Incorporation of dA opposite *N*3-Ethylthymidine Terminates in Vitro DNA Synthesis†

Opinder S. Bhanot,* Peter C. Grevatt, Jean M. Donahue, Christine N. Gabrielides, and Jerome J. Solomon
Department of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, New York 10016
Received May 1, 1990; Revised Manuscript Received August 9, 1990

ABSTRACT: *N*3-Ethylthymidine (*N*3-Et-dT) was site specifically incorporated into a 17-nucleotide oligomer to investigate the significance of DNA ethylation at the central hydrogen-bonding site (N3) of thymine. The 5′-(dimethoxytrityl)-protected *N*3-Et-dT was converted to the corresponding 3′-phosphoramidite and used to incorporate *N*3-Et-dT at a single site in the oligonucleotide during synthesis by the phosphite triester method. The purified *N*3-Et-dT-containing oligomer was ligated to a second 17-mer to yield a 34-nucleotide template with *N*3-Et-dT present at position 26 from the 3′-end. The template DNA, which corresponds to a specific sequence at gene G of bacteriophage φX174, was used to study the specificity of nucleotide incorporation opposite *N*3-Et-dT. At 10 μM dNTP and 5 mM Mg^{2+}, *N*3-Et-dT blocked DNA synthesis by *Escherichia coli* polymerase I (Klenow fragment): 96% immediately 3′ to *N*3-Et-dT and 4% after incorporation of a nucleotide opposite *N*3-Et-dT (incorporation-dependent blocked product). DNA replication past the lesion (postlesion synthesis) was negligible. Incorporation opposite *N*3-Et-dT increased with increased dNTP concentrations, reaching 35% at 200 μM. Postlesion synthesis remained negligible. DNA sequencing of the incorporation-dependent blocked product revealed that dA is incorporated opposite *N*3-Et-dT consistent with the "A" rule in mutagenesis. Formation of the *N*3-Et-dT·dA base pair at the 3′-end of the growing chain terminated DNA synthesis. These results implicate *N*3-Et-dT as a potentially cytotoxic lesion produced by ethylating agents.

N-Nitroso compounds form a large group of alkylating agents widely present in the environment (Bartsch & Montesano, 1984). Many of these agents are potent mutagens and carcinogens (Miller, 1979; Singer & Grunberger, 1983; Saffhill et al., 1985) that exert their biological activity, in part, by interacting with genomic DNA. Alkylating agents react with DNA at several sites, but alkylation at the ring nitrogen and exocyclic oxygen atoms of DNA bases is of particular biological importance. A growing body of evidence has implicated alkylation at the O^6-position of deoxyguanosine (O^6-alkyl-dG)[1] to be important with respect to alkylation mutagenesis and carcinogenesis (Basu & Essigmann, 1988). The O^6-alkyl-dG lesion can mispair with thymidine (dT) during DNA synthesis, giving rise to G·C → A·T transition mutations (Loechler et al., 1984; Bhanot & Ray, 1986). The presence of the activating G·C → A·T mutations in Ha-*ras* oncogenes in mammary carcinomas induced in pubescent rats by a single dose of *N*-methyl-*N*-nitrosourea (MNU) has implicated O^6-alkyl-dG lesions in chemical carcinogenesis (Sukumar et al., 1983; Zarbl et al., 1985).

The biological significance of dT alkylation in DNA is not fully established. Although the majority of thymine alkylation occurs at base nucleophilic oxygen sites (O^4 and O^2), adducts at the nucleophilic ring nitrogen site (N3) are also formed (Singer & Grunberger, 1983). Many studies have indicated that mammalian cells lack the well-defined *Escherichia coli* mechanisms for the repair of alkylpyrimidine lesions (Brent et al., 1988). No enzyme has been isolated from eukaryotic sources that can measurably repair or remove O^4- and O^2-alkyl-dT. These adducts are among the most persistent DNA alkylation products in both cultured mammalian cells and animal tissues (Den Engelse et al., 1987) and may contribute to the mutagenesis and carcinogenesis processes long after the original exposure has occurred.

Alkylation of dT in DNA may alter its base-pairing pattern to form a miscoding lesion such as O^4-alkyl-dT (Saffhill et al., 1985; Singer, 1986). Alternatively, it may compromise the ability of dT to serve as a template during DNA replication, producing a noncoding lesion such as *N*3-alkyl-dT (Huff & Topal, 1987; Grevatt et al., 1990) or O^2-alkyl-dT (Saffhill et al., 1985; Singer, 1986; Grevatt & Bhanot, 1990). Noncoding lesions that impede the progress of in vivo DNA replication require the "error prone" functions of the SOS response to produce mutations in bacteria (Walker, 1984). Transversion mutations at A·T base pairs form an important component of alkylating agent induced SOS-dependent mutagenesis in *E. coli* (Foster & Eisenstadt, 1985; Eckert & Drinkwater, 1987; Zielenska et al., 1988; Cuoto et al., 1989; Eckert et al., 1989).

In human cells, *N*-ethyl-*N*-nitrosourea (ENU) induces a significant number of transversion mutations at A·T base pairs (Eckert et al., 1988) in addition to the same G·C → A·T and

† This work was supported in part by National Science Foundation Grant DMB-8607556, National Institute of Environmental Health Sciences Grant ES 00260, Center for Indoor Air Research Grant 90-12, and Special Institutional Grant SIG 9A from the American Cancer Society. At the initial stage of this work P.C.G. and J.M.D. were supported by National Institute of Environmental Health Sciences Training Grant 2T 32 ES 07081. P.C.G. is a recipient of a Helen Q. Woodard Graduate Scholar Award. J.M.D. is a recipient of a Shell Graduate Scholar Award.
* To whom correspondence should be addressed.

[1] Abbreviations: O^6-alkyl-dG, O^6-Alkyldeoxyguanosine; MNU, *N*-methyl-*N*-nitrosourea; O^4-alkyl-dT, O^4-alkylthymidine; *E. coli*, *Escherichia coli*; O^2-Et-dT, O^2-ethylthymidine; ENU, *N*-ethyl-*N*-nitrosourea; *N*3-alkyl-dT, *N*3-alkylthymidine; AP, apurinic/apyrimidinic; *N*3-Me-dT, *N*3-methylthymidine; *N*3-Et-dT, *N*3-ethylthymidine; Kf Pol I, Klenow fragment of *E. coli* polymerase I; dT, thymidine; ATP, adenosine 5′-triphosphate; CI, chemical ionization; MS, mass spectrometry; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; DMTr-dT, 5′*O*-(4,4′-dimethoxytrityl)thymidine; DMTr-(*N*3-Et)dT, *N*3-ethyl-5′-*O*-(4,4′-dimethoxytrityl)thymidine; DTT, dithiothreitol; UV, ultraviolet; NMR, nuclear magnetic resonance.

