Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6394–6397, August 1991
Biochemistry

Allosteric and catalytic binding of *S*-adenosylmethionine to *Escherichia coli* DNA adenine methyltransferase monitored by ^3H NMR

(DNA binding protein/oligonucleotide/GATC sequence/conformational rearrangement/monomeric enzyme)

AGNÈS BERGERAT, WILHELM GUSCHLBAUER, AND G. VICTOR FAZAKERLEY

Service de Biochimie et de Génétique Moléculaire, Bat. 142, Département de Biologie Cellulaire et Moléculaire, Centre d'Etudes de Saclay, 91191 Gif-sur-Yvette Cedex, France

Communicated by Pierre-Gilles de Gennes, March 19, 1991 (received for review October 5, 1990)

ABSTRACT **Adenine methylation of GATC sequences in DNA is carried out by the DNA adenine methyltransferase with the methyl group source being the cofactor *S*-adenosylmethionine. We report ^3H NMR studies on the interaction of DNA adenine methyltransferase with *S*-adenosylmethionine and the reaction when the ternary complex is formed with an oligonucleotide containing a GATC site. The methylation reaction was also studied in the presence of a competitive inhibitor and this showed two successive stages involved in the methylation and two sites of binding for *S*-adenosylmethionine.**

The DNA adenine methyltransferase (Dam) catalyzes the *S*-adenosylmethionine (AdoMet)-dependent methylation of the amino group of the adenine of the specific GATC target sequence of DNA (1). The state of methylation of the GATC sequences plays a regulatory role in many biological processes. These include postreplicative mismatch repair, replication, expression of certain genes, transposition, and chromosomic segregation (2, 3). The Dam is a 32-kDa protein that acts as a monomer (4).

Previous studies have shown that AdoMet has two roles in its interaction with the Dam. First, it displays an allosteric interaction whereby AdoMet binding increases the affinity of the Dam for its GATC target site, and the simultaneous binding of AdoMet and an oligonucleotide containing a GATC sequence induces a conformational change in the Dam (5). Second, it is the methyl donor for the methylation reaction. To fulfill these two roles, the AdoMet could be bound in two different environments. For the allosteric role, the AdoMet binding is very strong ($K_d \approx 0.1$ μM) and analogues of AdoMet have been shown to be unable to bind at this site (5) for analogue/AdoMet ratios up to 10. For the catalytic role, the K_d for AdoMet binding has been estimated as 6 μM and analogues of AdoMet are competitive inhibitors. However, two mechanisms could explain the two roles for AdoMet. Either the allosteric site rearranges upon DNA binding to become a catalytic site or there are distinct allosteric and catalytic sites.

It has been shown (ref. 6 and refs. therein) that ^3H NMR is a powerful tool for monitoring enzyme reactions. We have used AdoMet, the methyl group of which is almost totally tritiated, to investigate the environment when complexed with Dam, this binary complex in the presence of an inhibitor (sinefungin) and in the ternary complex formed with an oligonucleotide containing the GATC sequence. Furthermore, we have monitored the methylation reaction in the presence of the inhibitor, which shows that AdoMet fulfills its roles simultaneously and is bound on two nonequivalent sites.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

EXPERIMENTAL

Dam was purified to electrophoretic homogeneity from *Escherichia coli* strain HB101/pDOX, obtained from Klaus Hülsmann (Free University of Berlin), as described by Herman and Modrich (4) and had a specific activity of 8×10^5 units/mg in the usual assay (7). Protein content was determined by the method of Bradford (8), using bovine serum albumin as a standard. A molecular mass of 32 kDa for Dam was used for determining molar yield. Enzyme was concentrated in ultracentrifugation cones from Amicon. [^3H]AdoMet (85 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. AdoMet was lyophilized and redissolved in ^2H_2O. The pH was adjusted to 7 with 0.1 M NaOH. Sinefungin (adenosylornithine) was a generous gift from M. Gero (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). The oligonucleotides were synthesized, purified, and quantified as described (5) to give the duplex 5′-d(GCGCGATCATGGCG)·d(CGCCATGATCGCGC). The samples were dissolved in a solution containing 100 mM Tris·HCl (pH 7.5) (at 25°C), 25% ^2H_2O, 4 mM dithiothreitol, and the total ionic strength was 100 mM.

