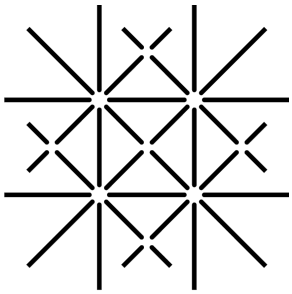

Promiscuous Behaviour of the Bacterial Metallohydrolase DapE: An Evolutionary and Mechanistic Perspective

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dedicated to my parents

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Abbreviations

ACE	Angiotensin I Converting Enzyme
A.proteolytica	*Aeromonas proteolytica*
Asp (D)	Aspartic acid
Asp-Leu	Aspartic acid-Leucine
C.elegans	*Caenorhabditis elegans*
C.glutamicum	*Corynebacterium glutamicum*
Dab	Diaminobutyric acid
DAP/mDAP	Diaminopimelic acid/meso-Diaminopimelic acid
Dap	Diaminopropionic acid
DapE	*N*-Succinyl-L,L-Diaminopimelic acid desuccinylase
DapE-C6His	C-terminus polyhistidine-tagged DapE
DMSO	Dimethyl Sulfoxide
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
G.stearothermophilus	*Geobacillus stearothermophilus*
HBTU	*O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate
HDAC	Histone Deacetylase
H. influenza	*Haemophilous influenzae*
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
k_{cat}	Catalytic constant
K_M	Michaelis constant
LB-medium	Luria Broth
Leu (L)	Leucine
L.pneumophila	*Legionella pneumophila*
MM	Michaelis-Menten
N.meningitides	*Neisseria meningitides*
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction

pNPP	*para*-Nitrophenyl Phosphate
RMSD	Root-Mean-Square-Deviation
SAHA	suberoylanilide hydroxamic acid
S.aureus	*Staphylococcus aureus*
S.enterica	*Salmonella enterica*
SDAP	*N*-Succinyl-L,L-Diaminopimelic acid
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
VB-medium	Vogel & Bonner medium
V.cholerea	*Vibrio cholera*
V_{max}	Maximal initial velocity

Abstract

Enzyme promiscuity, defined as functional properties other than those for which they are evolved, is considered a key factor in the evolution of new enzyme functions. Many metalloproteins can be alternatively metallated, which may lead to metal-dependent promiscuity. The mechanisms and evolutionary implications of metal-mediated promiscuity appear to be underexplored, especially considering that approximately one-third of structurally characterized proteins are thought to be metalloproteins.

Here, we investigated the bacterial binuclear metallohydrolase, *N*-Succinyl-L-L-Diaminopimelic acid desuccinylase DapE (EC 3.5.1.18) of *S.enterica*. DapE is an essential enzyme in the late stage of the lysine biosynthetic pathway that also provides a crucial building block of the peptidoglycan cell wall. Since DapE is essential for most Gram-negative and many Gram-positive bacteria and it is not present in humans, it has been proposed as a very good target for antibiotic development. It was also reported that DapE has a metal dependent promiscuous aspartyl dipeptidase activity, in which incorporation of either zinc or manganese to the enzyme leads to activity with different substrates and this phenomenon occurs both *in vivo* and *in vitro*.

We addressed the reaction mechanism of the native desuccinylase activity as well as the Mn^{2+}-dependent aspartyl dipeptidase promiscuous activity of DapE, by investigating a series of substrate analogues and potential inhibitors. We postulated a plausible mechanism for metal-dependent promiscuity, based on subtle differences in coordination preferences between Mn^{2+} and Zn^{2+},which may be widely applicable to other enzymes. We revealed why a promising inhibitor of the enzyme *in vitro*, L-captopril, fails to exert antibiotic activity and propose broad practical implications of this discovery for drug-design, as well as fundamental evolutionary implications. We described kinetic cooperativity in DapE, which offers clues on structural rearrangements that occur during catalysis and is also of relevance to inhibitor design.

Finally, we explored the evolutionary aspects of functional robustness of native activity over the promiscuous activity using DapE as a model enzyme and addressed the molecular mechanisms underlying the emergence of functional robustness through laboratory evolution.

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CHAPTER 1

Introduction

Approximately one-third of structurally characterized proteins and approximately half of all proteins are thought to be metalloproteins.[1-5] All living organisms require a variety of different metals, each of which has a rich and unique chemistry that is exploited to confer specific structural, regulatory and catalytic properties to proteins.

Selective metal utilization suggests that it is crucially important to incorporate the right metal for the right task and that this is a process that has been tailored carefully by evolution. For example, redox-inert metal ions like Zn^{2+} are often used in enzymes to stabilize negative charges and to activate substrates by virtue of their Lewis acid properties. In contrast Cu^{2+}, which is also a strong Lewis acid, is seldom used in Nature as such, perhaps because the stability of Cu^{2+}–ligand bonds can make it a relatively inefficient Lewis-catalyst; instead, copper is used in natural enzymes mostly for its redox-activity.[6]

Cells use both thermodynamic and kinetic mechanisms to incorporate the right metal into metalloproteins. The elucidation of these intricate control mechanisms remains a question of great fundamental interest.[7] However, biology is notoriously messy [8] and the "correct metals" may not always be incorporated into proteins.

Enzyme promiscuity

What are the consequences of a "wrong" metal being incorporated into a protein? Here we argue that such metal-substitution can lead to very different outcomes: metallation can be a source of toxicity and metabolic disruption, but also a source of phenotypic diversity, thus conferring potential evolutionary innovation through metal-mediated promiscuity.

Enzyme promiscuity has been defined by Khersonsky and Tawfik as the occurrence of enzyme activities other than those for which an enzyme evolved and that are not part of the

organism's physiology.[9] We aim to understand in detail the particular case of changes in enzyme specificity arising from metal-substitution, *i.e.* metal-mediated enzyme promiscuity.

Thermodynamic mechanisms	**Environmental factors**
• Type, number and orientation of ligands: a) Ligand preference of metals, *e.g.* Irving-Williams series [10] b) Metal-preference of coordination geometry	• Type of medium for the cell growth • Location of enzyme activity in the cells • Global availability of metals • Bio-availability of the metals • pH • Redox status
Kinetic mechanisms	**Genetic variations**
• Location of the protein folding in the cells • Effect of metallo chaperones	• Point mutations • Enzyme isoforms • Species variations [11]

Table 1: Factors that influence metal promiscuity

The adequate incorporation of metals into proteins takes advantage of both thermodynamic and kinetic control-mechanisms.[12-16] Thermodynamic mechanisms depend on metal availability in the protein environment and the intrinsic properties of metals. A common example of metal properties is reflected in the well-known Irving-Williams series of first-row divalent transition metals.[10] This series states that, in general, at equal concentrations of metal ions, the binding of Zn and Cu to protein ligands prevail over Mn-binding. However, such general thermodynamic preferences can be modulated in many additional subtle ways, for example by adapting the type, number and orientation of the ligands to suit the soft/hardness of the target metal and its preferred coordination geometry.[17] On the other hand, kinetic factors can dominate the distribution of metals in cells. *In vivo*, for example, the location of protein-folding may determine metallation and metallo-chaperones may also help to deliver metals to specific targets (Table 1).

Metallation of proteins *in vivo*

Exact knowledge of the metallation state of proteins *in vivo* is often thwarted with experimental difficulties.[5,18,19] Moreover, because metallation of proteins arises from complex interactions between thermodynamic and kinetic interactions (Table 1), the metal species bound to a protein may exhibit strong dependence on cell-context, differing for example in different media, cell-locations or in different species.[7] Such a cell-context dependence is

acutely illustrated by manganese superoxide dismutase (Mn-SOD) of *E.coli*,[20] an enzyme that requires Mn (not Fe) for activity. When this enzyme was over-expressed recombinantly in *E.coli* grown in medium supplemented with ferrous salts, Fe substituted for Mn in the active site.[21] The X-ray structure of this Fe-isozyme revealed a substrate-access channel blocked by solvent (either water or hydroxide), which provides a plausible explanation for lack of catalytic activity in the wrongly-metallated form.[22] Conversely, the incorporation of a metal that is not thought to be physiological may also lead to increased activity: a classical example of such enzyme activation is the metallation of *Aeromonas* leucine aminopeptidase with copper instead of zinc.[23] These studies show that one should be very cautious of concluding the nature of the physiologically-relevant metal based on enzyme activation alone.

Cambialistic enzymes: superoxide dismutases

The low metal-selectivity of proteins, which leads to alternatively metallated forms with differing activities according to the cellular environment, has been referred to as "metal promiscuity".[16] A more common term found in the literature on metalloproteins is "cambialism", which mostly refers to enzymes that are active with different metals. For example, there are a few "cambialistic superoxide dismutases" that retain measurable activity with various metals,[24,25] typically shown for Fe and Mn. In the case of many metalloproteins with cambialistic behaviour, the most physiologically relevant metallo-isoform may be difficult to establish and it may be that both (Fe & Mn) are used by the organism according to environmental availability, which may be dependent on the redox state of the cell. Under more oxidative conditions there may be a lowered availability of Fe due to formation of the less soluble Fe(III) species and therefore the Mn isoform of SOD may predominate.

Therefore, change in metallation may be environmentally-controlled in a physiologically-relevant manner without invoking genetic change. However, cambialism may be a careful balance between genetic and environmental factors.

Curiously, in *E.coli* Mn-SOD, there are mutations in the second-coordination sphere that modulate metal-selectivity, such as Glu170Ala. This genetic variant lacks an outer-sphere cross-link between active-sites in adjacent subunits and was isolated as an Fe-isoform even upon Mn-supplementation of bacterial cultures.[26]

Similarly, a mutation ~10Å away from the active site, Gly155Thr in the SOD from *P.gingivalis,* changed the activity ratio of Fe *vs* Mn from 0.6 in the wild type to 11.2 in the mutant.[27] Remarkably, such change of metal-specificity was accompanied by a doubling of the specific activity of the Fe-enzyme *in vitro* compared with the wild type, suggesting an evolutionary adaptation for properties other than maximal activity and, we speculate, the possibility of an evolutionary advantage for low metal-selectivity. Trade-offs, such as sub-optimal activation in exchange for adaptability to metal-availability, may be common in biology.[28]

Cambialism in evolution: a cadmium-containing carbonic anhydrase

The term "cambialism" has also been used in the context of putative changes in metal-cofactor usage by enzymes through evolution, for example due to changing environmental metal-availability, such as is thought to have occurred with availability of Fe upon advent of photosynthesis and the resulting change to a more oxidative atmosphere.[29] An example of such cambialism in evolution is the apparent adaptation of a carbonic anhydrase from marine diatoms to the scarcity of Zn in their environment: this enzyme was shown to exchange readily cadmium and zinc at its active site, a property that may provide a selective advantage to the organism when competing for metal-availability, particularly because Zn is scarce in its environment.[30] There has been an intense discussion on whether Zn-containing metalloenzymes may have been most abundant at the dawn of life.[31,32] Considering that Fe may have been much more abundant, together with the biochemical possibility of cambialism between Fe^{2+} and Zn^{2+}, we suggest that it is very plausible that some metalloenzymes that require Zn^{2+} today may have been evolved from enzymes that were mostly Fe^{2+}-containing in the past.

Metal-mediated substrate promiscuity *in vitro*

Enzymatic characterization of metalloproteins *in vitro* may lead to a change in substrate-selectivity according metal-supplementation. An example of such metal-mediated substrate promiscuity is the L-carbamoyl amino acid hydrolase from *Geobacillus stearothermophilus* CECT43.[33] Mn^{2+} activation of the metalloenzyme gave similar levels of hydrolysis for *N*-L-acetyl methione and *N*-L-carbamoyl methione substrates. However, when activated with Ni^{2+}, this promiscuous hydrolase exhibited a doubling of activity for *N*-L-acetyl methione.

Remarkably, this doubling of activity was substrate-specific, because the Ni^{2+} enzyme retained comparable levels of hydrolysis for *N*-L-carbamoyl methione as found for the Mn^{2+} isoform. Only the Ni^{2+} isoform, therefore, had a preference for the acetylated substrate compared with the methylated substrate.

Metal substitution may also lead to activity with new substrates. Cappielo *et al.* reported that the Mn/Zn isoform of the binuclear leucyl aminopeptidase from bovine lens generated a novel activity toward Cys-Gly, a substrate which is not hydrolysed by the Zn/Zn enzyme.[34,35] Although the Cys-Gly substrate was not hydrolysed by the Zn/Zn isoform, this compound was a competitive inhibitor for the more physiologically relevant Leu-Gly substrate, with a K_i value of 16.4 μM. Resolution of the crystal structure of the Zn/Zn isoform bound to the sulfhydryl-drug Zofenoprilat, together with modeling studies, provided a plausible explanation for such substrate-selectivity: the presence of the S atom at the Zn^{2+} coordination site 1, displaced the water molecule fundamental for catalysis and did not allow a Cys-Gly orientation within the enzyme active site that is productive for dipeptide hydrolysis. In contrast, it was surmised that a Mn^{2+} ion in site 1, an ion that prefers oxygen or nitrogen-containing ligands, allowed a productive orientation of the sulfur containing compounds, thus making them good substrates.

A cancer-associated protein phosphatase 2C was also shown to exhibit metal-mediated substrate selectivity *in vitro*.[36] The Mn^{2+} ion was most effective in activating the enzyme for dephosphorylation of the artificial substrate *para*-nitrophenyl phosphate (pNPP). For comparison, in this assay the Mg^{2+}-enzyme exhibited about 7-fold less activity than the Mn^{2+} enzyme. Surprisingly, Mg^{2+} was clearly a much better cofactor (leading to about 7-fold more activity than Mn^{2+}) when using the protein casein as substrate. Other distantly related type 2C phosphatases from bacteria also showed substrate preferences according to metallation either with Mn^{2+} or Mg^{+}. For example, the PrpZ phosphatase from *Salmonella enterica* was shown to exhibit a strong preference for Mn^{2+} when assaying dephosphorylation of pNPP *in vitro*. However, the Mn^{2+} metalloenzyme was only two-fold more effective than the Mg^{2+}isoform when using myelin basic protein as a substrate.[37] Similarly, the Stp1 phosphatase from *Bacillus anthracis* clearly exhibited higher activity for pNPP with Mn^{2+}, rather than Mg^{2+}, which worked less effectively for hydrolysis.[38] The same preference for Mn^{2+} was found when using the phosphopeptide RRA(pS)VA as substrate. Remarkably, there was no such clear preference for either metal when using the closely related phosphopeptide RRA(pT)VA

as substrate. Cumulatively, these biochemical data measured *in vitro* suggest that Mn^{2+}/Mg^{2+}-mediated substrate promiscuity may be common in type 2C phosphatases, even in distantly-related species. Moreover, although the concentrations of metals used in these studies were rather high (Mn^{2+} often between 1 and 10 mM), these findings suggest the possibility that the ratio of the available pool of Mn^{2+} and Mg^{2+} present intracellularly could be used as a mechanism to regulate the preferred substrates of these enzymes *in vivo*.

Metal-mediated substrate promiscuity *in vivo*

The relevance of the novel activities of the different metallated forms of enzymes such as leucyl aminopeptidase or phosphatases 2C *in vivo* is unclear, because these enzymes were expressed in recombinant form and characterized *in vitro*. However, there is increasingly compelling evidence that mixed-metallation can occur in some cases *in vivo*, which may be relevant for activity. For example, the Mn/Zn homologous enzyme from *Pseudomonas putida* was shown to be more active than the Zn/Zn isoform when using L-phenylglycine amide as a substrate. This activity assay was used to show that recombinantly expressed in *E.coli* contained Mn in site 1, even though Zn was thought to be the preferred metal and despite that neither the medium nor any of the buffers used in the purification procedure had been supplemented with Mn.[39] An even more illustrative example is given by the *dapE*-encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE), which cleaves the *N*-succinyl-L,L-diaminopimelic acid (SDAP) to form L,L-diaminopimelic (L,L-DAP) acid and succinate.[40] This binuclear metalloenzyme is thought to contain co-catalytic Zn ions in its active site in physiological conditions. However, DapE presents also a promiscuous dipeptidase activity.[41] In the presence of manganese ion (Mn^{2+}), one of the Zn^{2+} is exchanged by Mn^{2+}, which allows DapE to cleave aspartyl dipeptides *in vitro*.[41] In contrast, the Zn^{2+} enzyme, whether mononuclear or binuclear, is totally inactive for the promiscuous aspartyl-hydrolase activity. Importantly, enzyme promiscuity was also suggested to occur *in vivo*. In a series of elegant experiments using a bacterial strain deficient for all other aspartyl-dipeptidases and also unable to synthesize leucine, Broder & Miller showed that the growth of the bacteria on medium containing only the dipeptide Asp-Leu as a leucine source was strictly dependent on DapE expression.[41] The observation of such DapE-dependent growth led to the inescapable conclusion that sufficient Mn must be present inside the cell to lead to DapE promiscuous activity, even in minimal medium without any additional metal supplementation.

R. *Kpn*I is a Type II restriction enzyme, cleaving double-stranded DNA in the presence of many metal cations. It is the nature and concentration of the cation leads to various degrees of activity and fidelity of substrate recognition. Ca^{2+} and Zn^{2+} both induce robust and specific hydrolytic activity. However, it is a highly promiscuous enzyme in Mg^{2+}- and particularly in Mn^{2+} catalyzed reactions. It was speculated that the flexibility in metal ion utilization by R.*Kpn*I could provide the organism with an evolutionary advantage since the restriction enzyme could function regardless of the availability of a particular metal ion.[42] Moreover, considering that the free intracellular concentrations of Mg^{2+} range from 0.3 to 2 mM, it is feasible that facile exchange with Mg^{2+} is an evolved mechanism to modulate the degree of promiscuity *in vivo*. Clearly, the biological relevance of the promiscuous DNA cleavage is an important question that needs further investigation.

Metal-mediated promiscuous reaction mechanisms.

In some cases, the nature of the metal in an enzyme active site appears to select not only the substrate, but also determined the type of chemical transformation that takes place. The non-heme Fe^{2+}-dependent dioxygenase Dke1 from *Acinetobacter johnsonii* converts 2,4-pentanedione and O_2 into methyglyoxal and acetate. The Fe is coordinated by the triad His_{62}, His_{64}, and His_{104} (Figure 1) and is absolutely required for oxygenase activity, since other metal ions, like Zn^{2+}, Ni^{2+} or Cu^{2+}, that compete with Fe^{2+} for binding are completely inactive in the enzymatic reaction with O_2. However, the enzyme is catalytically promiscuous as a metallohydrolase, cleaving 4-nitrophenylesters either in the Fe or Zn mononuclear isoform. The non-native Zn^{2+} form of the enzyme is the more active esterase, exhibiting about ten-times more activity than the Fe^{2+} enzyme. Most remarkably, substitution of His_{104} by Glu (Figure 1), which destroys the oxygenase activity, is compatible with the function as a Fe^{2+}/Zn^{2+} esterase, exhibiting more than 10% remaining catalytic proficiency (k_{cat}/K_M). Analysis of the reported kinetic data reveals metal-dependent substrate promiscuity, since the ratio of the second-order rate constant (k_{cat}/K_M) for pNPP between the Zn and Fe isoform is approximately half of that of pNPB hydrolysis between the Zn and Fe isoforms.[43]

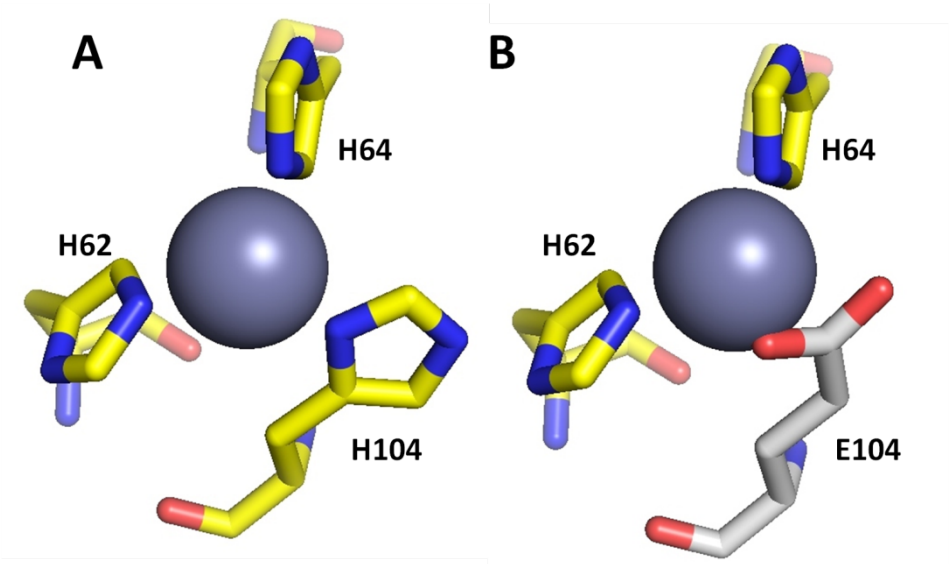

Figure 1. Active site of oxygenase Dke1 wild type (A) and modelled mutant H104E (B). The mutation destroys the Fe^{2+}-dependent oxygenase activity but is compatible with the promiscuous function as a Fe^{2+} or Zn^{2+} esterase, exhibiting more than 10% remaining catalytic proficiency (k_{cat}/K_M). Image created from Pymol using PDB: 3BAL file of wild type Dke1.

Conversely, the purple acid phosphatases (PAPs) are thought to be physiological hydrolases, some of which may exhibit promiscuous oxidase activity. These binuclear enzymes are found throughout all kingdoms of life and their colourful name originates from the chromophore generated by a charge-transfer between a tyrosinate and Fe^{3+} ion within the binuclear metal center.[44-47] The catalytic center and active-site domain are largely conserved despite the very low sequence similarity amongst different enzyme orthologs. Importantly, the nature of the second metal, a catalytically-important divalent cation in all cases, varies amongst the first-row transition metals: Fe^{2+}, Zn^{2+} and Mn^{2+}. It is notable that plants in particular have an abundance of PAPs with many different metallo-isoforms. As well as their well-characterised phosphatase activity, several PAPs have been reported to exhibit radical-forming activity, which requires a redox-active second metal, such as Fe in the case of human PAP.[48] Over expression of PAP increases oxygen-and nitrogen radical generation *in vivo* and increases the bactericidal activity of macrophages [49] and this radical-forming activity has been reported to be controlled independently of its phosphatase activity.[50] Moreover, preparations of a mammalian PAP, normally thought to be a mixed binuclear Fe^{3+}-Fe^{2+} active species have been isolated from bovine spleen containing trace amounts of a "contaminant" corresponding to a non-redox active Fe^{3+}-Zn^{2+} species, which is presumably incompetent for catalyzing production of oxygen and nitrogen radicals.[51] As an aside, it is noteworthy that the Fe^{3+} metal

in PAP has been substituted for an Al^{3+} ion to form a colourless enzyme species, which remains, however, catalytically active as a phosphatase. Although this Al-containing species is unlikely to be biologically-relevant, it remains to our knowledge the only example of a catalytically-active aluminium enzyme species.[52]

Metal-mediated moonlighting function

The significance of the various metallated forms of PAP *in vivo*, conferring different catalytic and regulatory properties remains speculative. Thus, it is unclear whether the di-iron and other redox-active isoforms truly have dual activities in *vivo* that may have been selected in evolution: if such is indeed the case rather than speaking of a promiscuous function, which by definition has not been selected in evolution, one could refer to such dual-enzymes as "moonlighting proteins".[53] "Moonlighting" has been defined as "the utilization of protein parts outside the active site for other functions, mostly regulatory and structural".[9] The regulation of such putative metal-mediated moonlighting proteins would be a worthwhile topic for exploration. For example, it would be interesting to investigate whether any of the PAPs are metallated differently in alternative compartments, with the metal used varying according to the required function. An example of "moonlighting protein" that is worthy of mention is the Iron-Regulated Protein 1 (IRP1), which alternates between an active cytosolic aconitase form that has an iron-sulfur cluster ligated to its active site and an apoprotein form that regulates iron homeostasis. [54] Thus, the apoprotein appears to contribute to the regulation of its own conversion to an enzymatically active metalloprotein: this regulation is achieved by binding to iron response elements (IREs) in the mRNA of ferritin, an important iron-storage protein.

Metallation of proteins *in vitro* for the creation of artificial metalloenzymes.

Metal-mediated enzyme promiscuity is very relevant for the expanding field of protein design and evolution, because this phenomenon can be exploited for the creation of new activities in the laboratory. We have shown several examples above, where the nature of the metal in the active site influences the chemical transformation. Already in the 1970's, Emil Kaiser and colleagues recognized that metal-ion substitution in metalloenzymes *in vitro* could yield novel activities that could act on biologically important substrates.[55,56] This postulate was demonstrated elegantly by preparing the Cu^{2+} form of carboxypeptidase A, which abolished

the hydrolytic activity of the enzyme but conferred oxidative activity on ascorbic acid to produce dehydroascorbic acid *in vitro*. More recently, a di-Cu^{+} substituted aminopeptidase was shown to acquire catechol oxidase activity.[57] Metal exchange of carbonic anhydrase II *in vitro* has been used successfully for the creation of a series of catalysts, including for epoxidation,[58] peroxidation[59] and hydroformylation.[60] In addition, effective sulphoxidation catalysts were achieved by substitution of the Zn^{2+} in thermolysin with tungstate.[61] As an aside, it has also been shown that direct coordination of metals into proteins that are not normally metal-binding can also give rise to nascent enzymatic activities *in vitro*, for example in enantioselective vanadium-mediated sulphoxidation[62] and osmium-mediated dihydroxylation[63] using the non-enzymatic protein scaffolds. There are also numerous examples of semi-synthetic metalloenzymes, constructed either by replacing a metal in a natural organic co-factor or by attaching a synthetic metal-ligand to a biomolecule.[64-67] It should be noted that in many cases the metals used for such catalysis *in vitro,* including noble metals like rhodium, are non-physiological and with no known biological relevance; nevertheless, these more exotic metals have attractive chemical properties that can be exploited for the design of artificial metalloenzymes.

In summary, control and prediction of metallation in proteins is thwarted with experimental difficulties and its effects on catalysis are often surprising and unpredictable.[5,18,19] Non-strict metal incorporation can be used as a facile way to create catalytic diversity [68] and, indeed, may be common in proteins and exploited as an evolutionary mechanism to generate phenotypic diversity. Finally, in a biomimetic spirit, such metal-mediated promiscuity may be exploited *in vitro*, for example in the context of biocatalysis.

	Enzyme	Metal	Enzyme Activity	Ref.
1	*E.coli* Mn-SOD, Wt	Mn^{2+}	Active	69
		Fe^{2+}	Inactive	21
	E.coli Mn-SOD, E170A	Fe^{2+}	Active	26
2	*Aeromonas* Leucine Aminopeptidase	Zn^{2+}	Active	23
		Cu^{2+}	More active than Zn^{2+}	
3	SOD	Fe^{2+}	Active	24
		Mn^{2+}	Active	
4	*P.gingivalis* SOD, Wt	Fe^{2+}	1600	27
		Mn^{2+}	2700 more than Fe^{2+}	
	P.gingivalis SOD, G155T	Fe^{2+}	2-fold more than wild type	
		Mn^{2+}	9-fold less than wild type	
5	Carbonic anhydrase	Zn^{2+}	Active	30
		Cd^{2+}	Active	
			***N*-L-acetyl methione**	
6	L-carbamoyl aminoacid hydrolase	Mn^{2+}	Active	33
		Ni^{2+}	2-fold more than Mn^{2+}	
			***N*-L-carbamoyl methione**	
		Mn^{2+}	Active	
		Ni^{2+}	Active	
7	Bovine lens leucyl aminopeptidase	Zn_1^{2+}/Mn_2^{2+}	Active	34,35
		Zn_1^{2+}/Zn_2^{2+}	Inactive	

			Oxygenase activity	[70]
8	Dioxygenase Dke1, Wt	Fe^{2+}	Active	
		Zn^{2+}, Ni^{2+} or Cu^{2+}	Inactive	
	Dioxygenase Dke1, H104E	Fe^{2+}	Inactive	
			Esterase activity	
	Dioxygenase Dke1, H104E	Fe^{2+}	Active as wild type	
		Zn^{2+}	10-fold more active than Fe^{2+}	
			pNPP as substrate	
9	2C Protein phosphatase	Mn^{2+}	Active	[71]
		Mg^{2+}	7-fold more than Mn^{2+}	
			Protein Casein as substrate	
		Mn^{2+}	Active	
		Mg^{2+}	7-fold increase than Mn^{2+}	
			SDAP as substrate	
10	DapE	Zn_1^{2+}/--	Active (Less active)	[72]
		Zn_1^{2+}/Zn_2^{2+}	More active than mono-Zn^{2+}	[72]
		Zn_1^{2+}/Mn_2^{2+}	More active than di-Zn^{2+}	This work
			Asp-Leu as substrate	[41]
		Zn_1^{2+}/--	Inactive	
		Zn_1^{2+}/Zn_2^{2+}	Inactive	
		Zn_1^{2+}/Mn_2^{2+}	Similar to SDAP activity	

Table 2. List of Metal-dependent Promiscuous enzymes discussed in this report. Wt is wild type. DapE is *N*-Succinyl-L-L-Diaminopimelic acid desuccinylase. SDAP is Succinyl Diaminipimelic acid. pNPP is *para*-Nitrophenyl Phosphate. SOD is Superoxide dismutase.

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CHAPTER 2

Selectivity of inhibition of *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE) *in bacteria*: DapE is not the target of L-captopril antimicrobial activity. [1]

Abstract

The emergence of bacterial strains that are resistant to virtually all currently available antibiotics underscores the importance of developing new antimicrobial compounds.[2,3] *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE) is a metallohydrolase involved in the meso-diaminopimelate (mDAP)/lysine biosynthetic pathway necessary for lysine biosynthesis and for building the peptidoglycan cell wall.[4] Because DapE is essential for Gram-negative and some Gram-positive bacteria, DapE has been proposed as a good target for antibiotic development.[5] Recently, L-captopril has been suggested as a lead compound for inhibition of DapE, although its selectivity for this enzyme target in bacteria remains unclear.[5] Here, we tested the selectivity of L-captopril against DapE *in bacteria*. Since DapE knockout strains of gram-negative bacteria are viable upon chemical supplementation with mDAP, we reasoned that the antimicrobial activity of compounds targeting DapE should be abolished in mDAP-containing media. To our surprise, although we confirmed that L-captopril has modest antimicrobial activity *in bacteria*, the inhibition of bacterial growth was independent both of mDAP supplementation and DapE over-expression, in *Escherichia coli* and in *Salmonella enterica*. We conclude that DapE is not the main target of L-captopril inhibition in these bacteria. The methods implemented here will be useful for screening for DapE-selective antimicrobial compounds directly in bacterial cultures.

Introduction

Most novel antibiotics that are developed are simply broad-spectrum, structural variants of a limited set of known of bioactive compounds, many targeting the same enzymatic pathways. Consequently, the risk that pathogenic bacterial strains eventually evolve resistance against new antibiotics is very high.[2,3] To help combat the serious problem of antibiotic resistance, it is imperative that new enzymatic targets are identified and that their specific inhibitors are developed. DapE, *N*-succinyl-L,L-diaminopimelic acid desuccinylase of the meso-diaminopimelate (mDAP)/lysine biosynthetic pathway of bacteria has been identified as an attractive potential antibiotic target.[6] DapE is a terminal enzyme for the hydrolysis of the *N*-succinyl-L,L-diaminopimelic acid (SDAP) (Scheme 1) into succinate and DAP.[7] Two of the products of this pathway (mDAP and lysine) are essential components of the cell: lysine is a protein-amino acid and DAP is a necessary component of the peptidoglycan cell wall of all Gram-negative and many Gram-positive bacteria.[4,6] Since there is no enzyme in mammals similar to DapE, the inhibitors of DapE could potentially provide selective toxicity against bacteria and have little or no effect on humans.

DapE is a homo-dimeric enzyme, with each monomer (41.6 KDa) containing two structural domains: a dimerization domain and a catalytic domain with a di-zinc active site.[7,8] Sequence alignment of all known DapE enzymes, including of *E.coli* and *S.enterica,* with the structurally characterized DapE of *H.influenzae* and *Neisseria meningitides,* point toward the very strict conservation of all the amino acids that function as metal ligands and putative substrate binding sites.[7,9-11]

Strong inhibition of metalloenzymes has often been achieved by direct coordination of catalytic metals within the active site. Three examples of such inhibition employed successfully in clinical drugs are the sulphonamides (as carbonic anhydrase inhibitors),[12] suberoylanilide hydroxamic acid (SAHA) as histone deacetylase inhibitor[13] and L-captopril (Scheme 1), which was the first marketed anti-hypertension drug, targeting angiotensin I converting enzyme (ACE).[14] Captopril binds to the catalytic zinc of ACE through coordination by a sulfhydryl group. Although captopril also shows some inhibitory activity towards other zinc metalloproteases, this is typically several orders of magnitude weaker than with ACE.[14]

Recently, Becker and colleagues [6] also identified captopril amongst the best inhibitors of *H. influenzae* DapE in a screen biased toward compounds containing zinc-binding groups (including thiols, carboxylic acids, boronic acids, phosphonates and hydroxamates). Captopril was found to be a low-micromolar inhibitor of DapE (IC_{50} = 3.3 μM, Ki = 1.8 μM) and had antimicrobial activity *in bacteria* (against *E.coli*). However, here we present evidence that DapE is not the main target of L-captopril antimicrobial activity *in bacteria*.

Bacterial strains, plasmids, media and other materials

Escherichia coli XL1-Blue was purchased from Stratagene. *Salmonella enterica* serovar Typhimurium DapE knockout-strain (TN5911) and the plasmid, pCM655/DapE, were kindly provided by Prof. Charles G. Miller.[15] E-medium[16] supplemented with 0.4% glucose and a 0.4 mM concentration of the appropriate amino acids (Bachem) was used as a minimal medium, and LB-medium was used as a rich medium. As a supplement, *meso*-diaminopimelate (mDAP) was added at 1 mM (Bachem). L-captopril was purchased from Sigma Aldrich. Sodium ampicillin, tetracycline and chloramphenicol were used at final concentrations of 60, 5 and 34 μg/ml, respectively, when added to either liquid or solid medium. Liquid cultures were aerated by shaking on a rotary shaker (250 rpm), and all growth incubations were at 37°C for 19 hours. Electro competent cells of *S.enterica* and chemical competent cells of *E.coli* were prepared by standard protocols. IPTG and mDAP were used at a final concentration of 1 mM. Agar (used at 1.5 %) and agarose (used at 1 %) were purchased from Invitrogen. T4-DNA ligase and *HindIII* from New England Biolabs. All other chemicals were purchased from Applichem, unless otherwise specified.

Methods

An empty plasmid, pCM699, was derived from the plasmid, pCM655/DapE,[15] by deleting the DapE gene by *HindIII* digestion and re-ligation of the vector backbone (Table 1). The strain TN5911 (chloramphenicol resistance), is a knock out strain for DapE and for several dipeptidases; therefore, mDAP has to be supplemented in both minimal and enriched media. An appropriate source of leucine and proline is also required in minimal medium and supplementation with lysine provides better growth (Prof. Charles G. Miller; personal communication). Two strains were derived from the knockout-strain TN5911 by transforming the plasmids pCM655/dapE and pCM699 (strains TN5935 and TN5959, respectively). The

plasmids (pCM655/dapE and pCM699) were transformed into TN5911 by electroporation (1.8Kv, 0.1cm cuvettes) and recovered with 1 ml of SOC medium containing mDAP and chloramphenicol for 1 hour at 37°C in a shaking incubator and selected for plasmid encoded ampicillin resistance. A single colony of each strain TN5935 and TN5959 was picked and cultured in 5 ml of LB containing appropriate amounts of chloramphenicol, ampicillin, and mDAP over night at 37°C in a shaking incubator. The cultures of the two strains were diluted by 10 times (to dilute-out any remaining mDAP) and each strain was plated in a minimal medium supplemented or not with mDAP. In a similar manner, *E.coli* strains, TN5960 and TN5962, were derived from wild-type XL1-Blue by transforming the plasmid pCM699 and pCM655/DapE respectively. Analysis of soluble cell extracts of TN5960 and TN5962 by SDS-PAGE revealed the presence of an additional species of about 42KDa in cells harbouring pCM655/DapE (*i.e.* strain TN5962), corresponding to the expected size of the recombinant *S.enterica* DapE in *E.coli*.

To test L-captopril inhibition, appropriate dilutions of each strain (e.g. TN5935 & TN5959) were spread on selection plates with and without mDAP. After spreading the cultures, sterile paper discs soaked in several concentrations of L-captopril were placed on each plate; alternatively, a given amount of compound was placed in powder form directly at a defined site on the agar, such that it could be covered by a paper disk, and subsequently 10µl of sterile water were added carefully on the disk. These selection plates were incubated over-night at 37°C.

Plasmids	**Genotype**	**Remarks**
pCM655/DapE	Plasmid with *dapE* gene	Allows over-expression of DapE, thus rescuing DapE knockout strains (even in absence of mDAP supplementation). Also confers ampicillin-resistance
pCM699	Plasmid without *dapE* gene	Confers ampicillin-resistance, but does not genetically rescue DapE knockout strains

Strains	Genotype	Remarks
S.enterica		
TN5911	*dapE* knockout strain	Strain does not grow in absence of mDAP supplementation
TN5935	TN5911 + pCM655/DapE	DapE is over-expressed from plasmid and strain can grow in absence of mDAP supplementation
TN5959	TN5911 + pCM699	Plasmid does not contain DapE and knockout strain does not grow in absence of mDAP supplementation
E.coli		
XL1-Blue	Wildtype (from Stratagene)	Has endogenous DapE and can grow in absence of mDAP supplementation
TN5960	XL1-Blue + pCM699	Plasmid does not contain DapE, but strain can grow in absence of mDAP supplementation due to endogenous DapE
TN5962	XL1-Blue + pCM655/DapE	In addition to endogenous DapE, this strain has plasmid-encoded DapE over-expression (from *S.enterica*).

Table 1. Summary of strains and plasmids used in this work.

Results

First, we compared the growth of *E.coli* in the presence of L-captopril, with and without mDAP supplementation in the agar-medium, using a disk-diffusion assay (Figure 1 and Table 2). We confirmed the modest inhibitory activity of L-captopril in *E.coli*, as previously reported. However, to our surprise, L-captopril antimicrobial activity was independent of mDAP supplementation in *E.coli*. Moreover, the extent of inhibition was also unaffected by heterologous expression of DapE (from *Salmonella enterica*) in *E.coli* (Figure 1c & 1d).

