UV-induced cross-linking of Tet repressor to DNA containing *tet* operator sequences and 8-azidoadenines

Rainer Meffert, Gabriele Rathgeber, Hans-Jochen Schäfer and Klaus Dose*
Institut für Biochemie, Johannes Gutenberg-Universität Mainz, Becherweg 30, D-6500 Mainz, FRG

Received July 16, 1990; Revised and Accepted October 8, 1990

ABSTRACT

The synthesis of 8-azido-2'-deoxyadenosine-5'-triphosphate is described. The photoreactive dATP analog was characterized by thin layer chromatography, proton resonance spectroscopy, infrared spectroscopy and UV spectroscopy. Its photolysis upon UV irradiation was studied. After incorporation of this dATP analog into DNA containing the *tet* operator sequence the investigation of the interactions between *tet* operator DNA and Tet repressor protein by UV photocross-linking becomes possible. Photocross-linking of protein to DNA was demonstrated by the reduced migration of the DNA in SDS polyacrylamide gel electrophoresis. Addition of the inducer tetracycline prior to UV irradiation significantly reduces the DNA-protein cross-linking rate. The long wave UV light applied here does not significantly alter the DNA or the protein under the photocross-linking conditions.

INTRODUCTION

Photoreactive nucleotide analogs have been applied to study specific interactions of proteins with their ligands by photoaffinity labelling (1−3). Especially the substrate binding sites of enzymes have been characterized by photoactivatable substrate analogs (4, 5). Various methods have been used to produce covalent cross-links between proteins and DNA: UV irradiation (6−8), γ-irradiation (9), chemical methods (10−12) and even vacuum or extreme dryness (13). To date only a few successful attempts have been made to study the specific interactions between proteins and nucleic acids by photocross-linking using photoreactive deoxynucleotides. 5-bromo and 5-azido-2'-deoxyuridine-5'-monophosphate have been incorporated into nucleic acids (14, 15). Upon UV irradiation these compounds form radicals or nitrenes, respectively. These highly reactive intermediates bind covalently to adjacent proteins. In a preceding paper the incorporation of 8-azido-2'-deoxyadenosine-5'-triphosphate into DNA by DNA polymerase I and its application to plasmid pBR322 − histone photo cross-linking have been described (16). Here we report the synthesis and characterization of 8-azido-2'-deoxyadenosine-5'-triphosphate and present evidence that 8-azidoadenine-modified DNA containing the *tet* operator sequence can be cross-linked to the Tet repressor protein by irradiation with long wave UV light (λ = 366 nm).

MATERIALS AND METHODS

dATP (disodium salt) was purchased from Boehringer Mannheim. All other chemicals were of the highest purity available from commercial sources.

8-Bromo-2'-deoxyadenosine-5'-triphosphate: dATP (117.8 mg, 0.2 mmole) was dissolved in 1.6 ml potassium acetate buffer (1 M, pH 3.9) and 15 μl bromine (0.29 mmole) were added. The reaction vessel was kept in the dark at room temperature for 6 hours. The reaction was followed spectroscopically. The absorbance maximum shifted from 256 nm to 262 nm. The excessive bromine was reduced by addition of traces of $Na_2S_2O_5$. The reaction mixture was poured into 20 ml cold ethanol and allowed to stand at −20°C for 30 minutes. The precipitated deoxynucleotide was collected and redissolved in 0.5 ml H_2O. Further purification was achieved by ion exchange chromatography on a DEAE-Sephadex A-25 column (50×2 cm) with a linear gradient of 1000 ml each of water and 0.7 M triethylammonium bicarbonate at pH 7.3. 8-BrdATP (triethylammonium salt) was obtained by lyophilization. Yield: 65% (spectroscopically).

8-Azido-2'-deoxyadenosine-5'-triphosphate: 8-BrdATP (triethylammonium salt, 87.3 mg, 0.1 mmole) was dissolved in 3 ml freshly distilled dimethylformamide. A solution of 34.4 mg hydrazoic acid (0.8 mmole HN_3) in 800 μl benzene and 80.8 mg (0.8 mmole) freshly distilled triethylamine were added. The reaction mixture was allowed to stand at 75°C in the dark for 7 hours. The reaction was followed spectroscopically. The absorbance maximum shifted from 262 nm to 280 nm. The solvent was evaporated in the vacuum and the residue redissolved in 1 ml H_2O. Further purification was achieved by ion exchange chromatography as described above. 8-N_3dATP triethylammonium salt was obtained by lyophilization. Yield: 30% (spectroscopically).

