Articles

Dynamics and Binding Mode of Hoechst 33258 to d(GTGGAATTCCAC)$_2$ in the 1:1 Solution Complex As Determined by Two-Dimensional ^{1}H NMR†

Agostino Fede,‡,§ Alexander Labhardt,‡ Willi Bannwarth,‡ and Werner Leupin*,‡

Pharma Division, Preclinical Research and New Technologies, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland, and Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

Received June 24, 1991; Revised Manuscript Received September 9, 1991

ABSTRACT: We have investigated the interaction of the bisbenzimidazole derivative Hoechst 33258 with the self-complementary dodecadeoxynucleotide duplex d(GTGGAATTCCAC)$_2$ using one-dimensional (1D) and two-dimensional (2D) proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. To monitor the extent of complex formation, we used the imino proton region of the 1D ^{1}H NMR spectra acquired in H_2O solution. These spectra show that the DNA duplex loses its inherent C_{2v} symmetry upon addition of the drug, indicating that the two molecules form a kinetically stable complex on the NMR time scale (the lifetime of the complex has been measured to be around 450 ms). We obtained sequence-specific assignments for all protons of the ligand and most protons of each separate strand of the oligonucleotide duplex using a variety of homonuclear 2D ^{1}H NMR experiments. The aromatic protons of the DNA strands, which are symmetrically related in the free duplex, exhibit exchange cross peaks in the complex. This indicates that the drug binds in two equivalent sites on the 12-mer, with an exchange rate constant of 2.2 ± 0.2 s^{-1}. Twenty-five intermolecular NOEs were identified, all involving adenine 2 and sugar 1′ protons of the DNA and protons in all four residues of the ligand, indicating that Hoechst 33258 is located in the minor groove at the AATT site. Only protons along the same edge of the two benzimidazole moieties of the drug show NOEs to DNA protons at the bottom of the minor groove. Using molecular mechanics, we have generated a unique model of the complex using distance constraints derived from the intermolecular NOEs. We present, however, evidence that the piperazine group may adopt at least two locally different conformations when the drug is bound to this dodecanucleotide.

In order to increase our understanding of the parameters which govern the way ligands interact with DNA, many biophysical studies about ligand–DNA complexes have been undertaken, both in the crystalline state and in solution. The ligands have been small proteins or low molecular weight compounds, e.g., drug molecules. These drugs represent good model compounds for the DNA-binding proteins, both in their way of interacting with the DNA and in their way of exhibiting similar sizes of contact surfaces between the DNA and the ligands. Thus the drug–DNA systems may serve as good models to study gene expression and to investigate the various structural changes of the DNA exerted by a DNA-binding ligand.

The structure of DNA fragments and their complexes with ligands in solution is best determined by the application of two-dimensional ^{1}H NMR[1] spectroscopy (Wüthrich, 1986). Several studies of drug/DNA complexes have been reported in the literature, whereby the drugs have been intercalators, e.g., nogalamycin (Zhang & Patel, 1990), bisintercalators, e.g., echinomycin (Gao & Patel, 1988; Gilbert et al., 1989), minor groove binders, e.g., distamycin A (Pelton & Wemmer, 1989, 1990), the bisquaternary ammonium heterocycle SN 6999 (Leupin et al., 1986), or covalently attached ligands, e.g., (+)-CC-1065 (Hurley et al., 1990).

A prerequisite for the study of all these complexes is the assignment of all resonances in the NMR spectra of the free ligand and the DNA and of both components in the complex. The strategies for the sequence-specific assignment of the proton resonances of oligodeoxynucleotides have been reported (Feigon et al., 1983; Scheek et al., 1983, 1984; Hare et al., 1983; Chazin et al., 1986) and are routinely used. Finally, the search for intermolecular NOEs remains one of the most important tasks in such studies, providing the basis for the localization of the ligand relative to the DNA fragment.

The study of DNA complexes involving the bisbenzimidazole Hoechst 33258 (Loewe & Urbanietz, 1974) was started after the first reports that Hoechst 33258 (H33258) can be employed in DNA and chromosome staining [see, e.g., Latt and Wohlleb (1975), Latt and Stetten (1976), Bontemps et al. (1975), Steiner and Sternberg (1979), Stokke and Steen (1985), and Loontiens et al. (1990, 1991)], whereby the large increase of the fluorescence quantum yield upon binding to DNA is used as a spectral indicator of DNA binding. Various experiments demonstrate that H33258 binds in the minor groove of the DNA with two different modes of binding dif-

† Presented in part at the 23rd Jerusalem Symposium in Quantum Chemistry and Biochemistry, May 14–17, 1990, and at the Keystone Symposium on Frontiers of NMR in Molecular Biology-II, April 8–14, 1991.

* Author to whom correspondence should be addressed.
‡ F. Hoffmann-La Roche Ltd.
§ ETH-Hönggerberg.

[1] Abbreviations: NMR, nuclear magnetic resonance; COSY, two-dimensional correlated spectroscopy; 2Q(F), two quantum (filtered); TOCSY, two-dimensional total correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; ROESY, two-dimensional rotating frame NOE spectroscopy; JR, jump and return; TPPI, time-proportional phase incrementation; TSP, 3-(trimethylsilyl)propionate; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate.

0006-2960/91/0430-11377$02.50/0

ferentiated by their binding affinities. It has recently been shown that Hoechst 33258 binds to DNA with multiple stoichiometries and that the tight binding mode exhibits a wide spectrum of site affinities (Loontiens et al., 1990). However, a detailed photophysical analysis of the fluorescence and UV/vis absorption spectra of H33258 and its changes upon binding to DNA has not been performed yet. On the other hand, several footprinting studies have been published (Martin & Holmes, 1983; Harshman & Dervan, 1985; Murray & Martin, 1988; Jorgenson et al., 1988; Portugal & Waring, 1988; Churchill & Suzuki, 1989; Bathini et al., 1990). These studies revealed that H33258 covers about 5 base pairs whereby it seems that 4 consecutive AT base pairs are needed for maximal binding. In these studies the sequence dNAAT (with N equal to T or A) has been shown to represent a very strong binding site. There are three structures of 1:1 complexes with H33258 and dodecanucleotides as determined by X-ray crystallography reported in the literature, two with the dodecanucleotide d(CGCGAATTCGCG)$_2$ (Pjura et al., 1987; Teng et al., 1988) and the third one with the DNA fragment d(CGCGATATCGCG)$_2$ (Carrondo et al., 1989).

Surprisingly, the two structures determined for the same ligand and the same oligonucleotide are not identical, differing (i) in the location of the ligand relative to the AATT segment and (ii) in the fact that Pjura et al. (1987) find a second complex with the same population, whereby the two benzimidazole moieties of H33258 are rotated by 180° relative to each other. The structure of the third complex is again different from the other two structures [see Carrondo et al. (1989) for a detailed discussion].

The method of choice for structure determinations of proteins, DNA, and their complexes in solution is based on two-dimensional NMR mehods (Wüthrich, 1986). The only reports about NMR studies of complexes between H33258 and defined oligonucleotides have been about the 2:1 complex with the dodecanucleotide d(CTTTTGCAAAAG)$_2$ (Searle et al., 1990) and a preliminary account about the 1:1 complex between H33258 and d(CGCGAATTCGCG)$_2$ (Parkinson et al., 1989). Thus, in order to gain insight into the structure of a complex with H33258 with a DNA fragment containing the dAATTC sequence and to compare it to the known structures derived from results of X-ray and NMR studies, we have set out to examine the 1:1 complex between H33258 and the dodecanucleotide d(GTGGAATTCACC)$_2$ in aqueous solution by two-dimensional NMR methods. In this paper we present the results of these studies, which lead to interesting data about the static and dynamic aspects of this complex.

In the final stages of the preparation of this publication two additional papers appeared in the literature reporting the results of NMR studies of complexes between oligonucleotide duplexes and H33258 and a derivative thereof (Parkinson et al., 1990; Kumar et al., 1990).

Materials and Methods

The bisbenzimidazole derivative Hoechst 33258 (H33258) was purchased from Fluka (Buchs, Switzerland) and used without further purification. The nomenclature used throughout this paper for the molecule is given in Figure 1a.

DNA Synthesis. The dodecanucleotide d(GTGGAATTCCAC) was synthesized on controlled pore glass (CP6) (Adams et al., 1983) in a 19-μmol scale using β-cyanoethyl phosphoramidites (Sinha et al., 1984) as building blocks (5-fold excess) and our standard technologies (Bannwarth, 1987). After synthesis, it was treated with concentrated ammonia at 343 K for 2 h, filtered off, and evaporated. The pellet was redissolved in 80% acetic acid, precipitated with ether, and

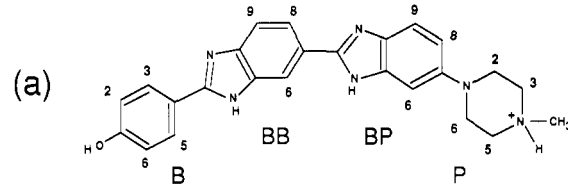

FIGURE 1: (a) Structure of Hoechst 33258. Individual protons are identified by the letter for the ring and the ring position, e.g., BP8. (b) Sequence and residue numbering for the dodecanucleotide duplex. For the (+)-strand, the nucleotides are consecutively numbered from the 5′ end to the 3′ end. For the (−)-strand, the nucleotides have the same numbers as the paired bases.

centrifuged. The pellet was dissolved in 0.6 M TEAB and applied to a DEAE-Sephadex column (30 × 2 cm). A gradient of 0.6–1.4 M TEAB was applied to the column in order to obtain the desired fragment. The derived oligonucleotide was repeatedly dissolved in aqueous ethanol solution and the solvent evaporated under reduced pressure. After the addition of a 10-fold excess of NaCl, it was dialyzed three times against water in a dialysis tube (1000 MW cutoff). The tube content was evaporated, taken up in water, and applied to a small Sephadex column (NAP25). The fraction containing the DNA was lyophilized, yielding 10.5 mg of DNA. The purity of the fragment was checked by reversed-phase HPLC.