^3H NMR spectra were recorded on a Bruker AM600 spectrometer operating at 640 MHz. For all spectra, the sample volume was 0.3 ml contained in thick-walled 5-mm NMR tubes. Spectra were recorded at 4°C and where titrations were carried out care was taken that the sample did not warm up to preserve the enzyme activity. Calibration experiments were carried out to determine the temperature of the solution after putting the tube into the probe by careful monitoring of the ^2H lock signal. For kinetic experiments started after 3 min, the solution was observed to be at 4°C ± 0.2°C. The probe temperature was regulated by using a nitrogen flow cooled by a thermostat bath Haake with the maximum flow rate to most rapidly achieve thermal equilibrium.

RESULTS AND DISCUSSION

Binding of AdoMet to Dam. The ^3H spectrum of AdoMet recorded at 4°C is shown in Fig. 1*A*. We have measured the ^3H spectrum of AdoMet at different AdoMet/Dam molar ratios. The Dam concentration was kept constant at 12 μM. Upon addition of 3 μM AdoMet, its methyl resonance is found at 2.99 ppm, 0.04 ppm downfield relative to that of free cofactor. This shift is very small—it corresponds to 25 Hz at the observation frequency—but is entirely reproducible from one preparation to another. Furthermore, the linewidth of the resonance in the presence of Dam is only 3.4 Hz so that chemical shifts can be measured very accurately. Spectra recorded at total AdoMet concentrations of 6, 9, and 12 μM

Abbreviations: Dam, DNA adenine methyltransferase; AdoMet, *S*-adenosylmethionine.

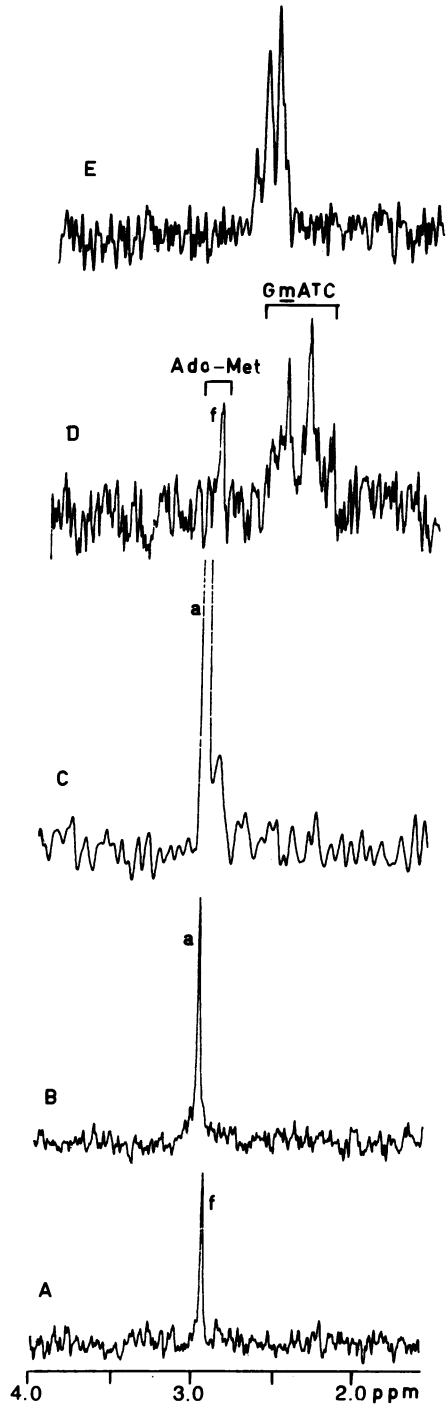

FIG. 1. ^{3}H NMR spectra recorded at pH 7.5 (total ionic strength, 100 mM at 4°C). (*A*) Spectrum of AdoMet. (*B*) Spectrum of the binary AdoMet/Dam complex (ratio, 1:1) with all the AdoMet bound in the allosteric site. The spectrum was recorded over 2 hr. (*C*) Spectrum recorded with an AdoMet/Dam ratio of 1.2:1. The spectrum has been resolution enhanced to show the broad peak at ≈2.95 ppm. (*D*) Spectrum after addition of an equimolar quantity of the GATC-containing oligonucleotide duplex. The spectrum was acquired during the first 2 hr after addition of the oligonucleotide. (*E*) Spectrum recorded after extraction of the enzyme.