8-N_3dATP was stored frozen in aqueous solution, pH 7.0, at −20°C in the dark. Thin-layer chromatography was carried out on silica gel plates F_{254} or cellulose plates F (Merck, Darmstadt) and developed in either solvent system A (isobutyric acid/H_2O/ammonia 66:33:1 v/v) or solvent system B (n-butanol/H_2O/acetic acid 5:3:2 v/v).

The proton resonance spectra were recorded with a Bruker WH 200 spectrometer (solvent D_2O). Infrared spectroscopy was performed with a Beckman IR 4220 infrared spectrophotometer.

* To whom correspondence should be addressed

The UV absorbance spectra were recorded with a Cary 118 spectrophotometer.

The photolysis of 8-N_3dATP was followed spectroscopically. Irradiation was performed with a Mineralight handlamp UVSL 25 at position 'long wave' (maximum 366 nm). The distance between the lamp and the samples was 3.8 cm. The fluence rate at this position was 4 J m^{-2} s^{-1}.

Tet repressor protein and modified plasmid pWH 106 were kindly supplied by K. Tovar and W. Hillen, Institute of Biochemistry and Microbiology, Erlangen, FRG. Tet repressor protein was prepared as described in (17). Plasmid pWH 106 was purified as described in (18). This plasmid contains a six-fold insertion of a 187 bp DNA fragment each containing in turn two *tet* operator sequences (19).

Azido-modified and ^{32}P-labelled DNA was prepared by nick translation as described (16). Non-photoactive DNA (controls) was prepared accordingly, but dATP was added instead of 8-N_3dATP during nick translation. ^{32}P-end-labelling of nick-translated pWH 106 by Klenow polymerase after EcoRI cleavage was achieved to increase the sensitivity of DNA fragment detection in autoradiography. In a typical experiment 100 ng of plasmid pWH 106 (0.03 pmole) were mixed with 230 ng Tet repressor (10 pmole). The DNA-protein samples were preincubated in 10 mM Tris/HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ and 15% glycerol for 30 minutes (37°C) before irradiation. Tetracycline was added before preincubation in 200-fold molar excess related to Tet repressor in the case of experiments testing the influence of the inducer.

Photocross-linking experiments were performed in principle as described earlier (16). Polyacrylamide gel electrophoresis to monitor the formation of repressor-operator complexes was carried out as described in (20, 21). Repressor-operator samples were 10% in glycerol. They were applied to polyacrylamide gels containing 10% glycerol. The electrophoresis buffer was 90 mM boric acid, 20 mM EDTA and 90 mM Tris adjusted to pH 8.0 with HCl. Denaturing gels and denaturing electrophoresis buffer additionally contained 1% SDS. After electrophoresis the gels were silver stained (22), dried and exposed to X-ray films (Pharmacia, NO. RPN 6) for 2 hours to 2 days at −70°C.

The quantification of gel bands in the autoradiograms after polyacrylamide gel electrophoresis was achieved by measuring the intensities of the respective gel bands densitometrically (Desaga, Type CD 50, transmission modus).

RESULTS AND DISCUSSION

8-N_3dATP was synthesized from dATP via 8-BrdATP (Figure 1). dATP reacts with bromine to yield 8-BrdATP which can be purified by ion exchange chromatography on DEAE-Sephadex A-25. 8-BrdATP is obtained as triethylammonium salt. In contrast to the alkali salts the triethylammonium salt is soluble in dimethylformamide. This is of advantage in the subsequent substitution of bromine by the azido group yielding 8-N_3dATP. This exchange reaction requires absolute dryness. Usually the formation of 8-N_3dAMP and 8-N_3dADP is also observed due to a limited hydrolytic cleavage of 8-N_3dATP. In addition traces of unreacted 8-bromoadenine deoxyribonucleotides are also found.

