Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6834–6837, November 1983
Biochemistry

Sequence-specific double-strand cleavage of DNA by penta-*N*-methylpyrrolecarboxamide-EDTA·Fe(II)

(minor groove binding/netropsin/distamycin/restriction enzymes)

PETER G. SCHULTZ AND PETER B. DERVAN*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

Communicated by John J. Hopfield, August 8, 1983

ABSTRACT **In the presence of O_2 and 5 mM dithiothreitol, penta-*N*-methylpyrrolecarboxamide-EDTA·Fe(II) [P5E·Fe(II)] at 0.5 μM cleaves pBR322 plasmid DNA (50 μM in base pairs) on opposite strands to afford discrete DNA fragments as analyzed by agarose gel electrophoresis. High-resolution denaturing gel electrophoresis of a ^{32}P-end-labeled 517-base-pair restriction fragment containing a major cleavage site reveals that P5E·Fe(II) cleaves 3–5 base pairs contiguous to a 6-base-pair sequence, 5′-T-T-T-T-T-A-3′ (4,323–4,328 base pairs). The major binding orientation of the pentapeptide occurs with the amino terminus at the adenine side of this sequence. In the presence of 5 mM dithiothreitol, 0.01 μM P5E·Fe(II) converts form I pBR322 DNA at 0.22 μM plasmid (1.0 mM in base pairs) to 40% form II, indicating the cleavage reaction is catalytic, turning over a minimum of nine times. This synthetic molecule achieves double-strand cleavage of DNA (pH 7.9, 25°C) at the 6-base-pair recognition level and may provide an approach to the design of "artificial restriction enzymes."**

Restriction endonucleases (type II) cleave double-helical DNA on opposite strands at or close to a defined recognition sequence typically 4–6 base pairs in size (1–3). The ability of these enzymes to cleave double-stranded DNA into unique DNA fragments is useful for DNA sequence determinations, chromosome analyses, gene isolation, and recombinant DNA manipulations. We describe here our initial efforts toward the design of synthetic, sequence-specific, double-strand DNA-cleaving molecules. Attachment of EDTA·Fe(II) to a sequence-specific DNA-binding molecule creates a sequence-specific DNA-cleaving molecule (4, 5). The natural product distamycin A is a tripeptide containing three *N*-methylpyrrolecarboxamides that binds in the minor groove of double-helical DNA with a strong preference for A+T-rich regions (6–11). The sequence specificity of distamycin binding presumably results from hydrogen bonding between the amide NHs of the antibiotic and the O-2 of thymine or the N-3 of adenine (12–15). Distamycin-EDTA·Fe(II) [DE·Fe(II)] and EDTA-distamycin·Fe(II) [ED·Fe(II)], which contain EDTA tethered to the amino or carboxyl terminus of an *N*-methylpyrrole tripeptide, respectively, cleave DNA adjacent to 5-base-pair A+T recognition sites (4, 5). DE·Fe(II) and ED·Fe(II) cleavage of double-helical DNA is confined mostly to single-strand scission (4, 5). The question arises whether a DNA-binding molecule with increased specific base-pair interactions would efficiently cleave DNA on opposite strands.

We report the synthesis of a sequence-specific, double-strand DNA-cleaving molecule, penta-*N*-methylpyrrolecarboxamide-EDTA·Fe(II) [P5E·Fe(II)] (Fig. 1). This bifunctional molecule has EDTA tethered to the amino terminus of an *N*-methylpyrrole pentapeptide (16, 17). In the presence of O_2 and dithiothreitol, P5E·Fe(II) cleaves double-helical pBR322 plasmid DNA (4,362 base pairs) on opposite strands to afford discrete DNA fragments.

