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Crosslinking of Dam methyltransferase with *S*-adenosyl-methionine

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Highly purified DNA-adenine methyltransferase was irradiated in the presence of different concentrations of radiolabelled *S*-adenosyl-methionine (AdoMet) with a conventional Mineralight UV-lamp from several minutes up to 1 h while incubating in ice. Incorporation of radioactivity was monitored by electrophoresis of the crosslink between *S*-adenosyl-methionine and Dam methylase on SDS-polyacrylamide gels followed by fluorography. Crosslinking reached a maximum in presence of 10 μM *S*-adenosyl-methionine; it was inhibited in the presence of substances which competitively inhibit methylation of DNA by Dam methylase, like sinefungin or *S*-adenosyl-homocysteine, but not in the presence of non-inhibitors like ATP or *S*-isobutyl-adenosine. The crosslink obtained was resistant against a wide range of even drastic conditions commonly used in protein and peptide chemistry. Proteins, which do not bind *S*-adenosyl-methionine, as well as heat inactivated Dam methylase were not photolabelled. After limited proteolysis the radioactive label appeared only in certain of the peptides obtained. From Western blots carried out with polyclonal antibodies produced against a synthetic peptide corresponding in its sequence to amino acids 92–106 of the Dam methylase, the crosslinking of AdoMet could be tentatively mapped at a position after amino acid 106.

Dam methylase; *S*-Adenosyl-methionine; Photocrosslinking; Sinefungin; Proteolysis

1. INTRODUCTION

Dam methylase of *E. coli* recognizes specifically the sequence GATC and transfers a methyl group from AdoMet to the amino group of adenine [1]. Many systems involved in e.g. postreplicative mismatch-repair, replication, transcription of certain genes, transposition and segregation of the chromosome are regulated by the state of methylation of the GATC-site *via* activating or inhibiting DNA-binding of other proteins [2,3].

Although the biological role of Dam methylase is partially understood, very little is known so far about its structure and mechanism. Dam methylase has a molecular mass of 32 kDa and acts as a monomer. Only one methyl group is transferred to the GATC-site per binding event [4] and the next 3 base pairs flanking the site both at the right and left modulate the rate of methylation [5]. Recently it was shown that AdoMet serves, besides its role as a substrate for the methylation reaction, as an allosteric activator which stimulates Dam-binding to its target sequence GATC [6]. To fulfill these two different roles AdoMet binds with very different affinities to two different binding sites, as shown by tritium-NRM [7].

A widely used technique to undertake studies on the molecular mechanism of protein–substrate interactions is to link the components covalently and irreversibly by UV-irradiation [8,9]. Covalent complexes from methylases and other AdoMet-dependent enzymes with either the natural occurring substrate [10–13] or the photoactivable 8-azido derivative [14–16] have been described in the literature. In two cases it was possible to isolate and characterize regions of DNA methyltransferases involved in AdoMet binding [16,17].

To study the mechanism of AdoMet binding to Dam methylase we have searched for suitable conditions to obtain crosslinks with Dam and its substrate, either radioactively labelled with ^{3}H on the methyl group or with ^{14}C at positions 3 and 4 in the methionine, with low-dose UV-irradiation to assure only a minimal disturbance of Dam methylase structure during the experiment. We have obtained crosslinks with both substrates which were stable under a wide range of conditions. Their specificity was proven by experiments taking into account several aspects: heat inactivated Dam methylase does not react with AdoMet; the crosslink reaction is inhibited by competitive inhibitors

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Abbreviations: AdoMet, *S*-adenosyl-methionine; AdoHcy, *S*-adenosyl-homocysteine; SIBA, *S*-isobutyl-adenosine; [^{3}H]AdoMet, $^{3}H_3C$-*S*-adenosyl-methionine; [^{14}C]AdoMet, *S*-adenosyl[3,4-^{14}C]methionine; Dam methylase, DNA-adenine-methyltransferase; DTT, dithiothreitol.

of the methylase reaction; saturation of labelling was reached in the presence of 10 μM AdoMet. Immunological detection of the proteolytic fragments obtained after limited proteolysis of crosslinked Dam methylase was carried out with antibodies produced against a synthetic peptide corresponding to the amino acid sequence from position 92–106 of Dam methylase. This permitted a tentative mapping of the crosslink reaction site after position 106.

