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UV-induced cross-linking of proteins to plasmid pBR322 containing 8-azidoadenine 2′-deoxyribonucleotides

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An efficient method of cross-linking DNA to protein is described. The method is based on the incorporation of photoactive 8-azidoadenine 2′-deoxyribonucleotides into DNA. We have found that 8-N_3dATP is a substrate for *E. coli* DNA polymerase I and that 8-N_3dATP can be incorporated into plasmid pBR322 by nick-translation. Subsequently we were able to cross-link a set of different proteins to 8-azido-2′-deoxyadenosine-containing pBR322 by UV irradiation (366 nm). No DNA-protein photocross-linking was observed under the same conditions when the non-photoactive plasmid pBR322 was used.

Azidoadenine; Plasmid pBR322; DNA polymerase I; Nick-translation; DNA-protein cross-link; Ultraviolet irradiation

1. INTRODUCTION

The recognition of nucleic acid structures and their base sequences by proteins plays a central role in the regulation of gene expression. The majority of protein-nucleic acid interactions is based on electrostatic and hydrogen bonding as well as on stacking and hydrophobic phenomena [1]. So far no perfect method is known that allows the identification of contact regions and contact groups between proteins and nucleic acids without modifying or destroying the native shape and conformation of the complex. Native covalent bond formation appears to play only a minor role in gene regulation. It has been described for very few proteins only, e.g. for topoisomerases [2] and gyrases [3]. Experimental formation of covalent cross-links usually aims at freezing the position of the associated nucleic acid and protein molecules in order to reveal the interactions within their binding domains.

Various methods have been used to produce covalent cross-links between DNA and proteins: UV irradiation [4–6], γ-irradiation [7], chemical methods [8–10], and even vacuum or extreme dryness [11]. 5-Bromouracil-substituted DNA has been applied in order to lower the UV dose required to produce DNA-protein cross-links, because the Br–C bond is readily split by UV light [12,13]. However, the free radicals thus produced increase the cross-linking efficiency only unsatisfactorily [14].

Photoaffinity labelling experiments with 8-azidoATP have shown that the azido group is very efficient in forming covalent bonds to proteins through the UV-induced generation of reactive nitrene intermediates [15]. We therefore anticipated that the incorporation of 8-azidoadenine 2′-deoxyribonucleotides into DNA would provide a higher DNA-protein cross-linking rate than any other method. Earlier Evans et al. [14] stated that 8-azidopurine nucleotide analogs are not substrates for template-directed enzymatic synthesis of DNA or RNA, because in C8-substituted purine nucleotides the syn conformation prevails

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Abbreviations: 8-N_3dATP, 8-azido-2′-deoxyadenosine-5′-triphosphate; UV, ultraviolet

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[16]. This renders the nucleotides unacceptable as a substrate for *E. coli* DNA polymerase I [17] and other template-directed polymerases [18–20]. In view of these restrictive assumptions, Evans et al. [14] have incorporated 5-azidouracil 2′-deoxyribonucleotides into *lac* operator. Although they successfully photocross-linked the modified *lac* operator to its repressor, it must be noted that the evaluation of protein-nucleic acid interactions is in this case additionally complexed by the incorporation of an analog (azidopyrimidine versus pyrimidine) of an analog (uracil versus thymine).

Here we describe the successful synthesis of 8-N_3dATP and its incorporation into plasmid pBR322 by nick-translation (involving *E. coli* DNA polymerase I). This azidoplasmid is shown to be cross-linked to a number of different proteins by long-wave UV irradiation (366 nm). The cross-linking efficiency depends on various parameters as reported below.

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

Unlabelled deoxyribonucleotides, all proteins and enzymes were purchased from Boehringer Mannheim (DNase I spec. act. 2000 U/mg, DNA polymerase I spec. act. 5000 U/mg). α-^{32}P-labelled dCTP was obtained from Amersham Buchler. Plasmid pBR322 was prepared from *E. coli* cultures. All other chemicals were of the highest purity available from commercial sources.

2.2. *Synthesis of 8-N_3dATP*

8-N_3dATP (triethylammonium salt) was synthesized (24% yield) from dATP via 8-BrdATP principally following the synthesis of 8-azidoATP [21]. Synthesis conditions, in particular the characterization of 8-BrdATP and 8-N_3dATP will be described in detail elsewhere. 8-N_3dATP was stored frozen in aqueous solution, pH 7.0, at −20°C in the dark.

