Kinetics of Methylation of DNA by a Restriction Endonuclease from *Escherichia coli* B

(methylase specificity/heteroduplex DNA)

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ABSTRACT The restriction endonuclease from *E. coli* B is both an endonuclease and a DNA methylase. Both activities either require or are stimulated by Mg^{+2} , adenosine triphosphate, and S-adenosyl-L-methionine. The particular activity which the enzyme exhibits depends upon the nature of the SB sites, the genetic sites that identify substrate DNA. Enzymatic treatment of DNA that has an unmodified, wild-type SB site results in either rapid restriction of the DNA or very slow methylation of the SB site. On the other hand, a hybrid SB site (modified), which protects the DNA molecule from restriction, results in rapid methylation of that SB site.

A DNA modification methylase that confers resistance to restriction by endonuclease R·*EcoB** has been previously isolated (2-4). In addition, a B-specific modification activity is also associated with purified endo R·*EcoB* (Vovis, Horiuchi, and Zinder, in preparation) as predicted (5). Likewise, in the allelic K-specific restriction-modification system (6), highly purified preparations of endo R·*EcoK* contain K-specific modification activity (5, 7). At the same DNA and enzyme concentrations, the methylation by these two related endonucleases is much slower than the nucleolytic activity (refs. 5 and 7, and this report). The nucleolytic reaction occurs in minutes while the methylating reaction requires hours. Similarly, methylase M·*EcoB* modifies DNA very slowly *in vitro* (4).

We have been studying how hybrid SB sites† of bacteriophage f1 are treated *in vitro* by the B-specific restriction-modification system (refs. 11 and 12, and Vovis *et al.*, in preparation). A hybrid DNA duplex with one strand containing an SB° mutation and the other an unmodified, wild-type SB site (SB°/SB+) is neither restricted nor methylated (ref. 11 and Vovis *et al.*, in preparation). However, a DNA duplex in which one strand has a modified, wild-type SB site and the other an unmodified, wild-type SB site (a hybrid SB^m/SB+

site) is not restricted but is modified (refs. 11 and 13, and Vovis *et al.*, in preparation). One methyl group is added for each such site per molecule (Vovis *et al.*, in preparation). Since modification undoubtedly follows DNA replication and DNA replication is semi-conservative, hybrid SB^m/SB+ sites should be the physiological substrate for modification. However, hitherto, only completely unmodified DNA molecules have been used as substrates for studies of *in vitro* modification. We report here that *in vitro* methylation of hybrid SB sites (modified) by endo R·*EcoB* is not only much faster than that of unmodified, wild-type SB sites but that this methylation is also as rapid as the phosphodiester hydrolysis of unmodified DNA by this endonuclease.

MATERIALS AND METHODS

The bacterial and f1 phage strains are the same as those used previously (11). The isolation of endo R·*EcoB* has been described (14). The hydroxylapatite fraction was used throughout these experiments. Endo R·*Hind* was purified by the method of Smith and Wilcox (15); however, the nucleic acids were removed by streptomycin sulfate precipitation instead of by Bio-Gel filtration. The preparation of covalently linked, circular double-stranded DNA (RFI) of f1 phage was described previously (16). The DNA duplexes containing hybrid SB sites were prepared (Vovis *et al.*, in preparation) essentially as before (11). RFI was enzymatically converted to a full length, linear double-stranded DNA molecule (RFIII) by endo R·*Hind*II which cleaves f1 DNA at a single site (Horiuchi, unpublished results). The RFIII was isolated and alkali-denatured, a 10- to 20-fold molar excess of f1 viral strands (single-stranded circular molecules) was added, and after annealing, the circular, double-stranded DNA which contains at least one single strand nick (RFII) was isolated by sucrose velocity sedimentation. DNA methylation was measured by the method used by Lautenberger and Linn (4). [*Methyl*-³H]-S-adenosyl-L-methionine (SAM) (4.8 Ci/mmol as determined by isotope dilution) was from New England Nuclear Corp.

RESULTS

Fig. 1 shows the kinetics of restriction with an SB = 1† RFI (SB₂°). The restriction reaction was completed by 7.5 min, i.e., essentially all the RFI had been converted by this time to RFIII, the final digest product (see ref. 14).