Sample Preparation. The lyophilized dodecanucleotide was dissolved in 450 μL of an aqueous buffer solution (50 mM PO_4^{3-}, 100 mM NaCl, 0.1% NaN_3, pH 7.0). Complete duplex formation was achieved by annealing at 358 K for 15 min and slowly cooling to room temperature (Figure 1b gives the nomenclature used for the oligonucleotide duplex). After one lyophilization step, the material was redissolved in the same volume of 90% H_2O/10% D_2O (v/v).

Titration. For the titration, 10-, 30-, and 50-nmol aliquots of the drug were taken from a freshly prepared stock solution in DMSO. First, the solvent was removed under vacuum and then the DNA solution was added to each aliquot of the drug. The extent of the complex formation was followed by monitoring changes in the imino proton region in a 1D ^{1}H NMR spectrum (Leupin et al., 1986). These spectra were acquired at 293 K with one jump-and-return (JR) (Plateau & Guéron, 1982) read pulse on a 500-MHz spectrometer (Bruker AM-500). The JR delay was set to 70 μs, giving excitation maxima at ±3571 Hz from the carrier frequency set on the water resonance. For each titration step, 200–400 scans were collected per spectrum, with a 1-s relaxation delay between scans. With a sweep width of 10000 Hz, 4K data points were measured and zero-filled to 16K points. A shifted Gaussian window was applied to the free induction decay (FID) prior to Fourier transformation. The spectra were baseline-corrected in the region of interest using fifth-order polynomials. The proton chemical shifts were indirectly referenced to TSP using the HDO resonance previously calibrated in stock buffer solution.

Two-Dimensional ^{1}H NMR Spectra. All 2D spectra were acquired on a Bruker AM-500 spectrometer at 298 K. The D_2O sample was prepared by repeated lyophilization from 99.8% D_2O, followed by dissolution in 99.996% D_2O. The sample concentration was 0.7 mM, as determined by UV absorption on a small aliquot of the free DNA (the extinction coefficient was computed for this sequence using standard ϵ_{260} values).

The sweep width was 4545 Hz for D_2O spectra and 9615 Hz for H_2O spectra, acquired in 2K and 4K points, respectively ($t_{2,max}$ = 225 and 213 ms). The carrier frequency was always set on the water resonance and phase-sensitive acquisition was achieved using TPPI (Marion & Wüthrich, 1983). The 2QF-COSY (Rance et al., 1983) was acquired with 128 scans/increment, and the maximal t_1 value ($t_{1,max}$) was 112.6 ms. The mixing time (τ_m) for clean-TOCSY (Griesinger et al., 1988) in D_2O was 120 ms. The delay between pulses of the MLEV-17 sequence was 2.6 times the length of the 90° pulse, giving a field strength of the mixing radiofrequency (rf) of 6.7 kHz. A total of 128 scans/t_1 value were collected, and $t_{1,max}$ = 112.6 ms. For clean-TOCSY in H_2O, a rf field of 7.6 kHz was applied during 80 ms, 128 scans/t_1 were acquired, $t_{1,max}$ = 25.6 ms, and the water was presaturated during 0.8 s using DANTE (Morris & Freeman, 1978). The NOESY spectra (Jeener et al., 1979; Kumar et al., 1980) were recorded in D_2O with a 50-ms mixing period (192 scans/t_1, $t_{1,max}$ = 31.8 ms) and a 150-ms mixing period (192 scans/t_1, $t_{1,max}$ = 56.3 ms). The suppression of the water resonance in NOESY acquired in H_2O was achieved with one JR read pulse (Plateau & Guêron, 1982) with a delay of 63 μs. A total of 96 scans/increment were collected, and $t_{1,max}$ = 53.2 ms. For the 2Q spectrum (Rance et al., 1983), each increment was composed of 256 scans, the excitation delay was set to 33 ms (Grütter et al., 1988), and $t_{1,max}$ = 28.7 ms. Mixing in ROESY (Bothner-By et al., 1984; Bax & Davies, 1985) during 75 ms used a DANTE sequence of 30° pulses applied with a duty cycle of 10% (giving a rf field strength of 2.1 kHz). A total of 192 scans/t_1 value were acquired, and $t_{1,max}$ = 66 ms. The processing was done on a X32 workstation running the uxnmr package. Prior to Fourier transformation, the experiments were apodized with shifted, squared sine-bell windows in both time dimensions and zero-filled to 4K and 2K in ω_2 and ω_1, respectively, leading to a final resolution of 2.2 and 4.7 Hz along ω_2 for H_2O and D_2O spectra, respectively, and 4.4 and 9.4 Hz along ω_1.

Modeling. The model of the dodecanucleotide was constructed using the coordinates of the crystal structure of the Drew dodecamer (Brookhaven Protein DataBase entry 1BNA; Dickerson & Drew, 1981). The hydrogen atoms were attached to the heavy atoms using standard geometry for the nucleotides.

To dock the ligand onto the 12-mer, the intermolecular proton–proton distances were estimated using the cytosine 5H–6H distance (r = 2.45 Å) as a reference. The unknown distance r_a between a pair of protons can be directly related to a known distance r_b from

$$r_a = r_b(\mathrm{NOE}_b/\mathrm{NOE}_a)^{1/6}$$

(Wüthrich, 1986; Zhang & Patel, 1990).

A value of 0.5 Å (for NOESY data collected at a 50-ms mixing time) or 1.0 Å (for NOESY data collected at a 150-ms mixing time) was added to the calculated distances in order to define upper limits (Zhang & Patel, 1990) for the Hoechst 33258–d(GTGGAATTCCAC)$_2$ complex.

All atoms were treated explicitly during the energy refinement using our in-house force field implemented on a Silicon Graphics Iris 4D workstation (Müller, 1984; Gerber and Müller, to be published). The distance constraints were included as quadratic terms to the total energy expression.

After the docking, the ligand coordinates were relaxed without changing the coordinates of the DNA in order to relieve bad contacts. Thereafter, all atom positions were relaxed with the NOE distance constraints active. In the final stage of the energy minimization, the NOE distance constraints were suspended. For each minimization run, the procedure was stopped when the total energy change per iteration was smaller than 0.05 kcal/mol.

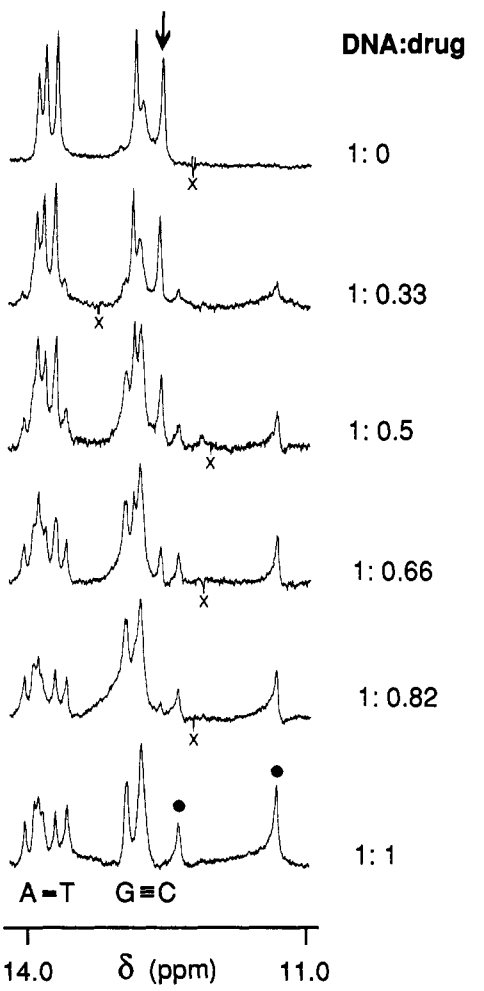

FIGURE 2: Titration of the DNA duplex of Figure 1b with Hoechst 33258. 1D 500-MHz ^{1}H NMR spectra were acquired after addition of increasing amounts of the drug. The region showing the imino protons of the DNA is presented (solvent H_2O, 50 mM phosphate buffer, 100 mM NaCl, 0.1% NaN_3, pH 7.0, T = 293 K). Water suppression was achieved using a jump-and-return excitation scheme. The DNA duplex-to-drug ratios are given on the right. The arrow indicates the resonance used to follow the extent of complex formation. The two resonances identified by a dot belong to exchangeable protons of the drug (BP,NH and BB,NH). The x's indicate instrumental artifacts.

Results

Titration. The imino proton regions of the different 1D NMR spectra acquired during the stepwise titration of the DNA duplex with the ligand are shown in Figure 2. In the free DNA, only six imino proton resonances are observed, due to the inherent C_{2v} symmetry of the free duplex. Three resonances for the thymine N3H and three for the guanine N1H protons of the AT and GC Watson–Crick base pairs can be discerned. As seen most clearly for the thymine N3H protons, the set of three lines is replaced by a new set of six lines during the titration. This indicates the formation of at least one new species in solution when the drug is added to the DNA. The appearance of distinct resonances for free and bound DNA in the same spectrum at intermediate drug:DNA ratios indicates that the complex is long-lived relative to the NMR time scale. We have used the guanine N1H resonance at 12.57 ppm to monitor the extent of the complex formation. The intensity of this line decreases during the titration, and the 1:1 lig-

and:DNA ratio is reached as this resonance disappears in the baseline. All imino proton resonances are shifted toward lower field as compared to the free duplex. This is already indicative of the drug binding in the minor groove of the DNA (Feigon et al., 1984). The two lines at 12.33 and 11.30 ppm arise from the labile NH protons of H33258 (see below).