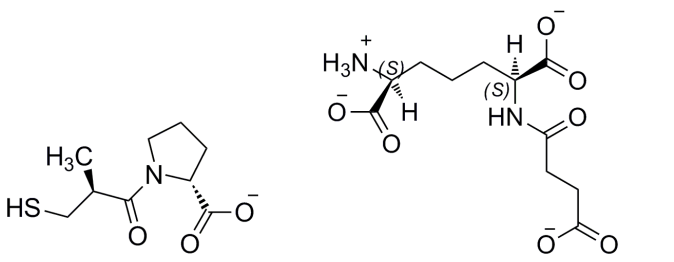
Second, considering that over-expression of DapE from *S.enterica* in *E.coli* did not affect L-captopril inhibition, we set-out to confirm that the DapE plasmid was functional for DapE expression. As previously reported [15], the DapE knock-out strain (TN5911) of *S. enterica* only grows when harbouring the plasmid carrying *dapE* gene (pCM655/DapE, within strain TN5935) or by supplementing the medium with mDAP, which is derived from the native product of DapE hydrolytic activity, L,L-DAP. Consequently, strain TN5935 (which harbours a plasmid-encoded *dapE* gene) grew even in the absence of mDAP supplementation, since it can produce its own DAP for cell-wall synthesis. As negative control, we corroborated that the knockout strain harbouring the corresponding "empty plasmid" (conferring ampicillin resistance but with no *dapE* gene; strain TN5959) did not grow in absence of mDAP supplementation.

Third, we tested the growth inhibiting-activity of L-captopril in these various recombinant strains of *S.enterica*. We hypothesised that if L-captopril was inhibiting DapE *in bacteria,* then mDAP supplementation or DapE over-expression would alleviate the antimicrobial effects of the drug. Surprisingly, L-captopril inhibited the growth of TN5959 (harbouring an empty plasmid) despite the addition of mDAP (Figure 2 and Table 3). We also tested whether the modest L-captopril inhibition could be overcome by DapE over-expression. Here also, we were surprised to find that similar L-captopril inhibition of strain TN5935 (over-expressing DapE), even when mDAP was additionally supplemented in the medium.

The zone of inhibition was slightly more in strain TN5935 compared to TN5959, although this difference was very subtle and could only be seen when the paper disks were soaked at the concentrations 25mg/ml and 50mg/ml, but not when 20mg were added (Table 3); at the latter amount of compound, the extent of inhibition was significant and identical to that

observed in TN5959. We speculate that this marginally higher L-captopril inhibition in the strain over-expressing DapE (TN5935) compared with TN5959 is due to the high metabolic load of the cell caused by over-expression of DapE.

Taken together, these data strongly suggest that L-captopril modestly inhibits both *S.enterica* and *E.coli* to a similar extent, but in a DapE-independent manner.



Scheme 1. Structures of L- captopril and *N*-succinyl-L,L- diaminopimelic acid.

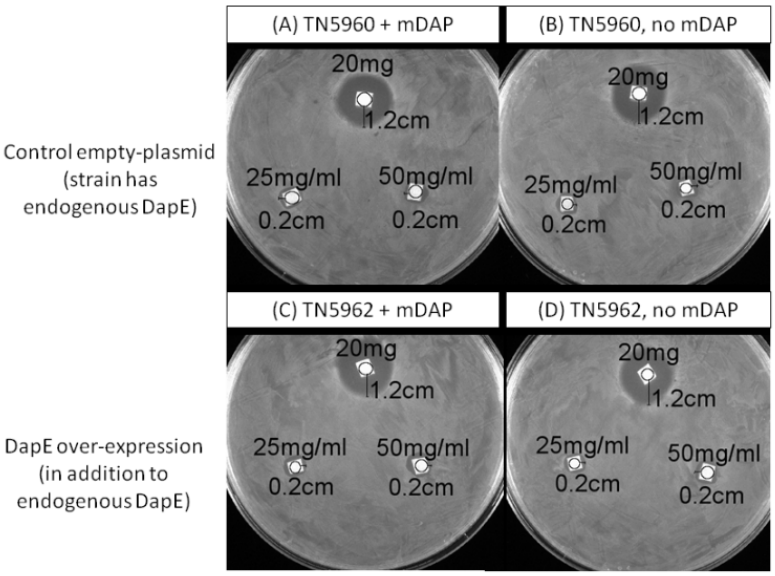


Figure 1. DapE independent inhibition of *E.coli* by L-captopril. (A) the strain has constitutively expressed endogenous *dapE* gene and the medium was also supplemented with mDAP, (B) the strain has constitutively expressed endogenous *dapE* gene but the medium was not supplemented with mDAP, (C) the strain has over-expressed plasmid encoded DapE (in addition to the constitutively expressed endogenous DapE) and the medium was also supplemented with mDAP, (D) the strain has over-expressed plasmid encoded DapE (in addition to the constitutively expressed endogenous DapE) but the medium was not supplemented with mDAP.

Strain with endogenous DapE	**TN5960** (Plasmid without *dapE* gene)		**TN5962** (Plasmid with *dapE* gene)	
Supplemented with mDAP	Yes	No	Yes	No
Radius of zone of inhibition by L- **captopril**				
25mg/ml	0.2cm	0.2cm	0.2cm	0.2cm
50mg/ml	0.2cm	0.2cm	0.2cm	0.2cm
20mg	1.2cm	1.2cm	1.2cm	1.2cm

Table 2. Inhibition of *E.coli* growth by L-captopril.

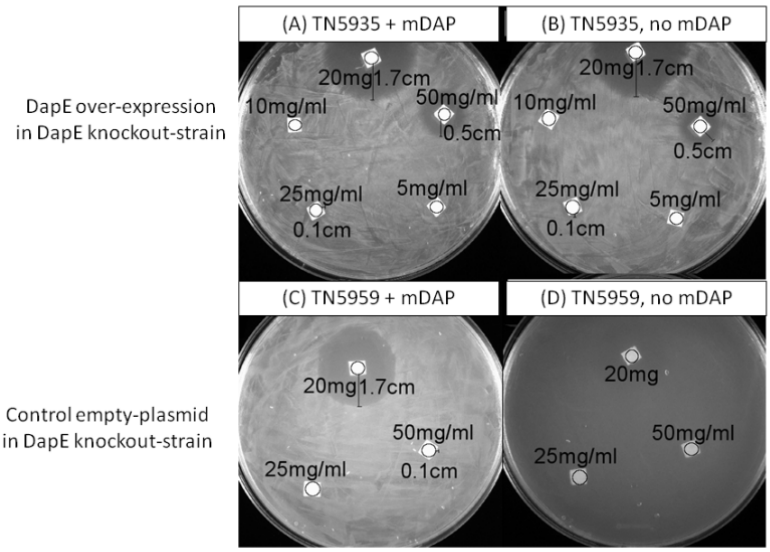


Figure 2. DapE independent inhibition of *Salmonella enterica* by L-captopril. The knockout-strain has plasmid encoded *dapE* gene but; (A) the medium is also supplemented with mDAP, (B) the medium is not supplemented with mDAP. The knockout-strain has no plasmid encoding *dapE* gene but; (C) the medium is also supplemented with mDAP, (D) the medium is not supplemented with mDAP.

DapE-knockout strains	**TN5935** **(**Plasmid with *dapE* gene)		**TN5959** (Plasmid without *dapE* gene)	
Supplemented with mDAP	Yes	No	Yes	No
Radius of zone of inhibition by L- **captopril**				
25mg/ml	0.1cm	0.1cm	0.0cm	No growth
50mg/ml	0.5cm	0.5cm	0.1cm	No growth
20mg	1.7cm	1.7cm	1.7cm	No growth

Table 3. Inhibiton of *Salmonella enterica* growth by L-captopril.

Discussion

Diaminopimelic acid is an essential precursor for cell wall synthesis in many bacteria, including *E.coli* and *S.enterica.*[4,17] Inhibition of DapE from *H.influenzae in vitro* and inhibition in *Escherichia coli* by L-captopril *in bacteria* was recently reported.[5] In the process of enzymatically characterizing mutant variants of *Salmonella enterica* DapE, we decided to test the inhibition of DapE with L-captopril *in bacteria*. We hypothesised that if L-captopril was inhibiting bacterial growth by inhibiting DapE*,* then mDAP supplementation in the growth media would overcome drug-inhibition. However, to our surprise we found that L-captopril inhibits the bacterial strains (*S.enterica* & *E.coli*) in a DapE-independent manner.

The putative substrate binding site and the metal coordinating residues are very much conserved in the DapE of *E.coli*, *S.enterica* and *H.influenzae,* all of which are thought to have similar binuclear metal-centres and identical catalytic mechanism.[7,9-11] Consequently, considering such structural and functional conservation among DapE from these various bacteria, it would be expected that L-captopril is able to inhibit the different homologs in a similar manner, proving potentially active as a broad-selectivity antibiotic. It is noteworthy that different isoforms of DapE (e.g. from different organisms or with different metal contents) could be inhibited differently, thereby making it difficult to find a single broad-spectrum antibiotic targeting DapE. Our data show that L-captopril is not a broad-spectrum antibiotic targeting DapE in bacteria, because it neither targets DapE in *E.coli* nor in *S.enterica*. Since the extent of inhibition by L-captopril *in bacteria* was similar in all of our experiments in *E.coli* and *S.enterica* and independent of DapE, we suggest that the modest antimicrobial activity of this compound is probably due to inhibition of hitherto-unidentified metalloproteins other than DapE.

In summary, our results show that, despite the reported inhibition of DapE from *H.influenzae* by L-captopril *in vitro*, it is very unlikely that DapE inhibition contributes to any significant antimicrobial activity in gram-negative bacterial cultures. Therefore, L-captopril is a modest antibiotic, inhibiting gram-negative bacteria at high doses, but its mechanism of action or molecular target remains unknown.

Finally, considering that DapE is a promising antibiotic target, the failure of a lead-compound that inhibits DapE *in vitro* to show any measurable anti-DapE effect *in bacteria* provides a sobering reminder of the difficulty of translating *in vitro* data to effects *in vivo,* even in pure microbiological cultures. However, despite our finding that captopril does not lead to DapE inhibition in bacteria, the development of other (more effective) DapE inhibitors *in vitro* and *in vivo* continues to be a very worthy goal and a promising line of research toward new antibiotics. We suggest that mDAP supplementation, as described here, will offer a facile and very robust method for confirming the selectivity of novel antibiotics targeting DapE *in bacteria*.

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CHAPTER 3

Zinc-selective Inhibition of the Promiscuous Bacterial Amide-Hydrolase DapE: Implications of Metal Heterogeneity in Evolution and Antibiotic Drug-Design [1]

Summary

The development of resistance to virtually all current antibiotics makes the discovery of new antimicrobial compounds to novel protein targets an urgent challenge. The *dapE*-encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE) is an essential metallo-enzyme for growth and proliferation in many bacteria, acting in the desuccinylation of *N*-succinyl-L,L-diaminopimelic acid (SDAP) in a late stage of the anabolic pathway towards both lysine and a crucial building block of the peptidoglycan cell wall. L-captopril, which has been shown to exhibit very promising inhibitory activity *in vitro* against DapE and has attractive drug-like properties, nevertheless does not target DapE in bacteria effectively. Here we show that L-captopril targets only the Zn^{2+}-metallo-isoform of the enzyme, whereas the Mn^{2+}-enzyme, which is also a physiologically-relevant isoform in bacteria, is not inhibited. Our finding provides a rational for the failure of this promising lead-compound to exhibit any significant antibiotic activity in bacteria and underlines the importance of addressing metallo-isoform heterogeneity in future drug design. Moreover, to our knowledge, this is the first example of metallo-isoform heterogeneity *in vivo* that provides an evolutionary advantage to bacteria upon drug-challenge

Introduction

It has been estimated that about 25 000 people die in Europe alone every year from an infection due to common antibiotic-resistant bacteria, causing an economic burden in excess of 1.5 Billion Euro per year.[2] Similar alarming statistics are reported around the world, making the current inevitable rise of antibiotic-resistance to our limited subset of clinically useful drugs an issue of great concern.[3,4] It is therefore crucially important and urgent that new protein targets are identified and that their specific inhibitors are developed.

DapE, *N*-succinyl-L,L-diaminopimelic acid desuccinylase of the meso-diaminopimelate (mDAP)/lysine biosynthetic pathway of bacteria has been identified as an attractive potential antibiotic target for two main reasons:[5] i) the growth of many bacteria is highly dependent of the biosynthesis of lysine and diaminopimelic (DAP) acid, which are essential for protein synthesis and elaboration of their peptidoglycan cell wall, respectively [6-8] and ii) both DAP and lysine are synthesized by the dap operon bacterial enzymes and no similar pathways take place in humans.

L-captopril was identified as a low-micromolar inhibitor of the *H.influenzae* DapE enzyme *in vitro*.[9] Considering that L-captopril is a marketed drug against hypertension[10] one may suppose that the compound already exhibits attractive pharmacokinetic properties. Moreover, considering the structural and functional conservation among DapE from various bacteria, one would expect that L-captopril may inhibit various bacterial homologues in a similar manner, thus potentially proving to be a broad-selectivity antibiotic. Disappointingly, however, L-captopril exhibited only very modest antimicrobial activity when tested in bacteria (including strains of *S.enterica* and *E.coli*). Moreover, this weak activity was revealed to be totally DapE-independent.[11]

The failure of a promising drug-like compound to exhibit any significant antimicrobial effect *in vivo* or to target the enzyme target identified *in vitro* is perplexing and acts as a sobering reminder of the difficulty of translating *in vitro* data to effects *in vivo*. In an attempt to overcome this initial set-back, we aimed at understanding why L-captopril should fail to have any significant antimicrobial potency in bacteria. Here, we present a simple answer to this conundrum, based on the surprising metal-selectivity of L-captopril and its inability to inhibit all microbiologically relevant metallo-isoforms. Further, our findings imply that heterogeneity

in metal content of metallo-enzymes may confer selective advantages.

Materials and Methods

Construction of C-terminus polyhistidine-tagged DapE (DapE-C6His)

A pET28b-based plasmid (Novagen) for DapE over expression was constructed according to published methods.[12] Briefly, the 1.2kb *dapE* gene was PCR amplified from the plasmid pCM655dapE by using the primers from microsynth;

F-SEQpCM655 (5' GCGTACGTCGCGACCGCGGACATGT 3')

R-pCM655XhoI (5' AGCCAAGCCTCGAGGGCGACGAGCTGTTCC 3')

Pfu turbo polymerase (Stratagene) and sub-cloned into pET28b plasmid (Novagen) at the restriction sites *Hind*III and *Xho*I (Biolabs).

Expression and purification of recombinant DapE-C6His

The expression plasmid, pET28b-DapE, was transformed into strain BL21(DE3), Stratagene; the pre-culture was made in LB-medium with 30 µg/ml kanamycin and 2% glucose at 37°C and the over-expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the OD_{600} is between 0.5 and 0.8, in LB-medium containing 30µg/ml kanamycin at 20°C throughout. The cells were harvested after 6 hours of IPTG induction and the cell pellet was frozen at -20°C, which was then thawed on ice and re-suspend in lysis buffer (50 mM tricine pH 7.8, 0.5 M NaCl, 1 mM PMSF, 3 µg/ml DNaseI and 5 µg/ml lysozyme) and the cell-lysis was carried out at 37°C, 200 rpm for 1 hour. The soluble protein fraction was transferred into a fresh bottle at 4°C after the centrifugation at high speed (9000 *g*) for 30 minutes in a pre-cooled (4°C) centrifuge.

The soluble protein fraction was filtered by 0.45 µm filter & the loading buffer (50 mM tricine pH 7.8 & 0.5 M NaCl) and elution buffer (50mM tricine, 0.5 M NaCl & 0.5 M imidazole and the final pH was adjusted to 7.8) were filtered by 0.2 µm filter. Filtered DapE-C6His protein was purified on HiTrap 5 ml chelating HP columns (Cat. # 17-0409-03, from GE) loaded with 0.1 M $ZnCl_2$ in 50 mM tricine with ÅKTA Purifier-10, from GE.

Removal of the labile metal from the purified protein solution was achieved by 3 step sequential dialysis. First, dialysis against 10 L of 50 mM tricine pH 7.8 with 1 mM 1,10-phenanthroline, second dialysis against 10 L of 50 mM tricine pH 7.8 with 5 mM ethylenediaminetetraacetic acid (EDTA) and third dialysis with 50 mM de-metallated tricine pH 7.8. Each dialysis is for 8 to 12 hours at 4°C using the dialysis bags with an approximate molecular weight cut-off of 6 to 8 KDa (Cat. # 132655, Spectrumlabs). Chelex-100 (Biorad) chelating ion exchange resins were used for the de-metallation of the buffer, 50 mM tricine pH 7.8 for dialysis.

Purified DapE protein from *S.enterica* exhibited a single band on SDS-PAGE, indicating a molecular weight of approximately 42 KDa. Protein concentrations were determined by using the Pierce BCA protein assay kit (Cat. # 23227, Thermo scientific). The yield of the purified DapE protein was about 1 mg/L of bacterial culture.

Synthesis of SDAP.

N-succinyl-L,L-diaminopimelic acid (SDAP) was synthesized in liquid phase through a standard coupling procedure between mono-*tert*-butyl succinate and enantiopure mono-*tert*-butyl protected L,L-diaminopimelic dimethyl ester (protected DAP), using *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as coupling agent. The product of the coupling was then conveniently deprotected by standard cleavage of the protecting groups. DAP was previously synthesized according to published protocols *via* olefin cross-metathesis between enantiopure Boc-L-Allylglycine-OMe and Cbz-L-Vinylglycine-OMe, in yields above 70%.[13]

DapE kinetic assays.

The enzymatic assays were carried out in triplicate in 50 mM tricine pH 7.8 at 37°C, using the purified DapE-C6His to a final concentration of 1 ng/µl. The final substrate concentration of native substrate (SDAP) was 20 mM in a reaction volume of 50 µl. Initially the DapE enzyme was pre-incubated with metals (0.2 mM $ZnCl_2$ & 0.5 mM $MnCl_2$ for native substrate) at 37°C for an hour and then with the inhibitor for another 10 minutes. L-captopril was purchased from Sigma-Aldrich. In all cases the reactions were initiated by adding substrate. The enzymatic reaction was followed by measuring the hydrolysis of the peptide bond of the

substrates.[14] Readings were carried out every 2 minutes for about 3 hours at UV230nm in UV transparent 96-well plates (Cat # 7.675 801, Greiner) using a TECAN Safire plate reader.

Growth assays.

Bacterial growth assays were carried out with strain TN5911, a knock out strain for DapE and for several dipeptidases; therefore, mDAP has to be supplemented in both minimal and enriched media. An appropriate source of leucine and proline is also required in minimal medium and supplementation with lysine provides better growth (Prof. Charles G. Miller; personal communication). For DapE expression, strain TN5911 was transformed with the plasmid pCM655/DapE to form strain TN5935.[11,12]

The metal-dependent anti-microbial activity of L-captopril was monitored by measuring the growth curves of the TN5935 in LB-medium containing L-captopril (0, 1 mM, 5 mM, 25 mM, 100 mM & 300 mM) under native activity selection (no mDAP supplementation) and under no selection (supplemented with 1 mM mDAP) and with and without 0.35 mM metal ($MnCl_2$ or $ZnCl_2$) supplementation. All measurements were carried out at least in duplicate. The main cultures were grown and the OD_{600} was measured in flat bottom 96-well plate (200 µl culture/well) in a plate reader (TECAN Safire) at 37°C for every 16 minutes. The main cultures were started by adding the pre-culture of OD_{600} ~1 to an initial OD_{600} between 0.1 and 0.2.

The effect of the metals on DapE enzyme activities in bacteria was monitored by measuring the growth curves of the TN5935 under no selection, selection for native & promiscuous activities with and without 0.35 mM metal salt ($MnCl_2$ or $ZnCl_2$) supplementation. These growth curves were measured form the cultures of minimal medium (1% $MgSO_4.7{\cdot}H_2O$, 10% citric acid$\cdot H_2O$, 50% K_2HPO_4, 17.5% $NaHNH_4PO_4{\cdot}4H_2O$, 0.4% glucose, 0.4 mM proline, 0.4 mM lysine, 0.4 mM leucine or Asp-Leu, 1 mM IPTG and 1mM mDAP) by following the OD_{600} every 4 hours at 37°C at 200 rpm. The cultures were started by adding the pre-culture of LB-medium of OD_{600} ~1 to an initial OD_{600} between 0.1 and 0.2, which corresponded in this case to 0.4 ml of overnight LB-medium culture in 100 ml of minimal medium in a 1 L Erlenmeyer flask. We carried out a control culture to confirm that at this dilution of rich LB-medium in minimal medium (250-fold dilution), there is insufficient carry-over of leucine to allow for any detectable growth of the *dapE* knockout strain (TN5911) when supplementing

with mDAP.

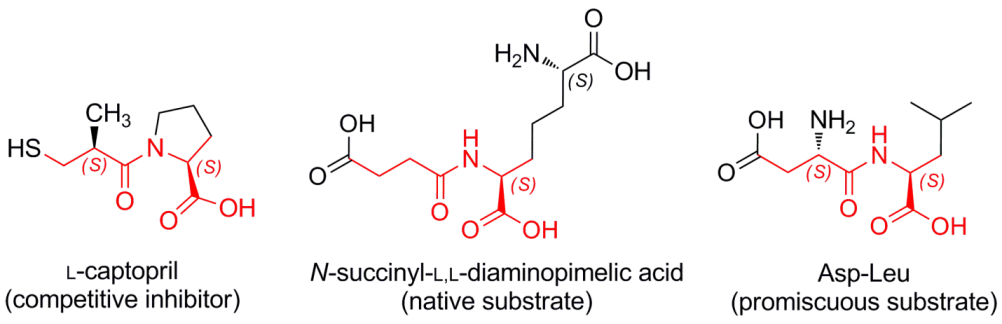
Structural modelling.

A homology model of the *Salmonella enterica* DapE model was built with SWISS-MODEL.[15] The di-zinc *Haemophilus influenzae* X-ray structure [PDB: 3IC1] sharing 61% identity with *S.enterica* without waters was used as a modelling template. The resulting model was energy minimized using the Yasara energy minimization server.[16] The L-captopril structure [PDBe: X8Z] was then placed manually into the active site and the resulting mono- and di-zinc structures energy minimized with fixed zinc ions using Maestro.[17] Only few ligand conformations without steric clashes were found satisfying the constraints of metal-coordination imposed and only the orientation in the final model occupies the binding pocket to any large extent, thus favouring free energies of binding. Model figures were all drawn with Pymol.[18]

Results

Metal substitution changes L-captopril inhibition *in vitro*.

DapE was active as an SDAP desuccinylase *in vitro* both in its Zn^{2+} mono-metallated form (Zn/-) or as a Zn^{2+} binuclear (Zn/Zn) or mixed (Zn/Mn) form, with comparable V_{max} (Scheme 1 & Table 1). Addition of L-captopril *in vitro* inhibited the catalytic activity of the (Zn/-) or (Zn/Zn) metallated isoforms of DapE, with an IC_{50} of 10 µM and 28 µM at 20 mM substrate, respectively (Figure 1). However, no significant inhibition of the Zn/Mn isoform was found even at 1 mM L-captopril.



Scheme 1. L-captopril inhibitor and substrates of DapE used in this study. Elements common to the three molecules are highlighted in red.

metallo-isoform DapE	V_{max} (µmol/L s^{-1})	k_{cat} (s^{-1})
Zn/-	18.23	309
Zn/Zn	39.81	677
Zn/Mn	64.99	1104

Table 1. DapE desuccinylation activity *in vitro*. [a] V_{max} and k_{cat} values of desuccinylase activity of different metallo-isoforms of recombinant DapE from *Salmonella enterica* typhimurium.

Manganese isoform is present *in vivo*

Traditionally, it has been considered that Zn^{2+} is the native and physiologically-relevant metal of DapE. However, the enzyme presents also a promiscuous dipeptidase activity that is dependent on the presence of manganese ions (Mn^{2+}), which can also be selected *in vivo* in an leucine auxotroph strain using Asp-Leu as a substrate.[12] Since this non-native dipeptidase activity occurs *in vitro* only when the labile, co-catalytic Zn^{2+} is replaced by Mn^{2+} to form a mixed Zn/Mn binuclear centre, then it is surmised that the DapE-dependent promiscuous activity in bacterial cultures also arises from Mn^{2+}-metallo-isoforms of DapE *in vivo*.[12]

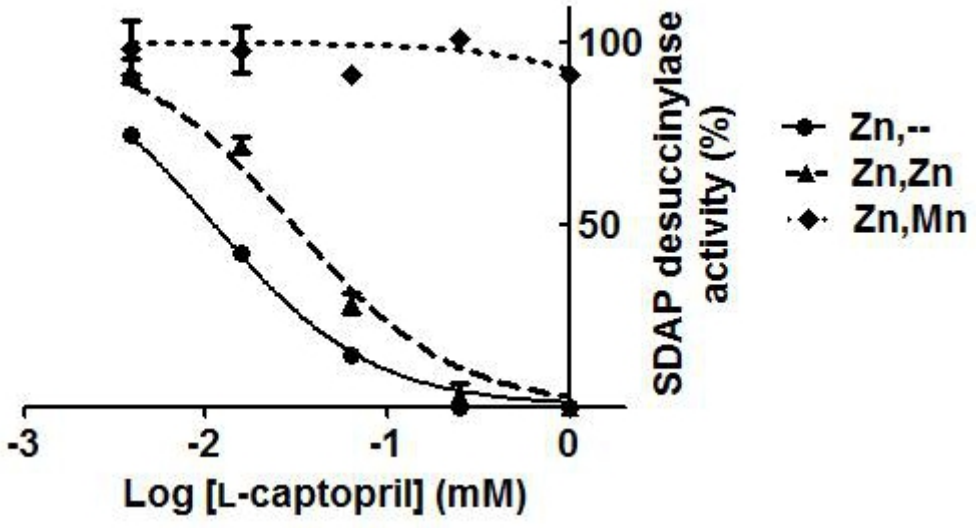


Figure 1. Inhibition of desuccinylase activity of different DapE metallo-isoforms by L-captopril. Measurements were carried out in triplicate and error bars represent the standard deviation from the mean.

When selecting for the native desuccinylase activity in bacteria, the conditions in which no metal was added and where Zn^{2+} was supplemented showed similar growth; in contrast Zn^{2+} slowed-down the growth of cell-cultures when selecting for dipeptidase activity, which suggests that Zn^{2+} is deleterious for the promiscuous activity of DapE, but not for its native activity (Figure 2). DapE was able to desuccinylate SDAP in its Zn/-, Zn/Zn and Zn/Mn metallo-isoforms *in vitro*, albeit to different extents, with the following trends for k_{cat}: Zn/Mn > Zn/Zn > Zn/- (Table 1). *S.enterica* grew appreciably better in Mn^{2+}-supplemented media when selected either for native or promiscuous activity (Figure 2). Therefore, it appears that addition of Mn^{2+} promotes dipeptidase activity of DapE both *in vivo* and *in vitro*, whereas Zn^{2+} inhibits the dipeptidase activity, presumably by competing with Mn^{2+} for the labile metal-binding site of DapE.

We conclude from these observations that: i) metallation of DapE can be influenced by metal-supplementation with either Zn^{2+} or Mn^{2+}; ii) Mn^{2+}-metallation is growth-limiting in non-supplemented medium, particularly when selecting for the dipeptidase activity and iii) that the Mn^{2+} isoform may be normally present *in vivo* but is not the only metallated species of DapE that occurs.

Zn^{2+} supplementation makes bacteria more susceptible to L-captopril inhibition compared to Mn^{2+}

Having concluded from growth profiles that metal supplementation of bacterial cultures can influence the state of metallation of DapE, we next investigated whether metal-supplementation also affected susceptibility to L-captopril in bacterial cultures. We reasoned, since L-captopril targets only the Zn^{2+}-isoform of DapE *in vitro* and since metal supplementation can influence the metallation of DapE *in vivo*, that we may be able to influence the sensitivity of bacterial cultures to L-captopril through metallation of DapE.

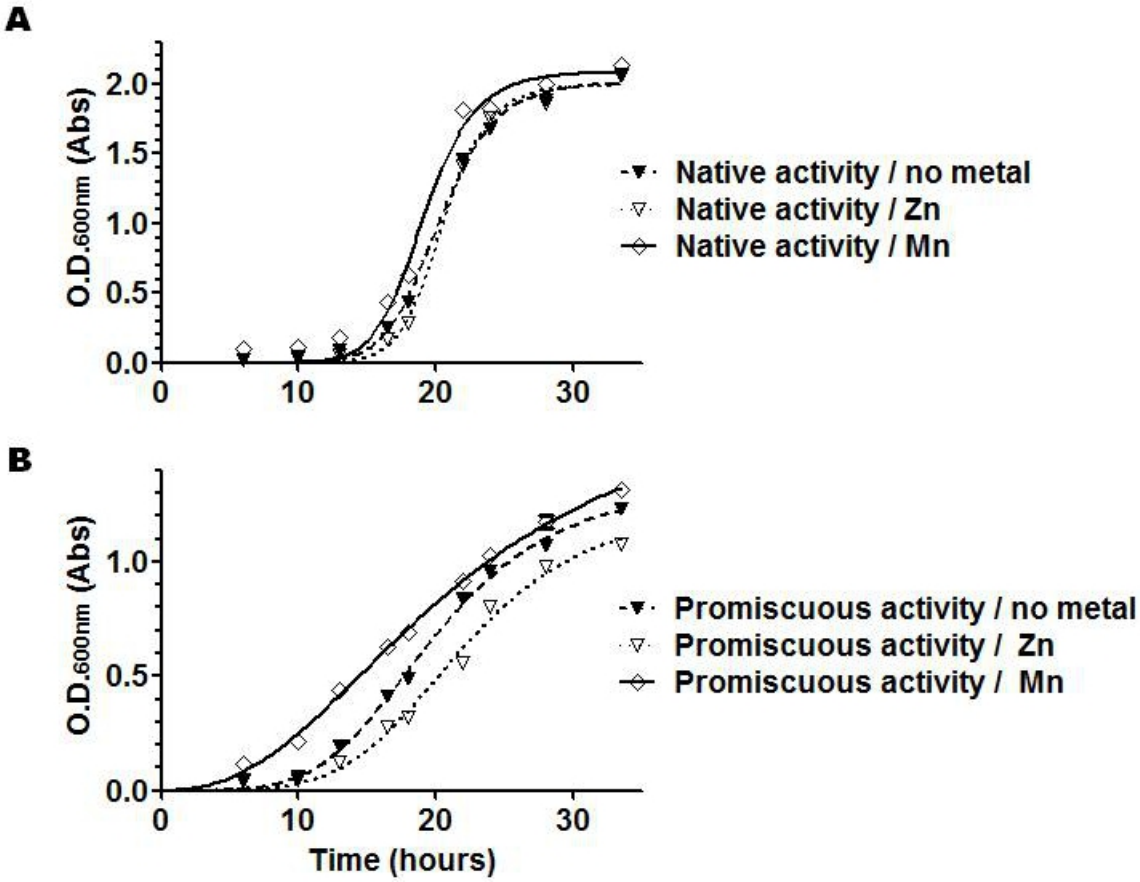


Figure 2. Growth curves of bacterial cultures selected for native (desuccinylase) DapE activity (A) or promiscuous (dipeptidase) DapE activity (B) in absence or presence of Zn^{2+} or Mn^{2+} supplementation. Note that Mn^{2+} accelerates growth of the culture appreciably when selecting both for native and promiscuous activity. In contrast, Zn^{2+} slows down growth of the culture appreciably when selecting for promiscuous activity but has little effect on proliferation when selecting for native activity. Plotted are fitted curves from means of duplicate cultures, with individual values comprised in the symbols. The values plotted are representative from two independent experiments with similar findings.

First, we confirmed that L-captopril was toxic in a dose-dependent manner, shown by bacterial growth-curves in rich medium at different inhibitor concentrations (Supplementary Figure S1). Second, in the growth conditions tested, in rich medium and 25 mM L-captopril, metal supplementation with either divalent Zn or Mn lowered L-captopril toxicity. The influence of metal supplementation on bacterial growth under these conditions was much larger in presence of 25 mM L-captopril than in the absence of the drug (Figure 3): Mn^{2+}-supplemented cultures clearly showed better growth as compared to Zn^{2+} supplemented or especially non-metal-supplemented cultures, which were more susceptible to L-captopril inhibition. The growth-profile in presence of L-captopril was Mn^{2+}>Zn^{2+}>no metal.

Intriguingly, L-captopril inhibition of DapE *in vitro* has exactly the complementary activity profile: L-captopril inhibition Mn^{2+}<Zn^{2+}<no metal.

Despite the metal-dependent effect of L-captopril, bacterial growth in absence of selection for DapE activity (*i.e.* with mDAP supplementation) showed that the toxicity of this drug is largely independent of DapE activity (Figure 3B), confirming our previous finding that this essential enzyme is not the main target of inhibition of L-captopril *in vivo* and suggesting that other metalloproteins may also be the main targets of L-captopril.[11]

We conclude that at least some of the targets of L-captopril are metal-dependent, *i.e.* metal-supplementation decreases sensitivity to L-captopril. The finding that Mn^{2+} supplementation is more protective than Zn^{2+} supplementation is consistent with the notion that L-captopril has a preference for Zn^{2+}-containing targets, at least some of which may also be functionally active with Mn^{2+}. We also surmise that the failure of L-captopril to exhibit any significant DapE-selective antibiotic activity in bacteria is due to the existence of sufficient Zn/Mn isoform, which is not susceptible to L-captopril inhibition.

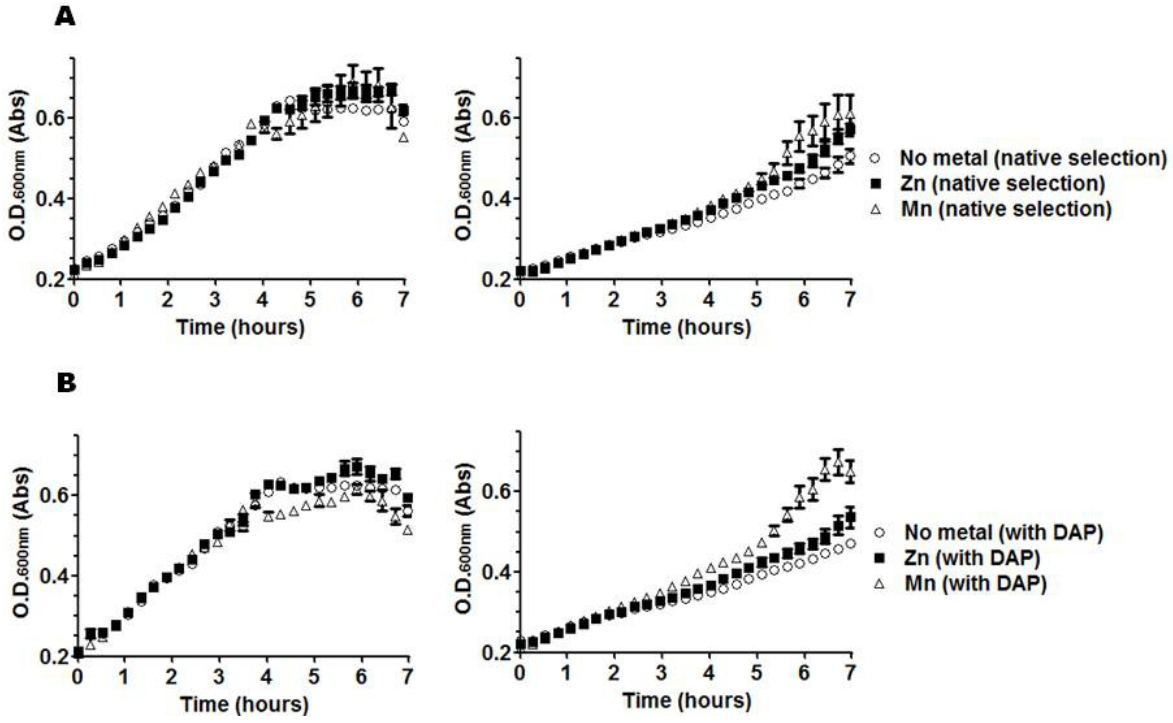


Figure 3. Effect of metal supplementation on growth curves of bacterial cultures selected for DapE activity (A) or non-selected (B), in absence of L-captopril (left) or in presence of 25 mM L-captopril (right). Cultures were grown in LB broth either in presence or absence of mDAP for selection of native (desuccinylase) activity of DapE. The effect of divalent Zn or Mn was tested and compared to the

absence of metal supplementation (no metal). Note that L-captopril toxicity is dependent on metal-supplementation and that Mn^{2+} is more protective than Zn^{2+}, although the toxic effect of the drug is largely independent of DapE activity. Means of measurements from quadruplicate cultures are plotted with standard deviations shown as error bars.

Model of L-captopril inhibition supports that thiol is the Zn^{2+}-binding group.

We created a model of *S.enterica* DapE (Figure 4) based on the high-resolution crystal structure of the homologous enzyme from *Haemophilus influenzae* reported by Nocek *et al.*.[19] The DapE enzyme is a dimer, each monomer of which contains two domains: a dimerization domain and a catalytic domain. The active site of the enzyme can contain either two or one zinc ions. The strongly-bound Zn^{2+} is referred to as Zn1, whereas the Zn^{2+} occupying the site of the labile metal (M2, which can be either Zn^{2+} or Mn^{2+}), is referred to as Zn2.

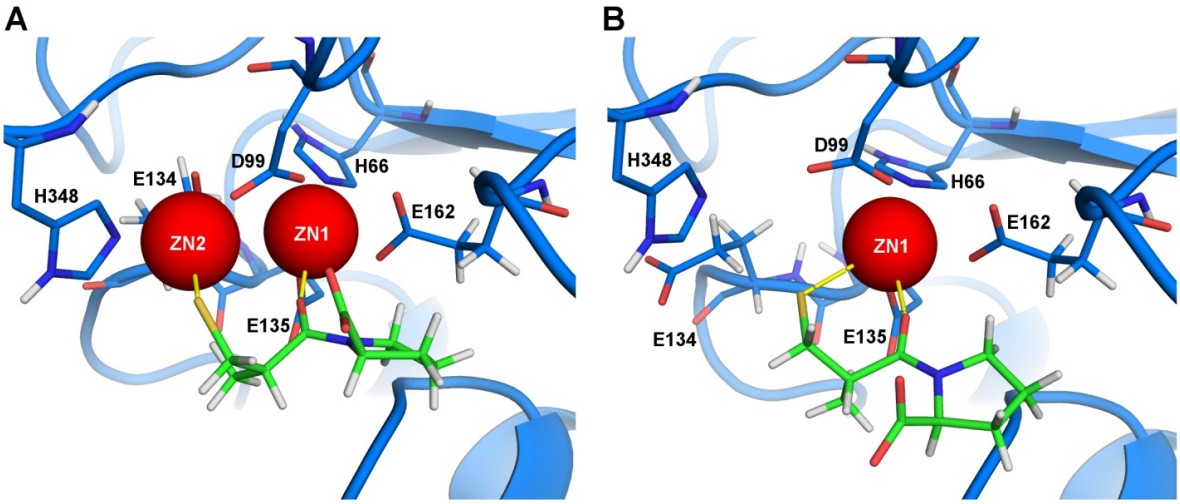


Figure 4. Model of *S.enterica* DapE based on the crystal structure of *H.influenzae* DapE (PDB: 3IC1), with L-captopril docked to the (A) di-zinc and (B) mono-zinc catalytic metal center. Only the side-chains of important residues for catalysis are drawn and labelled, *i.e.* coordinating amino acids and the catalytic base (E134). L-captopril is drawn in stick-form with each type of atom colored differently: green carbon; blue nitrogen; red oxygen; yellow sulfur and white hydrogen. The coordination bonds of L-captopril to the active-site metals are drawn in yellow: in both the mono- and di-zinc form, the L-captopril carbonyl group of the amide coordinates to the Zn1, whereas the L-captopril thiol group can additionally coordinate either to Zn2 in the di-zinc structure (A) or to Zn1 in the mono-zinc structure, to form a 6-membered ring (B). Figure courtesy of Dr. Tobias Schmidt & Dr. Stefan Nicolet.