0006-2960/90/0429-10357$02.50/0

A·T → G·C transition mutations it produces in *E. coli* (Richardson et al., 1987). The two transition mutations induced in human cells are consistent with the predicted mispairing of the DNA lesions O^6-alkyl-dG (Loechler et al., 1984; Bhanot & Ray, 1986) and O^4-alkyl-dT (Preston et al., 1986). The importance of A·T → T·A transversion mutations in the activation of cellular protooncogenes has been demonstrated (Bargmann et al., 1986; Perantoni et al., 1987; Stowers et al., 1988). The DNA lesions responsible for transversion mutations at A·T base pairs are not known.

The induction of A·T transversion mutations in SOS-induced bacteria and mammalian systems suggests that dA or dT adducts and/or a breakdown product of these adducts is responsible for transversion mutations at the A·T base pairs. We report the in vitro specificity of nucleotide incorporation opposite *N*3-Et-dT present at a single site in the DNA sequence that corresponds to bacteriophage φX174 DNA. At low dNTP concentrations, *N*3-Et-dT blocked DNA synthesis by the Klenow fragment of *E. coli* polymerase I (Kf Pol I), primarily 3′ to the lesion in the template DNA. At high dNTP concentrations, dA was incorporated opposite *N*3-Et-dT, consistent with the "A" rule in mutagenesis (Strauss et al., 1982). Formation of the *N*3-Et-dT·dA base pair at the growing end of the chain was resistant to further extension, causing termination of DNA synthesis at this point. These results suggest a role for the *N*3-Et-dT lesion in the cytotoxic properties of ethylating agents.

Materials and Methods

Ultrapure-grade dNTP and dT were purchased from Pharmacia P-L Biochemicals. γ-^{32}P-labeled adenosine 5′-triphosphate (ATP) was obtained from Du Pont-New England Nuclear. Kf Pol I and T4 DNA ligase were purchased from Boehringer Mannheim. One unit of Kf Pol I (as reported by the supplier) incorporates 10 nmol of total nucleotides into acid-precipitable material in 30 min at 37 °C with poly[d(A-T)] as the primed template. T4 polynucleotide kinase was obtained from New England Biolabs. Silica gel (Merck, grade 60, 230–400 mesh, 60 Å), *N*,*N*-diisopropylethylamine, and the reagents used in DNA sequencing were purchased from Aldrich. Ultrapure electrophoresis reagents were purchased from Bio-Rad. EM Science prepared thin-layer chromatography (TLC) plates, silica gel 60 F_{254}, were obtained from VWR Scientific. Chloro(*N*,*N*-diisopropylamino)(cyanoethoxy)phosphine was purchased from American Bio Nuclear. Diisopropylamine and other high-purity solvents were dried by distillation from calcium hydride and stored over 4-Å molecular sieves (Fisher Scientific) under nitrogen atmosphere. All other chemicals were of high-grade quality and purchased from different sources.

Synthesis and site-specific incorporation of *N*3-Et-dT into the oligodeoxynucleotide are described below. Silica gel column chromatography was performed in the presence of 0.1% pyridine. TLC plates were developed in the following solvent systems: I, chloroform–methanol (90:10); II, benzene–acetone (40:60); III, chloroform–ethyl acetate–triethylamine (45:45:10); IV, 1-propanol–NH_4OH–H_2O (55:35:10).

Synthesis of N3-Ethyl-5′-O-(4,4′-dimethoxytrityl)thymidine [DMTr-(N3-Et)dT]. 5′-*O*-(Dimethoxytrityl)thymidine (DMTr-dT) was prepared as described (Narang et al., 1980). DMTr-dT (15 mM) in tetrahydrofuran–methanol (85:15) was treated with a 6-fold molar excess of diazoethane in four portions with efficient stirring. Diazoethane was generated by treating 1-ethyl-3-nitro-1-nitrosoguanidine with potassium hydroxide (Hudlicky, 1980) by use of a minidiazald apparatus (Aldrich). After each addition of diazoethane and subsequent stirring for 30 min, the reaction mixture was examined by TLC (solvent system II). The reaction was stopped when ≈10% of the unreacted DMTr-dT was left to minimize the formation of diethylated derivatives. After concentration under reduced pressure, the residue in chloroform (7 mL) was chromatographed on a silica gel column (2 × 50 cm) preequilibrated with chloroform. The elution used chloroform containing increasing amounts of methanol (1–5%). The fractions (5 mL/5 min) containing pure DMTr-(*N*3-Et)dT (see Figure 1) were detected by TLC (solvent system II), pooled, and concentrated under reduced pressure. The residue in chloroform or ethyl acetate (10 mL) was precipitated from petroleum ether (500 mL). The precipitate was collected by centrifugation, dried under vacuum, and stored at −80 °C over a desiccating agent. The yield was 45%. R_f (TLC, solvent system II) was 0.64.

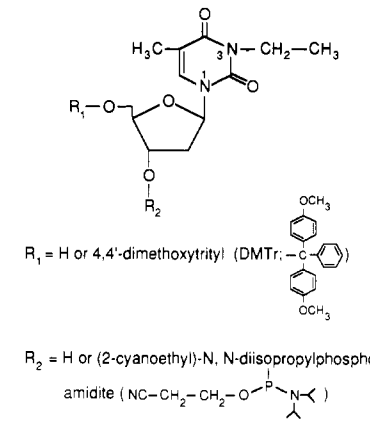

FIGURE 1: Structure of N3-ethylated dT derivatives. *N*3-Et-dT: R_1 = R_2 = H. DMTr-(*N*3-Et)dT: R_1 = DMTr; R_2 = H. 3′-Phosphoramidite of DMTr-(*N*3-Et)dT: R_1 = DMTr; R_2 = 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite.

DMTr-(*N*3-Et)dT was characterized by chemical ionization (CI) mass spectrometry (MS) with a VG-70SE high-resolution mass spectrometer as described (Solomon et al., 1988). As expected, the protonated molecular ion (MH) was observed at *m*/*z* 573, establishing the molecular weight as 572 and fragmentation revealing that ethylation had occurred on the base. The structure of DMTr-(*N*3-Et)dT was confirmed by nuclear magnetic resonance (NMR) spectroscopic analysis with a Varian XL300 spectrometer operating at 300 MHz for protons as described (Solomon et al., 1988). The chemical shift (δ) assignments of the protons of DMTr-(*N*3-Et)dT in dimethyl sulfoxide are as follows: 1.08 (t, 3, *J* = 7.0 Hz, *N*3-CH_2CH_3); 1.49 (s, 3, *C*5-CH_3); 2.13–2.32 (m, 2, 2′-CH_2); 3.14–3.26 (m, 2, 5′-CH_2); 3.73 [s, 6, $(OCH_3)_2$ of DMTr]; 3.84 (q, 2, *J* = 7.0 Hz, *N*3-CH_2CH_3); 3.88–3.93 (m, 1, 4′-H); 4.28–4.37 (m, 1, 3′-H); 5.34 (d, 1, 3′-OH); 6.25 (t, 1, 1′-H); 6.86–7.42 (m, 13, aromatic protons of DMTr); 7.57 (s, 1, *C*6-H). These assignments are based on one- and two-dimensional proton homonuclear correlation spectra.

