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NMR Assignments for the Amino-Terminal Residues of *trp* Repressor and Their Role in DNA Binding†

Cheryl H. Arrowsmith, Jannette Carey,* Lynda Treat-Clemons, and Oleg Jardetzky
Stanford Magnetic Resonance Laboratory and Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305

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ABSTRACT: The *trp* repressor of *Escherichia coli* specifically binds to operator DNAs in three operons involved in tryptophan metabolism. The NMR spectra of repressor and a chymotryptic fragment lacking the six amino-terminal residues are compared. Two-dimensional *J*-correlated spectra of the two forms of the protein are superimposable except for cross-peaks that are associated with the N-terminal region. The chemical shifts and relaxation behavior of the N-terminal resonances suggest mobile "arms". Spin-echo experiments on a ternary complex of repressor with L-tryptophan and operator DNA indicate that the termini are also disordered in the complex, although removal of the arms reduces the DNA binding energy. Relaxation measurements on the armless protein show increased mobility for several residues, probably due to helix fraying in the newly exposed N-terminal region. DNA binding by the armless protein does not reduce the mobility of these residues. Thus, it appears that the arms serve to stabilize the N-terminal helix but that this structural role does not explain their contribution to the DNA binding energy. These results suggest that the promiscuous DNA binding by the arms seen in the X-ray crystal structure is found in solution as well.

Escherichia coli trp repressor regulates transcription initiation by binding to operator targets within three operons involved in tryptophan metabolism (Klig et al., 1988). The X-ray crystal structure of the protein–DNA complex shows that the repressor uses a symmetry-related pair of helix–turn–helix modules to bind in successive major grooves of the DNA (Otwinowski et al., 1988). Several other DNA binding proteins use terminal "arms" to wrap around their DNA targets [summarized by Jen-Jacobson et al. (1986)]. The amino-terminal regions of *trp* repressor are disordered in the crystal structure of the protein in both the presence and absence of DNA (Schevitz et al., 1985). We have developed methods for selective, preparative removal of the arm residues using chymotrypsin. Comparison of the two-dimensional ^{1}H NMR spectra of intact and armless repressors allows the assignment of resonances from the arm, and confirms that the fine details of the *trp* repressor structure are preserved after removal of the arms. We have used the assignments to directly assess the dynamic behavior of the arms in solution and their role in DNA binding. The identification of independently mobile regions of proteins by NMR is a well-established method (Jardetzky et al., 1978; Wade-Jardetzky et al., 1979) and has been used recently to identify mobile N-terminal residues of λ repressor (Weiss et al., 1984).

Results and Discussion

The circular dichroism spectrum of *trp* repressor is typical of a protein with high α-helix content, with strong minima at 208 and 222 nm. Upon addition of chymotrypsin, the intensity of the 222-nm minimum decreases by about 7% over the first few minutes and then decreases further in a much slower reaction. SDS gel electrophoresis and Edman analysis show that the first phase correlates with removal of the first six residues, Ala-Gln-Gln-Ser-Pro-Tyr, from the N-terminus (Carey, 1989). Using the spectral change as a real-time assay, we prepared milligram amounts of armless repressor. Chymotrypsin was removed by purification through a column of phosphocellulose; unlike intact repressor (Joachimiak et al., 1983), the armless protein elutes during the 100 mM NaCl column wash.

The aliphatic regions of the two-dimensional *J*-correlated spectra of intact and armless repressors are shown in Figure 1, along with the corresponding one-dimensional spectrum of the intact protein. *trp* repressor is a 25 000-dalton dimer (107 residues per subunit) and therefore has a relatively long rotational correlation time, which gives rise to broad resonances in the ^{1}H NMR spectrum. As a consequence, not all the

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* Address correspondence to this author at the Department of Biochemistry, Stanford University Center for Molecular and Genetic Medicine.

