JOURNAL OF BACTERIOLOGY, Feb. 2003, p. 1284–1288 Vol. 185, No. 4
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.4.1284–1288.2003
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The Small Subunit of M·*Aqu*I Is Responsible for Sequence-Specific DNA Recognition and Binding in the Absence of the Catalytic Domain

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Received 12 August 2002/Accepted 16 November 2002

***Aqu*I DNA methyltransferase (M·*Aqu*I) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to the C5 position of the outermost deoxycytidine base in the DNA sequence 5′-CCCGGG-3′. M·*Aqu*I is a heterodimer in which the polypeptide chain is separated at the junction between the two equivalent structural domains in the related enzyme M·*Hha*I. Recently, we reported the subcloning, overexpression, and purification of the subunits (α and β) of M·*Aqu*I separately. Here we describe the DNA binding properties of M·*Aqu*I. The results presented here indicate that the β subunit alone contains all of the information for sequence-specific DNA recognition and binding. The first step in the sequence-specific recognition of DNA by M·*Aqu*I involves the formation of binary complex with the target recognition domain in conjunction with conserved sequence motifs IX and X, found in all known C5 DNA methyltransferases, contained in the β subunit. The α subunit enhances the binding of the β subunit to DNA specifically and nonspecifically. It is likely that the addition of the α subunit to the β subunit stabilizes the conformation of the β subunit and thereby enhances its affinity for DNA indirectly. Addition of *S*-adenosyl-L-methionine and its analogues *S*-adenosyl-L-homocysteine and sinefungin enhances binding, but only in the presence of the α subunit. These compounds did not have any effect on DNA binding by the β subunit alone. Using a 30-mer oligodeoxynucleotide substrate containing 5-fluorodeoxycytidine (5-FdC), it was found that the β subunit alone did not form a covalent complex with its specific sequence in the absence or presence of *S*-adenosyl-L-methionine. However, the addition of the α subunit to the β subunit led to the formation of a covalent complex with specific DNA sequence containing 5-FdC.**

It has been known that the DNA of most organisms contains minor bases. The most common minor bases are 5-methylcytosine (5-meC), *N*6-methyladenine (N6meA), and *N*4-methylcytosine (N4meC). The DNA of prokaryotes can possess 5-meC, N6-meA, and N4-meC, but higher eukaryotes contain 5-meC in their DNA. This modification is formed following replication, by enzymes known as DNA methyltransferases (MTases) that act on DNA duplex. All known DNA MTases use *S*-adenosyl-L-methionine (AdoMet) as a source of the methyl group (1). In prokaryotes, methylation of cytosine and adenine residues is primarily involved in restriction-modification systems, which serve to protect host cells from bacteriophage infection and invasion by foreign DNA. Restriction endonucleases selectively degrade exogenous DNA, while DNA MTase prevents degradation of host DNA (24). DNA of eukaryotic organisms is methylated at cytosine residues located at CpG-containing sequences and is associated with variety of biological roles, including gene expression, genomic imprinting, and X chromosome inactivation (20). There are currently three catalytically active mammalian DNA MTases, DNMT1, -3a, and -3b. (33). In eukaryotes, C5 DNA MTases methylate the 5-carbon of the pyrimidine ring of cytosine, creating 5-meC. In plant DNA, cytosine residues at CpXpG sequences are methylated in addition to CpGs (11). Methylation of these sites may involve more than one DNA MTase (8). Sequence comparison of nearly 50 bacterial C5 DNA MTases has shown that these enzymes share an overall common protein architecture. Ten conserved motifs (I to X), each 10 to 20 amino acids in length, have been identified, five of which are highly conserved (I, IV, VI, VIII, and X) (17, 23). Similar sets of motifs are found in the C terminus of mammalian CpG DNA MTases (5). The order of these motifs appears to be important, since of 45 sequences examined by Kumar et al. (15), the order is invariant. In addition, all of these enzymes have a hypervariable region lying between motifs VIII and IX called the target recognition domain, which is responsible for the specificity of DNA recognition and the choice of base to be methylated (2, 17, 21). The reaction catalyzed by DNA MTases involves the binding of substrate AdoMet and a specific recognition site. First, there appears to be a discrimination between the specific site to be methylated in the absence of methyl donor and AdoMet binding, which occurs when the MTase interacts specifically with its recognition site (27). All C5 DNA MTases are monomeric enzymes, with the exception of M·*Aqu*I and M·*Eco*HK31I. M·*Eco*HK31I consists of two polypeptides, and all of the conserved motifs in C5 MTases can be found in polypeptide α, except motif IX, which is present in polypeptide β. Both polypeptides are required for in vitro methylation (18, 19).

