Construction and Characterization of a Site-Directed CC-1065–N3-Adenine Adduct within a 117 Base Pair DNA Restriction Fragment†

Donald R. Needham-VanDevanter and Laurence H. Hurley*

Division of Medicinal and Natural Products Chemistry, Drug Dynamics Institute, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712-1074

Received June 17, 1986; Revised Manuscript Received August 14, 1986

ABSTRACT: The design, construction, and characterization of a site-directed CC-1065–N3-adenine adduct in a 117 base pair segment of M13mpI DNA are described. CC-1065 is an extremely potent antitumor antibiotic produced by *Streptomyces zelensis*. Previous studies have demonstrated that the cyclopropyl ring of CC-1065 reacts quite specifically with N3 of adenine in double-stranded DNA to form a CC-1065–DNA adduct. Following alkylation, the drug molecule lies snugly within the minor groove of DNA, overlapping with five base pairs for which a marked sequence preference exists [Hurley, L. H., Reynolds, V. R., Swenson, D. H., Petzold, G. L., & Scahill, T. A. (1984) *Science (Washington, D.C.) 226*, 843–844]. On the basis of the unique characteristics of the reaction of CC-1065 with DNA and the structure of the resulting DNA adduct, we have designed a general strategy to construct a site-directed CC-1065–DNA adduct in a restriction fragment. The presence of unique *Alu*I and *Hae*III restriction enzymes sites on each side of a high-affinity CC-1065 binding sequence (5′-GATTA) permitted the preparation of a partial duplex DNA molecule containing the CC-1065 binding sequence in the duplex DNA region. Since CC-1065 only binds to duplex DNA, potential CC-1065 binding sequences in the long single-stranded regions were protected from drug binding during the construction process. After purification of the CC-1065 partial duplex DNA adduct by differential melting of the modified and unmodified partial duplex DNA, DNA polymerase I was used to generate the full duplex DNA molecule, which contained a single site-directed CC-1065–N3-adenine adduct at adenine 6229 of the 117 base pair *Msp*I–*Bst*NI DNA restriction fragment of the *Escherichia coli lac* insert of M13mpI DNA. A CC-1065 thermal strand scission assay was used to confirm the unique binding site on the covalently modified strand. Methidiumpropyl-EDTA–iron(II) [MPE–Fe(II)] digestions were used to locate the binding site and the orientation of CC-1065 in the minor groove of DNA. MPE–Fe(II) footprinting revealed a slight enhancement of digestion on both DNA strands, but just to one side of the CC-1065–DNA adduct.

CC-1065 is an extremely potent antitumor antibiotic isolated from *Streptomyces zelensis* (Hanka et al., 1978; Martin et al., 1981). The antibiotic is composed of three benzodipyrrole subunits linked by amide bridges (Chidester et al., 1981), of which one contains an alkylating cyclopropane ring. These out of plane amide linkages impart a shallow twist to the CC-1065 molecule, which allows a discrete interaction within the minor groove of B-form DNA that leads to covalent binding to N3 of adenines (see Figure 1) in specific sequences (Hurley et al., 1984; Reynolds et al., 1985). In contrast, CC-1065 does not covalently bind to ssDNA,[1] RNA, or protein (Swenson et al., 1982). A comprehensive review of all aspects of CC-1065 including chemistry, synthesis, biosynthesis, and biological activity has recently been published (Reynolds et al., 1986).

A detailed knowledge of the chemical nature of the CC-1065–DNA adduct (Hurley et al., 1984; Reynolds et al., 1985) does not lead directly to an understanding of how CC-1065 might exert its potent cytotoxic potency (Bhuyan et al., 1982). It has been previously suggested (Reynolds et al., 1985) that CC-1065 may exert long-range effects on DNA structure and function resulting in cell death. Alternative hypotheses such as ineffective or deleterious DNA repair or modification of critical DNA sequences have been suggested as potential sequelae responsible for CC-1065 potency (Reynolds et al., 1985). Recently, we have reported on the potent and prolonged depletion of NAD levels in human cells exposed to CC-1065. This depletion of NAD apparently results from poly(ADP-ribose) synthesis, presumably in response to repair of CC-1065–DNA adducts (Jacobson et al., 1986). It is not clear, however, whether this depletion is responsible for the potent cytotoxic effects of CC-1065.

In order to study CC-1065–DNA adduct chemistry in a more defined system, we have developed a method for the construction of a site-directed CC-1065 adduct in a 117-bp DNA restriction fragment. Site-directed drug–DNA adducts avoid complications resulting from multiple adduct populations, locations, and types. Single drug adducts associated with short synthetic DNA oligomers have been previously employed to characterize the noncovalent minor groove ligand distamycin (Kopka et al., 1985) and the intercalater daunomycin (Quigley et al., 1980) by X-ray crystallography. A covalent adduct between the minor groove binder anthramycin and a synthetic

† Supported by grants from the National Cancer Institute, PHHS (CA-30349), and the Welch Foundation.

* Address correspondence to this author.

[1] Abbreviations: AAF, 2-(acetylamino)fluorene; ADP, adenosine diphosphate; AP, apurinic; BAP, bacterial alkaline phosphatase; bp, base pair(s); dNTP, deoxynucleotide triphosphate; dsDNA, double-stranded DNA; DSC, dilute saline citrate; DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MPE, methidiumpropyl-EDTA; NAD, nicotinamide adenine dinucleotide; PNK, polynucleotide kinase; Pu, purine; Py, pyrimidine; RF, reproductive form; ssDNA, single-stranded DNA; TBE, Tris–borate–EDTA; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-8430$01.50/0