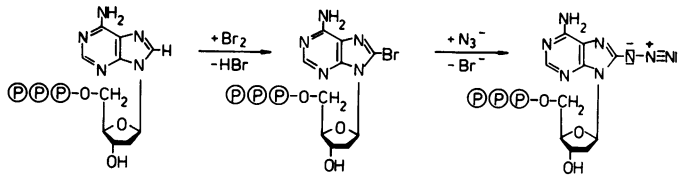

Figure 1. Principles of synthesis of 8-azido-2'-deoxyadenosine-5'-triphosphate.

The homogeneity of 8-N_3dATP was tested by thin layer chromatography on cellulose plates. The successful substitution of the hydrogen at position 8 of the purine ring was substantiated by proton resonance spectroscopy. In contrast to the NMR spectrum of dATP (two singlets at 8.18 ppm and 8.38 ppm) only one singlet at 8.05 ppm for the hydrogen at C2 was observed. The IR spectrum of 8-N_3dATP shows a characteristic band for stretching vibrations of the azido group at 2168 cm^{-1} which resembles that of 8-N_3ATP (23).

The UV absorption spectrum of 8-N_3dATP shows a maximum at 280 nm. The absorbance at 280 nm increases with decreasing pH value. The photolysis of 8-N_3dATP in aqueous solution (0.01 mM Tris/HCl pH 7.0) was followed spectroscopically (Figure 2). The occurrence of the isobestic points suggests that the photolysis is largely controlled by a single set of reactions.

Recently it has been demonstrated that 8-N_3dATP can be

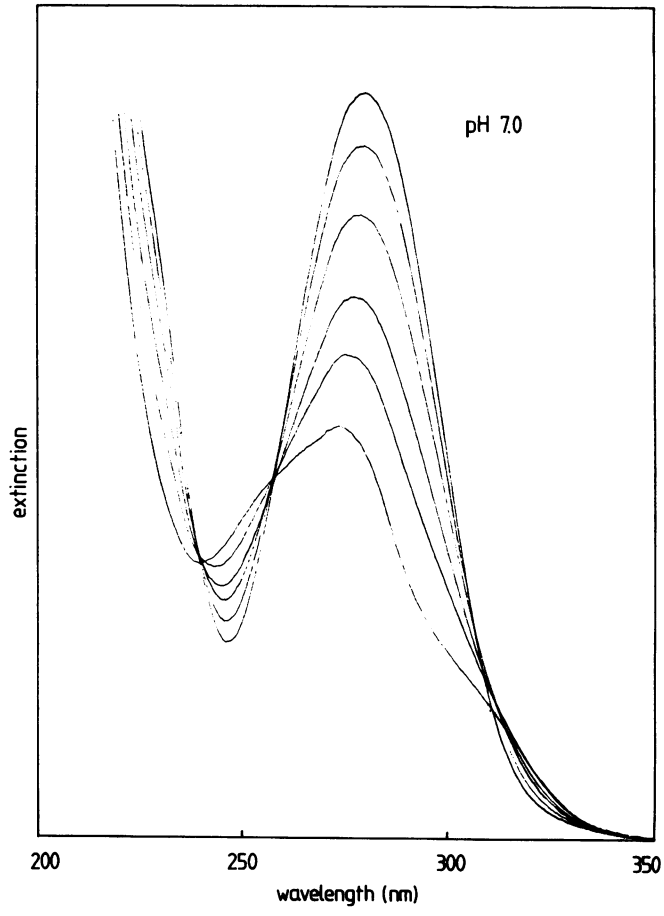

Figure 2. Alteration of the absorption spectrum of 8-N_3dATP upon irradiation with UV light (λ_{max} of lamp = 366 nm) in Tris buffer (0.01 M, pH 7.0 adjusted by HCl). The samples were irradiated at 20°C. The irradiation time between two subsequent UV recordings was initially 2 minutes. It was increased up to 10 minutes towards the end of photolysis. The final spectrum was taken after 30 minutes irradiation time.