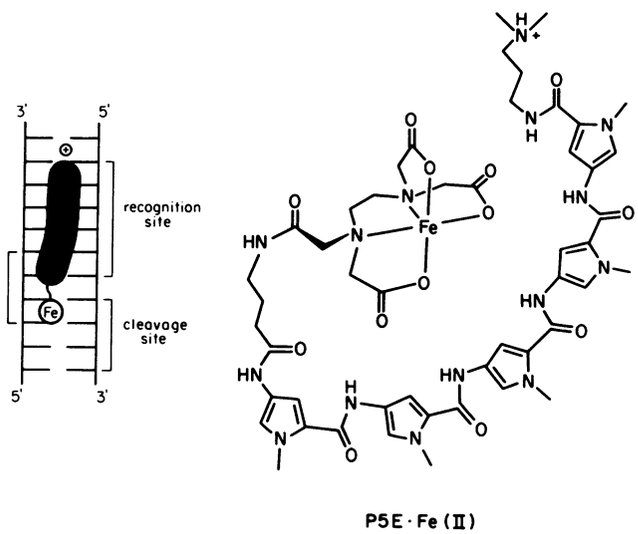

FIG. 1. Penta-*N*-methylpyrrolecarboxamide-EDTA·Fe(II).

MATERIALS AND METHODS

Penta-*N*-methylpyrrolecarboxamide-EDTA (P5E) was synthesized and purified by procedures analogous to those described for distamycin–EDTA (DE) and EDTA–distamycin (ED) (4, 5). The NMR, IR, UV, and mass spectral data are consistent with the assigned structure. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was obtained from Baker and dithiothreitol was from Calbiochem. Aqueous 5′-d[α-^{32}P]ATP triethylammonium salt (3,000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham, and aqueous 3′-d[γ-^{32}P]ATP (5,000–9,000 Ci/mmol) was from ICN. Nucleotide triphosphates were from Boehringer Mannheim. All enzymes were from New England BioLabs except bacterial alkaline phosphatase and T4 polynucleotide kinase, which were from Bethesda Research Laboratories. Solutions of $Fe(NH_4)_2(SO_4)_2$, dithiothreitol, and P5E were prepared freshly. P5E was characterized spectroscopically before use. DE and P5E were mixed immediately before use with Fe(II) at 1 mM concentrations and diluted appropriately. DNA for this investigation was isolated from the bacterial plasmid pBR322, whose entire sequence is known (18). Milligram quantities of the plasmid were grown in *Escherichia coli*, strain HB

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: kb, kilobase(s); MPE·Fe(II), methidiumpropyl-EDTA·Fe(II); P5E·Fe(II), penta-*N*-methylpyrrolecarboxamide-EDTA·Fe(II); DE·Fe(II), distamycin-EDTA·Fe(II); ED·Fe(II), EDTA-distamycin·Fe(II).

* To whom reprint requests should be addressed.

101, and isolated by procedures similar to those of Tanaka and Weisblum (19). Calf thymus DNA (Sigma) was sonicated, deproteinized, and extensively dialyzed.

Cleavage Efficiency Assay. Each DNA-cleaving reagent [DE·Fe(II) and P5E·Fe(II)] was allowed to equilibrate for 1 hr at 37°C with supercoiled (form I) pBR322 DNA (10 μM in base pairs) in a buffer containing 40 mM Tris base and 5 mM NaOAc at pH 7.9. The reaction was initiated by adding an aqueous solution of dithiothreitol. Final concentrations are given in Table 1. The cleavage reactions were allowed to run to completion (1.5 hr at 25°C), quenched with 4 μl of 50 mM Na_2EDTA/10% Ficoll, and electrophoresed on a 1% agarose gel at 120 V for 4 hr. Forms I, II, and III were analyzed by ethidium bromide staining, quantitated by densitometry, and corrected for decreased stainability of form I DNA and for the presence of 1.5% form II in the original sample.

Double-Strand-Cleavage Assay. Linear pBR322 plasmid DNA was obtained by digestion of superhelical plasmids with the restriction endonuclease *Eco*RI, followed by ethanol precipitation. P5E·Fe(II)/dithiothreitol reactions were carried out as described above. The final concentrations were: 0.5 μM and 1.0 μM P5E·Fe(II), 5 mM dithiothreitol, and 50 μM (in base pairs) DNA. Reactions were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The approximate P5E·Fe(II) cleavage sites were located by digestion of pBR322 with several restriction enzymes followed by P5E·Fe(II) (0.5 μM) cleavage. Changes in the lengths of the resulting DNA fragments could be correlated with the locations of the cleavage sites. Mapping restriction enzymes used were *Eco*RI, *Sal* I, *Nde* I, *Ava* I, *Hin*dII + *Ava* I, *Taq* I, and *Rsa* I.