Preparation of the Authentic Sample of N3-Et-dT. *N*3-Et-dT (see Figure 1) was prepared on an 8 mM scale as described (Kusmierek & Singer, 1986) except that the reaction was terminated when ≈10% of the unreacted material was left and that the purification of *N*3-Et-dT was performed by two successive rounds of silica gel column chromatography. In the first chromatography, the column (1 × 50 cm) was eluted (9 mL/8 min per fraction) with chloroform containing increasing concentrations of methanol (3–25%). This chromatographic step gave ≈95% pure *N*3-Et-dT. In the second chromatographic step, the column (1 × 45 cm) was developed with 3% methanol in benzene containing increasing amounts of acetone

(30–70%). Fractions (5 mL/5 min) containing pure N3-Et-dT (TLC; solvent system II) were pooled, concentrated under reduced pressure, precipitated from petroleum ether, and crystallized from benzene. The yield was 32%.

Comparison of N3-Et-dT Isolated from DMTr-(N3-Et)dT with the Authentic Sample. N3-Et-dT was derived from DMTr-(N3-Et)dT by removal of the 5′-(dimethoxytrityl) group with 3% trichloroacetic acid and purified as described above for the authentic sample. The deprotected material, N3-Et-dT, was found to be identical with the authentic sample when compared by TLC, high-pressure liquid chromatography (HPLC), UV spectra, and MS. The deprotected material comigrated with the authentic sample on silica gel TLC in solvent system I (R_f = 0.19) and solvent system II (R_f = 0.38). They also coeluted (retention time 21.5 min) on HPLC with a 4 × 250 mm C_{18} μBondapak column (Waters Associates) and the Waters' gradient 8 program at a flow rate of 1 mL/min. The UV spectrum for N3-Et-dT was identical with that of the authentic sample and had the correct λ_{max}(269) and λ_{min}(239) (Singer & Grunberger, 1983). The spectra in aqueous solutions were obtained at pH 1 (1 N HCl), pH 6 (H_2O), and pH 13 (0.1 N NaOH).

N3-Et-dT, isolated from DMTr-(N3-Et)dT, was further characterized by MS. The electron impact and CI-MS spectra revealed a molecular weight of 270 with ethylation occurring on the base. Confirmation of the assignment was established by an accurate mass measurement technique (Solomon et al., 1988), where diagnostic ethylated base containing ions could be measured. The most intense ion in the CI mass spectra of N3-Et-dT occurred at m/z 155, following protonation and cleavage of the glycosidic bond with hydrogen transfer (Wilson & McCloskey, 1979). This ion, corresponding to base plus two hydrogens, has the elemental composition of $C_7H_{11}N_2O_2$ with a calculated mass of 155.0821. The mass was measured as 155.0827, within 0.6 millimass unit of the predicted mass. The mass spectral data obtained with the authentic sample of N3-Et-dT were identical.

Preparation of N3-Ethyl-5′-O-(4,4′-dimethoxytrityl)thymidine 3′-(2-Cyanoethyl N,N-diisopropylphosphoramidite). The method described for phosphoramidites of normal nucleosides was adapted (Dorper & Winnacker, 1983). Under anhydrous conditions, chloro(N,N-diisopropylamino)(cyanoethoxy)phosphine (2 mmol) was added dropwise to the stirring solution of DMTr-(N3-Et)dT (1 mmol) in acid-free methylene chloride (3 mL) and N,N-diisopropylethylamine (5.7 mmol). After 30 min at room temperature, ethyl acetate (100 mL) was added, and the mixture was extracted (5×, 100 mL) with saturated sodium chloride. The organic layer containing the amidite was dried (anhydrous magnesium sulfate), concentrated under reduced pressure, and chromatographed on a silica gel column (2 × 40 cm) preequilibrated with solvent system III. The column was developed with the same solvent system. The fractions (5 mL/5 min), containing pure amidite (TLC, solvent system III), were pooled and dried under reduced pressure. The residue in toluene (10 mL) was precipitated from hexane (250 mL) at −80 °C. The white precipitate was filtered at −20 °C and washed immediately with 2 × 100 mL of hexane precooled to −80 °C. After being dried under vacuum, the phosphoramidite of DMTr-(N3-Et)dT (see Figure 1) was stored at −80 °C over a desiccating agent. The yield was 81%, and the product moved as a single sharp spot (TLC; solvent system III) with an R_f of 0.68.

Synthesis of Site-Modified Oligodeoxynucleotide. The 17 nucleotide long oligomer, 5′-TAAAAGTTT*AAAACATG (T* = N3-Et-dT) was synthesized on an Applied Biosystems Model 381A synthesizer by phosphite triester chemistry (Caruthers, 1985). This oligomer is complementary to the φX DNA sequence from position 2394 to position 2410 in gene G (Sanger et al., 1978). N3-Et-dT was introduced at position 2402 during synthesis by the use of N3-ethyl-5′-O-(4,4′-dimethoxytrityl)thymidine 3′-(2-cyanoethyl N,N-diisopropylphosphoramidite) (see Figure 1) synthesized above.

After synthesis, the detritylated oligomer was released from the solid support and further treated with concentrated ammonium hydroxide (2 mL) at 25 °C for 72 h to completely remove all protecting groups. The deprotected N3-Et-dT-containing oligomer was purified on silica gel TLC (solvent system IV). The slowest moving product band was located with a UV light, isolated, and eluted twice (2 mL each) with 25% ethanol in H_2O at 25 °C for 30 min. After concentration under reduced pressure, the product (≈90% pure) was purified to homogeneity by electrophoresis on a 20% polyacrylamide–8 M urea gel. The product band (visualized with UV light) was excised, crushed, and eluted by soaking in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 20 mM NaCl. The oligomer was recovered by passing the eluate through a Sep-Pak C18 cartridge (Waters Associates) as described by the supplier.

The site-modified oligomer was phosphorylated at the 5′-OH group with T4 polynucleotide kinase and [γ-^{32}P]ATP and fully characterized. Homogeneity of the oligomer was checked by electrophoresis on a 20% polyacrylamide–8 M urea sequencing gel. The oligomer was sequenced by the modified Maxam–Gilbert procedure (Maxam & Gilbert, 1980). To quantitate the N3-Et-dT adduct, the oligonucleotide (1.0 A_{260}) was digested at 37 °C for 4 h with snake venom phosphodiesterase (0.04 unit) and bacterial alkaline phosphatase (0.04 unit) in 50 μL of 10 mM Tris-HCl, pH 8.0, and 50 mM NaCl. The enzymatic digest was mixed with 300 μL of acetone, chilled at 4 °C for 30 min, and centrifuged to remove denatured proteins. The acetone solution containing nucleosides was dried under reduced pressure, dissolved in water, and analyzed by HPLC. HPLC analysis was kindly performed by Dr. J. Guttenplan of this department.