showed no further changes in either the linewidth or chemical shift of the single resonance. We do not observe any free AdoMet when the ratio is 1:1 (Fig. 1*B*); it must all be bound to the enzyme at a single site, which must be the high-affinity allosteric site. When the AdoMet concentration is raised to 15 μM, we observed a broad resonance centered around the chemical shift of free AdoMet (Fig. 1*C*). The broadening may be due to exchange with nonspecific binding sites on the enzyme. We observe no evidence for AdoMet binding at a second specific site. The system with a 1:1 ratio appears best defined and was thus chosen as a starting point for further experiments.

System AdoMet/Dam/GATC Oligonucleotide. To a solution containing a 1:1 (mol/mol) ratio of AdoMet and Dam at 4°C was added 1 mol of the 14-base-pair oligonucleotide containing the GATC sequence to give a solution concentration of 12 μM. At this AdoMet concentration, we could normally obtain a spectrum (with low signal/noise ratio) within 2–3 min. However, we observed no spectrum at all until data acquisition had lasted at least 30 min. Fig. 1*D* shows the spectrum after 2 hr accumulation. Resonances are observed in the range 2.2–2.7 ppm, which correspond to methyl groups now attached to the N-6 of adenine. In principle, two resonances could be expected, one for the doubly methylated oligonucleotide and one for the hemimethylated oligonucleotide as the AdoMet/oligonucleotide ratio is 1. A second resonance might be observed for each species if the chemical shift is not the same for methylation on each asymmetric strand. Clearly, there are a number of methyl resonances (which explains the low signal/noise ratio) and these probably rise through additional nonspecific binding of the oligonucleotide to the enzyme after methylation. A small peak is also observed at 2.95 ppm corresponding to free AdoMet. In a spectrum recorded during the period 3–4 hr after mixing, this free AdoMet peak had disappeared. Heating the sample could break up specific binding of the methylated oligonucleotides or nonspecific binding of oligonucleotide to the Dam but would be likely to melt the duplexes. We therefore removed the enzyme by phenol extraction and precipitated the DNA. When redissolved, and under otherwise identical experimental conditions, we observe three ^{3}H peaks (Fig. 1*E*) in a chemical shift range expected for amino methylated adenosine (9, 10). The chemical shift differences observed between Fig. 1 *D* and *E* show that most of the oligonucleotide duplexes had been bound in some way to the Dam. Spectra were recorded at different temperatures up to 70°C, where only a single resonance is observed at 2.94 ppm. The oligonucleotides show melting temperatures in the range 30°C–40°C.

It has previously been shown (4) that methyl transfer occurs singly and not in pairs and that the reaction is highly sequence specific (1). Although we have not characterized the reaction products, starting from a 1:1 ratio of AdoMet and Dam the major products will be the hemimethylated oligonucleotides. The minor peak in Fig. 1*E* will correspond to the fully methylated species for which either the two methyl groups are coincident or the second falls under one of the resonances of the major species.

System AdoMet/Sinefungin/Dam/Oligonucleotide. In the experiment described above, the rapid methylation prevented us from being able to observe the ternary AdoMet/Dam/oligonucleotide complex and any conformational rearrangements that may occur. We have, therefore, looked for a way to slow it down. Sinefungin is a competitive inhibitor for Dam as is the reaction product *S*-adenosylhomocysteine (5). To a 1:1 mixture of AdoMet/Dam (12 μM) we added 60 μM sinefungin. The methyl resonance corresponding to the binary AdoMet/Dam complex at 2.99 ppm was unaffected by the presence of sinefungin and no free AdoMet was observed in the spectrum even after 2 hr. A 5-fold excess of sinefungin is unable to compete with AdoMet for the allosteric AdoMet binding site and does not appear to affect the protein conformation around the AdoMet binding site. Addition of the GATC duplex to this system in the presence of sinefungin will result in two very different situations depending on how AdoMet plays its two roles. If the allosteric site is converted into a catalytic site, the sinefungin should displace ≈80% of

the AdoMet, as AdoMet and sinefungin have a similar K_d (6 μM) for the catalytic site, which will be seen free in solution. We should also expect to see AdoMet in the catalytic environment. If a second, catalytic, site appears, the free AdoMet concentration will be very low and the [^{3}H]methyl group will appear on the oligonucleotide. Similarly, the concentration of AdoMet in the catalytic site will be very low and undetectable.

