercalciuria that can develop in autosomal dominant hypocalcemia during such treatment, has led to the suggestion that therapy be reserved for those patients with symptoms definitely attributable to hypocalcemia (i.e., seizures) (Chattopadhyay *et al.*, 1996b).

CaR in Non-Homeostatic Organs/Cell Types

Although the CaR was originally considered to be involved primarily in the process of Ca_0^{2+} homeostasis among tissues such as parathyroid, C-cells, kidney, bone and intestine (Brown, 1991; Brown *et al.*, 1995), several recent findings suggest the existence of Ca_0^{2+}-sensing in various cells that do not have any obvious role(s) in the maintenance of the homeostasis of calcium and other mineral ions.

CaRs in the Brain

In adult rat brain the CaR has been localized by both immunocytochemistry and *in situ* hybridization (Ruat *et al.*, 1995; Rogers *et al.*, 1997). The receptor is expressed in most of the regions of the brain, albeit at varying levels. The highest levels are observed in the subfornical organ (SFO) and olfactory bulbs. High expression levels are also found in the hippocampus, striatum, cingulate cortex, cerebellum and ependymal zones of the cerebral ventricles and in cerebral arteries. The abundant expression of the CaR in the SFO, which is an important thirst center, implies its role(s) in the central control of fluid and electrolyte homeostasis. We have suggested that CaRs in the SFO may mediate the increased thirst observed in hypercalcemic individuals (Chattopadhyay *et al.*, 1996b). Moreover, increased thirst during incipient or actual hypercalcemia, coupled with CaR-mediated reduction in urinary concentration in the IMCD, may ensure that excretion of a calcium load takes place in a sufficiently large volume of dilute urine to minimize the risk of forming calcium-containing renal stones (Sands *et al.*, 1997).

The CaR is highly expressed in synaptic regions of all areas of the hippocampus, although it has not yet been established with certainty whether it is located predominantly in pre- and/or postsynaptic membranes (Chattopadhyay *et al.*, 1997a; Ruat *et al.*, 1995). Because its overall location is similar to those of the mGluRs and the NMDA type of ionotropic glutamate receptor channels, both of which play important roles in certain forms of long term potentiation (LTP), it is possible that the CaR could also contribute to cognitive functions, such as learning and memory, although direct evidence for such roles is at present lacking (Brown *et al.*, 1995; Chattopadhyay *et al.*, 1997a). The substantial levels of expression of the CaR in the cerebellar granule and Purkinje cells could indicate the receptor's involvement in cerebellar functions, such as motor coordination. The use of CaR knockout mice should eventually make it possible to establish whether or not the CaR plays any such postulated roles (Ho *et al.*, 1995).

How might the CaR regulate the function of hippocampal, cerebellar and other neurons? Recently we have shown the presence of a Ca^{2+}-permeable nonselective cation channel (NCC) that is modulated by the CaR in rat hippocampal pyramidal neurons (Ye *et al.*, 1996a). An NCC with similar characteristics is regulated in a CaR-dependent fashion in HEK293 cells stably transfected with CaR but not in nontransfected cells that do not express the

CaR (Ye *et al.*, 1996b). Furthermore, spermine and other CaR agonists stimulate the activity of a very similar NCC in neurons cultured from wild type mice but not those from mice with targeted disruption of the CaR gene (Ye *et al.*, 1997). The relationship of this channel to other Ca^{2+}-permeable nonselective channels, such as the NMDA type of ionotropic glutamate receptor channel that is known to play a key role in the induction of LTP in the hippocampus, is not yet entirely clear. CaR-mediated activation of this channel, however, could contribute to changes in cytosolic Ca^{2+} that are important for the development of LTP and other forms of synaptic plasticity. Our observations in the developing rat hippocampus that large increases in CaR expression occur at a time when LTP can first be induced could indicate that the CaR is involved in such key synaptic functions (Chattopadhyay *et al.*, 1997a). Again, however, studies on brain preparations from mice with targeted disruption of the CaR gene will be essential to evaluate further the CaR's role, if any, in such processes.

It should be recalled that synaptic vesicles contain abundant calcium, potentially enabling pre- and/or postsynaptic CaRs to sense changes in Ca_0^{2+} related to neurosecretion *per se* (perhaps in glutamatergic synapses calcium and glutamate could function, in effect, as cotransmitters) (for review, see Brown *et al.*, 1995). In addition, substantial, neuronal activity-dependent changes in Ca_0^{2+} have been documented within the extracellular brain microenvironment, owing to Ca^{2+} influx through various channels (e.g., NMDA channels) (Heinemann *et al.*, 1977). Thus the CaR might be involved in detecting local changes in Ca_0^{2+} in the vicinity of neurons, including within synaptic clefts, related to neuronal uptake of Ca_0^{2+}, thereby potentially regulating release of neurotransmitters or other synaptic processes (Vassilev *et al.*, 1997).

An elevation in cytosolic Ca^{2+} in the postsynaptic dendritic spines in hippocampus (as depicted in Figure 2) as a result of Ca^{2+} influx through NMDA channels is accepted to be a major factor in the induction of LTP (Malenka *et al.*, 1988; Jahr and Stevens, 1993). Available evidence supports the existence of one or more transsynaptic, retrograde messengers that modulate presynaptic glutamate release (Bliss and Collingridge, 1993). This putative retrograde signaling system acts to coordinate postsynaptic changes in Ca_i with presynaptic release of neuromediators, such that increases in postsynaptic Ca_i further stimulate glutamate release. Such a mechanism may underlie synaptic enhancement during the induction of LTP by high-frequency (100 Hz) electrical stimulation.

The synaptic cleft may be represented as a very thin disk of extracellular fluid (Figure 2). It is possible that at high stimulation frequencies the interval between pulses would be insufficient for diffusion of Ca^{2+} from the periphery of the cleft to replace extracellular Ca^{2+} depleted at the center of the cleft as a result of activation of postsynaptic, Ca^{2+}-permeable channels. We have employed computer modeling to assess the impact of the activation of glutamate receptor channels (GRCs) in the postsynaptic membrane on the level of extracellular Ca^{2+} within

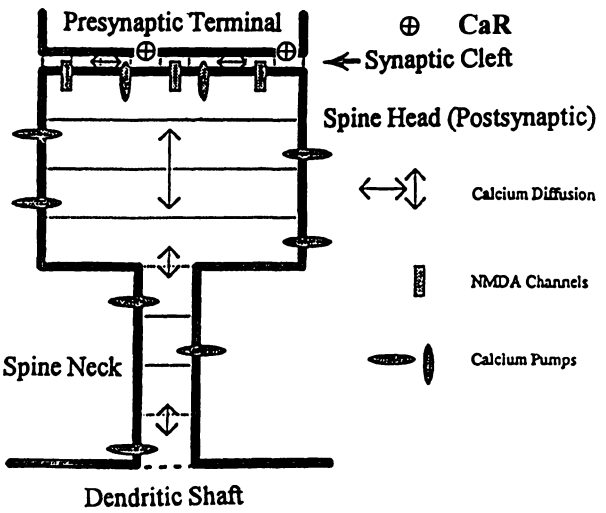

Fig. 2 Model of Ca^{2+} Dynamics in the Synaptic Cleft between a Hippocampal Presynaptic Terminal and a Dendritic Spine.
The CaR located on the presynaptic membrane could sense changes in Ca_0^{2+} in the cleft due to the operation of the Ca^{2+}-permeable NMDA and other glutamate receptor channels as well as Ca^{2+} pumps in the postsynaptic spines. The PLC- and G-protein coupled CaR might then modulate the secretion of glutamate or other neurotransmitters according to fluctuations in Ca_0^{2+}, thereby playing a key role in a putative retrograde messenger mechanism [see Vassilev *et al.*, (1997) for details]. The boundary between the dendritic shaft and spine neck is marked with a dashed line. See explanation in the text [reproduced with permission from Vassilev *et al.*, (1997), Biophys. J. *72*, in press].

the synaptic cleft (Vassilev *et al.*, 1997) (Figure 2). The model includes calcium influx from the synaptic cleft into the postsynaptic compartment through GRC and calcium efflux through calcium pumps. Concentrations of extracellular Ca^{2+} inside the cleft are estimated by using a compartmental model incorporating flux across the postsynaptic membrane and radial diffusion from the edge of the cleft. The simulations suggest that substantial depletion of extracellular Ca^{2+} can occur in the clefts during activation of GRCs, particularly at the high stimulation frequencies (~ 50–100 Hz) used to induce LTP (Figure 3). In contrast, only minimal transitory changes in extracellular Ca^{2+} are observed at low frequencies (~ 3–5 Hz) (Vassilev *et al.*, 1997). These frequency-dependent alterations in Ca_0^{2+} dynamics are a direct reflection of the activity of GRCs and could be involved in the modulation of presynaptic function via a retrograde messenger mechanism if there are extracellular Ca^{2+} sensors on the presynaptic membranes. The CaR that is known to be present in nerve terminals in hippocampus and other areas of the brain could potentially play such a role.

CaR in Keratinocytes

Additional non-homeostatic tissues expressing the CaR include human keratinocytes (Bikle *et al.*, 1996), a cell line derived from a human colon cancer (Kallay *et al.*, 1997), lens epithelial cells (Chattopadhyay *et al.*, 1997b), and the pituitary-derived, ACTH-secreting AtT-20 cell line (Emanuel *et al.*, 1996), providing additional indirect evidence for

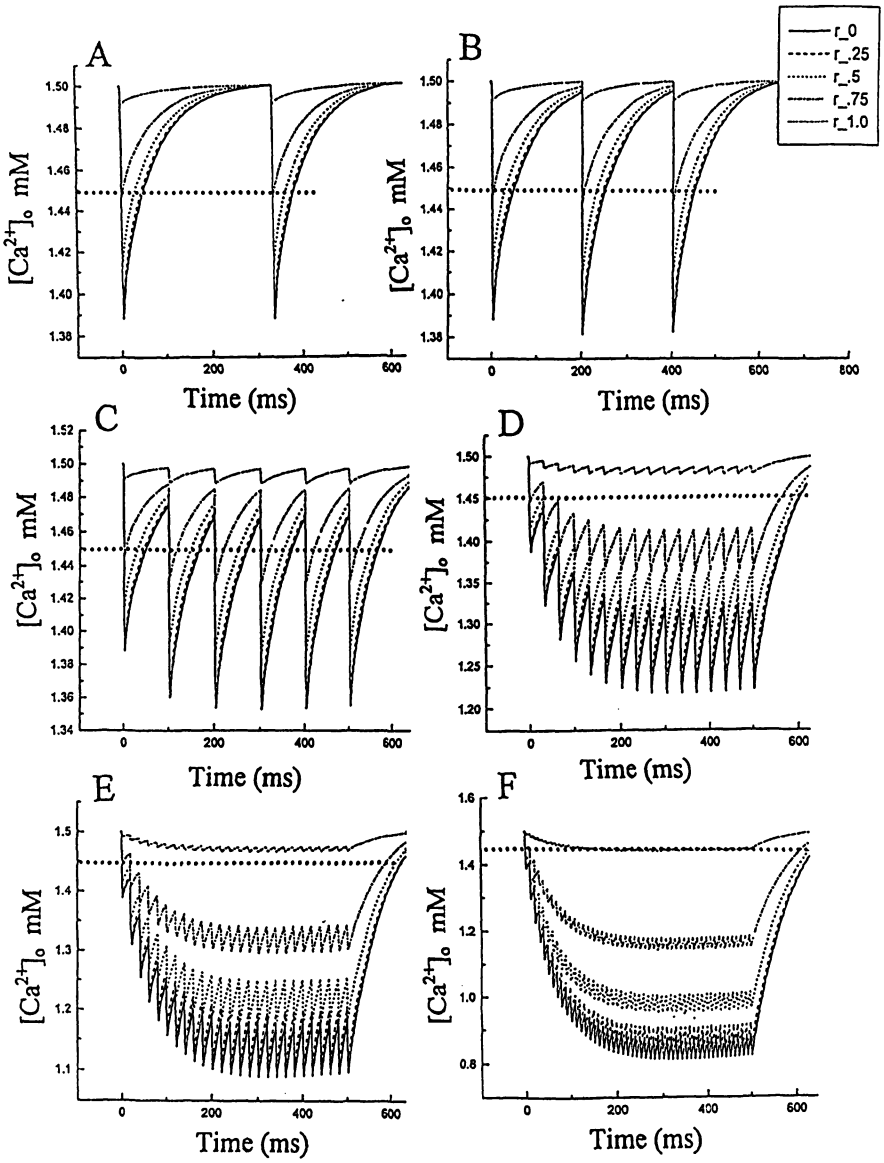

Fig. 3 Time Course for Changes in the Extracellular Calcium Concentration in the Synaptic Cleft at Various Stimulation Frequencies: 3Hz in A; 5 Hz in B; 10 Hz in C; 30 Hz in D; 50 Hz in E; and 100 Hz in F.
The profiles for the changes in Ca_0^{2+} at various distances from the center of the cleft are marked with different lines, as indicated in the insets. The center is marked with r_0 and the periphery with r_1.0. Other lines are as follows: r_.25, r_.50 and r_.75 represent 25%, 50% and 75% of the distance from the center of the cleft to the periphery, respectively. By sensing fluctuations in Ca_0^{2+} within the cleft occurring at different stimulation frequencies, the CaR might serve as a frequency detecting unit. [Reproduced with permission from Vassilev *et al.* (1997).]

a broader role for the CaR and the Ca_0^{2+} signal in the control of cellular differentiation, proliferation, neural transmission, apoptosis and potentially other processes. Human keratinocyte differentiation *in vitro* is known to be triggered by elevations in Ca_i in response to increases in Ca_0^{2+} through the involvement of (a) release of Ca_0^{2+} from intracellular stores and (b) Ca^{2+} influx through NCC (Bikle *et al.*, 1996). Moreover, human keratinocytes express transcripts for the CaR, and the differentiating stimulus of an increase in Ca_0^{2+} leads to an increase in CaR mRNA (Bikle *et al.*, 1996). Thus the CaR might mediate the effects of Ca_0^{2+} on keratinocyte differentiation through its known capacity to activate PLC and NCC (Bikle *et al.*, 1996; Ye *et al.*, 1996a, b).

CaR in Lens Epithelial Cells

It has long been appreciated that high Ca_0^{2+} has several effects on lens epithelial cells in culture, viz., disruption of plasma membrane integrity, loss of the cytoskeletal protein, vimentin, and activation of Ca-ATPase, leading to cataract formation (for review, see Delamere and Paterson, 1981). In addition, clinical observations, such as the development of osmotically-induced cataracts in hypoparathyroid patients and the high calcium content of these cataracts, are strongly suggestive of the role(s) of Ca_0^{2+} (and potentially the CaR) in the physiology of lens epithelial cells (Duncan and Bushell, 1975). We have recently shown that cultured human lens epithelial cells express the CaR (Chattopadhyay *et al.*, 1997b). The receptor is linked to a

Ca^{2+}-activated K^+ channel having a conductance of ~82 pS, with the open state probability of the channel being increased by addition of high Ca_0^{2+} or the polycationic CaR agonist, neomycin (Chattopadhyay *et al.*, 1997b). We previously characterized Ca^{2+}-activated K^+ channels in parathyroid cells, whose activity was stimulated by CaR agonists (Kanazirska *et al.*, 1995). In addition, a similar Ca^{2+}-activated K^+ channel is present in hippocampal neurons cultured from wild type mice as well as those from mice homozygous for targeted disruption of the CaR gene (*Ye et al.*, in preparation). This channel could only be activated by CaR agonists in the neurons from the wild type mice, however, and not in those from mice with inactivation of the CaR gene. Therefore, the presence of a similar, CaR-regulated K^+ channel in these three cell types suggests that receptor might be involved in membrane repolarization and associated voltage-dependent changes in cellular metabolism, neural transmission and secretion.

CaR in Cells Derived from Intestinal Cells

Ca_0^{2+} inhibits the proliferation of human colonocytes in culture, while reducing Ca_0^{2+} increases proliferation of the colonocytes in association with a rise in the expression of the c-myc proto-oncogene (Kallay *et al.*, 1997). We have shown that the human colon cancer-derived cell line, Caco-2, expresses the CaR, which might, therefore, be involved in the low Ca_0^{2+}-evoked upregulation of *c-myc* expression through the activation of the PKC pathway, since the use of H7, a PKC inhibitor, abolished low Ca_0^{2+} induced proliferation of these cells (Kallay *et al.*, 1997). It remains unclear, however, how the CaR would mediate an increase in PKC activity with a decrease in Ca_0^{2+}, since the receptor is generally positively coupled to phosphoinositide-specific PLC. It was further shown that application of low levels of Ca_0^{2+} to the apical but not to the basolateral side of the cultured Caco-2 cells leads to upregulation of c-myc expression (Kallay *et al.*, 1997). The lower one third of the lumen of the colonic crypts has been suggested to contain reduced levels of Ca_0^{2+} due to binding of Ca^{2+} by the mucus component of the luminal aspect of the cells (Whitfield, 1991). Therefore, Ca_0^{2+}-sensing by the CaR might represent a key cellular 'switch' that normally inhibits the proliferation and promotes the differentiation of the stem cells at the crypt bases as they migrate up the crypts (Kallay *et al.*, 1997; Sorenson *et al.*, 1988). The use of a specific CaR antagonist and/or colonic cells from mice with targeted disruption of the CaR gene, however, will be necessary to establish unequivocally the CaR's role in regulating colonic cell proliferation/differentiation.

Regulation of CaR Gene Expression

Relatively few studies are available on the regulation of CaR expression. CaR gene expression is dramatically reduced in cultured bovine parathyroid cells. A 75–80% loss of receptor mRNA takes place within 18 h of the initiation of culture, followed by a comparable loss of receptor protein that is accompanied by a marked reduction in high Ca_0^{2+}-induced inhibition of PTH secretion within 36–48 h (Mithal *et al.*, 1995). Conversely, as noted above, there is a dramatic upregulation of CaR mRNA and protein in rat kidney during the first week of postnatal life, mainly in the TAL and to a lesser degree in the CCD (Chattopadhyay *et al.*, 1996a). Substantial reductions in the immunoreactivity of the CaR protein have also been observed in parathyroid adenomas and parathyroid tumors from uremic subjects with severe secondary or tertiary hyperparathyroidism (Kifor *et al.*, 1996). Reduced CaR expression in hyperparathyroidism might potentially account, at least in part, for the abnormal Ca_0^{2+}-regulated PTH release that contributes importantly to the genesis of hypercalcemia in these patients. At this stage it is uncertain whether the receptor downregulation acts as the primary event leading to subsequent parathyroid cellular hyperplasia or is a secondary phenomenon resulting from predisposing somatic mutations in genes controlling growth. The latter is likely an important contributory factor, as recent studies have documented loss of tumor suppressor genes from several chromosomal loci in various types of parathyroid tumors (Arnold *et al.*, 1995).

Treatment of rats with $1,25(OH)_2D$ *in vivo* has given rise to divergent findings. Administration of $1,25(OH)_2D$ modestly increased CaR mRNA in parathyroid gland and kidney in one study (Brown *et al.*, 1996), while it had no significant effect in another (Rogers *et al.*, 1995). However, chronic alterations in serum calcium concentration had no effects on CaR gene expression in either tissue. In AtT-20 cells, in contrast, elevated levels of Ca_0^{2+} produced ~2-fold increases in CaR mRNA (Emanuel *et al.*, 1996). Moreover, hippocampus of developing rats, beginning during the second postnatal week and extending until about 30 days of age, displays ~3 fold higher levels of CaR expression relative to adult rats (Chattopadhyay *et al.*, 1997b). Thus the time course for the developmental expression of the CaR gene differs in rat hippocampus and kidney, occurring in the immediate postnatal period in the latter (Chattopadhyay *et al.*, 1996a) and achieving a level that remains constant into adulthood, rather than decreasing as in the hippocampus (Chattopadhyay *et al.*, 1997a). Finally, as noted before, changes in CaR mRNA expression occur in association with the induction of differentiation of keratinocytes by increases in Ca_0^{2+} (Bikle *et al.*, 1996). The physiological relevance of regulation of CaR gene expression in these various tissues is not clear; however, these observations raise the possibility that the regulation of the receptor in tissues involved in calcium homeostasis, such as parathyroid cell and kidney, may differ from that in other tissues, including the brain, AtT-20 cells and keratinocytes, that are not directly involved in systemic mineral ion homeostasis. In the former, recognition of the systemic Ca_0^{2+} signal (e.g., by parathyroid and C-cells as well as the CTAL) appears to be crucial for maintaining near constancy of Ca_0^{2+}. In the latter, in contrast, local changes in Ca_0^{2+} (or perhaps other CaR agonists, such

as Mg_0^{2+} or spermine) may provide signals that regulate cellular differentiation or regulate local Ca_0^{2+} homeostasis. For instance, neuronal activity-dependent changes in the brain microenvironment, by modulating the activity of nearby CaRs, might reduce the activity of Ca^{2+}-permeable channels (e.g., NCC) that contributed to the initial change in Ca_0^{2+} (Heinemann *et al.*, 1977).

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Review

Drug Efflux Proteins in Multidrug Resistant Bacteria

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Bacteria contain an array of transport proteins in their cytoplasmic membrane. Many of these proteins play an important role in conferring resistance to toxic compounds. The multidrug efflux systems encountered in prokaryotic cells are very similar to those observed in eukaryotic cells. Therefore, a study of the factors which determine the substrate specificity and energy coupling to drug translocation in bacteria has significance for the general field of multidrug resistance. Three issues will be dealt with in this review. First, an overview of the various classes of prokaryotic multidrug transporters will be presented. Second, the current understanding of the regulation of bacterial multidrug resistance will be summarized. Third, the present knowledge of the molecular mechanisms involved in drug transport processes will be discussed.

Key words: Antibiotics / ATP-binding cassette transporter / Transport mechanism / P-glycoprotein / Prokaryotes / Secondary transporter.

Multidrug Resistant Bacteria

The introduction of antibiotics into clinical use has brought about a spectacular decline in the prevalence of infectious diseases in humans. However, the emergence of resistant bacteria is changing this situation. Diseases such as tuberculosis and pneumonia, evocative of a former era when pathogenic bacteria were often deadly killers, are now re-emerging as major threats to public health (Cohen, 1992). In addition, the constant pressure of antibiotics in hospital environments has selected for drug resistant bacterial species which may not possess strong virulence but can infect debilitated patients (Neu, 1992).

Toxic compounds have always been part of the natural environment in which bacteria dwell. The development of strategies for life in this habitat has been crucial for survival of the organisms. As a result, bacteria have developed versatile mechanisms to resist antibiotics and other cytotoxic drugs. Examples are the enzymatic degradation or inactivation of drugs (Davies, 1994), and the alteration of drug targets (Spratt, 1994). In addition, mechanisms exist in which the entrance of a variety of cytotoxic compounds into the cell is prevented by the barrier and active transport function of the cell envelope which encloses the cytoplasm (Nikaido, 1994).