Formation of Site-Modified Primed Template. A 34 nucleotide long template, containing the N3-Et-dT adduct at a single preselected site, was constructed by ligating the site-modified 17-mer (100 pmol) to a second ^{32}P-labeled (3 Ci/mmol) 17-nucleotide oligomer (100 pmol) in the presence of an 18-nucleotide complementary oligomer (80 pmol) which held the ligating oligomers together. The three oligomers in 50 μL of 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM dithiothreitol (DTT) were heated at 55 °C for 15 min and slowly cooled to 4 °C (4 h). After addition of ATP to 1 mM, T4 DNA ligase (50 units as reported by the supplier) was added to the annealed mixture and incubated at 16 °C for 4 h. The reaction was stopped by the addition of EDTA to 12 mM and an equal volume of formamide. After being heating at 100 °C for 3 min, the ligated template was separated by electrophoresis on a 16% polyacrylamide–8 M urea gel. The ligated product band was visualized by autoradiography and excised. Urea was removed from the crushed band by extraction with methanol (4×, 10 mL) and the product eluted in 10 mM NaCl in 0.3× TE buffer. The site-modified template contains a single N3-Et-dT adduct at position 26 from the 3′-end. The low radioactivity present in the template did not interfere in the following experiments.

The primed template was obtained by annealing about a 2-fold molar excess of complementary 5′-^{32}P-labeled (3000 Ci/mmol) 17-nucleotide primer to the site-modified 34-mer

template in 20 mM Hepes buffer, pH 7.5, and 50 mM NaCl. The annealing mixture (10 μL/pmol of the template) was heated at 55 °C for 15 min followed by slow cooling to 4 °C (4 h). Formation of the primed template was checked by electrophoresis on a nondenaturing 12% polyacrylamide gel (>80% yield). A single batch of primed template was used in all DNA replication studies with *N*3-Et-dT.

The primed template with a 34 nucleotide long template, containing dT in place of *N*3-Et-dT, was constructed for use as a control in the DNA replication studies.

DNA Polymerase Reaction. Each polymerase reaction was conducted in 10 mM Hepes buffer, pH 7.5, containing 1 mM DTT and 0.1 pmol of primed template (total volume 5 μL) with 0.2 unit (as reported by the supplier) of Kf Pol I per reaction. The concentrations of dNTP and Mg^{2+}, incubation time, and temperature were varied depending upon the type of study conducted. Exact conditions are documented in the figure legends.

The reactions were stopped by adding EDTA to a final concentration higher than that of Mg^{2+} and an equal volume of formamide. After heating at 100 °C for 3 min, DNA synthesis products were separated by electrophoresis on a 16% polyacrylamide–8 M urea sequencing gel. Product bands were visualized by autoradiography and excised, and radioactivity was counted in 5 mL of scintillation cocktail (aqueous based). The radioactivity present in each product band was used to calculate the percent of DNA synthesis products in the polymerization reaction.

To prepare various DNA synthesis products in amounts sufficient for DNA sequencing, the polymerase reactions were performed at 37 °C with 5–10 pmol of the site-modified primed template in the presence of the desired dNTP concentrations and 5 mM Mg^{2+}. Products were separated by electrophoresis as described above except that a 1 mm thick gel was used. Product bands, visualized by autoradiography, were excised, crushed, and extracted with 4 × 10 mL of methanol to remove the urea. The products were then eluted with a minimum volume of 5 mM NaCl in 0.25× TE at 37 °C for 12 h.

Identification of Nucleotide Incorporated opposite N3-Et-dT. The identity of the nucleotide incorporated opposite *N*3-Et-dT in the DNA synthesis product was established by DNA sequencing with the modified Maxam–Gilbert method (Maxam-Gilbert, 1980).

Results

Chemical Synthesis of Oligodeoxynucleotide Containing N3-Et-dT. A 17 nucleotide long oligomer (5′-TAAAAG-TTT*AAAACATG, T* = *N*3-Et-dT), containing a single *N*3-Et-dT adduct, was synthesized by the solid-phase phosphite triester method (Caruthers, 1985). For synthesis of the required 3′-phosphoramidite (see Figure 1), DMTr-dT was chosen as the starting material. The presence of the DMTr group facilitated the separation by silica gel chromatography of DMTr-(*N*3-Et)dT obtained after ethylation. Furthermore, the DMTr group at the 5′-end of the amidite is needed for selective removal during oligonucleotide synthesis. Analysis of DMTr-(*N*3-Et)dT by CI-MS revealed the expected molecular weight of 572 with ethylation occurring on the base. The structure of DMTr-(*N*3-Et)dT was further confirmed by NMR. All NMR assignments, based on one- and two-dimensional proton homonuclear correlation spectram, were consistent with the expected structure of DMTr-(*N*3-Et)dT. *N*3-Et-dT, isolated from DMTr-(*N*3-Et)dT, was identical with the authentic sample, further confirming the structural identity of DMTr-(*N*3-Et)dT.

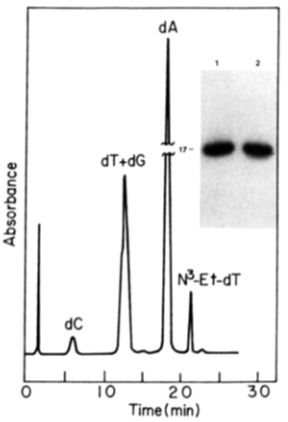

FIGURE 2: HPLC nucleoside analysis of *N*3-Et-dT-containing 17-mer. Nucleosides released by enzymatic digestion (see text) were chromatographed on a 4 × 250 mm long C_{18} μBondapak column (Waters Associates). The column was eluted with a Waters gradient 8 program at a flow rate of 1 mL/min. HPLC analysis confirmed the presence of *N*3-Et-dT in the primer. The molar ratio of *N*3-Et-dT as compared to dA or dC indicated that *N*3-Et-dT is present in >99.5% of the primer molecules. The figure inset represents an autoradiogram showing the purity of the *N*3-Et-dT containing 17-mer. The ^{32}P-labeled primer was electrophoresed on a 20% polyacrylamide–8 M urea gel. Lane 1, *N*3-Et-dT-containing 17-mer. Lane 2, 17-mer marker containing the same sequence except dT in place of *N*3-Et-dT.

DMTr-(*N*3-Et)dT is stable to conditions encountered during oligonucleotide synthesis (Caruthers, 1985). The facile conversion of DMTr-(*N*3-Et)dT to the corresponding 3′-phosphoramidite and the purification of the amidite by a single chromatographic step expedited the incorporation of *N*3-Et-dT at the desired site. The coupling efficiency for the modified amidite during synthesis was >95%, which is similar to that observed for normal nucleoside amidites.

