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Restriction and Modification in *Bacillus subtilis*: Two DNA Methyltransferases with *Bsu*RI Specificity

II. CATALYTIC PROPERTIES, SUBSTRATE SPECIFICITY, AND MODE OF ACTION*

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The properties of two DNA methyltransferases, termed M. *Bsu*RIa and M. *Bsu*RIb, whose isolation was described in the preceding paper (Günthert, U., Freund, M., and Trautner, T. A. (1981) *J. Biol. Chem.* 256, 9340-9345) were compared. Both enzymes recognize the same target sequence in double-stranded DNA, leading to methylation of the internal cytosine: 5'GG$\overset{*}{C}$C. The enzymes have identical reaction constants with their substrates, DNA (k_m = 2.7 nM for the 5' GGCC sequence), and *S*-adenosyl-L-methionine (k_m = 0.7 μM). Initial rates of methyl group transfer were proportional to enzyme concentration over a range of 50-fold, indicating absence of aggregation. The enzymes are different in their ionic strength requirements using Tris-HCl, pH 8.4. M. *Bsu*RIa is most active at 100 mM, M. *Bsu*RIb at 440 mM. As measured by incorporation kinetics and heat inactivation, M. *Bsu*RIa is the more stable enzyme of the two.

Equilibrium dialysis was used to study the mode of methyl group transfer to the DNA with either enzyme. The data indicate that initially *S*-adenosyl-L-methionine binds to methyltransferase. This complex attaches to either modified or nonmodified DNA. The methyl group will then be transfered to a nonmodified target sequence, leading to the dissociation of enzyme and *S*-adenosyl-L-homocysteine from the DNA.

Bacillus subtilis strains OG3R and R both express two different DNA cytosine methyltransferases with identical sequence specificity, M. *Bsu*RIa[1] and M. *Bsu*RIb. The preceding paper (Günthert *et al.*, 1981) describes the purification of the two enzymes. Following the purification, it became important to analyze the two *Bsu*RI specific methyltransferases with regard to their physicochemical properties. Such comparative studies would serve to obtain information on the relationship of the two enzymes. Furthermore, we were interested in characterizing the sequence of reaction steps involved when the M. *Bsu*RI enzymes transfer methyl groups from the methyl group donor AdoMet to the unmodified or semi-modified recognition sequence of its DNA substrate.

It is remarkable that relatively few investigations have been performed concerning the reaction mechanism of the type II modification enzymes in comparison to analogous studies with restriction enzymes. Until now only the *Eco*RI methyltransferase has been investigated in detail with respect to its reaction mechanism (Rubin and Modrich, 1977). We report here the physicochemical properties of the purified enzymes and their reaction mechanisms with the 5'GGCC target sequence.

EXPERIMENTAL PROCEDURES

Most of the materials and methods used here are described in the preceding communication (Günthert *et al.*, 1981), except for the following.

Materials

DNA—Chromosomal bacterial DNAs were isolated as described by Bron *et al.*, 1975. Phage DNA was prepared from purified phage stocks as described by Trautner *et al.*, 1974. Plasmid pC194 DNA was isolated as described by Gryczan *et al.*, 1978.

Enzymes—Methyltransferases M. *Bsu*RIa and M. *Bsu*RIb used in most of the experiments were the purified fractions X, unless otherwise stated. Fractions Xa (0.04 mg/ml) and Xb (0.08 mg/ml) have been diluted appropriately with storage buffer (100 mM Tris-HCl, pH 8.4, 1 mM dithioerythritol, 0.005% Brij 58, 50% glycerol). Restriction enzyme R. *Bsu*RI was the DNA affinity chromatography fraction, as described by Bron and Hörz (1980). All other restriction enzymes were from Boehringer Mannheim or BioLabs and used as indicated by the manufacturers.

Source of Materials—Nonradioactively labeled AdoMet and AdoHcy were obtained from Boehringer, Ado[carboxy-^{14}C]Met (specific activity 53 mCi/mmol) was from Amersham. The following substances were purchased from Serva: N^6-methyladenine, 5-methylcytosine, *N*-ethylmaleimide, and yeast RNA. *Micrococcus lysodeikticus* DNA was from Sigma. ATP and adenine were obtained from Boehringer. Cytosine, guanine, and thymine were from Merck.