To generate the model of L-captopril binding to the active site, we imposed some constraints based on prior knowledge of DapE and L-captopril as a substrate analogue (Scheme 1). First, as in the suggested catalytic mechanism of DapE,[19] we propose that the carbonyl group of the amide coordinates to the catalytic metal that acts as a Lewis acid (Zn1). Second, since captopril binds to the catalytic zinc of Angiotensin Converting Enzyme (ACE) through coordination by the sulfhydryl group[10] and since we found experimentally that the nature of the co-catalytic metal (M2, either Zn^{2+} or Mn^{2+}) influences inhibition by L-captopril, we chose to place the sulfhydryl group oriented toward this second metal.

Divalent manganese behaves similar to magnesium in having a preference for oxygen ligands, although Mn^{2+} is more receptive of nitrogen ligands; in contrast, zinc prefers nitrogen and sulfur as ligands and, in general, a lower coordination number.[20] Consequently, our model provides a reasonable and straightforward qualitative explanation for the metal-dependent selectivity of L-captopril inhibition of DapE: the presence of Mn^{2+} does not allow strong coordination by the sulfhydryl group, whereas either the absence of a co-catalytic metal or the presence of a second Zn^{2+} allows coordination with the sulfur liganding either Zn2 or Zn1, respectively.

Discussion

Implications for drug development.

L-captopril was the first marketed anti-hypertension drug, targeting the Zn-metalloprotease Angiotensin I Converting Enzyme (ACE) through coordination of the catalytic metal by a sulfhydryl group, with low nM affinity.[10] However, L-captopril has also shown inhibitory activity towards a variety of other zinc metalloproteases, although this is typically several orders of magnitude weaker than with ACE.[21] Recently, L-captopril was identified also as a low-micromolar inhibitor of *H.influenzae* DapE (IC_{50} = 3.3 μM). We found that L-captopril inhibited the Zn/Zn isoforms of *S.enterica* DapE with IC_{50} values comparable to those of the *H.influenzae* enzyme (i.e. IC_{50} of 10μM *vs* 3.3 μM, respectively), providing further evidence for the similarities in structure and function of the active sites of DapE of different species.[22-26] The low-micromolar affinity of L-captopril for the Zn/- and Zn/Zn isoforms of DapE could be seen as a reasonable starting point for further optimisation of this lead compound,[5,6] akin to what succinyl-proline was for ACE. [10,21] However, note that L-captopril at the concentrations

at which it inhibits DapE might cause hypotension in normo-tensive individuals, which would warrant further development of its antibiotic properties. Moreover, at this low-micromolar level of affinity some off-targets are likely to occur, as indeed suggested by the poor activity of L-captopril [11] and by the general protective effect of Zn^{2+} and especially Mn^{2+} supplementation (Figure 3).

We were nevertheless initially very surprised that we could detect no evidence of L-captopril inhibiting DapE activity *in vivo* and that the compound had such a modest antimicrobial activity, despite low-micromolar affinity of this drug *in vitro* for an essential enzyme. We now forward the proposal that a crucial contribution to this low antibiotic activity is that L-captopril does not target one of the physiologically relevant isoforms of DapE, *i.e.* the Zn/Mn form of the enzyme.

It is generally acknowledged that any screen (or selection) should reflect the desired result as closely as possible, because 'you get what you screen for'.[27] Becker and colleagues [6] identified L-captopril as an inhibitor of DapE in a screen biased toward compounds containing zinc-binding groups, which included thiols, carboxylic acids, boronic acids, phosphonates and hydroxamates and using the Zn-form of DapE *of H.influenzae* as a target *in vitro.* Consequently, a Zn-selective compound was identified with low micromolar affinity *in vitro*. Our discovery offers a plausible explanation for the failure of an apparently promising compound to show any DapE-targeted antimicrobial activity:[11] the Zn/Mn mixed-metal isoform, which is not inhibited by L-captopril, is normally present *in bacteria*, as originally suggested by Broder and Miller [12] judging by the presence of a selectable Mn^{2+}-dependent promiscuous activity, which we confirmed here also by exploring bacterial growth upon metal-supplementation of cultures.

Exact knowledge of the metallation state of proteins *in vivo* is often thwarted with experimental difficulties.[28-30] Moreover, metallation may exhibit strong dependence on cell-context, differing for example in different media, cell-locations or in different species.[31,32] Several other examples have been reported where incomplete knowledge of the physiologically-relevant metal has hindered drug development. For example, potent inhibitors of methionine aminopeptidase discovered *in vitro*, which were active against either Co^{2+} or Mn^{2+}-isoforms of the enzyme, failed to have any of the expected antibacterial activity *in vivo.*[33-35] It was later suggested that Fe^{2+} is the native cofactor in *E.coli*, prompting a move

either toward Fe^{2+}-selective inhibitors that exhibited antimicrobial activity [36,37] or, in what may be called a "bet-hedging move" in absence of a clear candidate, toward exploring selectivity against a plethora of different metallo-isoforms.[38] Similarly, peptide deformylase is another bacterial metalloenzyme and promising antibiotic target [39-41] that was originally thought to be a Zn-metalloenzyme in *E.coli* [42] but was later shown instead to contain Fe^{2+}, a metal that due to its tendency to oxidize in air, leads to facile loss of enzyme activity.[43] However, the precise identity of the metal-in peptide deformylase may be species-specific, since another bacterial pathogen, *Borrelia burgforderi*, does use Zn^{2+} in this enzyme.[44] Despite considerable interest in peptide deformylase inhibitors in recent years [41] it appears that little attention has been paid to the issue of metallo-selectivity of drugs targeting this essential enzyme.

Implications for evolution.

In general, the presence of different metal ions in metalloenzymes can maintain the overall structure of the active site, but provide alternative coordination interactions with substrates and inhibitors. It has already been pointed out that non-strict metal incorporation can be used as a facile way to create catalytic diversity.[45] For example, metal-heterogeneity has been proposed to have evolved in the carbonic anhydrase of marine diatoms that are challenged with low Zn-availability, thereby acquiring the capability of using either Zn^{2+} or Cd^{2+} in the active site of this essential enzyme.[46] Indeed, we suggest that such metal-ambiguity and the resulting promiscuous behaviour *in vivo*, which may be considered a further example of "messiness in biology",[47] is in fact a more general survival strategy, for example to avoid facile drug inhibition of an essential enzyme like DapE. To our knowledge, this may be the first example of metal-promiscuity providing a source of (non-genetic) diversity that confers an intrinsic survival advantage in defending against potential inhibitors, namely to the synthetic drug L-captopril, but also likely against other naturally-occurring dipeptide-like compounds. Considering the importance of diversity as a driver of Darwinian evolution, we speculate that such metal-promiscuity may be much more common than hitherto thought, not only to provide substrate promiscuity in the development of new catalytic function, but also promiscuity for inhibitor-escape.

Conclusions

In summary, DapE in bacteria is heterogeneous, containing various metallated forms –mono-metallated with Zn^{2+} or binuclear with Zn^{2+} or Mn^{2+}– all of which are active as a desuccinylase, the essential and native function of the enzyme. We also conclude that the Zn/Mn binuclear isoform, which is very likely to present *in vivo* as judged by bacterial selection of the promiscuous dipeptidase activity of DapE, is not inhibited by L-captopril, thus providing a mechanism of escape against this potential antibiotic. Such metal heterogeneity and resulting promiscuous properties may confer important selective advantages, including escape of inhibition, and therefore may have been selected for in evolution. Finally, the discovery of metal-selective inhibition of DapE that we describe here paves the way toward the discovery of compounds with improved antimicrobial properties. Our efforts are now directed toward finding novel inhibitors that target also the physiologically-relevant Mn^{2+} isoform of DapE.

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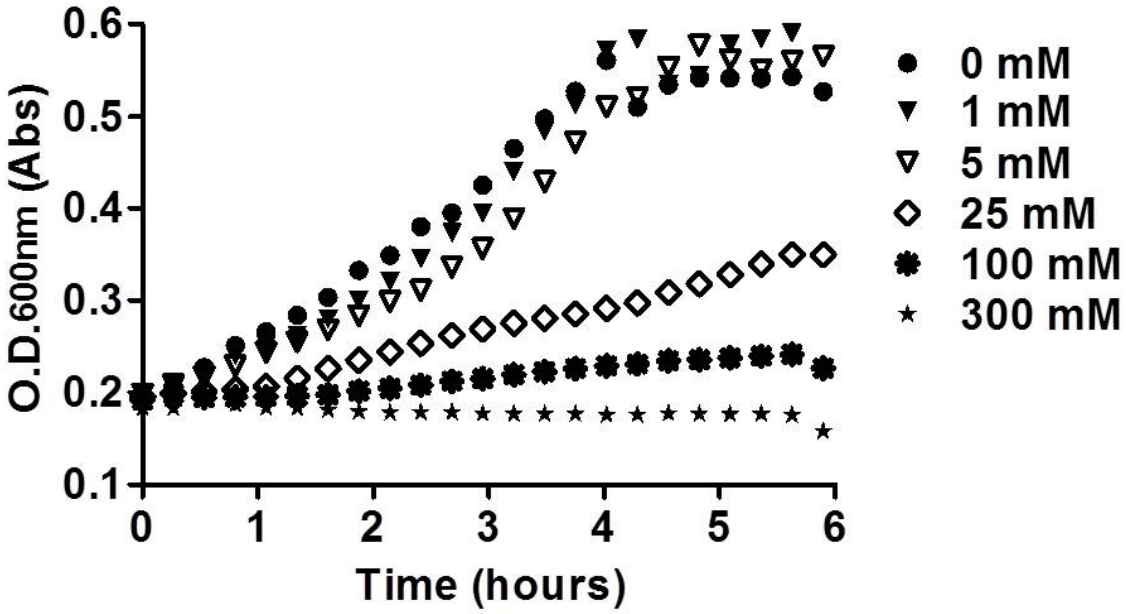

Supplementary Figure S1. Concentration-dependent inhibition of bacterial growth with L-captopril. S.enterica containing a plasmid expressing DapE (strain TN5935) was grown in L.B. medium in absence of mDAP supplementation, i.e. under selection for native DapE activity.

CHAPTER 4

Enzymatic Activation by Inhibition: Kinetic Cooperativity in the Bacterial Amide-Hydrolase DapE and Implications for Antibiotic Drug-Design

Abstract.

There is an urgent need to discover and develop novel antibiotics to fill the repertoire of drugs available to fight infections, which is being depleted alarmingly by the development of drug resistance. The *dapE*-encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE) is an essential enzyme of the anabolic pathway toward lysine and the peptidoglycan cell wall in many bacteria and thus a promising target for antibiotic drug-design. The understanding of the exact enzymatic mechanism of this enzyme may thus facilitate the development of novel effective antimicrobial drugs. Here we show that DapE exhibits positive kinetic cooperativity, which we postulate arises from activation of one of the two monomers upon binding of a substrate or substrate analogue to the other monomer. Such cooperativity leads to the counterproductive effect of effective activation of enzyme activity by suboptimal dosage of competitive inhibitors occurring within a range of substrate concentration that is expected to be physiologically relevant. We suggest that such cooperativity as observed for DapE provides a general evolutionary strategy for bacteria for maximizing escape of inhibition by substrate analogues. Moreover, such mechanism of activation by naturally-occurring dipeptides at concentrations that may be physiologically relevant provides an additional mechanism of control of enzyme activity. Finally, our findings also imply the necessity to search for drugs that do not induce cooperativity or, alternatively, that it may be easier to develop effective drugs against isoforms of DapE that do not exhibit such positive cooperativity.

Introduction

There is an urgent need to develop novel antibiotics. In Europe alone, every year 25'000 people die in hospitals due to bacteria that are antibiotic resistant. As well as the human tragedy that it entails, antibiotic resistance is estimated to cost in excess of €1.5 billion each year.[1]

The bacterial amide metallohydrolase DapE is an essential enzyme in most Gram-negative and many Gram-positive bacteria, being involved in anabolism of both the protein-amino acid lysine and of diaminopimelic acid (DAP), which is a crucial cross-linker of the peptidoglycan cell wall (Figure 1). The enzyme's native function is desuccinylation of *N*-succinyl-L,L-diaminopimelic acid.[2] Considering its importance for many bacteria, including many pathogens, and that it is involved in a metabolic pathway that does not occur in humans, this enzyme has been postulated as a potential antibiotic target.[3] As well as its native desuccinylase activity, the DapE enzyme of *S.enterica* typhimurium also exhibits a promiscuous manganese-dependent aspartyl dipeptidase activity.[4] The structure of several putative bacterial DapE enzymes are available; of these, the best-characterised isoform is a recombinant form from *H.influenzae*, [5-11] which is a dimer, with each monomer containing a dimerisation domain and a catalytic domain. Structures were obtained with each active site either a monometallated-Zn or as a bi-metallated Zn/Zn form, both of which metalloisoforms are catalytically active [6].

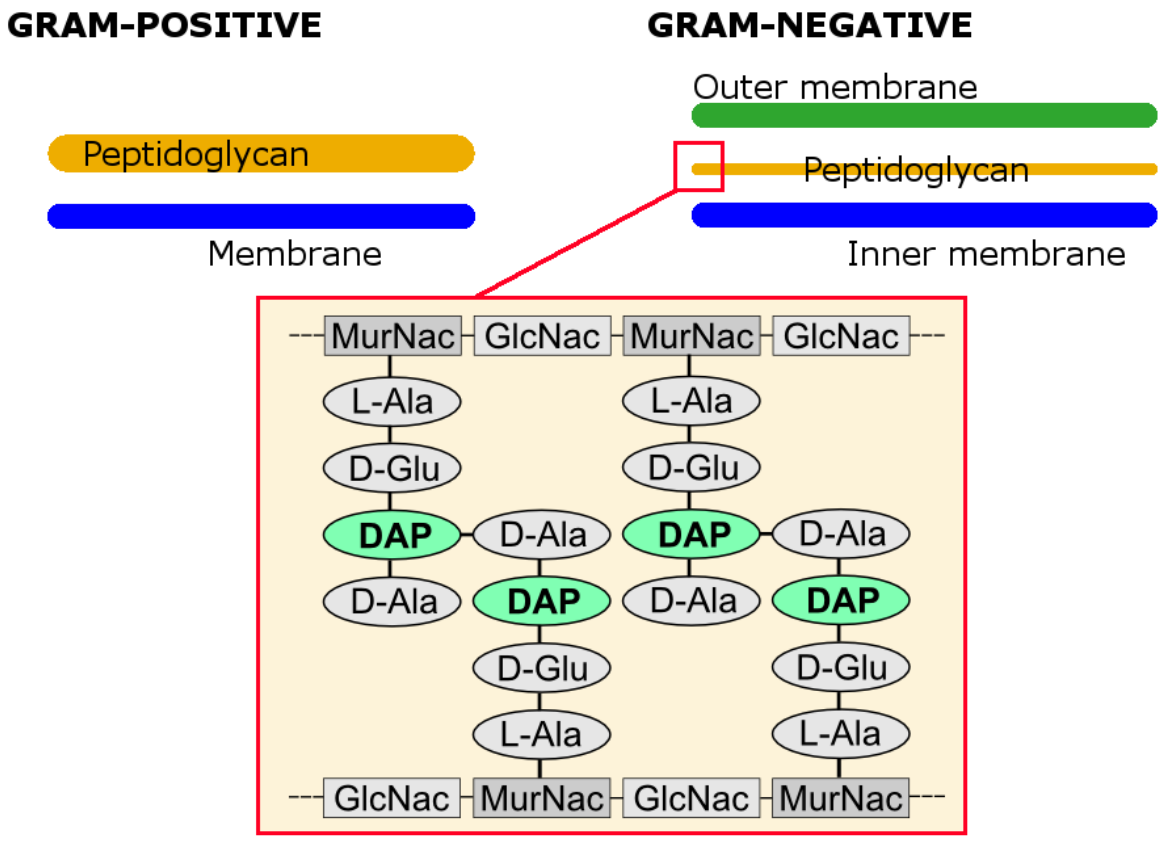

Figure 1. Overview of bacterial cell-wall composition in Gram-positive and Gram-negative bacteria. Graphics courtesy of Dr. Stefan Nicolet.

We have been interested in detailing mechanistic aspects of DapE in view of its potential as an antibiotic target. In particular, we have been intrigued by the failure of a low-micro molar inhibitor of the enzyme *in vitro* to target DapE *in vivo*.[12] Here, we identify enzyme cooperativity as potentially contributing to the failure of a promising lead-compound in vitro to exhibit any detectable DapE-targeted antimicrobial activity.

Organism	**PDB Number**
Legionella pneumophila	3PFE
Haemophilus influenza	3IC1, 3ISZ, 4H2K (for the latter, only catalytic domain)
Staphylococcus aureus	3KHZ, 3KHX, 3KI9
Vibrio cholerea	3T68, 3T6M (both only catalytic domain)
Corynebacterium glutamicum	3TX8
Neisseria meningitides	1VGY

Table 1. List of proteins labeled as DapE in PDB database.

Materials and Methods

Cloning and expression of C-terminus polyhistidine-tagged DapE (DapE-C6His)

A pET28b-based plasmid (Novagen) for DapE over expression was constructed according to published methods.[4] The 1.2kb *dapE* gene was amplified by PCR by using the primers from microsynth, F-SEQpCM655 (5' GCGTACGTCGCGACCGCGGACATGT3') & R-pCM655XhoI (5' AGCCAAGCCTCGAGGGCGACGAGCTGTTCC 3') from the plasmid pCM655dapE, with *Pfu* turbo polymerase (Stratagene) and sub-cloned into pET28b plasmid (Novagen) at the restriction sites *Hind*III and *Xho*I (Biolabs). Bacterial strain BL21(DE3), Stratagene, was used for the protein expression. For the over-expression of DapE protein the LB-medium with 30 µg/ml kanamycin was inoculated with overnight pre-culture in LB with 2% glucose at 37°C. When the OD_{600} reached between 0.5 and 0.8, the main culture was induced with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the temperature before and after induction was maintained at 20°C. The cells were harvested after 6 hours of IPTG induction and the cell pellet was frozen at -20°C.

Purification of recombinant DapE-C6His

The frozen cell pellet of the eprotein expression was then thawed on ice and re-suspend in lysis buffer (50 mM tricine pH 7.8, 0.5 M NaCl, 1 mM PMSF, 3 µg/ml DNaseI and 5 µg/ml lysozyme). The cell-lysis was done at 37°C, 200 rpm for 1 hour. And the soluble fraction was separated by centrifugation (pre-cooled, 4°C, centrifuge) and the clear soluble protein fraction was further centrifuged at high speed (9000 *g*) for 30 minutes and transferred into a fresh bottle at 4°C. The soluble protein fraction was filtered by 0.45 µm filter and the loading buffer (50mM tricine pH 7.8 & 0.5 M NaCl) and elution buffer (50mM tricine, 0.5 M NaCl & 0.5 M imidazole and the final pH was adjusted to 7.8) were filtered by 0.2 µm filter. DapE-C6His protein was purified on HiTrap 5 ml chelating HP columns (Cat. # 17-0409-03, GE health care) loaded with 0.1 M $ZnCl_2$ in 50mM tricine pH 7.8 with an ÅKTA Purifier-10 from GE Life Sciences.

Removal of the 2nd and labile metal from the active site was achieved by 3 step sequential dialysis. First, dialysis in 10L of 50mM tricine pH 7.8 containing 1mM 1,10-phenanthroline, second dialysis in 10L of 50mM tricine pH 7.8 containing 5mM ethylenediaminetetraacetic

acid (EDTA) and third dialysis was in 50mM de-metallated tricine pH 7.8 and each dialysis step was for about 8 to 12 hours at 4°C. Dialysis bags were from Spectrumlabs (Cat. # 132655), approximate molecular weight cut-off of 6 to 8 KDa. Chelex-100 chelating ion exchange resins were from, Biorad. Purified DapE protein from *S.enterica* was analysed on SDS-PAGE and found a single band at a molecular weight of approximately 42 KDa. Protein concentrations were determined by using the Pierce BCA protein assay kit (Cat. # 23227, Thermo scientific). The yield of the purified DapE protein was about 1 mg/L of bacterial culture.

Synthesis of SDAP.

N-succinyl-L,L-diaminopimelic acid (SDAP) was synthesized in liquid phase through a standard coupling procedure between mono-*tert*-butyl succinate and enantiopure mono-*tert*-butyl protected L,L-diaminopimelic dimethyl ester (protected DAP), using *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as coupling agent. The product of the coupling was then conveniently deprotected by standard cleavage of the protecting groups. DAP was previously synthesized according to published protocols *via* olefin cross-metathesis between enantiopure Boc-L-Allylglycine-OMe and Cbz-L-Vinylglycine-OMe, in yields above 70%.[13]

DapE enzyme activity assays.

The kinetic assays were performed in triplicate in 50mM tricine pH 7.8 at 37°C, using the purified DapE-C6His to a final concentration of 100ng/100µl. The final substrate concentration of native substrate (SDAP) was 20mM in a reaction volume of 50 µl. DapE enzyme was pre-incubated with metals (0.2mM $ZnCl_2$ & 0.5mM $MnCl_2$ for native substrate and 1mM $MnCl_2$ for the promiscuous substrate) at 37°C for an hour and then with the inhibitor for another 10 minutes. The reactions were initiated by adding substrate. The enzyme activity was measured by following the hydrolysis of the peptide bond of the substrates.[11] Readings were taken for every 2 minutes for about 3 hours at UV230nm in UV transparent 96-well plates (Cat # 7.675 801, Greiner) using a TECAN Safire plate reader.

Results

DapE from *S.enterica* typhimurium exhibits non-Michaelis-Menten behaviour both with native and promiscuous substrates.

Kinetic analysis of DapE activity was found to exhibit non-Michaelis-Menten behavior, i.e. sigmoidal behaviour consistent with positive cooperativity, both when using the native substrate SDAP and the promiscuous substrate Asp-Leu dipeptide, (Figure 2). The Hill coefficients for SDAP and Asp-Leu were calculated as h = 3.17 and 2.61, respectively.

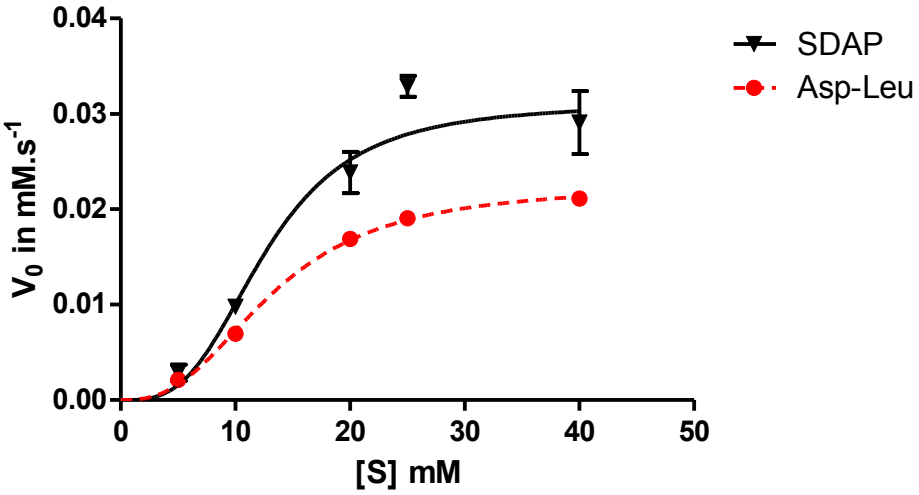

Figure 2. Positive cooperativity in both native and promiscuous activities of DapE, with Zn/Zn and Zn/Mn forms of the metalloenzyme, respectively. SDAP: succinyl diaminopimelic acid. For native desuccinylation activity, k_{cat} = 528 s^{-1} and Hill coefficient = 3.17. For promiscuous dipeptidase activity, k_{cat} = 383 s^{-1} and Hill coefficient = 2.61. All datapoints were measured in triplicate.

Activation of native activity by a metal-selective promiscuous substrate

The dipeptide Asp-Leu cannot be cleaved by the monometallated Zn/- or bimetallated Zn/Zn form of DapE, but is hydrolysed efficiently by the mixed Zn/Mn form of the enzyme. Since the native and promiscuous activities bind to the same active site, the substrates appear to behave as competitive inhibitors of each other.[4]

We also detected modest inhibition of the native activity with high concentrations (25mM) of Asp-Leu using the Zn/Zn isoform. Surprisingly, however, kinetic analysis of desuccinylation in presence of 25mM Asp-Leu revealed near-Michaelis-Menten behavior of the enzyme, as shown by a hyperbolic curve with h = 1.07 (Figure 3). As corresponds to a competitive inhibitor, the k_{cat} for SDAP was not significantly affected, showing that saturating concentrations of substrate outcompete the inhibitor (k_{cat} = 528 s^{-1} *vs* 578 s^{-1}). Surprisingly, the shift from sigmoidal to hyperbolic behavior had the consequence of *higher* activity in presence of the competitive inhibitor at lower concentrations of substrate, below approximately 15mM SDAP (Figure 4). At higher substrate concentrations, Asp-Leu inhibition was modest, as expected from increasing saturation by the substrate.

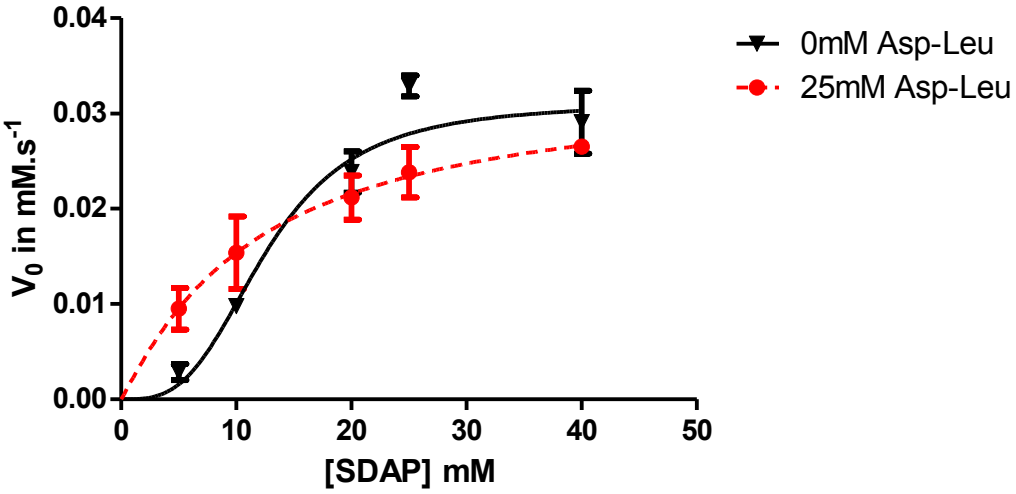

Figure 3. Effect of Asp-Leu dipeptide on the native activity of Zn/Zn isoform of DapE. In presence of 25mM of the dipeptide, hyperbolic, i.e. Michaelis-Menten, behaviour of the desuccinylation activity is observed, with Hill coefficient = 1.07. Note that Asp-Leu is not a substrate for the Zn/Zn form of the enzyme and that, as expected from a competitive inhibitor, the k_{cat} is largely unaltered compared the non-inhibited enzyme (k_{cat} = 570 s^{-1}).

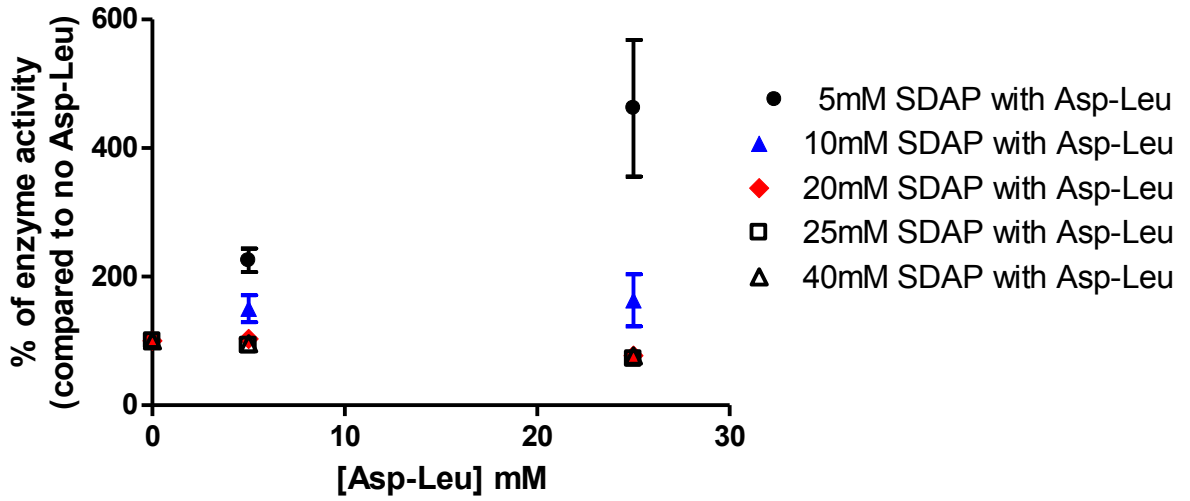

Figure 4. Relative activity of Zn/Zn isoform of DapE in presence of 0, 5 and 25 mM Asp-Leu dipeptide. In presence of 5 mM dipeptide, there is up to x 2.5 fold activation of desuccinylase activity at low substrate concentrations, at 25 mM dipeptide, activation is x4 to x6 fold at 5 mM substrate. In comparison, the dipeptide only exerts very marginal inhibitory effects even when present at high concentration and only observable to any extent at high substrate concentration.

Activation of native activity by the competitive inhibitor L-captopril.

L-captopril has been reported as a low-micromolar inhibitor of the *H.influenzae* form of DapE *in vitro*.[7] Considering the high-conservation of *H.influenzae* and *S.enterica* DapE, it was expected that inhibitors like L-captopril would work for both forms of the enzyme.[12] We confirmed this prediction by measuring inhibition by L-captopril of *S.enterica* DapE *in vitro* with an IC_{50} of approximately 30 μM, in presence of 20 mM SDAP as substrate. We then hypothesized that L-captopril, by virtue of binding to DapE competitively, would also be able to activate the enzyme at lower substrate concentrations, in a fashion analogous as found for Asp-Leu; however, we expected that this activation would occur at much lower concentration of inhibitor compared with the poor inhibitor Asp-Leu, considering the low micromolar IC_{50} for L-captopril. Indeed, carrying out kinetic analysis of desuccinylation at 30 μM L-captopril, at which half the enzyme sites would be expected to be bound, revealed Michaelis-Menten-like hyperbolic behavior (h = 0.91), figure 5. The k_{cat} of the enzyme that we measured was only modestly lower than the non-inhibited form compatible with a mechanism of competitive inhibition (k_{cat} = 438 s^{-1} *vs* 528 s^{-1}). Importantly, at 5 mM SDAP substrate, the enzyme was *activated* approximately 5-fold by L-captopril.

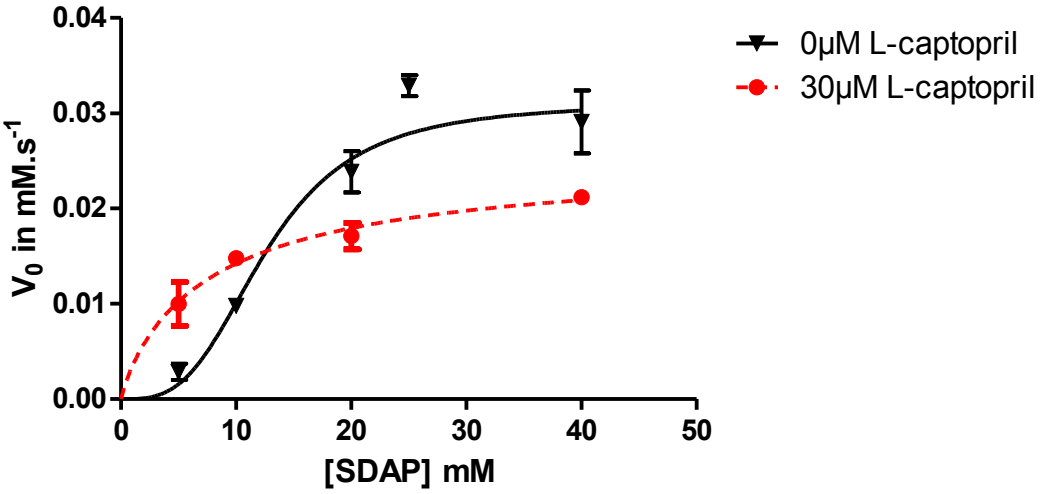

Figure 5. Effect of L-captopril on the native activity of Zn/Zn isoform of DapE. In presence of 30 μM L-captopril, which was previously established as the IC_{50} in presence of 10 mM substrate, the kinetic curves become hyperbolic, i.e. following Michaelis-Menten behavior, with Hill coefficient = 0.91. Note that L-captopril is an inhibitor of the Zn/- and Zn/Zn isoforms of DapE, but not of the Zn/Mn isoform of the enzyme. As corresponds to competitive inhibition, the k_{cat} is largely unaltered compared the non-inhibited enzyme (k_{cat} = 438 s^{-1} vs 528 s^{-1}).

Dimeric form of DapE

Cooperativity in catalysis most commonly arises from structural rearrangements in oligomeric enzymes.[14] Considering the high sequence similarity with the DapE of *H.influenzae*, which is reported as a dimer, a homology model of a DapE dimer from *S.enterica* typhimurium can be constructed with high confidence.[15] Gel filtration-chromatography suggests a molecular weight for our recombinant DapE of 74 KDa (Figure 6), which is only slightly lower than theoretically expected for a dimer (84 KDa) and compatible with DapE being a stable homodimeric complex in solution. Our measurements coincide closely with the measured native molecular mass of the partially-isolated dipeptidase activity, retrospectively identified as DapE activity,[4] which was reported as 70 kDa.[16]

Introduction of mutations I236K and I236D by site-directed mutagenesis, which are predicted to disrupt dimerisation by introducing a buried charged residue in place of a hydrophobic contact, led to the inability of the expressed enzyme to complement DapE deficiency in *S.enterica*. This result is compatible with the notion that DapE dimerisation is necessary for activity.

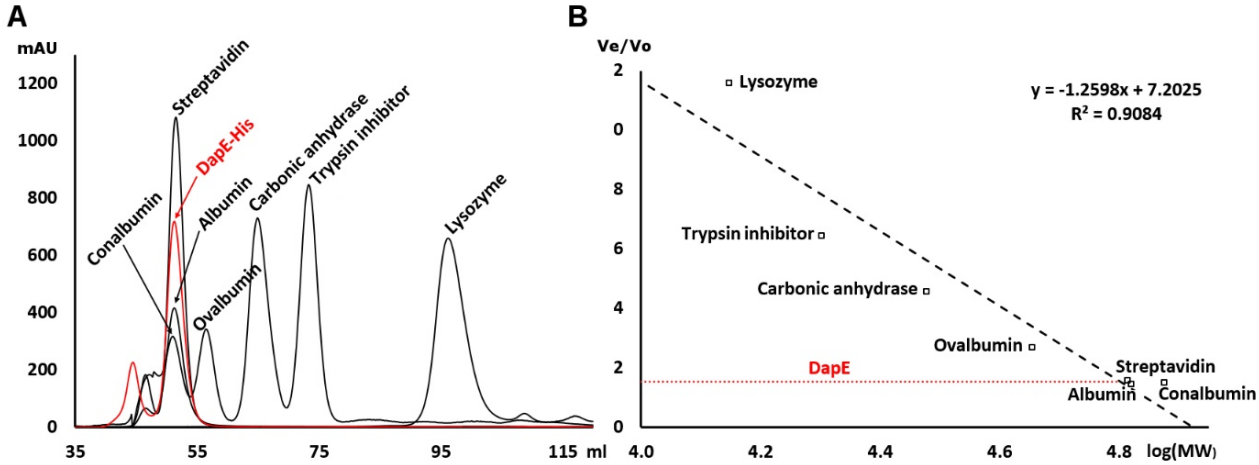

Figure 6. Molecular weight estimation of DapE-His by size-exclusion chromatography on a GE Superdex 75 16/600. In (A) the UV absorbance at 280 nm [mAU] of conalbumin (75 kDa), albumin (66 kDa), streptavidin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 kDa) is plotted versus the elution volume [ml]. The DapE-His protein peak, in red, has a maximum at 51.3 ml of elution volume. In (B) the ratio of elution volume by void volume (44.5 ml) was plotted against the logarithm of the molecular mass [Da] for each protein with a known mass. The linear regression (dashed-line) equation calculated from this points was used to estimate DapE-His molecular weight from its Ve/Vo =1.15 ratio. The calculated molecular weight of DapE-His is 74 kDa. Figure kindly provided by Dr. Stefan Nicolet.

Structural comparisons of DapE

To analyse potential substrate binding interactions of DapE, in particular the binding of the carboxyl of corresponding to the second amino acid in Asp-Leu, we compared the structure of DapE with those of two structurally-related enzymes crystallised with either an informative inhibitor or reaction product, i.e. carnosinase 2 in complex with the inhibitor bestatin (PDB:2zog)[17] and a catalytic mutant of beta-alanine synthase in complex with reaction product beta-alanine (PDB:1r3n).[18]

Superimposition of the structures of 3isz:A 2zog:A and 1r3n:A showed good alignment particularly at the catalytic domain with an RMSD of 1.59Å over 182 amino acids.[19] Although the dimerisation domains also conserve the same overall topology, despite lower sequence identity compared with the catalytic domains, the structural alignment does not cover the whole peptide chain chiefly because of different relative orientations of catalytic and dimerisation domains in the different structures.

Unlike the structure of DapE (3isz), which is in the putative "open conformation", the structures of the catalytic mutant of beta-alanine and of carnosinase 2 are closed conformations, as corresponds to ligand-bound representing catalytically-competent enzymes. The superimposed structures suggest the functional conservation of the following substrate-binding residues:

- Interaction with substrate backbone-amide. The backbone carbonyl of G396 in beta-alanine synthase shows a H-bonding to the product amine (arising from hydrolysis of the amide substrate). A similar interaction with the side-chain of the equivalent residue S417 is observed in carnosinase with its inhibitor bestatin and, finally, T325 in dapE was suggested potentially to have also this function.