Assignment of Hoechst 33258 in the Complex. A combination of 2QF-COSY and clean-TOCSY spectra acquired in D_2O was used to assign the nonlabile protons in the four spin systems of H33258. In the 2QF-COSY spectrum, three cross peaks in a spectral region where the DNA does not show scalar-coupled protons were attributed to the aromatic moieties of the drug (Leupin et al., 1986). The clean-TOCSY spectrum (τ_m = 120 ms) allowed completion of the benzimidazole spin systems. Indeed, the meta-coupled protons of these moieties also give rise to cross peaks which are better observed in clean-TOCSY than in 2QF-COSY.

In a NOESY spectrum acquired in D_2O (τ_m = 150 ms), the higher field benzimidazole 6 and 8 protons were assigned to the BP benzimidazole moiety because they displayed NOEs to a set of protons with chemical shifts of 4.34 and 3.40 ppm (the latter chemical shift corresponds to a broad resonance line where we could in fact assign two different chemical shifts of 3.38 and 3.43 ppm. These resonances are assigned to the same methylene protons for different conformations of the piperazine ring (see also below and Discussion)). Since these protons are scalar coupled to each other, as evidenced in clean-TOCSY, we have assigned the respective resonances to the piperazine P2;6 and P3;5 methylene protons. This was further confirmed by additional NOEs to resonances appearing at 2.96 and 2.56 ppm. These lines are assigned to the *N*-methyl group in two different chemical environments, thus indicating that the piperazine ring can adopt at least two forms when H33258 interacts with the DNA duplex.

The phenol B2;6 and B3;5 protons were also distinguished via an NOE from one NH proton of one benzimidazole group as observed in the NOESY spectrum acquired in H_2O. Thus, this NH resonance at 12.33 ppm has been assigned to the BB benzimidazole moiety. The intraresidue cross peak expected between the BB,NH and the BB6 proton could not be unambiguously identified since the BB6 proton resonance overlaps with the resonance line of an adenine 2 proton [ω(BB6) = ω(A_6,2H)]. The second NH resonance (δ = 11.30 ppm) has been assigned to the BP benzimidazole group as it exhibited an intraresidue cross peak to BP6. Other labile protons of H33258 (phenol OH, piperazine NH) could not be identified, probably because of fast exchange with the solvent water protons.

A fast ring-flip motion of the phenol moiety makes it impossible to distinguish between the B2 (B3) and B6 (B5) protons. Similarly for the piperazine ring, a flip around the piperazine–benzimidazole bond is likely, thus impeding to give precise stereochemical assignments for the methylene protons.

The chemical shifts of all assigned drug resonances are reported in Table I.

Sequential Assignments of the Dodecanucleotide Duplex. The individual spin systems of the deoxyribose moieties have been identified using 2QF-COSY and clean-TOCSY in D_2O. The problem of overlapping resonances in the 2′ and 2″ proton region was overcome by employing the remote connectivities of the 1′ protons to the 2′/2″ protons observed in the 2Q spectrum acquired in D_2O (Grütter et al., 1988) (Figure 3). The clean-TOCSY spectrum allowed a straightforward assignment of all TCH_3 resonances from their cross peaks to T6H. The 2QF-COSY spectrum has been used to identify

Table I: Proton Chemical Shift Assignments of Hoechst 33258 in the 1:1 Complex Formed with $d(GTGGAATTCCAC)_2$[a]

drug protons	chemical shift[b]	drug protons	chemical shift[b]
B2;6	7.32	BP6	7.60
B3;5	8.09	BP8	7.18
BB,NH	12.33	BP9	7.75
BB6	8.28	P2;6	4.34
BB8	7.95	P3;5	3.38, 3.43[c]
BB9	8.20	PCH_3	2.96, 2.56[c]
BP,NH	11.30		

[a] Experimental conditions: T = 298 K, 100 mM NaCl, 50 mM phosphate buffer, 0.1% NaN_3, pH 7.0. [b] In ppm (±0.02 ppm) relative to TSP. [c] Two different resonances were observed (see text for discussion).

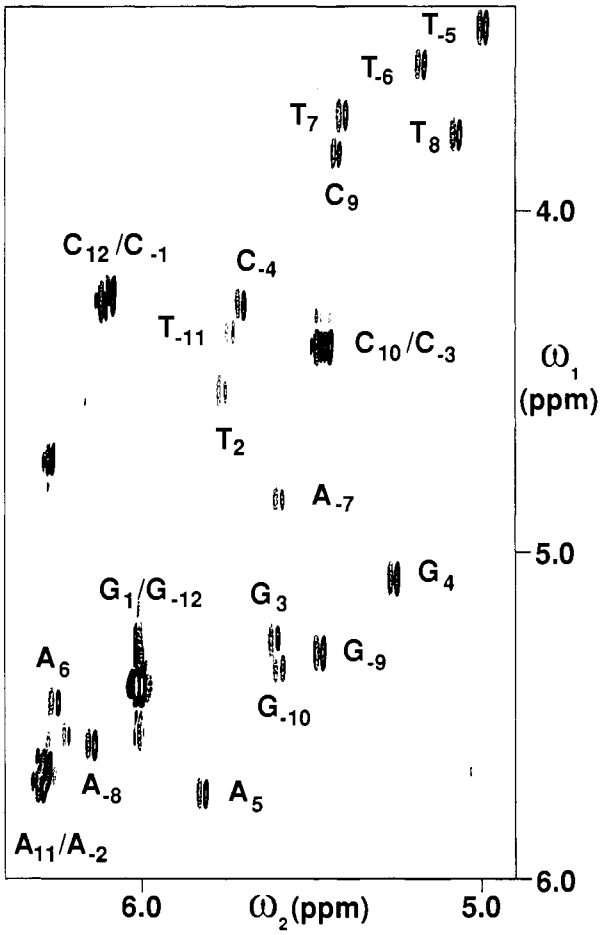

FIGURE 3: Phase-sensitive 2Q spectrum of the 1:1 complex formed between the DNA duplex of Figure 1b and Hoechst 33258 (500 MHz, excitation period 33 ms, solvent D_2O, T = 298 K, other conditions as in Figure 2). The region shown contains the remote peaks at ω_1 = ω(2′H) + ω(2″H) and ω_2 = ω(1′H). The assigned peaks are labeled with the one-letter code of the base and its sequence number (see Figure 1).

the cytosine C5H and C6H resonances. The sequential assignment of the spin systems was based on the observation of short sequential and intranucleotide connectivities in the NOESY spectrum recorded in D_2O at 150 ms. For this purpose, we have used the intraresidue d_i(2′;6,8)/d_i(1′;6,8) connectivities, involving the 1′/2′/2″ sugar protons and the 6/8 protons of the same base, as well as sequential connectivities d_s(2″;6,8)/d_s(1′;6,8) to the next base in the 3′ direction (Feigon et al., 1983; Hare et al., 1983; Scheek et al., 1983, 1984; Chazin et al., 1986). Only the protons of the 3′-terminal AC dinucleotide and the 5′-terminal G in both strands had equivalent chemical shifts. It was possible to sequentially assign all spin systems for each complete DNA strand sepa-

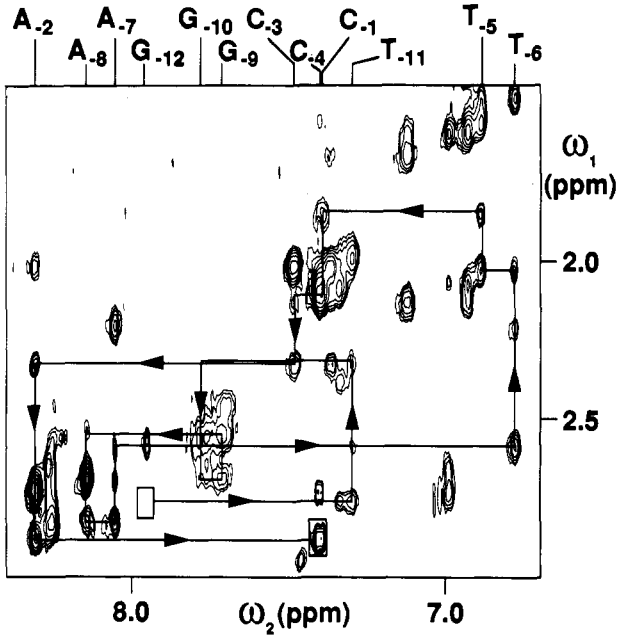

FIGURE 4: Phase-sensitive NOESY spectrum of the 1:1 complex formed between the DNA duplex of Figure 1b and Hoechst 33258 (500 MHz, τ_m = 150 ms, other conditions as in Figure 3). The region containing sequential NOEs $d_s(2'';6,8)$ and intraresidual NOEs $d_i(2';6,8)$ is shown. The sequential assignment pathway for the (−)-strand is indicated with lines and arrows, and the start and the end are identified by boxes. The missing cross peaks were observed at lower contour levels. The chemical shift positions for the 6H/8H base resonances are indicated at the top using the one-letter code and the sequence number (see Figure 1).

rately using these $d_i(2';6,8)/d_i(1';6,8)$ and $d_s(2'';6,8)/d_s(1';6,8)$ connectivities. The assignment pathway using the 2′H/2″H to base 6H/8H NOEs is given for the (−)-strand in Figure 4. The pattern of observed intensities reflects the fact that the duplex retains a right-handed, B-form DNA structure (Van de Ven & Hilbers, 1988) in the complex. NOEs corresponding to sequential connectivities $d_s(1';5)$ have also been observed. In addition, sequential and interstrand adenine 2H to 1′H NOEs, as well as sequential adenine 2H–2H contacts $d_s(2;2)$, have also been observed using the NOESY spectra (Grütter et al., 1988). The combined use of all mentioned connectivities allowed assignment of most of the nonlabile protons of both strands of the 12-mer in the complex. A survey of the used connectivities is given in Figure 5.