Methods

Equilibrium Dialysis—The method was performed in general as described by Teraoka and Nierhaus (1979). The two chambers of the dialysis apparatus were separated by a dialysis membrane (Spectrapor, Spectrum Medical, IN) with a cutoff of M_r = 6,000 to 8,000. 40 μl of reaction mixture or buffer, respectively, were placed into either of the two chambers. Dialysis was either at 4 or 37 °C. The reaction mixture contained 25 nCi of Ado[*Me*-^{3}H]Met (specific activity 15 Ci/mmol) or 2.5 nCi Ado[*carboxy*-^{14}C]Met, 5 μg of DNA (from calf thymus or SPP1.R) and methyltransferase in appropriate dilutions. After two days of dialysis, 30-μl aliquots of the two chambers were withdrawn and radioactivity was determined in Aquasol Scintillation liquid (New England Nuclear).

RESULTS

DNA Sequence Specificity of M. BsuRIa and M. BsuRIb

We have previously reported that cell-free extracts of *B.*

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[1] The abbreviations used are: M. *Bsu*RI, bacterial DNA modification enzyme of *B. subtilis* r^+m^+ strains; R. *Bsu*RI, bacterial DNA restriction enzyme of *B. subtilis* r^+m^+ strains; r^+m^+, *Bsu*RI restricting and modifying bacterial genotype; AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; SPP1.O, unmodified phage SPP1, grown on *B. subtilis* SB1207 (r^-m^-); SPP1.R, modified phage SPP1, grown on *B. subtilis* TB804 (r^+m^+).

TABLE I

Base analysis of in vitro methylated SPP1.0 DNA

SPP1.0 DNA was methylated *in vitro* using the standard methylation assay (Günthert *et al.*, 1981), hydrolyzed to bases with formic acid and analyzed by thin layer chromatography, as described by Günthert *et al.*, 1976.

Strain	Enzyme fraction	Total cpm incorporated	Percentage of label contained in					
			Ade	N^6-mAde[a]	Cyt	5-mCyt[a]	Gua	Thy
OG3R	Va	48,805	0.3	0.3	0.2	93.3	0.3	5.6
	Vb	73,377	0.1	0.2	0.2	94.7	0.1	4.6
R	Va	134,635	0.2	3.7[b]	0.6	87.4	0.6	7.1
	Vb	51,150	0.1	0.1	0.2	97.8	0.1	1.8
TB804	Vb	117,326	0.2	0.2	0.2	96.3	0.2	2.7

[a] N^6-mAde, N^6-methyladenine; 5-mCyt, 5-methylcytosine.

[b] In addition to the overall cytosine methylating activity, fraction Va from strain R showed some contaminating adenine methylating activity.

subtilis OG3R predominantly methylated the internal cytosine of the *Bsu*RI recognition sequence to produce 5′GG$\overset{*}{C}$C[2] (Günthert *et al.*, 1978). Also, both methyltransferases showed this specificity, thus denominated M. *Bsu*RIa and M. *Bsu*RIb. This was demonstrated with the following experiments. Base analysis of SPP1.O DNA, methylated *in vitro* by either enzyme, indicated methylation of cytosine (Table I). The only cytosine methylated in this reaction was the central cytosine of the sequence 5′GG$\overset{*}{C}$C, following direct sequence analysis[3] according to Günthert *et al.* (1978). Preincubation of SPP1.O DNA with M. *Bsu*RIa or M. *Bsu*RIb rendered the DNA resistant to subsequent digestion with restriction enzymes R. *Hae* III or R. *Bsu*RI (both specific for 5′GG↓CC), without affecting its sensitivity to R.*Eco*RI, R. *Hpa* II, or R. *Hin*dIII. When SPP1.O DNA was first degraded with R. *Hae* III or R. *Bsu*RI, its ability to become methylated was reduced by more than 99%, while R. *Hpa* II preincubation had only a marginal effect. After completely methylating SPP1.O DNA with either one of the *Bsu*RI methyltransferases, such DNA did not serve any more as a substrate for the other enzyme.