RFI of f1, SB₁°, or SB₂° is methylated instead of being restricted by endo R·*EcoB* if Mg^{+2} and ATP are omitted from the reaction mixture. However, the rate of modification is much slower than that of restriction (compare Fig. 1 with

Abbreviations: RFI, a covalently linked, circular double-stranded DNA molecule; RFII, a circular, double-stranded DNA molecule containing at least one single-strand nick; RFIII, a full length, linear double-stranded DNA molecule; SAM, S-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetate.

* The nomenclature proposed by Smith and Nathans (1) for restriction-modification enzymes has been used, i.e., endo refers to endonuclease, meth to methylase, R to restriction, M to modification, *Eco* to *E. coli*, B and K to the *E. coli* strains from which these enzymes are isolated, etc.

† An SB site is a genetic site which confers susceptibility to restriction by *E. coli* B. Bacteriophage f1 has two SB sites, SB₁ and SB₂ (8-10). SB = 1 mutants of f1 (SB₁° or SB₂°) have lost one of the two SB sites by mutation.

FIG. 1. Kinetics of restriction with SB_2° RFI. The 600- μ l reaction mixture contained 100 mM Tris·HCl at pH 7.4, 4 mM $MgCl_2$, 1 mM ATP, 5 μ M SAM, 10.9 μ g [3H]RFI of R140R (SB_2°) (1.8×10^4 cpm/ μ g), and 76.8 μ g of endo R·*EcoB*. The reaction mixture, prewarmed at 37° before enzyme addition, was incubated at 37° and sampled at 1 (B), 2.5 (C), 5 (D), 7.5 (E), and 15 min (F) by removing 100 μ l to a tube containing 20 μ l of 0.5 M ethylenediaminetetraacetate (EDTA) pH 8 and 7.5 μ l of 20% (w/v) sodium dodecyl sulfate. A control reaction was incubated for 15 min at 37° in the absence of SAM (A). The samples were layered onto 5 ml of a 5–20% (w/v) preformed neutral sucrose gradient (1 M NaCl, 0.1 M sodium phosphate, pH 7) and centrifuged for 4 hr (45,000 rpm at 18°) in the SW50.1 Spinco rotor. Six drop fractions were collected onto Whatman GF/A discs which were dried and counted (14). The arrows indicate, from left to right, the positions of RFI, RFII, and RFIII, respectively. Sedimentation was from right to left.

Figs. 2 and 3A). With the same substrate and enzyme concentrations, modification of an $SB = 1$ mutant RFI required more than 9 hr (Fig. 3A) as compared to 7.5 min for restriction (Fig. 1). In addition, there appears to be a difference in the rate at which the two SB sites of f1 are methylated. The SB_2 site seems to be modified somewhat faster than the SB_1 site (Fig. 2). Consistent with this interpretation is the observation that the wild-type f1 RFI which contains both SB sites is modified at a rate intermediate between the SB_1° and SB_2° RFI DNAs (Figs. 2 and 3A). The comparative methylation experiments were done with substrate concentrations such that the total number of methyl groups that could be added to the different DNA substrates was the same.

Although an unmodified, wild-type SB site in RFI is methylated very slowly by endo R·*EcoB*, a hybrid site is methylated very rapidly (Fig. 3). Approximately 30 min was required to methylate completely the SB sites in RFII in which the viral strand came from modified phage and the complementary strand came from unmodified DNA. The fact that there is no difference in the rate of methylation between RFII, prepared from unmodified wild-type viral and comple-

FIG. 2. Kinetics of methylation with f1, SB_1° , and SB_2° RFI. The 450- μ l reaction mixtures contained 100 mM Tris·HCl at pH 7.4, 0.2 mM EDTA, 5 μ M [3H]SAM, 15.3 μ g of endo R·*EcoB*, and either 6.72 μ g of RFI of f1 ($\circ$), or 13.6 μ g of RFI of R119R (SB_1°) (Δ), or 13.5 μ g of RFI of R140R (SB_2°) ($\square$), or no DNA (the control). The reaction mixtures were divided into 4 aliquots of 100 μ l each and these samples were incubated at 37° for the indicated times. The extent of DNA methylation was determined by the method used by Lautenberger and Linn (4). The results obtained with the control (no DNA) were subtracted from the other experimental values. The "normalized cpm" are the cpm/ μ g of DNA per total number of methyl groups able to be added per DNA molecule, i.e., four methyl groups for f1 RFI and two methyl groups for SB_1° or SB_2° RFI. The cpm/ μ g of DNA per methyl group added per DNA molecule in these experiments (Figs. 2 and 3) is approximately 920.