To the solution of AdoMet, sinefungin, and Dam we added 12 μM GATC duplex. In this experiment, we reduced the dead time before beginning spectral accumulation to the minimum, 3 min, even though the magnetic homogeneity had not been fully optimized. Spectra were stocked in blocks corresponding to 100 acquisitions (2 min 40 sec) for the first four spectra and subsequently in blocks corresponding to 200 acquisitions. In the first spectrum, we observe that the methyl resonance has shifted downfield to 3.03 ppm. During the first 16 min of the experiment, the methyl resonance shifts upfield, becoming stable after 20 min at a chemical shift very close to that of the binary complex. Extrapolating back to $t = 0$ (Fig. 2), we find that binding of the oligonucleotide induces a fast conformational change resulting in a downfield shift ($\approx$0.07 ppm) for the methyl group. This period of 20 min in which the resonance then shifts upfield does not correspond to the methylation reaction, as after 16 min the resonance has at least 80% of its intensity before addition of the oligonucleotide. The imprecision is due to the available signal/noise ratio. The chemical shift jump is not due to a temperature change. The ^{3}H chemical shift for the AdoMet/Dam complex is unchanged in the temperature range 0°C–10°C. Furthermore, in calibration experiments we have measured that the solution temperature was 4°C $\pm$ 0.2°C at the start of the acquisition.

The ternary complex formed immediately upon adding the oligonucleotide to the AdoMet/Dam complex is evidently not the active form necessary for methylation of the DNA to occur. The enzyme first undergoes a conformational rearrangement as monitored by the change in chemical shift of the methyl group. While we could observe this process during 15 min, it is 80% complete after 5 min at 4°C. By gel electrophoresis, we have previously observed that binding of a GATC-containing oligonucleotide in the presence of AdoMet induces a conformational change in Dam involved in the ternary AdoMet/Dam/GATC site complex to produce a more compact protein. This conformational change was not induced by nonspecific DNA (5). After rearrangement, the magnetic environment of the AdoMet is very similar to that in the binary complex. The process that we observe during the first 20 min could be the fit-induced rearrangement of the protein around the GATC site to produce the active complex.

In the subsequent 10 spectra, each lasting 5 min 20 sec, the chemical shift remains constant and a decay in the intensity of the resonance of AdoMet bound at the allosteric site is observed. From the time course of the decay we can estimate the half-time of the reaction as $\approx$30 min. In these spectra, we are not yet able to observe the oligonucleotide products. After 60 min, $\approx$75% of the ternary complex has disappeared and $\approx$30% of the unreacted AdoMet is observed at the chemical shift of free AdoMet (Fig. 3*a*). This shows that no more than 8% of the AdoMet has been displaced by sinefungin and that the methyl group must have been transferred to the GATC duplex. We do not observe these methyl groups because the acquisition time is too short. The reaction was allowed to continue and a spectrum was recorded over 15 hr (Fig. 3*b*). The resonances corresponding to the methylated oligonucleotide are now observed. As seen before (Fig. 1*D*), the methyl group exists in a number of magnetic environments. Resonances are observed for free AdoMet and also for AdoMet in the allosteric site, but these are absent in subsequent spectra. Sinefungin considerably slows down the methylation reaction that we have been able to observe over at least 1 hr, but, more importantly, it has allowed us to monitor the premethylation rearrangement. If methyl transfer occurred directly from the observed AdoMet site to the DNA, it would not be possible to explain the inhibitory role of sinefungin. AdoMet must therefore pass through another environment, and in this environment sinefungin is an efficient competitor.