Influence of Reaction Conditions on Methyltransferase Activity

Substrate Dependence—Nucleic acids from different sources were tested for their substrate specificity with M. *Bsu*RIa and M. *Bsu*RIb. DNA from calf thymus, *M. lysodeikticus*, or the nonrestricting and nonmodifying *B. subtilis* strains SB1207 and TB101 were readily methylated by both enzymes. RNA from yeast did not accept methyl groups. Neither did the DNA from *B. subtilis* r^+m^+ strains, such as TB804, R, and OG3R. Also, the DNAs of the temperate *B. subtilis* phases SPβ and ϕ3T did not serve as substrates for the *Bsu*RI methyltransferases. These phages code for their own DNA methyltransferase with the same sequence specificity for 5′GGCC (Trautner *et al.*, 1980; Noyer-Weidner *et al.*, 1981).

Influence of Salt Concentration—The standard methyltransferase assay had been performed with increasing concentrations of Tris-HCl or KCl. M. *Bsu*RIa and M. *Bsu*RIb showed a remarkably different influence on the concentration of Tris-HCl, pH 8.4 (Fig. 1). While M. *Bsu*RIa had a distinct optimum at 80 to 100 mM Tris-HCl, M. *Bsu*RIb had a broad optimum at around 400 mM Tris-HCl. At a concentration of 50 mM Tris-HCl, pH 8.4, the two methyltransferases also revealed a different dependence on the concentration of KCl:

[2] Asterisk denotes methyl group.

[3] U. Günthert and S. Jentsch, unpublished experiments.

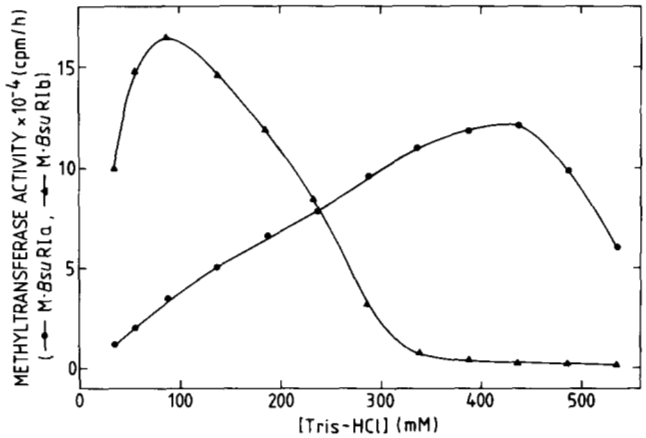

FIG. 1. **Effect on Tris-HCl (pH 8.4) concentration on the activity of M. *Bsu*RIa and M. *Bsu*RIb.** Methyltransferase activity was measured in the standard assay, except for variation of the Tris-HCl concentration as indicated, with 13 units of M. *Bsu*RIa and 10.5 units of M. *Bsu*RIb for each reaction condition.

M. OBsuRIa had an optimum at 90 to 100 mM KCl, while M. *Bsu*RIb had two optima, one at 50 mM and a second at 200 mM KCl.

Influence of pH—The pH optima for M. *Bsu*RIa and M. *Bsu*RIb were determined in the Tris-HCl buffer system using optimal salt concentrations. M. *Bsu*RIa revealed activity between pH 7.6 and pH 9.0 with an optimum at pH 8.4, while M. *Bsu*RIb was active between pH 7.3 and pH 8.7, with nearly the same optimum as M. *Bsu*RIa (pH 8.3), but with an additional shoulder at pH 7.7. The same values were obtained when these assays were performed at 270 mM Tris-HCl.

Influence of Temperature—The temperature optimum for the methylation reaction was tested by performing the standard assay at different temperatures. There was a gradual increase in activity for either enzyme from 17 °C to the activity peak between 40 and 43 °C. Beyond 44 °C the activity of both enzymes decreased very rapidly, with complete inactivation at 55 °C. At 37 °C, 86% activity of the optimal temperature was present. Of the two enzymes, M. *Bsu*RIa is the more stable one at 46 °C, as derived from temperature inactivation kinetics (Fig. 2). At this temperature the half-life of M. *Bsu*RIb is 3.5 min and of M. *Bsu*RIa is 10 min.