incorporated into plasmid pBR322 by nick translation with *E. coli* DNA polymerase I (EC 2.7.7.7.). UV irradiation of this modified plasmid in the presence of different proteins results in efficient formation of DNA-protein cross-links (16). To demonstrate that *tet* operator-Tet repressor complexes can be covalently stabilized by this technique, 8-N_3dATP was incorporated into plasmid pWH 106 by nick translation. After EcoRI cleavage two types of azido-modified DNA fragments are obtained, one of 3848 bp which does not contain *tet* operator sequences and a second of 187 bp which is present as six copies in the original plasmid each of which contains two *tet* operator sequences (19). UV irradiation of these DNA fragments in the presence of Tet repressor protein results in a reduced migration of the 187 bp fragments due to covalent operator-repressor cross-linking (Figure 3). These covalent complexes show a migration behaviour during denaturing SDS polyacrylamide gel electrophoresis that is similar to that of the native complexes in nondenaturing polyacrylamide gel electrophoresis (Figure 4).

Figure 3 shows that in addition some covalent 3848 bp fragment-repressor complexes are also detectable after irradiation with high fluences of UV light (more than 1000 J m^{-2}) although the 3848 bp fragment of pWH 106 does not carry any *tet* operator sequences. This effect is obviously caused by unspecific interactions.

No operator-repressor photocross-linking is observed when the non-photoactive plasmid pWH 106 (dATP incorporated instead of 8-N_3dATP by nick translation) was investigated (Figure 3, lane 2). The appearance of traces of radiolabelled material (Figure 3, lanes 1–4) migrating slightly faster than fraction d is not understood in detail. However, it could be demonstrated that they are not due to the formation of DNA-protein-complexes and that they do not represent DNA fragments resulting from incomplete EcoRI-cleavage.

Tetracycline dissociation experiments were carried out to examine the effect of tetracycline on the production of covalently bound DNA-protein complexes generated during UV-photolysis. Tetracycline induces the dissociation of the Tet repressor from the *tet* operator by producing an allosteric effect on the Tet repressor dimer (24). In order to demonstrate the influence of tetracycline on the binding of the Tet repressor to DNA fragments, solutions of the azidomodified EcoRI-cleaved pWH 106 and Tet repressor were irradiated in the presence of tetracycline. The denaturing SDS polyacrylamide gel electrophoresis of these samples (30′ irradiation time) is presented in Figure 3, lanes 8–11. Irradiation in the presence of tetracycline (varying molar excess related to Tet repressor) leads – as expected – to the complete disappearance of DNA-repressor cross-linking. If tetracycline is added after UV-irradiation (data not shown) photocross-linking efficiency resembles that of UV-irradiation in the absence of tetracycline (Figure 3, lanes 3–7). The transmission of the irradiated samples (90%) is not significantly lowered by the addition of tetracycline at the concentrations applied here.

Densitometric quantification of the gel bands of the autoradiogram (Figure 3, lanes 4–7) gave evidence that up to 20% of the EcoRI-cleaved azidomodified pWH 106 could be covalently bound to Tet repressor.