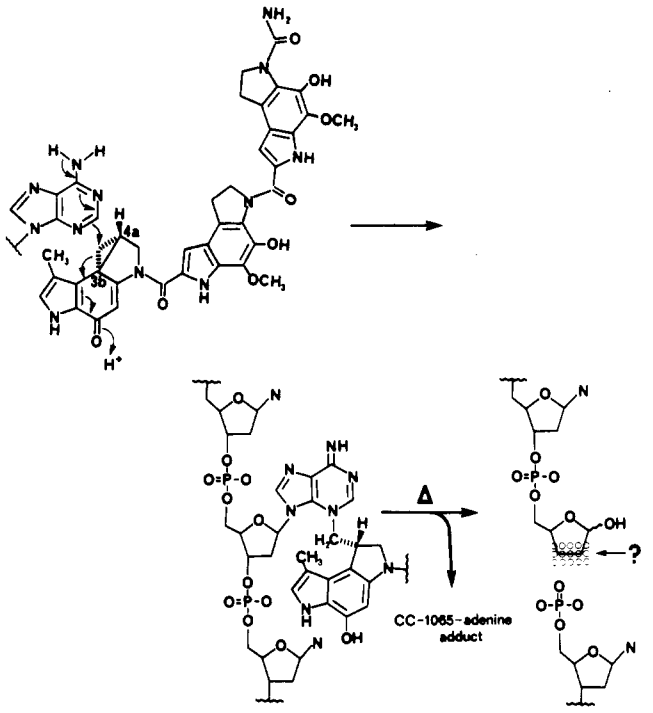

FIGURE 1: Reaction of CC-1065 with DNA to form the CC-1065–N3-adenine adduct and the consequences of thermal cleavage (Hurley et al., 1984; Reynolds et al., 1985).

hexamer duplex has been examined by ^{1}H and ^{13}C NMR (Graves et al., 1984, 1985). Additionally, site-directed drug–DNA adducts of O^6-methylguanine (Essigmann et al., 1982; Chambers et al., 1984) and AAF (Johnson et al., 1986) in circular plasmid DNA have been constructed to examine the biological effects of these DNA adducts.

Materials and Methods

Materials

CC-1065 was obtained from the Upjohn Co., Kalamazoo, MI. Electrophoretic reagents [acrylamide, TEMED, ammonium persulfate, and bis(acrylamide)] were from Bio-Rad. BAP, dNTPs, and lysozyme were from Sigma. All other enzymes [restriction endonucleases, T4-PNK, and DNA polymerase I (large fragment)] were from New England Biolabs. M13mp1 vector was obtained from Professor J. Messing, University of Minnesota. MPE was a generous gift of Professor P. Dervan, California Institute of Technology. M13mp1 RF DNA was isolated from infected *Escherichia coli* JM101 cells by $CsCl_2$ ultracentrifugation as described by Messing (1983). [γ-^{32}P]ATP was from ICN. X-ray film, intensifying screen, and developing chemicals were from Kodak.

Methods

DNA Restriction Digestion. DNA restriction digestions were performed in 50-μL volumes with supplier-recommended buffer and temperature conditions. Digestions were stopped with 10 μL of 0.1 M EDTA and ethanol-precipitated (Maniatis et al., 1982).

5′-^{32}P End Labeling of DNA Restriction Fragments. DNA restriction fragments were 5′-^{32}P end labeled by sequential BAP and T4-PNK treatments. Samples were suspended in 50 μL of 50 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.1 unit of BAP and incubated for 1 h at 65 °C. Incubations were extracted sequentially with 1:1 phenol/chloroform and chloroform and ethanol-precipitated. DNA pellets were resuspended in 25 μL of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM spermidine, and 0.5 mCi of [γ-^{32}P]ATP to which 4 units of T4-PNK was added. Samples were incubated 20 min at 37 °C and ethanol-precipitated.

Polyacrylamide Gel Electrophoresis. DNA fragments were purified by polyacrylamide gel electrophoresis with gels containing 19:1 acrylamide–bis(acrylamide) cross-linking and TBE (50 mM Tris–borate, 10 mM EDTA, pH 8.3) running buffer. Denaturing gels also contained 50% urea. DNA samples were suspended in neutral (50% v/v glycerol, 25 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) or alkaline (80% v/v formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol) tracking dyes and loaded into wells by glass syringe. Alkaline samples were heated at 90 °C for 30 s and quick cooled before loading. Gel dimensions were 360 × 300 × 0.4 mm, and gels were electrophoresed at 1000 V. DNA bands were visualized by ethidium bromide staining (unlabeled fragments) or autoradiography (labeled fragments). DNA bands were purified from acrylamide slabs by electroelution within dialysis bags, filtration through glass wool, and ethanol precipitation.

Densitometric Analysis of Autoradiograms. Autoradiograms obtained from polyacrylamide electrophoresis gels were scanned with laser densitometer (LKB 2202) coupled to a recording integrator (LKB 2220).

DNA Sequencing Reactions. Singly 5′-^{32}P-labeled DNA fragments were sequenced by the Maxam and Gilbert (1980) base-specific cleavage method and suspended in alkaline tracking dye for subsequent polyacrylamide gel electrophoresis.

CC-1065 Binding to DNA and Determination of Covalent Binding Sites by Thermal Treatment. CC-1065 was bound to DNA fragments in 20-μL volumes containing DSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.4) buffer at 4 °C for 24 h. Noncovalently bound CC-1065 was removed by two sequential ethanol precipitations.

CC-1065 binding sites on DNA fragments were identified by the method of Reynolds et al. (1985), in which samples were suspended in 50 μL of DSC, heated at 90 °C for 30 min, and ethanol-precipitated. DNA pellets were suspended in alkaline tracking dye and electrophoresed on denaturing polyacrylamide gels adjacent to Maxam–Gilbert sequencing reactions.

Generation of Singly 5′-Labeled M13mp1 117-bp DNA Fragments. M13mp1 RF DNA (200 μg) was restriction-digested with either *Bst*NI or *Msp*I restriction endonuclease. The 250-bp product of *Msp*I digestion (MspIJ) and 137-bp product of *Bst*NI digestion (BstNIE) were isolated and purified by 5% polyacrylamide gel electrophoresis and 5′-^{32}P end labeled. Labeled MspIJ DNA was digested with *Bst*NI endonuclease to generate singly a 5′-(+)-labeled 117-bp fragment, and labeled BstNIE DNA was digested width *Msp*I endonuclease to generate a singly 5′-(−)-labeled 117-bp fragment, both of which were purified by 8% polyacrylamide gel electrophoresis.