Influence of Various Assay Conditions—Data on the effects of variation of reaction conditions are summarized in Table II. They indicate differences in the requirements of the two enzymes. The activity of both methyltransferases is highly dependent on the presence of thiol-reducing agents, such as dithioerythritol. This is consistent with the finding that 0.4 mM *N*-ethylmaleimide can completely inhibit the methylation reaction. Omission of glycerol slightly reduces the activity of both enzymes. EDTA at 5 mM, as present in the standard assay buffer, was needed for full activity of M. *Bsu*RIa, but not for M. *Bsu*RIb. At a 10-fold higher concentration of EDTA, the activity of M. *Bsu*RIb was reduced, while M. *Bsu*RIa was activated. Addition of Mg^{2+} ions or ATP had no effect, as expected for type II modification enzymes.

Enzyme Kinetics

Influence of Enzyme Concentration—Both methyltransferases showed a linear dependence over 15 min of the initial rate of methyl group transfer on enzyme concentration (Fig. 3). This result, taken together with the finding reported above that native and denatured enzymes had the same molecular weight (Günthert *et al.*, 1981), indicates that the enzymes are active as monomers without cooperative action of subunits.

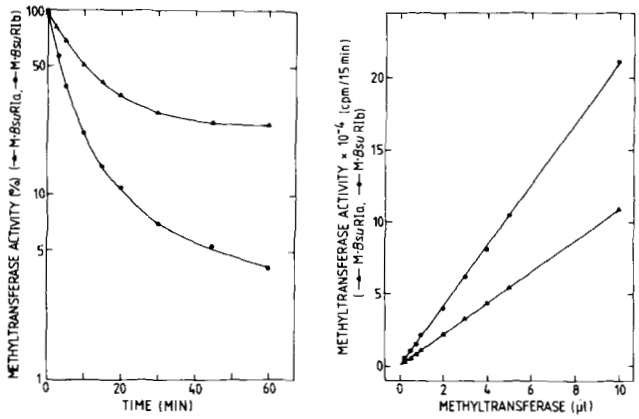

FIG. 2 (*left*). **Heat inactivation of the M. *Bsu*RI methyltransferases.** 190 units of M. *Bsu*RIa and 260 units of M. *Bsu*RIb were diluted to a total volume of 50 µl with storage buffer, and incubated at 46 °C, and 5-µl samples were withdrawn at the indicated times. Residual methylating activity was determined by adding these samples to the standard methyltransferase assay.

FIG. 3 (*right*). **Correlation between initial rate of methylation and concentration of M. *Bsu*RI methyltransferases.** The indicated amounts of enzyme were tested in the standard methyltransferase assay, except that the incubation time was decreased to 15 min. M. *Bsu*RIa was diluted 1:20 and M. *Bsu*RIb 1:30.

TABLE II

DNA methyltransferase activity at various conditions

Condition[a]	M.*Bsu*RIa activity	M.*Bsu*RIb activity
	%	
Standard	100[b]	100[c]
− dithioerythritol	9	11
− glycerol	74	88
− EDTA	65	100
+ EDTA (50 mM)	159	35
+ Mg^{2+} (10 mM)	108	93
+ ATP (1 mM)	99	107

[a] Reactions were performed under the standard conditions (Günthert *et al.*, 1981) with the indicated variations.

[b] 100% incorporation corresponds to 8.8 units methyltransferase activity.

[c] 100% corresponds to 15.0 units.

Time Course of the Methylation Reaction—Independent of the salt concentrations used, M. *Bsu*RIa showed a constant incorporation rate over a period of at least 5 h, while the rate for M. *Bsu*RIb is only constant for 1 h (Fig. 4).

Influence of Distance between Recognition Sites—Incorporation of methyl groups is within the limits of our experiments not affected by the average distance between 5′GGCC target sites. This was tested using phage SPP1.O DNA, having one 5′GGCC site/400 base pairs (Günthert *et al.*, 1975) and plasmid pC194 DNA, carrying one such site per 2700 base pairs (Gryczan *et al.*, 1978). Kinetics with these two substrates at equimolar amounts of 5′GGCC sequences did not show any differences. Therefore, we have used SPP1.O DNA as substrate for Michaelis-Menten kinetics with a molecular weight of $M_r = 28.6 \times 10^6$ (Behrens *et al.*, 1979), representing 220 mol 5′GGCC sites per 1 mol of SPP1 DNA.