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TABLE 1. Oligodeoxynucleotides used in this study

Oligodeoxynucleotide	Sequence[a]
AQ7/8 (specific duplex)	5′-GTACGAGCAGCT**CCCGGG**TCAGTCTGCCTA-3′
	3′-CATGCTCGTCGA**GGGCCC**AGTCAGACGGAT-5′
Msp5/6 (nonspecific duplex)	5′-CATGCTCGTCGACGGCCGAGTCAGACGGAT-3′
	3′-GTACGAGCAGCTGCCGGCTCAGTCTGCCTA-5′
AQ8/9F (specific fluoro-substituted duplex)	5′-TAGGCAGACTGA**CCCGGG**AGCTGCTCGAAC-3′
	3′-ATCCGTCTGACT**GGGCC^{F}C**TCGACGAGCTTG-5′

[a] Underlined sequences are sequence-specific recognition sites for M·*Aqu*I. ^{F}, position of 5FdC.

M·*Aqu*I recognizing the palindromic sequence 5′-CCCGG G-3′ was identified from *Agmenellum quadruplicatum*. DNA sequencing studies revealed two open reading frames, ORFα and ORFβ, which encode the M·*Aqu*I C5 MTase (12). ORFα encodes a 248-amino-acid polypeptide, while ORFβ codes for a 139-amino-acid polypeptide. ORFα corresponds to the N terminus, while ORFβ corresponds to the C terminus of a typical C5 DNA MTase. All 10 motifs are present in the combined polypeptides (motifs I to VIII on the α polypeptide and TRD plus IX and X on polypeptide β). M·*Aqu*I shows remarkable sequence similarities to other C5 DNA MTases. According to the crystal structure of M·*Hha*I (6, 14), the α subunit corresponds to the catalytic domain, while the β subunit corresponds to the DNA binding domain.

We have previously described the subcloning, overexpression, and purification of the separate subunits (α and β) of M·*Aqu*I as His-tag fusions and shown the reconstitution of an active enzyme from the individual subunits (22).

Here we describe the nature of the DNA binding and methylation properties of M·*Aqu*I with independently purified α and β subunits. The effect of cofactor AdoMet and the AdoMet analogues *S*-adenosyl homocysteine (AdoHcy) and sinefungin on the binding of M·*Aqu*I to DNA has also been examined. In addition, the incorporation of cytidine analogue 5-fluorodeoxycytidine (5FdC) into oligodeoxynucleotides allowed us to examine the binding properties of the β subunit of M·*Aqu*I to cognate DNA and the influence of the α subunit upon this binding.

MATERIALS AND METHODS

Reagents and enzymes. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham, and AdoMet, AdoHcy, and sinefungin were obtained from Sigma. Bacteriophage T4 polynucleotide kinase was purchased from NEB (United Kingdom). Unless otherwise stated, all other methods were performed as described by Sambrook et al. (25).

Radiolabeling of oligodeoxynucleotides and gel retardation assay. Oligodeoxynucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer, model 392. The sequences of the oligodeoxynucleotides used in this study are given in Table 1. The oligodeoxynucleotides were annealed by being heated to 90°C followed by slowly cooling to room temperature and then were end-labeled with [γ-^{32}P]ATP by using the T4 polynucleotide kinase reaction. Unincorporated radioactivity was removed by gel filtration with G25 resin, and the spin column procedure (25). In this work, His-tag fusion α and β subunits of M·*Aqu*I were used. Overexpression and purification of the separate subunits as His-tag fusions were performed as described in detail elsewhere (22). Reaction mixtures containing reaction buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1 mM dithiothreitol), a specified amount of protein samples, and oligodeoxynucleotide duplexes, as described in the figure legends, were incubated at room temperature for 1 h, mixed with the appropriate volume of agarose gel loading buffer, and run on a 6% nondenaturing polyacrylamide gel in Tris-borate-EDTA (TBE) buffer, electrophoresed at 100 V, and autoradiographed.