Preparation of Specifically Labeled DNA Fragments. Superhelical pBR322 plasmid DNA was digested with the restriction endonuclease *Eco*RI and then labeled at the 3′ end with d[α-^{32}P]ATP with the use of the Klenow fragment of DNA polymerase I (20). A second enzymatic digest with the restriction endonuclease *Rsa* I yielded two end-labeled fragments, 167 and 517 nucleotides in length. These were isolated by gel electrophoresis on a 2-mm thick 5% polyacrylamide (1:30 crosslinked) gel. Isolation of the two fragments from the gel and subsequent procedures were similar to those of Maxam and Gilbert (20). Cleavage of pBR322 with *Eco*RI and successive treatment with bacterial alkaline phosphatase, d[γ-^{32}P]ATP and T4 polynucleotide kinase (20), followed by digestion with *Rsa* I, yielded the 517- and 167-bp DNA fragments labeled with ^{32}P at the 5′ end.

High-Resolution Denaturing Gel. The cleavage reactions were run as described above with >600 cpm of ^{32}P-end-labeled restriction fragments made up to a total DNA concentration of 100 μM (in base pairs) with sonicated calf thymus DNA. Final concentrations were 1.2 μM P5E·Fe(II) and 1 mM dithiothreitol. The reactions were run at 25°C for 1 hr, terminated by freezing (−78°C), lyophilized and suspended in 4 μl of 100 mM Tris borate/50% formamide at pH 8.3. These samples were heat-denatured and loaded on a 0.4-mm thick, 40-cm long, 8% polyacrylamide (1:20 crosslinked)/50% urea gel and electrophoresed at 1,500 V (20, 21). Autoradiography of the gels was carried out at −50°C on Kodak, X-Omat AR film, and the autoradiograms were scanned at 485 nm on a Cary 219 spectrophotometer. The relative peak area for each site was equated to the relative cleavage efficiency.

RESULTS

Cleavage Efficiency. The DNA cleavage efficiency of P5E·Fe(II) was followed by monitoring the conversion of supercoiled pBR322 plasmid DNA (form I) to open circular (form II) and linear forms (form III). Unlike DE·Fe(II), P5E·Fe(II) required equilibration (37°C for 1 hr) with the DNA before initiation of cleavage with dithiothreitol for optimum efficiency. In the presence of O_2 and dithiothreitol (5 mM), P5E·Fe(II) at 0.01 μM concentration efficiently cleaved DNA (10 μM in base pairs) at almost 2 orders of magnitude lower concentration than required for efficient DE·Fe(II) cleavage (4, 5). Importantly, P5E·Fe(II) at 0.01 μM concentration in the presence of 5 mM dithiothreitol cleaved form I pBR322 DNA at 0.22 μM plasmid (1 mM in base pairs) to 40% form II DNA (Table 1). If one assumes the conversion of form I to form II represents a minimum of one-strand scission, this result corresponds to a minimum of nine single-strand cleavage events per P5E·Fe(II) molecule.

Table 1. Cleavage of pBR322 plasmid DNA (10 μM in base pairs) in the presence of 5 mM dithiothreitol

Reagent	Concentration, μM	Form % I	II	III
EDTA·Fe(II)	1.0	96	4	0
DE·Fe(II)	1.0	32	68	0
P5E·Fe(II)	0.1	3	59	38
P5E·Fe(II)	0.01	48	47	5
P5E·Fe(II)*	0.01	60	40	0

All reactions were run to completion; P5E was preequilibrated at 37°C for 1 hr.
* DNA at 1 mM in base pairs.