Influence of DNA Concentration—M. *Bsu*RIa and M. *Bsu*RIb were tested for their DNA-binding affinity under optimal reaction conditions. The initial rate kinetics were plotted according to Hofstee (Fig. 5). The k_m values for both enzymes were 2.7 nM. Modified SPP1.R DNA was a competitive inhibitor of the methyltransferase reaction with an inhibition constant of $k_i = 2.5$ nM, as shown graphically according to Lineweaver-Burk (Fig. 6). Semimodified SPP1 DNA (heteroduplex DNA constructed of one SPP1.O strand and one SPP1.R DNA, Spatz and Trautner, 1970) yielded the same k_m value as unmodified DNA, but the reaction proceeded with twice the velocity (Fig. 6). This finding is biologically significant when one considers that semimodified DNA is the natural substrate for the modification enzyme *in vivo*.

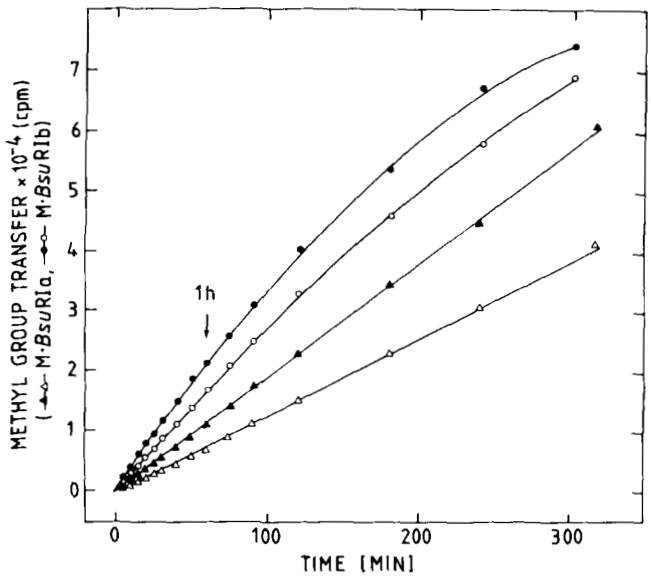

FIG. 4. **Time course of methylation.** Reaction mixtures (800 µl) contained 80 µg of calf thymus DNA, 3.3 µM Ado[*Me*-^{3}H]Met (15 Ci/mmol), and 14 units of M. *Bsu*RIa or 26 units of M. *Bsu*RIb. Both enzymes were tested under different ionic strength conditions with Tris-HCl, pH 8.4: M. *Bsu*RIa at 100 mM (▲——▲) and at 270 nM (△——△); M. *Bsu*RIb at 440 mM (●——●) and at 270 mM (○——○). 50-µl aliquots were withdrawn at the indicated times and methyltransferase activity determined. The time of the standard methyltransferase reaction is indicated by an *arrow*.

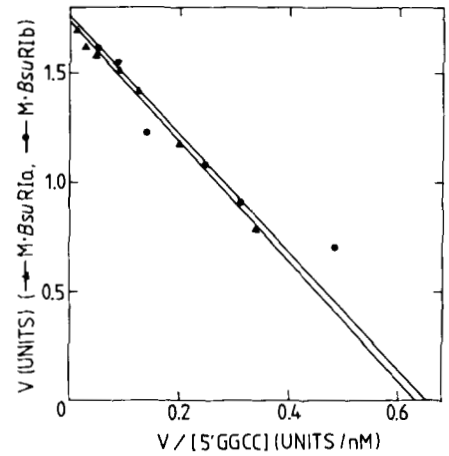

FIG. 5. **Determination of k_m for DNA.** For each indicated amount of SPP1.O DNA, time courses were performed. Each reaction mixture (400 µl) contained different amounts of SPP1.O DNA, fixed concentrations of Ado[*Me*-^{3}H]Met (3.3 µM), and 95 units of M. *Bsu*RIa or 90 units of M. *Bsu*RIb. At times 5, 10, 15, 20, 30, 45, and 60 min, 50-µl samples were withdrawn; methyl group transfer and initial reaction velocity were determined. SPP1.O DNA concentrations are in terms of moles of 5′GGCC recognition sequences (M_r = 130,000).