2. MATERIALS AND METHODS

2.1. *Materials*

Dam methylase was purified from the overproducing strain HB 101 (pDOX) [18] to >98% purity as estimated from silver-stained SDS-polyacrylamide gels [19,20]. Concentration of purified enzyme was determined spectophotometrically using ϵ_{278} = 1.165 [21] calculated from the Trp and Tyr content of Dam methylase [22]. The enzyme had a specific activity of about 800 000 U/mg [23].

^3H_3C-*S*-adenosyl-methionine ([^{3}H]AdoMet, 15 Ci/mmol) and ^{14}C-labelled rainbow-coloured protein molecular weight markers were purchased from Amersham. *S*-adenosyl-*L*[3,4-^{14}C]methionine ([^{14}C]AdoMet, 59 mCi/mmol) was from C.E.A.. *S*-adenosyl-methionine (AdoMet) and *S*-adenosyl-homocysteine (AdoHcy) were from Boehringer Mannheim, sinefungin and *S*-isobutyl-adenosine (SIBA) were generous gifts from Dr M. Géro (CNRS, Gif-sur-Yvette, France). All proteases used were purchased from Boehringer Mannheim.

2.2. *Crosslinking experiments*

Indicated amounts of ^{3}H-labelled or non-labelled AdoMet and its analogues were pipetted into Eppendorf-tubes, HCl was removed by evaporation in a Speed vac. This treatment does not affect the stability of AdoMet as proved by Dam methylase activity tests carried out with dried substrate. H_2SO_4 was neutralized with $BaCO_3$, and $BaSO_4$ removed by centrifugation prior to lyophilization. Dam methylase solution was added to the dried material and samples were preincubated on ice for 10 min (sample volume was always 20 μl). They were spotted into microtiter plates precooled on ice. Irradiation with UV-light was carried out for the indicated times placing a Mineralight handlamp (4 W, 254 nm) directly on the plate, while keeping the temperature of the irradiated samples always around 0°C. Buffer conditions were 50 mM K/Na-phosphate pH 7.6, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, and 5% glycerol. DTT is known to hinder crosslink by its radical scavenging capacity, but did not affect the reaction in this case as verified by varying DTT concentrations.

2.3. *Gel electrophoresis, fluorography*

Gel electrophoresis was carried out according to Laemmli [19] on 12.5 and 15% polyacrylamide gels, respectively. Gels were Coomassie-blue stained destained as usual. Gels of radioactively labelled enzyme were either stained/destained or fixed in 25% isobutylalcohol/10% acetic acid prior to impregnation with fluorographic reagent (Amplify; Amersham). Dried gels were exposed to Kodak Xomat S films with one Cromex HI plus intensifying screen for 2–5 days.

2.4. *Limited proteolysis*

The AdoMet/Dam methylase complex was subjected to enzymatic digestion under limited conditions. Buffer conditions were as described under crosslink experiments, except for trypsin where an elevation of phosphate buffer concentration to 200 mM led to a remarkable stabilization of the generated fragment. Proteases were added to indicated enzyme/substrate ratios and reactions were carried out at 4°C for the indicated time intervals. Higher temperatures resulted in fast degradation of Dam methylase without leading to a reproducible pattern of proteolysis products on the SDS-gel indicating a destabilization of protein structure. The enzyme is known to loose its activity rapidly at temperatures above 4°C. Reactions were stopped by adding phenylmethyl-sulfonyl fluoride to a final concentration of 1 mM and freezing samples immediately at −20°C.

2.5. *Western blot*

Proteins were blotted from SDS-polyacrylamide gels onto polyvinyl-difluorid-membranes (Millipore) using the Millipore SDE semidry electroblotting system (Millipore). The membrane was saturated with 3% bovine serum albumine in TBS-buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), washed for 1 min in TBS and incubated for 2 h with polyclonal antibodies (produced against a synthetic peptide with the sequence YQFREEFNKSQDPFR corresponding to the sequence 92–106 of Dam methylase, supplied by Prof. M. Marinus, Univ. of Massachussetts) in TBS plus 0.05% Tween 20. After three 5-min washes in TBS, the membrane was incubated for 2 h with alkaline phosphatase conjugated swine immunoglobulins to rabbit immunoglobulins (Dakopats, Copenhagen). After three additional washes the membrane was stained with 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue tetrazolium essentially as described by Blake et al. [24].