Double-Strand Cleavage. The sequence-specific double-strand cleavage of DNA by P5E·Fe(II)/dithiothreitol was examined on linear pBR322 plasmid DNA (4,362 base pairs) obtained by cleavage of supercoiled pBR322 plasmid with *Eco*RI. P5E·Fe(II) (0.5–1.0 μM) was allowed to equilibrate at 37°C for 1 hr with the linear plasmid DNA (50 μM), followed by addition of dithiothreitol (5 mM) to initiate reaction. After 1 hr the reaction mixture was analyzed by agarose gel electrophoresis (Fig. 2). The major observation was that P5E·Fe(II) (P5E per base pair = 0.01) cleaved linear pBR322 DNA into discrete DNA fragments. Restriction mapping indicated that major double-strand cleavage sites are centered at ≈4.3, 4.2, 3.3, and 3.2 kilobases (kb) with minor sites at 2.6, 2.4, 2.0, and 1.8 kb. At higher P5E concentrations (≥1.0 μM), the specificity of the cleavage reaction diminished.

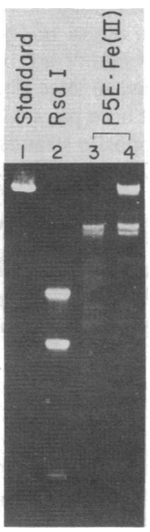

FIG. 2. Agarose gel (1%) of pBR322 plasmid DNA digest (50 μM in base pairs). Lanes: 1, *Eco*RI digest, 4,362-base-pair fragment; 2, *Rsa* I digest, 2,117-, 1,565-, and 680-base-pair fragments; 3, *Eco*RI digest/P5E·Fe(II) at 1.0 μM; 4, *Eco*RI digest/P5E·Fe(II) at 0.5 μM.

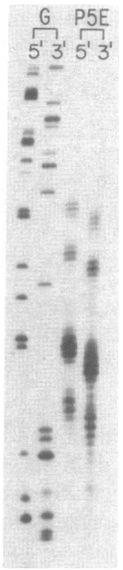

FIG. 3. Autoradiogram of high-resolution denaturing gel. Lanes: 1 and 2, Maxam–Gilbert G reactions on 5′ and 3′ end-labeled 517-base-pair DNA fragment, respectively; 3 and 4, P5E·Fe(II) (1.2 μM) cleavage reactions on 5′ and 3′ end-labeled 517-base-pair DNA fragment (100 μM in base pairs), respectively.

DNA Cleavage-Pattern Analyses. The cleavage sites of P5E·Fe(II) could be resolved in greater detail by analysis of the DNA cleavage patterns on ^{32}P-end-labeled restriction fragments with high-resolution denaturing gel electrophoresis. A 517-base-pair *Rsa* I–*Eco*RI restriction fragment from pBR322 (nucleotides 3,848–4,362) containing two major P5E·Fe(II) cleavage sites (4.3 and 4.2 kb) was chosen for study. The *Eco*RI site was labeled separately with ^{32}P on the 5′ and 3′ ends. The resulting DNA fragments were allowed to react with P5E·Fe(II) (P5E per base pair = 0.012) for 1 hr, stopped by freezing, lyophilized, and suspended in formamide buffer. The ^{32}P-end-labeled DNA cleavage products were analyzed by Maxam–Gilbert gel methods of sequence determination (Fig. 3). A histogram of the DNA-cleavage patterns obtained from densitometric analysis of the autoradiogram shows major cleavage sites covering 3–5 base pairs contiguous to a 6-base-pair region of A+T-rich DNA (base pairs 4,323–4,328) (Fig. 4). The cleavage sites flanking this region are of unequal intensity, with major cleavage on the adenine side of a 6-base-pair 5′-T-T-T-T-T-A-3′ sequence. Minor cleavage sites flank the sequence 5′-T-A-A-T-A-A-T-3′, located at base pairs 4,300–4,306. The cleavage patterns produced on opposite strands are asymmetric, shifted to the 3′ side of each DNA strand.

DISCUSSION

Cleavage Efficiency. P5E·Fe(II)/dithiothreitol cleaves supercoiled pBR322 DNA (10 μM in base pairs) at 2 orders of magnitude lower concentrations than does DE·Fe(II)/dithiothreitol. This higher efficiency presumably results from increased DNA binding affinity and specificity arising from additional hydrogen bonding interactions associated with the pentapeptide P5E·Fe(II) versus the tripeptide DE·Fe(II) (12–15). At 0.01 μM P5E·Fe(II) and high DNA concentrations (1 mM in base pairs), the cleavage reaction is found to be catalytic, turning over a minimum of nine times. However, we do observe inactivation of P5E·Fe(II) over a 2-hr period, which may be due to the degradation of P5E·Fe(II), perhaps by hydroxyl radical.