Isolation of 82- and 61-Base Single-Stranded DNA Fragments. Doubly 5′-^{32}P end-labeled MspIJ (250 bp) DNA was digested with *Hae*III, while doubly 5′-^{32}P end-labeled BstNIE (137 bp) DNA was digested with *Alu*I. Products from digestions were ethanol-precipitated, suspended in alkaline tracking dye, and electrophoresed on an 8% denaturing polyacrylamide gel until the dye front reached 250 mm. The gel was autoradiographed and bands corresponding to the 82-base (+) strand product of the *Hae*III digest of MspIJ DNA and the 61-base (−) product of the *Alu*I digest of BstNIE DNA were cut out, electroeluted into dialysis bags, dialyzed extensively against 20 mM NH_4HCO_3, and lyophilized to dryness. Remaining NH_4HCO_3 was removed by three sequential lyophilizations from 100 μL of deionized water.

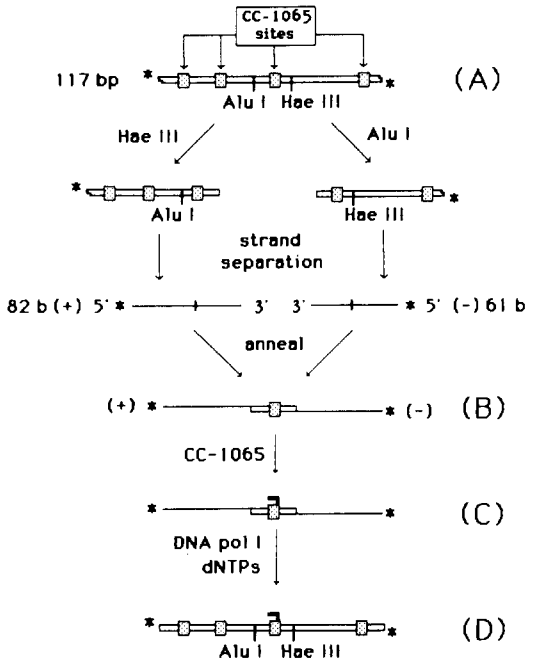

FIGURE 2: Strategy for construction of a site-directed CC-1065–DNA adduct. The 117-bp *Msp*I–*Bst*NI DNA restriction fragment (bases 6160–6276) of the *E. coli lac* insert of M13mp1 DNA was doubly 5′-^{32}P end-labeled (species A) and digested with either *Alu*I or *Hae*III restriction endonucleases. The resulting fragments were separated by denaturing polyacrylamide electrophoretic gel to isolate the 82-base (+)-ssDNA strand of *Hae*III digestion and the 61-base (−)-ssDNA strand of *Alu*I digestion. These two semicomplementary DNA strands were annealed to form a partial DNA duplex containing a single CC-1065 binding site at 6229 of the (+) strand (species B). CC-1065 was bound to the partial duplex DNA (species C), which was then extended to a complete duplex (species D) with DNA polymerase I (large fragment) and dNTP's and then purified by gel electrophoresis.

Construction of Partial Duplex DNA. Doubly 5′-^{32}P end-labeled partial duplex DNA was generated by annealing equal quantities (as determined by scintillation counting) of labeled 82-base (+) and 61-base (−) ssDNA strands. The ssDNA strands were suspended together in 20 μL of 100 mM NaCl, 1 mM Tris-HCl, pH 7.4, and 1 mM EDTA, warmed to 60 °C, and cooled slowly to 10 °C over about a 12-h period.

Singly 5′-^{32}P end-labeled partial duplex DNAs were generated by removing the 5′-terminal [^{32}P]phosphates of one or the other single DNA strands before annealing. Either 82-base (+) or 61-base (−) DNA was suspended in 50 μL with 50 mM Tris-HCl, pH 8.0, and 0.1 unit of BAP and incubated at 65 °C for 1 h. Incubations were phenol/chloroform- and chloroform-extracted and dialyzed extensively against 20 mM NH_4HCO_3. Dialyzates were lyophilized to remove NH_4HCO_3. An equal amount of 5′-^{32}P-labeled complementary strand was added to the samples, which were suspended and annealed as above.

RESULTS

Strategy for Construction of a Site-Directed CC-1065–N3-Adenine Adduct. Construction of a site-directed CC-1065–DNA adduct within a short DNA restriction fragment benefited from earlier observations that CC-1065 forms a relatively nondistortive DNA adduct (Swenson et al., 1982), exhibits a sequence selectivity of DNA binding (Reynolds et al., 1985), and will only bind to dsDNA (Swenson et al., 1982). Consequently, it was proposed that a short DNA duplex containing a high-affinity CC-1065 binding sequence (5′-PuNTTA; Reynolds et al., 1985) with long 5′ single-stranded extensions on opposite strands would serve as a template for

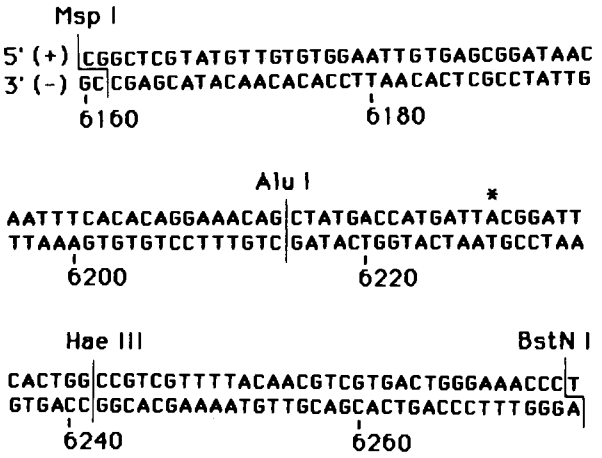

FIGURE 3: Sequence of the 117-bp *Msp*I–*Bst*NI fragment of M13mpI RF DNA (Messing 1983). *Alu*I and *Hae*III restriction sites and the adenine (*) modified in the site-directed adduct are identified.

site-directed adduct construction (species B in Figure 2). A partial duplex DNA molecule of this type can be constructed from a DNA restriction fragment containing a high-affinity CC-1065 binding site flanked by two unique restriction enzyme sites (species A in Figure 2). Saturation binding of CC-1065 to a short oligodeoxynucleotide duplex without 5′ ssDNA tails has been previously described (Needham-VanDevanter et al., 1984). Following saturation binding of CC-1065 to the lone drug binding site within the partial DNA duplex (species C in Figure 2), the DNA molecule could be extended to a complete duplex by the large fragment of *E. coli* DNA polymerase I (C to D in Figure 2).