- Interaction with substrate carboxyl.
    - A conserved arginine, interacting within the same monomer that binds the substrate forms an ionic bridge with the substrate carboxyl (R343 in carnosinase and R322 in beta-alanine synthase). This position corresponds to R258 in DapE; however, in the case of DapE,it is likely that a substantial domain-movement around the putative hinge would be required to approach this residue closer to the active site. R258 is found structurally-close to R329, with which it forms the lining of a "pocket"; it is noteworthy that in one of the monomers of the de-metallated structure of DapE (3IC1), both of the side-chains of these residues form an ionic interaction with a sulfate ion, which was suggested as a possibly mimicking a carboxylic group of the substrate.[6]

    - A conserved histidine, interacting with the opposite monomer that binds the substrate, forms a hydrogen-bond with the substrate carboxyl (H228 in carnosinase, which has been shown to be crucial for catalysis, and H262 in beta-alanine synthase). This position most likely corresponds to H194 in DapE, or possibly to the nearby-residue H199; however, H194 is not observed in the crystal structures of *H.influenzae* DapE due to low electron density around this loop, which is consistent with the notion that it is a flexible loop that can adopt different conformations.

- A conserved asparagine (N309 in beta-alanine synthase) or threonine (T330 in carnosinase), interacting with the opposite monomer that binds the substrate, form a hydrogen-bond with the substrate carboxyl. This position most likely corresponds either to N244 or N245 in DapE. Note that residues 241–245 were not modelled in chain B in the crystal structures of *H.influenzae* DapE due to disorder, again consistent with the notion that it is a flexible loop that can adopt different conformations.

Recently, the crystal structure of the L-carbamoylase of *Geobacillus stearothermophilus*, another member of this M20 family of dimeric metallo-hydrolases, revealed unambiguously that dimerisation is necessary for the activity of this enzyme and pointed also to the involvement of the conserved residues discussed above for substrate binding.[20]

SAXS analysis of DapE

Attempts at detecting structural rearrangements of DapE upon L-captopril binding by small angle X-ray scattering did not reveal any significant difference between the bound and free form of the enzyme, (Figure 7); the curves, however, were also compatible with a dimeric structure in solution (Dr Stefan Nicolet, personal communication). However, we cannot rule out that such postulated domain movement does not occur during the catalytic reaction coordinate.

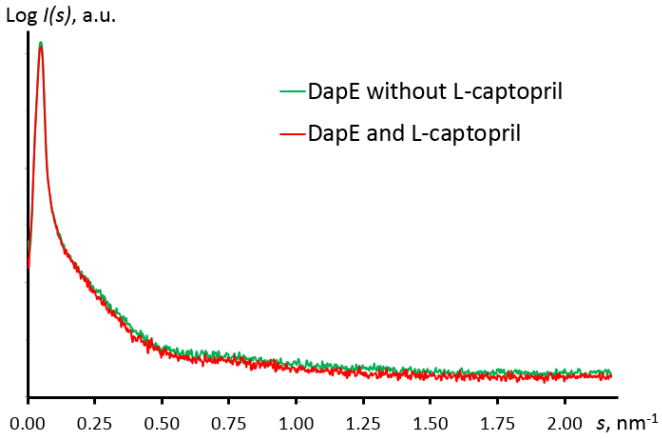

Figure 7. Supplementation of 10µg/ml of the L-captopril inhibitor to the *S. enterica* DapE-His protein (2.0 mg/ml) does not change the SAXS intensity curve and therefore excludes any large-scale rearrangement of the protein. The momentum transfer *s* is defined as $4\pi \sin(\theta)/\lambda$ [nm^{-1}], with the wavelength λ = 1.54Å. Figure kindly provided by Dr. Stefan Nicolet and Prof. Thomas Pfohl.

Note that, in carnosinase, ultracentrifugation analysis of the free enzyme or the enzyme bound to the bestatin inhibitor failed to find any conformational differences, despite the putative domain movements postulated upon substrate (or inhibitor) binding. One possible reason forwarded by the authors of that study was the possible small difference in axis ratio between both postulated forms.

Discussion

Evidence for cooperativity in *S.enterica* DapE

Here we report on positive cooperativity in the kinetic behavior of DapE, a potential antibiotic target,[3] as revealed by four lines of evidence: i) sigmoidal behavior in kinetic analyses with the native substrate SDAP, with the bimetallic Zn/Zn enzyme isoform; ii) sigmoidal behavior in kinetic analyses with the promiscuous substrate Asp-Leu, with the bimetallic Zn/Mn enzyme isoform; iii) recovery of Michaelis-Menten behavior upon addition of the weak competitive inhibitor Asp-Leu for the Zn/Zn isoform desuccinylase activity and iv) recovery of Michaelis-Menten behavior upon addition of low-micromolar concentrations of the competitive inhibitor L-captopril. To our knowledge, this study represents the first description of cooperative behavior of any DapE enzyme, which we conclude from detailed and comprehensive kinetic analysis of the enzyme from *S.enterica*. It will be interesting to explore whether other DapE enzymes also exhibit similar cooperative behavior; we suspect that cooperativity may be a property that, together with structure, function and inhibition profiles may also be conserved amongst isoforms of DapE.

Hinge-domain movements and cooperativity in M20 metallohydrolases

Although cooperativity may exist in monomeric enzymes through kinetic effects, [21] most cases of cooperativity in proteins (including enzymes) that have been reported are due to long-range conformational effects, as originally described for the classic experiments in haemoglobin.[22] DapE is classified as belonging to the M20 family of metallohydrolases,[23] in which hinge-domain movements have been reported or postulated on mechanistic grounds for many of its members. Domain movements are described for monomeric members like Sapep,[24] PepV,[25] auxin amidohydrolase,[26] methionine aminopeptidase from *E.coli* [27] and the aminopeptidase of *Aeromonas proteolytica*[28] as well as for its dimeric members like allantoate

amidohydrolase[29] (which is activated allosterically by anion binding),[30] carnosinase,[17] yeast beta-alanine synthase,[18,31] L-carbamoylase[20,32,33] and human aminoacylase 1.[34] PepD is structurally related to PepV, but exists as a dimer postulated to exhibit domain movements, although with a different spatial arrangement compared to other dimeric M20 dipeptidases like DapE.[35] Finally, higher-order arrangements that may maintain hinge-like motions are also known and, for example, a metallohydrolase from *S.aureus* has been shown to be a tetramer[36] and the *C.elegans* beta-alanine synthase, which is an M20-family member with Michaelis-Menten behavior, is a dodecamer.[37]

Despite the many reports of domain-movements of M20 family-members, examples of cooperativity in these enzymes appear to be exceedingly rare, perhaps because this issue may have been underexplored. Human Liver beta-ureidopropionase (*i.e.* beta-alanine synthase) was reported to have a Hill coefficient of 2,[38] which may be related to the formation of polymers.[39] The rat liver beta-alanine synthase is also allosteric with Hill coefficient of 1.9 at low substrate concentrations; however, at higher substrate concentrations, Michaelis Menten-kinetics were followed. Substrate binding also induced polymerization, to hexamers, which may be reason for cooperativity.[40] However, the best characterized beta-alanine-synthase is from yeast, which structure shows open and closed conformations.[18,31] Although for the yeast enzyme open and closed states appear to exist in equilibrium, the enzyme appears to have a very high K_M (around 60mM) and there is no evidence of cooperativity.[39]

Metalloenzymes of other families, such as insulysin of the M16 family, also show dimer-dependent allostery involving large-domain hinge-motions. However, in the case of insulysin, binding of a peptide-activator occurs in a non-catalytic domain. This movement is proposed to be rate-limiting and both open and closed conformations appear to exist in solution for the unliganded enzyme.[41] Mixed-dimers were used to abolish allostery.[42]

Mechanism of cooperativity in DapE

How does positive cooperativity arise in DapE of *S.enterica*? The enzyme appears to be a dimer in solution, as would be expected from structural homology with DapE from *H.influenzae*.[6] By sequence and structural conservation of key residues predicted to bind the substrate, in particular R258, H194 and N244, which are predicted to bind the carboxyl at the second position of SDAP or Asp-dipeptides, it is reasonable to predict that the enzyme

requires large-domain movements during catalysis, which allow these key residues to approach the active site. In particular, two of these residues (H194 and N244) are predicted to interact with the substrate bound to the active site of the opposite monomer, thus providing a rational for the requirement of a dimeric enzyme. Such intermonomeric contacts could also provide a reasonable mechanism by which cooperativity between active sites could arise: binding of a substrate (or inhibitor) in one monomer may lead to a conformational change that facilitates binding to the other monomer. Large hinge-motions of domains may be rate-limiting, as described for insulysin of the M16 family and it is possible that in DapE, ligand binding reduces the extent of such motions. Alternatively or additionally, a "closed" conformation on one monomer may lower the K_M or increase k_{cat} of the other monomer through electrostatic effects, for example.

It is possible that DapE in solution exists as an equilibrium of both open and closed conformations, as has also been suggested for insulysin.[41] In carnosinase, despite the postulated existence of open and closed forms for unliganded or liganded enzyme, ultracentrifugation analysis failed to detect any large conformational isoforms.[17] Our gel filtration analysis showed a single peak of enzyme eluting at a position compatible with a dimer and we failed to detect differences between ligand-free and ligand-bound by SAXS. Further structural and mutational analyses will be necessary to elucidate the exact details of how positive cooperativity is achieved in DapE.

Function of cooperativity in DapE

An additional question of interest is why does cooperativity exist in DapE at all? Cooperativity may offer a facile way to adapt affinity in evolution and arises often through formation of dimers or oligomers.[43,44] Thus, positive cooperativity facilitates an "all or nothing behavior". Although the concentrations of SDAP occurring in vivo are difficult to estimate, the presence of cooperativity at the low-mM range of substrate suggests strongly that this phenomenon is of physiological relevance for DapE. It has also been argued that cooperativity may allow kinetic control at the expense of maximal efficiency.[45] For DapE, one could speculate that there is a suboptimal activity of the enzyme under normal conditions, because physiological concentrations of substrate apparently would not lead to maximal possible activation; instead, one could imagine the first monomer being as catalytically active as the second even at very low substrate concentrations. Care must be taken with this

argument, because it is conceivable that there is no accessible way of reaching such high efficiency except in the form of cooperativity by dimerisation. However, assuming that one could conceive a very active monomer or very active independent monomers in a dimer, *i.e.* that DapE could be more "catalytically perfect" (defined as maximum possible k_{cat}/K_M) in the absence of cooperativity, then less enzyme would be required to maintain the same flow through the pathway, thus providing potential energetic benefits, *i.e.* in the form of lowered requirement of protein synthesis. Consequently, if positive cooperativity is the product of evolution and more efficiency could be achieved at low substrate concentration, then whatever advantages this phenomenon confers would be a trade-off for low efficiency. Therefore, what advantages could cooperative behavior confer to DapE?

Conceivably, cooperativity could be a mechanism for specificity, maximizing activity with useful substrates whilst minimizing unwanted catalytic transformation. However, the K_{50} both for the native substrate SDAP and the promiscuous substrate Asp-Leu appear to be similar, at around 15mM. Consequently, little selectivity appears to be gained, unless the normally available concentration of SDAP would exceed that of dipeptides like Asp-Leu, which seems unlikely. A possibly advantageous scenario for selectivity could be envisaged with large fluctuations of substrate, where the "activation" levels of the unwanted substrate, presumably an unwanted non-specific peptidase activity, would seldom be reached.

However, one may speculate about an alternative benefit for cooperativity, namely to protect against inhibition of this essential enzyme activity, as follows: DapE is a crucial activity for a bacterial cell; endogenous competitive inhibitors of the enzyme, such as aspartate dipeptides, are likely to exist physiologically and it is very likely that competing organisms could easily synthesise competitive inhibitors of DapE with potential antimicrobial activity; to survice, the bacterial cell would need to respond by increasing DapE expression, which is a slow process that may endanger survival; instead, it would be of benefit to have an "excess" amount of DapE ready for immediate release in case of necessity, *e.g.* in presence of an inhibitor; however, considering the potential toxic promiscuous activity of the enzyme as a dipeptidase, activation should be only when necessary and easily reversible. It is obvious that positive cooperativity provides a facile solution to this potential threat, at a presumably acceptable energetic and evolutionary cost.

Implications for cooperativity in drug design

In vivo, cooperativity may be a buffering system to prevent inhibition by internal promiscuous binders if they sporadically become abundant, such as Asp-Leu or by external inhibitors. The implications for inhibitor design are of relevance: a competitive inhibitor will only be really effective when it has extremely strong affinity for the enzyme *and* inhibits in near-stoichiometric amounts. The reason for these two requirement is that inhibiting the enzyme only partially and leaving many sites unbound would be insufficient, because the remaining sites would remain very active, thereby compensating for the inhibition. The failure of L-captopril to exert DapE-targetted antimicrobial activity may be due in part to incomplete targeting of all available cellular enzyme, thereby leading to unaffected overall cell growth. Note that this argument would not hold for monomeric enzymes exhibiting cooperative behavior from kinetic effects alone, because binding to the active site would be sufficient to prevent activation. Consequently, the monomeric form of the predicted SDAP desuccinylase from *S.aureus*, SaPEP,[24,46] may provide an easier target for inhibition. For the dimeric *S.enterica* DapE, alternatives to finding useful competitive inhibitors include: i) exploring other types of inhibitors that do not lead to cooperative activation (e.g. uncompetitive, non-competitive inhibitors); ii) inhibitors that simultaneously target both monomers of the same dimer; iii) inhibitors that destroy enzyme activity in other ways, such as preventing dimerisation or iv) inhibitors of activation itself, for which an atomic description of this allosteric phenomenon would be very helpful.

Cooperativity also poses additional practical challenges for screening for inhibitors *in vitro*. Typical screening conditions are carried out at non-saturating concentration of substrate, not just for cost-saving reasons but also because finding a competitive inhibitor at high substrate concentrations is intrinsically difficult, due to inhibitors being easily "outcompeted". However, in the case of positive cooperativity, finding an inhibitor at lower substrate concentrations is also difficult, because molecules that bind effectively to the active site may not be identified at all because they do not change the read-out of the assay or even be detected as "activators" *in vitro*. Consequently, it would be difficult to find lead-compounds for further improvement, which would otherwise be found easily in the absence of cooperative behavior.

Conclusions

In summary, we describe for the first time positive cooperativity in *S.enterica* DapE, which may arise from its dimeric structure and postulated large domain movements that cause the interaction of one monomer with the substrate bound to the active site of the opposite monomer. Cooperativity appears to ensure that even when inhibiting species are bound, the overall steady-state of the enzyme remains relatively unaffected and, therefore, may be an evolutionary adaptation to protect the function of this essential enzyme in many bacteria. The cooperative behavior of DapE poses practical challenges for the development of drugs against this potential antibiotic target.

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CHAPTER 5

Facile functional recombination of mutations by pairwise heteroduplex formation by PCR in experimental protein evolution

Abstract

Genetic recombination is a ubiquitous general strategy for generating sequence diversity. Sexual-reproduction appears to have evolved as a consequence of selective advantage of genetic recombination, although the exact cost-benefit of having males in a population are still a matter of debate.[1] Recombination has two main advantages: i) rapid testing of combinations of individual mutations that are known to have been previously selected, thereby exploring functionally-enriched sequence-space and ii) removal of deleterious mutations, thus preventing the potential slow decline in fitness of asexual populations that has become known as Muller's ratchet.[2] Directed evolution experiments commonly use *in vitro* recombination to improve the property of the protein being evolved, as pioneered by Stemmer in so-called "sexual PCR".[3,4] Of the plethora of genetic recombination techniques available to the experimenter,[5-7] a facile and in our view underexploited method is *in vivo* repair of heteroduplexes.[8,9] Here, we establish a platform for laboratory evolution of an enzyme, the bacterial amide hydrolase DapE,[10,11] using novel protocols for heteroduplex-formation for pairwise recombination of mutations in bacteria.

Introduction

There are many reports of sequence diversity arising as an artefact of PCR and methods to minimise them, for example recombination arising from *in vivo* repair of heteroduplexes. In *E.coli*, such artefactual recombination *in vivo* is thought to occur through the host's nick-directed mismatch repair system MutHLS, known as "long-patch" system.[12,13] However, such a repair system may also be exploited to generate useful diversity in directed evolution experiments.[8,9] Here we present a novel PCR based protocols for facile recombination in laboratory evolution experiments.

Materials and methods

PCR method A

pCM655 plasmid[11] containing variants of dapE were PCR co-amplified by using one or more templates in a single pot PCR using the primers F & R pCM655 for 25 cycles at 60°C annealing temperature using Mutazyme II polymerase. The resulting PCR product was cloned into empty pCM plasmid and then transformed into *S.enterica* electrocompetent TN5911 cells lacking *dapE* gene and platted on AMP-LB plates containing DAP (no selection pressure) and without DAP supplementation (selection for the DapE enzyme activity).

PCR method B

pCM655-dapE plasmids harbouring stop codons at various positions and tested to be non-functional for DapE activity were PCR co-amplified by using overlapping primers downstream to the gene. PCR reaction of 35 cycles was done using the following primers

5' CGTGGGCCGTGCACCTTAAGCTTAAGG 3' and

5' GCACGGCCCACGTGGCCACTAGTACTTC 3' at 50°C annealing temperature using *TurboPfu* polymerase. The resulting PCR product was transformed in *S.enterica* electrocompetent TN5911 cells lacking *dapE* gene and platted on AMP-LB plates containing DAP (no selection) or not (DapE activity required).

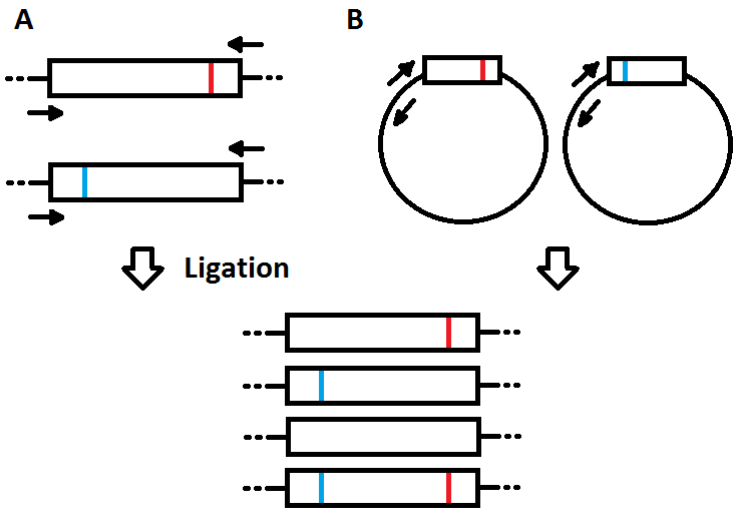

Figure 1. Overview of recombination protocols. The first method (A) implies co-amplification by PCR of two *dapE* gene variants containing different mutations depicted in red and blue; digestion with the restriction enzyme *Hind*III and cloning into the pCM655 vector[11] followed by transformation into *S.enterica.* The resulting sequenced plasmids isolated from single colony clones present recombination events. The second method (B) consist in co-amplifying the whole plasmids by PCR using primers downstream to the *dapE* gene, followed by transformation of the PCR product into *S.enterica*. The same type of recombination as with method A were observed. Figure courtesy, Dr. Stefan Nicolet.

Results

From the PCR method A (Figure 1), 9 single pot PCRs were carried out, in which each PCR had two templates consisting of DapE variants with defined point mutations. From the 9 randomisations 181 variants (i.e. 20 variants from each randomisation) were sequenced to identify the number of recombination events and the distances between the recombinations.

From the PCR method B (Figure 1), 11 PCR mixes of two different plasmids were carried out. A total of 104 colonies were found to grow in LB-agar AMP plates supplemented with DAP. Only 3 wt colonies grew in LB-agar + AMP plates (2.9% of recombinations yielding back DapE wild-type activity).

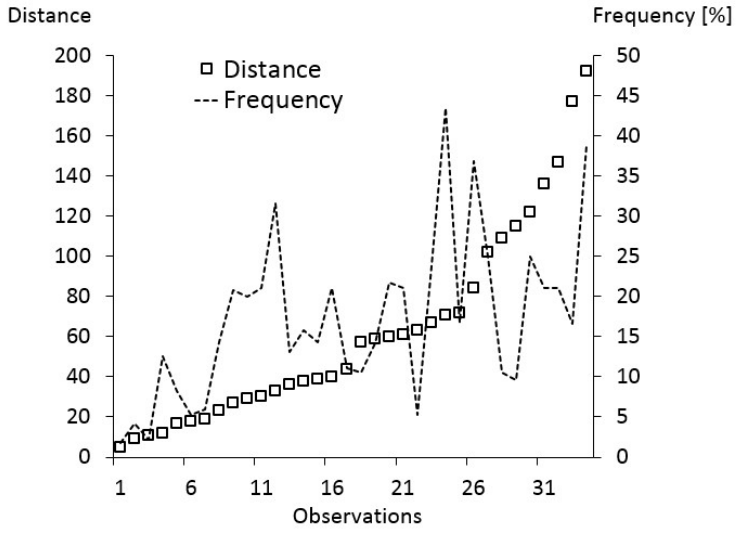

Figure 2. Distance in amino acids (codons) between possible DNA recombination events plotted with corresponding frequency of recombination. The frequency is defined by the ratio of observed recombinations by the total number of sequenced genes. The data suggest that there are very few recombinations at mutations that are a short-distance apart, whereas the few cases observed of neighbouring mutations separated by a large distance recombine more frequently. However, frequency of recombination shows a lot of variability. The frequency of events never exceeded 50%, as predicted by probabilities (25% template A, 50% template A+B, 25% template B). Figure courtesy, Dr. Stefan Nicolet.

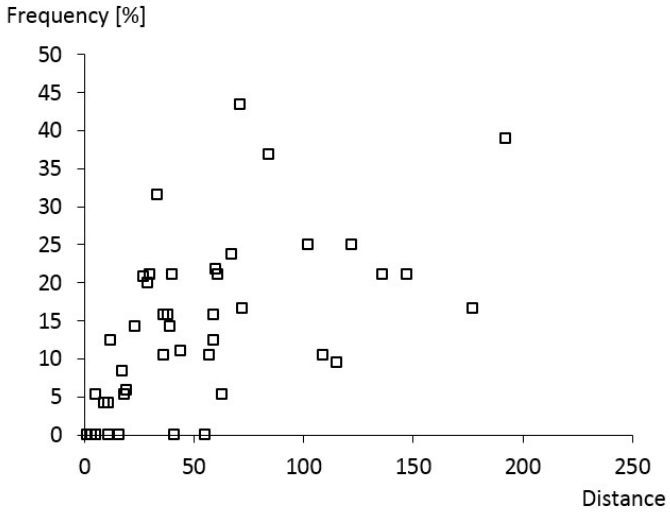

Figure 3. Plot of all the frequencies of observed recombination events, where these are possible, versus distance in amino acids. The data suggest a higher frequency of recombination with larger distance, although only the smallest possible distances of feasible recombinations were considered. Figure courtesy, Dr. Stefan Nicolet.

Discussion and conclusions

The method of heteroduplex formation and repair *in vivo* reported here mimics sexual recombination in that "shuffling" only occurs pairwise*, i.e.* between two strands each arising from their corresponding parental templates, regardless of the diversity of sequences in the original reaction. In classical "shuffling" protocols, where typically parental genes are fragmented and then reassembled by PCR, extreme sequence divergence can arise rapidly as a consequence of offspring from multiple parents. In contrast, with heteroduplex recombination *in vivo* only mixes of two variants are chosen, regardless of the initial diversity: consequently, extreme sequence divergence is minimised and only near-neighbours are explored. This property of heteroduplex recombination may be a distinct advantage, particularly where only relatively small libraries are explored, for example due to limited transformation efficiency.

We have simplified the protocols previously reported [8,9] in two ways:

A. For ligation of heteroduplex inserts, we implemented a one-pot PCR, in which two or more templates can be mixed, *i.e.* the inserts do not need to be amplified separately. Moreover, we also implemented the use of a *mutazyme* polymerase, which allowed incorporation of random mutations at this step at the same time that pairwise recombination could occur, thus more closely mimicking diversification by sexual reproduction and minimising parental templates. Finally, we explored the number and distance of recombinations comprehensively, using larger mixed-libraries than previously reported (*e.g.* previously, no recombination nearer than 24 bp was tested, whereas we explored possible recombinations arising "randomly" in our selected variants).

B. For whole-plasmid heteroduplex construction, we implemented a one-step PCR method using partial overlapping primers.[14] The novel protocol has several advantages including: i) the reaction is "one-pot", rather than having to treat plasmids separately before re-annealing; ii) it circumvents the requirement of restriction digestion altogether.

In both protocols above, the limitation of primer-availability at the end of PCR reaction, probably ensures the enrichment of heteroduplexes;[12] however, we suggest that an additional cycle of re-annealing without further extension may facilitate heteroduplex formation. In all cases, parental homoduplexes may be present, although these can be also minimised by statistical dilution, simply by incorporating many diverse templates in the one-pot PCR mixture. Finally, a limitation of these protocols is that hybrid duplexes may not form well with only-distantly-related sequences that may pair-poorly into defined double-helixes, thus limiting drastically transformation and recombination efficiency. However, the protocol does have hitherto under-exploited and great potential for facile and truly biomimetic "sexual evolution". In summary, the data suggest that there are very few recombinations at mutations short-distances apart, whereas the fewer cases observed of neighbouring mutations separated by a large distance recombine more frequently (Figure 2). However, frequency of recombination shows a lot of variability (Figures 2 & 3).

Perspectives

It may be relevant to assess whether such recombination events also occur in *E.coli in vivo*. Further, we plan to investigate if recombination events can also occur with more distantly-related genes, by PCR co-amplifying *E.coli* and *S.enterica dapE* genes sharing 94.4% identity at the protein level and 83.3% identity at the DNA level.

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CHAPTER 6

A Mechanistic Proposal for the Curious Metal-Dependent Substrate Promiscuity of the Bacterial Amide-Hydrolase DapE

Abstract

DapE (*N*-succinyl-L,L-diaminopimelic acid desuccinylase) is an essential metalloenzyme for most Gram-negative and many Gram-positive bacteria, which has been proposed as a good target for antibiotic development. Apart from its native desuccinylase activity, the enzyme exhibits Mn-dependent promiscuous aspartyl dipeptidase activity both *in vitro* and *in vivo*. Toward the development of novel DapE-targeting compounds, here we sought to explore the mechanism of such curious metal-dependent promiscuity and substrate selectivity, by exploring a range of compounds as potential substrates or inhibitors. We propose a model of substrate or inhibitor binding that qualitatively explains metal-selective activities of the enzyme. We conclude that the substrate binds in a different orientation to what has been hitherto accepted and also provide insights for the development of novel inhibitors.

Introduction

Emergency against antimicrobial resistance

Antimicrobial resistance (AMR) arises as a consequence of the use and misuse of antimicrobial medicines and develops when a microorganism mutates or acquires a resistance gene.[1] Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. For example, about 440 000 new cases of multidrug-resistant tuberculosis emerge annually, causing at least 150 000 deaths. Extensively drug-resistant tuberculosis has been reported in 64 countries to date.[2]

An effective strategy against AMR doesn't require only a global response in terms of policies and practices for the prevention and control of infections, but also the finding of new antimicrobials. While antimicrobial resistance is rapidly spreading, few pharmaceutical companies are still researching and developing new antibiotics. In 2004, 506 drugs in development by 15 large pharmaceutical companies and seven major biotechnology companies, only six were antibiotics. Approval of new antibacterial agents by the United States Food and Drug Administration decreased by 56% between 1998 and 2002. In 2008, a study of antibiotic development involving small firms as well as large pharmaceutical companies revealed that only 15 of 167 antibiotics under development had a new mechanism of action. If the current trend continues, before long there may not be effective antimicrobials with which to treat patients with serious infections.[3]

It is known that inhibitors of cell wall biosynthesis (like β-lactams, for example) show a very strong antibiotic activity and that enzymes involved in cell-wall synthesis are similar in structure and function, so that certain antibiotics can kill a broad range of different bacterial species.[4] Moreover, essential amino acids are synthesised only in plants and microorganisms, but not in humans or other higher organisms. For example, lysine is synthesized in *E.coli* from aspartate by an 11-step path which offers many potential antimicrobial enzyme targets.[5] Lysine is required in protein synthesis and in the formation of the peptidoglycan layer of Gram-positive bacterial cell walls. Meso-DAP, an intermediate in this pathway, is also an essential component of the peptidoglycan cell wall for Gram-negative bacteria.[6] Since lysine is an essential amino acid and is not synthesized by humans, it must be ingested. However,

most bacteria, plants, and algae synthesize lysine and m-DAP from aspartic acid through three related pathways that diverge after the production of L-tetrahydrodipicolinate (Figure 1).[5]

The primary biosynthetic pathway in bacteria, used by almost Gram-negative and many Gram-positive bacteria is the succinylase pathway.[7,8] The dehydrogenase pathway forms m-DAP directly from L-tetrahydrodipicolinate, but this is a high-energy transformation and is limited to only a few *Bacillus* species. The acetylase pathway is also a minor biosynthetic pathway for m-DAP production and is also limited to only a few *Bacillus* species.

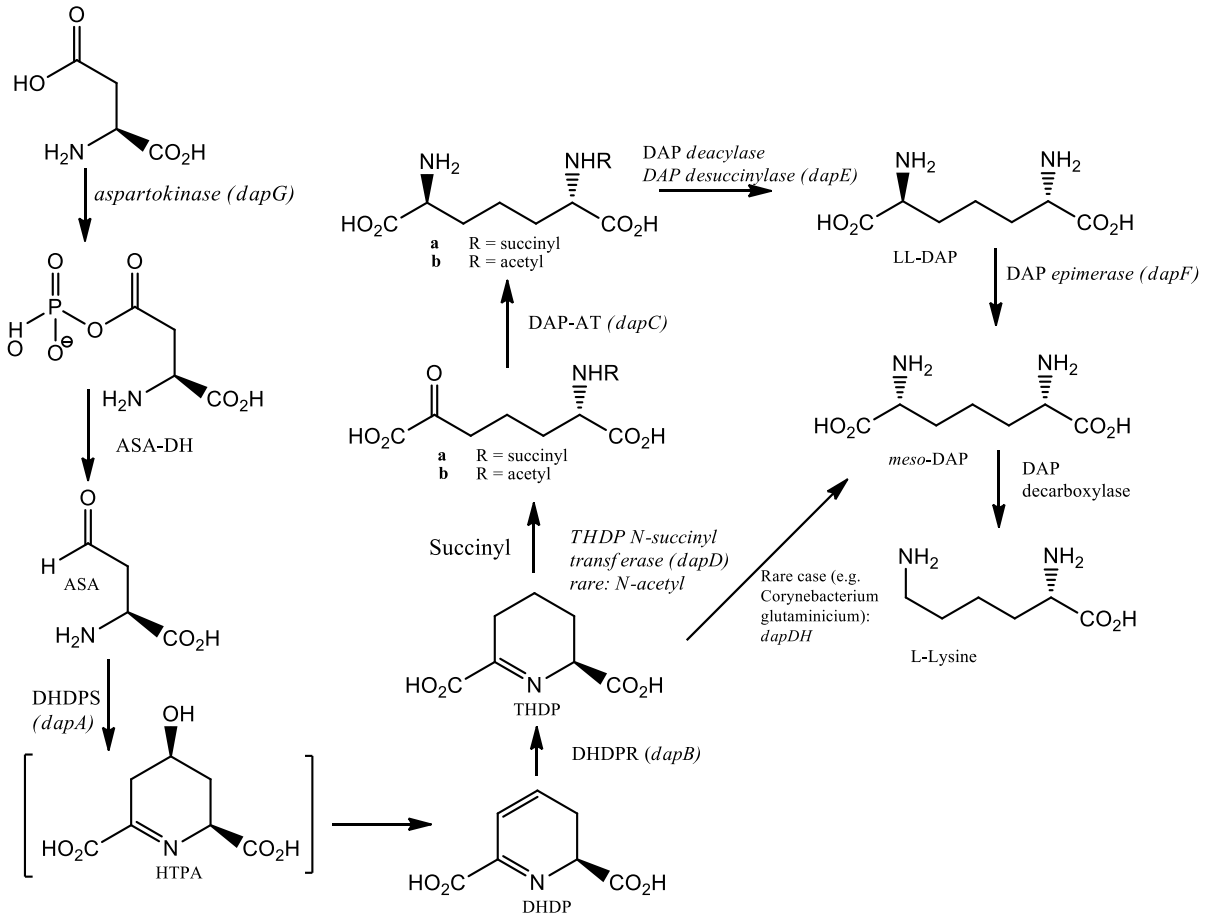
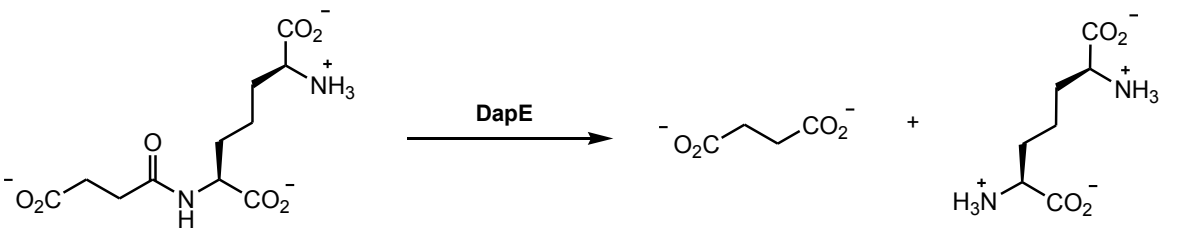


Figure 1. Pathways to lysine biosynthesis. Graphics courtesy, Dr. Gaetano Angelici.

DapE is a good target for new potential antibiotics

One of the enzymes in the succinylase pathway, the dapE encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE), is a Zn(II)-containing metallohydrolase. More specifically, DapE catalyzes the hydrolysis of *N*-succynyl-L,L-diaminopimelic acid (SDAP), forming L,L-diaminopimelic acid and succinate (Scheme 1). DapE deletion is lethal to

Helicobacter pylori and *Mycobacterium smegmatis*.[9] Therefore, DapE enzymes appear to be essential for cell growth and proliferation and are part of a biosynthetic pathway that is the only source of lysine in most bacteria. Since there are no similar biosynthetic pathways in mammals, DapE enzymes appear to be potential targets for inhibitors that may possess antimicrobial activity, *i.e.* inhibitors of enzymes in the mDAP/Lysine pathway could provide selective toxicity against bacteria whilst potentially having no side-effects on humans.



Scheme 1. Hydrolysis reaction of SDAP catalyzed by DapE

Structure of DapE and proposed mechanism

The high-resolution crystal structure of the DapE from Haemophilus influenza has been recently reported by Nocek et al.[10] showing that DapE includes two domains: a dimerization domain and a catalytic domain. The active site of the enzyme can contain either two or one zinc ions (Zn^{2+}) of which only one is tightly bound to the active site. The core of the catalytic domain consists of an eight-stranded twisted β-sheet that is sandwiched between seven α-helices. The location and the architecture of the active site in Zn/Zn_DapE are strikingly similar to the dinuclear active sites of CPG2[11] and AAP. The two zinc ions in Zn/Zn_DapE are 3.36 Å apart compared with 3.45 Å for AAP and 3.25 Å for CPG2.[12] Like AAP and CPG2, each of the zinc ions in Zn/Zn_DapE adopts a distorted tetrahedral or trigonal bipyramidal (TBP) geometry with the Nε nitrogen of His67 for Zn1 and H349 for Zn2 along with a bridging water/hydroxide oxygen atom making up the axial positions of a potential TBP geometry. Identical with CPG2 and AAP, each zinc ion is coordinated by one imidazole group (H67 for Zn1 and H349 for Zn2) and the carboxylate oxygens of E163 (OE1 and the dangling oxygen OE2) for Zn1 and E135 for Zn2 (OE1 and the dangling oxygen OE2). Both zinc ions are bridged by D100 and a water/hydroxide (Figure 2).

Based on the proposed mechanism for AAP[13,14] and on the crystal structure, Nocek at al. proposed a mechanism for both the mono-Zn and di-Zn (Scheme 2) forms of DapE. The first step in catalysis for DapE's is likely recognition of the L,L-SDAP side chain by the smile-shaped cavity adjacent to the Zn1 site. Next, the peptide carbonyl oxygen of L,L-SDAP coordinates to Zn1, expanding its coordination number from four to five, activating the substrate for nucleophilic attack. Deprotonation of the metal-bound water molecule by E134 to form a nucleophilic hydroxide moiety is consistent with the postulated pKa of the zinc-bound water molecule.[8] Once the zinc-bound hydroxide is formed, it can attack the activated carbonyl carbon of the substrate, forming an η-1-μ-transition-state complex. Once the products are released, a water molecule bridging the two metal ions is replaced. In the presence of a dinuclear site, the second metal ion likely coordinates either the peptide carbonyl oxygen in a bridging fashion or a carboxylate side chain of the substrate.

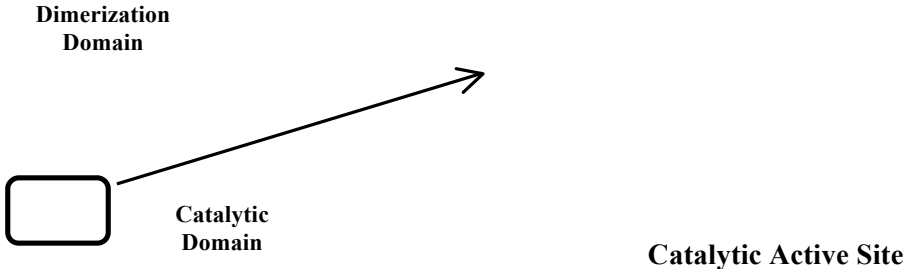
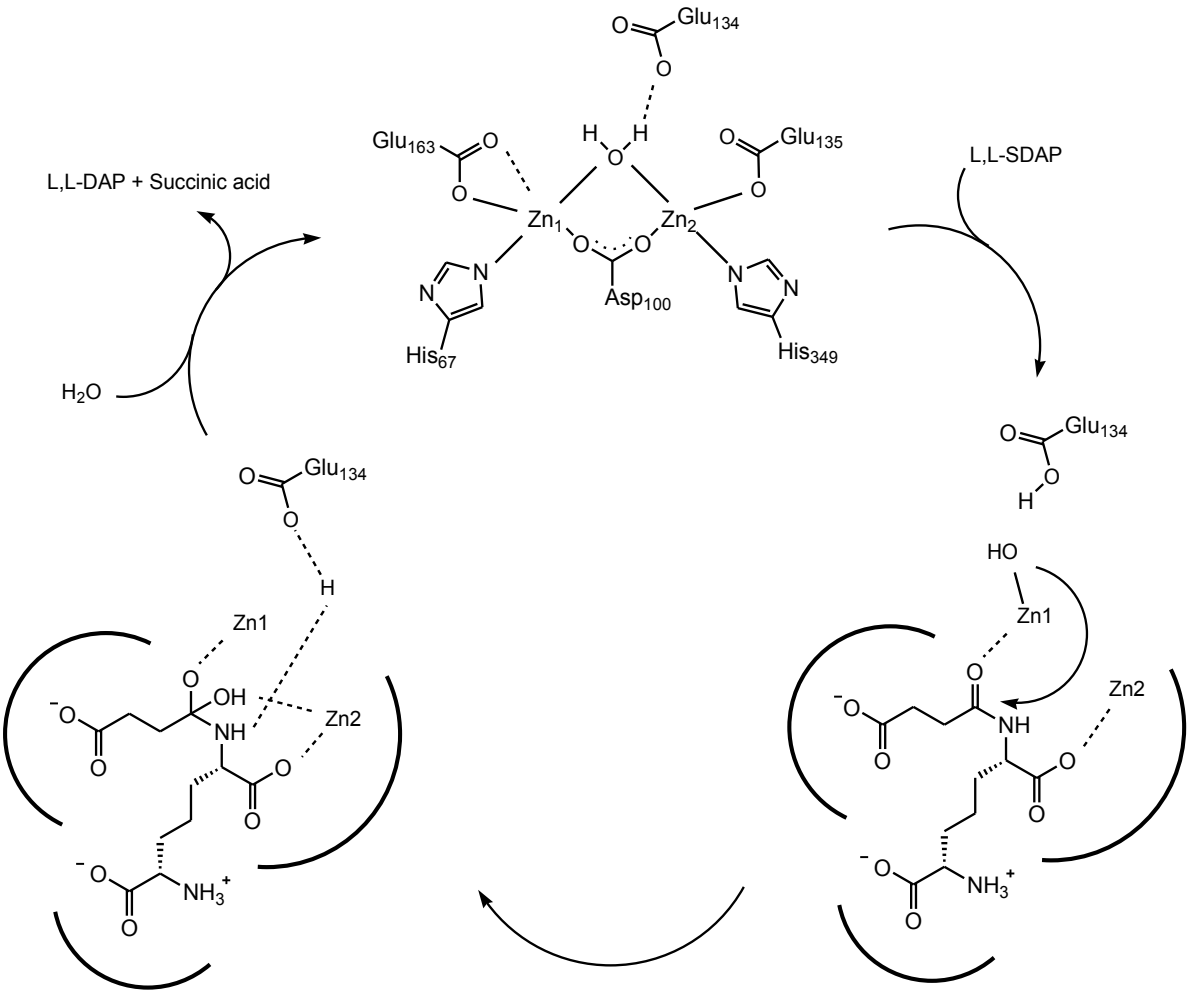


Figure 2. Structure of DapE (*Haemophilus Influenzae*) proposed by Nocek *et al.* and enlargement of the corresponding active site. Graphics courtesy, Dr. Stefan Nicolet.