The completely deprotected site-modified oligomer was purified in two steps. TLC purification gave ≈90% pure material which was purified further by electrophoresis on a polyacrylamide gel. The purified oligomer, labeled with ^{32}P at the 5′-terminus, moved as a single sharp band on a 20% polyacrylamide–8 M urea gel and had the same mobility as a standard 17-nucleotide primer synthesized in this laboratory (Figure 2, inset). HPLC analysis (Figure 2) of the enzymatically released nucleosides revealed that *N*3-Et-dT was present in over 99.5% of the oligonucleotide molecules. The oligonucleotide, sequenced by the modified Maxam–Gilbert procedure, contained the expected sequence (Figure 3). Since the 5,6-double bond in the thymine base moiety is available for reaction with hydrazine, the *N*3-Et-dT adduct behaved like dT in the sequencing reaction (Figure 3). In the site-modified oligomer the *N*3-Et-dT adduct corresponds to position 2402 (second position of the third codon of gene G) in the (−) strand. In addition to in vitro DNA replication studies, the same DNA sequence will be used in a separate in vivo site-specific mutagenesis study using the φX174-based mutagenesis system (Bhanot & Ray, 1986) to facilitate a comparison.

In Vitro DNA Replication System. An in vitro DNA replication system was used to study the response of Kf Pol I on replication past *N*3-Et-dT. The replication system utilizes the following primed template (T* = *N*3-Et-dT):

```
17-mer primer  5'- 32pTTATTTCTCGCCATAAT

34-mer template 3'-HO-AATAAAGAGCGGTATTAGTACAAAAT*TTGAAAAT5'
                                          |       |       |
                                         17      26      34
```

The replication system contains a 34-nucleotide site-modified template hybridized to a 5′-^{32}P-labeled 17-nucleotide com-

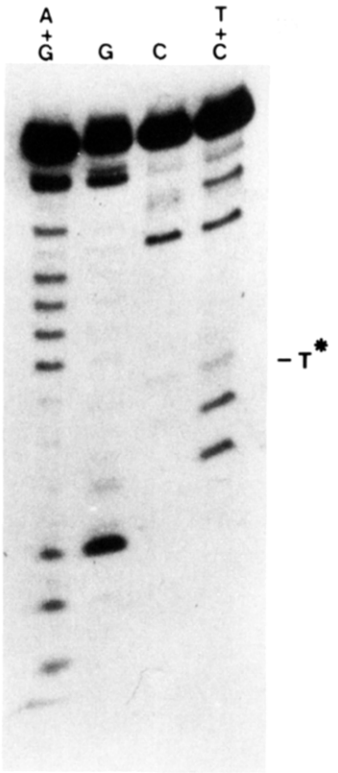

FIGURE 3: DNA sequence analysis of N3-Et-dT-containing 17-mer by the Maxam–Gilbert procedure. T* = N3-Et-dT. Sequencing gel confirmed the expected DNA sequence, pTAAAAGTTT*A-AAACATG-3′.

plementary primer. The N3-Et-dT adduct is present at template position 26 from the 3′-end. The 3′-terminus of the primer is eight nucleotides away from the N3-Et-dT lesion present in the template. This system represents a "running start" (Mendelman et al., 1989) for N3-Et-dT in DNA replication in that synthesis occurs prior to the polymerase reaching the N3-Et-dT lesion. In this replication system, each DNA synthesis product is labeled only once at the 5′-end, facilitating quantitation by counting radioactivity associated with each product band after separation by electrophoresis on a polyacrylamide gel.

The control primed template contained unmodified dT in place of N3-Et-dT in the template.

DNA Replication Block by N3-Et-dT. In this DNA replication system, when Kf Pol I encounters the N3-Et-dT adduct, the progress of the polymerase may be blocked immediately 3′ to the adduct. DNA synthesis is terminated without incorporation of a nucleotide opposite N3-Et-dT, producing a 25-nucleotide "preincorporation blocked product". Alternatively, the polymerase progress may be obstructed after incorporation of a nucleotide opposite the N3-Et-dT adduct, generating a 26-nucleotide "incorporation-dependent blocked product". Any synthesis that is not blocked by N3-Et-dT may proceed to the 5′-terminus of the template, yielding a 34 nucleotide long "postlesion synthesis product". Using polyacrylamide gel analysis, we have analyzed the products of DNA synthesis on the site-modified template.

By use of this primed template system, N3-Et-dT was found to block DNA synthesis by Kf Pol I in the presence of Mg^{2+} and 10 μM dNTPs (Figure 4B). In the control, DNA synthesis proceeded to the 5′-terminus of the template without interruption, yielding a 34-nucleotide product (Figure 4A). The results are consistent with the published reports that N3-Me-dT randomly present in synthetic polymers impedes DNA synthesis by Kf Pol I and AMV reverse transcriptase

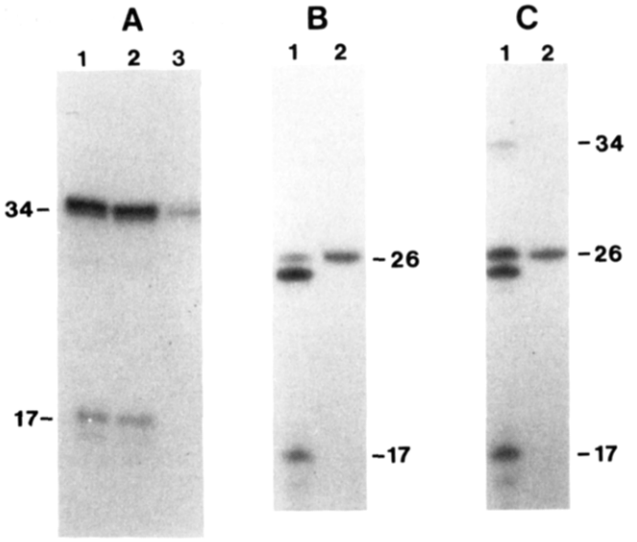

FIGURE 4: Analysis of DNA products synthesized by Kf Pol I on a primed template. The primed template contained a normal nucleoside (dT) or a single N3-Et-dT adduct at template position 26 from the 3′-end. The primed template was incubated at 37 °C for 15 min with 0.2 unit of Kf Pol I in the presence of 5 mM Mg^{2+}. Products were analyzed by gel electrophoresis and autoradiography as described in the text. A 25-nucleotide product corresponds to the preincorporation (adjacent to N3-Et-dT) blocked product; 26-nucleotide product depicts incorporation- (opposite N3-Et-dT) dependent blocked product; 34-nucleotide postlesion synthesis product represents no block during DNA replication with the synthesis proceeding entirely to the 5′-end of the 34-nucleotide template. (A) Control primed template containing normal nucleoside dT: lane 1, polymerization in the presence of 10 μM dNTP; lane 2, polymerization in the presence of 200 μM dNTP; lane 3, standard 34-nucleotide marker. (B) Primed template containing an N3-Et-dT adduct: lane 1, polymerization in the presence of 10 μM dNTP; lane 2, standard 26-nucleotide marker. (C) Primed template is the same as in (B): lane 1, polymerization in the presence of 200 μM dNTP; lane 2, standard 26-nucleotide marker.

