Press, W. H., Flannery, B. P., Teukolsky, & Vetterling, W. T. (1986) *Numerical Recipes*, Cambridge University Press, Cambridge.
Ratkowsky, D. (1983) *Nonlinear Regression Modeling*, Marcel Dekker, New York.
Reizer, J., Saier, M. H., Jr., Deutscher, J., Grenier, F., Thompson, J., & Hengstenberg, W. (1988) *CRC Crit. Rev. Microbiol. 15*, 297-338.
Reizer, J., Sutrina, S. L., Wu, L.-F., Deutscher, J., & Saier, M. H., Jr. (1991) *J. Biol. Chem.* (in press).
Ringe, D., & Petsko, G. (1985) *Prog. Biophys. Mol. Biol. 45*, 197-235.
Roder, H. (1989) *Methods Enzymol. 176*, 446-473.
Saier, M. H., Jr. (1989) *Microbiol. Rev. 53*, 109-120.
Saier, M. H., Jr., Novotny, M. J., Comeau-Fuhrman, D., Osumi, T., & Desai, J. D. (1983) *J. Bacteriol. 155*, 1351-1357.
Shaka, A. J., Barker, P. B., & Freeman, R. (1985) *J. Magn. Reson. 64*, 547-552.
Shoji, A., Ozaki, T., Fujito, T., Deguchi, K., Ando, S., & Ando, I. (1989) *Macromolecules 22*, 2860-2863.
Shoji, A., Ozaki, T., Fujito, T., Deguchi, K., Ando, S., & Ando, I. (1990) *J. Am. Chem. Soc. 112*, 4693-4697.
Smith, G. P., Yu, L. P., & Domingues, D. J. (1987) *Biochemistry 26*, 2202-2207.
Sutrina, S. L., Reddy, P., Saier, M. H., Jr., & Reizer, J. (1990) *J. Biol. Chem. 265*, 18581-18589.
Vold, R. R., & Vold, R. L. (1976) *J. Chem. Phys. 64*, 320-332.
Wagner, G. (1983) *Q. Rev. Biophys. 16*, 1-57.
Welch, G. R., Somogyi, B., & Damjanovich, S. (1982) *Prog. Biophys. Mol. Biol. 39*, 109-146.
Williams, R. J. P. (1979) *Biol. Rev. 54*, 389-437.
Williams, R. J. P. (1989) *Eur. J. Biochem. 183*, 479-497.
Wootton, J. C., & Drummond, M. H. (1989) *Protein Eng. 2*, 535-543.
Wright, L. L., Palmer, A. G., III, & Thompson, N. L. (1988) *Biophys. J. 54*, 463-470.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, p 24, Wiley, New York.
Zang, L., Laughlin, M. R., Rothman, D. L., & Schulman, R. G. (1990) *Biochemistry 29*, 6815-6820.

Two-Dimensional NMR Studies of d(GGTTAATGCGGT)·d(ACCGCATTAACC) Complexed with the Minor Groove Binding Drug SN-6999†

Shiow-meei Chen, Werner Leupin,‡ Mark Rance, and Walter J. Chazin*
Department of Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037
Received October 11, 1991; Revised Manuscript Received March 6, 1992

ABSTRACT: The dodecadeoxynucleotide duplex d(GGTTAATGCGGT)·d(ACCGCATTAACC) and its 1:1 complex with the minor groove binding drug SN-6999 have been prepared and studied by two-dimensional ^{1}H nuclear magnetic resonance spectroscopy. Complete sequence-specific assignments have been obtained for the free duplex by standard methods. The line widths of the resonances in the complex are greater than those observed for the free duplex, which complicates the assignment process. Extensive use of two-quantum spectroscopy was required to determine the scalar correlations for identifying all of the base proton and most of the 1′H–2′H–2″H spin subsystems for the complex. This permitted unambiguous sequence-specific resonance assignments for the complex, which provides the necessary background for a detailed comparison of the structure of the duplex, with and without bound drug. A series of intermolecular NOEs between drug and DNA were identified, providing sufficient structural constraints to position the drug in the minor groove of the duplex. However, the combination of NOEs observed can only be rationalized by a model wherein the drug binds in the minor groove of the DNA in both orientations relative to the long helix axis and exchanges rapidly between the two orientations. The drug binds primarily in the segment of five consecutive dA–dT base pairs d($T_3T_4A_5A_6T_7$)·d($A_{18}T_{19}T_{20}A_{21}A_{22}$), but surprisingly strong interactions are found to extend one residue in the 3′ direction along each strand to G_8 and C_{23}. The observation of intermolecular contacts to residues neighboring the AT-rich region demonstrates that the stabilization of the bis(quaternary ammonium) heterocycle family of AT-specific, minor groove binding drugs is not based exclusively on interactions with dA–dT base pairs.