3. RESULTS AND DISCUSSION

3.1. *Specificity of the crosslink*

The yield of crosslink obtained depended on the time interval of irradiation in a linear manner up to one hour, while longer irradiation did not lead to further labelling (Fig. 1). Under the conditions used in these experiments Dam methylase lost only half of its activity in one hour of irradiation (not shown). Irradiation induced crosslinks between AdoMet and Dam methylase occurred only when both components were present in the solution in intact form. If either Dam methylase or AdoMet were irradiated prior to addition of the other component no labelling was observed (not shown). Other proteins which have no affinity for AdoMet, like phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin were not labelled, nor was Dam methylase inactivated by heating at 37°C for 15 min prior to addition of AdoMet and irradiation (not shown). In the presence of 100-fold excess of unlabelled AdoMet the yield of radioactive crosslink obtained was drastically reduced while the presence of the same concentration of

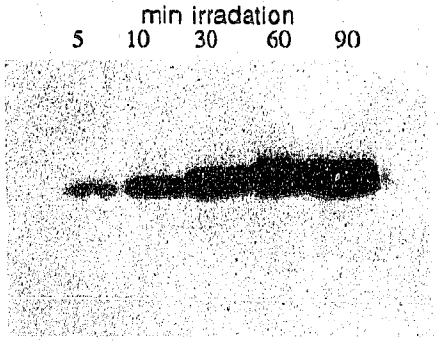

Fig. 1. Time dependence of crosslink reaction. 10 μM [^{3}H]AdoMet and 5.7 μM Dam was irradiated for various time intervals as indicated.

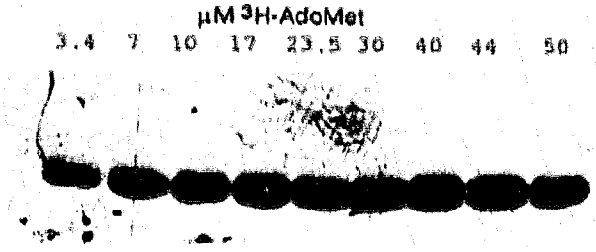

Fig. 2. Influence of AdoMet concentration. 110 pmol (5.7 μM) of Dam methylase were irradiated for 60 min in presence of different concentrations of [³H]AdoMet as indicated.

ATP did not affect the crosslink reaction (not shown).

To study the dependence of the crosslinks on [³H]AdoMet concentration, 110 pmol (5.7 μM) of Dam methylase was irradiated in the presence of various concentrations of [³H]AdoMet from 3.4–50 μM (Fig. 2). With less than 10 μM [³H]AdoMet the yield of crosslink depended on substrate concentrations, while higher concentrations did not lead to further labelling. This indicates that covalent attachment of the substrate to the enzyme depended upon the formation of a specific complex.

Complex formation was inhibited by sinefungin and AdoHcy. Both substances are competitive inhibitors for the methylase reaction [6], while SIBA, a specific inhibitor of certain eucaryotic cytosine-methyltransferases [25] had no influence on the kinetics of DNA methylation by Dam methylase [6] and showed only very little influence on the yield of crosslink formation (Fig. 3). At the present state we can not decide whether crosslinking of AdoMet takes place in the catalytic or in the allosteric site of the enzyme [6,7].

To demonstrate that radioactive labelling was due to the covalent attachment of the whole AdoMet molecule and not the result of methylation of Dam methylase by AdoMet, experiments were carried out with AdoMet ^{14}C-labelled in positions 3 and 4 of methionine. The yield of crosslinks depended upon the time of irradiation and the AdoMet concentration in a manner similar to the one observed using AdoMet ^{3}H-labelled in the methyl group (Fig. 4). The lower signal on the film in Fig. 4 is due to a specific activity used in these experiments about 250 times less compared with that of [³H]AdoMet. These experiments establish the covalent binding of probably the entire AdoMet molecule to the enzyme.

3.2. *Stability of the crosslink*

The crosslinked product obtained remained stable under conditions commonly used for peptide purification (precipitation with trichloracetic acid, presence of hydrophobic solvents like acetonitrile) as well as prolonged incubation with proteases in several buffers. Boiling of samples for 3 min in sample buffer [19] prior to loading them onto SS-gels did not affect the intensity of the band observed in fluorogram compared with a non-boiled sample (Fig. 5, fluorogram, lane 4).

3.3. *Limited proteolysis*

For a preliminary mapping of the crosslink site, crosslinks of Dam methylase with AdoMet and [³H]AdoMet were subjected to limited proteolysis with proteases of several specificities. Analysis was either by fluorography or immunological detection in a Western blot, carried out with polyclonal antibodies produced against a synthetic peptide corresponding to a large part of region II [27] of the amino acid sequence of Dam methylase (see legend of Fig. 5). The limited digestion by various proteases suggests that Dam methylase possesses a domain structure where the larger fragment is about 20–21 kDa. A second, 12 kDa fragment is visible on the Coomassie-stained gel in the elastase and papain digestion. The same pattern was observed in thermolysin digestion (not shown).