The envelope of Gram-negative bacteria consists of the cytoplasmic (or inner) membrane, a peptidoglycan layer, and the outer membrane that contains lipopolysaccharides (Nikaido and Vaara, 1985). Gram-positive bacteria are not enclosed by an outer membrane. Instead, their cytoplasmic membrane is directly surrounded by a thick peptidoglycan layer. Both the peptidoglycan layer of certain Gram-positive bacteria [e.g. mycobacteria (Jarlier and Nikaido, 1994)] as well as the outer membrane of Gram-negative bacteria are able to serve as a barrier against rapid penetration of drugs (Thanassi *et al.*, 1995). However, these barriers cannot prevent the drugs from exerting their action once they have entered the cell. Additional mechanisms are therefore needed to achieve significant levels of drug resistance. One of the major mechanisms to lower the cytoplasmic drug concentration is based on direct efflux of drugs across the cytoplasmic membrane.

Drug Transport Proteins

Microorganisms possess membrane proteins which catalyze transmembrane drug transport (Levy, 1992; Lewis, 1994; Nikaido, 1996). Some of these drug transporters are fairly specific for a given drug or class of drugs, but the so-called multidrug transporters have specificity for compounds with very different chemical structures and cellular targets. On the basis of bioenergetic and structural criteria, these transport systems can be divided into:

(i) Secondary transporters, which mediate the extrusion of drugs from the cell in a coupled exchange with ions (Paulsen *et al.*, 1996a), and
(ii) ATP-binding cassette [ABC] transporters, which utilize the release of phosphate bond-energy by ATP hydrolysis to pump drugs out of the cell (Higgins, 1992).

Secondary Multidrug Transporters

The number of secondary multidrug transporters discovered in prokaryotic and eukaryotic cells is vast and rapidly expanding. Secondary multidrug transporters have been detected in pathogenic bacteria such as methicillin-re-

sistant *Staphylococcus aureus* [QacC] (Littlejohn *et al.*, 1992), *Mycobacterium smegmatis* [LfrA] (Liu *et al.*, 1997), and *Neisseria gonorrhoeae* [MtrCD] (Hagman *et al.*, 1995). Computer-based sequence analyses have revealed that secondary drug transporters belong to one of three distinct families of transport proteins: the Major Facilitator Superfamily [MFS] (Marger and Saier, 1993), the Resistance-Nodulation-Cell Division [RND] (Saier *et al.*, 1994) family, and the Small Multidrug Resistance (SMR) family (Paulsen *et al.*, 1996b) (Table 1).

Examples of each type of secondary drug transporter, *L. lactis* LmrP [MFS family] (Bolhuis *et al.*, 1995), *S. aureus* QacC [SMR family] (Littlejohn *et al.*, 1992), and *Pseudomonas aeruginosa* MexB [RND family] (Poole *et al.*, 1993), are presented schematically in Figure 1. LmrP and QacC have specificity for an exceptionally wide range of amphiphilic, cationic drugs including antibiotics, quaternary ammonium compounds, aromatic dyes, and phosphonium ions. MexB confers resistance to amphiphilic anionic compounds such as antibiotics, basic dyes and detergents. In Gram-negative bacteria, transport of drugs from the interior of the cell to the external medium requires the translocation of solutes across the cytoplasmic and outer membrane. Therefore, some drug transporters [e.g., MexB] in such organisms are found in association with an accessory protein which spans the periplasmic space and interacts with a porin in the outer membrane. These auxiliary proteins belong to the Membrane Fusion Protein family (Saier *et al.*, 1994) and Outer Membrane Factor family (Dinh *et al.*, 1994).

Table 1 Multidrug Transporters in Bacteria.

Transporter Family	Protein	Organism	Accession No.[a]
ABC family[b]	LmrA	*Lactococcus lactis*	GB U63741
MFS family[c]	LmrP	*Lactococcus lactis*	GB X89779
	Bmr	Bacillus *subtilis*	SW P33449
	QacA	*Staphylococcus aureus*	EM X56628
	EmrB	*Escherichia coli*	SW P27304
	LfrA	*Mycobacterium smegmatis*	GB U40487
RND family[d]	MexB	*Pseudomonas aeruginosa*	GB L11616
	MtrD	*Neisseria gonorrhoeae*	SW P43505
	AcrB	*Escherichia coli*	EM U00734
SMR family[e]	QacC	*Staphylococcus* aureus	SW P14319
	QacE	*Klebsiella aerogenes*	PR S25583
	EmrE	*Escherichia coli*	SW P23895

[a] Accession number: GB, Genbank; SW, SwissProt; EM, EMBL; PR, PIR
[b] ATP-binding cassette family (Higgins, 1992)
[c] Major Facilitator Superfamily (Marger and Saier, 1993)
[d] Resistance-Nodulation-Cell Division family (Saier *et al.*, 1994)
[e] Small Multidrug Resistance family (Paulsen *et al.*, 1996b)

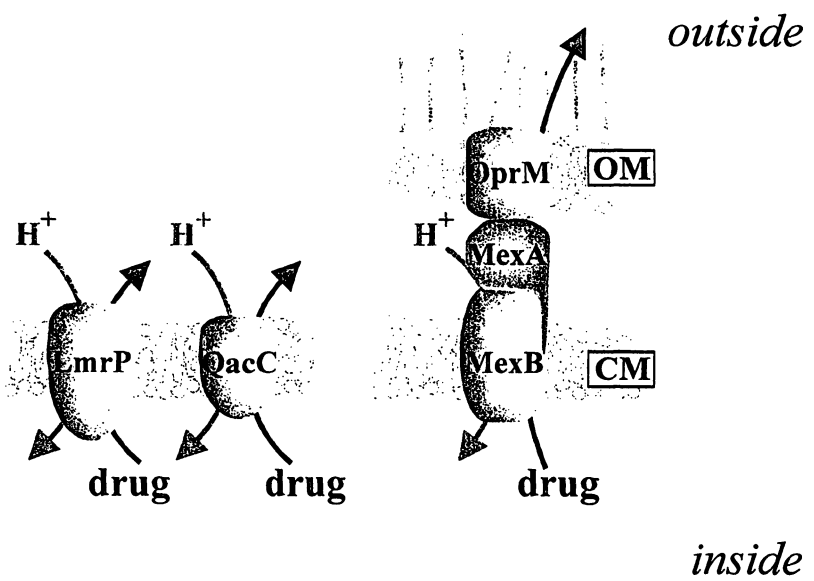

Fig. 1 Schematic Presentation of Representative Secondary Multidrug Transporters in Prokaryotes.
LmrP in *Lactococcus lactis* (Major Facilitator Superfamily), QacC in *Staphylococcus aureus* (Small Multidrug Resistance family) and MexB in *Pseudomonas aeruginosa* (Resistance-Nodulation-Cell Division family) are driven by the proton motive force which is present across the cytoplasmic membrane (CM). MexB is associated with the accessory protein MexA (Membrane Fusion Protein family) and the outer membrane porin OprM (Outer Membrane Factor family). These additional proteins enable the MexB-mediated transport of drugs across the outer membrane (OM) in the Gram-negative bacterium.

Energetics of Drug Transport

Bacterial secondary transporters are located in the cytoplasmic membrane, across which usually a proton motive force [interior negative and alkaline] exists. Based on (i) the structural similarity between secondary drug transport proteins and known proton motive force-dependent transport systems, and (ii) the sensitivity of drug transport to agents that dissipate the proton motive force, it is assumed that secondary drug transporters function as drug/proton antiporters. The direct involvement of the proton motive force as driving force was demonstrated for the *E. coli* tetracycline transporter TetA(B) [MFS family], which mediates an electroneutral exchange reaction of a $metal^{2+}$-$tetracycline^{1-}$ complex and one proton (Yamaguchi *et al.*, 1991), and the lactococcal multidrug transporter LmrP which mediates an electrogenic drug/nH^+ antiport reaction [$n \geq 2$] (Bolhuis *et al.*, 1996a).

Structure-Function Relationships

Analysis of the topography of secondary drug transporters suggest the presence of either 12 or 14 membrane-spanning segments in members of the MFS family, and 12 membrane-spanning segments in members of the RND family. The transmembrane segments are most likely in α-helical conformation, and are connected by hydrophilic loops protruding into the cytoplasm or periplasmic space. The structure of TetA(B) has been studied most extensively, by limited proteolysis (Eckert and Beck, 1989), site-directed antibody binding (Yamaguchi *et al.*, 1990), PhoA-fusion analysis (Allard and Bertrand, 1992), and site-directed chemical labeling (Kimura *et al.*, 1997). A relatively large cytoplasmic loop in the middle of the protein separates TetA(B) in an N- and C-terminal half, each containing 6 transmembrane segments. Both halves are evolutionary related, presumably by a gene duplication event, and are well conserved among TetA proteins of various classes (Rubin *et al.*, 1990). The N- and C-terminal halves of TetA(B) represent separate domains in the drug transporter (Curiale and Levy, 1982). The functional interaction between these domains is suggested by

(i) the intragenic complementation between mutations in the first and second halves of the *tet*A(B) gene (Curiale *et al.*, 1984; McNicholas *et al.*, 1995),

(ii) the ability of hybrid tetracycline efflux transporters containing N- and C-terminal halves of different classes of tetracycline efflux proteins (e.g., class A and C), to confer resistance (Rubin and Levy, 1990) and

(iii) the functional reconstitution of tetracycline resistance upon co-expression of the N- and C-terminal halves of TetA(B) as separate polypeptides (Rubin and Levy, 1991).

Members of the SMR family contain 4 putative transmembrane α-helices. This topological model has recently been confirmed for QacC by genetic fusion using alkaline phosphatase and β-galactosidase as reporters of subcellular localization (Paulsen *et al.*, 1995), and for *E. coli* EmrE by transmission fourier-transform infrared spectroscopy (Arkin *et al.*, 1996). In view of the 12 transmembrane models proposed for members of the MFS and RND families, members of the SMR family may function as a homotrimer (Yerushalmi *et al.*, 1995).

Multiple alignment of the amino acid sequences of the MFS family members reveals the presence of two conserved sequence motifs that are located at similar positions within the putative secondary structure of the proteins. Motif A [G*XXX*DR*X*GR(K/R)] is found in many members of the MFS family (Marger and Saier, 1993) and is present in the cytoplasmic loop between transmembrane segment 2 and 3. Motif A may be of structural importance by mediating opening and closing of the translocation pathway. Motif B [G*XXX* GP *XX* GG], the drug extrusion consensus sequence, is found at the end of transmembrane segment 5, and is typical for LmrP and other drug export systems of the Major Facilitator superfamily (Bolhuis *et al.*, 1995; Griffith *et al.*, 1992). At present, the role of motif B in drug recognition or binding is unclear.

It has been suggested that amino residues within transmembrane segments of the human multidrug resistance P-glycoprotein MDR1 play a role in drug specificity (for review see Gottesman *et al.*, 1995). Observations on secondary drug transporters in bacteria are consistent with this notion. The MFS members QacA and QacB in *S. aureus* are homologous proteins [98% identical amino acid residues]. QacB confers resistance to monovalent organic cations but differs from QacA by conferring no resistance to the divalent organic cations pentamidine isothionate and propamidine isothionate. This phenotypic difference between QacA and QacB is solely due to the presence of the acidic Asp^{323} within the putative transmembrane segment 10 of the QacA protein, instead of the neutral Ala^{323} in QacB (Paulsen *et al.*, 1996c). The ability of the MFS member Bmr in *Bacillus subtilis* (Neyfakh *et al.*, 1991) to interact with reserpine and other drugs is strongly affected by substitutions of Val^{286} (Ahmed *et al.*, 1993), and of Phe^{143} or Phe^{306} (Klyachko *et al.*, 1997), which are located within transmembrane segments. Thus, both charged and aromatic residues located within the phospholipid bilayer appear to interact with drugs and may be involved in drug recognition or binding. Interestingly, it has been shown that quaternary ammonium compounds and other cations can bind to the π face of the aromatic ring structures of tyrosine, phenylalanine or tryptophan residues in the hydrophobic environment of the membrane (Dougherty, 1996).

Regulation of Gene Expression

The expression of drug-specific efflux transporters is usually induced by the drugs themselves through their interaction with DNA-binding regulatory proteins. The best understood example is the regulation of bacterial tetracycline-efflux transporter TetA(B), where the binding of tetracycline to a specific repressor protein (TetR) causes the repressor to dissociate from the promoter of the transporter gene thereby allowing transcription (Hinrichs *et al.*, 1994; Kisker *et al.*, 1995). Studies on the regulation of the

expression of *B. subtilis* Bmr, *E. coli* EmrAB (Lomovskaya and Lewis, 1992), and *N. gonorrhoeae* MtrCD suggest that specific regulator proteins also play a role in the regulation of the expression of multidrug transporters.

Drugs recognized by Bmr enhance the expression of this multidrug transporter. In drug resistant mutants, Bmr can be overexpressed by gene amplification. In wild type cells, however, the regulation of gene expression is dependent upon expression of the regulatory protein BmrR which is encoded downstream of the *bmr* gene (Ahmed *et al.*, 1994). BmrR shows sequence homology and functional similarity with several known bacterial transcription activator proteins and contains a conserved N-terminal DNA binding domain. Upon binding of drugs to the C-terminal drug-binding domain, the protein binds to the *bmr* promoter and induces Bmr expression (Markham *et al.*, 1996). Interestingly, the *B. subtilis* genome encodes two additional multidrug efflux transporters:

(i) Blt, which shares 51% amino acid sequence identity with Bmr (Ahmed *et al.*, 1995), and
(ii) Bmr3, which shares 21% amino acid sequence identity with EmrB (Ohki and Murata, 1997).

Bmr, Blt and Bmr3 have an overlapping drug specificity but are differentially expressed in response to drugs. The expression of Blt is regulated by BltR, a transcriptional activator (Ahmed *et al.*, 1995). Although the DNA-binding domains of BmrR and BltR are related, their putative drug-binding domains are dissimilar. Apparently, the expression of Bmr and Blt is mediated by regulatory proteins which, like their regulated transporters, are capable of recognizing structurally diverse compounds.

Like Bmr and Blt, the expression of EmrAB in *E. coli* is enhanced in the presence of the drugs which are recognized by this multidrug efflux system. However, regulation of the expression of EmrAB is dependent on a negative regulator, EmrR, which is encoded upstream of *emrAB* (Lomovskaya *et al.*, 1995). Whether the transcriptional repression caused by EmrR is due to direct binding to the *emrAB* promoter is not known. In contrast to BmrR and BltR, EmrR does not contain known DNA-binding motifs.

MtrCD expression in *N. gonorrhoeae* is negatively regulated by a repressor protein, MtrR, which is homologous to TetR (Hagman *et al.*, 1995). Mutations that inactivate MtrR result in an increase in the level of MtrCD expression. Interestingly, the MtrR protein was found to be homologous to previously unidentified open reading frames (*acr*R and *env*R) that are upstream of the *E. coli acr*AB and *env*CD operons (Pan and Spratt, 1994). Although AcrAB expression is primarily regulated by global regulatory pathways (Okusu *et al.*, 1996), a major role of the repressor AcrR is to function in the fine tuning of this expression (Ma *et al.*, 1996). Mutations in *env*R may result in increased levels of the EnvCD proteins, and hence, in increased resistance of *E. coli* to antimicrobial agents.

Besides the specific regulatory mechanisms that affect the expression of single multidrug efflux systems, global regulatory mechanisms have evolved in bacteria that affect the expression of (dedicated) drug efflux systems and other proteins involved in the intrinsic resistance of the cell. The *mar* regulon of *E. coli* (Cohen *et al.*, 1993a; Rosner and Slonczewski, 1994) and various other bacteria (Zhanel *et al.*, 1995; George *et al.*, 1995) is involved in chromosomally mediated multiple antibiotic and superoxide resistance. The inducing agents of this regulon are often used in clinical situations: antibiotics such as tetracycline and chloramphenicol, and aromatic weak acids including salicylate and acetylsalicylate. The agents stimulate the transcription of the *mar*RAB operon (Cohen *et al.*, 1993a, b). Specifically, expression of *mar*A, encoding a transcriptional activator (Martin *et al.*, 1996), elevates the expression of about 10 unlinked genes that affect the outer membrane permeation (Cohen *et al.*, 1993a, b), antibiotic efflux (Seoane and Levy, 1995a), and the reducing potential of the cell (Ariza *et al.*, 1994). Although salicylate appears to induce the *mar* operon by binding to the negative regulator MarR, tetracycline and chloramphenicol do not bind to the regulator (Seoane and Levy, 1995b). The latter compounds must therefore induce the *mar* operon by an indirect mechanism (Martin and Rosner, 1995). Besides the *mar*RAB-dependent pathway for multiple antibiotic and superoxide resistance, the *sox*RS genes provide *E. coli* with a *mar*-independent pathway (Cohen *et al.*, 1993b). SoxS is a transcriptional activator homologous to MarA [39% identical residues], which elevates the transcription of the operons of the *mar* regulon (Martin *et al.*, 1996). SoxR is a [2Fe-2S] sensor of superoxides that positively regulates *sox*S gene-expression in its oxidized form (Greenberger *et al.*, 1990; Gaudu and Weiss, 1996). The dual regulation by *mar*RAB and *sox*RS of a common set of genes may enable different environmental signals to trigger responses (Rosner and Slonczewski, 1994).

ABC-Type Drug Transporters

Transport proteins belonging to the ABC transporter superfamily are involved in the tolerance to a wide diversity of cytotoxic agents in both prokaryotes and eukaryotes (Higgins, 1992). Included in this superfamily are the well-known human multidrug resistance P-glycoprotein (Gottesman and Pastan, 1993) and human multidrug resistance-associated protein MRP1 (Cole *et al.*, 1992) plasma membrane transporters, which catalyze the extrusion of antitumor drugs during the chemotherapy of human cancer cells.

A number of dedicated ABC-type antibiotic export systems have been found in prokaryotes. In *Streptomyces* strains, these transporters mediate the excretion of various antibiotics to ensure self-resistance to the antibiotics that they produce. A well-known example is, amongst others, DrrAB which is implicated in the efflux of daunorubicin/doxorubicin (Guilfoile and Hutchinson, 1991). ABC transporters dedicated to the efflux of specific drugs have also been detected in other bacteria such as *Bacillus* in which the BcrABC (Podlesek *et al.*, 1995) and TmrB (Noda *et al.*, 1992) proteins confer resistance to bacitracin and tunicamycin, respectively.

Remarkably few prokaryotic ABC-type efflux systems have been characterized that are able to transport multiple drugs (Table 1). To date, most multidrug transporters in bacteria utilize the proton motive force, rather than ATP as the driving force and act via a drug/H^+ antiport mechanism. The first example of an ABC-type multidrug transporter has been found in *L. lactis* (van Veen *et al.*, 1996). The gene encoding this transporter, termed *lmrA*, has been cloned and sequenced. It encodes a 590-amino-acid membrane protein, the hydropathy analysis of which suggests the presence of an N-terminal hydrophobic domain with 6 putative transmembrane regions and a C-terminal hydrophilic domain. The latter domain contains the ATP-binding cassette (Figure 2).

Comparison of LmrA and various ABC transporters clearly identifies the lactococcal transporter as a structural homolog of the human multidrug resistance P-glycoprotein (Gottesman and Pastan, 1993). This 1280-residue membrane protein is predicted to contain two homologous halves, each with 6 transmembrane regions and an ABC domain. Amino acid alignment of LmrA and each half of P-glycoprotein indicates that they share 32% identity with an additional 16% conservative substitutions for an overall similarity of 48%. Interestingly, the sequence identity includes particular regions which, in P-glycoprotein, have been implicated as determinants of drug recognition and binding. Indeed, the specificity of both transporters for amphiphilic organic cations appears to be identical, whereas the activity of both systems is reversed by a similar set of inhibitors (van Veen *et al.*, 1996). Thus, LmrA represents a naturally occurring, functional 'half-molecule' of the human multidrug resistance P-glycoprotein. On the basis of the general domain organization of ABC transporters, two ATP binding domains and two hydrophobic domains (Higgins, 1992), the lactococcal export system is predicted to function as a homodimeric complex (Figure 2).

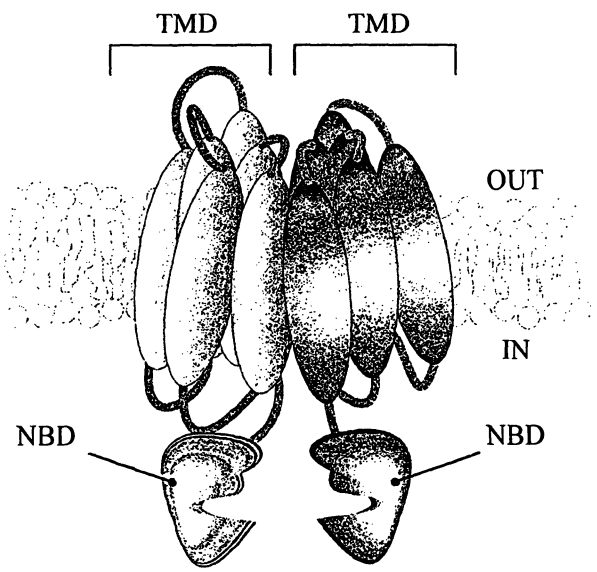

Fig. 2 Secondary Structure Model of the ABC-Type Multidrug Transporter LmrA of *Lactoccus lactis*.
LmrA consists of a transmembrane domain [TMD] with 6 transmembrane α-helices (depicted as ellipses), and a nucleotide-binding domain [NBD] containing the ABC family signature and Walker A/B motifs. In view of the twelve-transmembrane model proposed for the human multidrug resistance P-glycoprotein, LmrA may function as a homodimeric complex. OUT and IN refer to the outside and inside of the cytoplasmic membrane, respectively.

Besides the drug transporters LmrP and LmrA, *L. lactis* also possesses ATP-dependent drug extrusion systems with specificity for organic anions. One system in *L. lactis* has the ability to extrude the fluorescent pH indicator BCECF [2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein] (Molenaar *et al.*, 1991, 1992). BCECF efflux was directed against a concentration gradient and strictly correlated with the cellular ATP concentration. In addition, BCECF efflux was strongly decreased in the presence of ortho-vanadate, a well known inhibitor of P-type ATPases and ABC transporters. Most convincingly, an UV mutant with a strongly reduced efflux rate could be isolated from a BCECF-loaded and lactose-energized cell population by selection of highly fluorescent cells in a flow cytometer-cell sorter. Recently, evidence has been obtained for the presence of a second ATP-dependent transporter with specificity for the fluorescent pH indicator FTUG [N-(fluorescein thio-ureanyl)-glutamate] (Glaasker *et al.*, 1996).