(Huff & Topal, 1987). We found that the major product of DNA synthesis (96%) was a 25-nucleotide preincorporation blocked product, indicating that synthesis terminated immediately 3′ to the N3-Et-dT lesion in the template strand. This was confirmed by sequencing the preincorporation blocked product (data not shown). A small amount (4%) of the 26-nucleotide incorporation-dependent blocked product was also obtained, resulting from the DNA synthesis interruption after incorporation of a nucleotide opposite the N3-Et-dT lesion. No measurable 34-nucleotide postlesion synthesis product was observed (Figure 4B). This pattern of DNA synthesis interruption by N3-Et-dT was not affected by temperature (16–37 °C), reaction time (15–120 min), or concentration of the divalent cation, Mg^{2+} (1–10 mM), in the polymerization reaction. Unless stated, all polymerization experiments described in this paper were conducted in the presence of 5 mM Mg^{2+} at 37 °C for 15 min.

Degradation of the 17-nucleotide primer by the associated 3′ → 5′ exonuclease activity of Kf Pol I was observed (Figure 4). No degradation of DNA synthesis products was detected, indicating that the degradation is limited to the primer molecules that are not in the process of elongation. Degradation of oligomers by Kf Pol I in the absence of template and dNTPs has been observed (Bhanot et al., 1979).

Nucleotide Incorporation opposite N3-Et-dT. The effect of dNTP concentrations on the incorporation of a nucleotide opposite N3-Et-dT was significant. When the concentration of dNTPs was increased from 10 to 200 μM, the 26-nucleotide incorporation-dependent blocked product was increased from 4% (Figure 4B) to 35% (Figure 4C). Figure 5 represents the effect of dNTP concentration on the percentage of various

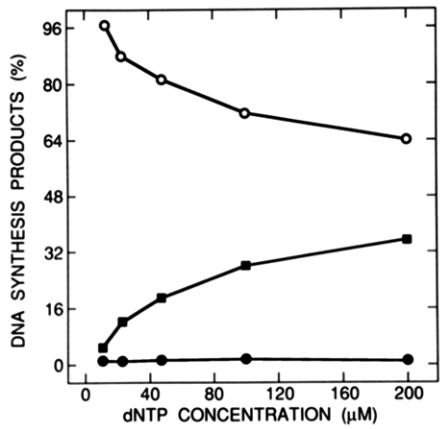

FIGURE 5: Effects of dNTP concentration on DNA synthesis products catalyzed by Kf Pol I on the primed template containing a single *N*3-Et-dT adduct. The primed template was incubated at 37 °C for 15 min with 0.2 unit of Kf Pol I in the presence of 5 mM Mg^{2+} and varying concentration of dNTP. The products were analyzed as described in the text. (○) 25-Nucleotide preincorporation blocked product; (■) 26-nucleotide incorporation-dependent blocked product; (●) 34-nucleotide postlesion synthesis product.

DNA synthesis products. Each point represents an average of three experiments with standard deviations of 2.2–3.0% for the preincorporation blocked and incorporation-dependent blocked products and 0.01–0.03% for the postlesion synthesis product. The high concentration of dNTPs induced the polymerase to incorporate a nucleotide opposite *N*3-Et-dT, extending the 25-nucleotide preincorporation blocked product by one nucleotide. The net result was a decrease of the 25-nucleotide preincorporation blocked product from 96% to 64% and an increase in the 26-nucleotide incorporation-dependent blocked product from 4% to 35% at 10 and 200 μM dNTP concentrations, respectively (Figure 5). Postlesion synthesis was negligible (<1%) even at 200 μM dNTPs (Figure 5). DNA sequencing of the 26-nucleotide incorporation-dependent product revealed that dA was incorporated opposite *N*3-Et-dT (Figure 6). The results are consistent with the preferential incorporation of dA opposite noncoding lesions during DNA replication (Strauss, 1985).

Discussion

The studies described in this paper reveal that a single *N*3-Et-dT adduct present in a DNA template blocks DNA synthesis by Kf Pol I either immediately 3′ to the *N*3-Et-dT adduct or after incorporation of a nucleotide opposite it. The *N*3-Et-dT adduct is formed in both single- and double-stranded DNA (Singer & Grunberger, 1983), and the repair activities of this DNA damage have not been identified. Our results suggest that alkylation of the central hydrogen-bonding site (N3) of thymine in DNA is biologically significant and may contribute to the cytotoxicity of alkylating agents.

At low dNTP concentrations (10 μM), DNA synthesis by Kf Pol I was terminated mainly (96%) at the base located immediately 3′ to the *N*3-Et-dT adduct, as confirmed by the isolation and DNA sequencing of the 25-nucleotide preincorporation blocked product. The time course (15–120 min) of the synthesis did not change the ratio of the different synthesis products, suggesting that the termination bands seen in our experiments (Figure 4B) represent permanent DNA synthesis blocks and not merely pause sites. The absence of postlesion synthesis (Figure 5) suggests impairment of the primer extension by Kf Pol I at the site of the *N*3-Et-dT lesion.

The results indicate that when Kf Pol I encounters an *N*3-Et-dT adduct on the template, the instructional mechanisms which position dNTP for subsequent base insertion are apparently unable to function normally. Studies of *E. coli* polymerase I have suggested a slow, rate-determining step in DNA synthesis which precedes phosphodiester bond formation (Mizrahi et al., 1985; Ferrin & Mildvan, 1986; Kuchta et al., 1987). This step may place the dNTP substrate in an appropriate position for phosphodiester bond formation through a first-order isomerization of the enzyme–polynucleotide–dNTP complex. This rate-limiting conformational change may be inhibited when an incorrect dNTP substrate is bound to the polymerase. The extent of inhibition may depend on the base-pairing free energies and stereochemical constraints. The central Watson–Crick hydrogen-bonding site in *N*3-Et-dT is blocked by ethylation, abolishing its ability to base pair. Any dNTP present in the enzyme–polynucleotide–dNTP complex will be an incorrect substrate for incorporation opposite *N*3-Et-dT and may inhibit the rate-limiting isomerization step and subsequent phosphodiester bond formation, causing the DNA synthesis to terminate. This is consistent with the observed 96% DNA synthesis interrupted immediately 3′ to the *N*3-Et-dT lesion.

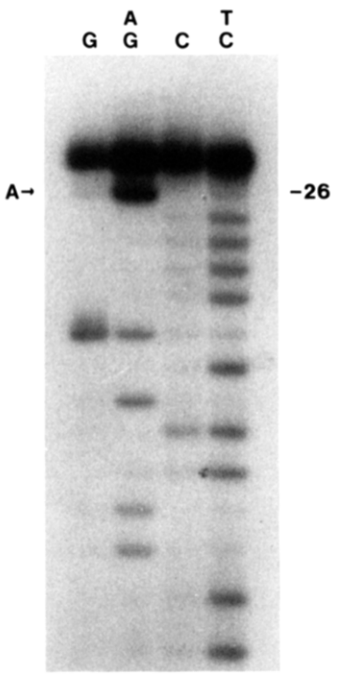

FIGURE 6: DNA sequence analysis of incorporation-dependent blocked product by Maxam–Gilbert procedure. The presence of a band only in the dA-specific lane at position 26 indicates exclusive incorporation of dA opposite *N*3-Et-dT. *N*3-Et-dT is present in the template at position 26 from the 3′-end.