Tryptic digestion led to only one fragment of about 29 kDa on the Coomassie-stained SDS-gel as well as on

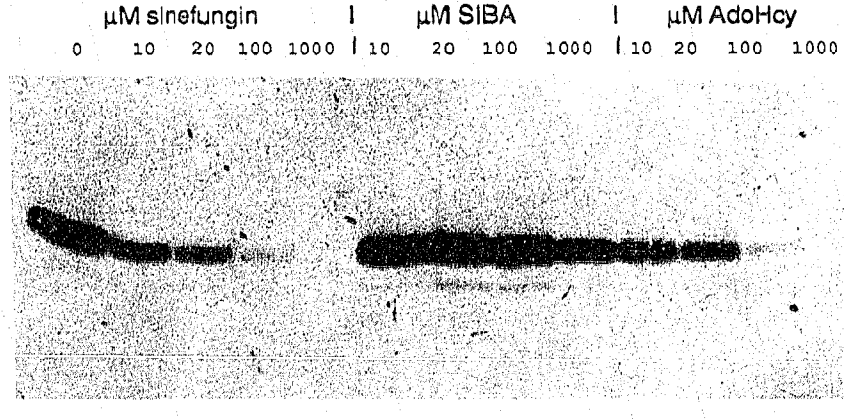

Fig. 3. Crosslinking in the presence of specific inhibitors for methylation. Dam methylase (5.7 μM) and [³H]AdoMet (10 μM) were irradiated for 60 min in the presence of indicated amounts of competitive methylase inhibitors.

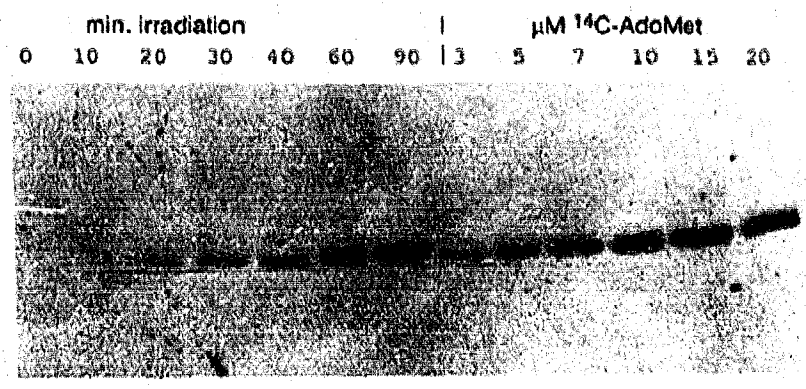

Fig. 4. Crosslinking in the presence of ^{14}C-labelled AdoMet. [^{14}C]AdoMet was photo-crosslinked with Dam methylase as a function of time (left) and of [^{14}C]AdoMet concentration (right). Dam concentration was always 5.7 µM, samples for studying the concentration dependence were irradiated for 60 min.

the more sensitive fluorography and Western blot. Papain digestion gave rise to two peptides of ca 21 and 19 kDa, which contain the AdoMet label and the antigenic site. Cleavage on either terminus is possible.

Elastase digestion yielded 3 peptides of ca 22, 19 and 17 kDa which contained the AdoMet label, but only the first reacted with the antibody. The 19- and 17-kDa peptides must derive from the 22 kDa domain. Whether the 22 kDa peptide contained the C-terminal (from amino acid ca 80 to 278) or the N-terminal (from amino acid 1 to ca 200), or neither (e.g. from amino acid 50–240) is presently unknown. A cut of 3 to 5 kDa to yield the 19- and 17-kDa peptides, respectively, however, eliminated the antigenic site. It is thus probable that the 22-kDa peptide is the C-terminal part and therefore, the AdoMet binding site is downstream of the antibody recognition site, i.e. after amino acid 106.