The timing of the cleavage events is currently not known. Double-strand cleavage may occur by sequential single-strand cleavages at the same site without P5E·Fe(II) dissociation or two single-strand cleavage events resulting from multiple binding events. The observation that one molecule of P5E·Fe(II) can single-strand-cleave nine supercoiled plasmids suggests that the latter mechanism may be important.

Specificity and Binding Site Size. P5E·Fe(II) at 0.5 μM cleaves linear pBR322 DNA (50 μM in base pairs) into discrete DNA fragments, presumably as a consequence of increased binding affinity or binding site size, or both. Restriction mapping indicates four major double-strand cleavage sites (4.3, 4.2, 3.3, and 3.2 kb) and four minor sites (2.6, 2.4, 2.0, and 1.8 kb)—all regions with poly(dA)·poly(dT) sequences. The DNA fragments shown in Fig. 2 (lanes 3 and 4) are the result of partial digests. At higher concentrations of P5E·Fe(II) (1.0 μM), the specificity decreases (Fig. 2, lane 3). This indicates that the four to eight preferred cleavage sites on pBR322 observed at low concentrations of P5E·Fe(II) are not absolute and that lower affinity sites are cleaved as the P5E·Fe(II) concentration is increased. Double-strand cleavage by P5E·Fe(II) is more specific and efficient than by DE·Fe(II) or ED·Fe(II), which double-strand-cleave DNA (50 μM in base pairs) only at concentrations > 0.1 mM.

The DNA cleavage patterns on the high-resolution gel are consistent with P5E·Fe(II) binding to a 6- to 7-base-pair recognition site and cleaving 3–5 bases flanking that binding site (Fig. 4). The multiple cleavage patterns, which are asymmetric on opposite DNA strands, presumably result from a diffusible oxidizing species, perhaps hydroxyl radical, generated from fixed placement of Fe(II) in the minor groove of right-handed double-helical DNA (Fig. 5). The average position of the EDTA·Fe(II) was assumed to be the center of symmetry of the asymmetric cleavage pattern. From this placement of the Fe(II) moiety, the binding sites for the pentapeptide were assigned (boxed areas in Fig. 4). In work not shown here, methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] cleavage inhibition patterns of the pentapeptide P5 (in which the amino end was acetylated) reveals two P5 binding sites on this sequence, corresponding to the binding sites from the P5E·Fe(II) DNA cleavage patterns (22). Like the tripeptide DE·Fe(II), P5E·Fe(II) apparently can adopt two binding orientations on the DNA helix. The unequal intensity of cleavage on each side of the 6-base-pair A+T site reveals major and minor orientations of the pentapeptide binding unit. The major binding orientation of the pentapeptide oc-

FIG. 4. Histogram of P5E·Fe(II) cleavage (Fig. 3). Arrows represent fragments generated by removal of the indicated base.

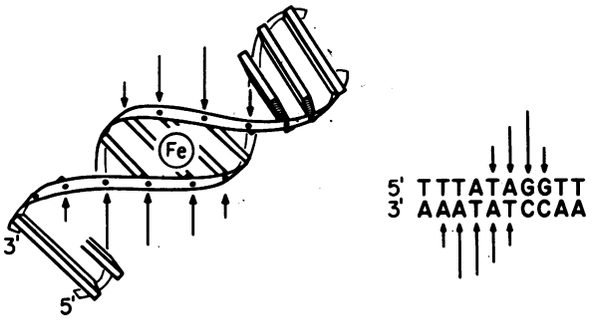

FIG. 5. Model for asymmetric DNA cleavage pattern.

curs with the amino terminus on the adenine side of the 5'-T-T-T-T-T-A-3' sequence (4,323–4,328 bp).