The therapeutic activity of one of the primary classes of antitumor drugs is known to occur by interference with DNA metabolism via reversible binding in one of the grooves of double-stranded DNA helices (Zimmer & Wähnert, 1986; Lambert & LePecq, 1987). One family of this class of antitumor agents is characterized by binding in the minor groove of duplex DNA and includes the well-known oligopeptide antibiotics distamycin and netropsin as well as the bis(quaternary ammonium) heterocycles (bQAHs).[1] This family of

† This work was supported in part by grants from the National Science Foundation (DMB 8903777 to M.R.) and from the American Cancer Society (JFRA-294 and CH-529 to W.J.C.).
‡ Present address: Pharma Division, Preclinical Research, F. Hoffmann La Roche, Ltd., Basel, Switzerland.

[1] Abbreviations: bQAH, bis(quarternary ammonium) heterocycle; CD, circular dichroism; UV, ultraviolet; NMR, nuclear magnetic resonance; 1D, one dimensional; 2D, two dimensional; COSY, correlated spectroscopy; 2Q, two-quantum spectroscopy; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; EDTA, ethylenediaminetetraacetic acid; d_i(A;B), intranucleotide distance between protons A and B; d_s(A;B), sequential distance between protons A and B, where A is in the 5′ direction relative to B.

0006-2960/92/0431-4406$03.00/0

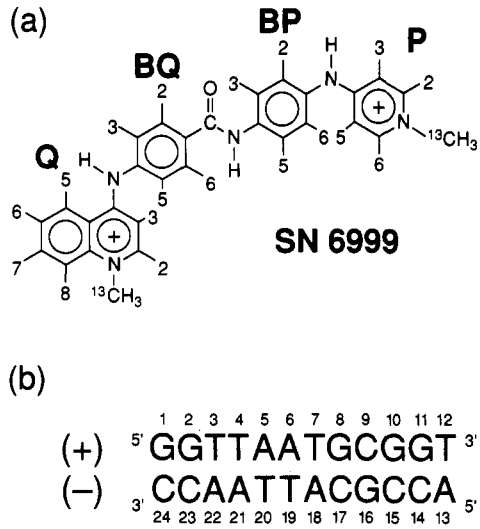

FIGURE 1: Molecular structures of (a) 4-[*p*-[*p*-(4-quinolylamino)-benzamido]anilino]pyridine SN-6999 and (b) the dodecadeoxyribonucleoside undecaphosphate duplex d(GGTTAATGCGGT)·d(ACCGCATTAACC). Each proton of the drug is identified by a letter specifying the aromatic ring (Q, quinolinium; BQ, benzamido; BP, anilino; P, pyridinium) and a number specifying its position in the ring, e.g., P-2H.

compounds invariably has an elongated multiunit structure, specific patterns of hydrogen-bond donors and acceptors, and a preference for four or more consecutive dA–dT base pairs. Despite a strong similarity in the nature of binding to DNA, the bis(quaternary ammonium) heterocycles have structural features that clearly distinguish them from the well-characterized oligopeptide antibiotics and that affect the details of their interaction with DNA (e.g., hydrogen-bonding donors/acceptors and formal cationic charge).