mentary strands, and RFI of f1 (Fig. 3A) demonstrates that it is not the absence of super-coils in RFII molecules that is affecting the rate of methylation but rather the nature of the SB site itself, i.e., hybrid as compared to completely unmodified SB site. Furthermore, the rapid methylation of hybrid sites is independent of which strand in the DNA duplex is modified. There was no gross difference in the rate of methylation of the hybrid SB sites when either the viral or the complementary strand was the modified strand of the substrate DNA (Fig. 4).

Since a hybrid SB site affords protection for a DNA molecule from restriction (11, 13), it is possible to test whether, in fact, the methylase associated with endo R·*EcoB* is stimulated by ATP and Mg^{+2} . The restriction activity requires both Mg^{+2} and ATP in addition to SAM as was first demonstrated by Meselson and Yuan (13). In the presence of ATP and Mg^{+2} (concentrations were those used in Fig. 1), the modification reaction was completed in one minute (Fig. 4). Thus, Mg^{+2} plus ATP are physiological effectors for both the restriction and modification activities of endo R·*EcoB*.

DISCUSSION

Bacterial restriction-modification systems have probably evolved as a protection against the introduction of foreign

FIG. 3. Kinetics of methylation with RFI and RFII. (A) The 750- μ l reaction mixtures contained 100 mM Tris·HCl at pH 7.4, 0.2 mM EDTA, 5 μ M [methyl- 3 H]SAM, 96 μ g of endo R·EcoB, and either 6.9 μ g of RFI of f1 (○), or 13.5 μ g of RFI of R119R (SB $_1$) (△), or 6.8 μ g of RFII prepared from unmodified, wild-type viral and complementary strands (●), or 13.8 μ g of RFII prepared from modified, wild-type viral strands and unmodified, wild-type complementary strands (■), or no DNA (the control). The reaction mixtures were divided into 7 aliquots of 100 μ l each and these samples were incubated at 37° for the indicated times. The extent of DNA methylation was measured by the method used by Lautenberger and Linn (4). The results obtained with the control (no DNA) were subtracted from the other experimental values. The "normalized cpm" are as defined for Fig. 2. (B) The reaction mixtures contained 100 mM Tris·HCl at pH 7.4, 0.2 mM EDTA, 5 μ M [methyl- 3 H]SAM, and: 6.8 μ g of RFII prepared from modified, wild-type viral strands and unmodified, wild-type complementary strands and 96 μ g of endo R·EcoB (■); or no DNA and 96 μ g of endo R·EcoB (the control) (final volume for both was 750 μ l); or 0.93 μ g of RFII prepared from unmodified, wild-type viral and complementary strands and 12.8 μ g of endo R·EcoB (●); or 0.9 μ g of RFI of f1 and 12.8 μ g of endo R·EcoB (○) (final volume for these latter two was 100 μ l). The reaction mixtures, preincubated at 37° for 3 min before enzyme addition, were incubated at 37° and sampled at the indicated times by removing 100 μ l to a tube at 0° containing 100 μ l of 5 mg/ml of albumin, 200 μ l of 100 mM sodium pyrophosphate, and, if necessary, [32 P]DNA (so as to be able to determine recovery; see ref. 4). Then 500 μ l 10% (w/v) trichloroacetic acid was added. The measurement of methylation, the treatment of the results, and the definition of "normalized cpm" were as for Fig. 2.