Using the known sequence-specific resonance assignments of the free duplex (W. Leupin, unpublished results), we tabulated the drug-induced chemical shift differences of specific DNA protons. Figure 6 shows such plots for 1′H, 2′H, 2″H and 6H/8H. These plots are symmetric relative to the center of the oligonucleotide, thus localizing the drug-binding site roughly to the center of the DNA. From these graphs follows that the drug-induced shift differences are quite different for different types of protons. Whereas all protons shown exhibit a drug-induced upfield shift of their resonances, the imino and adenine 2 protons are shifted downfield. Overall, these plots show that the protons of the DNA segment $d(A_5A_6T_7T_8)\cdot d(A_{-8}A_{-7}T_{-6}T_{-5})$ are mostly influenced by the ligand, indicating binding of H33258 to this region of the DNA duplex.

Additional cross peaks in NOESY spectra, which proved to be chemical-exchange cross peaks, have also been observed. As confirmed by ROESY, all protons which are symmetrically related in the DNA strands of the free duplex give rise to such cross peaks in the DNA/drug complex. This is best seen in the chemical shift region where the base 6 and 8 and adenine 2 protons resonate. Using a combination of NOESY and

FIGURE 5: Survey of the sequential NOEs used to assign both DNA strands of d(GTGGAATTCCAC)$_2$ in the 1:1 complex with Hoechst 33258. The different sequential connectivities are given on the left.

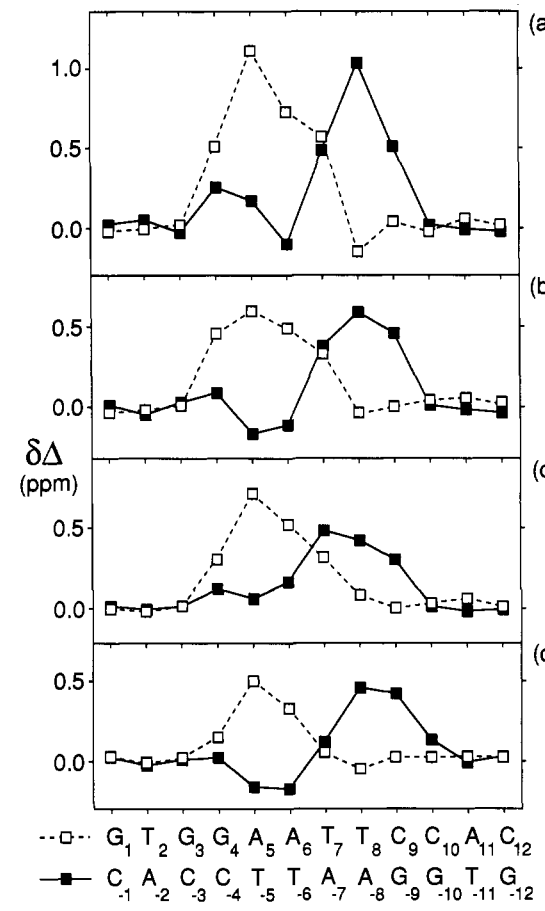

FIGURE 6: Plots versus the DNA sequence of chemical shift differences for corresponding protons of d(GTGGAATTCCAC)$_2$ in the free DNA duplex and in the complex with Hoechst 33258: (a) 1′ protons; (b) 6/8 protons; (c) 2′ protons; (d) 2″ protons.

ROESY (Figure 7), we can distinguish between cross peaks due to nuclear Overhauser enhancements and those due to chemical-exchange phenomena. Indeed, cross peaks caused by dipole–dipole interaction have in ROESY the opposite sign than the diagonal, and chemical-exchange cross peaks have the same sign (Bothner-By et al., 1984). All aromatic proton resonances, which were sequence-specifically assigned, show such exchange cross peaks.

The NOESY spectrum acquired in H_2O has allowed assignment of most of the amino and imino protons. The thymine T3NH and guanine G1NH imino proton resonances were

Table II: Chemical Shifts for the Assigned Protons of the Dodecanucleotide in the 1:1 Complex with Hoechst 33258[a]

base	1'H	2'H	2''H	3'H	C5H	TCH_3	6H	8H	A2H	$C4NH_2$[b]	G1NH/T3NH	A6NH_2/ G2NH_2[b]
						(+)-Strand						
G_1	5.99	2.61	2.77	4.82				7.94			nd	nd
T_2	5.75	2.11	2.40	4.85		1.36	7.35			nd	nd	
G_3	5.56	2.57	2.68	4.99				7.76			12.94	nd
G_4	5.24	2.47	2.58	4.98				7.70			12.79	nd
A_5	5.81	2.83	2.85	5.10				8.24	7.45			6.61/6.48
A_6	6.25	2.67	2.75	5.05				8.26	8.28			nd
T_7	5.41	1.60	2.08	4.45		1.32	6.98				14.01	
T_8	5.06	1.59	2.15	4.43		1.41	6.92				13.57	
C_9	5.42	1.68	2.15	4.57	5.34		7.11			8.04/6.39		
C_{10}	5.45	2.03	2.34	4.83	5.49		7.36			8.53/6.73		
A_{11}	6.27	2.74	2.89	5.05				8.30	7.95		nd	
C_{12}	6.11	2.15	2.15	4.47	5.45		7.36			nd		
						(−)-Strand						
G_{-12}	5.99	2.59	2.77	4.82				7.94			nd	nd
T_{-11}	5.74	2.01	2.33	4.85		1.36	7.29				nd	
G_{-10}	5.58	2.59	2.69	4.98				7.78			12.92	nd
G_{-9}	5.46	2.55	2.70	4.99				7.70			12.79	6.83
A_{-8}	6.13	2.70	2.83	5.07				8.13	7.61			nd
A_{-7}	5.58	2.22	2.60	4.98				8.04	8.36			6.50
T_{-6}	5.17	1.49	2.04	4.40		1.16	6.77				13.86	
T_{-5}	4.98	1.58	1.85	4.43		1.38	6.88				13.69	
C_{-4}	5.66	2.10	2.15	4.79	5.51		7.38			8.35/6.58		
C_{-3}	5.45	2.03	2.34	4.83	5.66		7.47			8.53/6.80		
A_{-2}	6.27	2.74	2.89	5.05				8.30	7.87			nd
C_{-1}	6.11	2.15	2.15	4.47	5.45		7.36			nd		

[a] In ppm (±0.02 ppm) relative to TSP. Experimental conditions: T = 298 K, 100 mM NaCl, 50 mM phosphate buffer, 0.1% NaN_3, pH 7.0. The 4',5' and 5'' protons are not assigned due to spectral crowding. nd = not determined. [b] The chemical shifts of the hydrogen-bonded amino protons are underlined.

assigned through two independent pathways. The first pathway involved sequential imino proton–imino proton NOEs and permitted the identification of 8 out of the 12 imino proton resonances. The fact that the imino proton resonances of the duplex termini are missing can be explained by fraying of the ends. The second pathway (Otting et al., 1987) involved internucleotide NOEs between imino protons and the previously assigned adenine 2H resonances. In this way, it was possible to assign the imino proton resonances of the tetranucleotide sequence $d(AATT)_2$. The previous assignments of the guanosine N1H imino proton resonances have been confirmed by the NOE to the cytosine amino protons, which in turn exhibit intranucleotide NOEs to their C5H protons (Wüthrich, 1986). The assignments of the DNA proton resonances are reported in Table II.

Intermolecular NOEs. On the basis of the known assignments of both ligand and oligonucleotide protons, we have been able to locate and identify a number of intermolecular NOEs. The regions of two NOESY spectra (τ_m = 150 ms, H_2O and D_2O) displayed in Figure 8 show most of these intermolecular NOEs. They involve exclusively the deoxyribose 1' protons and the adenine 2 protons of the dodecanucleotide, thus definitely locating the drug to the minor groove of the DNA duplex. Protons of all four spin systems of the ligand showed NOEs to protons of the DNA, demonstrating that the drug–DNA interaction occurs along the entire length of the drug. For both benzimidazole moieties, only the BP6, BB6, and the NH protons exhibit close contacts with protons of the dodecanucleotide duplex. The methylene protons of the piperazine ring show also close contacts to the duplex. Only the protons of the *N*-methyl group resonating at 2.96 ppm give rise to a NOE with A_5,2H (no such close contact was observed from the protons of the *N*-methyl group appearing at 2.56 ppm). In all, 25 intermolecular NOEs could unambiguously be identified (Table III). Their distribution is confined to the $d(A_5A_6T_7T_8C_9)\cdot d(G_{-9}A_{-8}A_{-7}T_{-6}T_{-5})$ segment of the 12-mer.

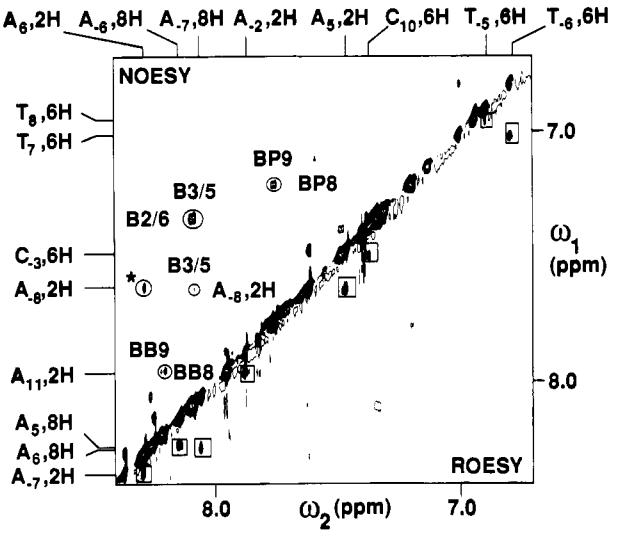

FIGURE 7: Combined plot of a ROESY spectrum and a NOESY spectrum (500 MHz, phase sensitive, τ_m^{ROESY} = 75 ms, τ_m^{NOESY} = 150 ms, other conditions as in Figure 3) used to distinguish cross peaks due to NOE transfer of magnetization from those due to chemical exchange. The aromatic region is shown. Cross peaks arising from different interactions are identified as follows: in the ROESY spectrum, positive cross peaks are drawn with multiple lines and negative cross peaks with a single contour, and peaks arising from chemical exchange (see text) are boxed. The aromatic protons of the DNA which are involved in the exchange process are labeled on the left and at the top using the numbering of Figure 1. In the NOESY spectrum, cross peaks arising from NOEs (see text) are circled and the interacting protons are identified. The peak marked with an asterisk could not be unambiguously assigned (see text).