Studies of complexes formed between DNA duplexes and other minor groove binding drugs by X-ray crystallography [netropsin (Coll et al., 1987, 1989), distamycin (Kopka et al., 1985), and Hoechst 33258 (Pjura et al., 1987; Teng et al., 1988; de C. T. Carrondo et al., 1989; Quintana et al., 1991a)] and 2D NMR spectroscopy [netropsin (Patel & Shapiro, 1986a,b), distamycin (Klevit et al., 1986; Pelton & Wemmer, 1988, 1989, 1990a,b), and Hoechst 33258 (Parkinson et al., 1989, 1990; Kumar et al., 1990; Searle & Embrey, 1990; Fede et al., 1991)] have identified a number of factors that are likely to determine the relative affinity, the AT specificity, and the molecular details of the drug–DNA interaction. Although these studies provide structural information for modeling complexes formed with the bis(quaternary ammonium) heterocycles, to fully understand the molecular mechanism for their activity and to design analogues with increased efficacy and specificity, it will be necessary to obtain structural information directly for bQAH complexes. Since efforts at crystallization of DNA complexes formed with bis(quaternary ammonium) heterocycles have been largely unsuccessful (Coll et al., 1987; Quintana et al., 1991b), solution-state NMR spectroscopy represents the method of choice for structural analysis.

We have previously reported on a series of NMR studies of the 1:1 complex of the bQAH SN-6999 (Figure 1a) with the decadeoxynucleotide duplex $d(GCATTAATGC)_2$ (Leupin et al., 1986, 1990). In that study, it was observed that the drug binds in the minor groove of the duplex, specifically in the AT-rich central region. The kinetics of binding was found to be intermediate to fast on the NMR time scale, with rapid exchange between the symmetrically equivalent binding sites on the palindromic duplex. Although a number of NOEs are observed between protons of the drug and the DNA, the detailed interpretation of these data is complicated by the 2-fold symmetry of the duplex, the fast exchange between equivalent sites, and the potential for sliding of the ligand along the minor groove. A general binding model, consistent with all of the experimental results, was proposed. In the study reported here, the DNA duplex has been redesigned in an effort to obtain more specific structural and dynamical details about the 1:1 complex with SN-6999. The nonpalindromic DNA duplex d(GGTTAATGCGGT)·d(ACCGCATTAACC) has been selected (Figure 1b) to simplify the interpretation of the NMR data of the drug–DNA complex. The drug-binding site has been optimized by reducing the AT-rich region to five base pairs to match the excluded-site size determined by optical spectroscopy (Braithwaite & Baguely, 1980) and thereby limit the possibility for sliding of the drug in the binding site. To further aid in the analysis, the two methyl groups of SN-6999 were labeled with ^{13}C, and heteronuclear-edited NMR spectra were acquired as demonstrated previously for the 1:1 complex between SN-6999 and $d(GCATTAATGC)_2$ (Leupin et al., 1990).

Materials and Methods

The methods used for the synthesis of SN-6999 and the oligonucleotides have been described elsewhere (Chazin et al., 1986; Leupin et al., 1986, 1990). The bis-quaternization of the free base was achieved by reaction with $^{13}CH_3I$ (99 atom % ^{13}C, Aldrich Chemical Co.) in *N*,*N*-dimethylacetamide at room temperature for 16 h. The two complementary oligonucleotides d(GGTTAATGCGGT) and d(ACCGCATTAACC) were mixed in equimolar amounts then lyophilized and redissolved in 420 μL of stock buffer (50 mM sodium phosphate buffer at pH 7.0, 100 mM NaCl, 0.1% NaN_3, 5% ^2H_2O). For the experiments to examine only nonlabile protons, the solution was repeatedly lyophilized from 99.6% ^2H_2O, followed by dissolution in 99.996% ^2H_2O (MSD Isotopes, Montréal, Canada). The 1:1 drug–DNA complex was prepared by adding the DNA solution into vials containing known amounts of solid SN-6999. The titration was monitored by following the imino proton resonances in the ^1H NMR spectrum. The final solution was lyophilized repeatedly from ^2H_2O, and then made up in 99.996% ^2H_2O. For the experiments to examine the labile protons of the complex, the solution was lyophilized and dissolved in 95% ^1H_2O/5% ^2H_2O.