By use of higher concentrations of dNTP, a nucleotide was incorporated opposite *N*3-Et-dT with subsequent termination of DNA synthesis (Figures 4C and 5). For effective recognition of the primer by the polymerase, the terminal deoxynucleotide and several of the adjacent bases must be correctly aligned. When dNTP is incorporated opposite *N*3-Et-dT, the 3′-terminus of the nascent chain may remain unpaired. This intermediate serves as primer for subsequent base addition, and its accumulation presumably reflects impairment of the primer extension. Following incorporation of a nucleotide opposite *N*3-Et-dT, Kf Pol I may dissociate from the primer–template complex, resulting in accumulation of the 26-nucleotide incorporation-dependent blocked product (Figure 5). Alternatively, the polymerase may excise the mispaired terminus through its proofreading exonuclease activity, regenerating the 25-nucleotide preincorporation blocked product.

The absence of postlesion synthesis strongly suggests that the nucleotide incorporated opposite N3-Et-dT does not form a stable base pair with the lesion and results in the termination of DNA synthesis.

DNA sequencing of the 26-nucleotide incorporation-dependent blocked product revealed that dA is incorporated opposite N3-Et-dT (Figure 6). The results indicate that the insertion of nucleotides at the noninstructional lesion, N3-Et-dT, does not occur at random. The nucleotide specificity observed can be understood as a reflection of the polymerase affinity for purines over pyrimidines under template-free conditions (Englund et al., 1969). Our data are consistent with the results reported for other noncoding lesions and support the hypothesis that, in the absence of instruction from the template, DNA polymerases tend to insert a purine (predominantly dA) opposite the noninstructional lesion (Strauss et al., 1982; Strauss, 1985).

Insofar as model systems can predict in vivo events, the findings reported in this paper provide additional support for the "A" rule in mutagenesis. When cells are confronted with noninstructional lesions, they tend to insert dA. If the noninstructional lesion is a modified thymidine such as N3-Et-dT, the incorporation of dA opposite the lesion is biologically significant only if the DNA synthesis is blocked by the lesion. The absence of postlesion synthesis strongly suggests that the N3-Et-dT·dA base pair is energetically and/or sterically unstable and, when present at the 3′-end of the growing chain, leads to termination of DNA synthesis. Under normal cellular conditions, replication past the N3-Et-dT·dA base pair may either not occur or occur at low efficiency, as observed in the bypass of AP sites and UV lesions by DNA polymerase III holoenzyme in vitro (Livneh, 1986; Hevroni & Livneh, 1988). The in vitro DNA replication studies reported in this paper implicate the N3-Et-dT lesion as being a potentially cytotoxic lesion produced by ethylating agents.

Processing of the noncoding N3-Et-dT lesion under SOS-induced conditions is not known. Specific SOS-induced proteins may facilitate the extension of the N3-Et-dT·dA base pair by the replication complex, yielding no mutations. Alternatively, SOS-induced specific proteins may decrease the fidelity of the replication complex, allowing the incorporation of dT, dC, or dG opposite N3-Et-dT, leading to SOS-dependent mutagenesis by this lesion. This is currently being investigated in SOS-induced cells with the φX174-based site-specific mutagenesis system (Bhanot & Ray, 1986).

References

Bargmann, C. I., Hung, M.-C., & Weinberg, R. A. (1986) *Cell 45*, 649–657.

Bartsch, H., & Montesano, R. (1984) *Carcinogenesis 5*, 1381–1393.

Basu, A. K., & Essigmann, J. M. (1988) *Chem. Res. Toxicol. 1*, 1–18.

Bhanot, O. S., & Ray, A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7348–7352.

Bhanot, O. S., Khan, S. A., & Chambers, R. W. (1979) *J. Biol. Chem. 254*, 12684–12693.

Brent, T. P., Dolan, M. E., Fraenkel-Conrat, H., Hall, J., Karran, P., Laval, F., Margison, G. P., Montesano, R., Pegg, A. E., Potter, P. M., Singer, B., Swenberg, J. A., & Yarosh, D. B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7173–7177.

Caruthers, M. H. (1985) *Science 230*, 281–285.

Cuto, L., Chaudhari, I., Donahue, B., Dempel, B., & Essigmann, J. M. (1989) *J. Bacteriol. 171*, 4170–4177.

Den Engelse, L., De Graaf, A., De Brij, R.-J., & Menkveld, G. J. (1987) *Carcinogenesis 8*, 751–757.

Dorper, T., & Winnacker, E. R. (1983) *Nucleic Acids Res. 11*, 2575–2584.

Dyroff, M. C., Richardson, F. C., Popp, J. A., Bedell, M. A., & Swenberg, J. A. (1986) *Carcinogenesis 7*, 241–246.

Eckert, K. A., Ingle, C. A., & Drinkwater, N. R. (1989) *Carcinogenesis 10*, 2261–2267.

Eckert, K. A., Ingle, C. A., Klinedinst, D. K., & Drinkwater, N. R. (1988) *Mol. Carcinog. 1*, 50–56.

Englund, P., Huberman, J., Jovin, T., & Kornberg, A. (1969) *J. Biol. Chem. 244*, 3038–3044.

Ferrin, L. J., & Mildvan, A. S. (1986) *Biochemistry 25*, 5131–5145.

Grevatt, P., & Bhanot, O. S. (1990) *Proc. Am. Assoc. Cancer Res. 31*, 100.

Hevroni, D., & Livneh, Z. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5046–5050.

Hudlicky, J. (1980) *J. Org. Chem. 45*, 5377–5378.

Huff, A. C., & Topal, M. D. (1987) *J. Biol. Chem. 262*, 12843–12850.

Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 8410–8417.

Kunkel, T. A., & Alexander, P. S. (1986) *J. Biol. Chem. 261*, 160–166.

Kunkel, T. A., & Soni, A. (1988) *J. Biol. Chem. 263*, 14784–14789.

Kusmierek, J. T., & Singer, B. (1976) *Nucleic Acids Res. 3*, 989–1000.

Livneh, Z. (1986) *J. Biol. Chem. 261*, 9526–9533.

Loechler, E. L., Green, C. L., & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6271–6275.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 497–560.

Mendelman, L. V., Boosalis, M. S., Petruska, J., & Goodman, M. F. (1989) *J. Biol. Chem. 264*, 14415–14423.

Miller, E. C. (1978) *Cancer Res. 38*, 1479–1496.

Mizrahi, V., Henerie, R. N., Marlier, J. F., Johnson, K. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 4010–4018.

Narang, S. A., Brousseau, R., Hsiung, H. H., & Michniewicz, J. (1980) *Methods Enzymol. 65*, 610–620.

Perantoni, A. O., Rice, J. M., Reed, C. D., Watatani, M., & Wenk, M. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84* 6317–6321.

Preston, B. D., Singer, B., & Loeb, L. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8501–8505.

Saffhill, R. (1985) *Chem.-Biol. Interact. 53*, 121–130.

Saffhill, R., Margison, G. P., & O'Connor, P. J. (1985) *Biochim. Biophys. Acta 823*, 111–145.