The DNA region chosen for site-directed CC-1065 adduct construction and analysis was the 117-bp segment of M13mpI DNA between the *Msp*I (6160) and *Bst*NI (6276) restriction endonuclease sites. This sequence (an insert of *E. coli lac* DNA; Messing, 1983) contains an isolated high-affinity 5′-GATTA CC-1065 binding site at adenine 6229 on the (+)-DNA strand flanked by unique *Alu*I and *Hae*III restriction sites (Figure 3). Figure 2 outlines the specific strategy proposed to construct the site-directed adduct within this DNA segment.

Location and Sensitivity on CC-1065 Binding Sites within the 117-bp DNA Fragment. To ensure that CC-1065 binding only occurred at adenine 6229 within the DNA region between *Alu*I and *Hae*III restriction sites, the 117-bp DNA fragment chosen for construction was analyzed for high- and low-affinity CC-1065 binding sites. Singly 5′-^{32}P end-labeled fragments on both strands were modified with several 10-fold dilutions of CC-1065 and heated at 90 °C for 30 min to generate drug-induced DNA strand breakage [see Figure 1 and Reynolds et al. (1985)]. Electrophoresis of CC-1065-modified fragments subjected to thermal treatment in lanes adjacent to unmodified DNA subjected to Maxam–Gilbert sequencing reactions (Figure 4) allowed identification of CC-1065 binding sites within this DNA fragment. As anticipated, the site most sensitive to CC-1065 binding within the 117-bp fragment was adenine 6229 of the (+) strand (lanes 4 and 5). Higher concentrations of CC-1065 induced binding at other adenines within the 117-bp DNA fragment. A second adenine between *Alu*I and *Hae*III restriction sites [6217 of the (+) strand] was modified at high CC-1065 concentrations. However, this adenine was not predicted to bind CC-1065 during the site-directed construction process, since the partial DNA duplex region would terminate two bases to the 5′ side of this residue and would therefore prevent drug binding. No significant drug binding was observed on the (−) strand between the *Alu*I and

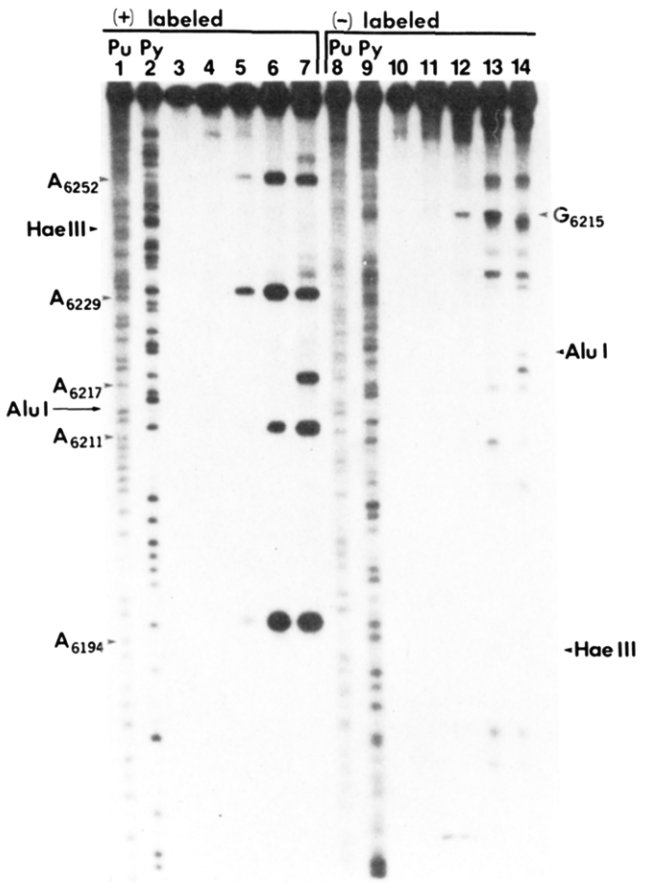

FIGURE 4: Distribution of CC-1065 binding sites in the 117-bp *Msp*I–*Bst*NI fragment of M13mp1 DNA. Singly 5′-^{32}P-labeled DNA fragments [lanes 1–7, 5′-^{32}P-(+)-labeled; lanes 8–14, 5′-^{32}P-(−)-labeled] were modified with 0 nM (lanes 3 and 10), 2.8 nM (lanes 4 and 11), 28 nM (lanes 5 and 12), 280 nM (lanes 6 and 13), or 2.8 μM (lanes 7 and 14) CC-1065. Modified DNA was heated at 90 °C for 30 min in DSC and electrophoresed adjacent to Maxam–Gilbert purine- and pyrimidine-specific DNA cleavage reactions (lanes 1, 2, 7, and 8). Adenines significantly modified by CC-1065 are noted. Guanine 6215 on the (−) strand is not a CC-1065 binding site but is apparently sensitive to thermal hydrolysis in the presence of adjacent CC-1065 adducts. A similar phenomenon in other sequences has been previously reported (Reynolds et al., 1985).

the *Hae*III restriction sites. At high CC-1065 concentrations, binding at adenine 6229 appears to decrease (lane 7), presumably due to multiple site occupation and competition by strand scission events occurring at adenines closer to the 5′-^{32}P label.

Construction of Partial Duplex Adduct and Differential Melting of Modified and Unmodified Partial Duplex DNA. Having established that the DNA sequence chosen was appropriate for the site-directed CC-1065–DNA adduct construction outlined in Figure 2, a partial duplex DNA molecule (B in Figure 2) was constructed. The 5′-^{32}P end-labeled single-stranded DNA molecules [the 82-base (+) and 61-base (−) products of *Hae*III or *Alu*I digestions, respectively] were purified from their unlabeled complementary strands by denaturing polyacrylamide gel electrophoresis (not shown). The (+)- and (−)-ssDNA species were annealed to generate the partial duplex DNA molecule (species B in Figure 2), which migrated slower than its ssDNA components on a nondenaturing polyacrylamide electrophoretic gel (compare lane 3 with lanes 1 and 2 in Figure 5). Incubation of the partial duplex with 280 nM CC-1065 for 12 h at 4 °C to form the CC-1065 adduct (species C in Figure 2) did not change the electrophortic migration of this new species (lane 4 in Figure 5). CC-1065 binding to the partial duplex DNA was confirmed