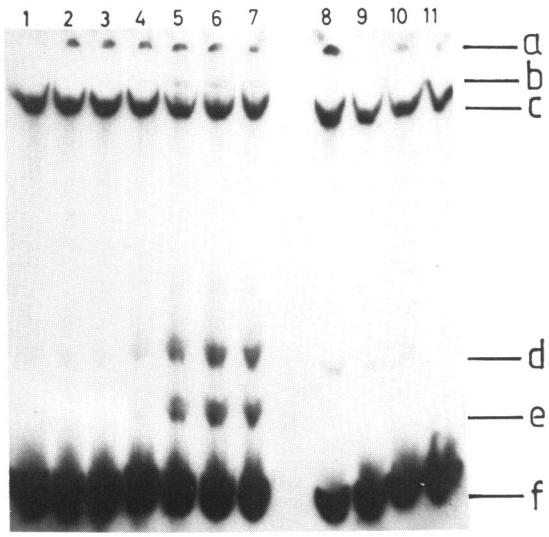

Figure 3. Autoradiogram of a denaturing 5% SDS-polyacrylamide gel electrophoresis showing photocross-linking of Tet repressor to azido-activated 187 bp and 3848 bp fragments obtained by cleaving of pWH 106 with EcoRI. ^{32}P-labelling was carried out as described in Materials and Methods. Each 187 bp fragment contains two *tet* operator sequences. The 3848 bp fragment contains no such sequence. UV irradiation in the presence of Tet repressor results in a reduced migration of the 187 bp fragments due to covalent DNA-protein photocross-linking. In each of lanes 1–7 0.06 pmole EcoRI-cleaved, azidomodified pWH 106 and 20 pmole Tet repressor were applied. The samples in lanes 8–11 in addition contained varying amounts of tetracycline added prior to UV-photolysis. Lane 1: photoactive fragments of pWH 106 without protein (30′ UV); lane 2: non-photoactive fragments of pWH 106 with Tet repressor (30′ UV); lanes 3–7: photoactive fragments of pWH 106 with Tet repressor (0′, 1′, 4′, 10′, 30′ UV); lanes 8–11: photoactive fragments of pWH 106 with Tet repressor and increasing amounts of tetracycline (40 pmole, 400 pmole, 4 nmole, 10 nmole) added prior to UV photolysis (30′ UV). The UV fluence rate was 240 J/m^2 per minute (λ_{max} of lamp = 366 nm). Fraction a) origin of sample-loading. Fraction b) 3848 bp-fragment covalently cross-linked to Tet repressor (lanes 4–7). Fraction c) 3848 bp-fragment, no Tet repressor bound. Fraction d) 187 bp-fragment covalently cross-linked to two Tet repressor dimers. Fraction e) 187 bp-fragment covalently cross-linked to one Tet repressor dimer 3384 bpfragment. Fraction f) 187 bp-fragment, no Tet repressor bound.

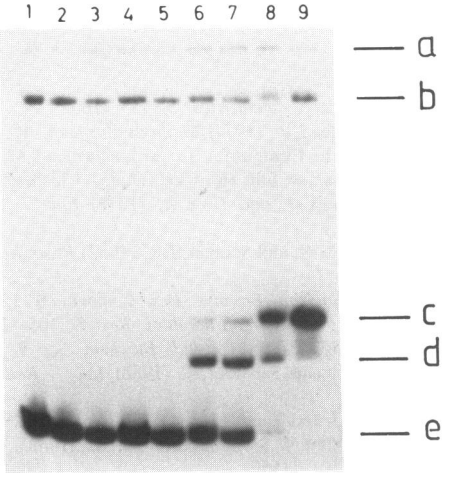

Figure 4. Autoradiogram of a 5% polyacrylamide gel electrophoresis showing titration of ^{32}P-labelled fragments of EcoRI-cleaved pWH 106 with Tet repressor. Each sample originally contained 0.03 pmole pWH 106 to which was added (lanes 1–9 respectively) 0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0 and 5.0 pmole Tet repressor. The two bands with reduced migration represent the binding of repressor dimers to one or both operator sequences of the 187 bp fragment respectively (for details see also Figure 3). Fraction a) origin of sample loading. Fraction b) 3848 bp-fragment, no Tet repressor bound. Fraction c) 187 bp-fragment binding two Tet repressor dimers. Fraction d) 187 bp-fragment binding one Tet repressor dimer. Fraction e) 187 bp-fragment, no Tet repressor bound.

The photoconversion of 8-azidoadenine-containing DNA causes fewer undesirable photolesions in DNA than any other photocross-linking technique largely because long wave UV light is applied here (15). This quality of UV light is less harmful to nucleic acids or proteins compared to the short wavelength UV light (254 nm) used for cross-linking of native nucleic acids and proteins (7) or for the photoactivation of bromouracil-containing nucleic acids (14, 25). In these two cases, moreover, substantial photocross-linking is only achieved with higher fluences.

The synthesis of highly photoactive 8-N_3dATP, its incorporation into nucleic acids and its use in photo cross-linking therefore opens new ways to the study of specific DNA-protein-interactions.

ABBREVIATIONS

UV, ultraviolet; dATP, 2′-deoxyadenosine-5′-triphosphate; 8-BrdAMP, 8-bromo-2′-deoxyadenosine-5′-monophosphate; 8-BrdADP, 8-bromo-2′-deoxyadenosine-5′-diphosphate; 8-BrdATP, 8-bromo-2′-deoxyadenosine-5′-triphosphate; 8-N_3dAMP, 8-azido-2′-deoxyadenosine-5′-monophosphate; 8-N_3dADP, 8-azido-2′-deoxyadenosine-5′-diphosphate; 8-N_3dATP, 8-azido-2′-deoxyadenosine-5′-triphosphate; TEA·HCO_3, triethylammonium bicarbonate; SDS, sodium dodecylsulfate; HN_3, hydrazoic acid.