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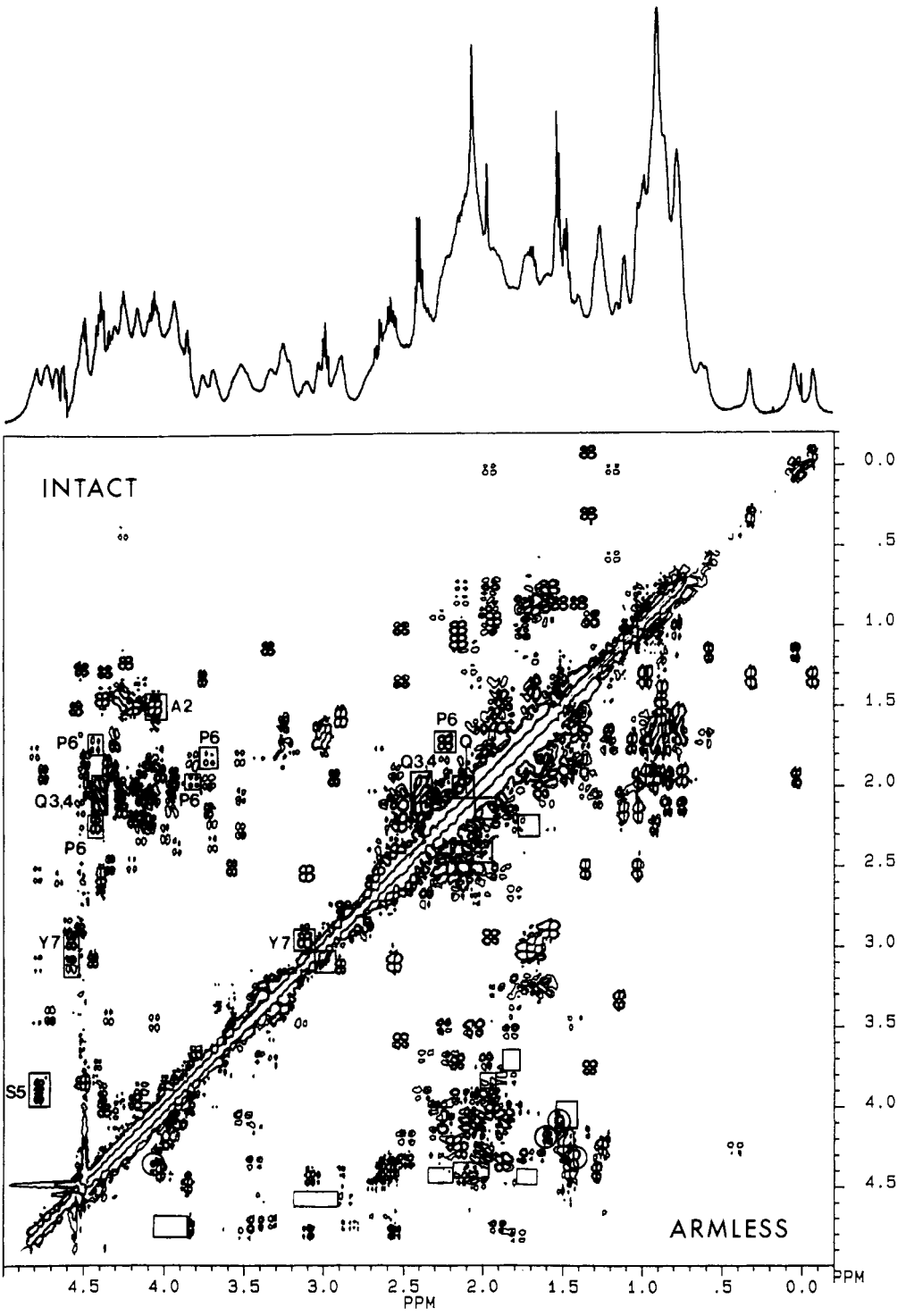