Although true P-glycoprotein homologs have not yet been found in bacteria other than L. lactis, there is little doubt that many more systems remain to be identified. Recently, physiological evidence has been obtained for the presence of an ATP-dependent multidrug transporter in *Enterococcus hirae* (Midgley, 1994), *Enterobacter cloacae* (Aspedon and Nickerson, 1993), and the archaeon *Haloferax volcanii* (Miyauchi *et al.*, 1992). In *E. coli*, the *mdl* gene is predicted to encode a member of the ABC transporter superfamily closely related to the human multidrug resistance P-glycoprotein (Allikmets *et al.*, 1993). Functional studies on this *E. coli* protein have not yet been performed.

Molecular Mechanism of Multidrug Transporters

Several models have been postulated for the pump function of multidrug transporters to explain their broad specificity for chemically unrelated compounds. Drug translocation may involve substrate transport from the cytoplasm to the exterior via an aqueous pore with flexible 'enzyme-like' substrate recognition sites (conventional transport hypothesis) (Altenberg *et al.*, 1994). Alternatively, multidrug transporters could recognize the lipophilic drugs by their physical property to intercalate into the lipid bilayer, and transport drugs from the lipid bilayer to the exterior (vacuum cleaner hypothesis) (Raviv *et al.*, 1990), or from the inner leaflet to the outer leaflet of the lipid bilayer [flippase hypothesis] (Higgins and Gottesman, 1992) (Figure 3).

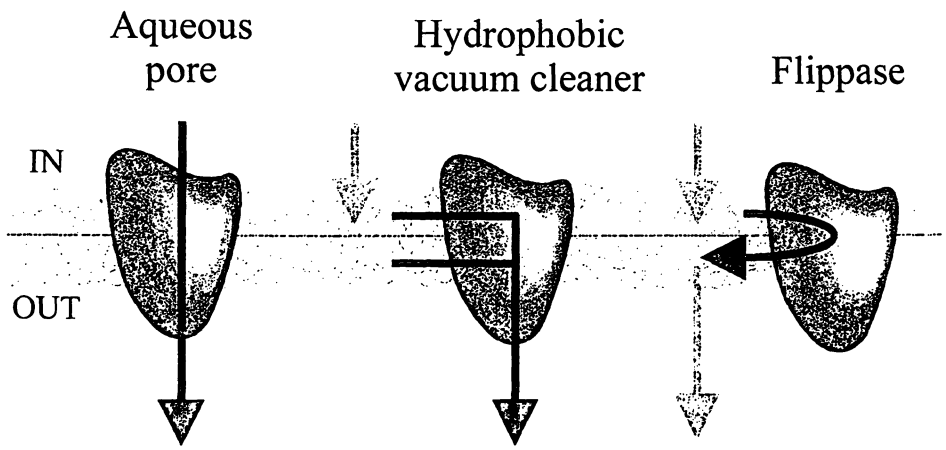

Fig. 3 Molecular Models for Drug Extrusion by Drug Transporters.
Partitioning of drugs between the water phase and phospholipid bilayer is indicated by a gray arrow. Protein-mediated drug transport is indicated by a black arrow. A drug transport protein may function as (i) an aqueous pore which transports drugs from the intracellular (IN) to the extracellular (OUT) water phase, (ii) a hydrophobic vacuum cleaner which transports drugs from the membrane to the extracellular environment, or (iii) a flippase which transports drugs from the inner to the outer leaflet of the phospholipid bilayer.

On the other hand, the extraordinary broad substrate specificity of multidrug transporters has led to the proposal of alternative, indirect mechanisms via which such proteins reduce the intracellular drug concentration. In one hypothesis, multidrug transporters may affect the electrochemical proton gradient across the plasma membrane (Simon *et al.*, 1994). Many of the transported drugs are weak bases having a positive charge at neutral pH. An increase in the transmembrane pH gradient (interior alkaline) and/or decrease in the transmembrane membrane potential (interior negative), observed in human multidrug resistant cancer cells (Roepe, 1992), would result in a decrease in the cellular drug concentration and, hence, in increased drug resistance. Currently, no information is available with respect to changes in the magnitude or composition of the proton motive force in multidrug resistant microorganisms. However, the observation of LmrA-mediated drug transport in the absence of a proton motive force argues against an indirect proton motive force-dependent mechanism, and favours a direct drug extrusion mechanism (van Veen *et al.*, 1996).

L. lactis cells expressing LmrA or LmrP were found to actively extrude the hydrophobic acetoxymethyl ester of BCECF (Bolhuis *et al.*, 1996b). Similar observations on the human multidrug resistance P-glycoprotein were taken as evidence for the transport of hydrophobic compounds from the plasma membrane (Homolya *et al.*, 1993). The transport mechnism of bacterial LmrA and LmrP has been studied in greater detail by using the amphiphilic membrane probe TMA-DPH [1-(4-[trimethylamino]phenyl)-6-phenylhexa-1,3,5-triene]. Intercalation of TMA-DPH in the phospholipid bilayer is based on a fast partitioning of the probe in the outer leaflet, followed by a slower transbilayer movement of the probe from the outer to the inner leaflet of the membrane. Remarkably, the effect of the partitioning of TMA-DPH in the membrane on the drug pumping activity of LmrA (Bolhuis *et al.*, 1996b) and LmrP (Bolhuis *et al.*, 1996a) suggests that both transporters extrude TMA-DPH from the inner leaflet of the membrane. Thus, LmrA and LmrP recognize drugs by their hydrophobic properties. In view of the structural and functional similarities between LmrA and Pgp, the LmrA-mediated transport of amphiphilic drugs from the inner leaflet of the phospholipid bilayer of *L. lactis* provides strong support for the hydrophobic vacuum cleaner model for the human multidrug resistance P-glycoprotein (Figure 3).

It has been proposed that the human multidrug resistance P-glycoprotein participates in the protection of human cells against hydrophobic xenobiotics by active excretion of these compounds from the membrane into bile, urine, or the intestinal lumen, and by preventing their accumulation in critical organs such as the brain (Schinkel *et al.*, 1994). Likewise, the physiological role of multidrug transporters in microorganisms could involve the efflux of amphiphilic compounds which originate from endogenous metabolism such as the oxidation of phospholipids or conjugation of xenobiotics, or amphiphilic compounds which are encountered in the extracellular environment. It is noteworthy that the natural environment of enteric bacteria is enriched in bile salts and fatty acids, and that these compounds are substrates for various multidrug transporters (Ma *et al.*, 1995; Thanassi *et al.*, 1997). Alternatively, microbial multidrug transporters may play a role in the transport of a common endogenous substrate, such as lipid (Smit *et al.*, 1993; Higgins, 1994; Ruetz and Gros, 1994; van Helvoort *et al.*, 1996), which remains to be established.

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Review

Avoidance of Iron Toxicity through Regulation of Bacterial Iron Transport[a]

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Under oxic conditions and at pH 7, ferric iron is insoluble, and complex formation of Fe^{3+} with ligands is required to supply cells with iron. Bacteria and fungi synthesize and secrete low-molecular-weight compounds, termed siderophores, that bind Fe^{3+}. Certain human pathogens take up iron from human transferrin, lactoferrin, hemoglobin, and heme. The ferric siderophores are actively transported into bacterial cells by highly specific transport systems. In Gram-negative bacteria, the ferric siderophores and iron released from the host proteins are actively transported across the outer membrane (OM). The electrochemical potential of the cytoplasmic membrane (CM) energizes transport across the outer membrane, which requires an energy-transducing device, consisting of the proteins TonB, ExbB and ExbD, from the CM to the OM. Active transport across the CM is energized by ATP hydrolysis. Transport is regulated at the level of gene transcription. In Gram-negative bacteria, this is controlled by the Fur protein, in most gram-positive bacteria, by the DtxR protein. Fur and DtxR act as repressors when loaded with Fe^{2+}. In the cytoplasm, iron is released from the siderophores by reduction to Fe^{2+}, and the siderophores are either inactivated or secreted. The intracellular iron is built into heme and non-heme iron proteins, and a small proportion is incorporated into bacterioferritin, but most of the iron is present in a poorly defined state. Iron overload results in iron toxicity, mainly due to the formation of hydroxyl radicals that strongly react with all kinds of biomolecules, of which DNA damage has the most deleterious consequences.

Key words: Iron / Regulation / Toxicity / Transport.

Introduction

The wide range of Fe^{2+}/Fe^{3+} redox potentials from –300 to +700 mV, depending on the iron ligands and the protein environment, may be the reason why iron is contained in many membrane-bound and soluble redox enzymes in almost all organisms. For the growth of 10^9 bacterial cells in 1 ml medium, 10^{14} iron ions are required. The concentration of free ferric ions in equilibrium with the ferric hydroxide polymer at pH 7 is 10^3 ions per ml, which demonstrates the extreme shortage of biologically available iron despite its great abundance in nature. To cope with iron limitation, bacteria synthesize and secrete siderophores, and they frequently can use siderophores synthesized and released into the surrounding by other bacteria and even fungi. The ferric siderophores have molecular masses of approximately 750 Da and they contain hydroxamate, catecholate, or carboxylate iron ligands. The formation constants of ferric siderophores is in the order of 10^{-23} to 10^{-35}, which enables them to complex the scarce iron ion in equilibrium with ferric hydroxide and with transferrin, lactoferrin, and ferritin in the human body. Some bacterial genera that are adapted to humans can use heme, hemoglobin, transferrin, lactoferrin, and hemopexin iron directly without interconnection of siderophores. For the uptake of ferric siderophores, heme, and iron released from iron-binding proteins, bacteria have developed highly sophisticated active transport systems. In Gram-positive bacteria, the iron complexes have direct access to the cytoplasmic membrane through which ferric iron and ferric siderophores are transported by a mechanism which in principle, but perhaps not in every detail, is common for all ferric iron sources in Gram-positive and in Gram-negative bacteria. The same mechanism is used for the transport of certain amino acids, peptides, sugars, and inorganic anions into bacteria. In Gram-negative bacteria, ferric iron and ferric siderophores also have to be transported across the outer membrane; this involves a unique transport mechanism.

[a] This article is an expanded version of the lecture given at the Meeting of the German Society of Biological Chemistry and Molecular Biology 1997 at the University of Tübingen.

Transport of Ferric Siderophores across the Outer Membrane of Gram-Negative Bacteria

Ferric siderophores are extracted from the culture medium and concentrated at the bacterial cell surface by binding to outer membrane receptor proteins. The receptors have a high specificity for the ferric siderophores, which mainly determines which ferric siderophore is used as iron donor for a particular strain. A certain receptor only binds ferric siderophores with very similar structural elements. *Escherichia coli* K-12, the standard laboratory strain, synthesizes two receptors for ferric hydroxamates, three receptors for

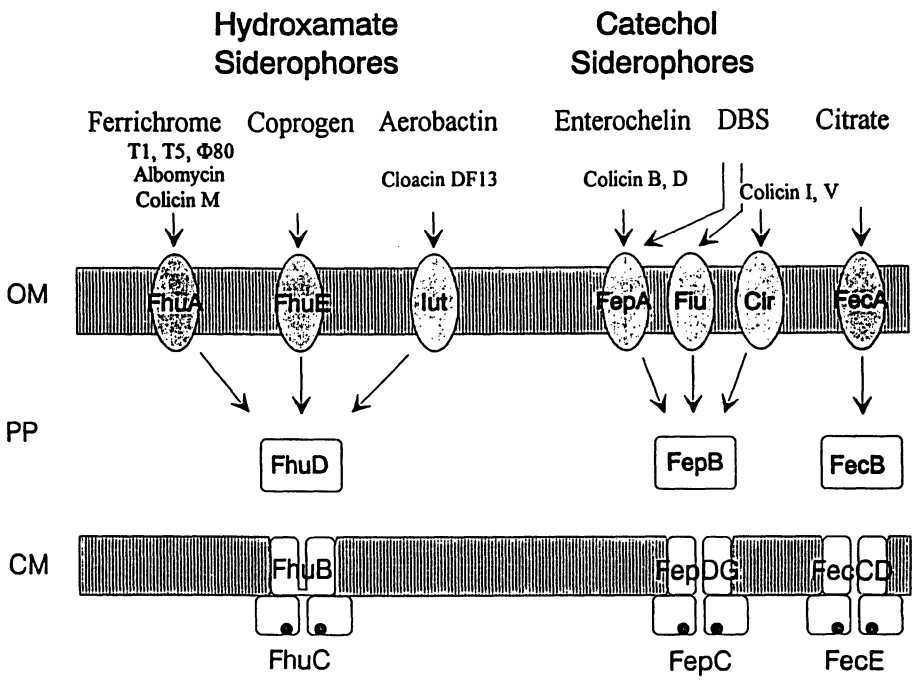

Fig. 1 Ferric Iron Transport Systems of *Escherichia coli* K-12.
Multifunctional receptor proteins in the outer membrane (OM) bind specifically the ferric siderophores and transport them into the periplasm (PP) where the ferric hydroxamates bind to the FhuD protein, the ferric catecholates bind to the FepA protein, and ferric citrate binds to the FecB protein. The periplasmic binding proteins donate the ferric siderophores to transport proteins in the cytoplasmic membrane (CM). Transport across the CM is energized by ATP hydrolysis catalyzed by the FhuC, FepC, and FecE proteins, which are associated with the CM. For the sake of clarity, the Ton system, composed of the TonB, ExbB, and ExbD proteins, and which catalyzes transport across the OM, is not included in the Figure. DBS, dihydroxybenzoyl serine. The receptors also serve for infection by the phages T1, T5, and ϕ80, and for the uptake of the antibiotic albomycin and the colicins B, D, I, M, and V, and cloacin DF13, which are bacterial protein toxins.

ferric catecholates, and one receptor for ferric citrate (Figure 1).

Once bound to the receptors, the ferric siderophores have to be transported into the periplasm. An idea how this might happen was obtained when a 34-amino-acid residue fragment of the FhuA receptor for the uptake of ferrichrome (Figure 1) was excised by genetic means. This fragment represents the largest FhuA loop at the cell surface (Koebnik and Braun, 1993) and its removal converted FhuA into a permanently open channel (Killmann *et al.*, 1993). Uptake of ferrichrome into cells that synthesized the FhuA deletion derivative (FhuAΔ322-355) is diffusion-controlled and no longer displays saturation kinetics. This demonstrates that within the concentration range used, the rate of ferrichrome uptake into cells that synthesize wild-type FhuA is determined by the transport rate across the outer membrane and not by the transport rate across the cytoplasmic membrane. Other substances, such as SDS and the antibiotic bacitracin, for which the outer membrane of wild-type cells forms a permeability barrier, diffuse through the FhuA deletion derivative and kill the cells. A fine-mapping of the region corresponding to the excised fragment (gating loop) with hexapeptides representing amino acid sequences of the gating loop disclosed three subdomains for uptake of ferrichrome, infection by the bacteriophages T1, T5 and ϕ80, and import of colicin M, all of which use FhuA as a receptor (Killmann *et al.*, 1995). The properties of other FhuA derivatives, in which fragments in the gating loop are excised, support the assignment of functional subdomains within the gating loop (Killmann *et al.*, 1996). The recent determination of the nucleotide sequence of the *fhuA* gene of *Salmonella paratyphi*, *Salmonella typhimurium*, and *Pantoea agglomerans* revealed that these FhuA proteins have deletions in the gating loop, as compared with the *E. coli* gating loop (Killmann *et al.*, unpublished results). The ferrichrome uptake and phage binding receptor properties of the FhuA proteins agree with the predictions derived from the *E. coli* FhuA studies. Incorporation of FhuAΔ322-355 into artificial black lipid membranes results in an open channel through which cations and anions diffuse at similar rates. Wild-type FhuA does not increase the conductance of lipid bilayer membranes. Single-channel conductance measurements of the open FhuA predict a channel three times as large as the diameter of the *E. coli* outer membrane porin channels (Killmann *et al.*, 1993). Addition of phage T5 to FhuA integrated in planar lipid bilayer membranes opens a channel (Bonhivers *et al.*, 1996), which exhibited electrophysiological properties similar to FhuAΔ322-355. This demonstrates that the phage creates the channel for the transfer of the phage DNA across the outer membrane and that the experimentally created channel reflects the properties of the naturally formed channel. If FhuA formed a permanently open channel of this size, *E. coli* would be vulnerable to deleterious compounds, such as bile salts in the gut.

Excision of 139 residues from the ferric enterobactin receptor FepA results in an open channel that allows nonspecific diffusion of unrelated compounds through the outer membrane of *E. coli* (Rutz *et al.*, 1992). The two receptors, FhuA and FepA, are probably typical for all regulated protein channels used for the transport of all ferric siderophores across the outer membranes of gram-negative bacteria.

Energy Transfer from the Cytoplasmic Membrane into the Outer Membrane

If FhuA forms a closed channel that is occasionally opened for the transport of ferrichrome, then the question arises how the channel is opened. This poses a very intriguing and interesting problem. Energy input is required for either opening or closing a channel. However, the outer membrane does not contain an energy source. Older data from the time when molecular biology was being founded indicated that an additional genetic locus, designated *tonB*, is required for infection of *E. coli* cells via the *fhuA* determinant (then designated *tonA*) by phage T1 (*ton* derived from *T-one*). It was later shown that the electrochemical potential of the cytoplasmic membrane is the energy source for adsorption of phage T1 to FhuA-containing cells, and it was proposed that TonB somehow transmits the energy from the cytoplasmic membrane to the FhuA receptor in the outer membrane (Hancock and Braun, 1976). This concept is still valid and is supported by many additional data, which, however, do not disclose the mechanism of TonB energization and of TonB-mediated energy transfer. The topology of TonB supports a function in energy transfer. TonB is inserted in the cytoplasmic membrane through its N-terminus, and most of the protein is located in the periplasm (Postle and Skare, 1988). TonB interacts with the FhuA receptor (Schöffler and Braun, 1989; Günter and Braun, 1990) and with the FepA receptor involved in the transport of ferric enterobactin (Skare *et al.*, 1993). Our current concept proposes that TonB senses the electrochemical potential of the cytoplasmic membrane and assumes an energized conformation. TonB binds to FhuA, which leads to the opening of the FhuA channel, and then TonB folds back into the nonenergized conformation. This cycle is then repeated with FhuA or with other ferric siderophore receptors. The activity of only those outer membrane proteins depends on TonB which transport ferric siderophores, transferrin, lactoferrin, hemoglobin iron, and vitamin B_{12} (Kadner, 1990).

Two additional proteins, ExbB and ExbD, are required for TonB activity (Ton system). ExbB spans the cytoplasmic membrane three times, and most of the protein is located in the cytoplasm (Kampfenkel and Braun, 1993). ExbD is arranged like TonB with the N-terminus in the cytoplasmic membrane and the remainder of the polypeptide in the periplasm (Kampfenkel and Braun, 1992). TonB, ExbB, and ExbD interact with each other (Fischer *et al.*, 1989; Larsen *et al.*, 1994; Braun *et al.*, 1996), which suggests that they form a functional complex for TonB energization and energy transfer from the cytoplasmic membrane to the outer membrane.

Transport across the Outer Membrane of Heme and of Iron Released from Transferrin and Lactoferrin

The first heme transport system was characterized in *Yersinia enterocolitica* (Stojiljkovic and Hantke, 1992, 1994). The system consists of an outer membrane receptor protein that requires the Ton system for heme transport across the outer membrane. Further transport across the periplasm and the cytoplasmic membrane is catalyzed by proteins that are similar to those of ferric siderophore transport systems (see below). Similar heme transport systems have been partially characterized in two other human pathogens, *Shigella dysenteriae* and *Vibrio cholerae*.

Other bacterial pathogens, such as *Neisseria meningitidis* (Stojiljkovic *et al.*, 1995), *Vibrio cholerae* (Torres and Payne, 1997), and *Haemophilus ducreyi* (Stevens *et al.*, 1996) extract heme in an unknown way from hemoglobin bound to outer membrane receptors. A very interesting case of heme mobilization is found in *Serratia marcescens,* which secretes a protein that is able to release heme from hemoglobin (Letoffe *et al.*, 1994). *Neisseria meningitidis* contains a receptor in the outer membrane that binds hemoglobin and the hemoglobin-haptoglobin complex (Lewis *et al.*, 1997). The receptor consists of two proteins, one of which is a lipoprotein with a covalently bound lipid of the type found in the murein lipoprotein (Hantke and Braun, 1973; Braun and Wu, 1994). The same type of two-component receptors of *N. meningitidis, N. gonorrhoeae,* and *Haemophilus influenzae* (Gray-Owen and Shrivers, 1996) bind iron-loaded transferrin (Anderson *et al.*, 1994) and iron-loaded lactoferrin (Pettersson *et al.*, 1994). It is not known how iron is released from transferrin and lactoferrin and how it is transported across the outer membrane. *Neisseria gonorrhoeae* when mutated in the *tonB* gene, is unable to grow on human transferrin, lactoferrin, and hemoglobin as sole iron source and is unable to take up radioactive iron from transferrin and lactoferrin (Biswas *et al.*, 1997). At present, it is not known which step of the iron uptake – release of iron from the proteins or the presumed opening of the receptor channel for translocation of iron across the outer membrane – depends on the Ton system and energization. Of interest is the high specificity of the receptors, which in the case of human pathogens, recognize transferrins and lactoferrins only from humans and primates (Gray-Owen and Shrivers, 1996).

Transport of Ferric Iron and Ferric Siderophores across the Cytoplasmic Membrane

All the ferric iron transport systems involve a periplasmic binding protein, one or two hydrophobic integral transport

proteins in the cytoplasmic membrane, and one or two proteins associated with the inside of the cytoplasmic membrane that contain the two Walker nucleotide binding motifs and function as ATP hydrolases (Figure 1). The periplasmic-binding-protein-dependent transport systems are the most frequently occurring substrate transport systems in bacteria. They belong to a superfamily of ATP-dependent transporters (traffic ATPases), which in bacteria also export certain proteins, polysaccharides, and toxic compounds. Eukaryotic members of the superfamily are the multidrug resistance protein, the cystic fibrosis transmembrane conductance regulator, and the a-factor secretory yeast protein. With the reconstituted maltose and histidine transport components in liposomes, it has been shown that the substrate-loaded binding proteins trigger ATP hydrolysis which is required for substrate translocation across the lipid bilayer (summarized and discussed by Boos and Lucht, 1996). For the ferrichrome transport system of *E. coli*, binding of ferrichrome to the binding protein FhuD (Köster and Braun, 1990; Rohrbach *et al.*, 1995a, b), interaction of FhuD with the integral transport protein FhuB (Rohrbach *et al.*, 1995a), and FhuB-mediated binding of the FhuC ATPase to the inner side of the cytoplasmic membrane (Schultz-Hauser *et al.*, 1992) has been demonstrated.