This other environment, the catalytic site, cannot arise via a rearrangement of the allosteric site as we do not observe a large and rapid displacement of AdoMet from the enzyme. We observe very little free AdoMet and that only when the reaction is largely complete; that is, when the ratio of

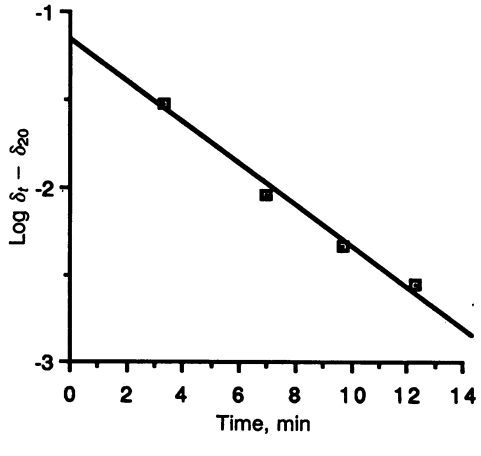

FIG. 2. Semilogarithmic plot showing the evolution of the difference in chemical shift of AdoMet at time t relative to that after 20 min as a function of time.

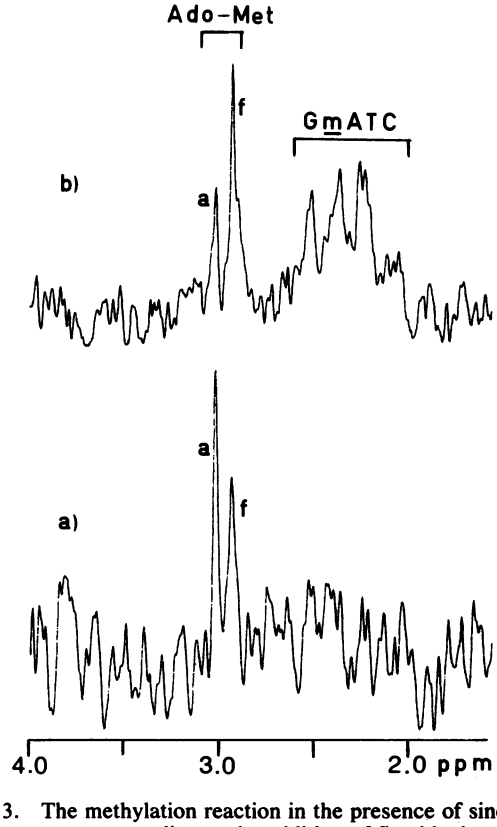

FIG. 3. The methylation reaction in the presence of sinefungin. (*a*) Spectrum corresponding to the addition of five blocks recorded between 30 and 60 min after the reaction was started. (*b*) Spectrum begun 1 hr after the reaction was started and lasting 15 hr.

inhibitor (sinefungin plus *S*-adenosylhomocysteine that has been produced) to AdoMet is very high. Our observations are in accord with the hypothesis of the existence of a second binding site for AdoMet to fulfill the role of methyl donor. As the starting ratio of AdoMet/Dam is 1 and the K_d is 0.1 μM for AdoMet at the allosteric site, and both Dam and AdoMet are at a concentration of 12 μM, very little AdoMet in solution is free to compete for the catalytic site. Under these conditions, we do not see any free AdoMet as its concentration is too low to be detected, nor can we detect AdoMet in the catalytic site for the same reason. Although methylation still takes place, it is very much slowed down by the presence of sinefungin.

After 1 hr ≈75% of the AdoMet has reacted with the oligonucleotide. However, all the remaining AdoMet is not found bound in the allosteric site; ≈8% of the initial AdoMet concentration is observed as free AdoMet. We stated above that addition of a 5-fold excess of sinefungin to the binary AdoMet/Dam complex did not result in the observation of free AdoMet. At first slight, these results appear to be contradictory. With a K_d of 0.1 μM of AdoMet for its allosteric site, the observation that ≈30% of the AdoMet present after 1 hr is free must mean that most of the enzyme molecules are no longer able to bind AdoMet to the allosteric site, which is occupied by an inhibitor molecule.