FIGURE 1: Composite of the phase-sensitive double-quantum-filtered *J*-correlated spectra of intact *trp* repressor (above diagonal) and *trp* repressor digested with chymotrypsin (below diagonal). The one-dimensional spectrum (top) is that of intact repressor. Digestion was carried out at 20 °C with 0.25 mg/mL repressor in 10 mM NaH_2PO_4, pH 7.6, 0.1 M NaCl, and 0.1 mM EDTA (P-11 buffer) by addition of 0.01 volume of 0.1 mg/mL α-chymotrypsin (Worthington) freshly dissolved in 10 mM Tris-HCl, pH 8.0. A portion of the reaction mixture was monitored continuously by circular dichroism at 222 nm on an Aviv 60DS spectropolarimeter, and sampled at 200-s intervals for SDS gel electrophoresis (Carey, 1989). After about 10 min, a plateau in the CD signal was reached, and the reaction was stopped by addition of 0.001 volume of phenylmethanesulfonyl fluoride from a 10 mg/mL solution in 2-propanol. The reaction mixture was loaded immediately onto a column of Whatman P-11 phosphocellulose equilibrated at 4 °C with P-11 buffer; typically, 10 mg was loaded onto a 50-mL column. The column was washed with P-11 buffer until the absorbance at 280 nm returned to zero. Column fractions were analyzed by SDS gel electrophoresis, pooled, concentrated, dialyzed into a D_2O buffer, then lyophilized, and resuspended in D_2O (99.96 atom %). Final NMR conditions were 1 mM *trp* repressor dimer, 500 mM KCl, and 100 mM NaH_2PO_4 in D_2O, pD 7.3. The two-dimensional spectra were acquired at 45 °C with a spectral width of 6493 Hz in both dimensions, 2K data points in F2, 512 in F1, and zero-filled to 2K for a final digital resolution of 6.3 Hz in both dimensions. Data were multiplied by a 45° shifted sine bell function in both dimensions before Fourier transformation. The residual HDO signal was suppressed by presaturation for 1.8 s before acquisition. Although differences other than those discussed in the text are seen between the two sides of the composite spectrum, these result from unavoidable differences in digital resolution in the two dimensions and slight asymmetric distortion of the base lines, and are thus an artifact only of the composite spectrum.

Table I: Chemical Shift Values of Resonances from the Amino-Terminal Arm of *trp* Repressor[a]

residue	α	β,β'	γ	δ
Ala-2[b]	4.02	1.48		
Gln-3	4.36	1.98, 2.10	2.37	
Gln-4	4.36	1.98, 2.10	2.37	
Ser-5	4.78	3.85, 3.92		
Pro-6[c]	4.40	1.72, 2.20	1.82, 1.95	3.64, 3.75
Tyr-7	4.56	2.94, 3.07	7.12	6.84

[a] Parts per million (ppm) relative to internal (trimethylsilyl)tetradeuteriosodium phosphate at 45 °C. Sample conditions are described in the legend to Figure 1. Residue numbers are based on the gene sequence (Gunsalus & Yanofsky, 1980); the encoded Met-1 residue is largely removed in vivo. [b] The positions of these peaks vary with pH in the range ~7–8, the α-proton being from 3.85 to 4.10 and the β-protons from 1.40 to 1.51 ppm. [c] The complete spin system of this residue was confirmed from a HOHAHA (Bax & Davis, 1985) spectrum of the intact protein.