Several peptides were observed in the V8 digestion. Only the largest one (27 kDa) contained both the AdoMet label and the antigenic site. Since the next

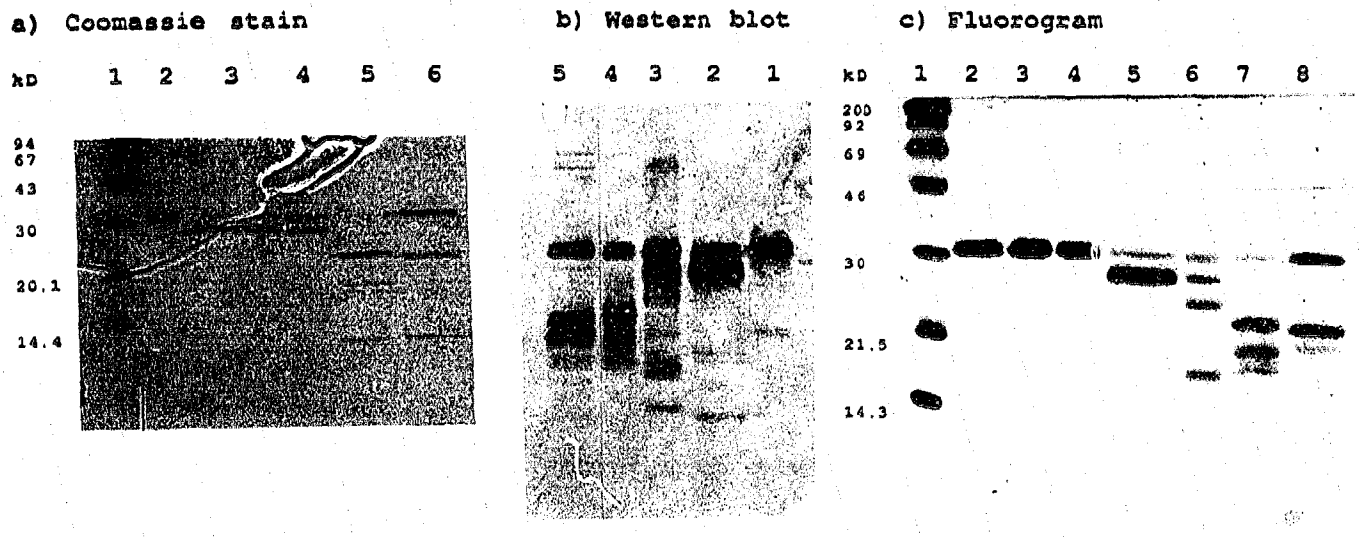

Fig. 5. Limited proteolysis of crosslinked enzyme with several proteases. Dam methylase crosslinked with [^{3}H]AdoMet (Fluorogram) or AdoMet (Coomassie-stained gel and Western blot) were digested with several proteases under limiting conditions. (a) Coomassie stain: lane 1, protein molecular weight markers; lane 2, Dam methylase; lane 3, tryptic digestion, enzyme/Dam = 1:100 (w/w), 4°C, 12 h; lane 4, V8 digestion, enzyme/Dam = 1:20 (w/w), 4°C, 36 h; lane 5, elastase digestion, enzyme/Dam = 1:100 (w/w), 4°C, 12 h; lane 6, papain digestion, enzyme/Dam = 1:100 (w/w), 4°C, 30 min. (Buffer conditions see section 2. (b) Western blot: polyclonal antibodies used for this experiment were produced against a synthetic peptide corresponding to the sequence 92–106 (YQFREEFNKSQDPFR) of Dam methylase. Lane 1, Dam methylase; lane 2, tryptic digestion; lane 3, V8 digestion; lane 4, elastase digestion; lane 5, papain digestion. (c) Fluorography: lane 1, ^{14}C-labelled rainbow-coloured protein molecular weight markers: lane 2, crosslinked Dam methylase; lane 3, crosslink, incubated at 4°C for 24 h; lane 4, crosslink, incubated and boiled in sample buffer for 3 min; lane 5, tryptic digestion; lane 6, V8 digestion; lane 7, elastase digestion; lane 8, papain digestion.

smaller peptide (23 kDa) did not appear on the Western blot, but contained the radioactive label, it appears that there is a cut *within* the antibody recognition site (the C-terminal peptide from position 100 to 278 has about 23 kDa). The AdoMet labelled 17-kDa peptide is derived from the 23-kDa fragment. Similar arguments as for the elastase reaction suggest that the AdoMet-labelled site is located downstream from amino acid 106, i.e. between amino acids 106 and ca 240. It is noteworthy that this fragment contains the two regions III and IV, highly conserved in all adenine methyltransferases [26,27]. Region IV has been suggested [27] to be the possible AdoMet-binding site.

The present data evidently do not allow further conclusions about the exact location of the AdoMet binding site. Purification of labelled peptides generated by limited and complete proteolysis, as well as their characterization by N-terminal sequencing and fast atom bombardment spectroscopy are under way. We are also investigating the possibility to obtain crosslinks by irradiation with an excimer laser.

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