RESULTS

In order to determine whether specific nucleoprotein complexes are formed by the β subunit, a competition assay was performed with unlabeled specific and nonspecific oligodeoxynucleotides. To determine specificity of binding, gel retardation assays were performed as outlined in Materials and Methods. The β subunit was incubated with a fixed amount (1 ng) of labeled specific duplex and a range of concentrations of unlabeled specific duplex in the ratios 1:0.01, 1:0.1, 1:1, 1:5, 1:10, 1:50, and 1:100. The nucleoprotein complex formed with the specific duplex was reduced in the presence of a 10-fold excess of unlabeled specific duplex (Fig. 1A, lane 7). In contrast, gel retardation experiments done with unlabeled nonspecific duplex (Msp5/6) in the ratios 1:0.01, 1:0.1, 1:1.0, 1:5, 1:10, 1:50, and 1:100 show that the β subunit remained bound to the labeled specific duplex even in the presence of 100-fold excess of nonspecific competitor DNA (Fig. 1B, lane 9). The results shown in Fig. 1 illustrate that the β subunit is capable of sequence-specific DNA recognition, and they confirm that the discrimination between specific and nonspecific duplexes is between 10- and 100-fold.

The influence of the α subunit on DNA recognition by the β subunit. Oligodeoxynucleotides containing a specific recognition site for M·*Aqu*I (AQ7/8) and both α and β subunits were included in the binding reaction mixtures. In the absence of a nonspecific competitor DNA species, poly(dI-dC), the addition of α subunit appears to induce the formation of a supershifted complex (Fig. 2, lanes 4 and 5). However, in the presence of a 1,000-fold excess of nonspecific DNA poly(dI-dC), the effect of the α subunit is clarified. Thus, in lanes 8 and 9, which are comparable to lanes 4 and 5, the presence of the competitor DNA reveals that the α subunit serves to enhance DNA recognition, both nonspecifically (lanes 4 and 5) and specifically (lanes 8 and 9). Whether the α subunit is present in the complex with the β subunit is not clear in this experiment.

The influence of cofactor and cofactor analogues on DNA binding of M·*AquI*. The effect of AdoMet and its analogues AdoHcy and sinefungin on the binding of M·*Aqu*I to the cognate DNA sequence was examined. Experiments were carried out with the β subunit alone and the α and β subunits in the same reaction mixture. The concentration of AdoMet, AdoHcy, and sinefungin was 300 μM. As can be seen in Fig. 3A, lane 2, specific binding of the β subunit to DNA does not require the α subunit or any cofactor. Moreover neither AdoMet nor its analogues AdoHcy and sinefungin have any observable effect upon this binding (lanes 3 to 5). However, where the α subunit was included in the reactions with the β subunit (Fig. 3B, lanes 1 to 3), the observed bands were sig-

