Site-Directed Mutagenesis of the T4 Endonuclease V Gene: Role of Tyrosine-129 and -131 in Pyrimidine Dimer-Specific Binding†

Donald G. Stump and R. Stephen Lloyd*

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received March 12, 1987; Revised Manuscript Received October 28, 1987

ABSTRACT: T4 endonuclease V incises DNA at the sites of pyrimidine dimers through a two-step mechanism. These breakage reactions are preceded by the scanning of nontarget DNA and binding to pyrimidine dimers. In analogy to the synthetic tripeptides Lys-Trp-Lys and Lys-Tyr-Lys, which have been shown to be capable of producing single-strand scissions in DNA containing apurinic sites, endonuclease V has the amino acid sequence Trp-Tyr-Lys-Tyr-Tyr (128–132). Site-directed mutagenesis of the endonuclease V gene, *den*V, was performed at the Tyr-129 and at the Tyr-129 and Tyr-131 positions in order to convert the Tyr residues to nonaromatic amino acids to test their role in dimer-specific binding. The UV survival of repair-deficient (*uvr*A *rec*A) *Escherichia coli* cells harboring the *den*V N-129 construction was dramatically reduced relative to wild-type *den*V$^+$ cells. The survival of *den*V N-129,131 cells was indistinguishable from that of the parental strain lacking the *den*V gene. The mutant endonuclease V proteins were then characterized with regard to (1) dimer-specific nicking activity, (2) apurinic nicking activity, and (3) binding affinity to UV-irradiated DNA. Dimer-specific nicking activity and dimer-specific binding for both *den*V N-129 and N-129,131 were abolished, while apurinic-specific nicking was substantially retained in *den*V N-129,131 but was abolished in *den*V N-129. These results indicate that Tyr-129 and Tyr-131 positions of endonuclease V are at least important in pyrimidine dimer-specific binding and possibly nicking activity.

T4 endonuclease V, which is the product of the *den*V gene, is an enzyme which binds and incises DNA at the site of UV light induced pyrimidine dimers [see Recinos and Lloyd (1988) for inclusive references]. The T4 *den*V gene has been cloned into a variety of plasmids which express endonuclease V activity in repair-deficient *Escherichia coli* (Valerie et al., 1985; Chenevert et al., 1986; Recinos & Lloyd, 1986; Recinos et al., 1986). Here, we report the use of oligonucleotide site-directed mutagenesis to evaluate DNA sequences within the *den*V gene which may be associated with the pyrimidine dimer specific binding of endonuclease V. The rationale for the selection of protein domains and specific amino acids which may be involved in dimer-specific binding is described below.

A consideration of the mechanism by which endonuclease V recognizes its substrate must focus on the properties of double-stranded DNA containing pyrimidine dimers which promote endonuclease V binding. Following thymine–thymine dimer formation, the two thymine bases are rotated 36° with respect to one another in the DNA and have an angular twist of approximately 4° for each thymine (Camerman & Camerman, 1968). The cyclobutane ring is slightly puckered with one carbon approximately 0.6 Å out of the plane. This in turn causes a puckering in the DNA and a disruption of hydrogen bonding at the dimer and potentially at adjacent bases (Hayes et al., 1971). In examining generalized structures within a protein which might facilitate protein binding at a DNA distortion, Helene's group demonstrated that the Lys-Trp-Lys tripeptide bound preferentially to denatured or UV-irradiated DNA by a two-step mechanism (Toulme et al., 1974; Dimicoli & Helene, 1974a,b; Toulme & Helene, 1977). The first step is postulated to be an electrostatic interaction between the Lys residues and the DNA. The second reaction is a stacking interaction of the indole ring of the Trp residue with denatured or UV-irradiated DNA and, to a 10-fold lesser degree, with native DNA. Peptides which contain Tyr or Phe in place of Trp have virtually no stacking potential with native double-stranded DNA while stacking does occur with single-stranded DNAs (Dimicoli & Helene, 1974b; Mayer et al., 1979). Examination of the amino acid sequence of endonuclease V reveals that the region spanning Trp-128 to Tyr-137 (Trp-Tyr-Lys-Tyr-Tyr-Gly-Lys-Ala-Ile-Tyr) contains four Tyr and one Trp (Valerie et al., 1984; Radany et al., 1984). This sequence is found in the C-terminal portion of the endonuclease V protein. Our working model suggests that Trp and/or Tyr residues cause the enzyme to stop sliding along the DNA through the formation of stacking interactions in regions where hydrogen bonding is disrupted around a pyrimidine dimer.