Sanger, T., Coulson, A. R., Friedman, T., Air, G. M., Barrell, B. G., Brown, N. L., Fides, J. C., Hutchison, C. A., III, Slocombe, P. M., & Smith, M. (1978) *J. Mol. Biol. 125*, 225–246.

Singer, B. (1986) *Cancer Res. 46*, 4879–4885.

Singer, B., & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.

Singer, B., Sagi, J., & Kusmierek, J. T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4884–4888.

Singer, B., Spengler, S. J., Fraenkel-Conrat, H., & Kusmierek, J. T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 28–32.

Solomon, J. J., Mukai, F., Fedyk, J., & Segal, A. (1988) *Chem.-Biol. Interact. 63*, 275–294.

Stowers, S. J., Wiseman, R. W., Ward, J. M., Miller, E. C., Miller, J. A., Anderson, M. W., & Eva, A. (1988) *Carcinogenesis 9*, 271–276.

Strauss, B. S. (1985) *Cancer Survey 4*, 493–516.
Strauss, B. S., Rabkin, S., Sagher, D., & Moore, P. (1982) *Biochimie 64*, 829–838.
Sukumar, S., Notario, U., Martin-Zanca, D., & Barbacid, M. (1983) *Nature (London) 306*, 658–661.
Walker, G. (1984) *Microbiol. Rev. 40*, 869–907.
Wilson, M. H., & McCloskey, J. A. (1979) *J. Am. Chem. Soc. 97*, 3436–3444.
Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D., & Barbacid, M. (1985) *Nature (London) 315*, 382–385.
Zielenska, M., Beranek, D., & Guttenplan, J. (1988) *Environ. Mol. Mutagen. 11*, 473–485.

Structure and Oxidation–Reduction Behavior of 1-Deaza-FMN Flavodoxins: Modulation of Redox Potentials in Flavodoxins†

Martha L. Ludwig,* Lawrence M. Schopfer, Anita L. Metzger, Katherine A. Pattridge, and Vincent Massey
Department of Biological Chemistry and Biophysics Research Division, University of Michigan, Ann Arbor, Michigan 48109
Received March 5, 1990; Revised Manuscript Received June 27, 1990

ABSTRACT: Flavodoxins from *Clostridium beijerinckii* and from *Megasphaera elsdenii* with 1-carba-1-deaza-FMN substituted for FMN have been used to study flavin–protein interactions in flavodoxins. The oxidized 1-deaza analogue of FMN binds to apoflavodoxins from *M. elsdenii* and *C. beijerinckii* (a.k.a. *Clostridium MP*) with association constants (K_a) of 1.0×10^7 M^{-1} and 3.1×10^6 M^{-1}, values about 10^2 less than the corresponding K_a values for FMN. X-ray structure analysis of oxidized 1-deaza-FMN flavodoxin from *C. beijerinckii* at 2.5-Å resolution shows that the analogue binds with the flavin atoms in the same locations as their equivalents in FMN but that the protein moves in the vicinity of Gly 89 to accommodate the 1-CH group, undergoing displacements which increase the distance between position 1 of the flavin ring and the main-chain atoms of Gly 89 and move the peptide hydrogen of Gly 89 by about 0.6 Å. The X-ray analysis implies that protonation of normal flavin at N(1), as would occur in formation of the neutral fully reduced species, would result in a similar structural perturbation. The oxidation–reduction potentials of 1-deaza-FMN flavodoxin from *M. elsdenii* have been determined in the pH range 4.5–9.2. The oxidized/semiquinone equilibrium (E'_0 = −160 mV at pH 7.0) displays a pH dependence of −60 mV per pH unit; the semiquinone/reduced equilibrium (E'_0 = −400 mV at pH 7.0) displays a pH dependence of −60 mV per pH unit at low pH and is pH independent at high pH, with a redox-linked pK of 7.4. Spectral changes of fully reduced 1-deaza-FMN flavodoxin with pH suggest that this latter pK corresponds to protonation of the flavin ring system (the pK of free reduced 1-deaza-FMN is 5.6 [Spencer, R., Fisher, J., & Walsh, C. (1977) *Biochemistry 16*, 3586–3593]. The pK of reduced 1-deaza-FMN flavodoxin provides an estimate of the electrostatic interaction between the protein and the bound prosthetic group; the free energy of binding neutral reduced 1-deaza-FMN is more negative than that for binding the anionic reduced 1-deaza-FMN by 2.4 kcal. In contrast, the redox-linked pK of native *M. elsdenii* flavodoxin, which is 5.8, is shown not to be associated with changes in the flavin absorbance, indicating that in the normal FMN flavodoxin structure, the pK determined in studies of the semiquinone/reduced equilibrium must be assigned to the protein rather than to FMN. These data are in agreement with the conclusion from NMR studies [Franken, H.-D., Rüterjans, H., & Müller, F. (1984) *Eur. J. Biochem. 138*, 481–489] that flavodoxins bind reduced FMN as the anionic species and suggest a mechanism in which unfavorable electrostatic interactions between the negatively charged ring and its protein neighbors play a major role in determining the redox potential of the semiquinone/reduced flavodoxin couple.

Flavodoxins typically perturb the oxidation–reduction potentials of FMN and display exceptionally low potentials for the one-electron equilibrium between semiquinone and reduced species (Mayhew & Ludwig, 1975). The sq/red[1] potentials are shifted from the free FMN value of −175 mV at pH 7 (Draper & Ingraham, 1968) to about −400 mV. Thermodynamics dictates that the association constant for binding of reduced FMN is therefore smaller than that for FMN semiquinone by a factor of 10^3–10^4, corresponding to a $\delta\Delta G$ of about 5 kcal. The effects of the protein on the potential for the ox/sq equilibrium are more dependent on the species of flavodoxin, ranging from almost no change, relative to free FMN, to increases of about 150 mV. As a consequence, the shifts of the two-electron midpoint potentials, produced by binding of FMN to protein, also vary appreciably with species.

The flavin–protein interactions that are responsible for lowering the sq/red potential have not been easy to identify. The structures of semiquinoid and reduced forms of *Clostridium beijerinckii* flavodoxins are very similar (Smith et al., 1978), indicating that conformation changes do not play a dominant role in the change in affinity for FMN. When the structure of a reduced flavodoxin was first determined (Ludwig et al., 1976) and the flavin ring found to be almost planar, free reduced flavins were thought to be nonplanar (Kierkegaard et al., 1971). It was presumed that the protein forced the cofactor to adopt a high-energy conformation (Ludwig et al.,

† This work was supported by U.S. Public Health Service Grants GM 11106 (V.M.) and GM 16429 (M.L.L.).

[1] Abbreviations: ox, oxidized flavin; sq, one-electron reduced (semiquinone) flavin; red, two-electron reduced flavin; flv, native flavodoxin; dflv, 1-deaza-FMN flavodoxin. Main-chain atoms are denoted as C_α, α-carbon; C, carbonyl carbon; O, carbonyl oxygen; N, peptide nitrogen.

0006-2960/90/0429-10364$02.50/0