Influence of AdoMet Concentration—The AdoMet binding constant for M. *Bsu*RIb was 0.7 µM (Fig. 7). The same k_m value was found for M. *Bsu*RIa. Addition of either unlabeled AdoMet or AdoHcy at 3-fold excess over the Ado[*Me*-^{3}H]Met concentration revealed that AdoHcy had a 3.5-fold higher

binding affinity than AdoMet (Fig. 7). AdoHcy was a competitive inhibitor of the methylation reaction with an inhibition constant of $k_i = 0.13$ μM.

Mode of Action of the Methyltransfer

Individual steps of the methylation reaction could be defined by measuring the binding between the substrates in-

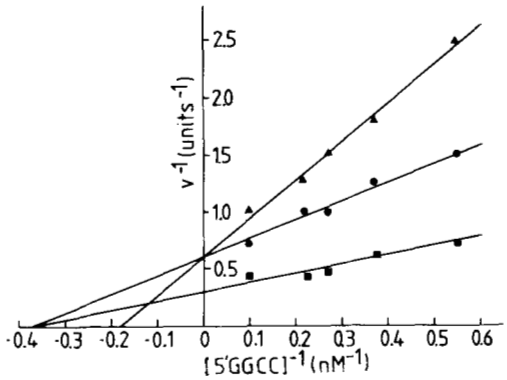

FIG. 6. **Determination of kinetic constants for modified and semimodified DNA.** Reactions were performed as described in the legend of Fig. 5, with M. *Bsu*RIb for SPP1.O DNA (●——●) and heteroduplex SPP1.O/R DNA (■——■). Influence of modified DNA was observed by adding constant amounts of SPP1.R DNA (2.7 nM) to the reaction mixtures with SPP1.O DNA (▲——▲).

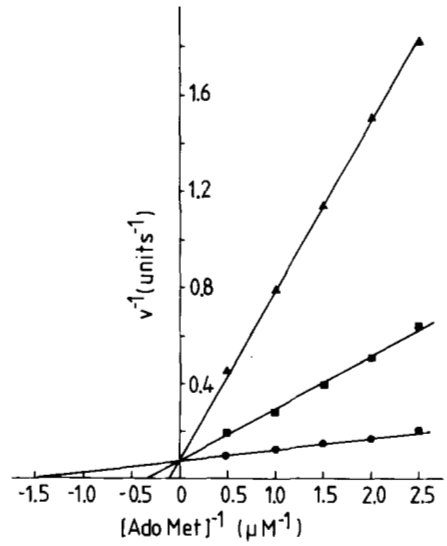

FIG. 7. **Determination of kinetic constants for AdoMet and AdoHcy.** Reactions were performed as described in the legend to Fig. 5 with M. *Bsu*RIb, except that the concentration of SPP1.O DNA was constant (16 μg) and the amount of Ado[*Me*-^{3}H]Met was varied as indicated (●——●). Competition of AdoHcy and nonradioactive AdoMet was observed by adding constant amounts of competitors. ■——■, kinetics with AdoMet (1.8 μM); ▲——▲, kinetics with AdoHcy (1.8 μM).

TABLE III
Equilibrium dialysis

RADIOACTIVE AdoMet	DNA SUBSTRATE	M.*BsuRI*	COMPETITOR[1]	TRIS-HCl, pH 8.4 100 mM	TRIS-HCl, pH 8.4 440 mM	TEMPERATURE 4 °C	TEMPERATURE 37 °C	BINDING OF RADIOACTIVITY[2]	DIVISION
Ado [Me-^{3}H] Met	●[3]	/	/	●[3] or	●	● or	●	-	1
	/	a or b	/	●		●		+	2
	/	a or b	/		●	●		+ +	
	/	a or b	/	●			●	(+)	
	/	a or b	/		●		●	(+)	
	/	a or b	AdoHcy		●	●		+ +	
	/	a or b	SPP1.R DNA		●	●		+ +	
	●	a	/	●		●		+ +	3
	●	a	/	●			●	+ +	
	●	b	/		●	●		+ +	
	●	b	/		●		●	+ +	
	●	b	AdoHcy		●	●		+	
	●	b	AdoHcy		●		●	(+)	
	●	b	AdoMet		●	●		(+)	
Ado [Carboxy-^{14}C] Met	/	a or b	/		●	●		+ +	4
	●	a or b	/		●	●		+ +	
	●	b	/		●		●	-	

[1] Competitors were added at equimolar amounts to Ado[Me-^{3}H]Met.