2.3. Production of azidoplasmid pBR322

Plasmid pBR322 was modified by simultaneously incorporating 8-N_3dATP and [α-^{32}P]dCTP (for radioactive labelling) using the nick-translation procedure [22,23]. The composition of the reaction medium was 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM dithiothreitol and 50 μg/ml bovine serum albumin. A typical 100 μl reaction mixture (preparation on ice) contained in addition: 17.3 pmol linearized pBR322, 50 nmol dTTP, 50 nmol dGTP, 500 pmol dCTP, 10 μCi of [α-^{32}P]dCTP (3000 mCi/μmol), 50 nmol 8-N_3dATP and 20 pg DNase I (prepared freshly by dilution of a stock solution of 1 mg DNase/ml in 0.15 M NaCl/50% glycerol). Non-photoactive plasmid (controls) was prepared accordingly, but 50 nmol of dATP were added instead of 8-N_3dATP. The reaction was started by adding 100 U *E. coli* DNA polymerase I (Kornberg enzyme) which contained definite amounts of DNase I (Boehringer Mannheim, No.104493). The reaction mixture was incubated for 1 h at 15°C in the dark. The reaction was stopped by the addition of EDTA (final concentration 20 mmol). Deoxyribonucleotides not incorporated during nick-translation were separated from photoactive ^{32}P-labelled pBR322 by gel filtration (Sephadex G-25, Amersham Buchler). Photoactive pBR322 was ethanol-precipitated, redissolved in twice distilled water and stored at −20°C in the dark.

2.4. *Photocross-linking*

Aqueous solutions containing the photoactive plasmid pBR322 (0.5 pmol) or the non-photoactive control (0.5 pmol) and the protein (10–50 pmol) to be cross-linked were prepared (typical volume 20–30 μl). The reaction mixtures were incubated at 37°C for 10 min and subsequently exposed to UV irradiation (5 s to 60 min) with a Mineralight ultraviolet lamp UVSL25. The emitted UV light (mainly 366 nm) allows the photoactivation of the azido group without any remarkable photodamage to DNA or protein. The lamp was placed above the samples at a distance of 3.8 cm. The fluence rate at the position of the sample was 4 J/m^2 per s. All solutions were kept under cover before and after photolysis and all subsequent steps were performed as quickly as possible.

2.5. *Gel electrophoresis and autoradiography*

SDS polyacrylamide gel electrophoresis of the samples was performed immediately after photocross-linking according to the procedure of Laemmli [24] with some variations. After adding 20 mg/ml bromophenol blue, the samples were loaded onto a SDS polyacrylamide gel of 5% polyacrylamide (separating gel) with an overlay of 3.5% polyacrylamide (stacking gel) containing 1% SDS, respectively. After the electrophoresis the gels were silver-stained according to Adams and Sammons [25], dried and exposed to X-ray films (Pharmacia, No.RPN 6) for 1 to 7 days at −70°C. The autoradiograms were developed with a Fuji X-ray film processor RG 2.

2.6. *Proteinase K digestion*

Immediately after the end of the photolysis procedure some samples were additionally digested by proteinase K. The reaction conditions were 1 mM Tris-HCl, pH 7.8, 0.5 mM EDTA. A typical 30 μl reaction mixture was prepared as follows: after photocross-linking, protein digestion was started by the addition of 100 pmol proteinase K. Incubation time was 1 h at 37°C in the dark. Subsequently the mixture was loaded onto a SDS polyacrylamide gel for analysis.

3. RESULTS

The structure of 8-N_3dATP (syn conformation) is shown in fig.1. The UV spectrum of 8-N_3dATP shows a typical red shift of the absorbance maximum (280 nm, pH 7) in comparison to dATP (259 nm, pH 7). A similar spectral shift has been observed for the couple 8-azidoATP/ATP [21].

Because of the C8-position of the azido group the syn conformation may prevail in 8-azidopurine 2′-deoxyribonucleotides as it does in

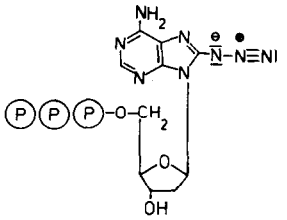

Fig.1. Structure of 8-azido-2′-deoxyadenosine-5′-triphosphate. The syn conformation is shown.