Peptides identical in sequence to a proposed periplasmic loop, and unexpectedly, corresponding to a transmembrane segment and a connected cytoplasmic region of the FhuB protein, bind to the isolated FhuD protein and inhibit ferrichrome transport into cells. These data suggest that FhuD not only interacts with regions of the FhuB protein which are exposed to the periplasm, but inserts deeply into and even crosses the cytoplasmic membrane. Moreover, the cytoplasmic loop of FhuB that interacts with FhuD is proposed to function as a binding site of the FhuC ATPase (Köster and Böhm, 1992). The available evidence suggests that the FhuD and FhuC binding sites on FhuB are close to each other but do not overlap. Triggering of FhuC-catalyzed ATP hydrolysis by ferrichrome-loaded FhuD would occur at the same cytoplasmic loop and would not require a signal transmitted through the cytoplasmic membrane via FhuB to FhuC, as has been proposed for induction of ATP hydrolysis by the cytoplasmic MalK through maltose-loaded periplasmic MalE (Davidson *et al.*, 1992).

It is likely that transport of ferric iron, ferric siderophores, and heme across the cytoplasmic membrane occurs basically by the same mechanism. Ferric iron transport across the cytoplasmic membrane was first demonstrated in *S. marcescens* (Zimmermann *et al.*, 1989; Angerer *et al.*, 1990). Sequence similarities of the genes of other human pathogens with the *S. marcescens sfuCDB* genes suggest that they determine proteins for ferric iron transport across the cytoplasmic membrane (Table 1). It is not known whether ferric iron as such or in complexed form is transported across the cytoplasmic membrane. It is likely, but not rigorously proven, that after release of iron from transferrin and lactoferrin and entry into the periplasm, iron is transported across the cytoplasmic membrane by the systems listed in Table 1.

Regulation of the Iron Content in Bacteria

Bacteria regulate their iron content for economic reasons and to avoid iron toxicity. The intracellular iron concentration is measured by the Fur protein in Gram-negative bacteria and by the DtxR protein in most (GC-rich) Gram-positive bacteria. Fur of *E. coli* is a histidine-rich protein composed of 148 amino acids (Schäffer *et al.*, 1985). DtxR of *Corynebacterium diphtheriae* contains 226 residues and shares only 25 % amino acid sequence similarity with the *E. coli* Fur protein (Boyd *et al.*, 1990). Fe^{2+} binds to Fur and DtxR and converts these proteins into repressors that inhibit transcription of genes that determine ferric siderophore synthesis and transport proteins. Footprinting within the promoter region of the biosynthesis genes for aerobactin, a bacterial siderophore, has revealed interaction of the Fur protein with a 19-bp-long degenerate palindromic sequence (de Lorenzo *et al.*, 1987), which is of the same minimal size that is covered by the DtxR protein in the promoter region of the *tox* gene that encodes diphtheria toxin (Tao and Murphy, 1994). X-ray analysis of DtxR crystals has revealed that the second and third helix forms the helix-turn-helix motif that binds to the iron-regulated promoter DNA. A similar region in the N-terminus of Fur is predicted to bind to DNA, based on NMR studies and sequence similarities to the known LexA protein (Holm *et al.*, 1994). Negative complementation of wild-type Fur by mutated inactive Fur (Braun *et al.*, 1990), and visualization of Fur bound to promoter DNA by electron microscopy and atomic force microscopy (Le Cam *et al.*, 1994) have shown that Fur functions as a multimer. Studies with genetically constructed Fur–λcI hybrid proteins indicate dimerization

Table 1 Ferric Iron Transport Systems of the Sfu Type.

Periplasm	Cytoplasmic membrane	Cytoplasmic ATPase	Strain	Reference
SfuA	SfuB	SfuC	*Serratia marcescens*	Angerer *et al.*, 1990
FbpA	FbpB	FbpC	*Neisseria gonorrhoeae*[a]	Adhikari *et al.*, 1996; Berish *et al.*, 1990
HitA	HitB	HitC	*Haemophilus influenzae*	Adhikari *et al.*, 1995
YfuA	YfuB	YfuC	*Yersina enterocolitica*	Saken and Heesemann, 1995; EMBL Acc. No. Z47200
AfuA	AfuB	AfuC	*Actinobacillus pleuropneumoniae*	Chin *et al.*, 1996

[a] also *Neisseria meningitidis*

of Fur, and interaction of the Fur N-terminus with promoter DNA (Stojiljkovic and Hantke, 1995).

Induction of Transport Genes by Ferric Siderophores: Induction from Without

Besides derepression of iron transport genes when transport is needed under conditions of iron deprivation, synthesis of transport proteins through induction of the genes by the transported ferric siderophores is another economical way to control the iron supply. Regulation of the ferric citrate transport system of *E. coli* K-12 has been studied in most detail (Braun, 1997). Transcription of the five transport genes *fecABCDE* is induced by ferric citrate in the culture medium. For induction, ferric citrate does not have to enter the cytoplasm (Zimmermann *et al.*, 1984). Ferric citrate bound to the outer membrane transport protein FecA induces transcription (Härle *et al.*, 1995). FecA occupied by ferric citrate transmits a signal into the periplasm where the regulatory protein FecR is located. Ferric citrate does not induce *fec* transcription in the absence of FecR. C-terminally truncated FecR derivatives no longer respond to ferric citrate and induce *fec* transcription constitutively (Ochs *et al.*, 1995). FecR is anchored to the cytoplasmic membrane such that residues 1–85 are located in the periplasm, residues 86–100 span the cytoplasmic membrane, and residues 101–317 extend into the periplasm (Ochs *et al.*, 1995). This arrangement enables FecR to transmit the induction signal across the cytoplasmic membrane into the cytoplasm. Transcription is initiated by the *fec*-specific sigma factor FecI, which directs the *E. coli* RNA polymerase core enzyme to the *fec* transport gene promoter upstream of the *fecA* gene. The *fecI fecR* regulatory genes are encoded upstream of the *fec* transport genes and are not autoregulated by FecI and FecR, but repressed by the Fur protein when Fur is loaded with Fe^{2+}. An *E. coli* mutant with a deletion of the entire *fec* operon transcribes in response to added ferric citrate a plasmid-encoded *fecA-lacZ* gene fusion or a *fecB-lacZ* operon fusion when transformed with the genes *fecI, fecR, fecA, tonB, exbB,* and *exbD*. Signal transduction across the outer membrane requires the same gene products as transport of ferric citrate across the outer membrane. However, transport can be uncoupled from induction by mutations in *fecA*. For example, removal of residues 47–101 (numbering includes the signal sequence from residues 1–33), which have been localized in the periplasm, abolishes signal transduction but fully retains the transport activity (Kim *et al.*, 1997). Overproduction of the N-terminal fragment from residues 1 to 127 of FecA (complete FecA contains 741 residues) interferes with ferric citrate induction, but not with ferric citrate transport. These data suggest that the N-terminus of FecA interacts with the C-terminus of FecR for induction of *fec* transport gene transcription.

Induction by ferric citrate and its transport requires the electrochemical potential of the cytoplasmic membrane. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibits induction and transport, while a *fecA* mutant that induces but does not transport independent of TonB induces also in the presence of CCCP (Kim *et al.*, 1997).

In vitro, transcription of a *fecA* DNA fragment depends on the presence of FecI and RNA polymerase core enzyme. Transcription is strongly enhanced in crude extracts of cells that synthesize FecA, FecI, FecR, TonB, ExbB, and ExbD and that are grown in a medium containing ferric citrate (Angerer *et al.*, 1995; Enz *et al.*, 1995). No covalent modification such as phosphorylation of the regulatory proteins has been found. These data are consistent with the following model: Binding of ferric citrate to FecA in the outer membrane of *E. coli* induces a conformational change in FecA. Induction-competent FecA changes the conformation of FecR by interaction through its N-terminus with the C-terminus of FecR. FecR in the induction-competent conformation activates FecI, which binds to the RNA polymerase core enzyme. The FecI – RNA-polymerase complex binds to the promoter upstream of *fecA* and initiates transcription of the *fec* transport genes.

The ferric siderophores pseudobactin BN7 and BN8 induce transcription of the ferric pseudobactin transport system in *Pseudomonas putida* by a mechanism that strongly resembles ferric citrate induction. Transcription of *pupB*, which encodes an outer membrane transport protein, is induced by the ferric pseudobactins. Two genes, *pupI* and *pupR*, with sequence similarities to *fecI* and *fecR*, respectively, regulate *pupB* transcription. The N-terminus of PupB is important for transcription induction since its replacement by the N-terminus of the similar PupA receptor abolishes induction, but retains transport activity for pseudobactin BN7. Replacement of the N-terminus of PupA by the N-terminus of PupB results in a hybrid protein that induces *pupB* transcription in response to pseudobactin 358, which is recognized by PupA (Koster *et al.*, 1994).

Induction of transport gene transcription by the cognate ferric siderophores seems to be the rule in *Pseudomonas* (Venturi *et al.*, 1995), and has been found in some other bacterial genera, but the control mechanism does not involve transcription initiation by ligand interaction at the cell surface and signal transfer to the cytoplasm.

Iron-Mediated Oxic Stress

Aerobic metabolism creates hydrogen peroxide and superoxide radicals (Figure 2). In a mixture of Fe^{2+} and H_2O_2, highly reactive hydroxyl radicals are formed. Superoxide reduces Fe^{3+} to Fe^{2+} and molecular oxygen. The sum of both reactions yields oxygen, hydroxyl radicals, and hydroxyl anions. The superoxide radicals are destroyed by superoxide dismutase; the manganese-containing form of superoxide dismutase of *E. coli* is encoded by the *sodA* gene, and the iron-containing form by the *sodB* gene. Fur is required for the synthesis of the iron superoxide dismutase during aerobic growth (Niederhoffer *et al.*, 1990). It is

$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad \text{Fenton}$$

$$Fe^{3+} + O_2^{-\cdot} \Rightarrow Fe^{2+} + O_2$$

$$O_2^{-\cdot} + H_2O_2 \Rightarrow O_2 + OH^{\cdot} + OH^{-} \quad \text{Haber-Weiss}$$

$$O_2^{-\cdot} + O_2^{-\cdot} \Rightarrow H_2O_2 + O_2 \quad \text{Superoxide dismutase}$$

$$H_2O_2 + H_2O_2 \Rightarrow 2\,H_2O + O_2 \quad \text{Catalase}$$

$$H_2O_2 + AH_2 \Rightarrow 2\,H_2O + A \quad \text{Peroxidase}$$

Fig. 2 Formation of Reactive Oxygen Radicals in the Fenton and Haber-Weiss Reactions and Their Enzymatic Destruction.

not clear whether Fur activates *sodB* transcription directly or via regulation of a control protein, for example, by repressing the transcription of a repressor. In cells grown anaerobically, Fur represses transcription of *sodA* (Tardat and Touati, 1993) which is presumably not needed under these conditions. In an *E. coli fur* mutant, iron uptake is no longer adjusted to the iron requirement; this results in iron overload. This mutant shows an increased oxygen-dependent mutation rate and is not viable under oxic conditions when it carries an additional mutation in the *recA* gene, which is required for DNA repair (Touati *et al.*, 1995). Reduction of ferric iron transport by a mutation in the *tonB* gene, overproduction of the iron storage protein FTNF, addition of ferrozine, which chelates intracellular iron, and supplementation with the hydroxyl radical scavengers dimethyl sulfoxide and thiourea prevent killing of the cells.

In *E. coli,* the superoxide radical induces transcription of *sodA* via the [2Fe-2S] protein SoxR, which binds to the *soxS* promoter and in the oxidized form activates *soxS* transcription (Hidalgo *et al.*, 1997). SoxS serves as a transcriptional activator of a number of stress-induced promoters. The iron-sulfur center of SoxR serves as a redox indicator that is not destroyed upon reduction, but functions reversibly.

Cell damage by intracellular iron also has to take into account intracellular iron metabolism, in particular iron storage in compounds that do not actively participate in intermediary metabolism. Mössbauer spectroscopy indicates that most of the iron in *E. coli* is not incorporated into redox enzymes, but is present in a poorly defined state, such as one compound that seems to be an acidic polysaccharide that contains 40% of the 'mobile iron pool' (Böhnke and Matzanke, 1995). A number of bacterial ferritins have been identified and sequenced, and even an X-ray structure is available. However, the amounts of the ferritins are too low to serve as major iron storage compounds and their role in iron metabolism still has to be elucidated (discussed in Braun *et al.*, 1998).

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Review

Transport of Glutathione Conjugates and Glucuronides by the Multidrug Resistance Proteins MRP1 and MRP2

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The search for the membrane proteins mediating the ATP-dependent transport of conjugates with glutathione, glucuronate, or sulfate has led to the identification of the multidrug resistance proteins MRP1 and MRP2. Both 190-kDa membrane glycoproteins were cloned in the recent years and shown to be unidirectional ATP-driven export pumps with an amino acid identity of 49% in human. MRP1 is detected in the plasma membrane of many cell types, including erythrocytes, whereas MRP2, also termed canalicular MRP (cMRP) or canalicular multispecific organic anion transporter (cMOAT), has been localized to the apical domain of polarized epithelia, particularly to the hepatocyte canalicular membrane. Physiologically important substrates of both transporters include glutathione *S*-conjugates such as leukotriene C_4, bilirubin glucuronides, 17β-glucuronosyl estradiol, dianionic bile salts such as 6α-glucuronosyl hyodeoxycholate, and glutathione disulfide. Both transporters have been associated with multiple drug resistance of malignant tumors because of their capacity to pump drug conjugates and drug complexes across the plasma membrane into the extracellular space. The substrate specificity of MRP1 and MRP2 is very different from MDR1 P-glycoprotein. MRP1 and MRP2 may be termed conjugate transporting ATPases functioning in detoxification and, because of their role in glutathione disulfide export, in the defense against oxidative stress.

Key words: ABC transporters / ATP-dependent transport / Glucuronides / Glutathione *S*-conjugates / Multidrug resistance proteins / Transport ATPases.

Introduction

It has long been known that glutathione *S*-conjugates and glucuronides are transported across cellular membranes (Combes, 1965; Billing, 1978; Wahlländer and Sies, 1979). This process has been characterized as a primary-active, ATP-dependent transport by studies using inside-out plasma membrane vesicles from many cell types including erythrocytes (Kondo *et al.*, 1980, 1982) and hepatocytes (Kobayashi *et al.*, 1988; Ishikawa *et al.*, 1990; Kitamura *et al.*, 1990; Akerboom *et al.*, 1991; Fernández-Chéca *et al.*, 1992). Identification of the membrane proteins mediating the ATP-dependent unidirectional transport of glutathione *S*-conjugates and glucuronides was closely linked to the characterization of the 190 kDa membrane glycoprotein mediating the ATP-dependent export of the endogenous glutathione *S*-conjugate leukotriene C_4 (LTC_4) (Leier *et al.*, 1994a). These studies resulted in the elucidation of the function of the multidrug resistance protein (MRP1) as a primary-active ATP-dependent membrane transporter for leukotriene C_4 and many other amphiphilic anionic conjugates (Jedlitschky *et al.*, 1994; Leier *et al.*, 1994b; Müller *et al.*, 1994; Jedlitschky *et al.*, 1996; Loe *et al.*, 1996a, b).

MRP1 is a membrane glycoprotein of an apparent molecular mass of 190 kDa that was identified on the basis of its overexpression in multidrug-resistant tumor cell lines (Cole *et al.*, 1992; Zaman *et al.*, 1993; Krishnamachary *et al.*, 1994). The original sequence analysis of MRP1 indicated that it is a member of the ATP-binding cassette (ABC) superfamily of transporters (Cole *et al.*, 1992). An isoform of MRP1 with a similar substrate specificity and a distinct, although related, sequence has been cloned recently and localized predominantly to the hepatocyte canalicular membrane (Büchler *et al.*, 1996; Paulusma *et al.*, 1996; Keppler and Kartenbeck, 1996; Taniguchi *et al.*, 1996; Keppler and König, 1997). This isoform has been termed MRP2, or canalicular MRP (cMRP), or canalicular multispecific organic anion transporter (cMOAT). MRP2 mediates the ATP-dependent transport of glutathione *S*-conjugates and glucuronides from the liver into bile (Keppler and König, 1997; Jedlitschky *et al.*, 1997) and across the apical membrane of kidney proximal tubules (Schaub *et al.*, 1997). Both MRP1 and MRP2 may be termed conjugate transporting ATPases with a broad substrate specificity.

Sequence Comparison and Localization of MRP1 and MRP2

Human MRP1 and MRP2 are composed of 1531 and 1545 amino acids, respectively. They contain two characteristic ATP-binding domains, and belong to the MRP family of

transporters within the superfamily of ABC transporters (Cole *et al.*, 1992; Keppler and König, 1997). Comparison between human MRP1 and MRP2 indicates an overall amino acid sequence identity of 49%; the highest degree of amino acid identity is in the carboxyl-terminal domain and, of course, in both nucleotide-binding domains (Büchler *et al.*, 1996). The molecular mass of unglycosylated MRP1 and MRP2 is 172 kDa (Cole *et al.*, 1992) and 174 kDa (Taniguchi *et al.*, 1996), respectively. Both are *N*-glycosylated in their mature form and exhibit an apparent molecular mass of about 190 kDa (Table 1). The number of transmembrane segments predicted for MRP1 has varied between 14 (Zaman and Borst, 1996) and 18 (Loe *et al.*, 1996c). The predicted topology model for MRP2 suggests 13 transmembrane segments (Keppler and König, 1997), and this topology model proposes an extracellular position of the amino-terminus (Büchler *et al.*, 1996). Four transmembrane segments are predicted between the first and second nucleotide-binding domain (Büchler *et al.*, 1996; Keppler and König, 1997).

Based on the amino acid sequence identity of the currently known members of the MRP family of transporters, human MRP2 and its homologs from rabbit and rat are closely related to human MRP1 and its homologs from mouse and rat (Keppler and König, 1997). The ABC transporters MRP1 and MRP2 from the soil nematode *Caenorhabditis elegans* are 43% identical and the conjugate-transporting cadmium resistance factor from yeast, termed YCF1 or YDR135c (Szczypka *et al.*, 1994) is 41% identical to MRP2. The long-known members of the P-glycoprotein family, some of which have been associated with multiple drug resistance, are only distantly related to MRP1 and MRP2. The amino acid sequence identity between MRP1 and MDR1 P-glycoprotein is only 15% (Cole *et al.*, 1992). The amino acid identity between MRP2 and the MDR1 and MDR2 P-glycoproteins is 25% and 24%, respectively.

Localization of the conjugate-transporting ATPases encoded by the *MRP1* and the *MRP2* (*cMRP/cMOAT*) genes by immunofluorescence microscopy and confocal laser scanning microscopy indicated the predominant localization of both export pumps in the plasma membrane, particularly of tumor cells (Hipfner *et al.*, 1994; Flens *et al.*, 1996; Büchler *et al.*, 1996; Keppler and Kartenbeck, 1996).

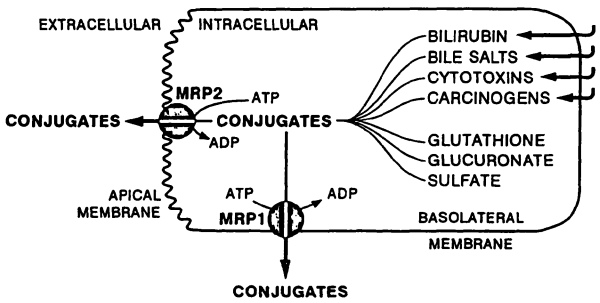

Fig. 1 Conjugation and ATP-Dependent Export of Endogenous and Xenobiotic Substances by the Multidrug Resistance Proteins MRP1 and MRP2.
These conjugate transporting ATPases are localized at the basolateral membrane (MRP1) or at the apical membrane domain (MRP2) of polarized cells.

In polarized epithelial cells, such as hepatocytes, MRP1 appears to be localized to the lateral membrane domain (Mayer *et al.*, 1995; Keppler and Kartenbeck, 1996; Roelofsen *et al.*, 1997), whereas MRP2 is strictly localized to the apical domains of hepatocytes (Büchler *et al.*, 1996) and proximal tubule epithelial cells of the kidney (Schaub *et al.*, 1997) (Figure 1).

Substrate Specificity of the Conjugate Transporting ATPases MRP1 and MRP2

The substrate specificity of recombinant human MRP1 determined in membrane vesicles from transfected cells is indistinguishable from that determined in membrane vesicles from drug-selected MRP1-overexpressing cells, such as the HL60/ADR line (Jedlitschky *et al.*, 1994, 1996; Leier *et al.*, 1994b). The glutathione *S*-conjugate LTC_4 is the substrate with the highest affinity known at present, whereas ATP-dependent transport of oxidized glutathione (GSSG) is a relatively low affinity process with a K_m value of 93 μM (Leier *et al.*, 1996). Reduced glutathione (GSH) itself is not a substrate for MRP1, however, it may undergo complex formation or serve as a co-substrate with cationic substances. This is indicated by the ATP-dependent transport of vincristine in the presence of GSH

Table 1 Comparison of the Human Conjugate Transporting ATPases MRP1 and MRP2.

	MRP1	MRP2 (cMRP/cMOAT)
Chromosomal localization of gene	16p13.1[a]	10q24[b]
Total amino acids	1531[a]	1545[b,c]
Cellular localization	plasma membrane (ubiquitous, basolateral)[d,e]	apical plasma membrane domain[f,g]
Glycoprotein mass	~190 kDa[d,e]	same[f,i]
Structure/topology	ABC transporter, 4+9 predicted transmembrane segments[c]	same[c]
Function	ATP-dependent transport of anionic conjugates[h,i,j,k]	same[c,f,g,i]
High-affinity substrates	LTC_4, estradiol 17β-glucuronide[j,k,l]	same[c,f,i]
Inhibitors	amphiphilic anions, e.g. LTD4 receptor antagonists[h,i,j]	same[f,i]

[a] Cole *et al.*, 1992; [b] Taniguchi *et al.*, 1996; [c] Keppler and König, 1997; [d] Loe *et al.*, 1996c; [e] Zaman and Borst, 1996; [f] Büchler *et al.* 1996; [g] Keppler and Kartenbeck, 1996; [h] Jedlitschky *et al.*, 1994; [i] Jedlitschky *et al.*, 1997; [j] Leier *et al.*, 1994b; [k] Jedlitschky *et al.*, 1996; [l] Loe *et al.*, 1996a.