DNA. The B- and K-specific restriction endonucleases are presumed to be composed of three different subunits (7, 17–21). Each of these enzymes is both a nuclease and a methylase. Both of these activities are enhanced by or require SAM, ATP, and Mg $^{+2}$ (Fig. 4; refs. 5 and 7, and Vovis *et al.*, in preparation). With the B enzyme and most probably also the K, the nature of the susceptibility site determines whether the enzyme will act as an endonuclease or as a methylase. A hybrid SB m /SB $^+$ site, which affords the DNA molecule protection from B-specific restriction (11, 13), is methylated (Figs. 3 and 4, and Vovis *et al.*, in preparation). On the other hand, an unmodified DNA molecule is restricted although the cleavage occurs at a site other than the SB site (22, 23).

How these two types of SB sites are treated by the enzyme *in vitro* is consistent with the way that they undoubtedly

FIG. 4. Kinetics of methylation of hybrid SB m /SB $^+$ sites in the presence of ATP and Mg $^{+2}$. The reaction mixtures contained 100 mM Tris·HCl at pH 7.4, 0.2 mM EDTA, 5.5 μ M [methyl- 3 H]SAM, and: 12.0 μ g of RFII prepared from modified, wild-type viral strands and unmodified, wild-type complementary strands and 83.2 μ g of endo R·EcoB (○) (final volume of the reaction mixture was 650 μ l); or 13.9 μ g of RFII prepared from unmodified, wild-type viral strands and modified, wild-type complementary strands and 96 μ g of endo R·EcoB plus (■) or minus (□) 1 mM ATP and 4.2 mM MgCl $_2$ (final volume for both was 750 μ l); or no DNA and 64 μ g of endo R·EcoB (the control) (final volume for this reaction mixture was 500 μ l). The reaction mixtures, prewarmed at 37° for 3 min, were incubated at 37° and sampled (100 μ l) at the indicated times as for Fig. 3B. The results obtained with the control (no DNA) were subtracted from the other experimental values. The "normalized cpm" were defined as for Fig. 2. The cpm/ μ g of DNA per methyl group added per DNA molecule in this experiment is about 800. (A different lot of [methyl- 3 H]SAM was used in this experiment.)

arise and are treated *in vivo*. In *E. coli* B, the DNA immediately after replication should possess only hybrid sites. Such sites must be modified rapidly. If not, another round of DNA replication would yield some completely unmodified SB sites which would make the DNA susceptible to restriction. On the other hand, if bacterial or viral DNA synthesized in a foreign bacterium should enter *E. coli* B, it would do so with unmodified SB sites. These sites would be expected to be poor substrates for methylation but excellent ones for eliciting restriction, the appropriate protective reaction. The *in vitro* action of endo R·EcoB, i.e., rapid restriction but very slow methylation of molecules with unmodified SB sites and rapid methylation but no restriction of molecules with hybrid sites, is entirely consistent with the above *in vivo* scenario and suggests that this enzyme is responsible for both restriction and modification *in vivo*.

There seems to be another *in vitro*–*in vivo* correlation. The degree to which f1 phage is restricted *in vivo* might be explicable by the rate at which endo R·EcoB methylation of unmodified SB sites occurs *in vitro*, although extrapolation from *in vitro* to *in vivo* conditions can be dangerous. The rate of adding the first methyl group to an SB = 1 mutant RF can be calculated (assuming the addition of the first methyl group to be rate limiting) to be about 0.7% of the rate of adding a

single methyl group to a hybrid SB site (Fig. 3). This is another way of saying that the frequency of methylation to restriction of an unmodified SB site is about 0.007. Thus, an SB = 1 mutant f1 phage grown on *E. coli* K would be expected to plate with an efficiency of about 0.7% on *E. coli* B. The actual value is about 2–3% (8, 10).

In addition to the B-specific methylase associated with endo R·EcoB, a B-specific methylase, which has only two of the three subunits found in endo R·EcoB and lacks any restriction activity (2–4), has also been purified from *E. coli* B. This enzyme, should it exist *in vivo*, could act as a backup or fail safe system for endo R·EcoB. In any event, the rapid nucleolytic or methylating action of endo R·EcoB that is exhibited only on the appropriate physiological substrate, strongly suggests that endo R·EcoB is the enzyme responsible for both restriction and modification *in vivo*. Although it remains to be shown, it is probable that similar substrate specificity will be found for the methylating and nucleolytic activities of other restriction–modification enzyme systems.

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