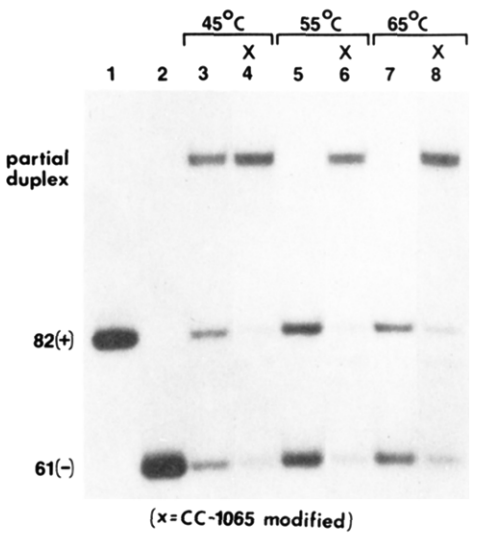

FIGURE 5: Thermal melting of CC-1065-modified and -unmodified partial duplex DNA. Doubly 5′-^{32}P-labeled partial DNA duplex molecules were modified with 280 nM CC-1065 (species C of Figure 2; lanes 4, 6, and 8) and compared to unmodified partial duplex DNA (species B of Figure 2; lanes 3, 5, and 7) with respect to thermal melting. Samples were warmed to the temperatures indicated for 10 min in 10 μL of 10 mM Tris-HCl and quickly cooled to 0 °C. A 10-μL aliquot of neutral tracking dye was added, and samples were electrophoresed on a polyacrylamide gel adjacent to 5′-labeled 82-base (+)-ssDNA (lane 1) and 61-base (−)-ssDNA (lane 2).

by differential thermal melting (Figure 5). Unmodified partial duplex DNA was observed to melt partially at 45 °C (lane 3) and completely at 55 °C (lane 5). In contrast, CC-1065-modified partial duplex DNA did not melt significantly at temperatures up to 65 °C (lane 8).

DNA Polymerase I Extension of CC-1065-Modified and CC-1065-Unmodified Partial Duplex DNA Molecules to Full DNA Duplexes. Both (+) and (−) singly 5′-^{32}P end-labeled partial duplex DNA species with and without CC-1065 modification were extended to full duplexes (species A or D in Figure 2) with dNTPs and the large fragment of *E. coli* DNA polymerase I. All four polymerase extension reactions yielded an identical new DNA species with a slower migration than the partial duplex DNA precursor (Figure 6). Contaminating reaction byproducts also appear in the reaction mixture (see arrows in Figure 6) and may result from polymerase action on ssDNA with secondary structure.

Characterization of Site-Directed CC-1065 Adduct in the 117-bp DNA Restriction Fragment. (*a*) *Thermal Treatment.* Heating of CC-1065-modified and -unmodified 117-bp DNA duplexes at 90 °C for 30 min yielded a drug-induced strand breakage corresponding to a single CC-1065 adduct located exclusively at adenine 6229 on the (+) strand (Figure 7, lanes 7–10). No other binding sites were occupied by CC-1065 (compare Figure 4 and Figure 7, lanes 7–10). The duplicity of species at the top of the gel in lanes 1 and 6 is due to a mixture of the single-stranded 117-base fragment either containing an apurinic acid site at A-6229 (lower species) or the CC-1065 adduct species (upper species). Even heating at 90 °C for 1 min at neutral pH prior to electrophoresis leads to some depurination at A-6229. Previous experiments showed that treatment of the residual material at the top of the gel with piperidine leads to quantitative conversion to a Maxam–Gilbert sequencing reaction for A-6229, consistent with an AP site at this position (Needham-VanDevanter et al., 1984).

(*b*) *MPE–Fe(II) Footprinting.* Both singly 5′-end-labeled site-directed CC-1065–DNA adducts were digested with MPE–Fe(II) both to confirm the location and to determine

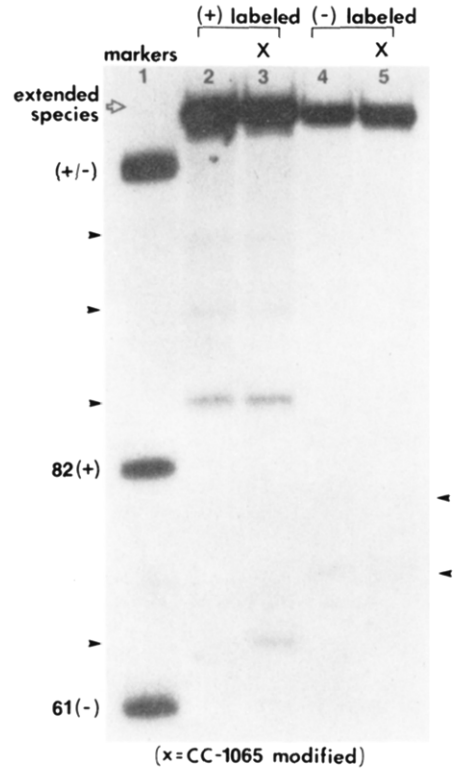

FIGURE 6: Extension of partial duplex DNA to complete duplexes with DNA polymerase I. Partial DNA duplexes with single 5′-^{32}P labels on the (+) strand (lanes 2 and 3) or the (−) strand (lanes 4 and 5) and either containing (lanes 3 and 5) or lacking (lanes 2 and 4) CC-1065–DNA adducts were suspended in 20 μL of 10 mM Tris-HCl, pH 8.0. Concentrated extension buffer (3 μL of 500 mM Tris-HCl, pH 7.2, 100 mM $MgSO_4$, and 1 mM DTT in 500 μg/mL BSA) was added, along with 5 μL of 2 mM dATP, 2 mM dTTP, 2 mM dCTP, 2 mM dGTP, and 4 units of *E. coli* DNA polymerase I (large fragment). Incubations were performed for 2 h at 16 °C, stopped with 10 μL of neutral tracking dye, and electrophoresed on a nondenaturing polyacrylamide gel adjacent to partial duplex DNA, 82-base (+)-DNA, and 61-base (−)-DNA standards (lane 1).

the orientation of the CC-1065–DNA adduct in the minor groove (Figure 7, compare lane 1 with 2 and lane 15 with 16). The presence of drug-induced strand breakage, as well as unreleased CC-1065 adduct, complicates the migration of DNA bands to the 3′ side of adenine 6229 on the (+) strand (lane 1). Densitometric comparison of modified and unmodified (−) strand MPE–Fe(II) digestions (Figure 8) shows that the CC-1065 molecule lies to the 5′ side of adenine 6229, as predicted by earlier sequence specificity studies (Hurley et al., 1984).