Summarising the proposed mechanism of Nocek *et al.*: Zn1 is thought to coordinate the carbonyl group of the peptidic bond and takes part to the hydrolysis reaction by coordinating water (or hydroxide), whereas Zn2 has the likely function of coordinating the carboxylic acid of the second amino acid (Scheme 2).[10]

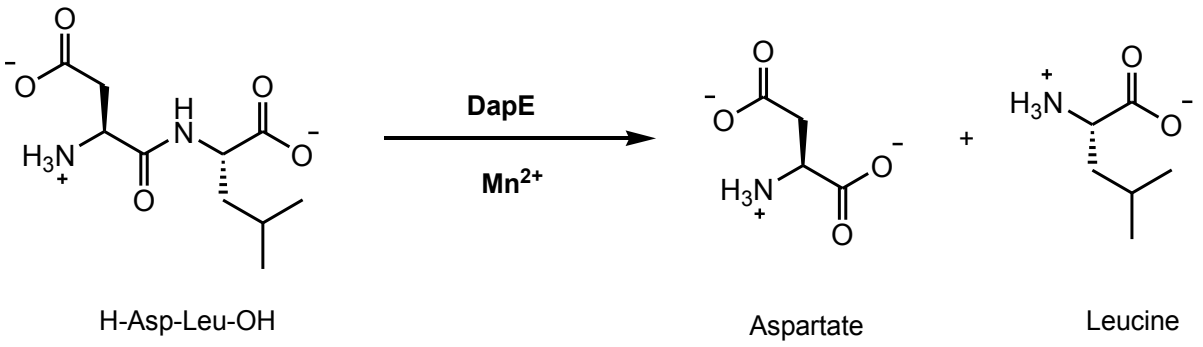


Scheme 2. Reaction mechanism proposed by Nocek *et al.*.[10] Graphics courtesy, Dr. Gaetano Angelici.

Metal-dependent promiscuity of DapE, consequences for potential antibiotics and evolutionary considerations

It is currently unclear whether the mechanism for substrate binding proposed by Nocek et al.[10] based on the X-Ray structure of DapE is correct. Crystal structures often provide an insufficient picture to understand function:[15] a relevant example where crystal structures do not explain why an enzyme fails to be catalytically active is provided by the metallohydrolase carnosinase CN2, which requires Mn^{2+} exclusively for its enzyme reaction; however the fine structures of the metal-binding sites in the Mn^{2+} and Zn^{2+} complexes with a transition-state analogue/inhibitor are highly similar.[16] Consequently, there is no obvious reason why the Zn-metalloisoform should fail to be catalytically-active or what genetic changes would be required to hydrolyse the substrate.

DapE presents also a promiscuous dipeptidase activity. In the presence of manganese ions (Mn^{2+}), DapE is able to cleave the dipeptide Asp-Leu (Scheme 3).[17]



Scheme 3. Metal-Dependant Substrate Promiscuity of DapE in presence of Mn^{2+}. Hydrolysis of dipeptide H-Asp-Leu-OH.

It is perhaps not surprising that DapE can act as a peptidase as well as a desuccinylase, being both amide hydrolysis reactions; moreover, aspartate can be viewed as a structural analogues of succinate, differing only by the presence of a charged alpha amino group. However, among a number of structural analogues of SDAP, Zn2-activated DapE hydrolyzed only *N*-succinyl-L,D-diaminopimelate at a rate comparable to that of SDAP, and most other succinylated analogues of L,L-DAP were not hydrolyzed at all. DapE has therefore been considered a highly specific enzyme.[18] Therefore, it was surprising that, for its best peptide substrates, the enzymatic efficiency of DapE was almost as great as that for its normal substrate, SDAP.[17]

The curious Mn-dependent dipeptidase activity of DapE is a unique and mysterious example of metal-mediated substrate promiscuity. We were thus interested in exploring the enzymatic mechanism as well as in designing potential inhibitors and antibiotics targeting the metal active site. A superficial interpretation of the mechanism or the assumption that the metalloisoform studied *in vitro* will be the same metallo-isoform as is targeted *in vivo* could indeed lead to the failure of a potential drug.

For example, L-captopril, which has been shown to exhibit very promising inhibitory activity in vitro against DapE, does not target DapE in bacteria effectively.[19] We previously demonstrated that L-captopril targets only the Zn^{2+}-metallo-isoform of the enzyme, whereas the Mn^{2+}-enzyme, which is also a physiologically-relevant isoform in bacteria, is not inhibited (Table 1). This finding provides a rational for the failure of this promising lead-compound to

exhibit any significant antibiotic activity in bacteria and underlines the importance of addressing metallo-isoform heterogeneity in drug design. Moreover, to our knowledge, this is the first example of metallo-isoform heterogeneity *in vivo* that provides an evolutionary advantage to bacteria upon drug-challenge.

	$\underline{Zn_1/--}$	$\underline{Zn_1/Zn_2}$	$\underline{Zn_1/Mn_2}$
Native activity (SDAP)	$k_{cat} = 300\ s^{-1}$	$k_{cat} = 700\ s^{-1}$	$k_{cat} = 1100\ s^{-1}$
Promiscuos activity (Asp-Leu)	$K_{cat} = 0$	$K_{cat} = 0$	$k_{cat} = 500\ s^{-1}$
Inhibiton of Native activity by L-Captopril	$IC_{50} = 10\mu M$	$IC_{50} = 28\mu M$	No inhibition even at 1mM L-captopril

Table 1. Native and promiscuous activities of different metallo isoforms of DapE. Inhibition of DapE native activity by L-captpril.

It is widely accepted that many enzymes evolved from pre-existing enzymes via gene duplication . Cumulative evidence suggests that nature has used common binding sites and common mechanistic features to catalyze the analogous reactions with different substrates and, further, has used common mechanistic features to catalyze different reactions. Particularly, the presence of different metal ions in metalloenzymes can maintain the overall structure of the active site, but provides alternative coordination interactions with substrates and inhibitors. It has already been pointed out that non-strict metal incorporation can be used as a facile way to create catalytic diversity.[20]

Methods

Expression and purification of recombinant DapE

Expression and purification of His-tagged DapE from *S.enterica* (DapE-C6His) was carried out as previously described in Chapter 3.

DapE kinetic assays

The enzymatic assays were carried out in 50 mM tricine pH 7.8 at 37°C, using the purified DapE-C6His to a final concentration of 1 ng/µl with a reaction volume of 50 µl or 100 µl. Initially the DapE enzyme was pre-incubated with metals (0.2 mM $ZnCl_2$ & 0.5 mM $MnCl_2$ for native substrate) at 37°C for an hour and then, if appropriate, with the inhibitor for another 10 minutes. In all cases the reactions were initiated by adding substrate. The enzymatic reaction was followed by measuring the hydrolysis of the peptide bond of substrate every 2 minutes for about 3 hours at UV230nm by using UV transparent 96-well plates (Cat # 7.675 801, Greiner) in a TECAN Safire plate reader.

Synthesis

All dipeptides (or analogues) were synthesized in liquid phase through a standard coupling procedures from commercially available compounds using *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as coupling agent. The product of the coupling was then conveniently deprotected by standard cleavage of the protecting groups. For DAP derivatives, protected DAP was previously synthesized according to published protocols via olefin cross-metathesis between enantiopure Boc-L-Allylglycine-OMe and Cbz-L-Vinylglycine-*O*Me, in yields above 70%.[21]

Results

We screened before the native and promiscuous activity for the different metalloisoforms of DapE, finding that the presence of Mn^{2+} relatively increased the native activity compared to Zn^{2+} or no metal.

As expected, only the Mn^{2+} metalloisoform showed the promiscuous peptidase activity, as shown by hydrolysis of the dipeptide Asp-Leu, (Table 1). In order to evaluate the binding mode of the native substrate SDAP and of other possible promiscuous substrates, we begun by designing several substrate-derivatives.

Influence of the second amino acid on succinate containing peptides on DapE metalloisoforms

In a first series, only the succinyl moiety was maintained (compounds 1-4, Figure 3) to evaluate the influence of the second amino acid on the activity of the three metalloisoforms of DapE. The diamino pimelic acid moiety (DAP) was substituted by the close analogue lysine, Leucine or ε-Lysine; we failed to detect activity with the latter, indicating that the interaction of the carboxylic acid in α of the second amino acid is essential in the active site. The values of the kinetic constants K_{cat} for the different metalloisoforms seems to suggest a increased activity in presence of manganese.

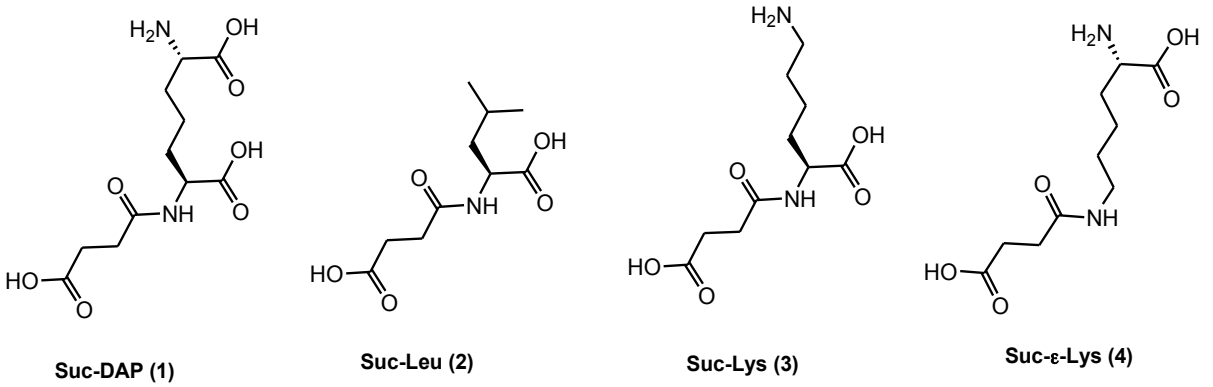


Figure 3. Native substrate (Suc-DAP) of DapE and its analogues.

Substrate	Zn/-- K_{cat} (s^{-1})	Zn/Zn K_{cat} (s^{-1})	Zn/Mn K_{cat} (s^{-1})
Suc-DAP	300	700	1100
Suc-Lys	NT	NT	NT
Suc-ε-Lys	ND	ND	ND
Suc-Leu	ND	80	168

Table 2. k_{cat} values of different metallo isoforms of DapE for native substrate and for the substrate analogues. ND is not detectable. NT is not tested.

Influence of the second amino acid on aspartate containing peptides on DapE metalloisoform

We also evaluated the influence of the second amino acid on the three metalloisoforms of DapE, whilst maintaining Asp in the first position (Figure 4).

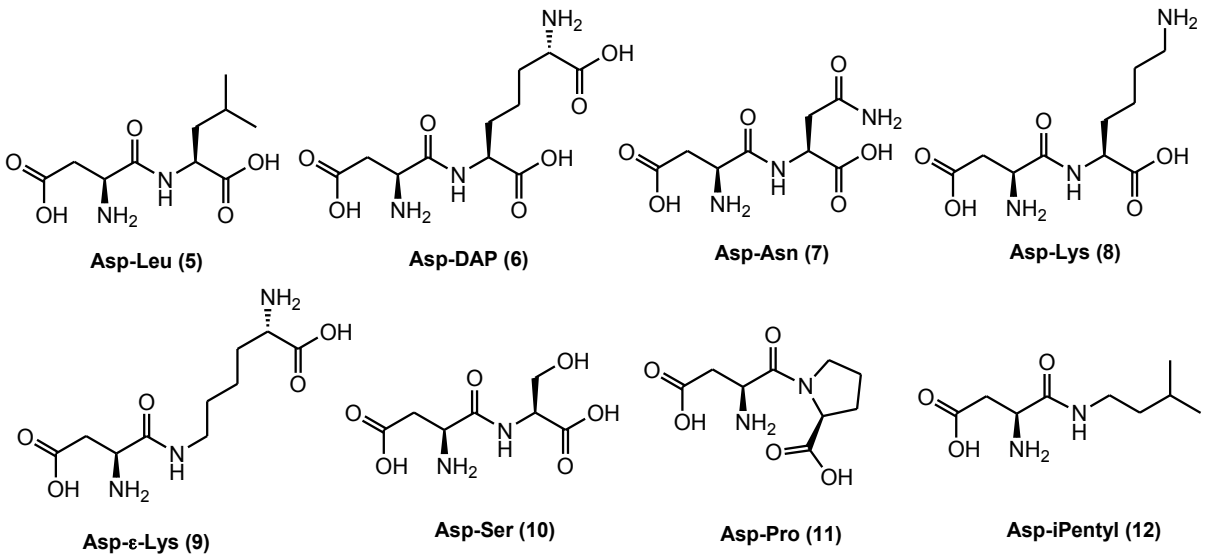


Figure 4. Promiscuous substrate (Asp-Leu) of DapE and its analogues.

Substrate	Zn/-- K_{cat} (s^{-1})	Zn/Zn K_{cat} (s^{-1})	Zn/Mn K_{cat} (s^{-1})
Asp-Leu	ND	ND	500
Asp-DAP	ND	ND	20
Asp-Asn	ND	ND	538
Asp-Lys	NT	NT	NT
Asp-ε-Lys	ND	ND	ND
Asp-Ser	ND	ND	927
Asp-Pro	ND	ND	ND
Asp-iPentyl	ND	ND	ND

Table 3. k_{cat} values of different metallo isoforms of DapE for promiscuous substrate and for the substrate analogues. ND is not detectable. NT is not tested.

None of the molecules tested in Figure 4 were active for the mono-Zinc and di-Zinc DapE, whereas compounds **5**, **6**, **7**, **8** and **10** were all hydrolyzed to different extents in presence of manganese, consistent with the essential role of this metal for the coordination of an aspartate-containing dipeptide and with potential bidentate coordination (Scheme 5). Notice how Asp-ε-Lys (**9**) and Asp-iPentyl (**12**) didn't hydrolize at all, in accordance with the already observed crucial role of the carboxyl acid in α of the second amino acid. Asp-Pro (**11**)

was not a substrate for the promiscuous activity of DapE, as previously reported,[17] probably for the presence of the more constrained proline which requires specific prolinase enzyme for hydrolysis. Prolidase and prolinase are thought to be the only known enzymes capable of degrading proline-containing dipeptides to produce free proline as a product.[22]

Moreover, Asp-DAP, more similar to the native substrate and therefore, probably more rich of interactions, shows a really low K_{cat}, suggesting that it could be a good competitive inhibitor.

Spatial configuration of aspartate isomers and space availability in the catalytic cavity for leucine containing peptides

Considering the proven secondary role of the second amino acid (as long the α carboxylic moiety remains) we synthesized and tested a series of Leu containing peptides at this position to test the effect of modifications at the first position (Figure 5), *i.e.* changing the configuration and the isomeric structure of aspartate, in order to better understand the nature of the putative bidentate coordination. Aspartate was also substituted with glutamic acid to explore the effect of chain length and to verify the space availability in the active site.

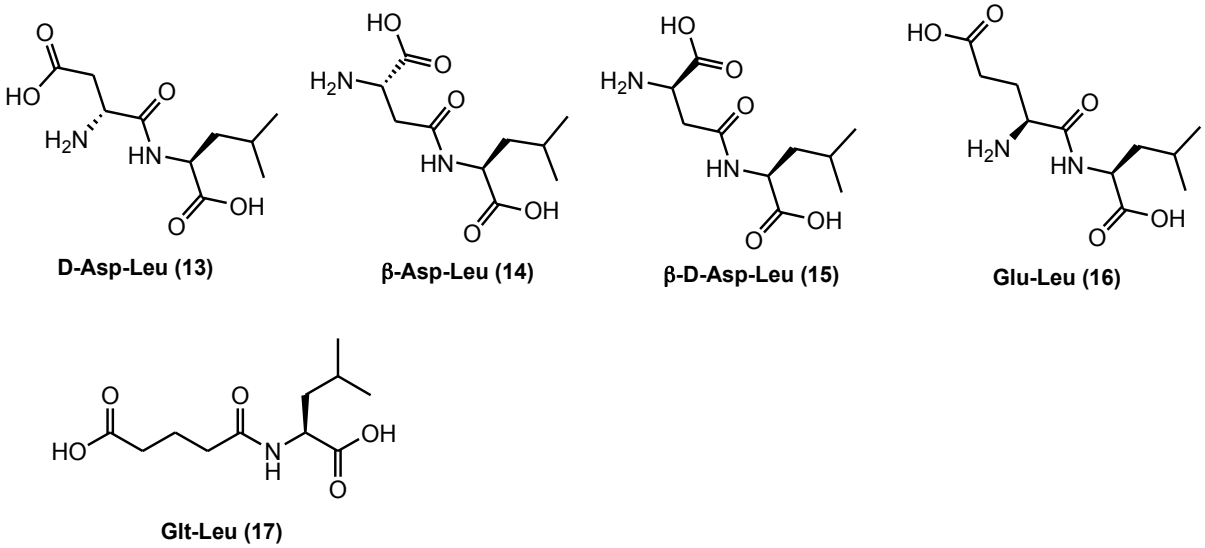


Figure 5. Analogues Promiscuous substrate (Asp-Leu) of DapE.

Substrate	Zn/-- K_{cat} (s^{-1})	Zn/Zn K_{cat} (s^{-1})	Zn/Mn K_{cat} (s^{-1})
D-Asp-Leu	ND	ND	Weak activity detected
β-Asp-Leu	ND	ND	ND
β-D-Asp-Leu	ND	ND	ND
Glu-Leu	ND	ND	ND
Glt-Leu	ND	ND	ND

Table 4, k_{cat} values of different metallo isoforms of DapE for promiscuous substrate analogues. ND is not detectable. NT is not tested.

DapE in presence of manganese is still able to hydrolyse D-Asp-Leu, even though extremely slowly, indicating the importance of keeping the right chirality. The structural isomers (**14**) and (**15**) and longer-chain derivatives (**16**) and (**17**) were not hydrolyzed, showing that length of the substrate side- chain and their regioisomers are important. .

Effects of Changing distance & types of ligands in the postulated inner coordination sphere

Zn^{2+} is considerably softer, therefore more polarizable, than Mn^{2+}.[23] The binding propensities of metal ions with respect to the three possible ligand atoms (O, N, S) found naturally in proteins varies depending on the nature of metal and it is also function of the inner coordination number of the complex. Mn and Zn have a similar tendency to bind sulphur at lower coordination numbers. The main difference for coordination numbers 6 or 7 is the larger probability of nitrogen to bind Mn^{2+}.[23] We therefore synthesized a series of compounds with alternative coordinating moieties (Figure 6), not only to test the difference between the ligands to identify other possible promiscuous substrates, but also with the aim of finding possible inhibitors of the promiscuous activity.

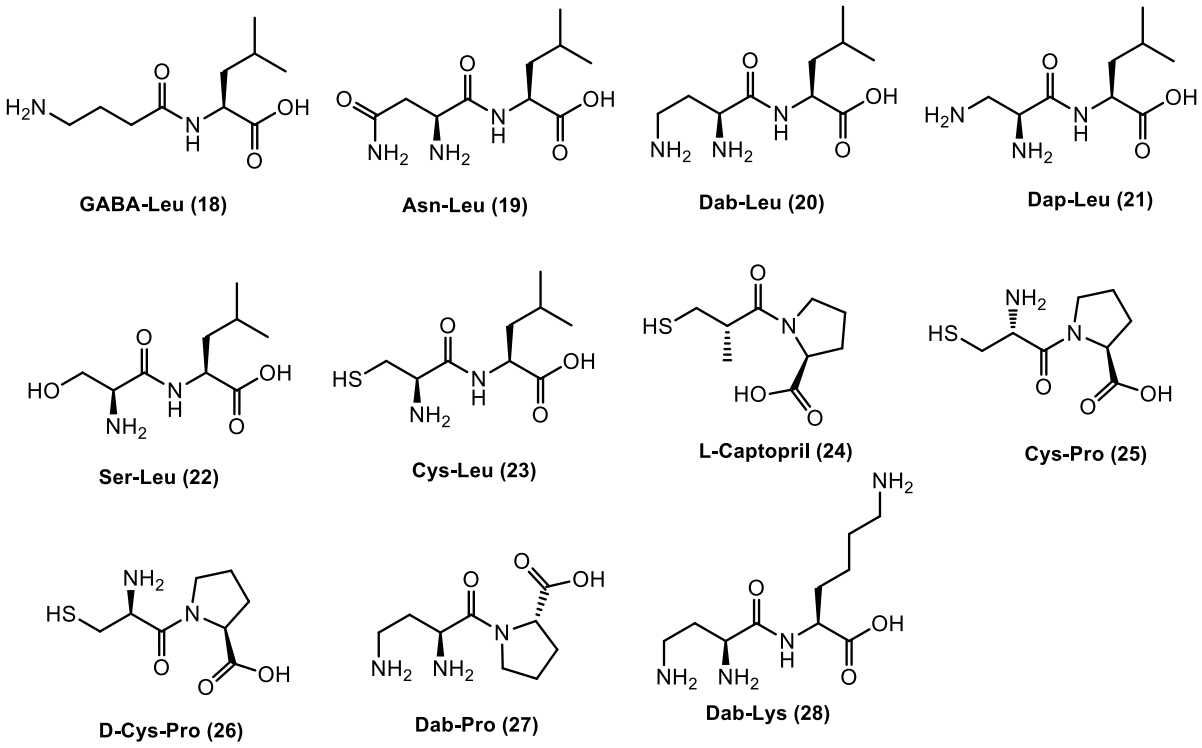


Figure 6. Compounds tested for the Inhibition of native and/or promiscuous activities of DapE.

These compounds were tested as substrate for the three metalloisoforms of DapE and surprisingly, only (**25**) and (**26**) showed to be slowly hydrolyzed in presence manganese. Even though the K_{cat} is very modest, it is the first time that some prolidase activity is observed in DapE. To our knowledge, this finding represents the first description of any promiscuous prolidase activity in the litarature.

Inhibition of the promiscuous activity of manganese-DapE

The compounds showed in Figure 4, 5 and 6 were tested as inhibitors of the peptidase activity on Asp-Leu (Scheme 3), in presence of Mn-DapE metalloisoform, to have some first qualitative evidence of which compounds could have the right properties at the first position to coordinate on manganese and fit with a maximum number of interactions in the active site. In Table 5 we report the behaviour of the listed compounds in inhibiting the hydrolysis of Asp-Leu.

Compound	Promiscuous activity Inhibition	Compound	Promiscuous activity Inhibition
Asp-Asn	ND	GABA-Leu	Yes (weak)
Asp-ε-Lys	ND	Asn-Leu	Yes (weak)
Asp-Lys	NT	Dab-Leu	Yes ($IC_{50} \approx 5$ mM)
Asp-Ser	NT	Dap-Leu	ND
Asp-Pro	ND	Ser-Leu	ND
Asp-iPentyl	ND	Cys-Leu	ND
D-Asp-Leu	ND	Dab-Lys	Yes
β-Asp-Leu	ND	L-Captopril	ND
β-D-Asp-Leu	ND	Dab-Pro	ND
Glu-Leu	ND	Cys-Pro	Yes
Glt-Leu	Yes ($IC_{50} \approx 5$ mM)	D-Cys-Pro	Yes

Table 5, Inhibiton or IC_{50} of promiscuous activity of DapEk using different compounds. ND is not detectable. NT is not tested.

The preliminary screening for inhibition of Asp-Leu hydrolysis (Table 5) revealed several useful insights: i) we confirmed that the carboxylic acid in α of the second amino acid is essential to interact with the active site; ii) the putative coordination of the ligand with manganese should form a six-member ring to interact effectively (Figure 7), as revealed by i) productive binding for aspartic acid (**5**) at the first position and unproductive binding for diamino-butyric acid in Dab-Leu (**20**) and ii) the failure to bind with the diamino-propionic acid in Dap-Leu (**21**), where a 5-member ring should be formed, or with glutamic acid on Glu-Leu where a 7-member ring should be formed.

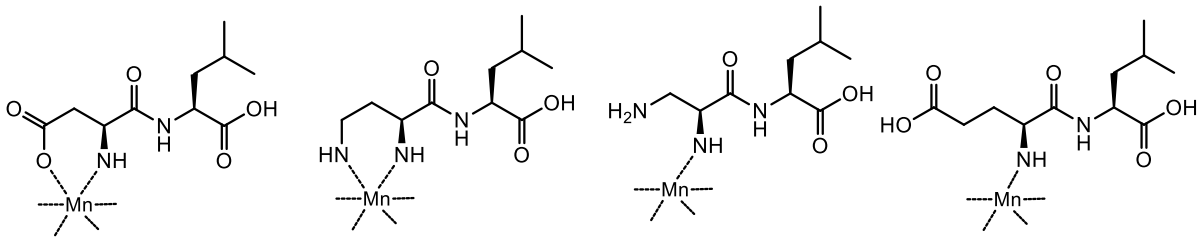


Figure 7. Promiscuous substrate and its analogues coordinating to the 2nd metal (Mn) of the binuclear metal centre of DapE.

Our screening suggests that in the position of the second amino acid a Lys (which is similar to DAP in the native substrate) was more effective that Leu. We compared the inhibition effect on promiscuous activity of mono-amino containing ligands and di-amino containing ligands (Figure 8). Dab-Leu (**20**), potentially forming a six-member ring with two amines, was a better inhibitor than Gaba-Leu (**18**) and Ala-Leu (**30**), both of which could coordinate with only one nitrogen to manganese. We also found a weak inhibition by Gaba-Leu (**18**), whereas we failed to detect inhibition by Ala-Leu (**30**), suggesting that the coordination to the nitrogen in the α amino group is less effective than an amino group at the γ position, which may be due to increased flexibility of the Gaba moiety to orient itself for coordination. We then speculated that the combination of diaminobutyric acid with diaminopimelic acid (DAP) could represent a good base for a potential inhibitor, because the putative chelating effect of the di-amino group would be added to the strong interactions of DAP (part of the native substrate) (Figure 8). To test this hypothesis, we synthesized and tested the molecules Dab-DAP (**29**), Ala-DAP (**31**) and Asp-DAP (**32**).

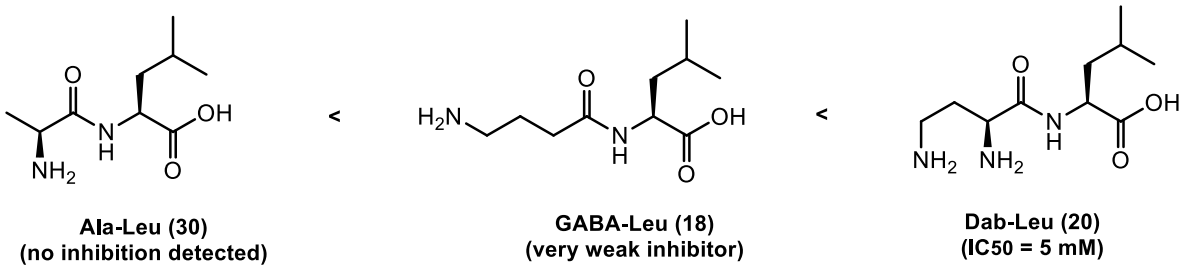


Figure 8. Inhibition profiles of compounds 18, 20 & 30 for DapE-mediated Asp-Leu hydrolysis.

The compounds **29** and **31** were not a substrate for any of the three metalloisoforms, whereas Asp-DAP (**32**) was a poor substrate of the Mn form (k_{cat} = 20 s^{-1}). Compounds **29** and **31** were found to be effective inhibitors of the promiscuous activity, in particular the diamino-butyric compound, suggesting that this potentially bidentate interaction on Mn could lead to non-productive binding, as previously found for Dab-Leu (**20**). Dab-DAP (**29**) was a better inhibitor than Ala-DAP (**31**), with an IC_{50} of approximately 50 μM (Figures 10 and 11), which is compatible with the notion of bidentate binding through a 6-membered ring being possible on Mn and providing a strong interaction through chelation. Conversely, of these three compounds, only Asp-Dap was a substrate and it appears therefore that the carboxylate of aspartyl or succinyl is necessary *in lieu* of an amino group for productive binding. The possibility of formation of a 6-membered ring on Mn with Asp-DAP, although compatible

with catalysis, appears to provide too strong interactions for efficient turnover; hence the Asp-DAP substrate had a poor turnover and was a competitive inhibitor of the Asp-Leu substrate. Surprisingly, however, we failed to detect any inhibition of the native activity with any of these compounds, even with the Mn isoform of DapE, suggesting that the enzyme is optimally evolved for hydrolysis of the native substrate, which outcompete even inhibitor such as Asp-DAP and Dab-DAP, especially designed to provide a maximum number of interactions with the enzyme. To our knowledge, this is the first example of *function-selective inhibition* of an enzyme, *i.e.* where the promiscuous activity is inhibited strongly but not the native activity. We speculate that such function-selective inhibition may be important *in vivo*, adding a whole layer of complexity in metabolic regulation.

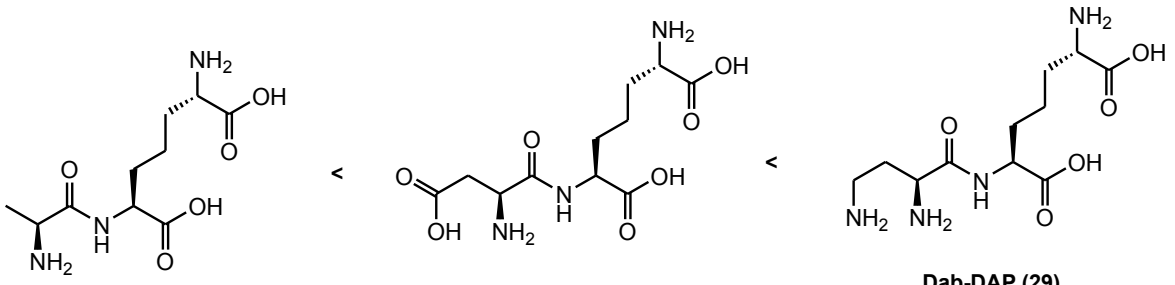


Figure 9. Inhibition profiles of compounds 29, 31 & 32 for DapE-mediated Asp-Leu hydrolysis.

Inhibitor	**IC_{50}**
Ala-DAP (31)	0.3 mM
Asp-DAP (32)	2.5 mM, 90% of activity inhibited*
Dab-DAP (29)	0.05 mM

Table 6. Inhibition of promiscuous activity. *We considered hydrolysis of Asp-Leu alone as representing 100% activity. However, both Asp-DAP and Asp-Leu were substrates and our assay could not distinguish their hydrolysis.

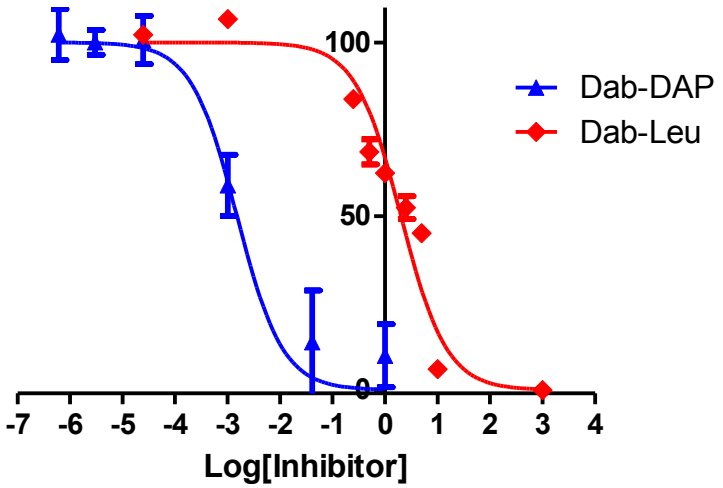


Figure 10. Inhibition of DapE-mediated hydrolysis of Asp-Leu in presence of Dab-DAP (**29**) or Dab-Leu (**20**). The vertical axis represents percent activity. Inhibitory concentrations are IC_{50} = 50 μM and about 5 mM, for Dab-DAP and Dab-Leu, respectively.

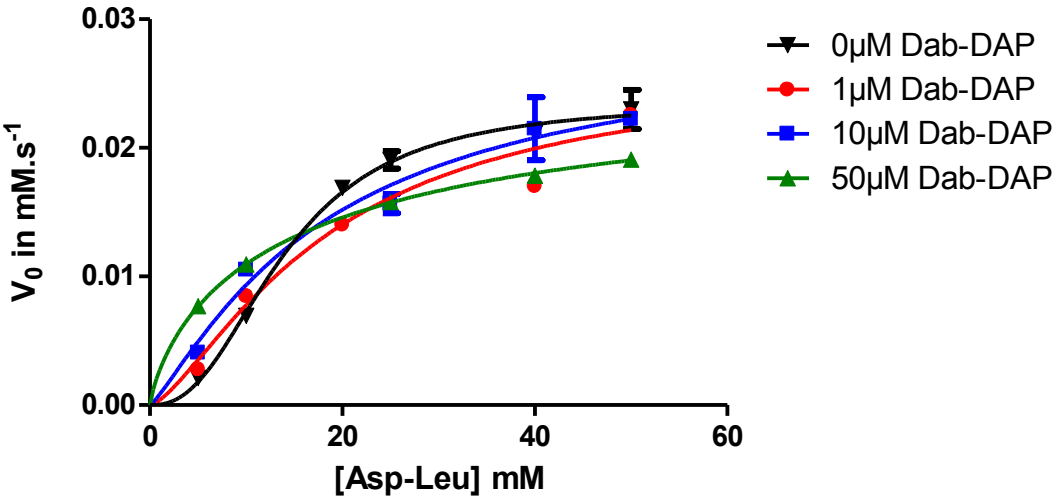


Figure 11. DapE-mediated hydrolysis of Asp-Leu in presence of Dab-DAP. The sigmoidal curve in absence of inhibitor, indicative of positive cooperativity, becomes more hyperbolic in presence of inhibitor closer to the IC_{50}, where approximately half of binding sites are occupied, *i.e.* tending to follow classical Michaelis-Menten behaviour.

Discussion

The promiscuous activity could be based on the different coordination shell of the metals in the active site

Divalent manganese, zinc and magnesium, have fundamental roles in biological systems, but despite their similar chemical properties (ionic radius, electronic stability), they differ mainly on the geometry of their coordination spheres. and molecular orbital and density functional calculations. A comparative study of the ligand properties of Zn and Mn, based on the analyses of data derived from the structural databases (from the Cambridge Structural Database and the Protein Databank) and molecular orbital and density functional calculations,[23] showed the distribution of coordination numbers for the two divalent metal cations in presence of different ligands. The preferred coordination number of divalent manganese is six (octahedral), while the preferred coordination is less definite for zinc and ranges from 4 (tetrahedral or distorted tetrahedral), to 5 and 6, to stabilize transition state or intermediates. Manganese generally binds to oxygen ligands although it is more receptive of nitrogen ligands, while zinc prefers nitrogen and sulfur, especially if the coordination number is low.