[2] Definition for binding symbols is expressed as percentage of total Ado[Me-^{3}H]Met input radioactivity in the reaction chamber: - = 50 % (+) = >50 to 55 %, + = >55 to 60 %, ++ = >60 %. Assignments for the distribution of radioactivity derived from Ado[Carboxy-^{14}C]Met were made by comparing the identical reactions with Ado[Me-^{3}H]Met given in division 2, line 2 and division 3, line 3.

[3] ● reaction performed
/ reaction component not added

volved and the enzymes using equilibrium dialysis (Table III). With either enzyme, we observed the following steps of the methylation reaction.

1) AdoMet alone does not bind to DNA under any conditions tested (Table III, division 1). Even in the absence of DNA, AdoMet binds to the methyltransferases (MT), which is presumably the initial step in the methyl transfer reaction. Neither AdoHcy nor SPP1.R DNA inhibit this complex formation.

$$MT + Ado^{*}Met \rightarrow MT \cdot Ado^{*}Met$$

2) The second step is probably the unspecific binding of the $MT \cdot Ado^{*}Met$ complex to nontarget sequences. We conclude this from the observation that SPP1.R DNA serves as a competitive inhibitor of the methylation reaction (Fig. 6).

$$MT \cdot Ado^{*}Met + DNA \rightarrow DNA \sim MT \cdot Ado^{*}Met$$

At low temperature, the DNA-bound MT·AdoMet complex was only weakly competitied by AdoHcy in contrast to nonradioactive AdoMet (Table III, division 3).

3) For methyl group transfer, recognition of the unmethylated target site must occur.

$$DNA \sim MT \cdot Ado^{*}Met \rightarrow [DNA\text{-}(GGCC)] \cdot MT \cdot Ado^{*}Met$$

4) At 37 °C, the methyl group transfer takes place as the rate-limiting step of the whole reaction sequence.

$$[DNA\text{-}(GGCC)] \cdot MT \cdot Ado^{*}Met \rightarrow [DNA\text{-}(GG^{*}CC)] \cdot MT \cdot AdoHcy$$

Only this step can be strongly inhibited by addition of the competitor AdoHcy (Table III, division 3).

5) Methyl group transfer leads to the separation of the modified DNA recognition sequence, AdoHcy, and the methyltransferase. This was demonstrated using AdoMet radioactively labeled in the carboxy group, leading to Ado[*carboxy*-^{14}C]Hcy and nonradioactive methylated DNA after the methyl group transfer (Table III, division 4). A complex of methyltransferase and AdoHcy could not be detected and is probably very unstable. This is in agreement with the finding that AdoHcy is not inhibitory to the binding of AdoMet to methyltransferase (Table III, division 2).

$$[DNA\text{-}(GG^{*}CC)] \cdot MT \cdot AdoHcy \rightarrow [DNA\text{-}(GG^{*}CC)] + MT + AdoHcy$$

Long time kinetics as shown in Fig. 4 demonstrated that the methyltransferases, after leaving the recognition sequence and releasing AdoHcy, can undergo another round of methylation at the same or at a different recognition sequence.

DISCUSSION

The preceding paper (Günthert *et al.*, 1981) describes the purification and physical characterization with respect to molecular weight and subunit composition of two cytosine-DNA methyltransferase activities of *B. subtilis*, termed M. *Bsu*RIa and M. *Bsu*RIb. In this paper, we characterized the activities further 1) with respect to their reaction properties, and 2) by analyzing the reaction steps of the methyl group transfer.

As both M. *Bsu*RI methyltransferases had unexpectedly the same sequence specificity, it was of importance to compare their reaction properties and physicochemical constants. A compilation of our data is listed in Table IV.

It has already been shown in the previous paper (Günthert *et al.*, 1981) that molecular weight, size, and shape of M. *Bsu*RIa and M. *Bsu*RIb are almost identical. The kinetic analyses of the two enzymes revealed the same affinities and reaction velocities with their substrates, DNA or AdoMet (Figs. 5 and 7). Also, the equilibrium dialysis studies showed a similar mode of action of the two enzymes.