8-azidoadenine nucleotides. This view, however, is contradicted by our data indicating that *E. coli* DNA polymerase I (which has been suggested to accept only 2′-deoxynucleoside triphosphates in anti conformation, see [14]) accepts 8-N_3dATP and allows a successful nick-translation. An experimental study of the conformation of 8-N_3dATP is underway in our laboratory. Nevertheless, the possibility exists that the steric requirements of the nucleotide binding sites of *E. coli* DNA polymerase are indeed less restrictive [26] than assumed [14].

In any case, here we present evidence that 8-azidoadenine 2′-deoxyribonucleotides are incorporated into plasmid pBR322 by nick-translation and that this photoactive plasmid can be cross-linked to proteins as demonstrated by SDS polyacrylamide gel electrophoresis. No DNA-DNA cross-links have been observed after application of fluences of up to 30 kJ/m^2 (irradiation time about 2 h, 366 nm).

Fig.2 shows the autoradiogram of a SDS polyacrylamide gel. In this case histone H1 was cross-linked to photoactivated and ^{32}P-labelled pBR322. Lanes 1, 2 and 4 demonstrate that no DNA-DNA cross-linking occurs by UV irradiation of photoactivated or control pBR322. Lanes 4 to 7 show that increasing UV exposure of photoactive pBR322 in the presence of H1 leads to a decrease in the migration of the DNA fraction caused by covalent bond formation between protein and DNA. The longer the photoactive DNA is irradiated, the more protein molecules are cross-linked to DNA. Non-photoactive pBR322 (dATP incorporated instead of 8-N_3dATP by nick-translation) is not cross-linked to H1 (see fig.2, lane 3).

Fig.3 shows that basic proteins such as histone H2a/b (lanes 8 and 9), histone H4 (lanes 12 and 13)

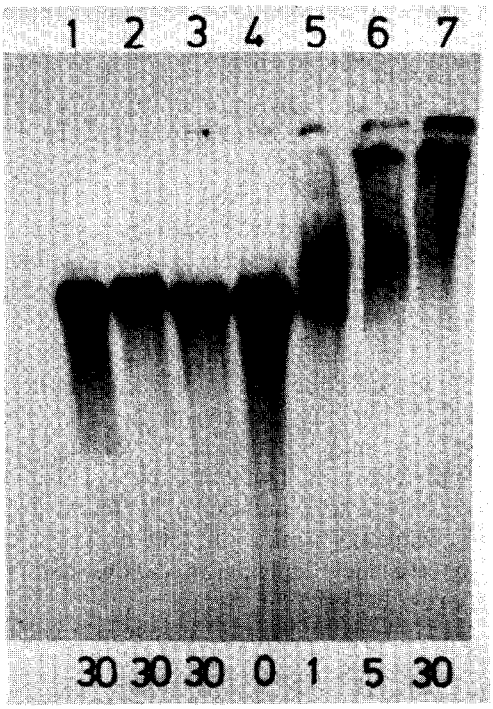

Fig.2. Autoradiogram of a SDS polyacrylamide electrophoresis gel showing photocross-linking of histone H1 to 8-N_3dATP-photoactivated and ^{32}P-labelled pBR322. Lane 1: photoactive pBR322 without protein; lane 2: non-photoactive pBR322 without protein; lane 3: non-photoactive pBR322 with histone H1; lanes 4–7: photoactive pBR322 with histone H1. At the bottom of the figure the times of UV irradiation are indicated in minutes (fluence rate 240 kJ/m^2 per min, 366 nm).

or lysozyme (lanes 4 and 5) can also be cross-linked to N_3dATP-labelled pBR322. But non-basic proteins such as bovine serum albumin or proteinase K could not be cross-linked, not even after UV irradiation periods of 1 h (not shown). Cross-linking efficiency was largely independent from salt concentrations in the irradiated solutions; e.g. H1 could be cross-linked to photoactive pBR322 in aqueous NaCl or $MgCl_2$ solutions up to 100 mM. However, at 1 M or higher concentrations of NaCl or $MgCl_2$ pBR322 could not be cross-linked to H1. The cross-linking efficiency was also reduced at pH below 3 and above 11.5 (0.01 mM Tris).