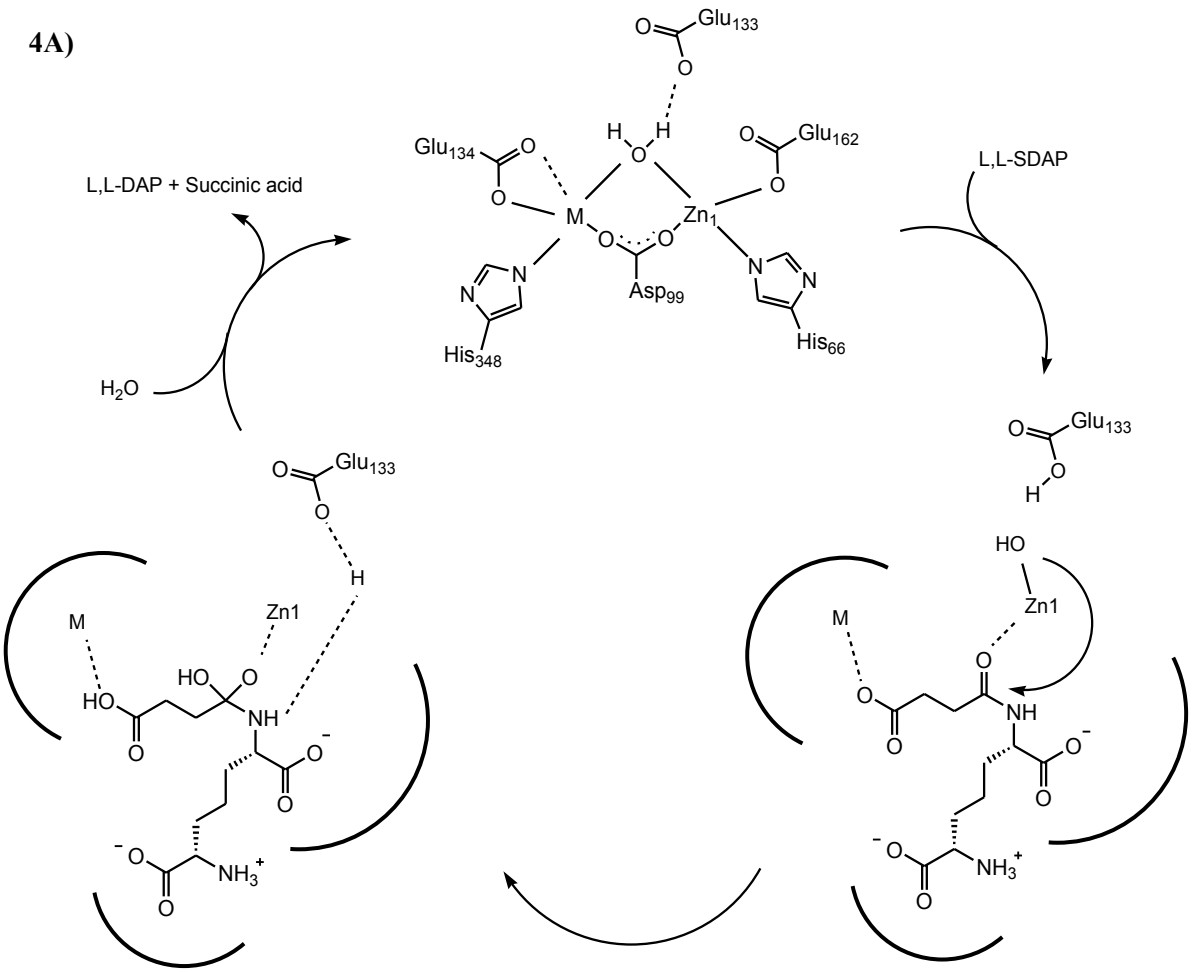
This crucial difference with Zn^{2+} means that Mn^{2+} may be able to bind the Asp-Leu substrate in a bidentate manner and still be able to expand the coordination sphere to 6-coordinate, to stabilize the transition state. In contrast, Zn^{2+} will not easily expand to a coordination number of 6, thereby being unable to hydrolyse the dipeptide, either because the bidentate-coordinated form does not allow for transition-state stabilization or because the monodentate coordination of substrate does not give sufficiently-strong binding (*i.e.* K_M is too high and the amount of substrate too low). We postulate that the binding orientation Asp-dipeptides allows complexation of the aspartic acid moiety with Mn^{2+}, with the leucine group occupying equivalent position of DAP. On the light of these observations, we also support the notion that, for the native activity (hydrolysis of SDAP), the Zn_2, which is not involved directly in the catalytic cycle is more likely bound to the carboxylic acid of the succinyl-moiety of the native substrate, rather than the carboxyl at the α position of DAP. We found that the latter appears to be essential for productive binding of the substrate, most probably by forming an ionic interaction with an Arg residue conserved at the dimerisation domain of M20 metallohydrolases, which corresponds to Arg257 of *S.enterica* DapE (see Chapter 4).

Regarding the role of the catalytic Zn_1 involved in the hydrolysis of SDAP for the native activity, our results agree with the proposed mechanism of Nocek, where the activated water coordinated by Zn_1 hydrolyses the peptidic bond (Scheme 4A). In the ground state, Zn_1 could either be on a tetrahedral or distorted tetrahedral (coordination number 4) or a trigonal bipyramidal (coordination number 5), depending on the mono or bi-coordination of Glu162. When the substrate SDAP enters the active site, either the carboxylic group of the peptidic bond replaces one coordination bond of Glu162, or Zn expands its coordination sphere from 4 to 5 (Scheme 4B).

In our proposed mechanism the second metal (Zn or Mn) doesn't bind to the terminal carboxylic group of the second amino acid in the case of native activity on DAP, but rather one carboxylic group of the succinic moiety of SDAP (Scheme 4C).

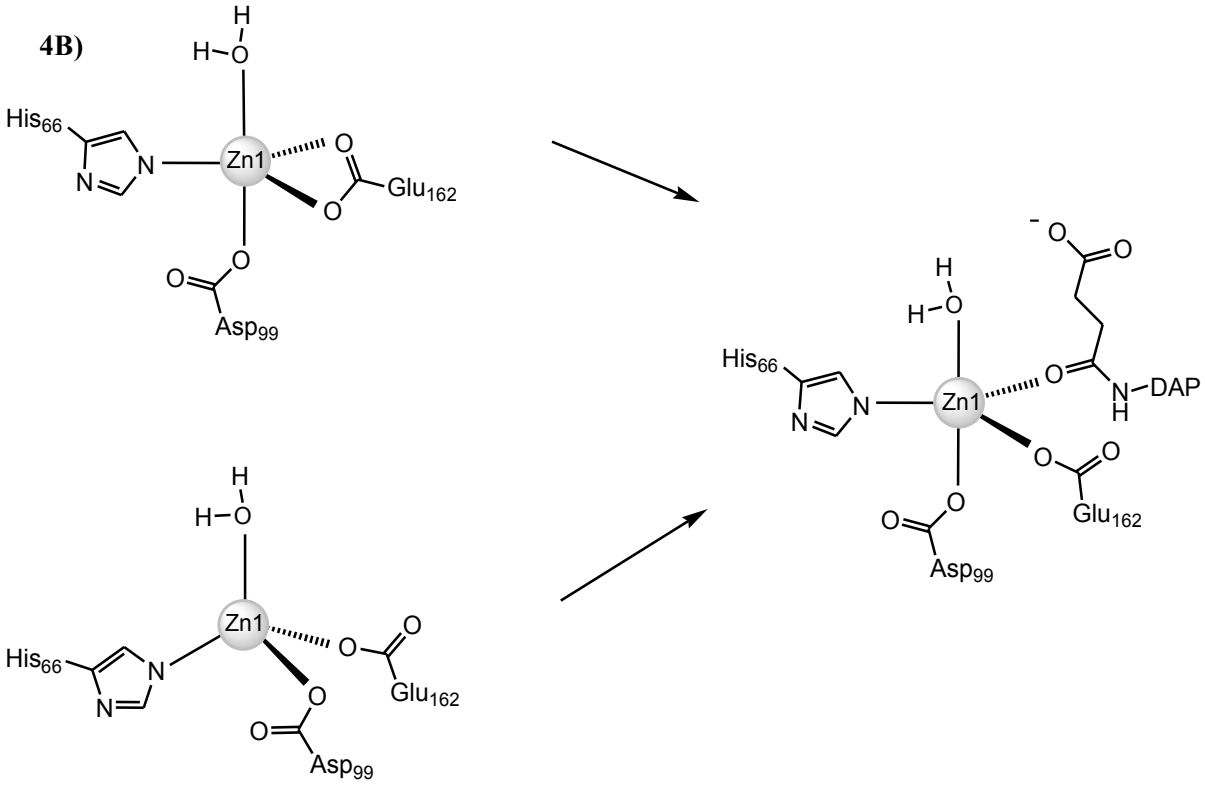
The energy required to expand the valence of Zn from 4 or 5 to 6 must be higher than for Mn, and a bidentate ligand like Aspartate in Asp-Leu, appears not to be able to coordinate easily to Zn with the aid of the sterically hindered amino group. We postulate that manganese, however, can easily expand its valence to 6 and bind a bidentate ligand forming an octahedral geometry (Scheme 5). Supporting this notion, Broder and Miller reported weak but detectable dipeptidase activity of DapE also in presence of Cd and Co,[17] both of which have a reported preference for octahedral geometry.[24]

Our mechanistic insight also provides a reasonable explanation to why carnosinase 2 (CN2), which is like DapE also a member of the M20 metallohydrolase family, fails to be catalytically active with Zn^{2+} but requires Mn^{2+} exclusively for its enzyme reaction, although the fine structures of the respective metal-binding sites with a transition-state analogue/inhibitor are highly similar:[16] although both Zn ions were crystallised in octahedral geometry and, consequently this geometry is undoubtedly thermodynamically feasible in the active site of the hydrolase, presumably the kinetic barrier of transition to this geometry is too high for productive catalysis in solution. Our postulated mechanism for the coordination geometry of the metals and the spatial position of the ligands in DapE are not based on direct structural observations such as crystallography, which could prove uninformative in this case, but are consistent with the available experimental observations of kinetic behaviour.

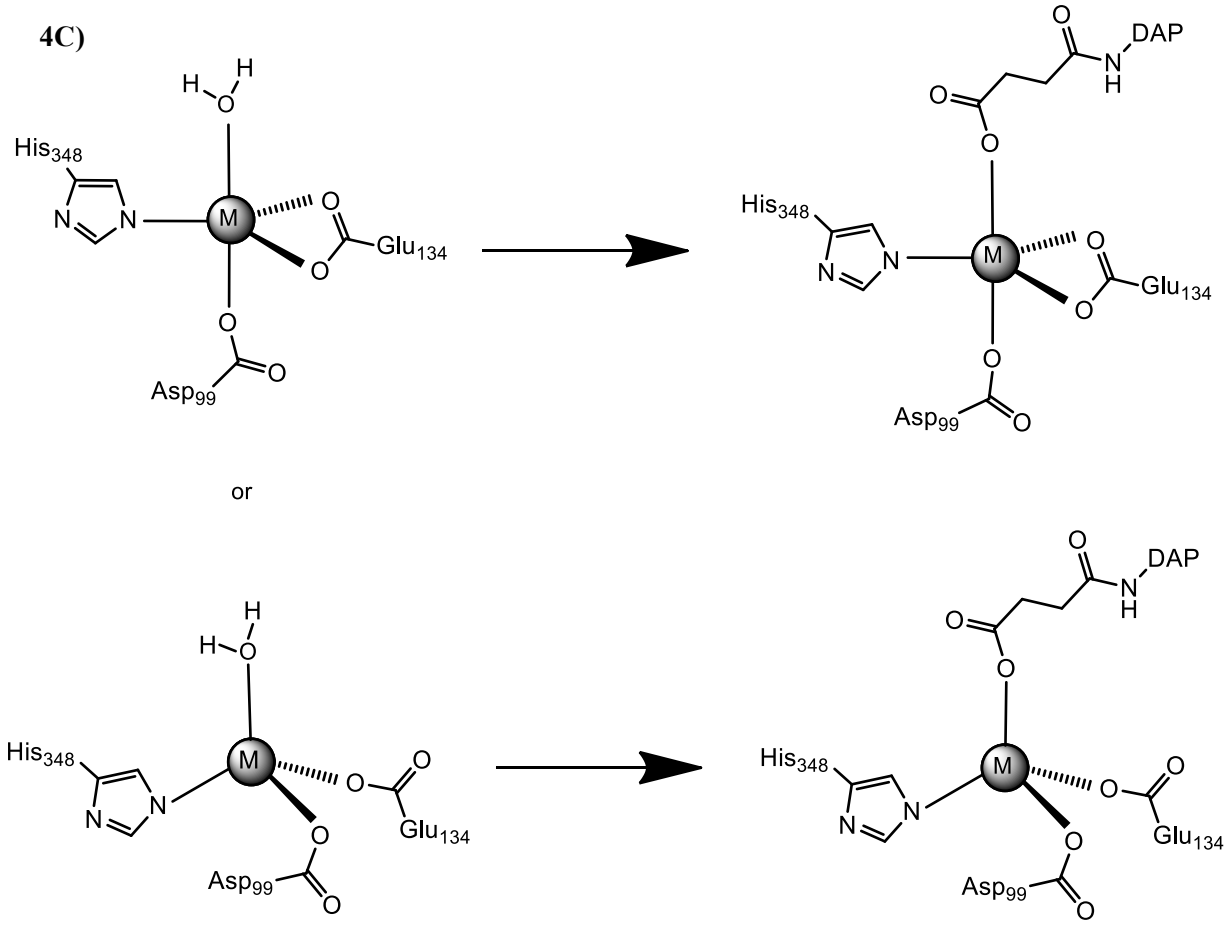


Scheme 4. A) Reaction mechanism for native activity of the Zn_1/M_2 metalloisoform of DapE. (M = Zn_2, Mn_2, or No Metal). B) Role of the catalytically active Zn_1 in its preferred coordination geometries. C) Coordination role of the second metal (M_2 = Zn_2 or Mn_2) on the succinic moiety of SDAP. Graphics courtesy, Dr. Gaetano Angelici.

4B)

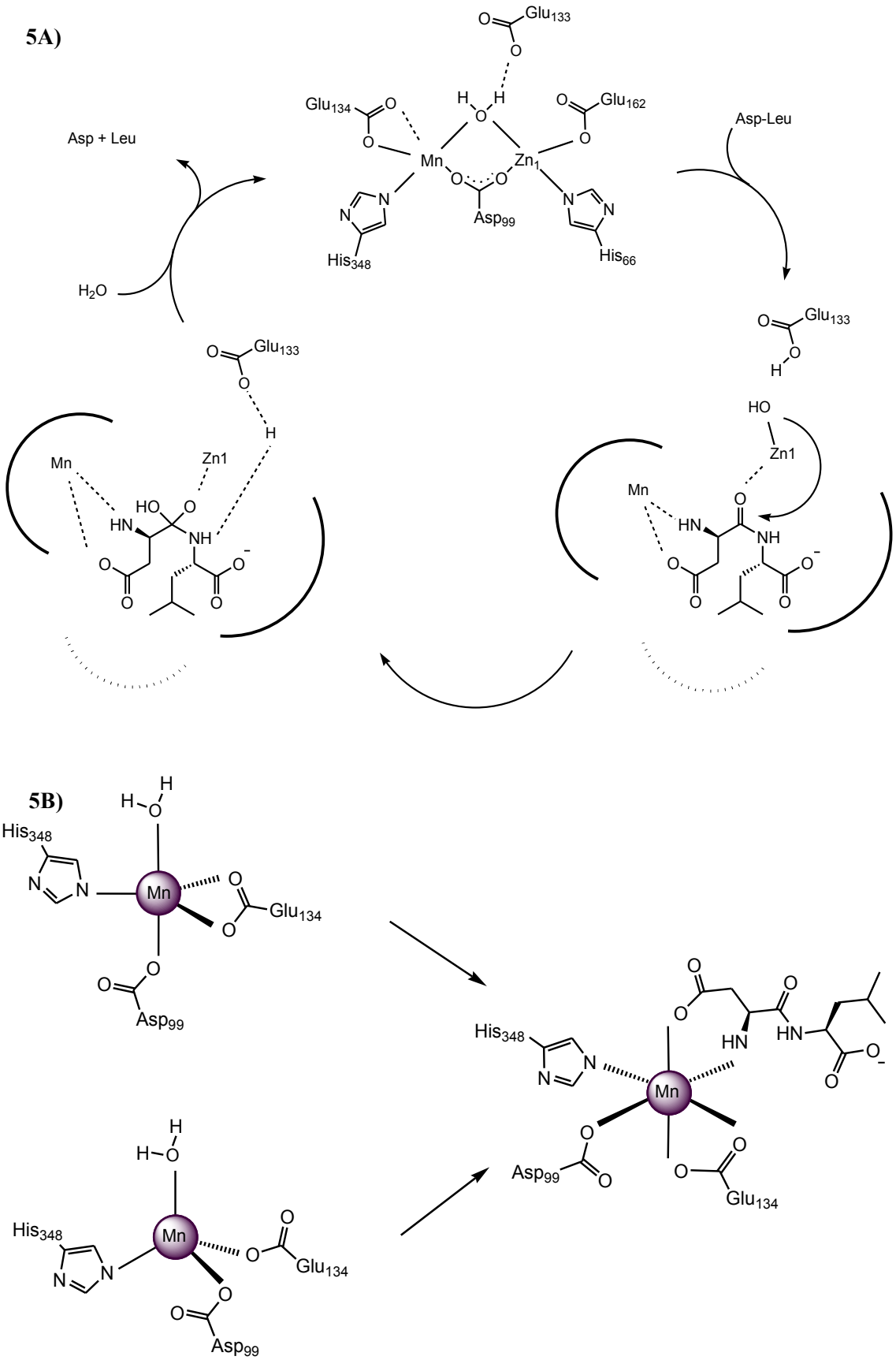

His66
Zn1
Glu162
Asp99
DAP

4C)


His348
M
Glu134
Asp99
DAP

or

His348
M
Glu134
Asp99
DAP



Scheme 5. A) Reaction mechanism for promiscuous activity of the Zn_1/Mn_2 metalloisoform of DapE. B) Coordination role of Manganese on the bidentate binding of Asp-Leu. Graphics courtesy, Dr. Gaetano Angelici.

Conclusions

In summary, we propose a qualitative explanation for the curious promiscuous dipeptidase activity of DapE in presence of manganese, based on our postulate of bidentate interaction of the substrate with Mn that cannot occur easily with Zn. Thus, subtle differences in coordination preferences can lead to very large differences in catalytic outcomes. A deeper understanding of the catalytic site of DapE is expected to provide insights for improved design of antibiotics that inhibit cell-wall synthesis in bacteria and may also assist in minimizing future antibiotic-resistance mediated by potential evolution of DapE.

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CHAPTER 7

The fragile birth of novel promiscuous function in the bacterial metallohydrolase DapE: why robustness favours the native substrate

Abstract

An important aim in protein engineering is to achieve a robust design. For example, for the creation of enzyme-like artificial catalysts, one should address not only how enzymes achieve such remarkable catalytic proficiencies, but also how they achieve such robustness. Laboratory selections for increased promiscuous enzyme-activity suggest that native catalytic activities are robust to mutagenesis. However, it remains unclear whether native catalytic activities are actually any more robust than their promiscuous activities. Moreover, little is known of the underlying molecular mechanisms leading to the emergence of functional robustness of enzyme activities or how these mechanisms relate to enzyme evolvability. Here we explore the robustness of two enzyme activities in an enzyme, native or promiscuous activity, and investigate the underlying molecular mechanisms for their robustness. We used neutrally evolved libraries of the bacterial metalloenzyme, DapE, *N*-succinyl-L,L-diaminopimelate desuccinylase, selected either for its native desuccinylase activity or for its promiscuous dipeptidase activity following random mutagenesis and placing the gene under expression of two different promoters. A knockout bacterial strain was used for the selection of native and promiscuous activities *in bacteria*. Variants selected for native desuccinylase activity, promiscuous dipeptidase activity and for both activities simultaneously were explored for their tolerance to varying DapE expression under either strong or weak promoters, enzyme mutational burden and also for metal availability in the culture-medium. We found that the native activity of DapE is more robust than its promiscuous activity on all variables tested. Our results support the notion that robustness is a systemic property, dependent on varied factors like metal availability and level of gene expression. Emerging promiscuous enzyme functions rely greatly upon their environment for survival when taking their first tentative steps in evolution and toward increased robustness. We predict that such insights on mechanisms of functional robustness can be exploited for drug design, biocatalyst design and synthetic biology.

Introduction

An important aim in engineering is to achieve a robust design. Robustness can be defined as the persistence of a system's characteristic behaviour under perturbations. Biological systems, such as enzyme pathways, exhibit remarkable phenotype resilience to genetic and environmental changes.[1] In genetic engineering, for example for the creation of enzyme-like artificial catalysts,[2] one should address not only how enzymes achieve such remarkable catalytic proficiencies, but also how they achieve such robustness.

Promiscuity is the capability of an enzyme to catalyse reactions and/or use substrates, in addition to its physiologically evolved activity. Laboratory selection for increased promiscuous enzyme-activity suggest that native catalytic activities are robust to mutagenesis.[3] However, it remains unclear whether native catalytic activities are actually any more robust than their promiscuous activities. Moreover, little is known of the underlying molecular mechanisms leading to the emergence of functional robustness of enzyme activities or how these mechanisms relate to enzyme evolvability. [4]

The underlying molecular mechanisms for functional robustness pose many fundamental problems [4]. For example, it has been commonly thought that native activities are driven by specific interactions, whereas promiscuous activities are entropy-dominated.[4,5] It has also been suggested that the evolution of drug-resistance can be minimized with strong and enthalpically-driven interactions.[6] Although in some cases promiscuous binding activities, standing alone, may also be highly specific,[7] in general, the available evidence suggests that enthalphy-driven, specific interactions are a thermodynamic hallmark of binding that is robust to mutagenesis. However, this view presents a paradox, because, intuitively, one would expect that a highly specific and enthalpically-driven interaction would be more prone to random mutagenic disruption than any promiscuous binding to a "sticky hydrophobic patch". Therefore, an important question to understand robustness is: what are the underlying molecular signatures of robust enzyme activities? A final important question that can be explored by neutral evolution of promiscuous enzyme activities relates to the design of functional robustness: how can knowledge of mechanisms of functional robustness be exploited for drug design, biocatalyst design and synthetic biology?

The promiscuous enzyme encoded by *N*-Succinyl-L,L-diaminopimelic acid desuccinylase (DapE) offers a useful tool to investigate functional robustness. In a promiscuous enzyme, more than one function co-exists in the same enzyme scaffold, thereby minimising the confounding effect of structural robustness in evolutionary studies. DapE is an essential enzyme for the synthesis of a key component (diaminopimelic acid –DAP-) of the peptidoglycan cell wall in gram negative bacteria. Deletion of *dapE* gene is lethal to several pathogenic bacteria and is therefore a target or antibiotic discovery.[8] Here we investigate two curiously independent activities of dapE in vivo: the native desuccinyse activity (DapE activity) and a manganese-dependent, promiscuous peptidase activity (Figure 1).[9]

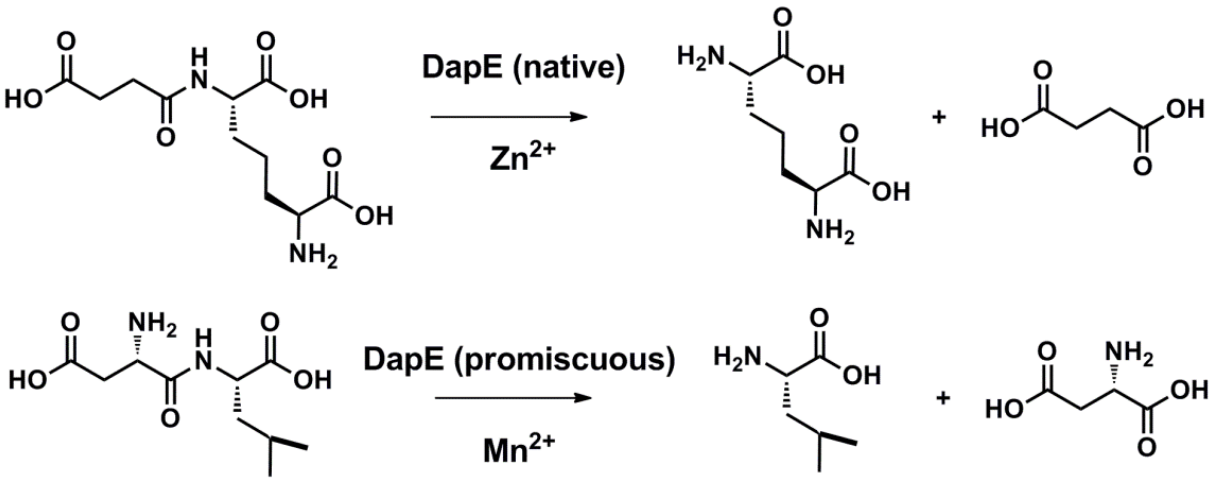

Figure 1. Native and promiscuous activities of dapE. The native desuccinylase activity is Zn^{2+}-dependent, although it is also functional with Mn^{2+}.[10,11] The promiscuous activity is strictly Mn^{2+}-dependent.[9]

The robustness of promiscuous or native activity can be assessed and quantified by selection in a genetically-modified bacterial strain.[9] We neutrally evolved the bacterial DapE for either its native desuccinylase activity or for its promiscuous dipeptidase activity using laboratory evolution to explore its functional robustness. Here we provide what is, to our knowledge, the first published direct evidence showing that a native activity is more robust than a promiscuous activity and provide insights for such robustness of the native enzyme activity.

Materials & Methods

Minimal medium, E-medium,[12] supplemented with 0.4% glucose and a 0.4 mM concentration of the appropriate amino acids and/or dipeptides (Bachem) was used as a minimal medium and LB-medium was used as a rich medium. As a supplement, 1 mM *meso*-diaminopimelate (mDAP) and IPTG were added (Bachem). Sodium ampicillin, Kanamycin, tetracycline and chloramphenicol were used at final concentrations of 30, 100, 5 and 34 µg/ml, respectively, when added to either liquid or solid medium. Electro competent cells of *S.enterica* and chemical competent cells of *E.coli* were prepared by standard protocols. Agar (used at 1.5 %) and agarose (used at 1 %) were purchased from Invitrogen. T4-DNA ligase, *XhoI HindIII* and other restriction enzymes are from New England Biolabs, unless specified. All other chemicals were purchased from Applichem, unless otherwise specified. Primers were synthesized from Microsynth, Switzerland and the gene sequencing was from Starseq, Germany.

Construction of Random mutagenesis libraries

In the first round of evolution the mutant library was constructed starting from a wild type *S.enterica dapE* gene, ~1.2Kb, was PCR amplified by GeneMorph® II Random mutagenesis kit, Stratagene, from the plasmid pCM655-*dapE* containing kanamycin resistance gene, primers (F & R-SEQ-pCM655). In second round two variants of the first round were used as templates and in the third round four variants of the second round were used as templates in a single-pot random PCR (Figure 2). The rate of mutagenesis at each round was controlled by using 0.5µg target DNA as a PCR template and by limiting the number of PCR cycles to 25, to obtain low to medium mutational frequency (0 to 4.5 mutations per kb).

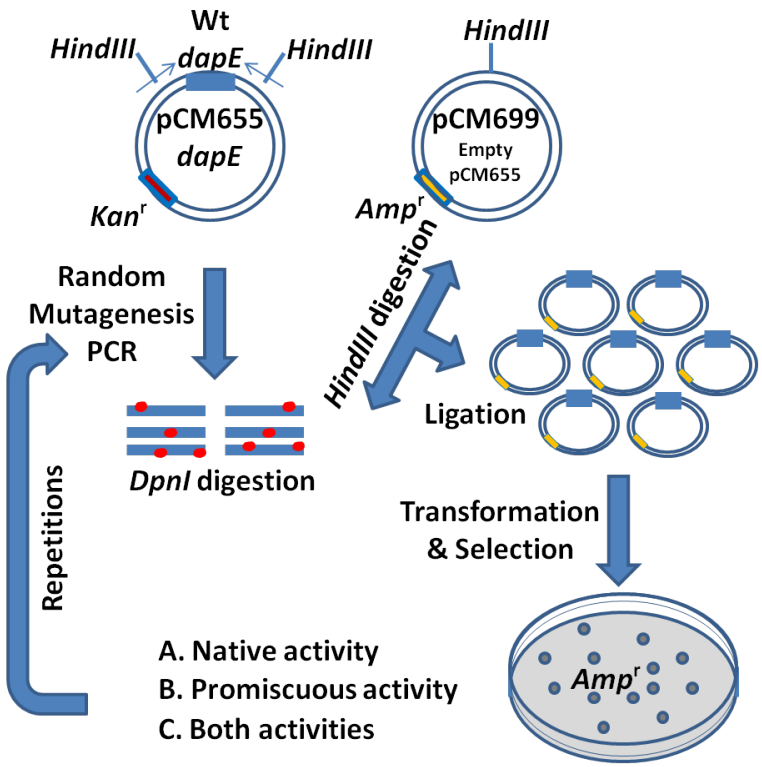

Figure 2. Overview of laboratory evolution by random mutagenesis, recloning and selection.

Cloning of random mutagenesis libraries

In the original plasmid, pCM655,[9] *S.enterica dapE* gene has a *HindIII* restriction site on either side of the *dapE* gene, which has a Ribosomal Binding Site (RBS) close to the start codon and the cloning strategy was designed in such a way that the gene is always cloned along with this RBS (Figure 3). Random mutagenesis with Genemorph II / normal PCR product with *pfu* Turbo polymerase was subjected to *DpnI* digestion to minimize the background of wild type gene and PCR template plasmid and then digested with *HindIII*, purified by PCR clean-up kit, (Machery-Nagel) and ligated (T4 DNA ligase, 2 hours at room temperature) with *HindIII* digested pCM699 vector containing Ampicilline resistance marker gene to avoid the background of the undigested and supercoiled plasmid template of the PCR random mutagenesis which contains kanamycin resistant marker gene, (Figure 2). The plasmid pCM699 was derived from pCM655, in which the *dapE* gene was deleted by *HindIII* restriction digestion and the empty plasmid vector was religated forming a single *HindIII* cloning site. The pCM699 has two promoters, a strong and IPTG inducible lac promoter and another weak and un-inducible phoB promoter, as assessed by BPROM (Softberry, Inc.), upstream and downstream of the single *HindIII* cloning site, respectively. Consequently, the

PCR amplified *dapE* gene / *dapE* mutant library digested with *HindIII* on either sides can be cloned into the *HindIII* digested plasmid vector, pCM699, in one of the two possible orientations. If the *dapE* gene is cloned in the forward orientation relative to the *lac* promoter it will be under the strong promoter (for wild type *dapE* gene pCM655) and if the gene is cloned in the reverse orientation it will be under the weak promoter (for wild type *dapE* gene pCM656) (Figure 3).

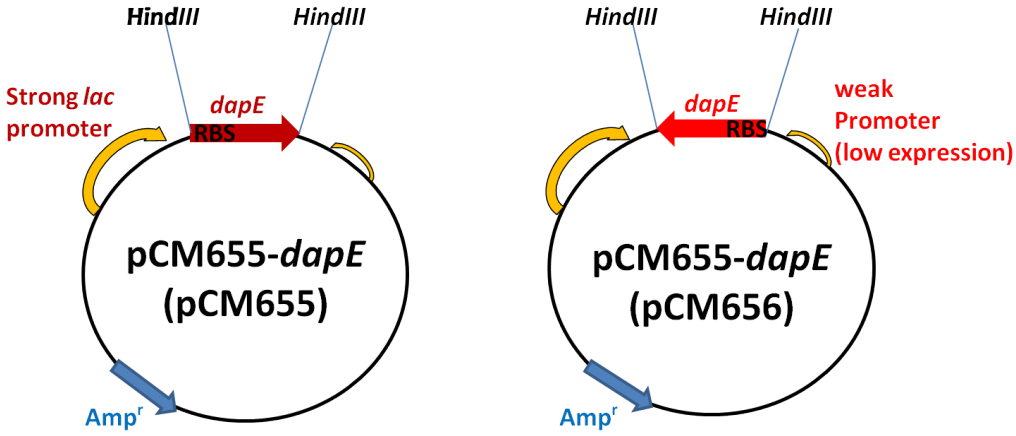

Figure 3. *dapE* gene under two different promoters in the plasmid. The two plasmids, pCM655 (gene under strong promoter) and pCM656 (gene under weak promoter).

Transformation

Transformation into *S.enterica* strain (TN5911),[9] which has all 7 genes of aspartyl dipeptidases and *dapE* gene knocked-out and it is also an auxotroph for leucine and proline; therefore, mDAP has to be supplemented in enriched media. In minimal medium, in addition to mDAP, supplementation of leucine and proline is also required. The strain TN5911 was used for the construction of various strains (Table 1) by transforming different plasmids (Table 1) through electroporation.

Electroporation

Electroporation of 100µl electro competent cells and 5 to 10µl ligation product or plasmid DNA in 0.1cm cuvettes (Axon labs) at 1.8Kv for ~5msec and the cells were recovered with 0.1 to 0,2ml of SOC medium and incubated for 1 hour at 37^0C in a shaking incubator of

200rpm. The recovered transformation mixture was briefly centrifuged for 1minute at 4000rpm to collect the cells or dilute to 100 to 1000 times in order to get rid of the rich medium as the further enzyme activity selections on minimal medium. The resuspended or diluted cells in sterile M.Q.H_2O were plated for single colonies under different selection conditions (selection for native activity, promiscuous activity and for both activities at the same time) and also with a plasmid marker, ampicillin, to check the transformation efficiency.

Selection for different enzyme activities in bacteria

The native activity selection of DapE were carried out without supplementation with mDAP, either in rich medium (LB-medium) or on minimal medium (VB-medium). Promiscuous activity was selected in minimal medium by supplementing Asp-Leu dipeptide as a source of leucine and mDAP for compensating the native activity. For selecting both native and promiscuous activities at the same time only Asp-Leu dipeptide was provided a leucine source but not mDAP source in the medium. If the selection was under minimal medium, it was also possible to check if the DapE enzyme was active / efficient enough to produce sufficient DAP for cell-wall synthesis as well as for the precursor of the lysine synthesis, when selected for native activity and for both activities at the same time by supplementing with or without lysine.

Effect of selection conditions for pCM655 and pCM656 on bacterial growth

Selection for native activity in rich medium (LB-medium) under the strong or weak promoter did not influence the growth when induced with or without IPTG, but the growth was consistently slower under the weak promoter compared to the strong promoter, irrespective of IPTG supplementation (Figure 4A). Bacteria selected for native activity in minimal medium (VB-medium) under the strong promoter without IPTG grew much better than with IPTG, which we interpret as arising from possible toxicity of over-expression of DapE. In minimal medium, selection for native activity under the weak promoter is largely unaffected by IPTG.

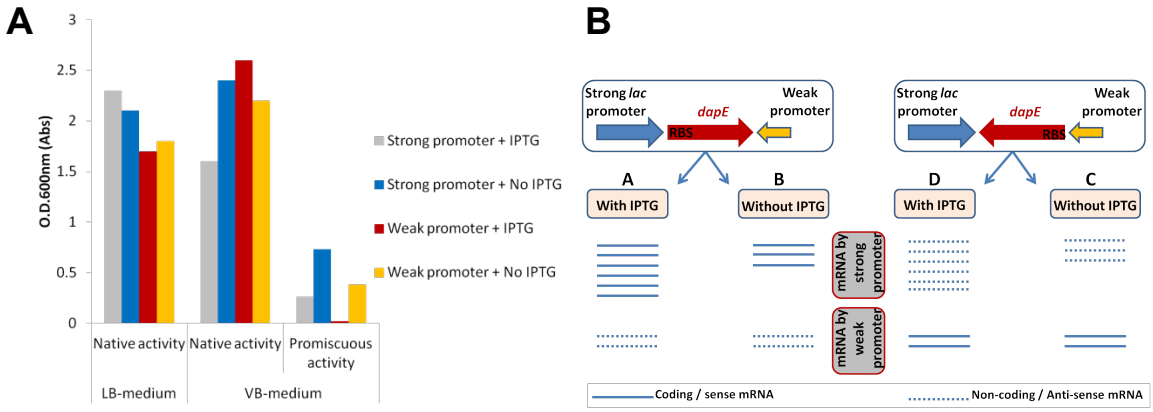

Figure 4. (A) Growth of the *dapE* knock-out of *S.enterica* containing plasmid-encoded DapE in regulated by strong or weak promoters, cultured under selection for native activity (in LB & VB-medium) and promiscuous activity (in VB-medium). Culture growth was monitored by measuring the OD_{600nm} after 10 hours in LB-medium and 24 hours in VB-medium. (B) Schematic drawing of different levels induction of both sense and antisense RNA.

Growth for promiscuous activity selection is very slow under the strong promoter with IPTG compared to without IPTG suggesting that the over-expression of DapE is toxic to the bacteria, as noticed also under the native selection in minimal medium. Growth under weak promoter with IPTG is ten times slower compared to without IPTG. We interpret this finding as IPTG inducing the transcription of anti-sense RNA from the strong promoter and this RNA can interfere with the RNA of the sense strand, thereby blocking the expression of DapE (Figure 4B). In contrast, in absence of IPTG, the amount of transcription of anti-sense RNA is likely lower than the transcription of sense mRNA, thereby allowing sufficient DapE expression for the growth of the bacteria (Figure 4).

Phenotypic validation of the selected variants

All selected plasmids were isolated and retransformed into TN5911 for phenotypic validation in selection media. This precaution was intended to ensure that the DapE-mediated phenotype was plasmid-encoded and not an artefact of any genomic mutations.

Cloning of dapE in pET28b for constructing C-terminus 6His-DapE

See Chapter 3 and Figure 5.

Primers	**Sequence (5‘ to 3‘)**
F-SEQpCM655	GCGTACGTCGCGACCGCGGACATGT
R-SEQpCM655	GCGTACGTCGCGACCGCGGACATGT
R-pCM655XhoI	AGCCAAGCCTCGAGGGCGACGAGCTGTTCC
F-pET28HindIII	CCCTCTAAAGCTTAAGGAGATATACC
R-pET28HindIII	GTTAGAAGCTTGATCTCAGTGGTGGTGGTG
F-DapE	AATTTTTGGGCATGGCGTGGGC
R-DapE	TCA GGC GAC GAG CTG TTC CAT
Plasmids	**Genotype**
pCM655[9]	Plasmid with Wt ***dapE*** gene under **strong** promoter, Ampicillin
pCM656 this work	pCM655 with Wt ***dapE*** gene under **weak** promoter, Ampicillin
pCM657 this work	pCM655 with Wt ***dapE*-C6His** under **strong** promoter, Ampicillin
pCM658 this work	pCM655 with Wt ***dapE*-C6His** under **weak** promoter, Ampicillin
pCM699 [13]	pCM655-Empty plasmid (without *dapE* gene), Ampicillin
pET28b	Kanamycin
pET28b-dapE-HindIII this work	pET28b with *dapE* between *HindIII* and *XhoI*. Kanamycin

Table 1. Summary of primers and plasmids used in this study.

***S.enterica* strains**	**Genotype**
TN5911[9]	Knockout strain for *dapE,* 7 aspartyl dipeptidases & leucine auxotroph
TN5935[9]	TN5911 + pCM655
TN5936 this work	TN5911 + pCM656
TN5937 this work	TN5911 + pCM657
TN5938 this work	TN5911 + pCM658
TN5959[13]	TN5911 + pCM699
***E.coli* strains**	**Genotype**
BL21DE3	BL21 (DE3)
BL2125 this work	BL 21 (DE3) + pCM655
BL2126 this work	BL 21 (DE3) + pCM656
BL2127 this work	BL 21 (DE3) + pCM657
BL2128 this work	BL 21 (DE3) + pCM658
BL2129 this work	BL 21 (DE3) + pCM699
BL2130 this work	BL 21 (DE3) + pET28b
BL2131 this work	BL 21 (DE3) + pET28b-dapE-HindIII

Table 2. Summary of bacterial strains used in this study.

PCR

Single colonies were re-suspended in 50µl of sterile M.Q.H_2O and 5µl of this cell suspension was used as a template for each colony PCR. For most of the PCR the following conditions were used, hot start PCR, with initial 95°C for 3 minutes, then 25 cycles of 95°C/ 1 minute, 60°C/ 1 minute and 72°C/ 2 minutes, with a final extension of 10 minutes at 72°C. Since the colony PCR was only for analytical purpose *Taq* DNA polymerase ($T_{elongation}$ 72°C) was used. However, whenever the PCR amplified product needed to be used for further cloning or sub-cloning *Pfu* Turbo DNA polymerase ($T_{elongation}$ 68°C) was used in order to avoid the errors of the Taq DNA polymerase. The genemorph II mutagenesis kit ($T_{elongation}$ 72°C) was used for random mutagenesis.