TABLE IV

Comparison data of M.BsuRI methyltransferases

Property	M.*Bsu*RIa	M.*Bsu*RIb
Molecular weight		
by SDS-gel electrophoresis	41,000	42,000 + 39,000
by gel filtration	37,000	40,000
by sedimentation	43,000	43,000
$s_{20,w}$ (10^{-13} s)	3.55	3.55
Stokes radius (nm)	2.6	2.8
Frictional coefficient (f/f_0)	1.1	1.2
k_m DNA (5'GGCC, nM)	2.7	2.7
k_m AdoMet (μM)	0.7	0.7
pH optimum (270 mM Tris-HCl)	8.4	8.3 (7.7)
Elution on phosphocellulose (M KCl)	0.21	0.32
Ionic strength optimum (mM Tris-HCl, pH 8.4)	100	440
Half-life at 46 °C (min)	10	3.5
Time of constant methylation rate (h)	>5	≤1
Content in exponentially growing cells (%)	10–20	80–90

Although the two enzymes have quite a few obvious similarities, there are also some significant differences. There were at least three conditions under which the enzymes reacted differently. M. *Bsu*RIa was completely inactive under the optimal Tris-HCl concentration of M. *Bsu*RIb, and M. *Bsu*RIb was reduced to 30% of its activity under optimal M. *Bsu*RIa conditions (Fig. 1). The two enzymes behaved differently in the presence of EDTA. M. *Bsu*RIa was activated at high EDTA concentrations, whereas M. *Bsu*RIb was inactivated (Table II). Additionally, the stability of the two enzymes was different with respect to temperature sensitivity (Fig. 3) and long time kinetics (Fig. 4). M. *Bsu*RIa, which reflected only 10–20% of the total methylating activity in exponentially growing cells (Günthert *et al.*, 1981), was more stable than M. *Bsu*RIb, which was the major methyltransferase in r^+m^+ *B. subtilis*.

Altogether, we cannot yet decide whether M. *Bsu*RIa and M. *Bsu*RIb represent the same enzyme in different forms encoded by one and the same gene, or whether distinct genes code for the two enzymes, whose activities are correlated with the cell cycle.

The phenomenon of two methyltransferases with identical sequence specificity shown in the same bacterial strain has been reported only in a few cases. *Escherichia coli* K12 (RII) cells contained two DNA cytosine methyltransferases with the same sequence specificity, but only one of them could be attributed to the host chromosome (M. *Eco*K12). The second was encoded by the RII plasmid (M. *Eco*RII). Although the two enzymes were chromatographically distinct, their enzymatic characteristics were the same (Hattman, 1977; Bogdarina *et al.*, 1979). Recently, Yoo and Agarwal (1980) reported the existence of two DNA methyltransferases with *Hpa* II specificity in *Haemophilus parainfluenzae* without finding differences in their enzymatic properties such as pH, temperature, and salt optima. The molecular weights of the two enzymes were almost the same. The authors assume that one enzyme is the precursor form of the other.

In general, we were interested to analyze further the interaction between a protein and its specific DNA target site. Relatively little is known about the mechanisms of interaction between type II modification enzymes and their DNA substrate sites. The only type II methyltransferase studied in more detail is the M. *Eco*RI modification enzyme. Rubin and Modrich (1977) demonstrated that the enzyme acts asymmetrically with its recognition sequence: it methylates the sequence of one strand as a monomer and dissociates from the

DNA prior to the second methylation. The binding affinities for DNA (with respect to the *Eco*RI recognition sequence) and AdoMet are almost similar to those of the *Bsu*RI methyltransferases reported here.

The *Bsu*RI methyltransferases also function as monomers. By equilibrium dialysis and kinetic studies, we proposed a sequence of reaction steps. From these data we cannot draw definite conclusions as to whether the enzymes dissociate from the DNA strand after each unspecific binding, or whether they move along the DNA by diffusion.

With the availability of semimodified DNA, representing the *in vivo* situation in the cell, we could demonstrate that semimodified recognition sequences were methylated faster than unmodified sites by the methyltransferases, although the binding affinity was not increased. This more rapid methylation was also demonstrated with M. *Eco*B, a type I enzyme (Vovis *et al.*, 1974).

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