Analysis of the MPE–Fe(II) footprinting histogram (Figure 8) also reveals a slight enhancement in MPE–Fe(II), cutting both to the 5′ side and 3′ side of the drug binding site on the (+) and (−) strands, respectively. Both the MPE–Fe(II) footprint of CC-1065 and the increased MPE–Fe(II) cutting are two to three bases offset to the 3′ direction on opposite strands, a characteristic of MPE–Fe(II) footprinting (Van Dyke & Dervan, 1982).

Discussion

In this paper we describe the construction of a site-directed adduct of CC-1065 in a 117-bp DNA fragment. The characterization of the construction shows that there is a single type of lesion incorporated specifically at A-6229 and virtually all the DNA molecules contain this lesion.

The novel construction of a site-directed CC-1065–N3-adenine adduct within a 117-bp DNA fragment described here was dependent on a number of unique CC-1065 characteristics.

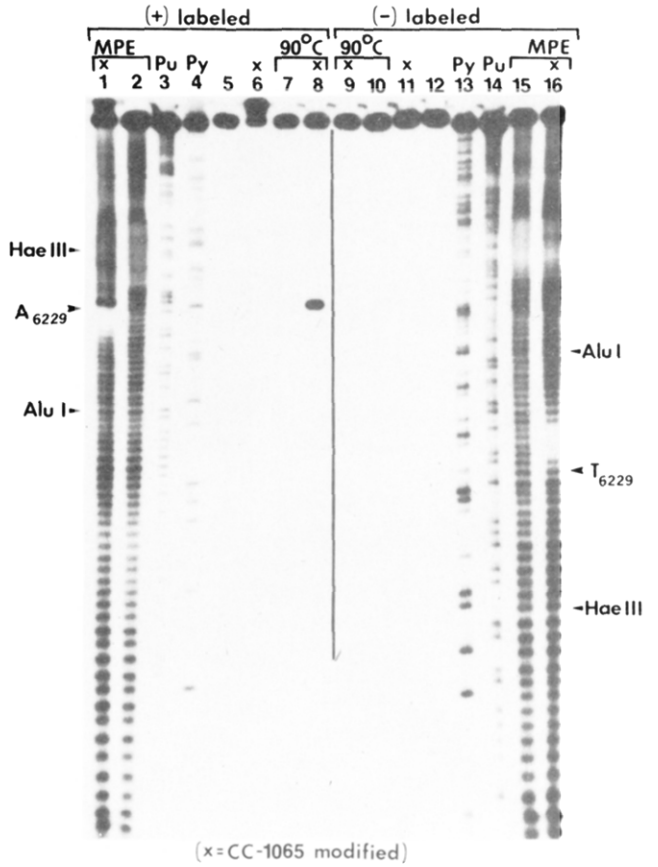

FIGURE 7: Thermal strand break and MPE–Fe(II) footprinting analysis of the site-directed CC-1065–DNA adduct. Singly 5′-^{32}P-labeled site-directed CC-1065–DNA adducts isolated from electrophoresis of partial duplex extension reactions (Figure 6) were analyzed for CC-1065 adduct location by thermal strand scission assay (lanes 7–10) and MPE–Fe(II) digestion (lanes 1, 2, 15, and 16). For MPE–Fe(II) digestion, DNA samples were suspended in 16 μL of 50 mM NaCl and 10 mM Tris-HCl, pH 7.5, and 2 μL of 100 μM MPE and 100 μM $Fe(NH_4)_2$ and equilibrated for 30 min at 37 °C. DTT (2 μL of 20 mM) was added and incubated 30 min at room temperature, and samples were lyophilized to dryness. DNA pellets were suspended in 10 μL of alkaline tracking dye for electrophoresis. Analyses were performed on 5′-^{32}P-(+)-labeled (lanes 1–8) and 5′-^{32}P-(−)-labeled (lanes 9–16) DNA fragments with (lanes marked ×) or without CC-1065 modification. Reactions were electrophoresed adjacent to unmodified DNA (lanes 5, 7, 10, and 12) and Maxam–Gilbert DNA sequencing reactions (lanes 3, 4, 13, and 14).

The previous demonstration of formation of a single adduct type, at N3 of adenine in specific sequences (Hurley et al., 1984), allowed reaction of CC-1065 with a DNA segment in situ, as opposed to the far more labor-intensive drug (carcinogen)–deoxynucleotide adduct synthesis prior to oligodeoxynucleotide synthesis. The latter method is required for site-directed adduct construction with agents that form multiple DNA adduct types (e.g., AAF; Johnson et al., 1986). The requirement of dsDNA for covalent CC-1065 binding allowed "protection" of alternate drug binding sites in ssDNA form, which could then be "deprotected" by DNA polymerase I after occupation of the desired duplex site. Finally, the helix-stabilizing nature of CC-1065–DNA adducts (Swenson et al., 1982) allowed binding to partial duplex DNA without distortion, which might result in duplex denaturation. Retention of DNA duplex character was perceived to be important for subsequent strand extensions by the large fragment of DNA polymerase I to generate a full DNA duplex species.

Specific site-directed modification of the 117-bp region of *E. coli lac* DNA at N3 of adenine 6229 of the (+) strand was confirmed by thermal strand break analysis (Figure 7), which