In addition to determining that the Lys-Trp-Lys tripeptide was capable of stacking with denatured or UV-irradiated DNA, Helene's group also demonstrated that at high concentrations of tripeptide, DNA containing apurinic sites was incised at the site of the missing base. In further analogy with those tripeptides, the *den*V amino acid sequence contains a Lys at position 130 which is flanked by two aromatic amino acids on each side. In the preceding paper (Recinos & Lloyd, 1988), we have investigated whether the Lys-130 residue is responsible for the apurinic nicking activity which is associated with endonuclease V.

Genetic and biochemical data support the hypothesis that the C-terminal portion of endonuclease V is associated with dimer-specific binding. The defect in the temperature-sensitive (ts)[1] *den*V mutant F431 (Sato & Sekiguchi, 1976) is a change of Gly-133 to Asp-133 (Valerie et al., 1984). Extracts of cells

† This research was supported in part by NIH Grant ES 00267 and by a University Research Council Award, Vanderbilt University. D.G.S. was partially supported by NIH Grant HD 07043.

* Address correspondence to this author at the Department of Biochemistry, Vanderbilt University School of Medicine.

[1] Abbreviations: RF, replicative form; AP, apurinic or apyrimidinic; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; ts, temperature sensitive.

0006-2960/88/0427-1839$01.50/0

infected with this mutant have reduced glycosylase and apurinic endonuclease activities at 30 °C which are more pronounced at 42 °C (Sato & Sekiguchi, 1976; Seawell et al., 1980b; McMillan et al., 1981). This reduction in activities probably reflects the equivalent reduction in UV-irradiated DNA binding which was measured by Seawell et al. (1980b). We suggest that the introduction of a negatively charged amino acid adjacent to the Tyr residues provides enough electrostatic repulsion to greatly reduce the accessibility of the enzyme to the dimer. Other genetic and biochemical data to support this hypothesis are as follows. Extracts of cells infected with the T4*v*1 mutant (*den*V1) have no capacity to incise either UV-irradiated DNA or depurinated DNA (Friedberg & King, 1969; Ohshima & Sekiguchi, 1975; Sato & Sekiguchi, 1976; Seawell et al., 1980b; Nakabeppu & Sekiguchi, 1981; Nakabeppu et al., 1982). The region of the *den*V gene which contains this mutation has been sequenced and found to have a thymine deleted in codon 83 resulting in a frameshift mutation (Valerie et al., 1984). The endonuclease V protein encoded by T4*v*1 would have a normal amino terminus through amino acid residue 82 and then an additional 33 residues comprising a hydrophobic tail (Valerie et al., 1984). These authors postulate that the amino-terminal portion of endonuclease V is responsible for DNA binding. This conclusion is difficult to reconcile with the results of Seawell et al. (1980a,b), in which it was demonstrated that there was no binding of the *den*V1 endonuclease V to UV-irradiated DNA even when 4-fold more *den*V1 protein was used relative to that used to show substantial binding by the wild-type protein. On the basis of these observations and data, we postulated that the substitution of other polar (but nonaromatic) amino acid residues at Tyr-129, -131, and -132 would greatly reduce the ability of the enzyme to bind UV-irradiated DNA, and concomitantly reduce both nicking activities on UV-irradiated DNA. The studies described herein were designed to test these predictions.

Materials and Methods

DNA Constructions and Oligonucleotide Site-Directed Mutagenesis. The construction of the *den*V gene in phage M13 mp18 and its use as template for site-directed mutagenesis are described in the preceding paper (Recinos & Lloyd, 1988). DNA oligonucleotides were synthesized by using a BioSearch Sam-One Series II DNA synthesizer and were purified as previously described (Lloyd et al., 1986).