(Loe *et al.*, 1996b). The glutathione moiety is not a structural determinant of the substrate properties of MRP1 as indicated by the efficient transport of the *N*-acetylcysteine *S*-conjugate *N*-acetyl-LTE_4 (Leier *et al.*, 1994b), and of the glucuronate conjugates 17β-glucuronosyl estradiol, monoglucuronosyl bilirubin and bisglucuronosyl bilirubin (Jedlitschky *et al.*, 1996, 1997). These results indicate that the term glutathione S-conjugate (GS-X) pump (Ishikawa, 1992) is not appropriate for the description of the function of MRP1. On the other hand, many organic anions, including monoanionic bile salts, are not transported by MRP1. Therefore, the term multispecific organic anion transporter (MOAT) suggests a substrate specificity much broader than the actual specificity of MRP1. A ranking of the substrates for MRP1 according to the V_{max}/K_m ratios was as follows:

LTC_4 > LTD_4 > *S*-(2,4-dinitrophenyl)glutathione > 17β-glucuronosyl estradiol > monoglucuronosyl bilirubin > 3α-sulfatolithocholyl taurine > GSSG.

The substrate specificity of MRP2 has been studied most extensively in inside-out hepatocyte canalicular membrane vesicles from normal rat liver in comparison with membrane vesicles from mutant rats lacking MRP2 (Ishikawa *et al.*, 1990; Büchler *et al.*, 1996). ATP-dependent transport of substrates by human MRP2 has been analyzed in membrane vesicles from HepG2 hepatoma cells cultured under conditions with a predominant expression of MRP2 (Jedlitschky *et al.*, 1997). The ranking of substrates for rat (and human) MRP2 according to the V_{max}/K_m ratio was as follows:

LTC_4 > LTD_4 > *S*-(2,4-dinitrophenyl)glutathione > monoglucuronosyl bilirubin > 17β-glucuronosyl estradiol > 3α-sulfatolithocholyl taurine > GSSG.

A similar spectrum of substrates for MRP2 in the rat liver canalicular membrane was suggested by *in vivo* measurements of the hepatobiliary elimination of substances in normal and transport-deficient mutant rats (Jansen *et al.*, 1985; Huber *et al.*, 1987; Oude Elferink *et al.*, 1995).

Substrate specificity differences between MRP1 and MRP2 are indicated by a higher affinity and transport rate of MRP1 for LTC_4 and by the preferential transport of monoglucuronosyl bilirubin and bisglucuronosyl bilirubin by MRP2 (Jedlitschky *et al.*, 1997). In addition, the quinoline-based LTD_4 analog MK571 more potently inhibits MRP1 (Leier *et al.*, 1994b) than rat MRP2 (Büchler *et al.*, 1996). These comparisons indicate that the substrate specificity of both conjugate transporting ATPases is quite similar, in spite of the differences in the amino acid sequence between MRP1 and MRP2.

Function of MRP1 and MRP2 in Detoxification and Defense Against Oxidative Stress

Formation and excretion of conjugates of endogenous and xenobiotic substances with glutathione, glucuronate, or sulfate is of vital importance in detoxification and cellular homeostasis. The broad substrate specificity of the conjugate export pumps MRP1 and MRP2 enables the terminal excretion of a multitude of conjugates and amphiphilic anions which are formed by a large number of relatively specific monooxygenases and transferases in phase I and phase II metabolism of endogenous and xenobiotic substances (Figure 1). This is indicated by the MRP1- and MRP2-mediated export of bilirubin glucuronides and bile salt conjugates (Jedlitschky *et al.*, 1996, 1997). With respect to carcinogens it is of interest that the glutathione *S*-conjugate of aflatoxin B_1 was recently shown to be a high-affinity substrate of MRP1 (Loe *et al.*, 1997). In addition, several glutathione and glucuronate conjugates of cytotoxic anticancer agents are substrates for human MRP1 (Jedlitschky *et al.*, 1996). The finding that GSSG, but not GSH, is transported by MRP1, as well as by rat MRP2 (Leier *et al.*, 1996), points to an important role of MRP1 and MRP2 in the export of GSSG from cells under conditions of oxidative stress, when the reduction of GSSG becomes rate-limiting and GSSG export must be increased (Ishikawa and Sies, 1989).

Hereditary Deficiencies of the Apical Conjugate Transporting ATPase MRP2

The human *Dubin-Johnson syndrome* represents an inherited defect in the secretion of amphiphilic anionic conjugates from hepatocytes into bile (reviewed by Roy Chowdhury *et al.*, 1994). Identification of the human canalicular membrane protein mediating the ATP-dependent transport of glutathione and glucuronate conjugates as MRP2 suggested that the absence of this transporter is the molecular basis of this hereditary disease (Büchler *et al.*, 1996; Kartenbeck *et al.*, 1996). Selective detection of human MRP2 by an antibody directed against the carboxyl-terminal sequence of this transporter has recently demonstrated that the MRP2 protein is only expressed in normal human liver but not in the liver of a patient with Dubin-Johnson syndrome (Keppler and Kartenbeck, 1996). MRP1 has been detected in erythrocyte membranes from patients with Dubin-Johnson syndrome (Kartenbeck *et al.*, 1996), in agreement with the normal ATP-dependent transport of glutathione *S*-conjugates into inside-out membrane vesicles from the patients red blood cells (Board *et al.*, 1992). The cDNA sequencing of human MRP2 and of the homologous rat cDNA (Büchler *et al.*, 1996; Paulusma *et al.*, 1996; Taniguchi *et al.*, 1996), together with our demonstration of the absence of the MRP2 protein from the hepatocyte canalicular membrane in Dubin-Johnson syndrome provide the basis for the elucidation of mutations in this disorder.

Two hyperbilirubinemic mutant rat strains have been described which are deficient in the transport of conjugates and amphiphilic anions across the canalicular membrane (Jansen *et al.*, 1985; Takikawa *et al.*, 1991; Oude Elferink *et al.*, 1995). ATP-dependent transport of glutathione *S*-conjugates by canalicular membrane vesicles

is below detectability in the Groningen yellow / transport-deficient (GY/TR$^-$) (Ishikawa *et al.*, 1990) and the Eisai hyperbilirubinemic (EHBR) mutant rats (Fernández-Chéca *et al.*, 1992), which are derived from the Wistar and the Sprague-Dawley strain of rats, respectively. Immunofluorescence microscopy and confocal laser scanning microscopy have revealed the absence of the MRP2 protein from the canalicular membrane domain of both mutant strains (Büchler *et al.*, 1996; Kartenbeck *et al.*, 1996). The mutation in the EHBR mutant rat (Ito *et al.*, 1997) has similar consequences as in the GY/TR$^-$ mutant (Paulusma *et al.*, 1996), as shown by Northern blotting, immunoblotting, and double-label immunofluorescence microscopy (Büchler *et al.*, 1996; Paulusma *et al.*, 1996). These mutants have promoted and facilitated the characterization of the rat canalicular conjugate export pump. Cloning and sequencing of the cDNA encoding rat MRP2 and the subsequent immunoblot analysis has proven that the absence of the 190 kDa membrane glycoprotein causes the transport deficiency in the mutant rats. Therefore, canalicular membrane vesicles from normal hepatocytes in comparison with those from EHBR or GY/TR$^-$ mutant liver provide a valuable tool to analyze in further detail the function, substrate specificity, and regulation of rat MRP2.

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Review

Multiple Roles of Glutathione in the Central Nervous System

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Glutathione is a storage form of cysteine and protects against reactive oxygen species and potentially toxic xenobiotics in the central nervous system. Marked reductions in intracellular or intramitochondrial glutathione are associated with cell death. Enzymes involved in glutathione metabolism are very active in the choroid plexus, and astrocytes maintain a high concentration of glutathione. Astrocytes probably play an important role in regulating cerebral sulfur/glutathione metabolism and in protecting the brain against noxious chemicals. Oxidative stress contributes to age-related neurodegenerative diseases. Patients with inborn errors of glutathione metabolism often exhibit progressive neurological problems. Therefore, increasing brain glutathione levels may have therapeutic benefits.

Key words: Anti-oxidants / γ-Glutamyl cycle / Glutathione / Glutathione disulfide / Reactive oxygen species.

Introduction

Glutathione (GSH) was first discovered by J. de Rey-Pailhade over 100 years ago and its structure (i.e. L-γ-glutamyl-L-cysteinylglycine) was deduced in the 1930s. [See Meister (1989) for a discussion of historical perspectives.] GSH is present in most mammalian cells at concentrations ranging from 0.5 to 12 mM (~ 2–3 mM in whole brain) (Cooper, 1997).

GSH may exert its anti-oxidant activity synergistically with both ascorbate (Jain *et al.*, 1994; Meister, 1995) and vitamin E. Vitamin E is essential for normal neurological function and is both a free radical scavenger and structural stabilizer (see discussion in Heslop *et al.*, 1996). The link between GSH and vitamin E is apparent from the fact that dietary selenium (an essential component of glutathione peroxidase) is known to prevent many symptoms of vitamin E deficiency (Heslop *et al.*, 1996). Dehydroascorbate resulting from oxidation of ascorbate is reduced by GSH (and NADPH) in brain (Rose, 1993). Patients with disorders of GSH metabolism apparently exhibit an increased reliance on ascorbate as an antioxidant, and many display progressive neurological disease. These observations underscore the importance of GSH for normal brain function. [See Cooper (1997) for a recent review].

Generally, GSH serves as:

a) A non-toxic storage form of cysteine,
b) an enzyme cofactor,
c) a component in metabolic (and transport) pathways, and
d) a protectant against reactive oxygen species (ROS) and potentially harmful xenobiotics.

(For discussions on the physiological roles of GSH see, for example, Meister and Anderson, 1983; Meister, 1989). A scheme that shows interrelationships among these functions is presented in Figure 1. The general properties of GSH are briefly discussed below, followed by a more detailed description of its metabolism in brain and possible therapeutic interventions designed to increase GSH.

Glutathione Is a Non-Toxic Storage Form of Cysteine

An important physiological role of GSH is as a carrier and storage form of cysteine. Elevated cysteine/cystine is excitotoxic, possibly by interfering with the *N*-methyl-D-aspartate (NMDA) receptor. Cysteine also has more generalized toxic effects through possession of a reactive sulfhydryl group that forms hemithioketals with α-keto acids and hemithioacetals with aldehydes. An example of reactivity with aldehydes is the formation of a hemithioacetal with pyridoxal 5′-phosphate. This adduct cyclizes to a thiazolidinone thereby inhibiting some key pyridoxal 5′-phosphate-containing enzymes, such as glutamate decarboxylase. In contrast, the sulfhydryl of GSH is less reactive, permitting concentrations in mammalian tissues to be ~10–100 times higher than those of cysteine. (Reviewed by Cooper, 1997).

Cofactor Functions

GSH is an essential cofactor for a number of enzymes including formaldehyde dehydrogenase, glyoxylase, maleylacetoacetate isomerase, dehydrochlorinase, and prostaglandin endoperoxidase isomerase. In these reactions, GSH is not consumed but presumably plays a critical role in the catalytic mechanism (Meister, 1989).

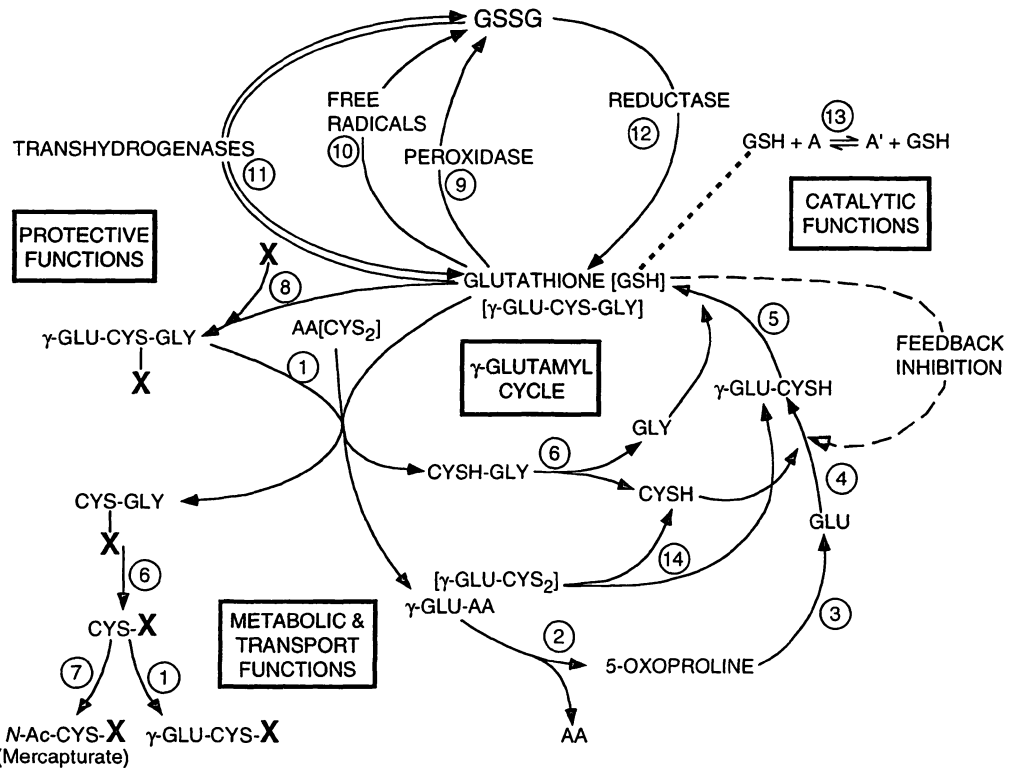

Fig. 1 Metabolism of Glutathione (GSH).
(1) γ-Glutamyltranspeptidase; (2) γ-glutamylcylotransferase; (3) 5-oxoprolinase; (4) γ-glutamylcysteine synthetase; (5) glutathione synthetase; (6) cysteinylglycine dipeptidase; (7) L-cysteine *S*-conjugate *N*-acetyltransferase; (8) glutathione *S*-transferase; (9) glutathione peroxidase; (10) free radical quenching (probably non-enzymatic); (11) glutathione transhydrogenase; (12) glutathione disulfide (GSSG) reductase; (13) enzymatic reactions in which GSH is required as a cofactor but is not consumed; (14) transport of γ-glutamylcystine and reduction to γ-glutamylcysteine. AA, amino acids; CYSH, cysteine; CYS2, cystine; **X** = compounds that form conjugates with glutathione. *Modified from Meister (1989).*

Metabolism of GSH and Possible Transport Functions

In 1970, Orlowski and Meister noted that enzymes involved in GSH metabolism may be linked in such a way as to form a process for the recycling of the constituent amino acids of the tripeptide. This process was named the γ-glutamyl cycle (Figure 1). GSH is synthesized by the consecutive actions of the ATP-dependent enzymes γ-glutamylcysteine synthetase (reaction 4) and glutathione synthetase (reaction 5). Levels of GSH are regulated in part by feedback inhibition of γ-glutamylcysteine synthetase by GSH. In the presence of a suitable amino acid [AA] acceptor, GSH is catabolized by the action of γ-glutamyltranspeptidase (reaction 1) to yield a γ-glutamyl amino acid [γ-GLU-AA] and cysteinylglycine [CYSH-GLY]. The γ-GLU-AA is converted to free amino acid and 5-oxoproline by the action of γ-glutamylcyclotransferase (reaction 2). 5-oxoproline is converted back to glutamate by the ATP-dependent 5-oxoprolinase reaction (reaction 3). The glutamate released in this process can then be reused in the synthesis of GSH, thus completing the cycle with the glutamate component of GSH. The cysteinylglycine released in the γ-glutamyltranspeptidase reaction is hydrolyzed by the action of a dipeptidase (reaction 6) to glycine and cysteine, completing the cycle with the glycine and cysteine components of GSH. Cystine is an especially active amino acid substrate of γ-glutamyltranspeptidase yielding γ-glutamylcystine [γ-GLU-CYS_2] (reaction 1, square brackets) which in turn is reduced to γ-glutamylcysteine [γ-GLU-CYSH] and cysteine (reaction 14). Note that when cystine is used as the amino donor, the cycle is short-circuited by bypassing reactions 3 and 4 (Thompson and Meister, 1975).

Orlowski and Meister (1970) suggested that the positioning of γ-glutamyltranspeptidase on the cell surface and of the other enzymes of GSH metabolism within the cell permits the γ-glutamyl cycle to play a role in the translocation of amino acids across the cell membrane. Certainly, strong evidence suggests that the cycle operates *in vivo* (Griffith and Meister, 1979). Moreover, the constituent enzymes of the γ-glutamyl cycle are most active in tissues, such as the kidney, where transport of amino acids is especially prominent (Meister and Anderson, 1983). However, many amino acid transporters have now been characterized and found to be unrelated to the γ-glutamyl cycle (e.g. Tate *et al.*, 1992). The cycle is now recognized to play a minor role in transport of amino acids across cell membranes. Perhaps the γ-glutamyl cycle is too energetically expensive (requiring the hydrolysis of 3 ATPs at steps

3, 4 and 5) and spacially too diffuse for efficient translocation of most amino acids. A possible exception is cystine, because as noted above this amino acid is an excellent substrate of γ-glutamyltranspeptidase.

Current opinion holds that the γ-glutamyl cycle has a major role in metabolism of leukotrienes, estrogens and prostaglandins (depicted as **X** in Figure 1) through *S*-conjugate formation. Here, we discuss the role of the γ-glutamyl cycle in leukotriene metabolism. Leukotriene A_4 (LTA_4) is formed from arachidonic acid that is released from membrane phospholipids in response to a variety of immunological and inflammatory stimuli. LTA_4 is either hydrolyzed to the leukocyte stimulator leukotriene B_4 (LTB_4) or is converted consecutively to glutathione *S*-conjugate (LTC_4), γ-glutamylcysteine *S*-conjugate (LTD_4) and cysteine *S*-conjugate (LTE_4) by the actions of a specialized form of glutathione *S*-transferase, cysteinylglycine dipeptidase and L-cysteine *S*-conjugate *N*-acetyltransferase, respectively (Figure 1, reactions 8→1→6). LTC_4. LTD_4 and LTE_4 are now known to be components of the 'slow acting substance of anaphylaxis' and are involved in pulmonary smooth muscle contraction, mucous secretion, vasoconstriction and vascular permeability (Nicholson, 1993). The physiological effects of the leukotriene *S*-conjugates are terminated by conversion of the cysteine *S*-conjugate to the mercapturate [*N*-acetyl cysteine *S*-conjugate (*N*-Ac-CYS-**X**); Figure 1, reaction 7] which is excreted. Alternatively, the cysteine *S*-conjugate may enter directly into the γ-glutamyl cycle as a substrate of γ-glutamyltranspeptidase. This reaction generates the γ-glutamylcysteine *S*-conjugate which in turn is a substrate of the cyclotransferase (reactions 1 and 2) effectively translocating the cysteinyl *S*-conjugate.

In addition, the γ-glutamyl cycle and associated reactions are involved in the detoxification of potentially poisonous xenobiotics (again depicted as **X** in Figure 1) (reactions 8→1→6→7). This pathway is discussed in more detail later in the review.

In brain, the enzymes of the γ-glutamyl cycle (Figure 1, reactions 1 through 6) are especially abundant in the choroid plexus (Tate *et al.*, 1973). The specific activities are comparable to those in the kidney, suggesting that the cycle is involved in some sort of transport process in the brain. GSH is present in the cerebrospinal fluid (CSF) at μM concentrations, and the choroid plexus plays a role in the formation and recycling of GSH in the CSF (Anderson *et al.*, 1989). γ-Glutamyltranspeptidase is present in brain capillaries and is regarded as a marker for capillaries. However, γ-glutamyltranspeptidase is also present in astrocytes and neurons (Makar *et al.*, 1994). Special aspects of GSH turnover and metabolism in brain are described later in the review.

Protective Functions

The mammalian, adult brain has a very high energy demand, normally relying almost entirely on the oxidative metabolism of glucose to meet its energy demands. Most (> 90%) of the glucose taken up by the brain is fully oxidized to CO_2. This very high capacity to oxidize glucose suggests that the brain may generate ROS at an appreciable rate. The brain contains relatively low levels of some anti-oxidant defenses (e.g., catalase) and a high lipid content. The combination of high levels of ROS production, highly susceptible targets, and relatively low levels of defense mechanisms suggests that the brain may be especially vulnerable to oxidative stress. GSH is a major protectant in the brain against oxidative stress by interacting directly with ROS or by participating in enzyme-catalyzed redox cycling reactions.

An important enzyme in glutathione redox reactions is glutathione peroxidase (eq. 1) which catalyzes the reduction of potentially toxic H_2O_2 (or lipid peroxides; ROOH) to H_2O (or ROH) with the concomitant conversion of GSH to glutathione disulfide (GSSG). Catalase also converts H_2O_2 to H_2O, but this enzyme is not present in mitochondria of most tissues (with the notable exception of heart) and cannot detoxify lipid peroxides. Therefore, glutathione peroxidase is especially significant in protecting these organelles against H_2O_2 generated from incomplete reduction of O_2 (Benzi and Moretti, 1995). [The importance of glutathione peroxidase is underscored by recent work of Zeevalk *et al.* (1997) who showed that mice overexpressing this enzyme are resistant to oxidative damage resulting from treatment with malonate (an inhibitor of succinate dehydrogenase). Addition of malonate to GSH-depleted mesencephalic cultures elicited a toxic response (Zeevalk *et al.*, 1997).] Mitochondria also contain glutathione disulfide reductase (eq. 2) completing a glutathione redox cycle. A relatively large pool of GSH is maintained in mitochondria through the action of a high-affinity GSH uptake system (Mårtensson *et al.*, 1990). Depletion of this pool in preweanling rats leads to damaged brain mitochondria and death (Jain *et al.*, 1991; Meister, 1995).

$$2\,GSH + H_2O_2\ (\text{or ROOH}) \rightarrow GSSG + 2\,H_2O\ (\text{or ROH} + H_2O) \quad (1)$$

$$GSSG + NADPH + H^+ \rightarrow 2\,GSH + NADP^+ \quad (2)$$

GSH has been known for many years to play a crucial role in mitochondria by helping maintain normal function and structure (Hunter *et al.*, 1964). If mitochondria are not suitably protected against metabolic insults (including those induced by ROS) the organelles may become irreversibly damaged through a process culminating with induction of a mitochondrial permeability transition (mPT). As summarized by Gunter *et al.* (1994), the mPT is characterized by a sudden increase in the permeability of the mitochondrial inner membrane to small ions and molecules (with M_r values < 1500). This increase is associated with the complete collapse of the mitochondrial membrane potential ($\Delta\Psi$) and colloid-osmotic swelling of the mitochondrial matrix.

Agents that can precipitate the mPT include compounds that oxidize pyridine nucleotides, deplete matrix GSH (Gunter *et al.*, 1994; Reed and Savage, 1995) or increase the GSSG/GSH ratio (Beatrice *et al.*, 1984) thereby creating stress. GSH detoxifies 4-hydroxyhexenal (a lipid peroxidation byproduct) – the most potent inducer of the mPT yet identified (Kristal *et al.*, 1996). Until recently, almost all work on the mPT was carried out on liver and heart mitochondria. However, work from our laboratory has shown that a PT can be induced in isolated rat brain mitochondria and in mitochondria *in situ* in primary cultures of astrocytes (Kristal and Dubinsky, 1997). Some evidence suggests that mitochondrial dysfunction is a primary event in the cascade leading to glutamate excitotoxicity (White and Reynolds, 1996; Schinder *et al.*, 1996). Taken together these findings suggest that GSH and the glutathione redox cycle play a crucial role in maintaining mitochondrial integrity in brain and other organs.