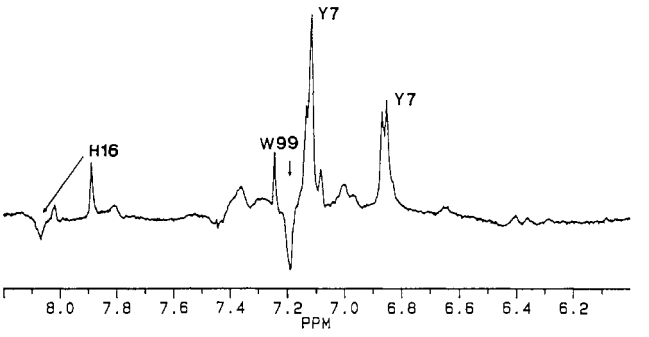

FIGURE 2: Aromatic region of a difference spectrum (intact – armless) taken at 35 °C. Sample conditions as in Figure 1. Assignments were made as described by Hyde et al. (submitted for publication). Positive peaks arise from intact repressor and are labeled by residue number; negative peaks marked with arrows are the corresponding shifted resonances of the armless repressor.

expected *J* cross-peaks are seen in the 2D *J*-correlated spectrum. Nevertheless, a sufficient fraction ($\sim^2/_3$) of cross-peaks is resolved to allow a structural interpretation of the data. With the exception of the nine sets of cross-peaks discussed below, the major spectral features of intact repressor seen in the 2D COSY spectrum are also displayed by the armless protein. For example, the peaks shifted to very high field are characteristic of *trp* repressor, and indicate a similar folded structure for the two forms of the protein. Some of these high-field peaks result from ring current effects on the methyl groups of Val-23 (0.03 ppm) and Val-103 (0.3 and –0.07 ppm) due to nearby aromatic residues (Trp-19 and Phe-22, and Trp-99, respectively; Hyde et al., submitted for publication). A detailed comparison of the two-dimensional spectra shows that in the armless protein five sets of cross-peaks are missing and four sets of cross-peaks have moved.

Cross-peaks that disappeared were assigned to the missing residues based on spin system type and chemical shift values (Wüthrich, 1986). These assignments are given in Table I. The corresponding cross-peaks are indicated on the spectrum of intact repressor in Figure 1 (above the diagonal), and the corresponding regions of the armless repressor's spectrum are outlined (below the diagonal). The chemical shifts are characteristic of amino acids in small unstructured peptides (Wüthrich, 1986), indicating that the amino-terminal region of the repressor is solvent-accessible and unstructured in solution.

The four sets of cross-peaks that move upon removal of the arm (circled in Figure 1) appear to arise from one serine and three alanine residues, as judged by their chemical shifts and spin system types. These results indicate an altered environment for several residues of this type in the armless protein. The new N-terminus of the armless repressor has the sequence Ser-Ala-Ala-Met-Ala, and these residues comprise the beginning of the first α-helix in the X-ray crystal structure of intact repressor (Schevitz et al., 1985). The armless repressor contains four other Ser and six other Ala residues but no other (Ser, Ala) sequences (Gunsalus & Yanofsky, 1980). The most likely explanation for the shifts in these resonance positions is a structural change in the amino-terminal portion of this first α-helix, adjacent to the site of cleavage. Except for these differences associated with the N-terminal region, both two-dimensional spectra are identical.

The aromatic region of the one-dimensional NMR spectrum also contains detailed structural information. Because four of the seven aromatic residues (His-16, Trp-19, Phe-22, and Tyr-30) of each subunit are buried in the stably folded core of *trp* repressor (Schevitz et al., 1985), their resonances are a sensitive probe for its folded structure. Figure 2 shows the aromatic region of a difference spectrum (intact minus armless). In addition to the prominent peaks due to the 2,6 and 3,5 protons of the missing Tyr-7, a relatively large shift is observed for the resonance of the H2 proton of His-16, consistent with a structural perturbation of the first helix. A very small shift (0.03 ppm) is also seen in the H2 resonance of Trp-99, which is partially exposed on the surface of the repressor. There is no corresponding change in the methyl peaks of Val-103, the chemical shift positions of which are determined primarily by ring current effects from the Trp-99 indole ring (Hyde et al., submitted for publication). No changes are seen in the resonances of Trp-19, Phe-22, or Tyr-30. Thus, it appears that there are no significant structural rearrangements at sites distant from the absent arm.