Expression and Characterization of Endonuclease V Proteins. The wild-type and mutant *den*V genes in M13 mp18 RF were subcloned into the *E. coli* plasmid pEMBL19(+). Each of the *den*V$^+$, *den*V N-129, and *den*V N-129,131 pEMBL19(+) constructions were transformed into repair-deficient *E. coli* AB2480 (*uvr*A, *rec*A). Colonies were selected for ampicillin resistance and then for the presence of desired *den*V sequences by probing with specific ^{32}P-labeled oligonucleotides. The constructions were verified by restriction enzyme analyses of the plasmids, Southern hybridization, and DNA sequencing. In all further manipulations, the AB2480 cells containing endonuclease V were grown at 30 °C to stationary phase in LB medium containing 100 μg/mL ampicillin (Recinos et al., 1986).

UV survival quantitation of endonuclease V, pyrimidine dimer specific binding, and nicking and glycosylase activities were performed with each of the mutants essentially as described in the preceding paper (Recinos & Lloyd, 1988).

Results

UV Survival As Measured by Colony-Forming Ability. In an attempt to alter the pyrimidine dimer specific binding of T4 endonuclease V, the technique of oligonucleotide site-directed mutagenesis was used to alter the primary sequence of the T4 *den*V gene. It was postulated that the C-terminal amino acid domain from Trp-128 to Tyr-137 (Trp-Tyr-Lys-Tyr-Tyr-Gly-Lys-Ala-Ile-Tyr) may associate through stacking interactions with DNA at the site of pyrimidine dimers. The amino acid substitutions were made to alter Y-129 to N-129 and alter both Y-129,131 to N-129,131. The mutant *den*V genes which were under the control of the λ hybrid promoter/operator O_LP_R were then inserted into pEMBL19(+). The altered *den*V structural genes were completely sequenced to verify that the DNA sequences had been accurately mutated and that no alterations occurred in other regions of the gene. These plasmids were transformed into *E. coli* AB2480 cells (*uvr*A *rec*A), and the colony-forming ability of repair-deficient cells harboring pEMBL-*den*V constructs was evaluated following UV exposure (Figure 1). The AB2480 containing the wild-type *den*V gene (closed circles) showed a large increase in survival relative to AB2480 cells which only contained pEMBL19(+) (open circles). The colony-forming ability of AB2480 pEMBL-*den*V N-129 (open triangles) was dramatically reduced relative to the level attained by wild-type *den*V complementation. In the case where both Y-129 and Y-131 were changed (open squares), the UV survival of these cells is indistinguishable from that of the parental strain containing pEMBL19. Thus, it appeared that these single and double amino acid substitutions had a dramatic negative affect on the ability of these mutant endonuclease V molecules to enhance UV resistance in these cells.

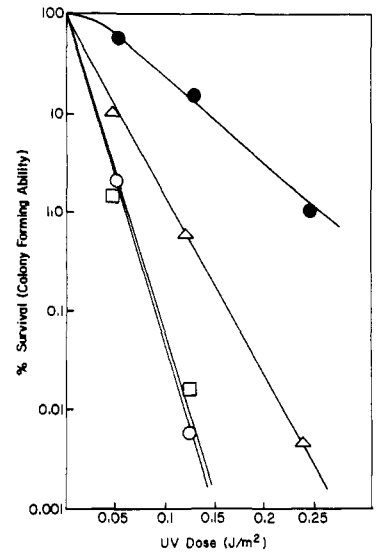

FIGURE 1: Colony-forming ability of UV-irradiated repair-deficient *E. coli* AB2480 cells containing *den*V$^+$ or mutant *den*V plasmid constructs. Stationary-phase cells were irradiated on agar plates and incubated at 30 °C for 30 h. (○) AB2480 cells with pEMBL19; (●) *den*V$^+$ cells; (△) *den*V N-129 cells; (□) *den*V N-129,131 cells.