The glutathione redox cycle is present in the cytosol of brain cells (Cooper, 1997). In addition, the brain contains a phospholipid hydroperoxide glutathione peroxidase. Moreover, certain cytosolic glutathione *S*-transferases also catalyze GSH-dependent reduction of lipid peroxides. Lipids are protected against free radical damage by α-tocopherol (vitamin E), which quenches free radical propagation by formation of α-tocopheroxyl radical (Figure 1, reaction 10), and by other lipophilic scavengers such as the ubiquinols. The α-tocopheroxyl radical does not accumulate, in part because it can be re-reduced nonenzymatically to α-tocopherol by GSH (Meister, 1989). This reaction and those catalyzed by glutathione peroxidase, glutathione *S*-transferases possessing peroxidase activity, phospholipid hydroperoxide glutathione peroxidase, and transhydrogenases (eq. 3) all result in the formation of GSSG.

$$R'SSR'' + 2\,GSH \longleftrightarrow R'SH + R''SH + GSSG \qquad (3)$$

Under normal conditions, oxidative stress puts little 'strain' on the glutathione redox cycle, and the concentration of GSSG in most tissues is usually low. Indeed, in rat forebrain the concentration of GSSG (in GSH equivalents) is normally < 1.0% that of GSH (Cooper, 1997; Cooper *et al.*, 1980). The low GSSG/GSH ratio is presumably due to strong GSSG reductase activity in brain and rapid synthesis of NADPH from $NADP^+$. About 3–5% of cerebral glucose is converted to CO_2 via the oxidative branch of the pentose phosphate pathway – providing a ready source of NADPH for reduction of GSSG (Baquer *et al.*, 1988). The pentose phosphate pathway is up-regulated in CNS cells exposed to H_2O_2 (Ben-Yosef *et al.*, 1996). In addition to the pentose phosphate pathway, other possible sources of NADPH for reduction of GSSG include the reactions catalyzed by the two malic enzymes and by isocitrate dehydrogenase.

In addition to its role in protecting tissues against ROS, GSH is important in the detoxification of potentially harmful xenobiotics. GSH forms *S*-conjugates with electrophilic compounds in reactions that are catalyzed by, or accelerated by, a large family of glutathione *S*-transferases. These enzymes are mostly cytosolic with the exceptions of a single microsomal enzyme and possibly a single mitochondrial enzyme. [Leukotriene C_4 synthase is a specialized membrane-bound glutathione *S*-transferase (Nicholson, 1993).] The glutathione *S*-conjugate formed from the action of these enzymes is converted to the corresponding L-cysteinylglycine-*S*-conjugate and then to the L-cysteine-*S*-conjugate by the consecutive actions of γ-glutamyltranspeptidase and dipeptidase. The L-cysteine-*S*-conjugate is then acetylated to the corresponding mercapturate (*N*-acetyl-L-cysteine-*S*-conjugate) which is excreted, completing the detoxification process. [The mercapturate pathway is depicted in Figure 1 to the side of the γ-glutamyl cycle as reactions 8→1→6→7).] However, the mercapturate pathway can also sometimes lead to bioactivation (generation of a toxin). Halogenated cysteine *S*-conjugates that generate highly reactive sulfhydryl-containing fragments through the action of cysteine *S*-conjugate β-lyases (eq. 4) are nephrotoxic. Dichloroacetylene is both nephrotoxic and neurotoxic. The compound is an excellent substrate of microsomal glutathione *S*-transferase yielding *S*-(1,2-dichlorovinyl)glutathione (DCVG) which is transformed to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). The latter conjugate is converted to pyruvate, ammonia and a reactive (toxic) sulfhydryl-containing fragment by the action of cysteine *S*-conjugate β-lyases. The neurotoxicity of dichloroacetylene may be due to formation of the glutathione *S*-conjugate and subsequent metabolism to a toxic fragment exclusively within the brain. However, it is also conceivable that dichloroacetylene is converted to glutathione- and cysteine *S*-conjugates elsewhere in the body and transported to the brain. The blood-brain barrier (BBB) possesses uptake systems for both DCVC (probably the L-type transporter) and DCVG. For a discussion see Cooper (1994).

$$\begin{array}{l} RSCH_2CH(NH_2)CO_2H + H_2O \\ \qquad\qquad\qquad\qquad \downarrow \\ CH_3C(O)CO_2H + NH_3 + HSR \end{array} \qquad (4)$$

The mercapturate pathway is present in the brain (see below), where it is presumably important for the metabolism of endogenous substances (such as leukotrienes) and in detoxifying reactions. However, the role of the pathway in detoxifying (and toxifying) reactions in the brain has not been extensively studied.

Origin of GSH in the Brain

The metabolism of GSH is intimately linked with overall sulfur homeostasis in the brain. The concentration of GSH in the brain (~2–3 mM) is much greater than that in the blood (~15 μM) or cerebrospinal fluid (~5 μM). Therefore, the brain must have an avid system for accumulating GSH, or more likely, of synthesizing it *in situ*. Some evidence suggests that the brain is able to take up GSH (Kaplowitz *et al.*, 1996), but other evidence suggests that uptake from

the blood is not the major source of brain GSH (Jain *et al.*, 1991). Cysteine is taken up into the brain on the L-type carrier (Oldendorf and Szabo, 1976; Wade and Brady, 1981). Cystine is apparently not transported by this mechanism, but cystine moieties may enter the brain as cystinyl bisglycine and γ-glutamylcyst(e)ine both of which are ready sources of free cysteine (Jain *et al.*, 1991). Cysteine taken up by the brain is used for protein synthesis and for the synthesis of GSH. Cysteine can also be released from brain. Isolated brain capillaries contain an ASC-type transporter on the abluminal surface that is presumably involved in the egress of cysteine (Tayarani *et al.*, 1987). Methionine is readily taken up into brain on the L-type transporter (Oldendorf and Szabo, 1976). This methionine is used for protein synthesis and as a source of methyl groups. The extent to which this methionine is a source of cysteine sulfur and indirectly of GSH sulfur in the brain is not clear.

Metabolic Compartmentation of GSH Metabolism within the Brain

In the brain, glutamine is synthesized in a small, rapidly turning over pool of glutamate (primarily in astrocytes) that is kinetically distinct from a larger, more slowly turning over pool of glutamate (primarily in neurons). Tracer studies, histochemical studies, and studies of cells in culture all suggest that GSH formation is similarly compartmented. In brain, GSH is synthesized from glutamate primarily, but not exclusively, in the small pool (astrocytes) and astrocytes maintain a large pool of this tripeptide. Nerve endings also probably contain appreciable levels of GSH. Several authors have measured GSH levels in cultured astrocytes and neurons. Without exception all these studies have found high levels of GSH in cultured astrocytes (2–20 mM). The reported levels in neurons tend to be more variable but generally appreciably less than in astrocytes. [Reviewed by Cooper (1997).] Enzymes of GSH synthesis and turnover are present in neurons in culture (Makar *et al.*, 1994). As described below, compartmentation of GSH metabolism in the brain has important physiological consequences. GSH metabolism in brain is shown schematically in Figure 2.

A Role for Astrocytic GSH in Neuroprotection

Astrocytes and the Detoxification of Glutamate and Cysteine

Excess glutamate in the extracellular space is neurotoxic (Olney *et al.*, 1971). Astrocytes contain relatively strong uptake systems for glutamate, glycine and cysteine. Dringen and Hamprecht (1996) have suggested that under physiological conditions the synthesis of GSH from glutamate in the presence of glycine and cysteine in these cells may be a way of disposing potentially toxic glutamate. The same system would also dispose of potentially neurotoxic cysteine.

Astrocytes and Metallothioneins

Metallothioneins are low M_r proteins that contain high levels of cysteine, serine and lysine residues but no aromatic amino acid residues. These proteins have been suggested to play a role as free radical scavengers and in protection against UV and X-ray damage (Dunn *et al.*, 1988), presumably as a result of high cysteine content. Metallothioneins are especially rich in mouse, rat, monkey and human astrocytes and are inducible by cytokines (Ebadi

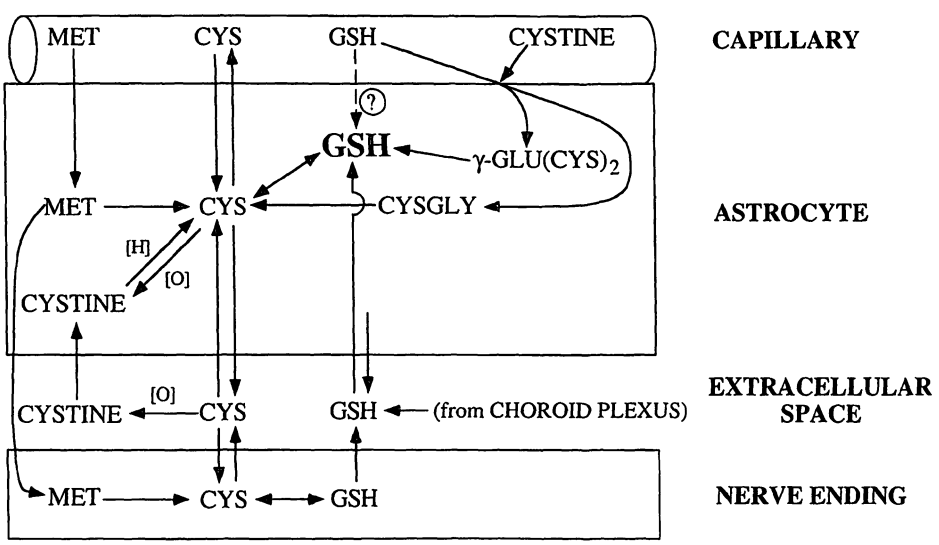

Fig. 2 Compartmentation of Sulfur Metabolism in the Brain.
Note the proposed central importance of GSH within the astrocytes. The transfer of sulfur from methionine to cysteine occurs via the transsulfuration pathway and is denoted as MET → CYS. Neurons contain GSH and the enzymes necessary for its synthesis (e.g. Makar *et al.*, 1994). However, this pool of GSH is probably small compared with that of astrocytes in the majority of neurons and is not depicted in the Figure.

et al., 1995). Four isoforms (MT-I, MT-II, MT-III, MT-IV) are known to be present in mammalian tissues. MT-I and MT-II are ubiquitously expressed, and these isoforms are known to protect against metal toxicity. MT-IV is expressed in tissues containing stratified squamous epithelia (Ebadi *et al.*, 1995, 1996).

MT-III is expressed almost exclusively in the brain and is thought to regulate brain zinc homeostasis, which itself appears to be regulated in part through redox reactions. One scenario is that an oxidative challenge to the brain will result in increased GSSG formation. GSSG causes the release of free zinc from MT-III, which in turn stimulates zinc-dependent processes and zinc-dependent enzymes. Up to a point this release of zinc may be protective, but beyond a threshold value the release of zinc may be deleterious (e.g. by inhibiting -SH-dependent enzymes) (Ebadi *et al.*, 1995, 1996; Maret, 1995). Inasmuch as astrocytes contain large pools of GSH and metallothioneins, these cells may be especially important in controlling the redox-driven zinc homeostasis in the brain.

Astrocytes and the Mercapturate Pathway

Earlier immunohistochemical studies suggested that glutathione *S*-transferases in the brain were present exclusively in astrocytes. However, some of these enzymes are now known to occur in the choroid plexus, oligodendrocytes and in selected neuronal cell populations. Nevertheless, it is apparent that glutathione *S*-transferases are particularly abundant in astrocytes (especially in the end feet) and ependymal cells (Lowndes *et al.*, 1994; Makar *et al.*, 1994; Cooper, 1997). This location of glutathione *S*-transferases suggests the possibility that astrocytes and ependymal cells in the brain form a first line of defense against potentially toxic xenobiotics that can diffuse across the BBB and CSF-brain barrier, respectively (Cammer *et al.*, 1989). Possibly, glutathione *S*-transferases also participate in transport of substances into and out of the brain. Such bidirectional transport could affect hormonal control over processes such as myelination and neuronal growth and could facilitate removal of endogenous as well as exogenous toxins from the central nervous system (CNS) (Cammer *et al.*, 1989). However, as noted above, the extent to which the glutathione S-transferases and the mercapturate pathway are involved in detoxification reactions in the brain is unknown and the pathway may actually lead, on occasion, to bioactivation of xenobiotics.

Possible additional roles of glutathione *S*-transferases in the brain include protection of the myelin sheath against toxic substances, removal of epoxyeicosatrienoic acid derivatives from cells, and participation in the local metabolism of prostaglandins and leukotrienes (Cammer *et al.*, 1989). LTC_4 and LTB_4 are present in brain and these compounds may have a role in the secretion of luteinizing hormone from the anterior pituitary (Sammuelsson *et al.*, 1987).

Quantitatively, in the whole body, the most important site for reaction of electrophiles with GSH is the liver. The glutathione *S*-conjugates formed by this process in the liver are released to the bile. In the intestinal lumen they are converted in part to the cysteine *S*-conjugate. The cysteine *S*-conjugates enter first the portal vein and then eventually the general blood circulation. In the kidney the cysteine *S*-conjugates are *N*-acetylated to the mercapturate and excreted (Dekant *et al.*, 1994). In future work it will be interesting to determine how the enzymes of the mercapturate pathway in the brain conform with this general scheme. As noted already, enzymes involved in the formation of glutathione *S*-conjugates and enzymes of the γ-glutamyl cycle are present in the choroid plexus. If cysteine *S*-conjugate *N*-acetylases are similarly located then the data would suggest excretion of mercapturates to the CSF is analogous to excretion of mercapturates from kidney into urine. Evidently, the role and dynamics of the mercapturate pathway in brain are fertile areas for further research.

Astrocytes and ROS

In addition to maintaining high levels of GSH, astrocytes in culture have relatively high levels of α-tocopherol and enzymes of GSH metabolism (Makar *et al.*, 1994). This finding suggests that astrocytes may have a special role in the brain in protecting neurons against ROS (cf. Han *et al.*, 1996). The ability of astrocytes to detoxify ROS may be particularly important for diffusible species such $NO^{\bullet}$. Astrocytes in culture are much more resistant to ROS than are neurons (Bolaños *et al.*, 1995; Han *et al.*, 1996; Ben-Yosef *et al.*, 1996) and recent evidence suggests that both catalase and glutathione peroxidase are important in these cells for detoxifying external H_2O_2 (Dringen and Hamprecht, 1997). GSH biosynthesis may be up-regulated in astrocytes exposed to ROS (Ben-Yosef *et al.*, 1996; Han *et al.*, 1996). β-Amyloid has been shown to induce apoptosis and oxidative stress in neurons *in vitro*. For example, depletion of GSH by Aβ25–35 leads to oxidative stress in rat cortical neurons (Müller *et al.*, 1997). Whether stimulation of the antioxidative defense systems of astrocytes in Alzheimer's disease is of therapeutic value remains to be determined.

Despite the generally protective role of astrocytes, under some pathophysiological conditions (e.g. reflow after stroke) these cells may exacerbate injury to neurons. The activity of inducible NO synthase may increase in stressed astrocytes. This induction would in turn be predicted to cause increased $NO^{\bullet}$ production and increased $ONOO^{-}$ formation, resulting in damage to nearby neurons (Barker *et al.*, 1996) and interference with mitochondrial energy metabolism (Bolaños *et al.*, 1997). Such mitochondrial dysfunction may occur in certain neurodegenerative diseases (Bolaños *et al.*, 1997).

Sulfur Trafficking between Astrocytes and Neurons

An understanding of the processes involved in sulfur homeostasis and sulfur trafficking in the CNS must take into account the following three observations:

1) Astrocytic end feet surround capillaries – controlling in part the flow of many substances, including sulfur-containing amino acids, across the BBB;
2) GSH is a major store of cysteine in the brain; and
3) astrocytes contain a large pool of GSH.

These observations underscore the central importance of GSH in sulfur homeostasis in the CNS (Figure 2). As noted above, some GSH may enter the astrocytes by transport intact across the BBB. It is likely, however, that the astrocytic GSH pool is maintained largely by ingress of cysteine which is converted to GSH directly. Inasmuch as cysteine movement across the BBB is bidirectional, the GSH pool in the astrocytes may be regarded as a cysteine/sulfur buffer that can be enlarged or depleted as dictated by the requirements of the whole brain. This buffering is disrupted by metabolic insults. For example, ischemia to the rat forebrain results in depleted GSH levels (Cooper *et al.*, 1980). This depletion is presumably due to lack of ATP which will seriously restrict GSH synthesis but not catabolism. In brain, the ischemia-induced loss of GSH is matched almost stoichiometrically by an increase in cysteine (Slivka and Cohen, 1993). The accumulation of cysteine may be substantial and contribute to ischemia-induced neurotoxicity (Slivka and Cohen, 1993).

Astrocytes also presumably act normally to buffer neuronal metabolism of sulfur-containing amino acids. Neuronal requirements for sulfur-containing amino acids are probably met in part by uptake of cysteine and methionine released from astrocytes (Figure 2). Cysteine in the extracellular space is readily oxidized non-enzymatically to cystine, which can be taken up by astrocytes, but not by neurons (Sagara *et al.*, 1996). Inasmuch as elevated levels of both cysteine and cystine are neurotoxic, the concentrations of these two compounds in the extracellular space must be tightly regulated.

Cysteine and GSH are readily interconvertible via reactions of the γ-glutamyl cycle, but cysteine is not formed anew by this process. Net synthesis of cysteine can, however, occur via the transsulfuration pathway (Figure 2, MET→CYS). Methionine is a source of methyl groups (via its conversion to *S*-adenosylmethionine), and as noted above, methionine is readily taken up across the BBB by the L-transporter (Oldendorf and Szabo, 1976). Methylation reactions are extensive in the brain, generating *S*-adenosylhomocysteine which in turn is converted to homocysteine. The complete transsulfuration pathway provides the machinery for the removal of potentially toxic homocysteine, generation of *de novo* cysteine, and recycling of methionine carbon and sulfur. Inborn errors of methionine adenosyltransferase, and thus abnormalities in the transsulfuration pathway, can lead to demyelinating disease (Ubagai *et al.*, 1995). As with cysteine, the astrocytes presumably control the flow of methionine from blood to neurons. Evidently, the importance of the transsulfuration pathway in the brain is yet another fertile area for further research.

Extracellular GSH in the CNS is provided by the choroid plexus (Anderson *et al.*, 1989), astrocytes (Yudkoff *et al.*, 1992; Sagara *et al.*, 1996; Dringen *et al.*, 1997), and possibly nerve endings (Zängerle *et al.*, 1992). Sagara *et al.* (1996) could find no evidence for uptake of GSH by neurons, suggesting that astrocytes regulate the levels of this tripeptide in the extracellular space.

The GSH in the extracellular space may be involved in neuronal signaling. Astrocytes have high-affinity binding sites for GSH that may regulate physiological responses such as G-protein coupling, activation of second messengers, regulation of protein kinases, and Ca^{2+} release (Guo and Shaw, 1992). Some evidence has also been presented that GSH may be a modulator of glutamatergic transmission by acting as a selective agonist for the *N*-methyl-D-aspartate (NMDA) recognition domain of the NMDA receptor ionophore (Ogita *et al.*, 1995). These observations suggest that the concentration of GSH may regulate physiological signals.

Finally, the central nervous system contains large amounts of taurine. This amino acid may be involved, for example, in osmoregulation and neurotransmitter modulation (Huxtable, 1989). Taurine is synthesized from cysteine via sulfur oxidation/decarboxylation. Inasmuch as GSH is a storage form of cysteine the tripeptide may have a regulatory role in the synthesis and disposition of taurine in the brain.

Cerebral GSH in Aging and Disease

Several studies have shown that the levels of GSH and of 'total' glutathione (i.e., GSH plus GSSG) in the rodent brain decline with age and that the brains of older rodents are more susceptible to peroxidative stress (reviewed by Benzi and Moretti, 1995). Several reports also suggest that the ratio of GSSG to GSH increases with age, but because GSH is easily oxidized during tissue extraction the results of these studies must be viewed cautiously (Cooper, 1997).

A large body of evidence suggests the involvement of ROS in several neurodegenerative diseases [e.g., Parkinson's disease (PD)]. GSH levels are decreased in substantia nigra of PD patients (Sian *et al.*, 1994) and of individuals with incidental Lewy bodies considered presymptomatic for PD (Dexter *et al.*, 1994). Although some evidence suggests that Alzheimer's disease (AD) is associated with ROS, no evidence has been found that GSH levels are altered in the brains of these patients compared with the levels in the brains of age-matched controls (Makar *et al.*, 1995). However, as noted above, Aβ25–35 causes a decrease in GSH in neuronal cells *in vitro*. Perhaps the contrast between AD and PD is due to differences in the chemical nature of the ROS or to the site(s) of their generation

in brain. Decreased levels of GSH are associated with decreased survival rate in patients with HIV (Herzenberg *et al.*, 1997). The concentrations of *S*-adenosylmethionine and GSH are decreased in the CSF of HIV patients exhibiting neurological symptoms, suggesting impaired cerebral GSH metabolism/transsulfuration (Castagna *et al.*, 1995).

Animal Models of GSH Deficiency

Many methods have been devised to interfere with the turnover of GSH. For example, GSH synthesis may be inhibited by buthionine sulfoxide, a specific inhibitor of γ-glutamylcysteine synthetase (reviewed by Meister, 1995; Cooper, 1997). Other methods designed to decrease GSH include administration of diamide (oxidizes GSH), cyclohexene-1-one or cycloheptene-1-one (deplete free GSH by forming glutathione *S*-conjugates) (for original references see Benzi and Moretti, 1995; Cooper, 1997). Interestingly, challenges with diethyldithiocarbamate (a copper chelator and inhibitor of superoxide dismutase), paraquat (induces superoxide formation), or aminotriazole (inhibits catalase) lead to a small, but significant increase in GSH in the forebrains of rats (Benzi and Moretti, 1995). Perhaps, brain tissue *in vivo*, as was noted earlier for astrocytes in culture, up-regulates GSH synthesis in the face of an oxidative challenge.

In addition to causing an oxidative stress, depletion of brain GSH can lead to enhanced toxicity toward xenobiotics. For example, the agrochemical intermediate L-2 chloropropionic acid is acutely neurotoxic to rats, causing cerebellar edema and locomotor dysfunction (Wyatt *et al.*, 1996). The compound destroys cerebellar granule cells. Toxicity toward these cells is exacerbated by depletion of GSH with buthionine sulfoximine. On the other hand, glutathione isopropyl ester (a precursor of GSH; see the following section) is protective (Wyatt *et al.*, 1996).