NMR experiments were performed using Bruker AM-500 and AM-600 spectrometers. The temperature was 301 K for all experiments on the duplex and 310 K for the complex, except where indicated otherwise. All spectra were acquired and plotted in the phase-sensitive mode. The transmitter was placed on the solvent resonance, and the TPPI method was used to achieve frequency discrimination in the ω_1 dimension (Marion & Wüthrich, 1983). The standard pulse sequence and phase cycling was used for 2Q spectra (Braunschweiler et al., 1983), 2QF-COSY (Rance et al., 1983), and 3QF-COSY (Rance & Wright, 1986). A total of 32 or 96 scans/t_1 value were acquired with a 30-ms preparation period and t_{1max} = 30 ms for 2Q, and t_{1max} = 128 ms for 2QF- and 3QF-COSY. An E-COSY spectrum was generated for the free duplex by the appropriate linear combination of the 2QF-COSY and 3QF-COSY spectra, and coupling constants were extracted as detailed in Griesinger et al. (1987). TOCSY (Braunschweiler & Ernst, 1983; Bax & Davis, 1985) spectra were acquired using the modifications described by Rance (1987) with Waltz-16 (Shaka et al., 1983) mixing. Typical acquisition parameters were ~80-ms mixing period, 32 scans/t_1 value, and t_{1max} ~50 ms. NOESY (Macura & Ernst, 1980) spectra

from ^1H_2O solutions were recorded with the last pulse replaced by a jump-and-return composite pulse (Plateau & Guéron, 1982) with the "return" pulse phase and flip angle being adjusted empirically for optimal water suppression. NOESY spectra from ^2H_2O solution were recorded with saturation of the residual HO^2H during the preparation period and the mixing time, and a short Hahn-echo period was employed to improve the quality of the baseline [e.g., Davis (1989)]. For these experiments, 32–160 scans/t_1 value were acquired with mixing times of 50–250 ms and t_{1max} = 30–60 ms. ^{13}C-directed (Rance et al., 1987) and ^{13}C-ω_1-half-filtered (Otting et al., 1986) NOESY spectra for the complex were acquired at 297 K with 64 scans/t_1 value, a 200-ms mixing time, and t_{1max} = 18 ms.

The NMR data were processed using a CONVEX C240 computer with FTNMR software (Hare Research, Inc., Woodinville, WA).

Results and Discussion

The strategies for sequence-specific assignment of the ^1H resonances of small DNA duplexes have been amply reviewed (Wemmer & Reid, 1985; Wüthrich, 1986; Patel et al., 1987; Reid, 1987; van de Ven & Hilbers, 1988) and the specific protocol used here has been described (Chazin et al., 1986, 1991). The basic strategy used for the drug–DNA complex requires certain minor modifications. The short-hand notation described by Wüthrich (1986) is used to specify interproton distances and corresponding NOEs.

^1H NMR Assignment and Characterization of the Free Duplex. Only a brief summary of the NMR studies of the free duplex is given here. Full details are provided in the Supplementary Material, including tables of chemical shifts and scalar coupling constants. The assignments of the labile and adenine 2H resonances were obtained in the usual manner from the NOESY spectrum acquired in ^1H_2O solution. The assignment of adenine 2H resonances was confirmed by the observation of $d_s(2;2)$ and $d_s(2;1')$ connectivities in a 200-ms NOESY spectrum.

All cytosine 5H–6H and thymine $5CH_3$–6H resonances were identified by scalar connectivities in both 2QF-COSY and 2Q spectra. The 1′H–2′H–2″H spin subsystems for 23 of the 24 deoxyribose sugar rings were readily identified in the 2QF-COSY, 3QF-COSY, and 2Q spectra. The 24th 1′H–2′H–2″H spin subsystem could only be identified after careful inspection revealed that the resonances of two spin subsystems are nearly degenerate (cf. C_{15} and C_{17}, Table S1). All 3′H resonances could be identified in the 2Q spectrum from remote peaks at ω_1 = 2′H + 2″H, ω_2 = 3′H (Figure S2). Extension of the deoxyribose spin system identification to the 4′H, 5′H, and 5″H resonances was made from analysis of the 2Q spectrum and confirmed in NOESY spectra, as described previously (Chazin et al., 1986, 1991). The stereospecific assignments of the 5′H resonances were made from $d_s(3';5')$ NOEs.