Quantitation of Mutant Endonuclease V Accumulation in Cells. When the biological activity of mutant enzymes is compared to that of the wild-type, an important consideration is the steady-state accumulation of the respective proteins. Various endonuclease V proteins may accumulate to different levels due to changes in protein half-life, differential transcription rates, mRNA stability, or differential translation initiation rates. In order to determine accumulation levels, wild-type *den*V, *den*V N-129, *den*V N-129,131, and control pEMBL19 were transformed into an *E. coli* minicell-producing strain, X1411. Following the purification of minicells, a constant number of minicells was radiolabeled and analyzed

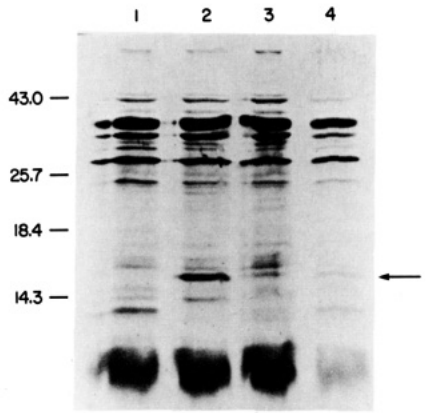

FIGURE 2: Quantitation of the accumulation of mutant endonuclease V in *E. coli* minicells. Plasmid constructs containing no insert, the *den*V$^+$ gene, and the mutant *den*V genes were transformed into the *E. coli* minicell-producing strain X1411. [^{35}S]Methionine incorporation into plasmid-encoded protein was examined following SDS–polyacrylamide gel electrophoresis (15%) and autoradiography. (Lane 1) *den*V$^-$; (lane 2) *den*V$^+$; (lane 3) *den*V N-129; (lane 4) *den*V N-129,131. Prestained molecular weight markers are indicated in $\times 10^{-3}$ at the left. The arrow indicates the position of T4 endonuclease V.

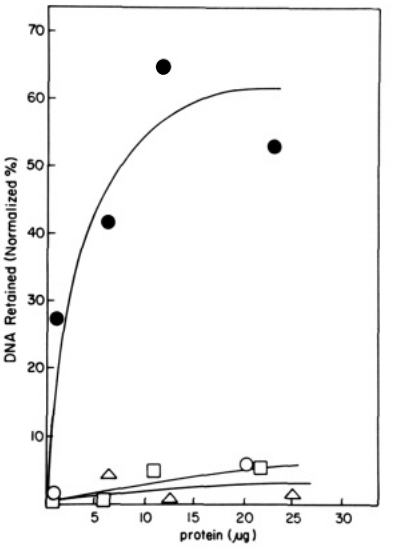

FIGURE 3: Endonuclease V binding to UV-irradiated DNA. The ability of *den*V$^+$, *den*V$^-$, or mutant cell lysates to bind to UV-irradiated DNA was measured by using the nitrocellulose filter binding assay of Seawell et al. (1980a). (●) *den*V$^+$ lysates; (□) *den*V$^-$ lysates; (△) *den*V N-129; (○) *den*V N-129,131. Dimer-specific binding was calculated by first correcting for background retention of unirradiated DNAs and then normalizing the percent DNA retained to the retention observed with saturating amounts of purified endonuclease V.

by SDS–polyacrylamide gel electrophoresis and autoradiography (Figure 2). Densitometric scans of the resulting films revealed that endonuclease V N-129 and endonuclease V N-129,131 were accumulated to 5.5 and 6.7 times less, respectively, than was the wild-type protein. In order to test whether less *den*V-specific mRNA was accumulated within the AB2480 *den*V N-129 cells, RNA was isolated from both wild-type and mutant cells. The relative amounts of mRNAs as measured by slot blot RNA–DNA hybridization showed that the cells containing the mutant plasmids accumulated *den*V-specific mRNAs to 95% of the level found for cells expressing wild-type *den*V (data not shown). Since these DNA changes which occur approximately 400 bases from the ribosome binding site would not be expected to alter translation initiation, the most likely explanation for decreased accumulation of the mutant endonuclease V proteins is that they are subject to increased proteolytic degradation. In the accompanying previous paper (Recinos & Lloyd, 1988), decreased levels of accumulation of two other mutant endonuclease V proteins (at the Lys-130 residue) were also detected. In the paper, mRNA levels were also indistinguishable from wild-type mRNA levels.