Possible Therapeutic Benefits Associated with Increasing Glutathione Levels

The role of GSH in neuroprotection suggests that raising tissue GSH levels may be therapeutically beneficial. Administration of relatively large amounts of GSH to experimental animals, however, does not result in appreciable increases in tissue GSH because of poor penetration into cells (Meister, 1995; Jain *et al.*, 1991). In animal models of GSH deficiency, GSH levels can be restored by administration of *N*-acetylcysteine (*N*-Ac) or 2-oxothiazolidine-4-carboxylate (OTC), two compounds that are converted to cysteine *in vivo*. GSH esters are also GSH precursors *in vivo* (reviewed by Cooper, 1997). In addition, as noted above, ascorbate has a sparing effect on GSH (Meister, 1995; Mårtensson and Meister, 1992; Jain *et al.*, 1994). As a result of these findings ascorbate, OTC, *N*-Ac and GSH esters have all been considered as possible therapeutic agents in diseases associate with oxidative stress. For example, ascorbate and *N*-Ac have been used to treat patients with hereditary glutathione synthetase deficiency (Mårtensson *et al.*, 1989; Jain *et al.*, 1994). Several studies have shown that GSH monoester, *N*-Ac and OTC suppress HIV virus expression in infected human cells (Harekeh *et al.*, 1990; Kabelic *et al.*, 1991). *N*-Ac is especially effective, presumably because it is an excellent precursor of GSH and is also an antioxidant (Raju *et al.*, 1994). Castagna *et al.* (1995) showed that the decrease in *S*-adenosylmethionine and GSH in the CSF of HIV patients could be redressed by parenteral administration of *S*-adenosylmethionine. The above mentioned studies suggest that regimens designed to correct the imbalance in sulfur-containing amino acids and increase tissue GSH status may be useful in treating HIV patients and patients with inborn errors of GSH metabolism. Whether such regimens will prove robust in treating other diseases associated with oxidative stress or exposure to toxic xenobiotics remains to be determined.

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Review

Lipid Mobilization and Gluconeogenesis in Plants: Do Glyoxylate Cycle Enzyme Activities Constitute a Real Cycle? A Hypothesis

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Glyoxysomes are specialized peroxisomes present in various plant organs such as germinating cotyledons or senescing leaves. They are the site of β-oxidation and of the glyoxylate cycle. These consecutive pathways are essential to the maintenance of gluconeogenesis initiated by the degradation of reserve or structural lipids.

In contrast to mitochondrial β-oxidation, which is prevalent in animal cells, glyoxysomal β-oxidation and the glyoxylate cycle have no direct access to the mitochondrial respiratory chain because of the impermeability of the glyoxysomal membrane to the reduced cofactors. The necessity of NAD^+ regeneration can conceivably be fulfilled by membrane redox chains and/or by transmembrane shuttles.

Experimental evidence based on the active metabolic roles of higher plant glyoxysomes and yeast peroxisomes suggests the coexistence of two mechanisms, namely a reductase/peroxidase membrane redox chain and a malate/aspartate shuttle susceptible to transfer electrons to the mitochondrial ATP generating system.

Such a model interconnects β-oxidation, the glyoxylate cycle, the respiratory chain and gluconeogenesis in such a way that glyoxysomal malate dehydrogenase is an essential and exclusive component of β-oxidation (NAD^+ regeneration). Consequently, the classical view of the glyoxylate cycle is superseded by a tentative reactional scheme deprived of cyclic character.

Key words: Beta-oxidation / Gluconeogenesis / Glyoxylate cycle / Glyoxysomes / Lipids / Peroxisomes.

Introduction

Germination is a critical step in the lifetime of a plant, when growth is reinduced after a period of metabolic inactivity. Reserve macromolecules, stored as proteins, lipids or carbohydrates during the maturation of seeds, are catabolized in order to provide carbon and energy to the seedling. Since lipids do not migrate from cell to cell, oily stores are first converted into carbohydrates, which are then translocated to the growing root and shoot.

The conversion of lipids involves several consecutive pathways occurring in various cellular compartments:

(1) Hydrolysis of triglycerides in oleosomes (Huang, 1992; Garcia-Agustin *et al.*, 1992),
(2) β-oxidation (Cooper and Beevers, 1969b) and
(3) glyoxylate cycle (conversion of C2 units into C4 units) in glyoxysomes (Breidenbach and Beevers, 1967),
(4) partial citric acid cycle (conversion of succinate into malate) in mitochondria (Cooper and Beevers, 1969a), and finally
(5) gluconeogenesis in the cytosol (Nishimura and Beevers, 1979).

A comparable reactional scheme involving structural lipids has also been observed in various senescing plant tissues. Senescence, which is initiated by natural or experimentally triggered photosynthate starvation, is an active and genetically programmed process leading to the organized disassembly of biological functions at various levels, from individual cells or specific organs to entire plants (for a review, see Noodén, 1988). Reserve carbohydrates are rapidly depleted during senescence, so that lipids become the major energy source for the process, as substrates of the mitochondrial respiration (Dieuaide *et al.*, 1992, 1993; Hooks *et al.*, 1995). Galactolipids, which are predominant components of thylakoid membranes, are degraded during senescence (Gut and Matile, 1988a, b). Most of the acyl residues are released as CO_2, but a portion is converted into metabolites such as sucrose and glucose (Wanner *et al.*, 1991). This conversion implies reinitiation of gluconeogenesis. Reactivation of peroxisomal β-oxidation has indeed been observed in senescent petals (De Bellis *et al.*, 1991), and glyoxylate cycle activities have been measured in senescent tissues such as leaves (Godavari *et al.*, 1973; Gut and Matile, 1988b; Pistelli *et al.*, 1991; Graham *et al.*, 1992; Pastori and Del Rio, 1994), cotyledons (De Bellis *et al.*, 1990; Vincentini and Matile, 1993; Mc Laughlin and Smith, 1995; Pistelli *et al.*, 1995), or petals (De Bellis *et al.*, 1991). Gluconeogenesis enzyme activities have also been detected in senescent cotyledons (Kim and Smith, 1994).

During post-germinative growth, the conversion of lipids into carbohydrates via β-oxidation and the glyoxylate cycle is essential to the development of the seedling, whereas two situations can be described for the similar conversion characterizing senescing tissues. For monocarpic plants, where whole plant senescence is associated with the transition from vegetative to reproductive growth, gluconeogenesis based on structural lipids provides carbon and energy to the maturing seeds. In contrast, gluconeogenesis associated with the seasonal senescence of leaves of a perennial plant results in the temporary storage of carbohydrates (in the trunk and roots), which subsequently sustain the chemoheterotrophic metabolism of the organism at the onset of the next growing season, i.e. before photosynthesis is reinitiated.

Mitochondrial and Peroxisomal/Glyoxysomal Metabolism during Lipid Mobilization

Peroxisomes are ubiquitous small spherical one-membrane organelles that contain H_2O_2 producing enzymes, such as urate oxidase, acyl-CoA oxidase, glycolate oxidase or L-amino acid oxidase, as well as H_2O_2 scavenging enzymes such as catalase or ascorbate peroxidase. They contain neither DNA, nor ribosomes or internal membrane systems. Their granular matrix is amorphous but sometimes contains a paracrystalline dense core, or a protein crystal (for a review, see Beevers, 1979). Peroxisomes are bounded by a fragile membrane that is easily broken during isolation procedures (for reviews, see Lazarow and Fugiki, 1985; Van den Bosch *et al.*, 1992; Sulter *et al.*, 1993). Glyoxysomes are plant specific peroxisomes first observed in germinating castor bean (Breidenbach and Beevers, 1967). They are the sites of two major metabolic pathways that are active in growing seedlings as well as in senescing tissues, namely β-oxidation and the glyoxylate cycle. The glyoxysome diameter may reach 2–4 μm, whereas the peroxisome typical diameter ranges from 0.1 to 1.7 μm. The final size of peroxisomes/glyoxysomes appears to depend on the amount of imported matrix proteins (Mullen and Trelease, 1996), which in turn is correlated with the nature and intensity of the prevalent metabolic pathways.

Several reports on the biogenesis of yeast and mammal peroxisomes describe them as self-perpetuating organelles essentially characterized by processes of proliferation/enlargement or enlargement/proliferation. The corresponding mechanisms for higher plant peroxisomes have not yet been elucidated to a comparable extent (for a review, see Mullen and Trelease, 1996).

The β-oxidation of fatty acids is composed of a recurring sequence of four reactions: first oxidation (dehydrogenation), hydration, second oxidation, and finally thiolysis. Each round of catalysis produces one $FADH_2$, one $NADH_2$ and one acetyl-CoA. In animal cells, β-oxidation is active in mitochondria (where it depends on the carnitine acyltransferase system for the import of fatty acids), as well as in peroxisomes. The main function of mitochondrial β-oxidation is to provide the cell with ATP, since it completely degrades fatty acids and feeds electrons into the respiratory chain. By contrast, animal peroxisomal β-oxidation serves to shorten fatty acids to medium chain compounds ($\sim C \geq 12$), which can be channelled into various metabolic processes.

In plant cells, β-oxidation mainly occurs in peroxisomes, where degradation of fatty acids reaches completion (Cooper and Beevers, 1969b; Hutton and Stumpf, 1969). At the onset of seed germination as well as in senescing tissues, redirection of the ensuing carbon flow toward gluconeogenesis is observed.

The initial dehydrogenation step of β-oxidation is performed by an acyl-CoA dehydrogenase in mitochondria and by an acyl-CoA oxidase in peroxisomes. Acyl-CoA dehydrogenase transfers redox equivalents to the respiratory chain and initiates production of ATP, whereas acyl-CoA oxidase directly uses molecular oxygen as electron acceptor without recovery of chemical energy (Masterson *et al.*, 1992; Dieuaide *et al.*, 1993) (Figure 1). Various types of plant acyl-CoA oxidases that show specific affinities for long, medium or short acyl-CoA chain lengths have been identified and purified. Coordinate expression of these enzymes provides a control of the levels of substrates and products of peroxisomal β-oxidation (Hooks *et al.*, 1995).

In animal systems, multifunctional proteins are known to participate in the β-oxidation pathway in mitochondria (Carpenter *et al.*, 1992; Uchida *et al.*, 1992), as well as in peroxisomes (for a review, see Hiltunen *et al.*, 1996), where proteins exhibiting multifunctional enzyme activities are involved in the degradation of the low amounts of unsaturated fatty acids. It has also been noted that some activities of the β-oxidation pathway in plant systems are similarly carried out by multifunctional proteins, or show multiple locations on several distinct proteins (Kindl, 1992). For example, enoyl-CoA hydratase, hydroxyacyl-

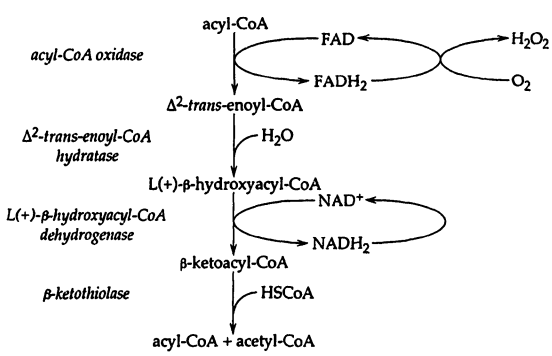

Fig. 1 The Peroxisomal β-oxidation Pathway (Saturated Fatty Acids), as Proposed by Cooper and Beevers (1969b).
In peroxisomes, the first oxidation is performed by an acyl-CoA oxidase, whereas the mitochondrial pathway is characterized by an acyl-CoA dehydrogenase (both enzymes are flavoproteins). It should also be noted that several activities of peroxisomal (as well as mitochondrial) β-oxidation are carried out by multifunctional proteins. The hydrogen peroxide produced by the oxidase can be degraded by catalase and/or peroxidase activities.

CoA dehydrogenase and hydroxyacyl-CoA epimerase activities are achieved by a trifunctional protein in the mold *Neurospora crassa* (Thieringer and Kunau, 1991) and in cucumber (Gühnemann-Schäfer and Kindl, 1995).

Plant β-oxidation is specifically characterized by the nature of its substrates, since saturated fatty acids, characteristic of animal triglycerides, are present in plants in very low amounts only. Degradation of unsaturated or polyunsaturated fatty acids therefore necessitates two additional enzymes, namely an isomerase and an epimerase that catalyze the conversions of the produced Δ^3-*cis*-enoyl-CoA and D(–)-β-hydroxyacyl-CoA to Δ^2-*trans*-enoyl-CoA and L(+)-β-hydroxyacyl-CoA respectively, the appropriate substrates of hydratase and hydroxyacyl-CoA dehydrogenase (Kindl, 1992, 1993).

Plant mitochondria are a minor site of β-oxidation, whose exact role is still unsolved (investigations on β-oxidation activities in plant tissues have shown that mitochondrial and peroxisomal enoyl-CoA hydratases have distinct kinetic and immunological properties; Miernyk *et al.*, 1991). The low capactiy of this mitochondrial pathway for long chain fatty acid degradation raises the question of its *in vivo* function. Compared to the corresponding peroxisomal activity, plant mitochondrial medium-chain fatty acid β-oxidation is however not negligible. This suggests some role for plant mitochondria in the oxidation of medium-chain fatty acids (Dieuaide *et al.*, 1993). These organelles possess carnitine acyltransferase activity (Masterson *et al.*, 1992) just as animal mitochondria do. This further suggests that fatty acids may be not entirely degraded in plant mitochondria since carnitine transferase would compete with β-oxidation enzymes for acyl-CoAs. This would induce export of shortened acyl-CoA chains. In other words, β-oxidation in plant mitochondria might not reach completion, the ensuing situation being the opposite of that assumed to prevail in animal cells.

At the onset of germination or senescence, acetyl-CoA produced by β-oxidation in plant peroxisomes (in this case more appropriately designated as glyoxysomes) is transferred in various proportions to the glyoxylate cycle, which can be considered as a short cut of the citric acid cycle. The glyoxylate cycle possesses two specific enzymes, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), and 'borrows' three enzyme activities (by gene duplication) from the citric acid cycle, namely citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3) and malate dehydrogenase (EC 1.1.1.37) (Figure 2). This pathway bypasses the two decarboxylative steps of the citric acid cycle, and redirects the 2-carbon units produced by β-oxidation toward gluconeogenesis. In the classical view, each turn of the cycle integrates two molecules of acetyl-CoA and produces one molecule of succinate, a citric acid cycle intermediate that is exported to the mitochondrion and subsequently initiates gluconeogenesis after oxidation. The glyoxylate cycle was initially described in *Pseudomonas* spp. (Saz and Hillary, 1956; Kornberg and Madsen, 1957), where it allows growth on acetate as the sole source of carbon. In plants, the pathway was first detected in the endosperm of germinating castor bean (Kornberg and Beevers, 1957).

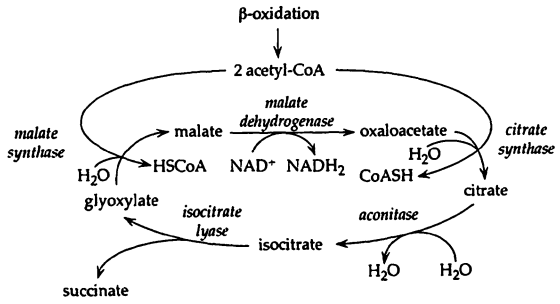

Fig. 2 Glyoxylate Cycle: Classical View.

The glyoxylate cycle has been localized in glyoxysomes (Breidenbach and Beevers, 1967), although aconitase activity has never been clearly measured in isolated organelles. The first interpretations assumed that aconitase is so soluble that the enzyme is lost during organelle isolation because of membrane disruption (Cooper and Beevers, 1969a). There is now evidence that glyoxysomes do not contain aconitase. The enzyme possesses an Fe-S cluster, and measurements by isotropic electronic paramagnetic resonance did not reveal any aconitase in the glyoxysomes (Courtois-Verniquet and Douce, 1993). One cytosolic aconitase isoform might participate in the glyoxylate cycle, since developmental changes in the abundance of this isoform are correlated with increases and decreases observed for other glyoxylate cycle enzymes during seedling growth (Hayashi *et al.*, 1995) and cotyledon senescence (De Bellis *et al.*, 1995). The absence of aconitase in glyoxysomes might be a necessity due to its strong sensitivity to H_2O_2 (Verniquet *et al.*, 1991). Since catalase shows a poor affinity for its substrate (Aebi, 1974), aconitase could not operate in peroxisomes/glyoxysomes, where continuous production of H_2O_2 occurs. The glyoxylate cycle thus requires a detour via the cytosol.

Glyoxylate cycle activities are also present in various animal tissues, such as the toad urinary bladder (Goodman *et al.*, 1980; Davis *et al.*, 1986), embryos, larvae, intestine and muscle of *Caenorhabditis elegans* (Kahn and McFadden, 1980; Liu *et al.*, 1995), marine bivalve mollusks (Benevides *et al.*, 1989), rat epiphyseal cartilage and liver (Davis *et al.*, 1989a, b), chicken liver (Davis *et al.*, 1990a), black bear brown adipose tissue (Davis *et al.*, 1990b), as well as human liver (Davis and Goodman, 1992). The presence of glyoxylate cycle activities in animal cells suggests that such organisms are able to convert fatty acids into carbohydrates. Particularly in the case of the bear brown fat tissue (a heat producing tissue), where peroxisomal β-oxidation activities increase and glyoxylate cycle activities appear during hibernation. Furthermore, incubation of the tissue in the presence of palmitate induces an increase of the glycogen content, which indicates that the complete gluconeogenesis pathway based on lipids is activated (Davis *et al.*, 1990b; by contrast, no such increase occurs in nonhibernating animals). What

might be the advantage of such a process versus a direct degradation of fatty acids by mitochondrial β-oxidation? Chemical energy initially contained in fatty acids would in the latter case be recovered as ATP in the mitochondrial matrix, via the electron transport chain and oxidative phosphorylation. In terms of ATP production, fatty acid degradation by peroxisomal β-oxidation is comparatively wasteful of chemical energy. It has been suggested that the heat directly produced by this loss of energy might participate in the thermoregulation of the organism (Kramar *et al.*, 1978) by a mechanism different from the dissipative proton pathway based on the mitochondrial uncoupling protein (thermogenin; for a review of the mitochondrial mechanism, see Wojtczak and Schonfeld, 1993).

Production of Reduced Cofactors

In peroxisomes, β-oxidation as well as the glyoxylate cycle (as postulated by the classical view; see Figure 2) produce reduced cofactors: two $FADH_2$ and three $NADH_2$ appear for each exported succinate molecule. These cofactors, which do not have direct access to the mitochondrial electron transport system, must nevertheless be reoxidized in order for both pathways to remain functional. Rat liver peroxisomes seem to be *in vitro* freely permeable to low molecular mass compounds (< 800 Da), such as $NADH_2$ or NAD^+ (Van Veldhoven *et al.*, 1983), as well as ATP, carnitine and HSCoA (Van Veldhoven *et al.*, 1987). This apparent permeability might however be due to the rupture of the membrane during organelle isolation, since yeast peroxisomes are *in vivo* closed compartments impermeable to $NADH_2$, acetyl-CoA or carnitine (Van Roermund *et al.*, 1995). These results suggest that regeneration of the oxidized cofactors must occur within the glyoxysomal matrix. The first oxidation step of peroxisomal β-oxidation is catalyzed by an acyl-CoA oxidase, and the redox equivalents are directly transferred to molecular oxygen (Figure 1). In contrast, $NADH_2$ produced by hydroxyacyl-CoA dehydrogenase and by malate dehydrogenase in the glyoxylate cycle accumulates in the matrix of isolated glyoxysomes (Cooper and Beevers, 1969b).

Two models have been proposed for the reoxidation of $NADH_2$:

(1) Oxidation by a membrane dehydrogenase, or
(2) transfer of redox equivalents to another cellular compartment via an appropriate shuttle.

These two systems will be explained and discussed in view of the overall equilibrium of the correlated metabolic pathways.

Regeneration of Oxidized Cofactors via a Membrane Redox Chain

The electron acceptors cyt c and ferricyanide have been instrumental in the characterization of the glyoxysomal redox system, in an approach similar to that already used in the investigation of the ER (endoplasmic reticulum) electron transport. $NADH_2$ and $NADPH_2$ dehydrogenase activities have thus been measured in glyoxysome membranes isolated on sucrose gradients (Hicks and Donaldson, 1982; Donaldson and Fang, 1987; Luster and Donaldson, 1987). The membranes were also shown to contain cyt b_5 and cyt P_{420}, a degradation product of cyt P_{450} (Fang *et al.*, 1987). These dehydrogenases would be capable of transferring reducing equivalents generated by the glyoxysomal matrix metabolism to an acceptor across the glyoxysomal membrane. The proposed model (Figure 3) is analogous to the electron transport chain found in mammalian liver microsomes, which consists of two components:

(1) A $NADH_2$ dehydrogenase flavoprotein able to reduce ferricyanide or to directly transfer electrons to cyt b_5, and
(2) a cyt b_5 which can reduce cyt c (Hicks and Donaldson, 1982; Luster and Donaldson, 1987; Struglics *et al.*, 1993).

Several tests were performed in order to validate the proposed system. Palmitoyl-CoA and malate oxidations can be coupled to ferricyanide or cyt c reduction, as demonstrated with the acceptors NAD^+, ferricyanide or cyt c (Donaldson and Fang, 1987). When membrane preparations (washed with 0.1 M Na_2CO_3 in order to completely remove the matrix) were combined with matrix fractions, matrix dehydrogenases were able to transfer reducing equivalents to membrane reductases ($NADH_2$ dehydrogenases; Fang *et al.*, 1987). The *in vitro* activities of these enzymes are sufficient to handle a considerable portion of the $NADH_2$ flux in glyoxysomes as evaluated by the activity of acyl-CoA oxidase, which catalyzes the β-oxidation rate-limiting step (Hicks and Donaldson, 1982). However, since the physiological acceptors of the redox components are not known, the actual capabilities of such a membrane electron transport system may be different from the rates measured with experimental acceptors.