Dynamic Properties. The exchange cross peaks measured by ROESY indicate that the drug can bind in two equivalent sites on the duplex. Considering a symmetrical two-site chemical-exchange case with equal populations and equal relaxation times (both T_1 and T_2) for the two sites (Ernst et

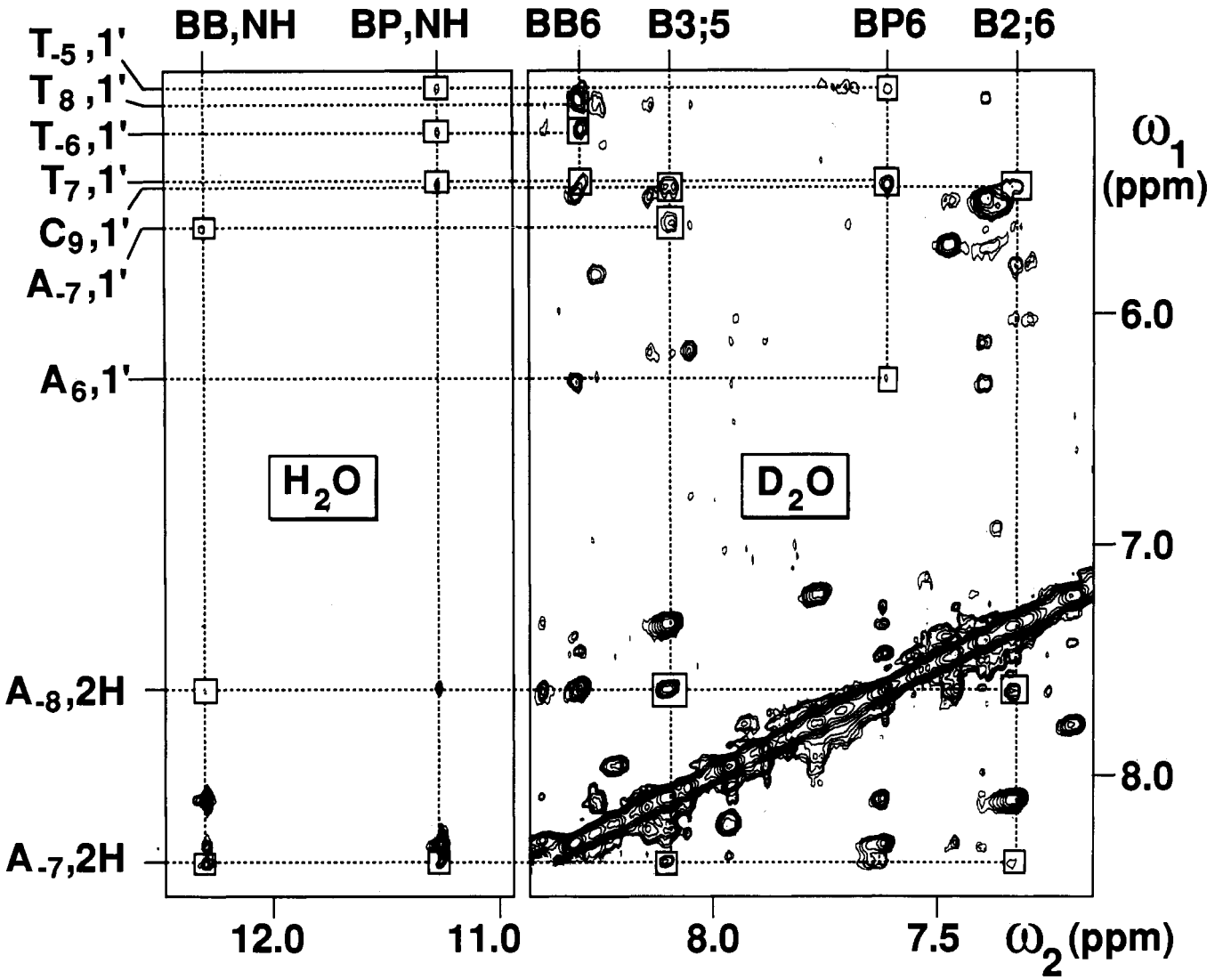

FIGURE 8: Parts of two phase-sensitive NOESY spectra of the 1:1 complex formed between the DNA duplex of Figure 1b and Hoechst 33258 (500 MHz, τ_m = 150 ms, other conditions as in Figure 3). The drug resonances are identified at the top, the DNA sugar 1′ and adenine 2 protons are identified on the left, and the intermolecular cross peaks are framed with a square.

al., 1987), the exchange rate can be determined from the ratio of peak intensities as

$$k_{ex} = \ln [(1 + R)/(1 - R)]/2\tau_m$$

where k_{ex} is the exchange rate (s^{-1}), R is the ratio of the cross peak to diagonal peak intensity, and τ_m is the mixing time. For small R, in the initial rate approximation, this formula simplifies to $k_{ex} = R/\tau_m$. Assuming identical line shapes for the diagonal and the cross peaks, we calculated R from the number of contour levels in the 2D plot of the NOESY spectrum (τ_m = 50 ms) acquired in D_2O, using the cross peaks and the diagonal peaks arising from the T_7,6H, T_{-6},6H, A_8,8H, and A_{-5},8H protons. The rate of exchange for this process is 2.2 ± 0.2 s^{-1} at 298 K, which is equivalent to a lifetime τ of the complex of 450 ± 50 ms at these temperature and solvent conditions. The free energy of activation for this exchange process can be determined from the Eyring equation by applying the formula (Günther, 1973)

$$\Delta G^* = 19.14T[10.32 - \log (k_{ex}/T)] \quad (J/mol)$$

with T being the temperature (in kelvin). The estimated ΔG^* is 71 ± 0.5 kJ/mol at T = 298 K.

We have also acquired a series of 1D spectra in D_2O solution at different temperatures (data not shown). From these data follows that the original C_{2v} symmetry of the free DNA duplex is restored in the complex above 330 K if, e.g., the methyl proton resonances of T_7 (δ = 1.32 ppm) and T_{-6} (δ = 1.16 ppm) are observed. These two lines coalesce to a single line at 1.24 ppm at 330 K, the coalescence temperature (T_{coal}). The simplified formula (Günther, 1973)

$$k_{ex}^{coal} = 2.22\Delta\nu$$

with $\Delta\nu$ equal to the difference in chemical shifts (hertz) measured at slow-exchange regime, allows estimation of k_{ex}^{coal} for our system. The free energy of activation for this process can be estimated using the equation

$$\Delta G^* = 19.14T_{coal}[9.97 + \log(T_{coal}/\Delta\nu)] \quad (J/mol)$$

(Sutherland, 1971; Günther, 1973), which is based on transition-state theory. With T_{coal} = 330 K and $\Delta\nu$ = 80 Hz (for T_7,CH_3 and T_{-6},CH_3), we estimated k_{ex}^{coal} = 180 s^{-1} (τ = 5.5 ms) and ΔG^* = 67 ± 1 kJ/mol.

The dynamic behavior of the phenol group has been assessed by clean-TOCSY (τ_m = 80 ms, H_2O) at T = 273 K. Under these experimental conditions, we have still observed a single cross peak correlating the B3;5 to the B2;6 protons. This indicates a fast ring flip of the phenol moiety pertaining to this low temperature (Wüthrich, 1976).

Molecular Modeling. In the relaxed model, the drug covers the four central base pairs (AATT stretch) of this oligonucleotide and is isohelical with the DNA. A large number of attractive hydrophobic interactions keep the molecule

Table III: Intermolecular NOEs and Upper Distance Limits in the Complex Formed between the DNA Duplex of Figure 1b and Hoechst 33258[a]

ligand proton	(+)-strand proton[b]	(−)-strand proton[b]
B2;6	C_9,1′H (3.8)	A_{-8},2H (3.4)
		A_{-7},2H (4.7)
B3;5	C_9,1′ (4.2)	A_{-8},2H (3.3)
		A_{-7},2H (3.6)
		A_{-7},1′H (3.8)
BB,NH		A_{-8},2H (4.3)
		A_{-7},2H (3.8)
		A_{-7},1′H (4.3)
		T_{-6},1′H (4.5)
BB6	T_8,1′H (3.8)	T_{-6},1′H (3.4)
	T_7,1′H (3.8)	
BP,NH	T_7,1′H (4.1)	A_{-7},2H (3.9)
		T_{-6},1′H (4.3)
		T_{-5},1′H (4.3)
BP6	T_7,1′H (3.6)	T_{-5},1′H (4.2)
	A_6,1′H (4.7)	
P3;5[c]	A_5,2H (5.5)	
P2;6[c]	A_6,1′H (5.5)	
	A_5,2H (5.5)	
PCH_3[c]	A_5,2H (5.5)	

[a] Observed in NOESY spectra acquired in D_2O (τ_m = 50 and 150 ms) or H_2O (τ_m = 150 ms), T = 298 K, 100 mM NaCl, 50 mM phosphate buffer, 0.1% NaN_3, pH 7.0. The intermolecular proton–proton distances were calculated using the cytosine 5H–6H distance (2.45 Å) as a reference. These distances were converted into upper limits by adding a value of 0.5 Å for NOESY data collected at a 50-ms mixing time or 1.0 Å for NOESY data collected at 150 ms (see Materials and Methods for more details). [b] Values in parentheses in Å. [c] Only the *N*-methyl protons resonating at 2.96 ppm give rise to an NOE with a DNA proton (see Results).

snugly fitted in the minor groove (Figure 9a). The piperazine ring lies centered at the $d(A_5A_6)\cdot d(T_{-6}T_{-5})$ dinucleotide, and the phenol moiety is at the T_8/A_{-8} base pair. Both benzimidazole moieties present the same edge to the bottom of the minor groove. Overall, the quality of the model is assessed, for example, through the absence of any repulsive van der Waals forces.