DNA Cleavage Products. Like MPE·Fe(II) and DE·Fe(II), the presumed mechanism of cleavage of DNA by P5E·Fe(II) is oxidative degradation of the deoxyribose on the DNA backbone (4, 23). In unpublished work, we found that MPE·Fe(II) strand scission of DNA releases free purine or pyrimidine bases with loss of the attached deoxyribose. The DNA product labeled at the 3' end resulting from reaction with MPE·Fe(II), DE·Fe(II), and P5E·Fe(II) comigrates on a sequence determination gel with the product from DNase I cleavage of DNA or a Maxam–Gilbert chemical sequence assay reaction and is presumed to have a 5' phosphate terminus (unpublished data). There are two cleavage products on the 3' terminus in roughly equal proportions, which from the electrophoretic behavior of the DNA products labeled at the 5' end are assigned as a 3' phosphate and a 3' glycolic acid esterified through its hydroxyl group to the phosphate terminus (unpublished data). This 3'-phosphodiester terminus is similar to that found from cleavage of DNA by the antitumor antibiotic bleomycin (24).

CONCLUSION

P5E·Fe(II) is a *synthetic* molecule achieving sequence-specific double-strand cleavage of DNA at the 6-base-pair recognition level in a catalytic reaction. P5E·Fe(II) falls short of restriction enzyme capabilities with regard to absolute sequence specificity and the specificity of the cleavage reaction itself. P5E·Fe(II) is a first step toward defining those elements necessary for the design of synthetic double-strand DNA-cleaving molecules of defined target sequence and binding site size.

We are grateful to the National Institutes of Health for grant support (GM-27681) and to IBM for a fellowship to P.G.S.

1. Smith, H. O. (1979) *Science* **205,** 455–462.
2. Modrich, P. (1982) *Crit. Rev. Biochem.* **13,** 288–323.
3. Roberts, R. J. (1983) *Nucleic Acids Res.* **11,** R135–R167.
4. Schultz, P. G., Taylor, J. S. & Dervan, P. B. (1982) *J. Am. Chem. Soc.* **104,** 6861–6863.
5. Taylor, J. S., Schultz, P. G. & Dervan, P. B. (1983) *Tetrahedron Symposium in Print* **38,** in press.
6. Reinert, K. E. (1972) *J. Mol. Biol.* **72,** 592–607.
7. Luck, G., Zimmer, Ch., Reinert, K. E. & Arcamone, F. (1977) *Nucleic Acids Res.* **4,** 2655–2670.
8. Nosikov, V. & Sain, B. (1977) *Nucleic Acids Res.* **4,** 2263–2273.
9. Zimmer, Ch. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **15,** 285–318.
10. Van Dyke, M. W., Hertzberg, R. P. & Dervan, P. B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5470–5474.
11. Van Dyke, M. W. & Dervan, P. B. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 347–354.
12. Wartell, R. M., Larson, J. E., Wells, R. D. (1974) *J. Biol. Chem.* **249,** 6719–6731.
13. Krylov, A. S., Grokhovsky, S. L., Zasedatelev, A. S., Zhuze, A. L., Gursky, G. V. & Gottihk, B. P. (1979) *Nucleic Acids Res.* **6,** 289–304.
14. Berman, H. M., Neidle, S., Zimmer, Ch. & Thrum, H. (1979) *Biochim. Biophys. Acta* **561,** 124–131.
15. Marky, L. A., Blumenfeld, K. S. & Breslauer, K. J. (1983) *Nucleic Acids Res.* **11,** 2857–2870.
16. Zimmer, Ch., Luck, G., Thrum, H. & Pitra, C. (1972) *Eur. J. Biochem.* **26,** 81–89.
17. Arcamone, F., Nicolella, V., Penco, S. & Redaelli, S. (1969) *Gazz. Chim. Ital.* **99,** 632–640.
18. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 77–90.
19. Tanaka, T. & Weisblum, B. (1974) *J. Bacteriol.* **121,** 354–362.
20. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
21. Sanger, F. & Coulson, A. R. (1980) *J. Mol. Biol.* **94,** 441–448.
22. Dervan, P. B. & Schultz, P. G. (1983) *J. Biomol. Struct. Dynam.*, in press.
23. Hertzberg, R. P. & Dervan, P. B. (1982) *J. Am. Chem. Soc.* **104,** 313–315.
24. Giloni, L., Takeshita, M., Johnson, F., Iden, C. & Grollman, A. P. (1981) *J. Biol. Chem.* **256,** 8608–8615.