	F-primer	R-primer	PCR amplicon	Gene orientation	Promoter type
1	F-DapE	R-SEQpCM655	Yes	Forward	Strong
2	F-SEQpCM655	R-DapE	Yes	Forward	Strong
3	F-SEQpCM655	R-SEQpCM655	Yes	Cannot distinguish	
4	F-DapE	F-SEQpCM655	Yes	Reverse	Weak
5	F-SEQpCM655	R-DapE	Yes	Reverse	Weak

Table 3: primers for the identification of the orientation of the gene in pCM plasmid constructs.

	Component	Quantity	Concentration	Note
1	DNA / cells	0.01µg to 0.5µg		5µl of cells for colony PCR
2	Buffer	5µl	1X	As per the DNA polymerase
3	DMSO	2µl	4%	
4	dNTP's	2µl	0.mM each	1.6mM dNTP's mix
5	F-primer	1.5µl	0.3µM	
6	R-primer	1.5µl	0.3µM	
7	DNA Polymerase	0.5µl to 1µl		*Taq* DNA polymerase *Pfu* Turbo DNA polymerase Genemorph II Mutagenesis kit
8	DNase free water	To 50µl		

Table 4: Components and concentrations of the PCR reactions.

Cloning of C6His-dapE from pET28b to pCM699

The C6His-tag DapE was cloned into the pCM699 vector backbone in order to validate the enzyme activity of the C-terminus 6xHistidine-tagged DapE in bacteria by phenotypic validation. The primers used for this cloning were, F-pET28HindIII and R-pET28HindIII with *pfu* Turbo polymerase and then the PCR amplified C6His-tag DapE and pCM699 vector were digested with *HindIII* restriction site. Since the gene could be cloned in two possible orientations in pCM699, the wild type C6His-tag DapE in forward orientation was placed under strong promoter (pCM657) and the gene in reverse orientation was placed under the weak promoter (pCM658). The 6xHistidine-tagged DapE complemented native and promiscuous activities under both the promoters comparable to non his-tagged DapE.

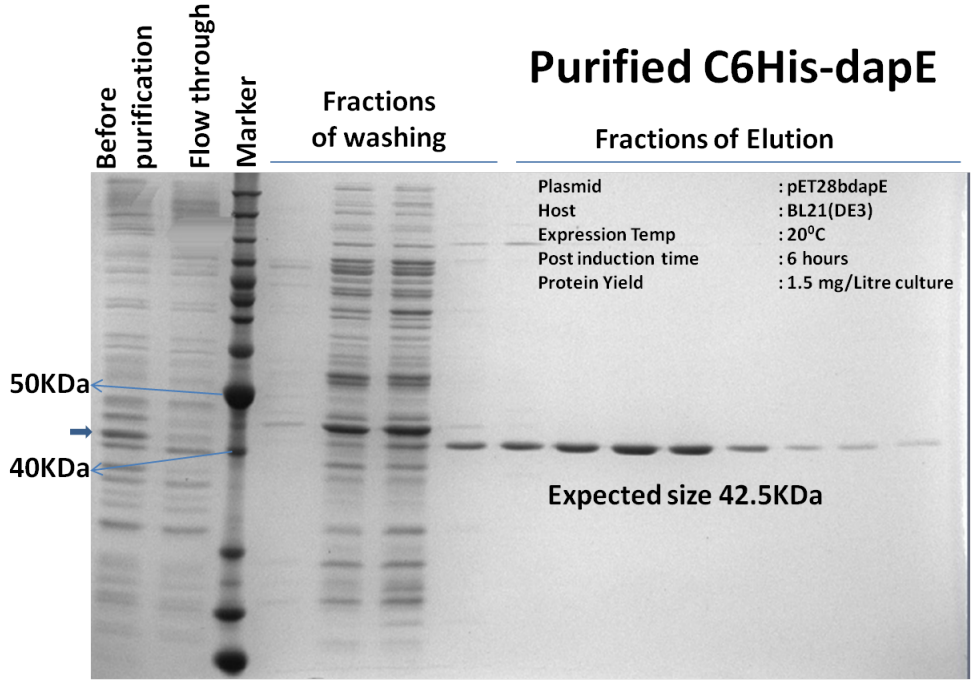

Figure 5. SDS-PAGE showing the purified C6His-tagged DapE of ~42.5KDa.

DapE kinetic assays.

See Chapter 3.

Results

Random mutagenesis leads to more survivors for native than for promiscuous activity

Genetic robustness of DapE enzyme function was assessed by random mutagenesis and by placing the gene under expression of two different promoters for selection (Figure 3).

When the *dapE* gene randomised library was selected for native activity there were consistently more colony-forming units (cfu) than when selected for promiscuous activity or for both activities at the same time (Figure 6). The number of colonies when selecting for promiscuous activity or for both activities selection was similar, but always less than under selection for native activity. This observation was independent for the type of PCR template used for randomisation, i.e. a single wild type *dapE* gene or one or more *dapE* variants, previously pre-selected for one or the other or both activities at the same time, amplified in one-pot PCR randomisation.

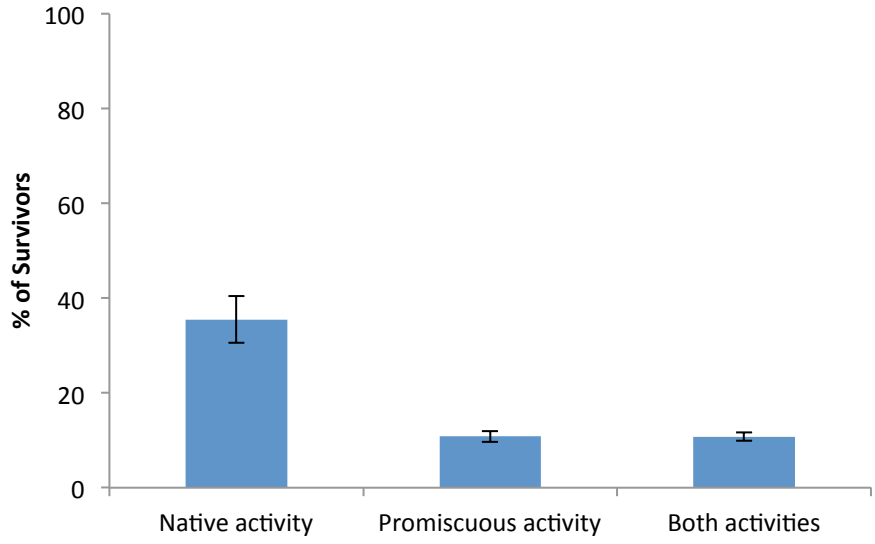

Figure 6. Percentage of survivors form a randomised library of DapE, selected for native activity, promiscuous activity and for both activities at the same time, compared to survivors in absence of any selection for DapE (only selected for plasmid transformation through ampicillin resistance). This experiment carried out in triplicates under identical conditions is representative of 4 different experiments carried out with different mutational rates leading to similar conclusions.

The finding that selection for both activities at the same time leads to similar survival rate as for promiscuous activity suggests that the native activity imposes little additional selection pressure to the promiscuous activity. Taken together the promiscuous activity is very sensitive

to mutational burden compared to the native activity; since selection for the native activity leads to more survivors than promiscuous activity, the native activity of DapE *in vivo* is more robust to mutagenesis than its promiscuous activity.

Selection pressure for DapE function biases the promoter preference

The cloning strategy that we used for the construction of the random gene libraries (Figures 2 & 3) is a novel selection method for screening the randomised libraries at a range of selection pressures controlled by protein-dosage (Figure 4) within a single experiment. The cloning strategy was designed in such a way that the *dapE* gene can be cloned either under the strong and inducible *lac* promoter or under the weak and un-inducible *phoB* bacterial promoter, which provides 50% chances for *dapE* wild type or variant gene to be cloned under any one of the two promoters. In order to prove that this concept is experimentally true, we selected the randomised library only for the plasmid marker gene (Ampicillin) and with no selection pressure for any of the enzyme activities. As expected, the gene insert was found in either orientation, with about 50% occurrence for each of the two possibilities (Figure 7).

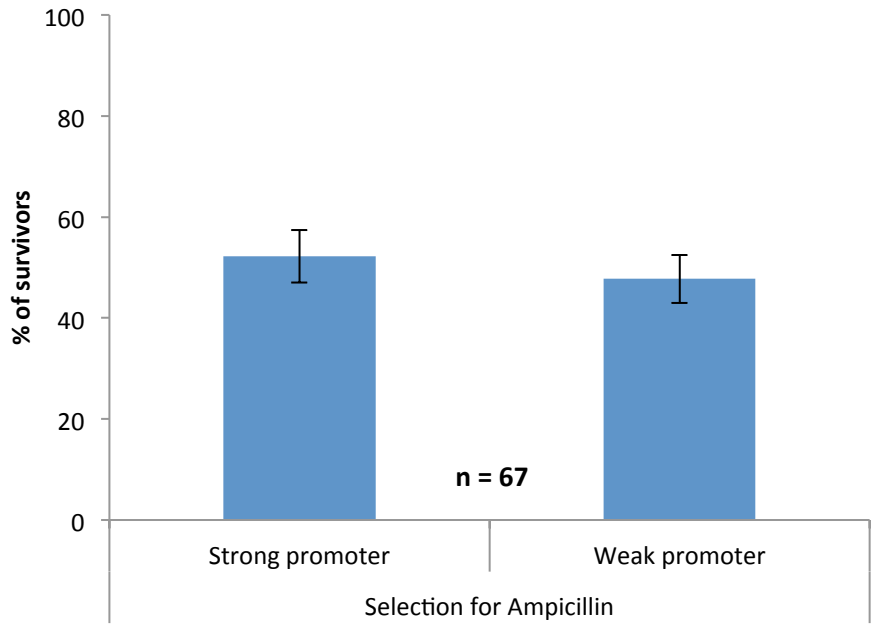

Figure 7. Percentage of survivors under strong and weak promoters when selected for plasmid marker gene, Ampicillin, resistance with no selection pressure for any of the enzyme activities.

When the randomised library was selected for native activity, almost 50% variants survived under each of the two promoters (Figure 8) in a similar manner to the percentage of survivors

under no selection pressure (Figure 7), suggesting that the native activity is on average insensitive to low expression level even under the mutational burden imposed in the experiments. Knowing that the wild type has native activity under the weak promoter, it is reasonable to assume that some variants may show a bias towards the strong promoter for compensating the mutational burden; however, our results showed that the percentage of survivors for native activity at each promoter are almost equivalent. It is noteworthy that in neutral evolution it is also possible to have (rare) variants with improved native activity under either promoter. However, we did not proceed to expressing and characterising the variants kinetically any further.

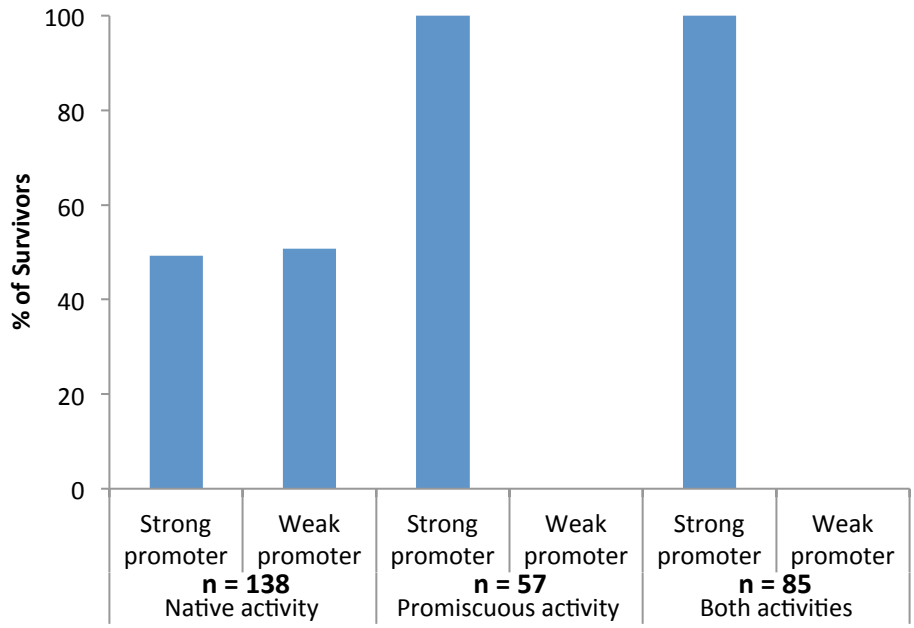

Figure 8. Promoter preference under selection pressure for native activity, promiscuous activity and for both activities at the same time.

When selected for promiscuous activity and also for the both activities at the same time, there are no survivors under the weak promoter. Our control experiments showed that wild type DapE had no promiscuous activity under the weak promoter at the very stringent selection condition (Figure 4). During selection of random libraries, we did not find any variant under the weak promoter with promiscuous activity (0/57 selected for promiscuous alone or 0/85 selected for both activities). However, we identified 1 variant, M4B3, out of 87 variants cloned in reverse and phenotypically cross-validated, that maintain promiscuous activity under the weak promoter even with stringent IPTG selection. We hypothesise that variant M4B3 may have improved promiscuous activity compared to the wild type, as it has been

selected under the very stringent selection conditions, *i.e.* under weak promoter with IPTG; however, we did not explore this variant any further, neither kinetically nor for robustness.

Selection for native activity led to a similar number of survivors cloned under the strong promoter and weak promoter. In contrast, the absence any survivors when originally selecting for promiscuous activity and found to be cloned under expression of the weak promoter strongly suggests that the native activity tolerates lower levels of expression than the promiscuous activity. Similar conclusions are suggested already from growth profiles under different selections (Figure 4) as well as the fact that IPTG induction under the weak promoter is incompatible with DapE wildtype selection of promiscuous activity, whereas these conditions do not hinder survival of DapE wildtype under native selection. In summary, bacteria need more DapE to survive when the dipeptidase activity is required than when DAP availability is required; since most mutations are deleterious, most DapE variants cannot survive at all under the weak promoter for promiscuous activity, in contrast to native activity.

Effect of lysine supplementation on the promoter preference

DapE is a metallo-hydrolase present in the lysine biosynthetic pathway producing mDAP, which is one of the essential components of the bacterial cell wall and also precursor for the lysine synthesis. The promiscuous activity in bacteria, throughout this chapter, is always referring to the hydrolysis of a dipeptide, Asp-Leu, producing the free amino acids, aspartic acid and leucine. The *dapE* knock-out bacterial strain used for the screening of the random libraries is also leucine auxotroph. Leucine has a huge requirement for the cellular metabolism compared to the amount of mDAP required for the cell wall synthesis and hence we wanted to know if the DapE native activity is rate limiting for lysine synthesis, which in turn may affect the difference in the percentage of survivors and also the promoter preference between native and promiscuous activities.

Although we found that, on average, genes selected for the native activity could be found either under expression of the strong or weak promoter with little preference (Figure 9), closer analysis revealed a bias for the weak promoter of variants selected in presence of lysine (44 % forward, n = 39) and a stronger bias for the strong promoter when variants were selected in absence of lysine (66% forward, n = 38). This bias suggests that the selection pressure for the native activity was different in both selection conditions (Figure 10). Similar trends were seen

cumulatively, even when considering very different mutational rates from a variety of different DapE libraries preselected for various conditions (Figure 11). We interpret the bias against a strong promoter in the presence of lysine to toxic effects of DapE overexpression, as suggested also in growth curves of wildtype DapE in minimal medium (Figure 4A). In contrast, it appeared advantageous to express DapE under the strong promoter for most variants when selecting stringently for native activity, *i.e.* in absence of lysine, possibly because most variants required high enzyme expression to cope with the requirement for sufficient DAP product, both for cross-linking the peptidoglycan cell wall as well as lysine-biosynthesis. It is noteworthy, when comparing stringent selection for native activity and for promiscuous activity, *i.e.* both in minimal medium and supplementing either with leucine or mDAP, respectively, that the native activity *still appears more robust to mutagenesis*. This is surprising, considering that in both of these conditions, DapE is selected to genetically complement the requirement for an essential amino acid (lysine or leucine).

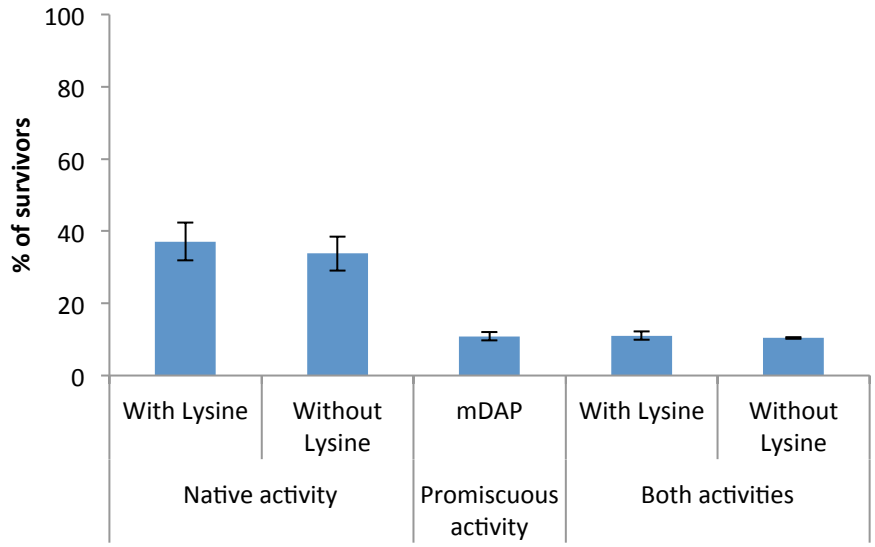

Figure 9. Survivors for native, promiscuous and both activities at the same time with and without lysine.

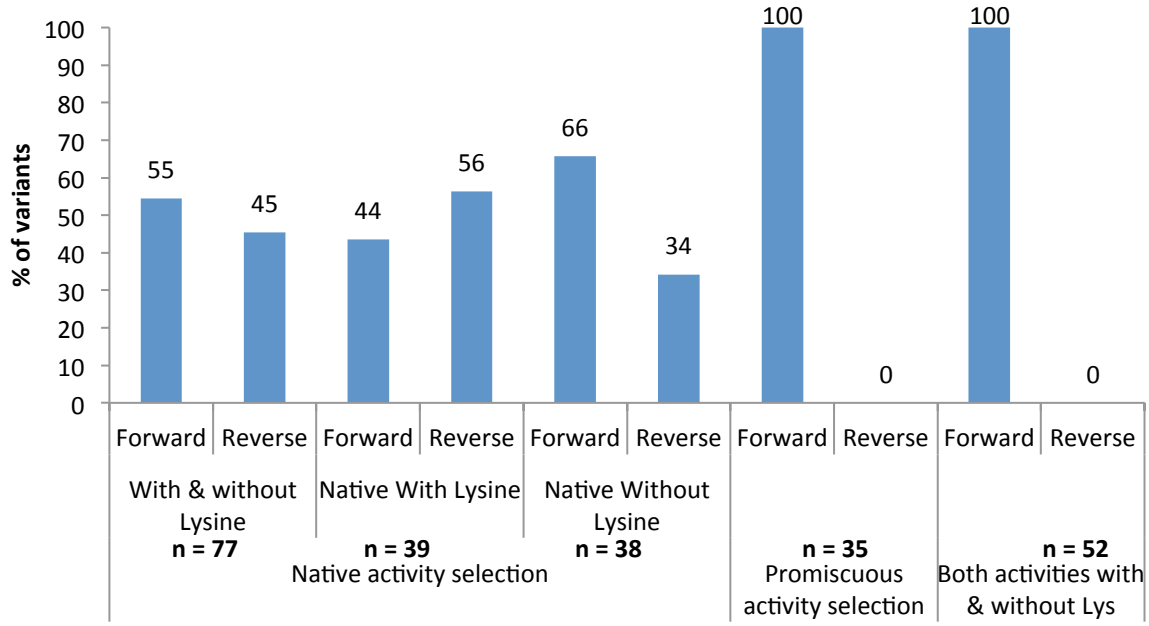

Figure 10. Promoter preference under selection for native activity either in presence or absence of lysine supplementation; for promiscuous activity and for both activities at the same time. Selection was carried out in presence of 1 mM IPTG in VB-minimal medium.

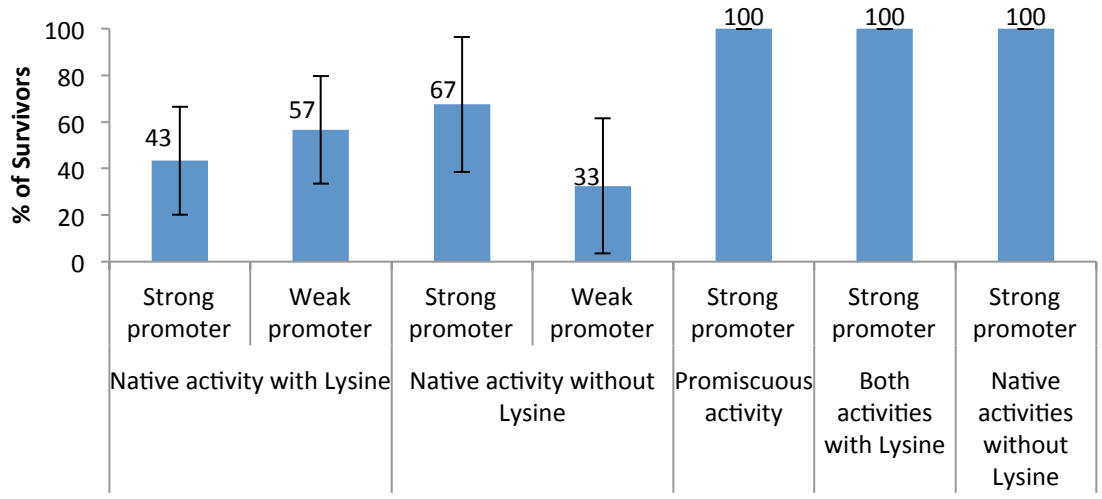

Figure 11. Percentage of survivors with and without Lysine supplementation for native activity and also for the promiscuous activity, based on 9 different randomised libraries arising from different templates (hence large error bars).

Overall, ratio of survivors for native activity over survivors of promiscuous activity is 3.5 times and 3.2 times with and without lysine in native selection, respectively (Figure 12). We conclude from these ratios: i) that survival under native activity compared to promiscuous activity (Figures 6 & 9) is not due exclusively to lysine supplementation and ii) nor to the possibility that the native activity can be under both the promoters but not the promiscuous activity, in which case one would predict a ratio of two if both were equally robust. Taken together, we conclude that the larger number of survivors for the native activity are due to the functional robustness of the native activity over the promiscuous activity, independent of lysine supplementation and promoter strength, although both these factors obviously contribute to survival upon selection for native activity.

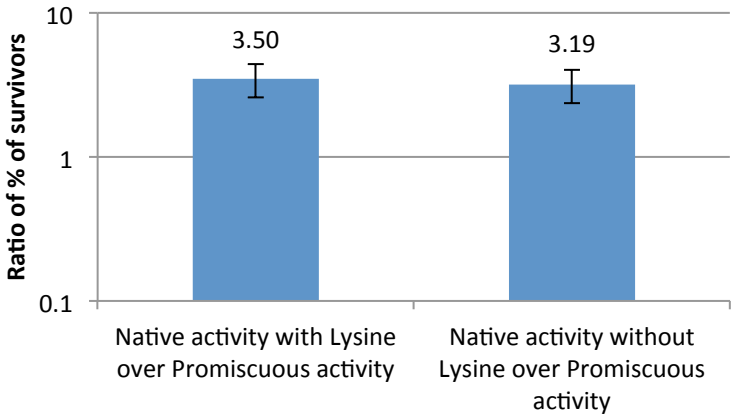

Figure 12. Ratios of percentage of survivors of native activity with and without lysine over the percentage of survivors of promiscuous activity.

Robustness of native activity over the promiscuous activity is maintained in directed evolution by sequential randomisations.

In the first two rounds, the percentage of survivors for native activity was more than three times more than for either promiscuous activity or both activities (Figure 13). The percentage of survivors for both activities was slightly lower than the survivors of promiscuous activities, which may be due to high selection pressure, as the enzyme has to perform both the activities at the same time and to mutagenic conditions of this experiment. The results of 1st round were reproduced in four independent experiments and the results of the 2nd and 3rd rounds of randomisations were reproduced each round in two independent experiments, with similar results.

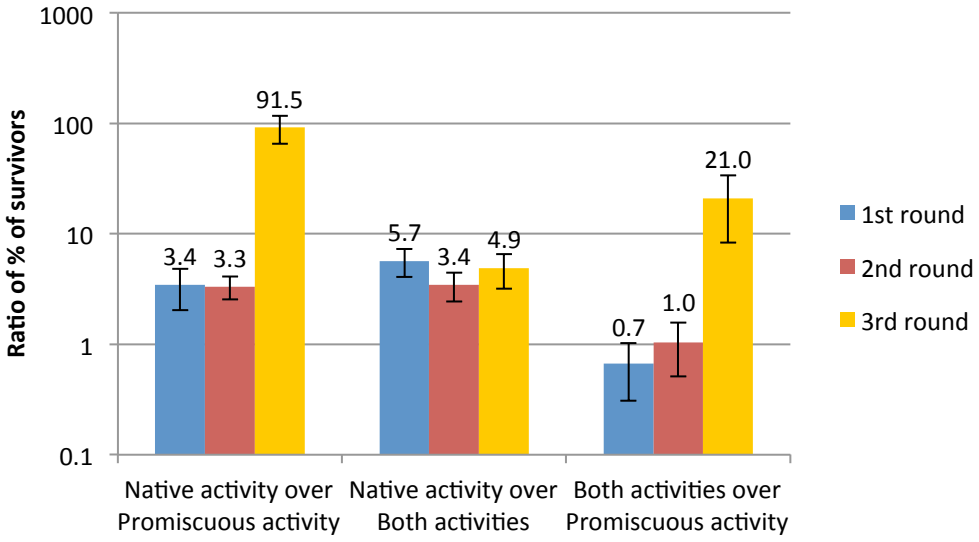

Figure 13. Survivors for the three rounds of randomisations, process of sequential evolution of DapE.

In the third round the percentage of survivors for native activity was 110 times more than the promiscuous activity and the percentage of both activities was 30 times more than the survivors of both activities; however, such extreme differences in survival rate in this particular experiment were due to the survival of only one variant for promiscuous activity in two replicate experiments; this one surviving variant at the 4th generation was identical to one of the four templates used for the one-pot PCR randomisation of the 3rd generation, suggesting the template variants have already reached the maximum mutational tolerance for the promiscuous activity and that further active mutants are not easily accessible. We confirmed that the loss of promiscuous activity in the 3rd round of randomisation was not a rare "founder effect": the initial founders (templates of the 2nd round) used for the randomisation reproducibly gave survivors for promiscuous activity at similar frequencies to controls from the 1st round of randomisation. These control experiments suggested that the template variants did not have drastic mutations. However, using the templates of the 3rd round in two independent experiments led to lineage extinction (or near-extinction). This conclusion in our evolutionary experiments supports the notion that the promiscuous activity is more sensitive, as it approaches the apparent maximum limit of mutational burden more easily than the native activity.

Promiscuous activity is more easily lost than native activity.

In order to check the robustness of the native activity over promiscuous activity, we cross-validated the survivors selected for one enzyme activity for the other enzyme activity and found that native activity was *always* maintained for the variants selected for promiscuous activity, *i.e.* 100% survivors of promiscuous activity maintain the native activity, whereas for native activity about 17% of the survivors under the strong promoter lost promiscuous activity (Figure 14); maintenance of the promiscuous activity under the weak promoter was extremely rare (only one variant, M4B3, as discussed above).

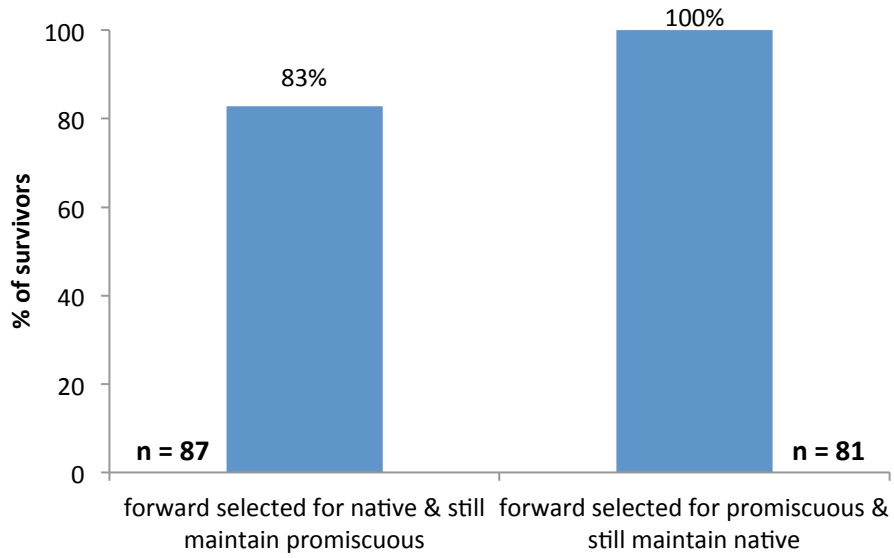

Figure 14. Cross validation of survivors of native activity for promiscuous activity and vice versa.

Promiscuous activity survivors always maintaining the native activity and native activity survivors often losing the promiscuous activity, supports the notion that the native activity is more robust to random mutagenesis than the promiscuous activity (Table 4).

	Variant	Genotype
1	10.A2	F114S , A208T & R297H
2	8+10.A9	W219R & A228V
3	3A5.A3	D44Y, G46V, F51L, A98V, T166S, L199P, E313D & A375V
4	3A5.A4	D44Y, G46V, F51L, A98V, T166S, L199P, S242T & E313D
5	3A8.A3	A98V, V142I, T166S, A228V, T320M & N344Y
6	3A8.A7	P71L, A98V, T166S & A228V
7	3A5+3A8.A9	A98V, T166S, A228V & R364H
9	M3B4	D171N
10	M4A2	T320K
11	M5A4	P16T, G46S, R57H, A105T & V252M
12	M6A1	H198D & V204A

Table 4. Variants that lose promiscuous activity under strong promoter and maintain the native activity.

Native activity is more robust to the mutational burden

The mutational burden (total mutations or non-silent mutations or drastic mutations per gene) for ampicillin selection is similar under both strong and weak promoters, which is expected as there is no selection pressure for the enzyme activity (Figures 14 and 15). When selected for native activity the mutational burden appears to be lower compared to no selection pressure. Moreover, within variants selection for native activity, those expressed under the strong promoter appear to tolerate more drastic mutations than the variants under the weak promoter (Figure 15). These trends suggest that the over-expression of protein can compensate for the mutational burden to some extent and, conversely, that lower expression lowers the tolerance to mutational burden.

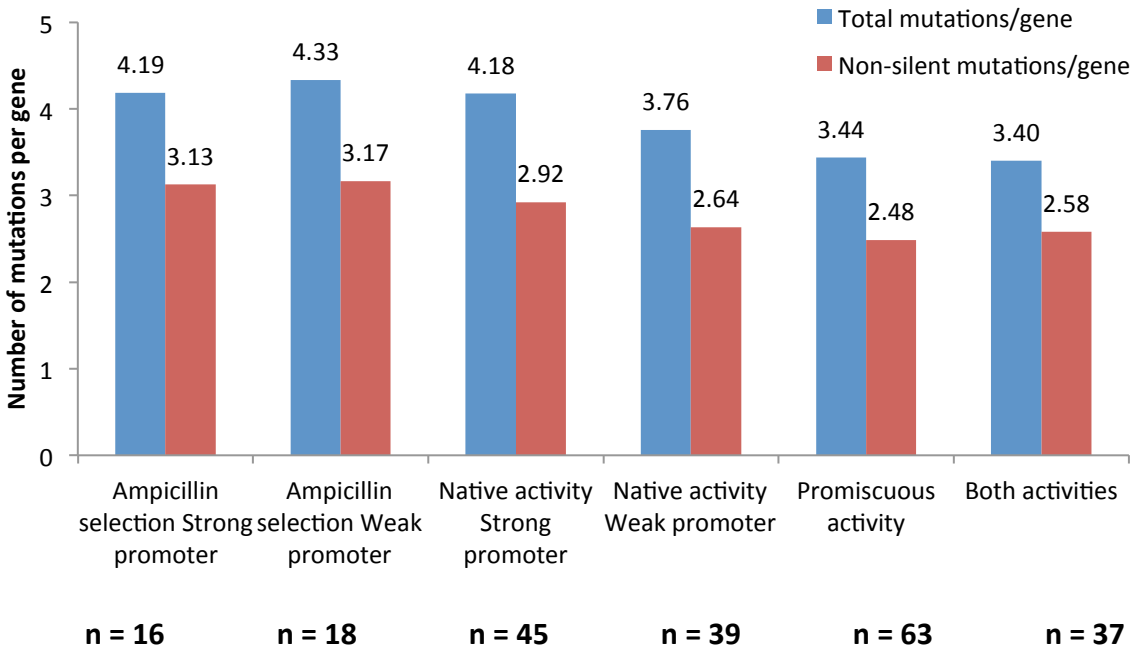

Figure 15. Mutational burden (number of mutations/gene) when selected for ampicillin (no selection pressure for the enzyme activity), native activity, promiscuous activity and for the both activities at the same time.

Native activity appears to tolerate more mutations than the promiscuous activity, especially drastic mutations. Further, the native activity under the weak promoter appears to tolerate more drastic mutations than the promiscuous activity (Figure 16) supporting the notion that the native activity is more robust to mutational burden compared either to promiscuous activity or to selection for both activities at the same time. Overall, the tolerance to mutational burden, particularly the drastic mutations, appears higher for native activity under strong promoter than weak promoter. The native activity under weak promoter has more tolerance to drastic mutations than promiscuous and than both activities at the same time, even though these were always found under strong promoter.

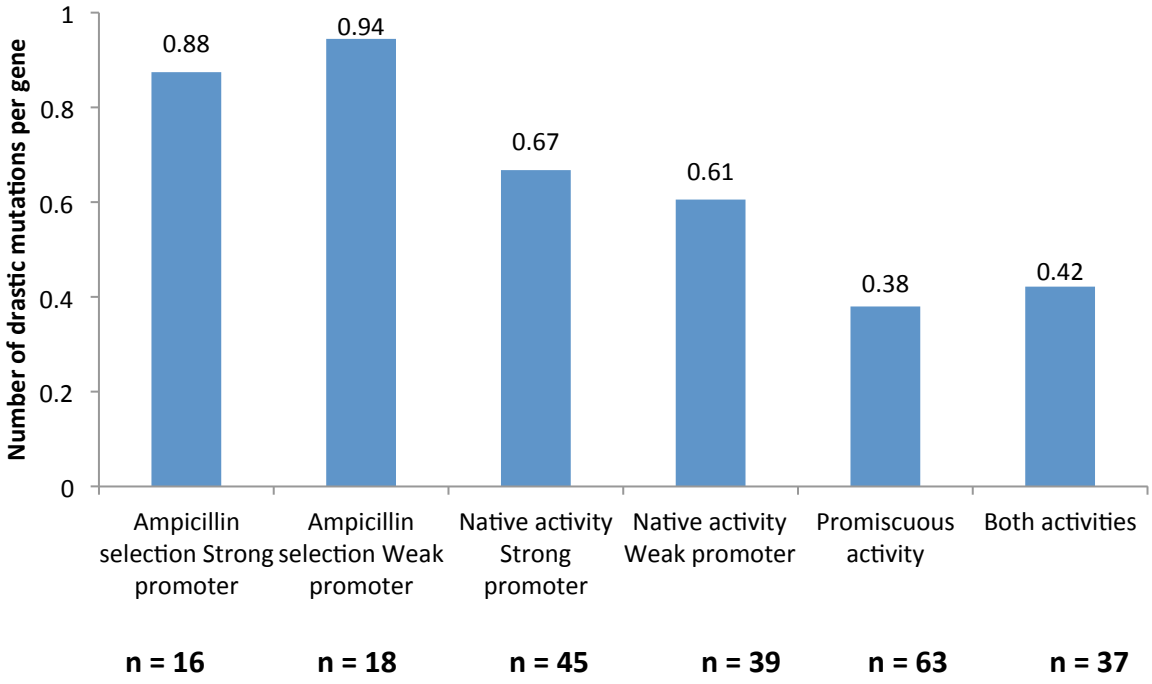

Figure 16. Tolerance to drastic mutations per gene when selected for ampicillin (no selection pressure for the enzyme activity), native activity, promiscuous activity and for the both activities at the same time. Drastic mutations were identified according to ClustalW.

Effect of lysine supplementation on mutational burden

Since we already found that the lysine supplementation allows the survival of more variants for native activity under the weak promoter compared to no lysine supplementation, we were curious to know if the lysine supplementation can also have an effect on the tolerance to mutational burden. Native activity under weak promoter without lysine supplementation appeared to tolerate both fewer non-silent and fewer drastic mutations compared to selectants with lysine (Figures 17 and 18). These data confirmed that lysine greatly influenced selection pressure, as determined by tolerance to mutational burden, which is consistent with our finding in having more survivors under weak promoter when supplemented with lysine.

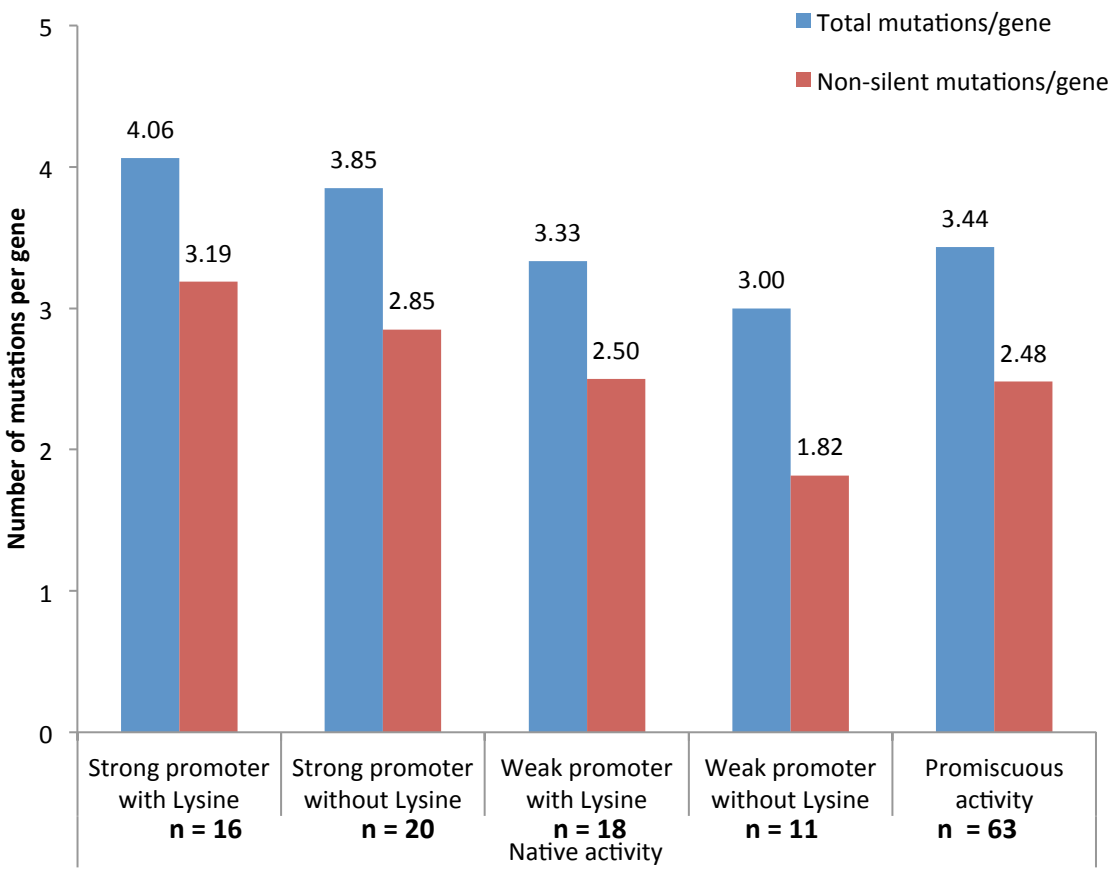

Figure 17. Mutational burden (number of mutations/gene) when selected for native activity with the effect of lysine supplementation on tolerance to mutational burden under the strong and weak promoters compared to the mutational burden of the promiscuous activity.