In this model, 8 of the 25 intermolecular proton–proton distances are larger than the imposed upper distance limits. These distance violations span a range from 0.2 to 0.6 Å, with a mean residual violation of 0.4 Å. All but two intermolecular proton–proton distances are smaller than 5 Å, the largest distance still giving rise to an observable NOE (Wüthrich, 1986).

Within this model, two intermolecular hydrogen bonds are present, involving the benzimidazole NH protons and two thymine carbonyl oxygens on the same strand of the DNA. The first bond is formed between BB,NH and T_8,O2 and the second between BP,NH and T_7,O2 (Figure 9c). A third hydrogen bond exists between the phenol OH proton and the O4′ oxygen of the A_{-8} deoxyribose moiety.

We have performed modeling of the piperazine ring of the drug in the complex. These studies show that the piperazine moiety fits in the minor groove of the DNA in at least two different conformations/configurations, irrespective of the remaining moieties of the ligand, thus underlining our previous assumption based on the observation of two resonance lines for the *N*-methyl protons.

Discussion

The binding of Hoechst 33258 to the oligonucleotide $d(GTGGAATTCCAC)_2$ was first assessed through the acquisition of 1D spectra in H_2O during the titration. The doubling of, for example, the imino proton resonances upon binding indicates that the addition of the (asymmetric) drug to the 12-mer lifts the inherent C_{2v} symmetry of the free oligonucleotide. The observation of drug-induced low-field-shifted imino proton resonances (Feigon et al., 1984; Leupin et al., 1986; Leupin, 1990) indicates that Hoechst 33258 binds in the minor groove of the DNA duplex.

The sequence-specific assignment of the proton resonances of both the DNA duplex and bound drug in the complex was achieved independently of the spectra of the free molecules. This is in contrast to the approach taken in an NMR study of the complex between Hoechst 33258 and the Drew dodecamer $d(CGCGAATTCGCG)_2$ (Parkinson et al., 1990). In this study, the spectra of a 0.5:1 (ligand:duplex ratio) complex were used to link the sequence-specific resonance assignments of the free oligonucleotide to its assignments in the 1:1 complex using exchange spectroscopy. We decided not to use this method because it requires a dissociation of the drug:DNA complex in the appropriate time range to observe exchange cross peaks.

A first indication of the binding of Hoechst 33258 to the center of the DNA duplex followed from an inspection of the plots depicting the drug-induced chemical shift differences of specific protons of the DNA duplex (Figure 6). The largest differences have been observed for the 1′ protons which are pointing into the minor groove. Interestingly, base 6 and 8 protons pointing into the major groove are also affected upon binding of the drug, reflecting some locally induced conformational changes of the nucleotides (e.g., base roll, propeller twist), which influences the chemical environment of protons in the major groove. This comparison of chemical shifts in free and bound DNA indicates also that qualitatively different effects of ligand binding are observed for the deoxyribose protons and the 6 and 8 base protons on one hand and for the imino and adenine 2 protons (plots not shown) on the other hand. The binding-site size of 5 base pairs for the binding of Hoechst 33258 to DNA derived from optical studies (Loontiens et al., 1990, and references cited therein) and from footprinting studies (Martin & Holmes, 1983; Harshman & Dervan, 1985; Murray & Martin, 1988; Jorgenson et al., 1988; Portugal & Waring, 1988; Churchill & Suzuki, 1989; Bathini et al., 1990) agrees well with the number of DNA protons influenced by the binding of the drug (Figure 6).

The most direct and precise information for the binding of Hoechst 33258 to the minor groove was given by the identification of 25 unambiguous intermolecular NOEs directed to 1′ and adenine 2 protons of different nucleotides on both DNA strands. A molecular model was obtained using these NOEs which define intermolecular distance constraints. From this model follows that the four central base pairs $d(A_5A_6T_7T_8)\cdot d(A_{-8}A_{-7}T_{-6}T_{-5})$ of the oligonucleotide form the binding site for the drug. The protons of the piperazine moiety exhibit NOEs to the adenine 2 and 1′ protons of the dinucleotide $d(A_5T_{-5})\cdot d(A_6T_{-6})$, thus defining the relative orientation of Hoechst 33258 to the DNA duplex. The model also shows that only one edge of the drug is involved in DNA recognition. In this way, the bound drug is crescent shaped and isohelical with the DNA.

The occurrence of intermolecular hydrogen bonds between BP,NH and T_7,O2 and between BB,NH and T_8,O2 cannot be derived directly using our ^1H NMR data. However, these hydrogen bonds are found indirectly on the basis of the appearance of numerous intermolecular NOEs involving the drug NH and the DNA nonlabile protons. In this way, both hydrogen-bond donors and acceptors are brought into correct juxtaposition. The magnitudes of the integrals over the singlet line of the two drug NH resonances observed in the 1D NMR spectra indicate that the hydrogen bond between BP,NH and

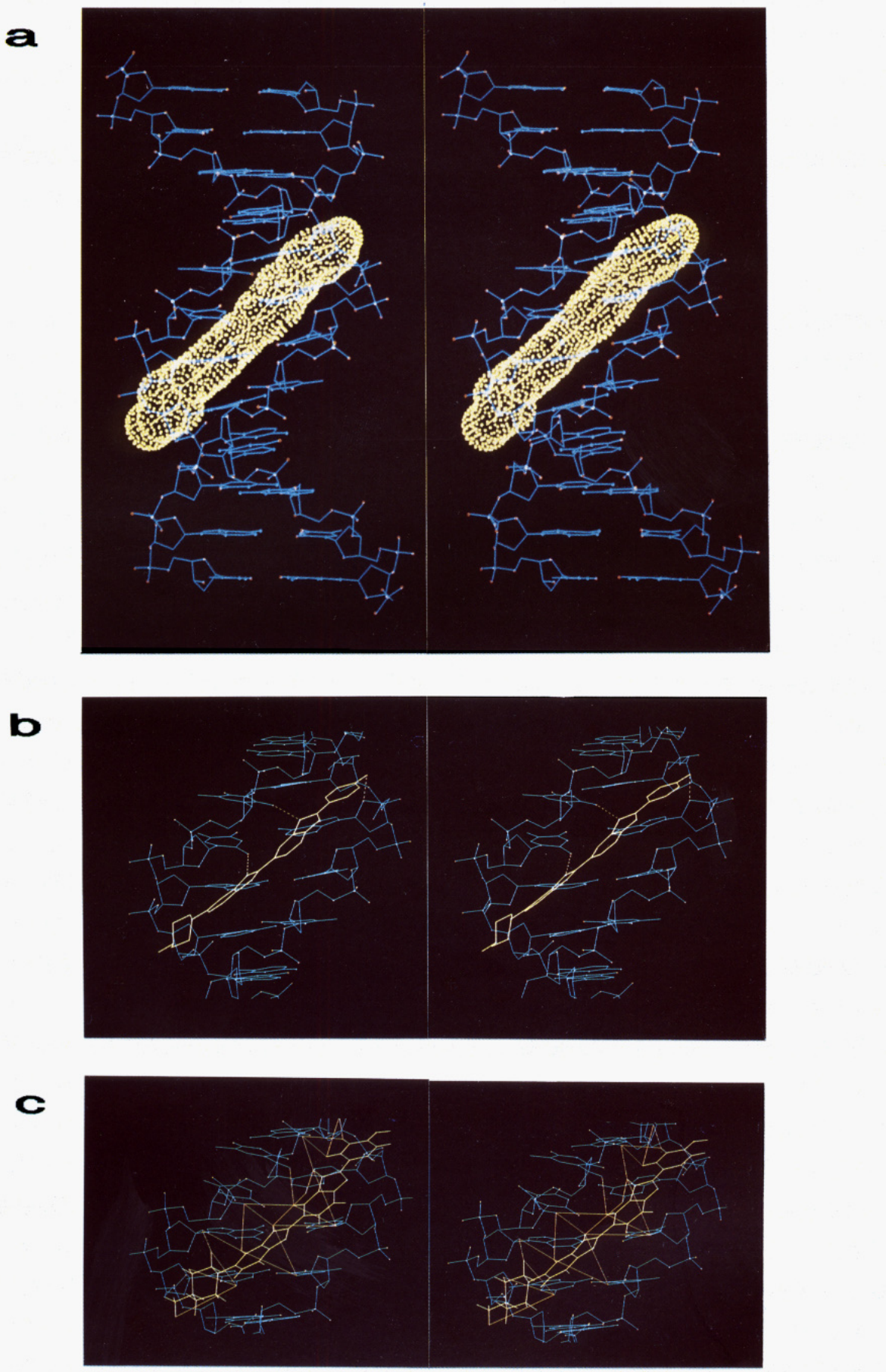

FIGURE 9: (a) Stereoview of the model for the 1:1 complex formed between d(GTGGAATTCCAC)$_2$ and Hoechst 33258. Only heavy atoms are shown. The docking was done using distance constraints generated from the intermolecular NOEs (Figure 8) followed by energy minimization. (b) Close-up at the binding site depicting in red the three intermolecular H-bonds BB,NH–T_8,O_2, BP,NH–T_7,O2, and phenol OH–A_{-8},O4′ as derived from this model (only heavy atoms are shown). (c) Close-up at the binding site depicting in red selected close intermolecular proton–proton contacts as observed by NOESY. All protons of the ligand but only the sugar 1′ and adenine 2 protons of the DNA are shown.