Endonuclease V Binding to UV-Irradiated DNA. The ability of wild-type and mutant enzymes to specifically retain UV-irradiated supercoiled DNA to a nitrocellulose filter was determined (Figure 3). The proteins encoded by *den*V N-129 and *den*V N-129,131 were unable to specifically bind dimers in these assays. The endonuclease V from *den*V$^+$ cells, however, demonstrated substantial binding capacities (Figure 3). Thus, 1.1 μg of protein lysate from *den*V$^+$ cells, which contained 4 and 3.3 times less endonuclease V than the highest concentrations used of enzymes N-129 and N-129,131 respectively, caused 27% of the DNA to be bound, while no binding was seen with either the N-129 or the N-129,131 proteins. These results show that the alterations in Y-129 and Y-129,131 result in dramatic decreases in the abilities of these mutants to bind DNA at the site of a pyrimidine dimer.

Endonuclease V Dimer-Specific Nicking Activity. Although no pyrimidine dimer-specific DNA binding activity could be detected, the dimer-specific nicking activity of the mutant endonuclease V proteins was evaluated relative to the wild-type enzyme (Table I). Dimer-specific nicking was also completely

Table I: Endonuclease V Dimer-Specific Nicking Activity

sample	protein (ng)	% form I remaining	% dimer-specific nicking[a]
*den*V$^+$	5700	6	100
	1140	10	93
	228	36	47
*den*V N-129	6250	80	0
	1250	84	0
	250	88	0
*den*V N-129,131	5075	78	0
	1015	87	0
	203	88	0

[a] Percent dimer-specific nicking = (form I_I − N − form I_R)/(form I_I − N) where form I_I = form I initial containing dimers, N = nonspecific nicking, and form I_R = form I remaining containing dimers.

abolished (Table I) using cell extracts containing wild-type, N-129, and N-129,131 enzymes. With 1140 ng of *den*V$^+$ lysate, 93% dimer-specific activity was seen. This amount of protein approximately corresponds to the same amount of endonuclease V as does 6250 ng of *den*V N-129 lysate and 1.5 times more endonuclease V than 5075 ng of *den*V N-129,131 lysate. However, these amounts of mutant enzymes show no nicking activity.

Endonuclease V AP DNA-Specific Nicking Activity. Although the pyrimidine dimer-specific binding and incision properties for both the *den*V N-129 and *den*V N-129,131 proteins were abolished relative to wild-type enzyme, the ability of these enzymes to incise DNA containing AP sites was investigated (Table II). As was expected for *den*V$^-$ cells, there was a residual level of EDTA-resistant AP nicking activity in the cell extracts. However, cell extracts from the wild-type *den*V$^+$ cells showed a substantial enhancement in AP nicking activity above this background due to wild-type endonuclease V activity. The *den*V N-129 cell extract was indistinguishable from that of the parental strain at all enzyme concentrations tested. In contrast, the *den*V N-129,131 lysate did maintain significant levels of AP-nicking activity for several enzyme concentrations. When this level of AP-specific nicking is

Table II: Endonuclease V Apurinic DNA-Specific Nicking Activity

sample	protein (ng)	% form I remaining	% AP-specific nicking[a]
*den*V⁻	1065	27	0
	213	48	0
	106	48	0
	53	48	0
	27	52	0
*den*V⁺	1140	0	100
	228	26	43
	114	34	30
	57	41	15
	28	48	8
*den*V N-129	1250	36	0
	250	48	0
	125	50	0
	62	52	0
	31	53	0
*den*V N-129,131	1015	17	37
	203	36	24
	102	45	7
	51	48	0
	26	52	0

[a] Percent AP-specific nicking = (form II − form II_{denV^-})/(form I_{denV^-} − DNA without AP sites).

normalized to account for the decreased relative accumulation of the *den*V N-129,131 mutant enzyme, the AP-nicking activity is approximately that of wild-type.