The sequence-specific assignments were obtained from a NOESY spectrum acquired with a short (50-ms) mixing time, minimizing spin diffusion so that only direct NOEs have significant intensity (Chazin et al., 1986). The combination of $d_i(6,8;2')$, $d_s(2'';6,8)$, $d_i(5CH_3;6)$, and $d_s(6,8;5CH_3)$ connectivities were sufficient for complete sequential resonance assignment (Figure 3S).

Measurements of the vicinal scalar coupling constants $J_{1'2'}$ and $J_{1'2''}$ (7.7–9.9 and 5.4–7.0 Hz, respectively, Table S2) show that the sugar ring conformations in the dodecamer duplex are typical for B-type DNA (Widmer & Wüthrich, 1986; Celda et al., 1989). In the 50-ms NOESY spectrum, the relative intensities of cross peaks [e.g., $d_i(6,8;2') > d_s(2'';6,8)$ and $d_i(5CH_3;6) > d_s(6,8;5CH_3)$] are characteristic of direct NOEs and, together with the coupling constant data, firmly establish that d(GGTTAATGCGGT)·d(ACCGCATTAACC) is a B-form DNA duplex with predominantly C2′-endo sugar ring conformations.

General Characterization of the Complex and Resonance Assignments. Upon titration of the DNA solution with one molar equivalent of drug, the two well-separated drug resonances near 8.6 ppm in the ^1H NMR spectrum show the changes in chemical shift and increased line width that are characteristic of the binding of the drug to the DNA duplex. Another readily discernible indication of drug binding is the substantial changes in the chemical shift of the imino proton resonances (cf. Table S1 and Table I). Given that there is only one AT-rich binding site, the absence of multiple sets of NMR signals upon binding of the asymmetric drug implies that either the drug binds in only one orientation on the DNA or that a single time-averaged NMR spectrum is observed due to fast exchange on the NMR time scale between two (or more) orientations of the drug.

One method to distinguish between these two possibilities is to look for evidence of selective broadening of resonances in the NMR spectrum as a function of temperature; thus, the 1D ^1H NMR spectrum was followed from 274 to 313 K. Some of the thymine methyl resonances in the high-field end of the spectrum are found to be broader than other resolved signals in the 1D spectrum. The line widths of these methyl signals vary with temperature, but even at the lowest temperature accessed (274 K) there was no clear sign of a separation of each of the broadened methyl resonances into two discrete signals as had been observed previously for the SN-6999–$d(GCATTAATGC)_2$ complex (Leupin et al., 1986). However, the analogy in line-broadening effects between the two complexes suggests that the lack of multiple sets of signals is due to the same phenomenon, intermediate to fast exchange (on the NMR time scale) of the drug between at least two DNA binding sites.

The acquisition of 2D NMR spectra over a range of temperatures proved important in the analysis of the complex because several key resonances could only be assigned at 310 K where line widths are narrow, whereas some of the intermolecular NOEs could only be identified at 276 K where the exchange rate of the drug is decreased. The complex was fully characterized at 276, 301, and 310 K; complete resonance assignments were made first at 310 K where nearly all expected scalar correlations are observed, and then assignments were transferred to the lower temperatures by comparison.