T_7,O2 is stronger than the one between BB,NH and T_8,O2. This is confirmed by the measurement of temperature-induced changes of the chemical shifts for these protons (data not shown). These coefficients differ by a factor of 2 (−3.2 Hz/K for BB,NH vs −1.7 Hz/K for BP,NH), thus supporting the above conclusion that BP,NH is involved in a stronger hydrogen bond than BB,NH.

We report some details of the dynamics of the drug binding to this 12-mer. We have shown that the drug can switch between the two equivalent binding sites of the DNA dodecamer and have calculated, from the exchange spectra, the rate for this process. The long residence time of Hoechst 33258 on the DNA of 450 ± 50 ms at 298 K is in agreement with the dissociation constants of up to 3×10^{-9} M^{-1} for specific DNA sequences (Loontiens et al., 1990; Parkinson et al., 1990). The lifetime of our complex is very similar to the lifetimes for the 2:1 and 1:1 complexes between distamycin A and DNA (Pelton & Wemmer, 1990; Klevitt et al., 1986) but is larger than the lifetimes for the 1:1 complexes of netropsin and DNA (Patel & Shapiro, 1985, 1986), and a bisquaternary ammonium heterocycle SN6999 and DNA (Leupin et al., 1986). The dynamic behavior of the phenol moiety was also investigated. These studies demonstrated that a fast ring flip for the phenol group of the bound drug is still possible, even at the low temperature used. We do not give any rate constant for this process as it is impossible to estimate the chemical shift values for the B2, B3, B5, and B6 protons for the immobilized ring. Knowing the chemical shift values for these protons is a prerequisite for calculating such a rate constant. In their studies of the 2:1 complex of Hoechst 33258 with d(CTTTTGCAAAAG)$_2$, Searle and Embrey (1990) report, however, an upper limit of 200 s^{-1} for this flip rate, assuming a chemical shift difference of 0.5 ppm between the B2 and B6 and between the B3 and B5 proton resonances, respectively, if the ring is immobilized in the minor groove.

The overall structure of the DNA does not change upon complexation of the ligand, being of the B-type family as the free duplex. This follows from an inspection of (i) the ratio of NOE intensities d_i(6,8;2′) and d_s(2″;6,8) and of (ii) the fine structure of the cross peaks due to $^3J_{1'2'}$ and $^3J_{1'2''}$ in phase-sensitive 2QF-COSY. All these data are similar to those found for the free duplex and characteristic of the values expected for nucleotides in $C_{2'}$-endo conformation (Chazin et al., 1986; Wüthrich, 1986).

Our data are in fair agreement with the ones from an NMR study of a 1:1 complex of Hoechst 33258 with d(CGCGAATTCGCG)$_2$ (Parkinson et al., 1989, 1990). In that study, the authors also claim that Hoechst 33258 binds to the central AATT site, as found by us and in the X-ray study of Teng et al. (1988). Whereas both Parkinson et al. (1990) and we detected 25 intermolecular NOEs, some of these NOEs involve different protons of the ligand and/or DNA. For example, we have found that the labile protons BP,NH and BB,NH of the drug give rise to intermolecular NOEs to 1′ protons of the oligonucleotide (see Table III), whereas Parkinson et al. (1990) do not report such NOEs. On the other hand, they listed one intermolecular NOE involving a guanosine 8 proton which points into the major groove. This can only be explained by spin diffusion, which is very effective at the long mixing time they used (300 ms). Parkinson et al. (1990) also report an intermolecular NOE between the BP9 proton of the drug (proton Hoechst H19 in their nomenclature) to an adenine 2 proton of the DNA. The occurrence of this NOE suggests that both edges of the BP benzimidazole moiety are in closs contact with the minor groove. This is only feasible by positioning the BP benzimidazole flat on the bottom of the minor groove or by assuming two rotamers of this benzimidazole moiety. We did not detect such an NOE. This is in agreement with our model, where the same two edges of both benzimidazoles expose their NH and 6 protons to the DNA. Because we report the assignment of all nonlabile protons of the piperazine ring, we have been able to reliably identify intermolecular NOEs involving these protons. In their study, Parkinson et al. (1990) report a single chemical shift value of 2.33 ppm for the piperazine *N*-methyl protons as opposed to the two values reported above (2.56 and 2.96 ppm, respectively).

In another NMR study, Searle and Embrey (1990) presented data on a 2:1 complex of Hoechst 33258 with d(CTTTTGCAAAAG)$_2$. In their final model of the complex, Hoechst 33258 also covers the two AAAA sites, with both piperazine moieties pointing toward the center of the duplex but not covering the central GC base pair. The lifetime of one Hoechst 33258 molecule in its binding site was estimated to be >100 ms. These data qualitatively and quantitatively agree well with ours and the ones of Parkinson et al. (1990).

The 1:1 complex of an analogue of Hoechst 33258 with the decadeoxynucleotide d(CATGGCCATG)$_2$ was also investigated by ^{1}H NMR (Kumar et al., 1990). This analogue contains a benzoxazole ring (instead of the BP benzimidazole ring) which is thought to be a GC base pair reading element and, in addition, contains a nitrogen atom in the benzene ring of the BP benzimidazole moiety. Kumar et al. (1990) indeed report binding of the analogue to the CCAT site of their decadeoxynucleotide with the piperazine ring situated roughly between the two GC base pairs. The partial selectivity of this analogue for GC base pairs has also been demonstrated by footprinting studies (Bathini et al., 1990). In contrast to our studies, Kumar et al. (1990) report the occurrence of a second complex containing the ligand rotated by 180° around the benzimidazole–benzoxazole bond. These two rotamers interchange even in the complex and exhibit a lifetime comparable to the NMR time scale. [Note that two complexes containing corresponding rotamers have also been found by Pjura et al. (1987) in their X-ray study of the 1:1 complex formed between Hoechst 33258 and d(CGCGAATTCGCG)$_2$.] A further comparison to our results is not feasible, because of the different ligands and different DNA fragments employed.

A point which has not been addressed up to now is the state of protonation of Hoechst 33258 and the equilibria between the tautomeric forms of the two benzimidazole moieties, both free in solution and bound to DNA. The knowledge of these values is essential, for example, when modeling complexes of Hoechst 33258 with DNA or when discussing the feasibility of complexes containing different rotamers as proposed by Pjura et al. (1986) and Kumar et al. (1990). Experiments to solve these questions are under way in this laboratory and will be published elsewhere (Leupin et al., in preparation). Our preliminary data clearly show that the p*K* values given by Bontemps et al. (1975), which are cited throughout the literature, are the values for free benzimidazole and phenol but not for Hoechst 33258.

Qualitative Comparison with X-ray Studies. Three different X-ray investigations have been published with Hoechst 33258 cocrystallized with different oligonucleotides (Pjura et al., 1987; Teng et al., 1988; Carrondo et al., 1989). Although Pjura et al. (1987) and Teng et al. (1988) have both studied the 1:1 complex formed between Hoechst 33258 and the Drew dodecamer d(CGCGAATTCGCG)$_2$, they surprisingly found

different binding modes with the ligand covering the ATTC (Pjura et al., 1987) or the central AATT site (Teng et al., 1988). In the structure by Teng et al. (1988), Hoechst 33258 covers 4 AT base pairs, with the piperazine ring interacting mainly with an AT base pair. In the structure of Pjura et al. (1987), the ligand is shifted in the binding site by 1 base pair, with the piperazine ring interacting mainly with a GC base pair.

In our NMR study, we have used an oligonucleotide containing the 9 inner base pairs of the Drew dodecamer, thus comprising both binding sites for Hoechst 33258 proposed by Pjura et al. (1987) and Teng et al. (1988). Our data on the 1:1 solution complex of Hoechst 33258 with d-(GTGGAATTCCAC)$_2$ indicate that the ligand is bound to the central AATT segment of the DNA, supporting the results of the crystal study reported by Teng et al. (1988).

Moreover, Pjura et al. (1987) propose a second mode of binding, in which Hoechst 33258 binds to the same ATTC site, with the BP benzimidazole moiety rotated around the BB,-C7–BP,C2 bond by 180°, thus presenting the opposite edge of the BP benzimidazole to the minor groove of DNA. On the basis of our NMR results, we can exclude such a binding mode as the 8 and the 9 protons of both benzimidazole moieties do not present any intermolecular NOEs to DNA, as expected for such a rotated BP benzimidazole.

To summarize, we want to emphasize that our data contain no evidence for an off-center bound ligand nor (as outlined in details above) for the occurrence of a second rotamer of the type advocated by Pjura et al. (1987).

Conclusion

In this paper, two-dimensional ^{1}H NMR spectroscopy has been used to determine the binding mode and the static and the dynamic properties of the 1:1 complex formed between Hoechst 33258 and the dodecanucleotide duplex d-(GTGGAATTCCAC)$_2$. In conjunction with molecular modeling, these data have allowed the determination of a detailed model of this complex at an atomic level. This provides an opportunity to better understand the sequence-specific interactions between low molecular weight ligands and oligonucleotides.

Acknowledgments

We thank Dr. Paul Gerber for assistance during the molecular modeling studies and Prof. Dr. K. Wüthrich for fruitful discussions.