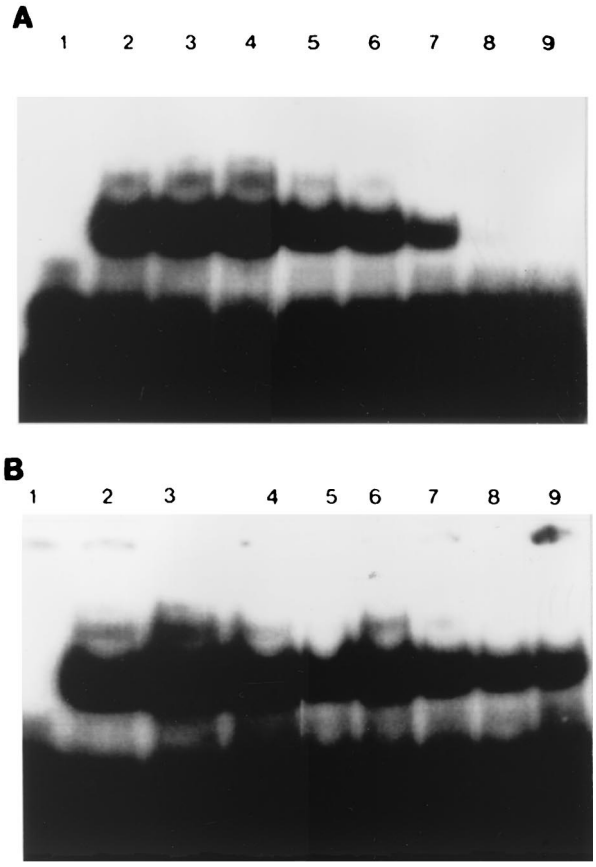


FIG. 1. (A) Sequence-specific DNA recognition by M · *Aqu*I β subunit determined by a competition assay done with unlabeled specific duplex (AQ7/8) Lanes: 1, AQ7/8; 2, AQ7/8 plus β; 3 to 9, as lane 2, but in the presence of 0.01-, 0.1-, 1-, 5-, 10-, 50-, or 100-fold excess of unlabeled AQ7/8, respectively. In all lanes, the concentration of labeled specific oligonucleotide (AQ7/8) was fixed at 1 ng (final concentration, 0.038 μM) and the concentration of β subunit was 0.1 μg (final concentration, 0.04 μM). (B) Sequence-specific DNA recognition by M · *Aqu*I β subunit determined by a competition assay done with unlabeled nonspecific duplex (Msp5/6). Lanes: 1, AQ7/8; 2, AQ7/8 plus β subunit; 3 to 9, as lane 2, but in the presence of 0.01-, 0.1-, 1-, 5-, 10-, 50-, or 100-fold excess of unlabeled Msp5/6, respectively. In all lanes, the concentration of labeled specific oligonucleotide (AQ7/8) was fixed at 1 ng (final concentration, 0.038 μM) and the concentration of β subunit was 0.1 μg (final concentration, 0.04 μM).

nificantly enhanced, suggesting that AdoMet, AdoHcy, and sinefungin stimulate the binding of the MTase to the specific DNA via the α subunit. AdoMet, AdoHcy, and sinefungin influenced the binding of M · *Aqu*I in an identical manner, as judged by the intensity of the retarded bands.

Binding to 5-FdC-containing DNA. In order to investigate whether M · *Aqu*I forms a covalent complex with an oligodeoxynucleotide duplex containing 5-FdC, AQ9F was synthesized in which the outermost cytosine base of the M · *Aqu*I recognition sequence was replaced by a 5-fluoro derivative (Table 1). Covalent complex formation is dependent upon the methylation reaction proceeding up to the β subunit elimination stage (7). The β subunit was incubated with labeled AQ8/9F in the presence and absence of AdoMet for 1 h at 37°C. Reactions were stopped by addition of an equal volume of sodium dodecyl sulfate loading buffer, boiled for 5 min, and run on a denaturing polyacrylamide gel. Following electrophoresis, the complex was visualized by autoradiography. It can be seen in Fig. 4 that covalent complex formation occurred only when both the α and the β subunits and cofactor AdoMet were present in the reactions (Fig. 4, lane 4).

DISCUSSION

The studies presented above describe the binding of M · *Aqu*I to DNA under a variety of conditions. They clearly indicate that the β subunit alone contains all of the information required to generate the discrimination between nonspecific and specific DNA duplexes in the absence of the α subunit and cofactor. The weak discrimination observed by DNA MTases against nonspecific DNA probably reflects the fact, unlike transcriptional modulators, these DNA binding proteins are able to couple thermodynamic recognition to catalysis. In the case of the sequence-specific enzymatic reaction catalyzed by R · *Eco*RV, equilibration of DNA at the active site is completely nonspecific; specificity in this case is intimately coupled to the formation of the ternary complex with magnesium ions around the scissile bond prior to hydrolysis (28, 31). In the case of the C5 MTase M · *Msp*I, the formation of the ternary complex between the recognition sequence and methyl donor generates a 20-fold enhancement of sequence-specific discrimination. We have observed similar effects in the case of M · *Aqu*I, but this enzyme has enabled us to dissect the two components of DNA sequence specificity. The structure of M · *Hha*I has revealed that sequence-specific DNA recognition by such enzymes is mediated by the C-terminal half of the protein and the fully functional recognition site (6). In the case of M · *Aqu*I, the β subunit includes the target recognition domain together with homology blocks IX and X, including the C-terminal helix-loop-helix region.