NMR relaxation measurements (Hahn, 1950; Carr & Purcell, 1954; Meiboom & Gill, 1958) show directly that the resonances assigned to the mobile termini of *trp* repressor have long T_2 relaxation times relative to the rest of the protein, indicating a higher degree of mobility in this region (Jardetzky & Roberts, 1981). Figure 3B shows the aliphatic region of a spin-echo spectrum of intact repressor taken with a delay time (2τ) of 180 ms. During this time, the amplitude of most signals has decayed (compare with Figure 1 top), leaving only those with the longest T_2 values. At this delay time, most of the resonances from the N-terminal arm are still visible (shaded peaks). Few other resonances have T_2 relaxation times as long as those of the arms (ca. 140 ms), indicating that, in solution as in the crystal, only the extreme termini are disordered. Spin-echo spectra of intact repressor with and without L-tryptophan are identical at delay times greater than 100 ms (not shown), confirming that crystal packing forces must be responsible for the differences seen at the amino termini in the X-ray structures of these two forms of repressor (Schevitz et al., 1985; Zhang et al., 1987).

One of the other slowly relaxing resonances detected is that tentatively assigned to Asp-108. The multiplets at 2.57 and 2.65 ppm give rise to prominent cross-peaks at 4.40 ppm in the *J*-correlated spectra, consistent with a mobile C-terminal Asp residue. This residue, which is also present in the armless protein spectrum, is disordered in the crystalline repressor (Schevitz et al., 1985; Otwinowski et al., 1988).

The spin-echo spectrum of the armless repressor at 180 ms (Figure 3C) lacks the resonances due to the arm residues, and shows new peaks indicating increased mobility for several additional residues. Among these are the resonances tentatively attributed to one Ser and three Ala residues above. In addition, the signal from a methionine methyl group shifts from 2.05 to 2.12 ppm, and its T_2 relaxation time nearly doubles,

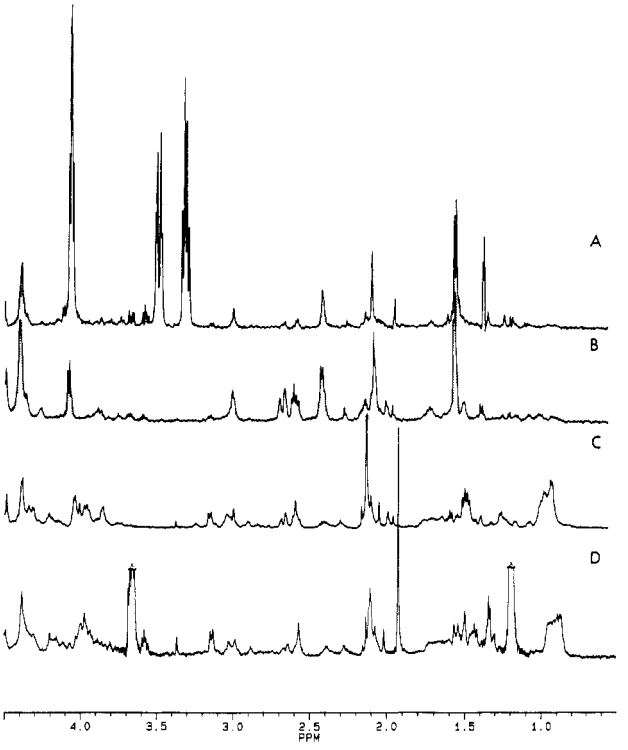

FIGURE 3: Carr–Purcell–Meiboom–Gill NMR spectra of intact *trp* repressor plus L-tryptophan (B), armless repressor plus L-tryptophan (C), ternary complex of intact repressor, L-tryptophan, and a 20 bp operator DNA fragment (A), and ternary complex of armless repressor, L-tryptophan, and DNA (D). Spectra were acquired at 35 °C using the Hahn (1950) spin-echo pulse sequence with the modifications of Carr and Purcell (1954) and of Meiboom and Gill (1958) with a delay time (2τ) of 180 ms. Residues assigned to the N-terminal arm of repressor are shaded in (A) and (B). Quantitative DNA binding assays showed that a sample of the protein used for experiments A and B had full activity. The ratio of DNA to protein in (A) and (D) was 1.1 to ensure that no free repressor remained in solution. The large peaks at 4.06, 3.49, and 3.3 ppm in (A) are due to excess free L-tryptophan. The peaks marked "X" in (D) are residual ethanol from cleaning the NMR tube.