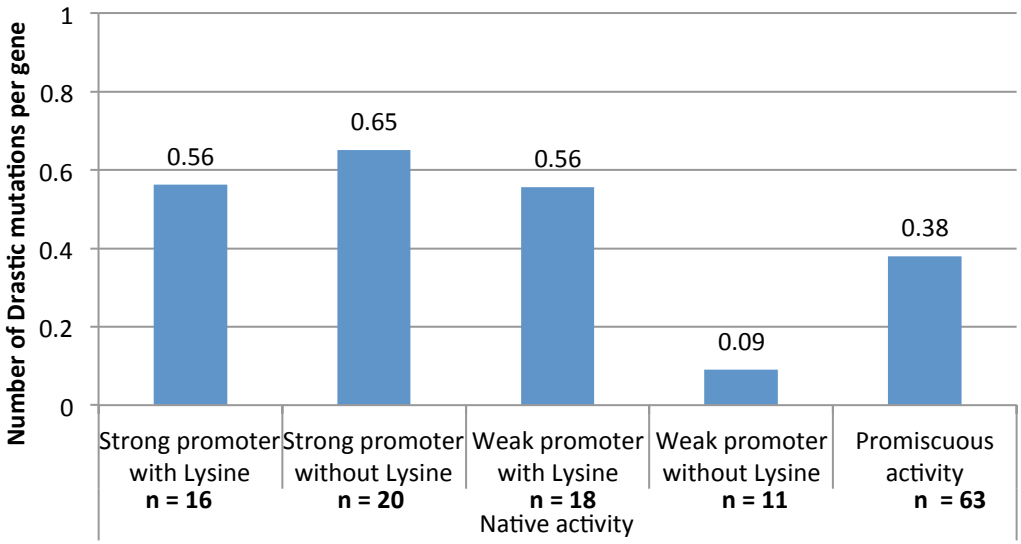

Figure 18. Tolerance to drastic mutations per gene when selected for native activity and the effect of lysine supplementation on mutational burden under the strong and weak promoters, compared to the mutational burden of the promiscuous activity. Drastic mutations were identified according to the ClustalW.

Role of metal availability on the native or promiscuous activities

Initially, the effect of metal supplementation was tested on the wild type DapE and found that Mn supplementation helps the growth under promiscuous activity selection as well as native activity selection compared to no metal supplementation, whereas the Zn supplementation does not affect the growth under native activity but certainly inhibits the growth under promiscuous activity selection (Figure 19). These data suggest that the native activity is robust to changes in metal availability and the promiscuous activity is sensitive, especially to Zn supplementation.

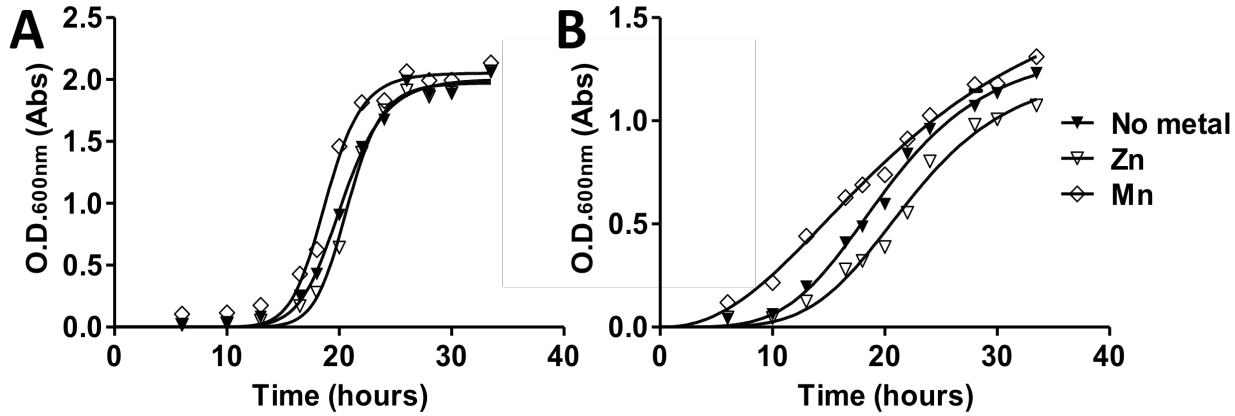

Figure 19. Growth curves of bacterial cultures selected for native desuccinylase activity (A) or promiscuous dipeptidase activity (B) with and without metal supplementation in minimal medium. Note that Mn^{2+} accelerates growth of the culture appreciably when selecting both for native and promiscuous activity. Plotted are representative curves from two independent experiments.

We also explored if metal supplementation had any effect on DapE variants under selection, by semi-quantitatively determining the size of the colonies in a blind-assessment.

We found that Mn supplementation allowed better growth of variants, compared to Zn or no metal supplementation, only when selecting for promiscuous activity, whereas the metal supplementation (Zn or Mn) did not provide any advantage for the growth under native activity selection (Figure 20).

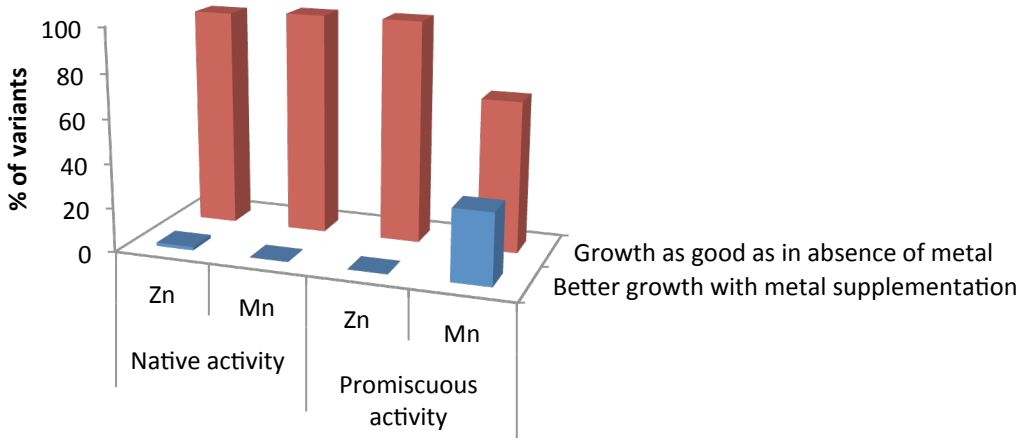

Figure 20. Growth of the DapE variants when selected for native and promiscuous activity with Zn and Mn supplementation. Number of variants observed in total: 134.

Role of metal availability at various DapE expression levels

For the wild type DapE and the other variants tested (Table 5) when selected for promiscuous activity, Mn supplementation helped the growth and Zn supplementation inhibited the growth under both promoters, particularly seen under the weak promoter (Figure 21B, colonies 2, 3, 5, & 8) compared to strong promoter (Figure 21B, colonies 1, 4, 6 & 7). In Figure 21B Colonies 9 and 11 were under strong promoter and did not show promiscuous activity even with metal supplementation (Zn or Mn) as these variants have already lost promiscuous activity during the three rounds evolution for native activity, as described above; it is obvious that their counterparts under the weak promoter (Figure 21B, colony 10 & 12) do not survive for promiscuous activity. Note that, in this experiment there is no IPTG supplementation and hence the wild type and the other variants can survive under the weak promoter, especially with Mn supplementation. In this experiment, we observed little difference in growth for the variant M4B3 (Figure 21B, colonies 3, 5 & 8) selected for promiscuous selection under weak promoter, compared to the wild type (Figure 21B, colony 2). However, this variant may have kinetically improved promiscuous activity compared to wild type, as M4B3 was *originally* selected under weak promoter with IPTG, which is the most stringent selection for promiscuous activity at which wild type cannot survive. For native activity, metal supplementation does not make any difference in the growth compared to no metal supplementation (Figure 21A), suggesting that the native activity is very robust to changes in

metal availability. Conversely, it appears than Mn bioavailability is limiting for survival of many variants only under promiscuous selection.

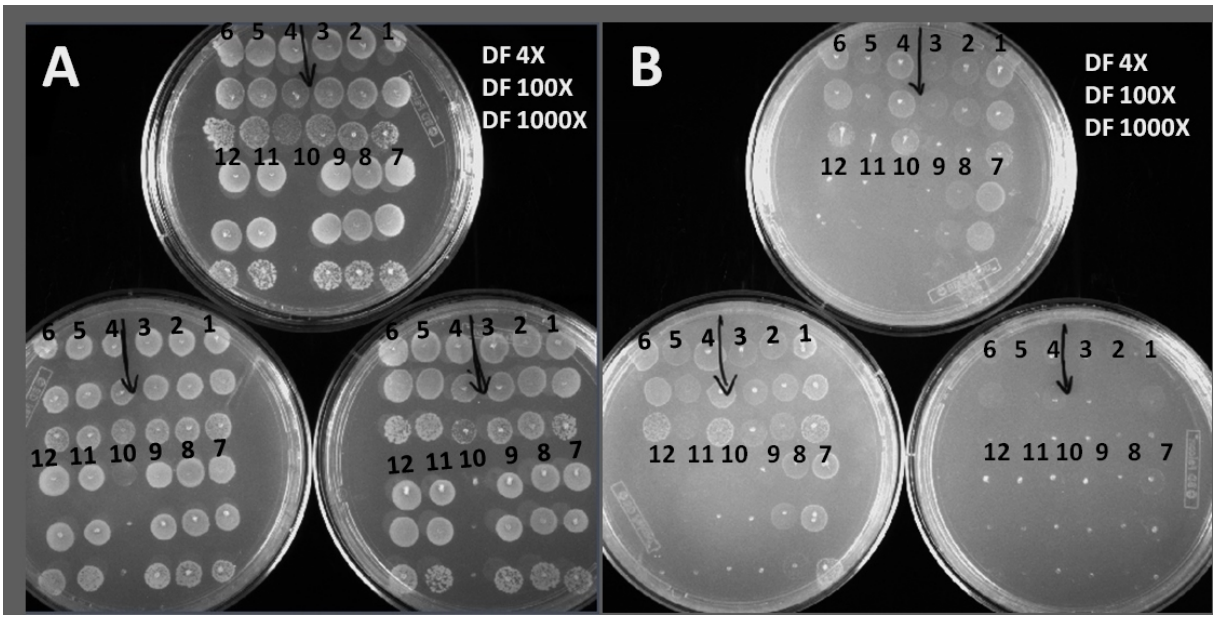

Figure 21. Growth of the wild type and variants of DapE selected for native activity (A) and selected for promiscuous activity (B) on minimal medium without IPTG. For each enzyme activity selection, the top plate is without any metal supplementation, left plate with 1mM Mn and right plate with 1mM Zn supplementation. Each plate has 12 variants, numbered 1 to 12, including the wild type, and each variant is at three dilution factors (DF), 4X, 100X and 1000X. Images were taken after 65 hours at 37°C. See Table 5 for a description of variants used here.

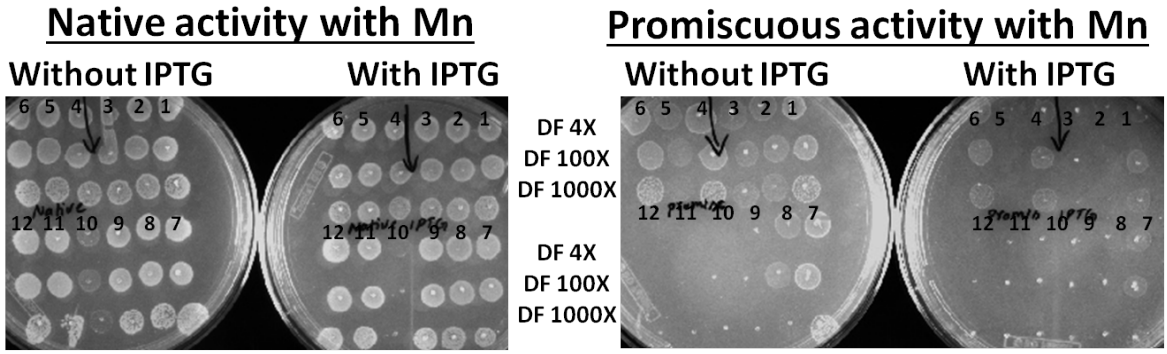

Figure 22. Effect of IPTG on the weak promoter. Growth of the wild type and variants of DapE in presence of Mn, selected for native activity (left) and selected for promiscuous activity (right) on minimal medium. Each plate has 12 variants, numbered 1 to 12, including the wild type and each variant is at three dilution factors (DF), 4X, 100X and 1000X. Images were taken after 65 hours at 37°C. See the table 4, for the labelling of the colonies.

Using on phenotypic validation of variants on solid media, we confirmed that IPTG induction under strong promoter (both for native and promiscuous activities) appears to have some inhibition on the growth, suggesting that the over expression of the DapE protein is may have high metabolic burden / toxic effect on the bacteria. Secondly, it is very evident that the IPTG induction under weak promoter always (including wild type) inhibits promiscuous activity (Figure 22, colonies 2, 3, 5, 8 & 10) and also the native activity of few variants, (Figure 22, colony 10), but never wild type.

Finally, we identified a few variants that lost native activity under the weak promoter, whilst maintaining activity in the forward, such as 3A1 (Figure 21A, colony 10). Loss of native activity under the weak promoter more pronounced with IPTG stringent selection condition (Figure 22, native activity with IPTG, colony 10), although it maintains native activity under the strong promoter (Figure 21A, colony 9). Besides losing native activity under weak promoter, 3A1 also loses promiscuous activity under the strong promoter (Figure 21B, colony 9), suggesting that the mutations of this variant are decreasing overall enzyme activity, perhaps due to instability.

Colony No.	**Variant name**	**Clone number**	**Promoter**
1	Wild type DapE	1	Strong
2		10.4	Weak
3	M4B3	M4B3	Weak
4		2.3	Strong
5		2.9	Weak
6		2.12	Strong
7		2.13	Strong
8		2.16	Weak
9	3A1	3A1	Strong
10		3.15	Weak
11	3A5	3A5	Strong
12		4.7	Weak

Table 5. List of the DapE wild type and variants selected for native and promiscuous activities with and without metal supplementation.

From figure 13, there were 17% of native activity variants that lost promiscuous activity even under the strong promoter, such as 3A5 (Figure 21B, colony 11) whilst maintaining native activity both under strong and weak promoters (Figure 21A, colony 11 & 12 respectively). Since 3A5 lost only promiscuous activity under strong promoter but maintained native activity under both promoters, it is most likely that the mutations of this variant are preferentially losing the promiscuous over native activity due to intrinsic mutational effects on DapE affecting catalysis.

2nd metal, Mn, is crucial for the promiscuous activity.

We found that mono-Zn form (Zn_1/--) and di-Zn form (Zn_1/Zn_2) of DapE can have only native activity and cannot perform promiscuous activity *in vitro*. Promiscuous activity can only occur in a mixed metal form (Zn_1/Mn_2) of the enzyme (Table 6), as previously reported.[9]

DapE metalloisoform	Native activity	Promiscuous activity
	k_{cat} (s^{-1})	k_{cat} (s^{-1})
Zn_1/--	300	0
Zn_1/Zn_2	700	0
Zn_1/Mn_2	1100	500

Table 6. k_{cat} values of native desuccinylase and promiscuous dipeptidase activity of different metalloforms of DapE.

Randomisation by site-directed mutagenesis around 2nd metal suggests promiscuous activity is especially sensitive to mutagenesis.

A library generated by the site specific random mutagenesis at the 2nd metal coordination residue H348 and two other residues in the close proximity of 2nd metal, that can potentially influence the metal coordination are N344 and I347, was screened for the native and promiscuous activity with the metal supplementation, either Zn or Mn. To simplify analysis, this site specific mutagenesis library was generated to have the DapE only under the strong promoter and therefore no effect of weak promoter. When selected for native activity the number of survivors was in the same order of magnitude for Zn and Mn supplementation, 360 & 430 respectively (Figure 23). These results are consistent with the notion that the 2nd metal

can either be Zn or Mn for native activity also *in vivo* and that native activity is robust for such changes in the metal. Congruently, *in vitro* (Table 5) the 2nd metal was not crucial for the native activity as the native activity can also occur with mono-Zn form of the enzyme, albeit less efficiently than the di-Zn form form of the enzyme. In marked contrast, when selected for promiscuous activity, Zn supplementation totally hindered survival of any mutants in the library in this experiment.

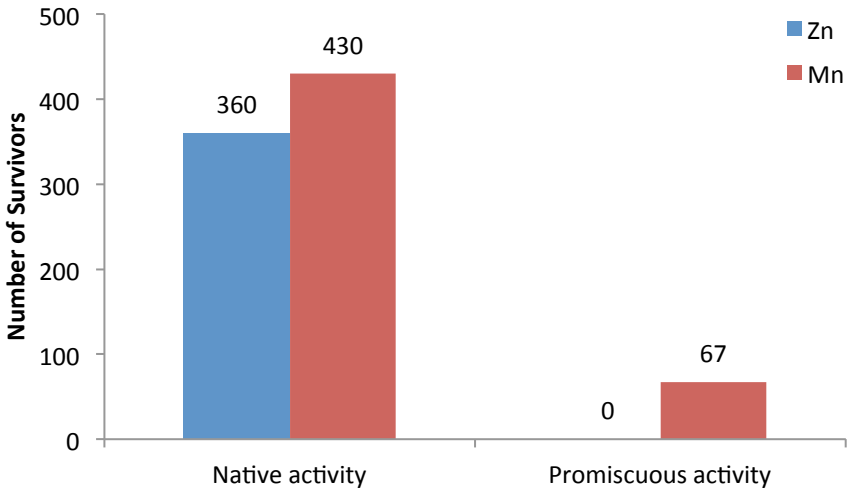

Figure 23. Number of survivors from the site specific randomisation of 2nd metal coordinating residue H348 and other nearby residues, N344 & I347, selected for native activity and promiscuous activity either with Zn or Mn supplementation in the minimal medium.

The library under selection for promiscuous activity had much fewer survivors even with Mn supplementation compared to native activity with Mn supplementation (67 vs 430). i.e. even with Mn supplementation, native activity is more robust to mutations than promiscuous activity, in this case by more than 6-fold. Native activity was largely unaffected by presence of Zn or Mn, determined by the similar number of selectants with either metal in this focused library, as found also for random mutant libraries. We conclude that the promiscuous activity is much more sensitive to metal supplementation and is "poisoned" by excess Zn, confirming that the native activity is more robust to fluctuations in metal content. Moreover, this preliminary finding of apparent extreme sensitivity to mutations near the Mn-binding site, which is particularly acute for the promiscuous activity, supports the notion that it is a crucial "catalytic hotspot" for the dipeptidase activity of the enzyme. A larger sampling of focused libraries may be required to assess whether it is much easier to identify mutations selectively affecting the promiscuous activity compared to the native, than the reverse.

Robustness of native activity to inhibition.

Our previous results of DapE activity *in vitro* suggest that it is relatively easy to inhibit the promiscuous activity with competitive inhibitors designed to bind selectively to Mn, whereas the native activity with the same metallo-isoform is unaffected (see Chapter 6). For example, Dab-DAP inhibits the promiscuous activity, but not native activity in any metalloisoform, suggesting that the native substrate mode of binding is more robust to competition by inhibitors. Moreover, although Zn-selective inhibitors can be identified *in vitro*, such as L-captopril (see Chapter 3), the function of the desuccinylase activity is unlikely to be affected, because the native activity is also catalysed by the Mn-isoform of DapE. Overall, these kinetic studies support that the native activity may be intrinsically or kinetically more robust, being a product of evolution.

Our mechanistic model of substrate interactions with DapE suggests that the promiscuous activity mostly relies on the bidentate binding of the dipeptide to Mn (Figure 24), whereas the native substrate distributes its binding energy to the enzyme more widely. We postulate that such a localised and specific interaction of the promiscuous substrate with DapE contributes to the apparent fragility of the reaction to genetic disruption or inhibition, due to lack of redundant interactions. In conclusion, besides systemic effects *in vivo*, such as metal availability, requirement for products and protein expression levels, that appear to make the native activity much more robust than the promiscuous activity, it is likely that the native activity has an intrinsic kinetic robustness compared to the promiscuous activity that may be the product of evolution.

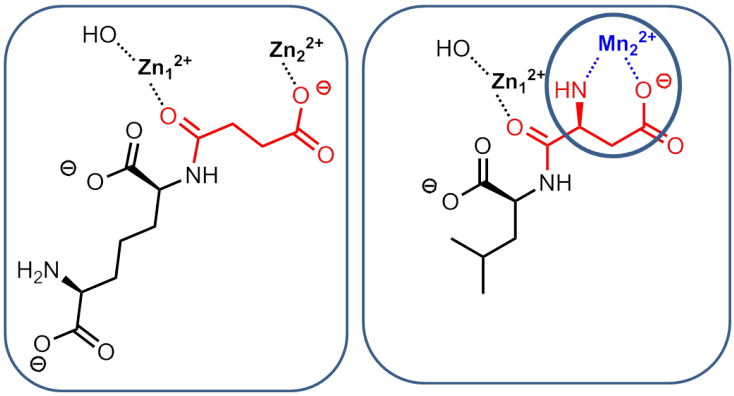

Figure 24. Hypothesis for the mode of substrate binding and metal coordination for native and promiscuous activities (see Chapter 6).

Interesting variants identified.

Variant 3A1 (G179S, L199P & D264N), loses native activity under weak promoter and promiscuous activity under strong promoter but maintains native activity under strong promoter, suggesting that the mutations of this variant are making the enzyme less stable than the wild type. All the three residues, G179S, L199P & D264N are found in the dimerization domain, G179S is in the close proximity of R177 which can have a potential interaction with the substrate and also G179 is very much conserved in DapE homologs, suggesting that it may have a role in loss of native activity, (Figure 24).

We found that variant 3A5 (D44Y, G46V, F51L, A98V, T166S, L199P & E313D) lost the promiscuous activity even under the strong promoter, whilst maintaining the native activity both under strong and weak promoters, suggesting that the native activity has an intrinsic robustness such that it can withstand the mutational burden that is responsible for the total loss of promiscuous activity *in vivo*; however, the variant also withstands lower expression under selection for native activity. Consequently, it is most likely that mutations of this variant cause intrinsic loss of promiscuous over native activity. The mutations at positions D44Y, G46V and F51L are in the vicinity of the metal (Zn_1) binding residue, H66. The mutation, A98V is adjacent to the bridging aspartate, D99, of the two metal ions (Zn_1 & Zn_2) and it is very well conserved in DapE-homologs. Residues T166S, L199P & E313D are in the dimerisation domain (Figure 25).

Variant M4B3 (V233L, A307T & R332H), gains promiscuous activity under weak promoter and maintains promiscuous activity under strong promoter and also native activity under both promoters. Variant M4B3 appeared to gain promiscuous activity under the weak promoter in very stringent selection with IPTG, unlike wild type. Residue V233L is in the dimerisation domain and the residues A307T & R332H are in the catalytic domain; both are moderately conserved residues in DapE homologs; in addition R332 is in the positioned close to the substrate binding residue 334 (Figure 25).

In summary all these variants that lose promiscuous activity under strong promoter, (Figure 25), apparently have more severe mutations, such as A98V, which is adjacent to the metal bridging residue D99 and mutations (P71L, A105T, D171N, V252M & T320M/K) that are close to the substrate binding residues. A most interesting mutation is N344Y, which is near the Mn-binding site. Besides the localization of these mutations around the catalytic site, it was also noted that these residues are in general well conserved in DapE homologs. It will be interested to explore whether these variants have improved/reduced native activity and/or promiscuous activity compared to wild type.

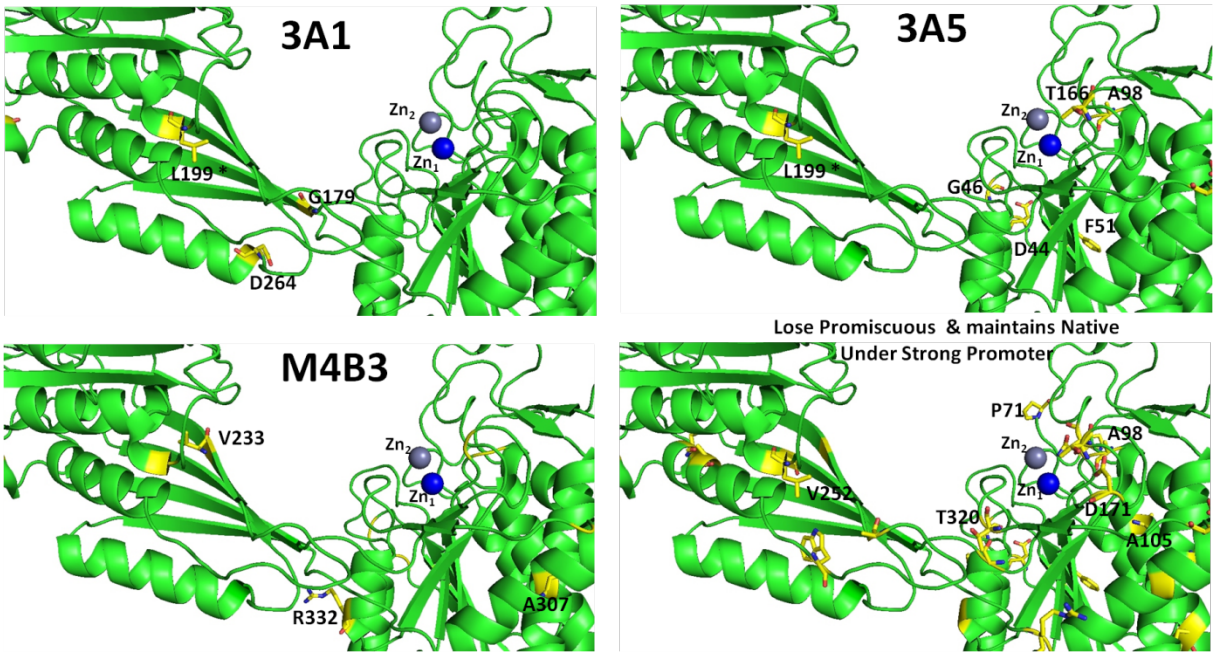

Figure 25. Structure of the *S.enterica* DapE, homology model obtained from Swiss Protein Model.[14] Upper; left is variant-3A1 and right is variant-3A5. Bottom; left is variant-M4B3 and right is representative of different variants. Residues are showed in sticks, colouring of atoms; Carbon (yellow), Hydrogen (gray), Nitrogen (blue), Oxygen (red) & Sulfur (green). Zn_1 (Blue) & Zn_2 (Light blue), numbers 1 & 2 represents the position of the metal ion and the 2nd metal should be Mn for promiscuous activity. (*) residue from the opposite monomer. The images were created by using Pymol.

Discussion

The randomized libraries of wild type DapE always have at least 3 times more survivors for the native activity compared to the promiscuous activity. We found that the lysine supplementation does not influence having more survivors for native activity over the promiscuous activity; however the lysine supplementation allowed increased survival under the weak promoter compared to no lysine supplementation. Our findings suggest that protein expression level can play a crucial role in the tolerance to mutational burden. If the templates used for the randomization were already pre-selected for one or the other activity, such pre-selection did not appear to give any advantage for having more survivors for that particular activity. Presumably, evolution of specialization and potentially increased robustness for a particular activity under selection requires larger libraries and more rounds of selection than explored here.

We conclude that the native desuccinylase activity of DapE is more robust than promiscuous dipeptidase activity, as determined by more survivors after random mutagenesis and selection; tolerance to lower protein expression levels; possibility of surviving a higher mutational burden and relative independence to metal availability. Consequently, the robustness of DapE native activity is largely a systemic property, dependent on a physiological environment that has been presumably highly-optimised by evolution.

Our kinetic and mutational analyses also suggest that the DapE enzyme itself may be intrinsically more robust for the native activity, as assessed by a slightly higher k_{cat} for the native substrate; by multiple isoforms maintaining native function *in vitro*; by the resistance of native function to competitive inhibition and the apparent inability to identify mutations that preferentially lose the native activity over the promiscuous activity. Besides all the systemic effects, the native activity thus appears also to exhibit intrinsic kinetic robustness compared to promiscuous activity. Importantly two independent sites (A_{98} and N_{344} I_{347} H_{348}), around the active site, preferentially disrupted the promiscuous activity but not the native activity. Conversely no mutations were identified that disrupted the native activity preferentially. The kinetic characterization of the variants selected for native activity and for the promiscuous activity and especially the unbiased variants (*i.e*. variants selected just for ampicillin) that have one or the other activity may provide more insights about the intrinsic

robustness of the native or promiscuous activity. Exploration of kinetic parameters will be crucial to ensure that the increased or decreased kinetic efficiency of the enzyme activity is not just due to expression or changes in stability of the variant protein compared to the wild type (ΔΔG), *i.e.* improved catalysis may compensate for lowered stability or vise versa.

Based on our conclusion that the native activity is more robust than the promiscuous activity, we postulate that the DapE native activity might have a much higher upper limit (excess fitness) compared to promiscuous activity. In this scenario, most or all the variants selected for native activity may have reduced fitness, but still be further from the minimum threshold / death line of the enzyme activity. In contrast, the fitness of the promiscuous enzyme may be much closer to the limits of disruption, i.e. the "death line" and hence almost any perturbations will affect survival (Figure 26). We propose that the native activity has "excess fitness" that effectively buffers the phenotypic effect of random disruption. In contrast, the promiscuous activity does not have such a buffering zone and is effectively working close to the survival threshold. The evolutionary consequence of the lack of tolerance to mutations is that the enzyme selected promiscuous activity is more likely to become extinct, *i.e.* a promiscuous function has a "fragile birth".

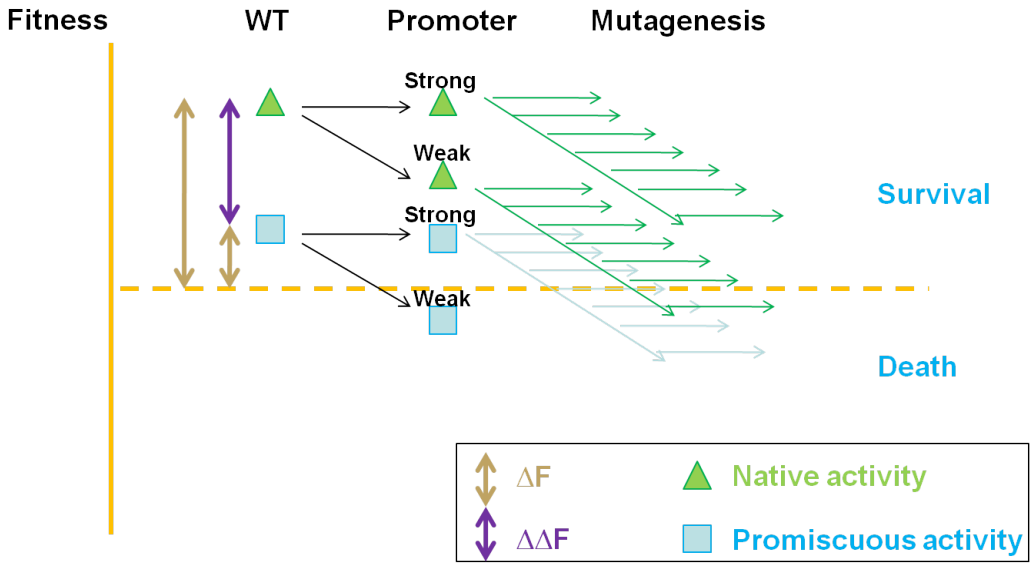

Figure 25. Model showing the relative fitness of native activity over promiscuous activity. ΔF is fitness and ΔΔF is relative fitness.

When selected for promiscuous activity as well as for "both activities" at the same time, one could expect that the native activity would lowered more than the promiscuous activity; however our results clearly indicated that the native activity is always maintained for all the variants selected for promiscuous activity, thereby suggesting that the native activity has more functional robustness compared to the promiscuous activity.

Outlook

Broader perspectives of planned research

Molecular insights underpinning robustness are of fundamental importance for the advancement of science, but also useful for practical applications such as the design of pharmaceuticals and in synthetic biology.[15] It is envisaged that the understanding of robustness at the molecular level will allow either the direct engineering of robustness in enzymes and networks, or alternatively allow to implement Darwinian protocols for the evolution of robustness from more primitive designs, in what may be called "systems-biology directed evolution".

Moreover, since robustness is a feature of many fields of human knowledge, including engineering, social sciences (e.g. economy) and information sciences,[16] such insights have potential to be transferable beyond chemistry and biology.

In summary, our neutral evolution experiments in the laboratory suggest that the native activity of DapE is more robust to random mutagenesis than its promiscuous activity. Moreover, our results support the notion that robustness is a systemic property, dependent on varied factors like metal availability and level of gene expression, as well as hinting at intrinsic genetic factors of enzyme mechanisms that contribute to robust catalysis. In conclusion, emerging promiscuous enzyme functions rely greatly upon its environment for its survival when taking their first tentative steps in evolution and toward increased robustness.

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CHAPTER 8

Outlook

1. Our findings of the mechanism of native and promiscuous enzyme activities of DapE are illustrative enough for understanding the reaction mechanism. However a crystal structure of DapE of *S.enterica* with an inhibitor bound in the active to one of the metalloforms, especially for the Zn/Mn form of the enzyme may provide an additional and visual evidence for the bi-dentate binding of the promiscuous substrate.

2. DapE is certainly a unique target for antibiotics as it is not found in humans. Much structural and functional information is known about DapE, especially compared to other enzymes of the lysine biosynthetic pathway. Lack of mechanistic insights of DapE has been a serious drawback for the design of potential inhibitors for DapE.[1] Our findings may provide impactful insights for the design of new classes of inhibitors targeting DapE.

3. A surprising and little-understood finding in this thesis is that strong-binding and selective binders of the Mn isoform of DapE, which inhibit the promiscuous activity, fail to inhibit the native activity. Such functional-selective inhibition has potentially wide implications for control of metabolism and evolution, because the phenomenon adds a whole additional layer of complexity in enzyme regulation. It will be interesting to establish the role of endogenous inhibitors for regulation of enzyme activities in physiological settings. Moreover, detailed molecular insights on how the native substrate manages to outcompete a bound inhibitor (which the promiscuous substrate fails to do) may be of fundamental importance to understand enzyme mechanisms and selectivity.

4. Apparently subtle differences in metal-coordination can lead to huge differences in catalytic outcome. For bacteria under selection for DapE activity, such small differences may be the difference between life and death. Considering that approximately one-third of structurally characterized proteins and approximately half of all proteins are thought to be metalloproteins,[2-6] there is an urgent need to get

increased detailed understanding of kinetic properties of metals and of metal-ligand exchange during catalysis. Such atomic and molecular detail is at the forefront of enzyme chemistry and will require an interdisciplinary approach, including not just X-ray crystallography (which has important limitations) but enzymology and computational chemistry.

5. Kinetic characterization of the various mutants of the neutrally evolved DapE library would provide crucial data that can be used for the quantification of the functional robustness of the native and promiscuous activities. Apart from kinetic characterization, it may be possible to explore the ΔΔG of all these variants compared to the wild type using the bioinformatics tools (e.g. FOLDX), to correlate kinetic data *in vivo* with the thermodynamic data, ΔΔG.

    a. For example, we found one variant, M4B3, that could survive the stringent selection under weak promoter, it could be interesting to see the kinetic properties of this variant compared to the wild type; our prediction is that this neutrally-evolved variant may have kinetically increased promiscuous activity compared to the wild type. It may be of interest also to follow the process of specialisation toward the dipeptidase activity of this variant, to explore whether it correlates with increasing functional robustness for the evolving activity (and loss of functional robustness for the desuccinylase activity).

    b. Another example is variant 3A5, which loses only promiscuous activity and maintains the native activity even under weak promoter; the kinetic properties of this variant may provide evidence if the robustness of the native activity can be evolved further or not.

6. We found that Mn supplementation can provide an additional advantage for the promiscuous enzyme activity, which can be implemented for the evolution of promiscuous activity under Mn supplemented selection medium, especially under the weak promoter. A similar strategy could be implemented for selection of other metal-activities, for example for the reported weak dipeptidase activity of DapE with Cd.[7] The laboratory evolution for Cd-dependent hydrolase activity could reproduce natural evolution of a Cd-dependent carbonic anhydrase in marine diatoms.[8]

7. Since we conclude that the native activity is more robust than the promiscuous activity, does it mean that the native activity has already reached the limit of evolvability or can it be evolved further? A way forward to find the further evolvability of the native activity is to use the stringent selection conditions for the native activity, *i.e.* under weak promoter with IPTG and/or in presence of a competitive inhibitor. If the native activity is evolvable further, it suggests that evolution may not reach the maximum optimisation as it may not require such extreme efficiency for survival or may even be a counterproductive strategy for evolvability.

8. The finding of robustness of native activity in case of DapE may not apply for all other promiscuous enzymes universally. In order to generalise this concept, it is important to explore robustness of native and promiscuous activity in other enzymes. Towards this direction, we have started working on another structurally similar enzyme, Sapep of *S.aureus*, which has a promiscuous beta-lactamase activity.[9,10]

9. Another fundamental question that arises from our findings of robustness of native activity as a systemic property is whether such systemic robustness is evolvable. We have already initiated this project by evolving the bacterial strain under the selection pressure for native activity, promiscuous activity and for both activities at the same time, together with a control without any selection pressure for DapE activity. However, these bacterial strains need to be further validated for their systemic evolution.

10. In 1967, Ohno proposed that gene duplication is an evolutionary approach that nature adapted for the creation of new enzyme activities.[11] However, little effort effort has been made since then to prospectively test such a hypothesis in laboratory evolution experiments. In our studies we have identified some variants that show gene duplications and these variants could be a potential resource for evolving one activity or the other activity, in presence of gene duplicates.

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