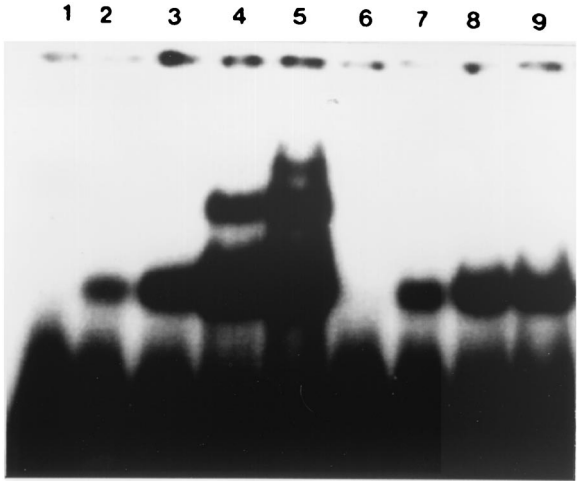


FIG. 2. The influence of the α subunit on DNA recognition by the β subunit determined by gel retardation analysis. Lanes: 1, AQ7/8 (0.038 μM); 2 and 3, as lane 1, but in the presence of 0.02 and 0.04 μM β subunit, respectively; 4 and 5, as lane 3, but in the presence of 0.01 and 0.02 μM α subunit, respectively; 6, as lane 3, but in the presence of 1 μM poly(dI-dC); 7 to 9, as lane 2, but in the presence of 0.01, 0.02, and 0.04 μM α, respectively, and poly(dI-dC).

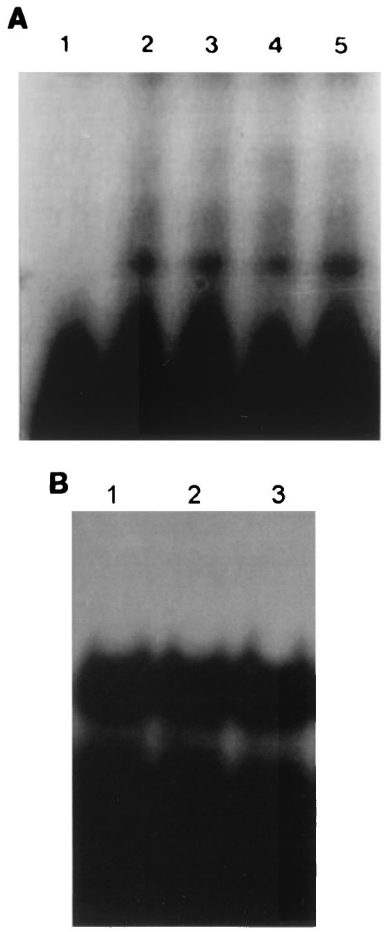


FIG. 3. The influence of AdoMet, AdoHcy, and sinefungin on the binding of M · *Aqu*I to DNA. (A)Lanes: 1, AQ7/8; 2, as lane 1, but in the presence of 0.02 μM β subunit; 3 to 5, as lane 2, but in the presence of AdoMet, AdoHcy, and sinefungin, respectively. (B) Lanes: 1 to 3, AQ7/8 plus 0.0.1 μM β subunit plus 0.01 μM α subunit and in the presence of AdoMet, AdoHcy, and sinefungin, respectively.

Primary sequence alignments between C5 DNA MTases have pointed to a major role for the hypervariable region of these enzymes in sequence-specific DNA recognition. However, the results of domain exchange experiments between M · *Msp*I, M · *Hha*I and M · *Hpa*II (13, 21) suggest that the monospecific enzymes are less flexible in coupling DNA recognition and catalysis than the multispecific C5 MTases. Indeed, the domain exchange experiments with the multispecific C5 MTases reported by Walter et al. (29) suggest that the hypervariable region alone may be an independently folded DNA binding domain.