upon removal of the arms. These data further support the notion that, in the absence of the arms, the N-terminal end of the first helix becomes less structured.

Addition of a slight excess of a 20 bp synthetic operator DNA in the presence of excess L-tryptophan had essentially no effect on the relaxation times of the terminal resonances of intact repressor (Figure 3A). Most of the DNA resonances have relatively short T_2 values (Lefevre et al., 1987), and, although they are not seen at the delay time shown here, changes consistent with complex formation were observed in those DNA resonances as well as in some protein resonances at shorter delay times. These results indicate that the arms remain mobile in the protein–DNA complex in solution, as in the crystal structure.

Measurement of the DNA binding energy for armless and intact forms of the protein (Carey, 1989) shows that, in the absence of the arms, approximately 2.3 kcal/mol, or about 18%, of the net DNA binding energy is lost. Thus, the arms contribute substantially to the binding energy without occupying a fixed position on the DNA. One explanation for this apparent paradox could be that a small change in the dynamic behavior of the armless protein, such as increased fraying at the N-terminal end of the first helix, consumes some of the DNA binding energy even though this conformational change occurs far from the DNA (Otwinowski et al., 1988). The spin-echo spectrum of armless repressor in the presence of DNA (Figure 3D) shows that complex formation does not reduce the relaxation times of the new mobile resonances. This observation strengthens our tentative assignment of these resonances to the newly exposed N-terminus. If these resonances arose from the DNA binding domain, where most of the other Ser and Ala residues of repressor are found, their relaxation times would likely be altered upon complex formation.

The results presented here suggest that the N-terminal arms of *trp* repressor serve a structural role in stabilizing the first α-helix. Such stabilization could arise by two general mechanisms: helix macrodipole effects and/or sequence-specific interactions. In the armless protein, the positively charged N-terminal α-amino group is much closer to the positive pole of the helix macrodipole than it is in the intact protein. Proximity of these like charges has been implicated as a destabilizing influence in model peptide helices (Shoemaker et al., 1987). Alternatively, specific interactions of arm residues with backbone (Presta & Rose, 1988) or side chain (Shoemaker et al., 1987) groups may be required to stabilize the N-terminal portion of the first helix. However, the energetic contribution of the arms to DNA binding apparently is not derived from this structural requirement. The present results demonstrate that stability of the N-terminal helix is not coupled to DNA binding directly. An indirect effect, via the overall stability of the repressor, is unlikely for two reasons. First, the intact and armless proteins have essentially the same midpoint for urea-induced denaturation (Carey, 1989), and therefore have no measurable difference in stability. Second, removal of the arms causes increased mobility for only a few residues, apparently restricted to the region of the protein adjacent to the cleavage site and distant from the dimer interface.

The X-ray structure of *trp* repressor (Otwinowski et al., 1988) shows that the arm residues make promiscuous contacts with DNA, although in the crystal lattice the proximity of neighboring DNA molecules may promote this mode of binding. The present results are consistent with this mode of binding in solution, suggesting that it is an intrinsic property of the arms rather than a fortuitous result of crystal packing effects.