Proteinase K digestion of some cross-linked samples (azido-pBR322 cross-linked to H1 or H4) were carried out to demonstrate that complete digestion of the histones results in pBR322-polyacrylamide gel bands showing migrations

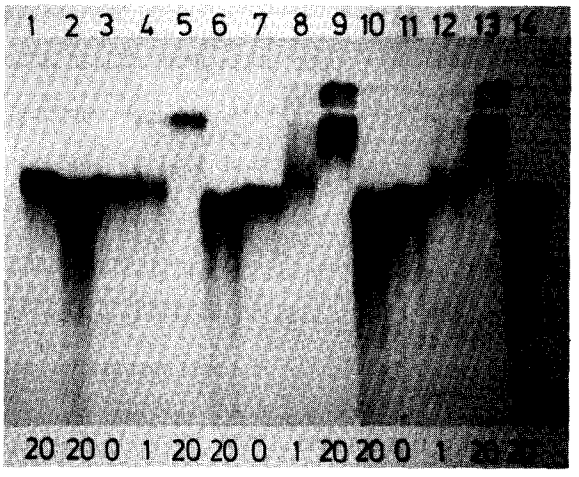

Fig.3. Autoradiogram of a SDS polyacrylamide electrophoresis gel showing photocross-linking (366 nm) of histones H2a/b, H4 and lysozyme to 8-N_3dATP-photoactivated pBR322. Lane 1: photoactive pBR322 without protein; lane 2: non-photoactive pBR322 without protein; lanes 3–5: photoactive pBR322 with lysozyme; lane 6: non-photoactive pBR322 with lysozyme; lanes 7–9: photoactive pBR322 with H2a/b; lane 10: non-photoactive pBR322 with H2a/b; lanes 11–13: photoactive pBR322 with H4; lane 14: non-photoactive pBR322 + H4; in each case the plasmid pBR322 had been ^{32}P-labelled by nick-translation with ^{32}P-dCTP. For more details see also fig.2.

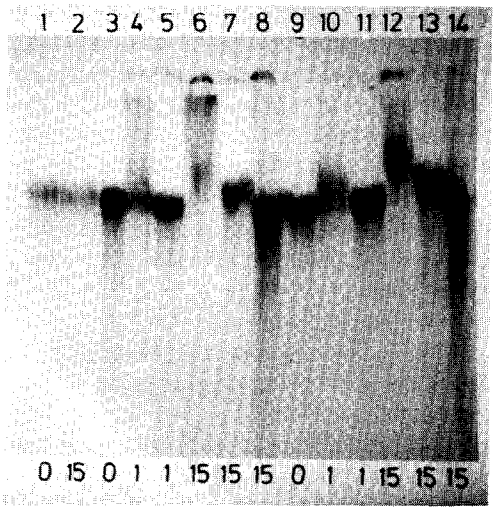

Fig.4. Autoradiogram of a SDS polyacrylamide electrophoresis gel showing effects of proteinase K digestion on photocross-linked pBR322-H1 (-H4) complexes. Lanes 1 and 2: photoactive pBR322 without protein; lanes 3–7: photoactive pBR322 with H1 (lanes 5 and 7: additional proteinase K digestion of cross-link); lane 8: non-photoactive pBR322 with H1; lanes 9–13: photoactive pBR322 with H4 (lanes 11 and 13: proteinase K digestion); lane 14: non-photoactive pBR322 with H4. For more details see also fig.3.

similar to those of unirradiated pBR322 (fig.4). These results also confirm that the slowed migration of photoactive pBR322 samples irradiated in the presence of histones is due to covalent attachment of protein molecules to DNA.

4. DISCUSSION

The synthesis of highly photoactive 8-azido-2′-deoxyadenosine-5′-triphosphate and its incorporation into DNA open new ways to the study of DNA-protein interactions. The advantage of the azido group incorporated into DNA is its efficient conversion into a reactive nitrene by long-wave UV (366 nm). Nitrenes react with nucleophilic groups (alcohols, amines) or directly insert into C–H bonds. The electron sextet can also add to multiple bonds or to aromatic rings [15]. The high reactivity of azidoadenine-photoactivated DNA therefore results in very efficient cross-linking to proteins upon UV irradiation. The efficient photoconversion of 8-azidoadenine-containing DNA causes fewer undesirable photolesions in DNA than any other photocross-linking technique [14].

Here we have shown only a few preliminary examples for the cross-linking of a photoactivated plasmid to various basic proteins. More experiments designed to study more detailed interactions between specific DNA sequences and their recognition proteins are to follow.

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