This series of experiments demonstrate that the first stage in sequence-specific recognition of DNA by M · *Aqu*I involves the formation of a DNA binary complex with the target recognition domain in conjunction with conserved elements IX and X, all of which are contained within the β subunit. Reinforcement of this recognition is most likely a consequence of the stabilization of this subunit by the α polypeptide and the formation of a ternary complex with the methyl donor, as has been demonstrated for M · *Msp*I (9). The results presented here indicate the α subunit serves to enhance DNA recognition, both specifically and nonspecifically. The α subunit appears to enhance the nonspecific recognition by M · *Aqu*I by one of two ways: by stimulation of binding of the β subunit or by forming an α subunit-DNA binary complex that migrates to the same position in our gel retardation assays. The enhancement of β subunit-specific DNA recognition by the α subunit is likely to be a consequence of either stabilization of the β polypeptide or acting cooperatively with it in DNA recognition. A similar situation was observed with the DNA MTase component of the type I restriction enzyme *Eco*RI24 when the DNA binding subunit, HsdS, was incubated with the modification subunit, HsdM (16). The results reported in this paper also indicate that the β subunit of M · *Aqu*I binds to DNA specifically in the absence of cofactor. Moreover the presence of cofactor does not affect the interaction of the β polypeptide to its cognate DNA sequence. However, since the α subunit contains most of the AdoMet binding site and the catalytic cysteine (C81 in M · *Hha*I), AdoMet and its analogues, AdoHcy and sinefungin, enhance the binding of M · *Aqu*I to cognate DNA. AdoMet has been shown to have a dual role as allosteric effector and methyl donor for the A-N6 MTase Dam of *Escherichia coli* (3, 4). Friedman (10) has shown that covalent complex formation of M · *Eco*RII and M · *Hha*I, but not M · *Msp*I and M · *Hpa*II, with 5-azaC-containing DNA is stimulated by AdoMet. The presence of AdoMet is essential for complex formation. A separate study of M · *Hha*I by Santi et al. (26) showed formation of a stable complex between M · *Hha*I and 5-azaC-containing DNA; the presence of AdoMet did not appear to affect either the formation or stability of this complex. A detailed kinetic study of M · *Hha*I (32) indicated that AdoMet bound to the protein-DNA complex, and following methyl transfer, AdoHcy was released prior to the dissociation of the enzyme from the methylated duplex. In the case of M · *Msp*I (9), DNA binding was promoted by AdoMet or the analogues AdoHcy and sinefungin; in the absence of cofactors, only very weak binding was observed. These results, in conjunction with the results presented here, also indicate that methyl transfer is not an integral part of the binding process. This is illustrated by the fact that both sinefungin and AdoHcy stimulate the binding of

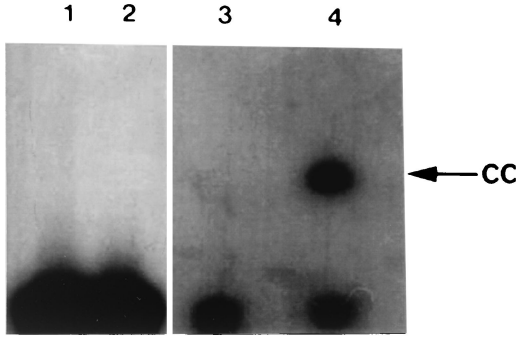


FIG. 4. Covalent complex formation of M · *Aqu*I with 5FdC-containing DNA. Lanes: 1, AQ7/8F plus 0.02 μM β subunit plus 1% SDS; 2, as lane 1, but in the presence of 300 μM AdoMet; 3, AQ7/8F plus 0.01 μM β subunit plus 0.01 μM α subunit plus 1% SDS; 4, as lane 3, but in the presence of 300 μM AdoMet. Samples were boiled and then analyzed by SDS-polyacrylamide gel electrophoresis. CC, covalent complex.

the MTase to a specific sequence. AdoHcy and sinefungin have essentially the structural features of AdoMet, the only changes being at the sulfonium center.

The results obtained by using a 5-FdC-containing oligonucleotide in gel retardation assays revealed that covalent complex formation between M·*Aqu*I and specific DNA sequences occur. Covalent complex formation between M·*Aqu*I and 5-FdC-containing DNA occurred only in the presence of cofactor AdoMet. Moreover, β subunit alone did not form a covalent complex either in the presence or absence of AdoMet. Since the α subunit contains the AdoMet binding site in this particular enzyme, addition of this subunit to the reaction led to formation of covalent complex in the presence of AdoMet. This observation demonstrates that 5-FdC inhibits the regeneration of active enzyme before the β subunit elimination step (30).

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