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Mechanism of Inhibition of DNA Gyrase by Quinolone Antibacterials: Specificity and Cooperativity of Drug Binding to DNA

Linus L. Shen,* John Baranowski, and Andre G. Pernet
Anti-Infective Research Division, Abbott Laboratories, Abbott Park, Illinois 60064
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ABSTRACT: Although the functional target of quinolone antibacterials such as nalidixic acid and norfloxacin has been identified as the enzyme DNA gyrase, the direct binding site of the drug is the DNA molecule [Shen, L. L., & Pernet, A. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 307–311]. As described in this paper, binding specificity and cooperativity of quinolones to DNA were further investigated with the use of a variety of DNA species of different structures and different base compositions. Results show that the drug binding specificity is controlled and determined largely by the DNA structure. The drug binds weakly and demonstrates no base preference when DNA strands are paired. The drug binds with much greater affinity when the strands are separated, and consequently, binding preference emerges: it binds better to poly(G) and poly(dG) over their counterparts including poly(dI). The results suggest that the drug binds to unpaired bases via hydrogen bonding and not via ring stacking with DNA bases. The weak binding to relaxed double-stranded DNA and the stronger binding to single-stranded DNA are both nonspecific as they do not demonstrate binding saturation and cooperativity. The specific type of binding, initially demonstrated in our previous publication with the supercoiled DNA and more recently with complex formed between linear DNA and DNA gyrase [Shen, L. L., Kohlbrenner, W. E., Weigl, D., & Baranowski, J. (1989) *J. Biol. Chem.* (in press)], occurs near the drug's supercoiling inhibition concentration. As shown in this paper, binding saturation curves of this type are highly cooperative (with Hill constant greater than 4). This form of binding represents a specific mode of drug binding which determines the drug's biological potency.

Quinolones, a series of nalidixic acid analogues, have become a major class of synthetic antibacterial agents which are under extensive clinical development (Hooper & Wolfson, 1985; Hooper, 1986; Neu, 1987; Fernandes, 1988). These drugs are considered to have a bright future owing to their extremely potent antibacterial activity, rapid bactericidal effects, and low incidence of resistance development (Wolfson & Hooper, 1985). Nalidixic acid, the first member of the quinolone family, was synthesized 25 years ago (Lesher et al., 1962). Little progress was made in the development of this class of drug until the discovery of DNA gyrase (Gellert et al., 1976). Subsequent extensive studies on the mechanism of the gyrase-catalyzed DNA supercoiling process and genetic analysis of quinolone-resistant bacterial mutants lead to the conclusion that the functional target of quinolone drugs is the A subunit of the enzyme [for reviews, see Cozzarelli (1980), Gellert (1981), and Wang (1985)]. In a recent preliminary publication, however, we demonstrated that the direct binding site of the drug is the DNA molecule (Shen & Pernet, 1985). The drug was shown to bind preferentially to single-stranded rather than double-stranded DNA. We also observed a saturable drug binding phase with supercoiled DNA which was probably due to binding to a localized underwound region of the DNA molecule. Using an indirect competition method, we further demonstrated that the binding affinity of a number of quinolones correlates well with the drug's potency to inhibit DNA supercoiling catalyzed by DNA gyrase. This indicates that this type of saturable binding is specific and is an important determinant of the inhibitory potency. At this preliminary stage of investigation, no answer was obtained concerning the exact role of drug binding during gyrase inhibition, as the saturable form of binding was observed only with supercoiled DNA (the product) but not with the relaxed DNA (the substrate) or the substrate–enzyme complex.[1] In this paper, we present some additional binding data obtained with various DNA species and the result of cooperativity tests of the drug binding curve in an attempt to gain better insight into the binding specificity and modes of bindings. The information is crucial for proposing the cooperative drug–DNA binding model of DNA gyrase inhibition presented in the following paper (Shen et al., 1989b).

Experimental Procedures

Materials. Deoxynucleoside monophosphates and chromosomal DNA preparations from *Escherichia coli*, *Micrococcus lysodeikticus*, and *Clostridium perfringens* were pur-

* To whom correspondence should be addressed at Department 47N, Building AP9A, Abbott Laboratories, Abbott Park, IL 60064.

[1] The binding experiment was carried out in the absence of ATP or its analogue.

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