Electron transport through the glyoxysomal membrane would require an appropriate orientation of the $NADH_2$ dehydrogenase in the membrane, and this has been evaluated using intact or deliberately broken glyoxysomes. Enzyme latency phenomena have been extensively used to assess the integrity of both outer and inner mitochondrial membranes, because these barriers prevent the immediacy of various enzymatic reactions. However, ma-

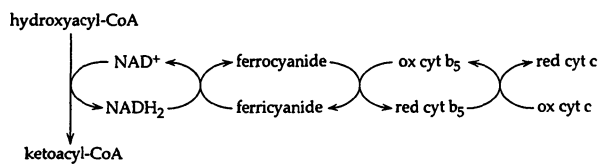

Fig. 3 Reoxidation of the Nicotinamide Coenzyme after its Reduction by the Second Redox Step of β-oxidation (Scheme 1). The reducing equivalents are transferred to an external acceptor by membrane redox compounds. This scheme is based on the $NADH_2$:ferricyanide and cyt c reductase activities measured *in vitro* in the glyoxysomal membrane.

trix enzymes can be organized into functional clusters (metabolons); this supposedly allows the direct interchange of metabolites, but might also create additional latency phenomena. Such 'reactional compartmentation' has for example been observed in leaf peroxisomes (Heupel *et al.*, 1991; Heupel and Heldt, 1994) and in cotyledon glyoxysomes (Guex *et al.*, 1995). In leaf peroxisomes, the suborganelle compartmentation remains functional after osmotic shock, so that isolated organelles lacking an intact boundary membrane can channel their photorespiratory metabolites within the remaining structures, and enzyme latency is still observed. In addition, it is well established that glyoxysomes are fragile organelles and that their membranes are damaged during isolation procedures. This could explain why electron donor and acceptor sites have been assumed to be located on both sides of the membrane, even when latency experiments suggested that the organelles were intact (Luster and Donaldson, 1987).

The evolution of $NADH_2$ dehydrogenase activities in the course of germination has already been determined. The results suggest that the ER and the glyoxysomal membranes are enriched with redox proteins during their development. It is observed that the increase and decrease of the redox activities are coordinated with the glyoxylate cycle activities, with a peak on the 5th day after germination (Alani *et al.*, 1990). This peak actually corresponds to a general maximum in the metabolism of the endosperm, which is strongly active at the onset of germination and then atrophies after depletion of its metabolic reserves.

More intriguing is the $NADPH_2$ dehydrogenase activity measured in the glyoxysomal membrane. Isocitrate dehydrogenase specific for $NADP^+$ is believed to exist in the matrix of animal peroxisomes (Masters and Crane, 1995). In plant glyoxysomes, the presence of such an enzyme concurrently with isocitrate lyase would be detrimental, since the organelle compartmentation of the glyoxylate and citric acid cycles is supposed to appropriately prevent the partition of isocitrate between nonhydrolytic cleavage and oxidative decarboxylation. The activity of a glyoxysomal isocitrate dehydrogenase would induce a net loss of organic carbon during germination, whereas the glyoxylate cycle precisely redirects the carbon flow to gluconeogenesis with a minimum loss. The very minor isocitrate dehydrogenase ($NADP^+$) activity measured in isolated glyoxysomes (Cooper and Beevers, 1969a) cannot actually be considered as significant, and the function of the high proportion (16%) of total cellular $NADP(H_2)$ in these organelles (Donaldson, 1982) therefore remains unclear.

All these experiments were performed on glyoxysomes isolated on density gradients. In order to control that the reductase activities measured in these membranes were not due to contamination by microsomal membranes, the rate of contamination was evaluated using marker enzymes. The purification of organelles does not result in the strict separation of organelles, but the observed level of contamination remains comparatively low. It appears in such a situation that the $NAD(P)H_2$ dehydrogenase activities measured in the glyoxysomal membranes were greater than what could be expected from contaminations by the ER (Fang *et al.*, 1987). However, when soybean glyoxysomes isolated on a sucrose gradient are gently resuspended and reloaded on a second gradient, residual cyt c oxidase activity (a mitochondrial marker) is detected as a sharp band corresponding to the density of mitochondria (C.L. Escher, unpublished results). This observation indicates that mitochondria contaminate the glyoxysomal fractions, even if their marker enzyme activity may first be overlooked. Similar contaminations were shown to be responsible for the mislocations of putative glyoxysomal membrane proteins (for a review, see Mullen and Trelease, 1996). Systematic cross-contamination of membrane material would of course mitigate the hypothesis of a glyoxysomal membrane redox chain involving cyt b_5, and might explain why the ER and glyoxysomal membrane redox systems appear to be similar, as observed after isolation on density gradients. Both membranes possess the same redox proteins [cyt b_5, cyt P_{420}, $NADH_2$ dehydrogenases; Hicks and Donaldson, 1982) and $NADPH_2$ dehydrogenases (Fang *et al.*, 1987)], although the ER is relatively deficient in flavin as compared to glyoxysomal membranes (Hicks and Donaldson, 1982). $NADH_2$:ferricyanide reductases isolated from both membranes are homologous (Luster *et al.*, 1988), and even immunologically indistinct (Struglics *et al.*, 1993). However, the glyoxysomal $NADH_2$:ferricyanide reductase is specific for the β-hydrogen of $NADH_2$ (Struglics *et al.*, 1993), whereas the ER enzyme is α-specific (You *et al.*, 1978). ER and glyoxysomal membrane proteins show similar resistance to carbonate extraction compared to KCl washing, similar elution profiles when submitted to reverse phase chromatography on a C-18 HPLC column, and comparable mobilities in SDS polyacrylamide gel electrophoresis (Donaldson and Gonzalez, 1989). Proteins recognized as common to glyoxysomal and ER membranes were found in similar amounts in both membranes. This observation is particularly intriguing in view of the postulated biogenesis of glyoxysomes, since the glyoxysomal membrane proteins are not synthesized on the ER but on cytosolic free polysomes (for reviews, see Trelease, 1984; Lazarow and Fugiki, 1985; Sulter *et al.*, 1993).

With respect to the supposed occurrence of reductase activities in the glyoxysomal membrane, it is sensible to consider that the performed experiments do not properly represent the functioning of the *in vivo* system, since ferricyanide is not a physiological compound, and cyt c is located *in vivo* within the mitochondrial membrane. A tentative hypothesis had previously suggested the transfer of reducing equivalents by primary electron acceptor(s) from glyoxysomes to secondary acceptor(s) in the cytosol, and finally to the mitochondrial respiratory chain. The ascorbate/dehydroascorbate pair would be a good candidate as an electron mediator, because of its apparent ubiquity in plant cells were it functions as redox buffer, and its favorable reduction potential with respect to the $NADH_2$/NAD^+ pair. $NADH_2$:dehydroascorbate reductase activity

has indeed been measured in glyoxysomal membranes, and this enzyme exhibits characteristics similar to those of the putative $NADH_2$:ferricyanide reductase, such as insensitivity to trypsin digestion or to inactivation by Triton X-100 (Bowditch and Donaldson, 1990). This suggests that dehydroascorbate may be reduced by the same enzyme that reduces ferricyanide *in vitro*. Reoxidation of ascorbate could conceivably be performed by an ascorbate peroxidase, but this would obviously preclude any electron transport to a respiratory chain. Very few membrane proteins have been proven to be peroxisomal/glyoxysomal, but immunogold labeling (using an antibody raised against a 31 kDa glyoxysomal membrane protein; Yamaguchi *et al.*, 1995a) definitely demonstrated the glyoxysomal nature of ascorbate peroxidase (Yamaguchi *et al.*, 1995b). Its active site is now predicted to be on the matrix side of the membrane, and it is assumed that all oilseed glyoxysomes possess such an enzyme (Bunkelmann and Trelease, 1996). The substrates of ascorbate peroxidase are ascorbate and H_2O_2; if the presence of ascorbate in the glyoxysomal matrix has yet not been reported, H_2O_2 is efficiently produced by oxidases and superoxide dismutases.

The latter enzymes have been immunocytochemically localized in the peroxisomal membrane (Del Rio *et al.*, 1983), where they constitute a protective mechanism against superoxide radicals (for a review, see Del Rio *et al.*, 1992). Superoxide radicals may be generated in these organelles by side reactions of the matrix xanthine oxidase and of membrane $NADH_2$ dehydrogenases (Sandalio *et al.*, 1988; Del Rio and Donaldson, 1995).

Dismutation of hydrogen peroxide by glyoxysomal catalase might not be very efficient, because of a K_m value in the 1–5 M range indicative of a low affinity between catalases of various origins and their substrate (Aebi, 1974; Huang *et al.*, 1983). This property mitigates the *in vivo* significance of the long established high molecular activity of such enzymes (Barman, 1969; Schonbaum and Chance, 1976). Consequently, low concentrations of hydrogen peroxide may be more effectively 'scavenged' by ascorbate peroxidase (Bunkelmann and Trelease, 1996), whose K_m value is in the 30–80 mM range (Chen and Asada, 1989). Moreover, since catalase is inhibited by semidehydroascorbate (Davison *et al.*, 1986), high concentrations of ascorbate would further favor the ascorbate peroxidase pathway for H_2O_2 degradation.

The coordinated effects of $NADH_2$:dehydroascorbate reductase and ascorbate peroxidase would thus simultaneously allow regeneration of NAD^+ and scavenging of residual H_2O_2 (Figure 4). This pathway does not involve any transfer of electrons across the glyoxysomal membrane toward an ATP producing system. Formally, the proposed mechanism is stoichiometrically correct if all the H_2O_2 produced by acyl-CoA oxidase is reduced by ascorbate peroxidase. However, when glyoxysomal β-oxidation is strongly active (resulting in the production of significant amounts of hydrogen peroxide), catalase efficiently competes with ascorbate peroxidase. The dehydroascorbate

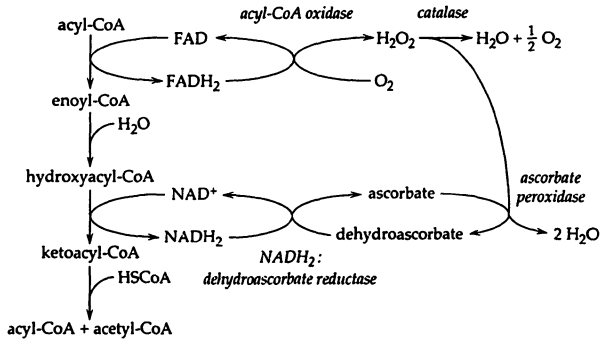

Fig. 4 Reoxidation of the Nicotinamide Coenzyme after its Reduction by the Second Redox Step of β-oxidation (Scheme 2). The reducing equivalents are transferred to matrix dehydroascorbate by an $NADH_2$:dehydroascorbate reductase. The produced ascorbate is then reoxidized by a membrane ascorbate peroxidase.

reductase/peroxidase system may not therefore constitute the primary mechanism for NAD^+ regeneration and H_2O_2 elimination in glyoxysomes, but it certainly provides an essential pathway for the protection of the glyoxysomal membrane against reactive oxygen species, and sustains the pool of NAD^+ required for postgerminative seedling growth (Bunkelmann and Trelease, 1996).

Regeneration of Oxidized Cofactors via a Transmembrane Shuttle

Another way to overcome a membrane barrier is to use a shuttle. Such systems have been described for the transfer of electrons from cytosol to mitochondria, which is mediated by a glycerol-3-phosphate/dihydroxyacetonephosphate shuttle, and by a malate/aspartate shuttle. A similar system may be considered with regard to the necessity of NAD^+ regeneration sustaining glyoxysomal/peroxisomal β-oxidation. The observation that specific activities of the glyoxysomal forms of malate dehydrogenase and aspartate aminotransferase are 10 to 100-fold higher than those of other glyoxysomal enzymes (Cooper and Beevers, 1969a; Schnarrenberger *et al.*, 1971) suggested indeed that malate and aspartate might be involved in the transfer of electrons between glyoxysomes or leaf peroxisomes and other cellular compartments (Schnarrenberger *et al.*, 1971; Tolbert, 1971). This hypothesis postulates that reducing equivalents are exported as malate, which is oxidized elsewhere in the cell, e.g. in mitochondria, where high malate dehydrogenase and aspartate aminotransferase activities are observed. In order to maintain the required carbon balance and thus to ensure a shuttle mechanism, oxaloacetate returns to the glyoxysome as aspartate, which is reconverted into oxaloacetate by aspartate aminotransferase (Figure 5; Mettler and Beevers, 1980). In this scheme, glyoxysomal malate is not converted into oxaloacetate as previously assumed in the case of an 'independent' glyoxylate cycle (Figure 2), since glyoxysomal malate dehydrogenase would now

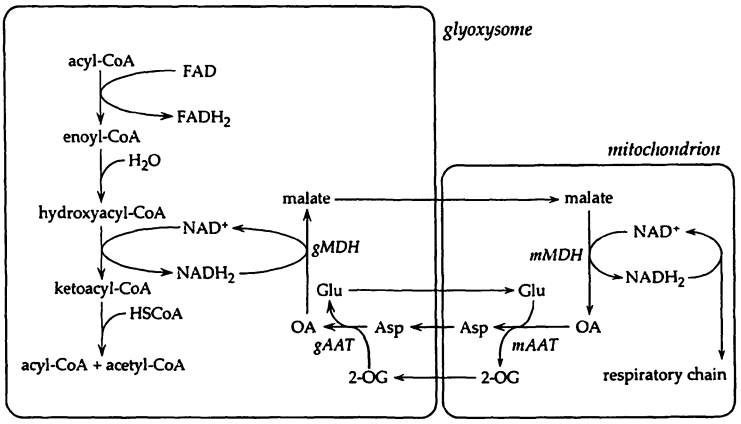

Fig. 5 Reoxidation of the Nicotinamide Coenzyme after Its Reduction by the Second Redox Step of β-oxidation (Scheme 3), as Proposed by Mettler and Beevers (1980).
The reducing equivalents are transferred to the mitochondrial matrix by a malate/aspartate shuttle. Nonstandard abbreviations: gAAT and mAAT, glyoxysomal and mitochondrial aspartate aminotransferases; gMDH and mMDH, glyoxysomal and mitochondrial malate dehydrogenases; OA, oxaloacetate, 2-OG, 2-oxoglutarate.

function in the reductive direction to consume $NADH_2$ and generate malate. Malate oxidation by malate dehydrogenase is a thermodynamically unfavorable reaction. Whether malate or oxaloacetate is formed by malate dehydrogenase actually depends on physiological parameters such as the $NADH_2/NAD^+$ balance. Since this ratio is maintained at a high level by glyoxysomal β-oxidation, oxaloacetate reduction to malate is favored over the reverse reaction. The opposite situation prevails in mitochondria because $NADH_2$ is readily reoxidized by an efficient electron transport chain.

Various tests were performed in order to assess the existence of this shuttle. Addition of aspartate and 2-oxoglutarate to purified glyoxysomes induces a rapid oxidation of accumulated $NADH_2$, and this oxidation is prevented by aminooxyacetate, which is an inhibitor of aminotransferase reactions (Mettler and Beevers, 1980).

The postulated shuttle requires regulated transports of malate, glutamate, aspartate and 2-oxoglutarate through the glyoxysomal and mitochondrial membranes. For transport studies, glyoxysomes purified on sucrose gradients at their buoyant density (1.25 g/cm^3; Huang, 1975) must first be resuspended, but since these organelles are very sensitive to osmotic shocks, no evidence could be obtained for specific transmembrane transport mechanisms for the shuttle intermediates (Mettler and Beevers, 1980). If the presence of transporters in the glyoxysomal membrane has not been proven, it has to be borne in mind that succinate transport from glyoxysomes to mitochondria is well accepted even if the corresponding transporter has not been found. Moreover, the photorespiratory pathway also involves intermediate transport through the membranes of different organelles, namely chloroplasts, peroxisomes and mitochondria (for a review, see Gietl, 1992). In this reactional mechanism, $NADH_2$ required for peroxisomal hydroxypyruvate reduction is equimolar to $NADH_2$ generated by mitochondrial glycine oxidation. Since malate dehydrogenase activity has been measured in leaf peroxisomes (Yamazaki and Tolbert, 1969), redox equivalents are assumed to be transferred from the mitochondrial matrix to the peroxisomal compartment by a malate/aspartate shuttle (Ebbighausen *et al.*, 1987; Reumann *et al.*, 1994). The transport of the shuttle intermediates across the peroxisomal membrane could be mediated by diffusion through specialized pores. The peroxisomal membrane contains a channel forming protein ('porin-like channel'), as demonstrated by purification from isolated organelles and reconstitution (Reumann *et al.*, 1995). The conductance of this channel is 10 to 20-fold lower than that of mitochondria or plastids, which are permeable to hydrophilic molecules up to 4–5 kDa (Fischer *et al.*, 1994). The diameter of peroxisomal channels has been estimated at about 1 nm, and thus appears to be just large enough to let photorespiratory metabolites pass through (Reumann *et al.*, 1995).

Isolation of glyoxysomes/peroxisomes is necessary in order to experimentally validate the hypothesis of a similar shuttle possibly required for NAD^+ regeneration (β-oxidation). In this process, partial tearing of the organelle membrane cannot be avoided, and the evolution of the observed reactions is further affected by the *in vitro* conditions, such as pH and intermediate concentrations. For example, malate oxidation by malate dehydrogenase will inevitably be detected (instead of the opposite reaction required by the shuttle) when electron acceptors are added in such a concentration that the overall equilibrium of the reaction is suitably altered (Donaldson and Fang, 1987). Therefore, the establishment of the *in vivo* pathway beyond any reasonable doubt definitely requires investigations on intact cells. Such experiments were carried out using transformed *Saccharomyces cerevisiae* strains. The inactivation of peroxisomal malate dehydrogenase by disruption of the corresponding gene results in an impaired β-oxidation capacity, which prevents cell growth on oleate and induces a strong accumulation of hydroxyacyl-CoA intermediates (Van Roermund *et al.*, 1995). In contrast,

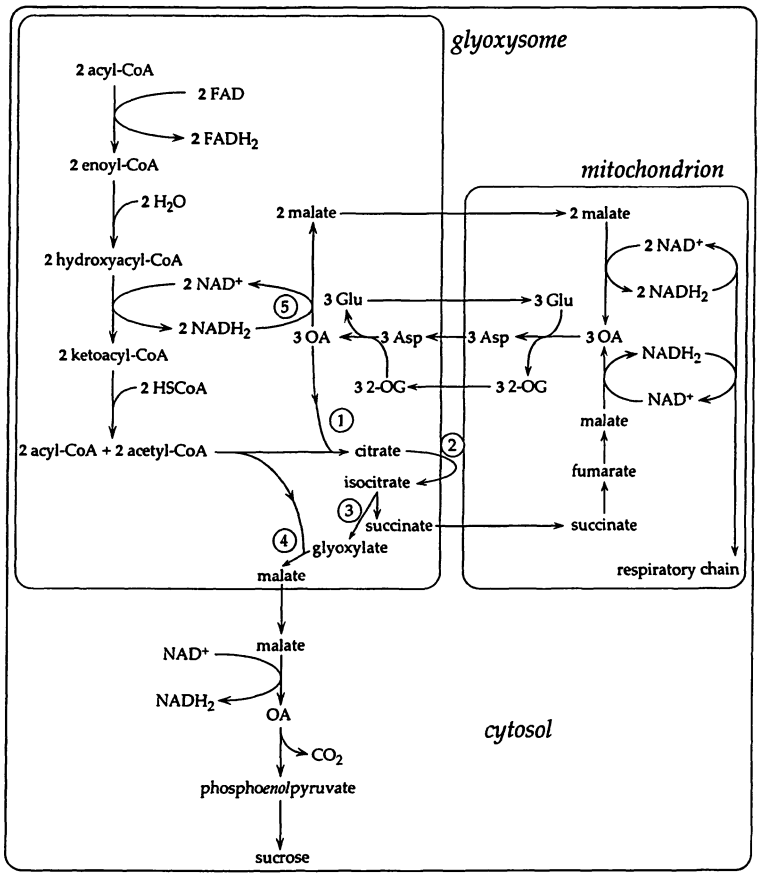

Fig. 6 Glyoxylate Cycle Activities as the Essential Intermediate between Fatty Acid Catabolism and Gluconeogenesis.
The circled enzymes are: (1) citrate synthase, (2) aconitase (cytosolic isoform), (3) isocitrate lyase, (4) malate synthase, and (5) malate dehydrogenase (its reduction of oxaloacetate results in the regeneration of glyoxysomal NAD^+). Nonstandard abbreviations: OA, oxaloacetate, 2-OG, 2-oxoglutarate.

growth of transformed yeast on C2 or C3 compounds, such as acetate, ethanol or glycerol (known to be 'upstream substrates' of the glyoxylate cycle) is unaffected (Van Roermund *et al.*, 1995). These results suggest that glyoxysomal/peroxisomal malate dehydrogenase is essentially involved in the reoxidation of $NADH_2$ generated by fatty acid β-oxidation and does not therefore constitute a critical component of a 'classical' glyoxylate cycle.

Interplay between Glyoxysomal, Cytosolic and Mitochondrial Reactional Pathways

The occurrence of the putative malate/aspartate shuttle is supported by the thermodynamic parameters affecting oxaloacetate↔malate interconversions as well as by metabolic needs, and this imposes a reassessment of the current views on the glyoxylate cycle. The initiation of the cycle is considered to be the citrate synthase catalyzed production of citrate from acetyl-CoA and oxaloacetate. Acetyl-CoA can readily be formed by β-oxidation of fatty acids, and it has been assumed that oxaloacetate is produced from malate by the highly active glyoxysomal malate dehydrogenase. However, since it cannot now be ruled out that malate dehydrogenase would function *in vivo* in the reductive direction (thus catalyzing conversion of oxaloacetate into malate), the interconnections between β-oxidation, the glyoxylate cycle, the respiratory chain and gluconeogenesis may be different and/or more intricate than previously thought. The *in vivo* occurrence of a glyoxylate cycle *stricto sensu* might in particular be questioned. Figure 6 (Escher, 1996) proposes that glyoxysomal malate dehydrogenase is an essential and exclusive component of β-oxidation (NAD^+ regeneration). Its substrate oxaloacetate derives from the malate/aspartate shuttle, which also meets the need of citrate synthesis. The transport of succinate to the mitochondrion would equilibrate the shuttle. In this overall scheme, it is also considered that the C4 metabolite initiating gluconeogenesis would formally be malate resulting from the malate synthase catalyzed condensation of acetyl-CoA and glyoxylate.

Concluding Remarks

The sustenance of active gluconeogenesis initiated by lipid degradation during the germination and senescence processes presupposes that the glyoxysomal matrix is the

site of efficient β-oxidation and acetyl-CoA condensation reactions. Since the organelle membrane is not permeable to produced reduced cofactors, their *in situ* reoxidation is required. The readily available acceptor for the electrons generated by the flavine containing acyl-CoA oxidase is molecular oxygen,with subsequent elimination of the produced hydrogen peroxide by catalase and/or peroxidase activities. It is reasonable to assume that the reoxidation of $NADH_2$ produced by hydroxyacyl-CoA dehydrogenase is not independent of this reactional scheme, since the membrane dehydroascorbate reductase/peroxidase system is a likely candidate as a regeneration mechanism of NAD^+, concurrently with the malate/aspartate shuttle connected to the mitochondrial respiratory chain. Both the reductase/peroxidase system and the shuttle might coexist in a dynamic equilibrium depending on the simultaneous and varying necessities of $NADH_2$ reoxidation, protection against damages by hydrogen peroxide and demand for ATP.

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