References

Adams, S. P., Kavka, K. S., Wykes, E. J., Holder, S. B., & Galluppi, J. R. (1983) *J. Am. Chem. Soc. 105*, 661–663.
Bannwarth, W. (1987) *Chimia 41*, 302–317.
Bathini, Y., Rao, K. E., Shea, R. G., & Lown, J. W. (1990) *Chem. Res. Toxicol. 3*, 268–280.
Bax, A., & Davies, D. G. (1985) *J. Magn. Reson. 63*, 207–213.
Bontemps, J., Houssier, C., & Fredericq, E. (1975) *Nucleic Acids Res. 2*, 971–984.
Bothner-By, A. A., Stephens, R. L., Lee, J., Warren, C. D., & Jeanloz, R. W. (1984) *J. Am. Chem. Soc. 106*, 811.
Carrondo, M. A. A. F. d. C. T., Coll, M., Aymami, J., Wang, A. H. J., van der Marel, G. A., van Boom, J. H., & Rich, A. (1989) *Biochemistry 28*, 7849.
Chazin, W. J., Wüthrich, K., Hyberts, S., Rance, M., Denny, W. A., & Leupin, W. (1986) *J. Mol. Biol. 190*, 439–453.
Churchill, M. E. A., & Suzuki, M. (1989) *EMBO J. 8*, 4189–4195.
Dickerson, R. E., & Drew, H. R. (1981) *J. Mol. Biol. 149*, 761–786.
Ernst, R. R., Bodenhausen, G., & Wokaun, A. (1987) *Principles of nuclear magnetic resonance in one and two dimensions*, Clarendon Press, Oxford.
Feigon, J., Leupin, W., Denny, W. A., & Kearns, D. R. (1983) *Biochemistry 22*, 5943–5951.
Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1984) *J. Med. Chem. 27*, 450–465.
Gao, X., & Patel, D. J. (1988) *Biochemistry 27*, 1744–1751.
Gilbert, D. E., van der Marel, G. A., van Boom, J. H., & Feigon, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3006–3010.
Griesinger, C., Otting, G., Wüthrich, K., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870–7872.
Grütter, R., Otting, G., Wüthrich, K., & Leupin, W. (1988) *Eur. Biophys. J. 16*, 279–286.
Günther, H. (1973) *NMR-Spektroskopie*, Georg Thieme Verlag, Stuttgart.
Hare, D., Wemmer, D. E., Chou, S. H., Drobny, G., & Reid, B. R. (1983) *J. Mol. Biol. 171*, 319–336.
Harshman, K. D., & Dervan, P. B. (1985) *Nucleic Acids Res. 13*, 4825–4835.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.
Jorgenson, K. F., Varshney, U., & van de Sande, J. H. (1988) *J. Biomol. Struct. Dyn. 5*, 1005–1023.
Klevitt, R. E., Wemmer, D. E., & Reid, B. R. (1986) *Biochemistry 25*, 3296–3303.
Kumar, A., Ernst, R. R., & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1–6.
Kumar, S., Yadagiri, B., Zimmermann, J., Pon, R. T., & Lown, J. W. (1990) *J. Biomol. Struct. Dyn. 8*, 331–357.
Latt, S. A., & Wohlleb, J. C. (1975) *Chromosoma 52*, 297–316.
Latt, S. A., & Stetten, G. (1976) *J. Histochem. Cytochem. 24*, 24–33.
Leupin, W. (1990) in *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions* (Pullman, B., & Jortner, J., Eds.) pp 579–603, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Leupin, W., Chazin, W., Hyberts, S., Denny, W. A., & Wüthrich, K. (1986) *Biochemistry 25*, 5902–5910.
Loewe, H., & Urbanietz (1974) *Arzneim.-Forsch. 24*, 1927–1933.
Loontiens, F. G., Regenfus, P., Zechel, A., Dumortier, L., & Clegg, R. M. (1990) *Biochemistry 29*, 9029–9039.
Loontiens, F. G., McLaughlin, L. W., Diekmann, St., & Clegg, R. M. (1991) *Biochemistry 30*, 182–189.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–974.
Martin, R. F., & Holmes, N. (1983) *Nature 302*, 452–454.
Morris, A. G., & Freeman, R. (1978) *J. Magn. Reson. 29*, 433–462.
Müller, K. (1984) *Chimia 38*, 249–253.
Murray, V., & Martin, R. F. (1988) *J. Mol. Biol. 201*, 437–442.
Otting, G., Grütter, R., Leupin, W., Minganti, C., Ganesh, K. N., Sproat, B. S., Gait, M. J., & Wüthrich, K. (1987) *Eur. J. Biochem. 166*, 215–220.
Parkinson, J. A., Barber, J., Douglas, K. T., Rosamund, J., & Sharples, D. (1989) *J. Chem. Soc., Chem. Commun.*, 1023–1025.
Parkinson, J. A., Barber, J., Kenneth, T. D., Rosamond, J., & Sharples, D. (1990) *Biochemistry 29*, 10181–10190.
Patel, D. J., & Shapiro, L. (1985) *Biochimie 67*, 887–915.

Patel, D. J., & Shapiro, L. (1986) *J. Biol. Chem. 261*, 1230-1239.
Pelton, J. G., & Wemmer, D. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5723-5727.
Pelton, J. G., & Wemmer, D. E. (1990) *J. Am. Chem. Soc. 112*, 1393-1399.
Pjura, P. E., Grzeskowiak, K., & Dickerson, R. E. (1987) *J. Mol. Biol. 197*, 257-271.
Plateau, P., & Guéron, M. (1982) *J. Am. Chem. Soc. 104*, 7310-7311.
Portugal, J., & Waring, M. J. (1988) *Biochim. Biophys. Acta 949*, 158-168.
Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479-485.
Scheek, R. M., Russo, N., Boelens, R., Kaptein, R., & van Boom, J. H. (1983) *J. Am. Chem. Soc. 105*, 2914-2916.
Scheek, R. M., Boelens, R., Russo, N., van Boom, J. H., & Kaptein, R. (1984) *Biochemistry 23*, 1371-1376.
Searle, M. S., & Embrey, K. J. (1990) *Nucleic Acids Res. 18*, 3753-3762.
Sinha, N. D., Biernat, J., McManus, J., & Köster, H. (1984) *Nucleic Acids Res. 12*, 4539-4557.
Steiner, R. F., & Sternberg, H. (1979) *Arch. Biochem. Biophys. 197*, 580-588.
Stevens, E. S., Sugawara, N., Bonora, G. M., & Toniolo, C. (1980) *J. Am. Chem. Soc. 102*, 7048-7050.
Stokke, T., & Steen, H. B. (1985) *J. Histochem. Cytochem. 33*, 333-338.
Sutherland, I. O. (1971) *The Investigation of the Kinetics of Conformational Changes by Nuclear Magnetic Resonance Spectroscopy*, Academic Press, London and New York.
Teng, M.-K., Usman, N., Frederick, C. A., & Wang, A. H. J. (1988) *Nucleic Acids Res. 16*, 2671.
Van de Ven, F. J. M., & Hilbers, C. W. (1988) *Eur. J. Biochem. 178*, 1-38.
Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland Publishing Co., Amsterdam and Oxford.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.
Zhang, X., & Patel, D. J. (1990) *Biochemistry 29*, 9451-9466.

Structure of the Pure-Spermine Form of Z-DNA (Magnesium Free) at 1-Å Resolution†,‡

Martin Egli,* Loren Dean Williams, Qi Gao,§ and Alexander Rich*
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received August 5, 1991; Revised Manuscript Received September 11, 1991

ABSTRACT: We describe the three-dimensional X-ray structure of a complex of spermine bound to a Z-DNA duplex, $[d(CGCGCG)]_2$, in the absence of any inorganic polyvalent cations. We have crystallized the DNA hexamer d(CGCGCG) in the exclusion of magnesium and other polyvalent ions and solved its structure at 1.0-Å resolution. In the crystal of this pure-spermine form of Z-DNA, the relative orientation, position, and interactions of the DNA differ from the arrangement uniformly observed in over a dozen previously reported Z-DNA hexamers. Moreover, the conformation of the Z-DNA hexamer in this structure varies somewhat from those found in earlier structures. The DNA is compressed along the helical axis, the base pairs are shifted into the major groove, and the minor groove is more narrow. The packing of spermine–DNA complexes in crystals suggests that the molecular basis for the tendency of spermine to stabilize compact DNA structures derives from the capacity of spermine to interact simultaneously with several duplexes. This capacity is maximized by both the polymorphic nature and the length of the spermine cation. The length and flexibility of spermine and the dispersion of charge–charge, hydrogen-bonding, and hydrophobic bonding potential throughout the molecule maximize the ability of spermine to interact simultaneously with different DNA molecules.

Condensation of DNA into compact structures is cation dependent. The focus of this report is spermine, a member of a complex and nearly ubiquitous family of biological cations known as the polyamines [for reviews, see Morris (1981), Pegg and McCann (1982), Tabor and Tabor (1984), and Feuerstein et al. (1991)]. Spermine is the largest polyamine found in eukaryotes and, with a positive charge of 4 at pH 7, also the most highly charged. A series of larger polyamines (pentamines and hexamines) have been isolated from thermophilic bacteria. Spermine stabilizes duplex DNA against thermal denaturation (Mandel, 1962; Tabor, 1962; Bloomfield & Wilson, 1981; Thomas & Bloomfield, 1984) and can condense DNA (Gosule & Schellman, 1978; Chattoraj et al., 1978; Wilson & Bloomfield, 1979; Widom & Baldwin, 1980) and chromatin (Sen & Crothers, 1986) into compact structures.

We describe the three-dimensional X-ray structure of a complex of spermine bound to a Z-DNA duplex. Z-DNA was first detected with CD spectroscopy (Pohl & Jovin, 1972), and the three-dimensional structure of the Z conformation of [d-

† This research was supported by grants from the National Institutes of Health, the American Cancer Society, the National Science Foundation, the Office of Naval Research, and the National Aeronautics and Space Administration. M.E. acknowledges fellowship support by the Geigy-Jubiläums-Stiftung. L.D.W. acknowledges fellowship support by the National Institutes of Health and the Medical Foundation, Boston, MA.
* To whom correspondence should be addressed.
‡ The atomic coordinates have been deposited with the Brookhaven Protein Data Bank (entry number 1D48).
§ Current address: Analytical Research Department, Bristol Myers-Squibb, Wallingford, CT 06492.

0006-2960/91/0430-11388$02.50/0