Discussion

In this study, we have investigated the functional role of two of the aromatic residues, Y-129 and Y-131, which are found near the C-terminal portion of the T4 endonuclease V enzyme. Each of the mutant enzymes was evaluated relative to wild-type endonuclease V for its biological activity, as well as its pyrimidine dimer-specific binding and DNA incising activities.

Although the accumulation of mutant endonuclease V molecules within cells was reduced, it was still possible to evaluate biological and enzymatic parameters. The UV survival of cells harboring *den*V N-129 and *den*V N-129,131 was significantly reduced compared to *den*V⁺ cells. The UV survival of AB2480 cells containing *den*V N-129,131 was so reduced, as to be indistinguishable from the AB2480 cells containing only pEMBL19. This decrease in survival correlates well with the absence of nicking and binding activities of *den*V N-129,131 cell lysates on UV-irradiated DNA. The *den*V N-129 cells, however, showed survival levels intermediate between *den*V⁺ and *den*V⁻ cells, even though no in vitro nicking or binding activities could be detected in crude cell extracts. This appears to indicate that some endonuclease V binding and nicking activities must be present in the *den*V N-129 cells, but at levels which are not detected in the in vitro cell lysate assays. In addition, the coupled *N*-glycosylase and AP nicking activities of *den*V N-129 and *den*V N-129,131 cell lysates are completely eliminated. This loss in activity for both mutants cannot totally be a result of the decreased amounts of endonuclease V in the mutant cell lysates since the total amount of enzyme was compensated for by the addition of increased total mutant protein. These reduced nicking activities are probably a reflection of dramatically reduced dimer-specific binding. Thus, losses in the ability of mutant enzymes to retain UV-irradiated DNA on nitrocellulose filters correlate well with losses in dimer-specific incising activity.

The AP-specific nicking activity of *den*V N-129,131 protein was similar to that from *den*V⁺ cells, while *den*V N-129 extracts had AP activity indistinguishable from *den*V⁻ lysates. These results are similar to those obtained in the preceding paper (Recinos & Lloyd, 1988) in which a structurally more drastic change of Lys-130 to Gly-130 resulted in AP activity greater than *den*V⁺, while changing to His-130 had little effect. Thus, there does not appear to be direct correlation between AP activity and either UV survival or dimer-specific binding capacity.

The results of this work demonstrate that alterations of the Tyr to Asn at position 129 and at positions 129 and 131 cause a dramatic decrease in both the specificity and activity of endonuclease V on UV-irradiated DNA. Although these changes in activity may simply reflect an overall conformational change in the enzyme which renders it nonfunctional, we consider this unlikely since the double amino acid mutant retains wild-type levels of apurinic nicking activity and the single amino acid mutant retains some detectable biological activity. On the other hand, these data may suggest that this highly aromatic portion of the repair enzyme may be crucial for the interaction with regions of DNA which are single-stranded in character. It should be noted that the predominant effects of amino acid substitutions at the Lys-130 residue of endonuclease V were a reduction in pyrimidine dimer-specific binding rather than effects on nicking activities [see Recinos and Lloyd (1988)]. Conservation of a positively charged amino acid at that position (Lys to His) resulted in a 3-fold reduction in binding while a Lys to Gly change resulted in an almost 10-fold reduction in dimer-specific binding. These data serve to bolster the hypothesis that the C-terminal portion of the T4 endonuclease V is integrally associated with the recognition and binding of endonuclease V to pyrimidine dimers.

Acknowledgments

We thank Dr. M. L. Dodson for many helpful discussions of this research. We also thank Tracy Wright for synthesizing the oligonucleotides needed to perform the site-directed mutageneses and Dr. Neil Osheroff for use of his densitometer. We especially thank Doris Harris for preparation of the manuscript.

Registry No. T4 endonuclease V, 52227-85-7; tyrosine, 60-18-4.