At this time, the concentration of free AdoMet is ≈1 μM and that of bound AdoMet is ≈2 μm. From the K_d of 0.1 μM for the allosteric site, we can calculate the apparent concentration of free enzyme able to fix AdoMet at this site. This gives a concentration of ≈2 μM. The rest (≈9 μM) is no longer available for AdoMet binding. The total concentration of inhibitor present (sinefungin and the *S*-adenosylhomocysteine that has been produced) is 69 μM. Knowing the concentrations of free and inhibitor-bound enzyme and the inhibitor concentration, we can calculate the K_d for binding of the inhibitor to the allosteric site. Under the assumption that the catalytic site is saturated by inhibitor (12 μM), the free inhibitor concentration (57 μM) gives a K_d value of 11 μM for inhibitor binding at the allosteric site, which is 2 orders of magnitude higher than that for AdoMet binding. This explains why we do not observe free AdoMet early in the reaction when the ratio of sinefungin to AdoMet is ≈5.

In the absence of sinefungin, we did not observe a resonance corresponding to that of AdoMet in either the allosteric or the catalytic site. The chain of events following the addition of the oligonucleotide will be the same as that observed in the presence of sinefungin, except that methyl transfer will be much faster once the fit-induced rearrangement has taken place. The presence of sinefungin will not influence the rate of the fit-induced rearrangement. In this case, >50% of the ternary complex has undergone the rearrangement during the dead time before the acquisition can be started. By the end of the acquisition of the first block, >80% has rearranged and the intensity of the AdoMet resonance will have fallen below the noise level if the methylation reaction of the rearranged complex is fast. This is what we observe and it shows that in the absence of inhibitor the fit-induced rearrangement is the rate-limiting step for methylation, whereas in the presence of sinefungin, the methylation reaction was the slowest step. The appearance of a free AdoMet resonance during the long accumulation is due to the formation of a competitive inhibitor, the reaction product *S*-adenosylhomocysteine, which, when the AdoMet concentration becomes low, competes for binding as described above for sinefungin.

CONCLUSION

We have shown in this paper that the two roles of AdoMet in the process of methylation of GATC sequences by Dam are mediated by association of this cofactor at two different sites on the enzyme. First, AdoMet is fixed to a high-affinity allosteric site on the enzyme even in absence of the GATC duplex. For this site, a 5-fold excess of sinefungin is unable to displace AdoMet. After binding of the GATC oligonucleotide to the binary AdoMet/Dam complex, this initial ternary complex undergoes a fit-induced rearrangement of the protein around the GATC site and the AdoMet returns to an environment close to that of the binary complex. We have shown that the fit-induced rearrangement of the Dam around the GATC site results not only in an increased affinity for the site but, above all, in the formation of an active complex capable of methylating the DNA. The complex is now active and methylation begins. The inhibitory action of sinefungin requires that there is a second site of binding for AdoMet, a catalytic site for which sinefungin competes for binding with AdoMet.

We are grateful to Drs. K. Hülsmann and U. Hahn (Berlin) for the HB101/pDOX strain. This work was partially supported by a Twining Grant ST2.0034 from the European Community.

1. Hattman, S., Brooks, J. E. & Masurekar, M. (1978) *J. Mol. Biol.* **126,** 367–380.
2. Messer, W. & Noyer-Weidner, M. (1988) *Cell* **54,** 735–737.
3. Barras, F. & Marinus, M. G. (1989) *Trends Genet.* **5,** 139–143.
4. Herman, G. E. & Modrich, P. (1982) *J. Biol. Chem.* **257,** 2605–2612.
5. Bergerat, A. & Guschlbauer, W. (1990) *Nucleic Acids Res.* **18,** 4369–4375.
6. Newmark, R. D., Un, S., Williams, P. G., Carson, P. J., Morimoto, H. & Klein, M. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 583–587.
7. Geier, G. E. & Modrich, P. (1979) *J. Biol. Chem.* **254,** 1408–1413.
8. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–252.
9. Fazakerley, G. V., Téoule, R., Guy, A., Fritzsche, H. & Guschlbauer, W. (1985) *Biochemistry* **24,** 4540–4548.
10. Fazakerley, G. V., Quignard, E., Téoule, R., Guy, A. & Guschlbauer, W. (1987) *Eur. J. Biochem.* **167,** 397–404.