ACKNOWLEDGEMENTS

This work was supported by Deutsche Forschungsgemeinschaft grant Scha 344/1−2 and by grant 01 QV 174 from the Federal Ministry for Research and Technology, Germany. It is part of the doctoral thesis of R.Meffert.

We thank K.Tovar and Prof. Dr W.Hillen from the Institute of Biochemistry and Microbiology in Erlangen/Germany both for numerous suggestions and for the generous gift of Tet repressor and modified plasmid pWH 106 containing the *tet* operator sequences.

REFERENCES

1. Knowles,J.R. (1972) *Acc. Chem. Res.* **5**, 155−160.
2. Bayley,H. and Knowles,J.R. (1977) *Methods Enzymol.* **46**, 69−114.
3. Schäfer,H.-J. (1987) In Eyzaguirre, J. (ed.), *Chemical Modifications of Enzymes, Active Site Studies*, Ellis Horwood Limited, Chichester, pp. 45−62.
4. Scheurich,P., Schäfer,H.-J. and Dose,K. (1978) *Eur. J. Biochem.* **88**, 253−257.
5. Czarnecki,J., Abbott,M.S. and Selman,B.R. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7744−7748.
6. Smith,K.C. (1982) *Biochem. Biophys. Res. Commun.* **8**, 157−163.
7. Shetlar,M.D. (1980) *Photochem. Photobiol. Rev.* **5**, 105−197.
8. Welsh,J. and Cantor,C.R. (1984) *Trends Biochem. Sci.* **9**, 505−508.
9. Ekert,B., Giocanti,N. and Sabattier,R. (1986) *Int. J. Radiat. Biol.* **50**, 507−525.
10. Lesko,S.A., Drocourt,J. and Yang,S.U. (1982) *Biochemistry* **21**, 5010−5015.
11. Wedrychowsky,A., Ward,W.S., Schmidt,W.N. and Hnilica,L.S. (1985) *J. Biol. Chem.* **260**, 7150−7155.
12. Summerfield,F.W. and Tappel,A.L. (1984) *Chem. Biol. Interact.* **50**, 87−96.
13. Dose,K., Bieger-Dose,A., Martens,K.-D., Meffert,R., Nawroth,T., Risi,S., Steinborn,A. and Vogel,M. (1987) *Proc. 3rd European Symposium on Life Sciences Research in Space* (ESA SP-271), pp. 193−195.
14. Lin,S.Y. and Riggs,A.D. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 947−951.
15. Evans,R.K., Johnson,J.D. and Haley,B.E. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5382−5386.
16. Meffert,R. and Dose,K. (1988) *FEBS Letters* **239**, 190−194.
17. Oehmichen,R., Klock,G., Altschmied,L. and Hillen,W. (1984) *EMBO J.* **3**, 539−543.
18. Hillen,W., Klock,G., Kaffenberger,I., Wray,L.V. and Reznikoff,W.S. (1982) *J. Biol. Chem.* **257**, 6605−6613.
19. Kleinschmidt,C., Tovar,K., Hillen,W. and Porschke,D. (1988) *Biochemistry* **27**, 1094−1104.
20. Fried,M. and Crothers,D.M. (1981) *Nucl. Acids Res.* **9**, 6505−6516.
21. Garner,M.M. and Revzin,A. (1981) *Nucl. Acids Res.* **9**, 3047−3060.
22. Adams,L.D. and Sammons,D.W. (1981) *Electrophoresis* **2**, 155−165.
23. Schäfer,H.-J., Scheurich,P. and Dose,K. (1978) *Liebigs Ann. Chem.* **1978**, 1749−1753.
24. Hillen,W., Gatz,C., Altschmied,L., Schollmeier,K. and Meier,I. (1983) *J. Mol. Biol.* **169**, 707−721.
25. Barbier,B., Charlier,M. and Maurizot,J.C. (1984) *Biochemistry* **23**, 2933−2939.