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Kinetic and Equilibrium Binding Studies of Actinomycin D with Some d(TGCA)-Containing Dodecamers†

Fu-Ming Chen

Department of Chemistry, Tennessee State University, Nashville, Tennessee 37203

Received June 30, 1987; Revised Manuscript Received September 18, 1987

ABSTRACT: Comparative kinetic, melting, and equilibrium binding studies of actinomycin D (ACTD) with d(ATATACGTATAT), four d(TGCA)-containing dodecamers, and poly(dG-dC)·poly(dG-dC) revealed that (1) the affinity of ACTD for the dC-dG sequence is much less than for the dG-dC sequence; (2) ACTD forms 1:1 and 2:1 drug–duplex complexes with d(TATATGCATATA) and d(TATGCATGCATA), respectively, and their SDS driven dissociations exhibit single-exponential characteristics with rates ($\sim 5 \times 10^{-4}$ s^{-1} at 20 °C) slightly slower than that of poly(dG-dC)·poly(dG-dC); (3) although the melting temperature of d(CATGCATGCATG) is 8–9 deg higher than that of d(TATGCATGCATA), the rates of ACTD dissociation from these two oligomers are not greatly different and binding constants of (1–5) $\times 10^7$ M^{-1} have been estimated for both; (4) a 3:1 stoichiometry is exhibited by ACTD binding to duplex d(TGCATGCATGCA) and the complex dissociates with two characteristic times, the fast component ($1/k = \sim 100$ s) comprising $^2/_3$ of the contribution and the slow process (~ 2000 s) contributing the other $^1/_3$; and (5) the slow dissociation kinetics of an oligomer appears to be correlated to the higher percentage of slow association kinetics detectable by non-stop-flow techniques. These results indicate that the d(TGCA) sequence is a stronger binding and a slower dissociation site than the d(CGCG) sequence and suggest that base pairs flanking the dG-dC intercalative site may modulate interactions of the pentapeptide rings of ACTD with the DNA minor groove. The fast ACTD dissociation from the near-end dG-dC sites in d(TGCATGCATGCA) is most likely due to the inability of one of the pentapeptide rings to anchor securely, a consequence of end-fraying effects.

Actinomycin D (ACTD) is an antitumor antibiotic that contains a 2-amino-phenoxazin-3-one chromophore and two cyclic pentapeptide lactones. The biological activity of ACTD is believed to be the consequence of its ability to bind to duplex DNA, which results in the inhibition of DNA-dependent RNA polymerase. Earlier binding studies with synthetic polynucleotides (Goldberg et al., 1962; Wells & Larson, 1970) had established the guanine specificity of this drug. Detailed spectroscopic and hydrodynamic studies led Muller and Crothers (1968) and Waring (1970) to conclude that ACTD binds to DNA via insertion of its phenoxazone chromophore between the DNA base pairs. On the basis of their X-ray diffraction results of a 2:1 complex of deoxyguanosine with ACTD, Sobell and Jain (1972) subsequently proposed a binding model with intercalation at the dG-dC sequence and specific hydrogen bonding between the 2-amino group of guanine and the carbonyl oxygen of the threonine of the peptide rings. NMR studies (Patel, 1974; Krugh et al., 1977; Brown et al., 1984) using oligonucleotides containing the dG-dC sequence had generally agreed with such a binding model. Recent DNase I footprinting experiments (Lane et al., 1983; Scamrov & Beabealashvilli, 1983; Fox & Waring, 1984a) have also confirmed the dG-dC binding specificity of ACTD. These observations are further supported by the finding that the most prominent RNA elongation inhibition sites are encoded by a consensus tetranucleotide sequence XGCY, where X is any nucleotide but G and Y is any nucleotide but C (Aivasashvilli & Beabealashvilli, 1983), suggesting possible effects of neighboring base pairs on the ACTD binding.

The combination of a planar intercalating phenoxazone chromophore and the two pentapeptide rings appears to be responsible for some of the unusual kinetic properties observed for the binding of ACTD to DNA. For example, the association of ACTD to natural DNA is characterized by five

† Research supported by USPHS Grant CA-42682 and by a subproject of MBRS Grant S06RR0892.

0006-2960/88/0427-1843$01.50/0