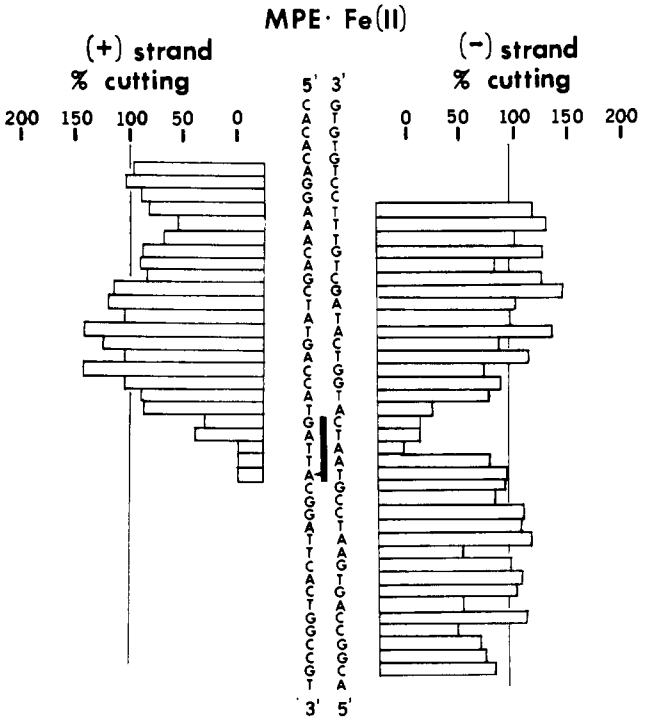

FIGURE 8: Histogram analysis of MPE–Fe(II) footprinting of site-directed CC-1065–DNA adducts. MPE–Fe(II) digestions of the 117-bp DNA fragments (lanes 1, 2, 15, and 16 of Figure 5) were scanned by an integrating laser densitometer. Following normalization of total lane areas, relative percentages of MPE–Fe(II) cutting were calculated by dividing individual peak areas in lane 1 or 16 by areas of corresponding peaks in lane 2 or 15 (respectively) and multiplying by 100.

relies on drug-dependent DNA strand scission (Reynolds et al., 1985). Denaturing polyacrylamide gel electrophoresis of the site-directed CC-1065–DNA adduct demonstrated that the adduct can be retained on ssDNA following denaturation, resulting in a reduced migration of the modified (+)-DNA strand (Figure 7, compare lanes 6 and 11). The retention of drug on ssDNA introduces significant complications in MPE–Fe(II) analysis to the 3′ side of the CC-1065–DNA adduct on the (+) strand (see Figure 7, lane 1). Adduct retention obscures the DNA banding pattern above adenine 6229 to the extent that analysis of densitometric traces (Figure 8) of the MPE–Fe(II) digestion pattern is not possible in this region. Complication of this type introduced by covalent DNA modification are common to many, but not all, DNA alkylators. Those agents that have labile covalent linkages (for example, the N2-guanine minor groove binding pyrrolo[1,4]benzodiazepines; Petrusek et al., 1981) can be removed from DNA fragments following analytical manipulations, but before electrophoresis, to avoid gel smearing (Hertzberg et al., 1986). Attempts at removal of CC-1065 adducts to generate only AP sites before electrophoresis have so far proved unsuccessful in our laboratory (unpublished results).

The utility of the site-directed CC-1065–DNA adduct in determining local CC-1065 adduct interactions with the DNA helix is demonstrated by the clear MPE–Fe(II) footprint on the 5′-(−)-labeled strands (Figure 8), which confirms earlier predictions (Hurley et al., 1984; Reynolds et al., 1985) of drug orientation in the minor groove. Additionally, a small enhancement of MPE–Fe(II) cutting to the 5′ side of the CC-1065 adduct on the (+) strand and to the 3′ side of the CC-1065 adduct on the (−) strand, which reaches maximum intensity at about one helix turn from the drug binding site (see Figure 8), was observed. Little if any effect on MPE–Fe(II) cutting on the 5′ side of the CC-1065 adduct on the (−) strand was apparent. The phenomenon of enhanced MPE–Fe(II) cutting adjacent to covalent drug binding sites has been observed with the non-site-directed pyrrolo[1,4]-benzodiazepine–DNA adducts and has been attributed to unwinding of the helix on either side of the drug binding sites (Hertzberg et al., 1986). A similar MPE–Fe(II) analysis of *non-site*-directed CC-1065-modified DNA would be complicated by adduct smearing and strand scission on *both* DNA strands.

The site-directed CC-1065–DNA adduct was incorported into a well-characterized DNA sequence, the *lac* operon of *E. coli*, with the anticipation that future studies will reveal the effect of a defined CC-1065 adduct on DNA structure and function. In addition, techniques developed during this study will prove essential for future constructions of site-directed CC-1065 adducts into larger, more experimentally flexible DNA molecules.

ACKNOWLEDGMENTS

We gratefully acknowledge informative discussions with our colleagues at the Upjohn Co., especially David Swenson and Mark Mitchell, during the course of this work. Last, we acknowledge the skill and patience of Lesley Koop, who prepared the manuscript.

REFERENCES

Bhuyan, B. K., Newell, K. A., Crampton, S. L., & Von Hoff, D. D. (1982) *Cancer Res. 42*, 3532–3537.

Chambers, R. W., Sledziewska-Gojska, E., Hirani-Hojatti, S., & Borowy-Borowski, H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7173–7177.

Chidester, C. G., Kreuger, W. C., Mizsak, S. A., Duchamp, D. J., & Martin, D. G. (1981) *J. Am. Chem. soc. 103*, 7629–7635.

Essigmann, J. M., Green, C. L., Croy, R. G., Fowler, K. W., Buchi, G. H., & Wogen, G. W. (1982) *Cold Springs Harbor Symp. Quant. Biol. 47*, 327–337.

Graves, D. E., Pattaroni, C., Balakrishnan, C., Ostrander, J. M., Hurley, L. H., & Krugh, T. R. (1984) *J. Biol. Chem. 259*, 8202–8209.

Graves, D. E., Stone, M. P., & Krugh, T. R. (1985) *Biochemistry 24*, 7573–7585.

Hanka, L. J., Dietz, A., Gerpheide, S. A., Keuntzel, S. L., & Martin, D. G. (1978) *J. Antibiot. 31*, 1211–1217.

Hertzberg, R. P., Hecht, S. M., Reynolds, V. L., Molineux, I. J., & Hurley, L. H. (1986) *Biochemistry 25*, 1249–1259.

Hurley, L. H., Reynolds, V. L., Swenson, D. H., Petzold, G. L., & Scahill, T. A. (1984) *Science (Washington, D.C.) 226*, 843–844.

Jacobson, M. K., Twehous, D., & Hurley, L. H. (1986) *Biochemistry 25*, 5929–5932.

Johnson, D. L., Reid, T. H., Lee, M. S., King, C. M., & Romano, L. J. (1986) *Biochemistry 25*, 449–456.

Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376–1380.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, p 461, Cold Springs Harbor Laboratory, Cold Springs Harbor, NY.

Martin, D. G., Biles, C., Gerpheide, S. A., Hanka, L. J., Kreuger, W. C., McGovren, J. P., Mizsak, S. A., Neil, G. L., Stewart, J. C., & Visser, J. (1981) *J. Antibiot. 34*, 1119–1125.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Needham-VanDevanter, D. R., Hurley, L. H., Reynolds, V. L., Therialt, N. Y., Kreuger, W. C., & Wierenga, W. (1984) *Nucleic Acids Res. 12*, 6159-6168.
Petrusek, R. L., Anderson, G. L., Garner, T. F., Fannin, Q. L., Kaplan, D. J., Zimmer, S. G., & Hurley, L. H. (1981) *Biochemistry 20*, 1111-1119.
Quigley, G. L., Wang, A. M. J., Ughetto, G., van der Morel, G., van Boom, J. H., & Rich, A. (1980) *Proc. Natl. Acad. Sci U.S.A. 77*, 7204-7208.
Reynolds, V. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H., & Hurley, L. H. (1985) *Biochemistry 24*, 6228-6237.
Reynolds, V. L., McGovren, J. P., & Hurley, L. H. (1986) *J. Antibiot. 39*, 319-334.
Swenson, D. H., Li, L. H., Hurley, L. H., Rokem, J. S., Petzold, G. L., Dayton, B. D., Wallace, T., Lin, A. M., & Kreuger, W. C. (1982) *Cancer Res. 42*, 2821-2828.
Van Dyke, M. W., & Dervan, P. B. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 347-353.

Inhibition of the Thermally Driven B to Z Transition by Intercalating Drugs†

Jonathan B. Chaires
Department of Biochemistry, The University of Mississippi Medical Center, Jackson, Mississippi 39216-4505
Received April 25, 1986; Revised Manuscript Received September 3, 1986

ABSTRACT: Poly(dG-m^{5}dC) in phosphate buffer containing 50 mM NaCl and Mg^{2+} will undergo a reversible thermally driven conversion from the B to the left-handed Z conformation. The temperature at the midpoint of the thermally driven B to Z transition (denoted T_z) is dependent upon the total Mg^{2+} concentration, with $[d(1/T_z)]/(d \ln [Mg]) = 0.0134\ K^{-1}$. The Mg^{2+} concentration at the midpoint of the equilibrium B to Z transition curve, denoted $[Mg]_{1/2}$, is dependent on temperature, with $(d \ln [Mg]_{1/2})/(d \ln T) = -1.02$. Binding of the anticancer drug daunomycin to the polymer results in a pronounced increase in T_z, dependent on the molar ratio of added drug. T_z is increased by 71.9 °C with nearly saturating amounts of drug bound. Transition profiles are biphasic at less than saturating amounts of bound drug. By experiments monitoring such biphasic curves at a visible wavelength sensitive to the binding of daunomycin, it may be demonstrated that no drug is released until the later phase of the transition. These results are analogous to the effects of intercalating drugs on the thermal denaturation of DNA and indicate that drug molecules preferentially interact with B-form DNA and are redistributed to regions in the B conformation over the course of the transition. Comparative studies show that some intercalators stabilize right-handed DNA more effectively than others. At similar initial binding ratios, the following order, from most to least effective, was experimentally observed: actinomycin > daunomycin > ethidium > proflavin.

The transition of DNA from the right-handed B to the left-handed Z conformation is of current interest, both as a striking example of DNA polymorphism and as a potential mechanism for gene regulation (Rich et al., 1984). Intercalators profoundly affect the stability of Z DNA and will, in general, inhibit the formation of Z DNA. Certain intercalators have been demonstrated to act as allosteric effectors on DNA conformation and will convert Z DNA to an intercalated right-handed form under solution conditions that would otherwise favor the Z form in the absence of the intercalator (Pohl & Jovin, 1972; Walker et al., 1985a,b; Chaires, 1985b, 1986). Mirau and Kearns (1983) noted striking quantitative differences in the ability of a variety of intercalators to inhibit the rate of the B to Z transition. The molecular details of the mechanism by which intercalators inhibit the B to Z transition and the origins of the observed quantitative differences among intercalators remain incompletely described.

The data presented here show that daunomycin and other intercalators inhibit the reversible thermally driven B to Z transition in poly(dG-m^{5}dC). Daunomycin increases the temperature at the midpoint of the transition in proportion to the amount of added drug. Below saturating drug binding ratios, transition profiles are biphasic and drug is not released until late in the transitions. These effects are entirely analogous to the effect of intercalators on the thermal denaturation of DNA, as explained by Crothers (1971) and McGhee (1976). Comparative studies show that intercalators vary in the effectiveness with which they stabilize the right-handed form of poly(dG-m^{5}dC). The method used thus provides additional insight into the mechanism by which intercalators inhibit the B to Z transition and provides a convenient means of assessing quantitative differences among intercalators.

†Supported by U.S. Public Health Service Grant CA 35635 and National Science Foundation Grant DMB-8421185.

Materials and Methods

Polynucleotides. Poly(dG-m^{5}dC) was purchased from Pharmacia (Milwaukee, WI) and used without further purification. Samples were dialyzed against BP buffer, consisting of 2 mM NaH_2PO_4, 6 mM Na_2HPO_4, and 50 mM NaCl, pH 7.0. A molar extinction coefficient of 16 800 M^{-1} cm^{-1} was used to calculate polymer concentration.

Equilibrium Mg^{2+} Titration Experiments. Ultraviolet absorbance spectra of poly(dG-m^{5}dC) in BP buffer were recorded in a Cary 219 spectrophotometer as a function of added Mg^{2+} concentration. Samples were thermostated, and the temperature was controlled by a Neslab circulating water bath. Following each addition of Mg^{2+}, absorbance at 300 nm was monitored continuously with time to establish that equilibrium was reached. The complete ultraviolet absorbance spectrum was then recorded. The fraction of Z form θ was calculated by

$$\theta = (A_{300} - A^0_{300})/(A^F_{300} - A^0_{300})$$

where A^0_{300} is the absorbance at 300 nm in the absence of Mg, A_{300} the absorbance in the presence of a given concentration of Mg, and A^F_{300} the maximal absorbance at 300 nm upon total

0006-2960/86/0425-8436$01.50/0