Obtaining resonance assignments for the complex proved much more difficult than for the free DNA because some of the line widths of the ^1H resonances of the complex are significantly larger. COSY-type experiments for identifying scalar correlations did not work well in this study, whereas 2Q and TOCSY experiments were more successful because the latter are less sensitive to the cancellation problems associated with larger line widths. In this study, 2Q spectroscopy was found to be the method of choice for identifying scalar correlations because the cross peaks in TOCSY spectra tend to merge in spectral regions that are very crowded, whereas the 2Q peaks are easier to resolve due to their antiphase multiplet structure in the ω_2 dimension. The 2Q spectrum for the complex provides scalar connectivities for all 5H, $5CH_3$, and 6H resonances and for 19 of the 24 1′H–2′H–2″H deoxyribose sugar ring spin subsystems of the drug–DNA complex. The spectral region of the 2Q spectrum containing the

Table I: Proton Chemical Shifts in the d(GGTTAATGCGGT)·d(ACCGCATTAACC)–SN6999 Complex at pH 7.0, 301 K

	DNA Chemical Shifts (ppm)[a]									
residue	N1H, N3H	NH_2	2H, 5H, $5CH_3$	6H, 8H	1′H	2′H	2″H	3′H	4′H	5′H/5″H
G_1	*b*		–	7.84	5.69	2.54	2.70	4.81	4.17	3.69, 3.69
G_2	12.82		–	7.84	6.07	2.66	2.83	4.98	4.43	4.11, 4.15
T_3	14.10		1.42	7.25	6.08	2.09	2.60	4.86	4.26	4.06, 4.16
T_4	13.73		1.66	7.37	5.59	2.16	2.46	4.84	4.08	
A_5	–		6.90	8.17	5.56	2.53	2.77	4.92	4.06	
A_6	–		7.84	7.94	5.70	2.25	2.62	4.83	4.02	4.06, 4.09
T_7	13.29		1.15	6.80	5.58	1.74	2.12	4.68	4.08	
G_8	12.52		–	7.69	5.68	2.43	2.54	4.82	4.08	4.16
C_9	–	6.22, 8.22	5.24	7.20	5.60	1.80	2.24	4.78	4.20	4.06
G_{10}	13.08		–	7.79	5.55	2.62	2.69	4.98	4.30	3.94, 4.08
G_{11}	*b*		–	7.76	6.05	2.58	2.70	4.95	4.38	4.16
T_{12}	*b*		1.57	7.38	6.21	2.24	2.24	4.53	4.08	
A_{13}	–		8.06	8.22	6.22	2.67	2.78	4.83	4.26	3.75, 3.75
C_{14}	–	6.57, 8.03	5.43	7.45	5.84	2.12	2.38	4.79	4.23	
C_{15}	–	6.69, 8.47	5.56	7.40	5.61	2.01	2.37	4.81	4.23	
G_{16}	12.92		–	7.85	5.85	2.60	2.70	4.99	4.35	4.02, 4.10
C_{17}	–	6.22, 8.16	5.41	7.32	5.52	2.02	2.38	4.82	4.22	4.18
A_{18}	–		7.72	8.30	6.26	2.69	2.90	4.99	4.37	4.08, 4.13
T_{19}	13.79		1.39	7.06	5.70	1.86	2.40	4.74	4.05	
T_{20}	13.55		1.57	7.16	5.31	1.91	2.28	4.69		4.02
A_{21}	–		6.89	8.09	5.60	2.46	2.69	4.85	4.08	
A_{22}	–		7.79	7.94	5.57[c]	2.39	2.56	4.80		
C_{23}	–		5.08	7.14	5.67	1.87	2.19	4.70	4.02	
C_{24}	–		5.61	7.53	6.21	2.23	2.23	4.52	3.99	

Drug Chemical Shifts (ppm)[d]			
Q-2H *8.56*	Q-7H *8.24*	BQ-2,6H *8.26*	P-2,6H *8.17*	Q/BQ NH 10.38
Q-3H *8.11*	Q-8H *8.33*	BQ-3,5H *8.16*	P-3,5H 7.12[e]	BQ/BP NH 9.62
Q-5H *8.71*	Q-CH_3 4.32	BP-2,6H *7.68*	P-CH_3 4.03	P/BP NH 10.08
Q-6H *8.05*		BP-3,5H *7.84*		

[a] Measured from a 1 mM solution containing 50 mM phosphate buffer, 100 mM NaCl, and 0.1% NaN_3. The chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt, using the HO^2H resonance previously calibrated in stock buffer solution. [b] Not observed due to rapid exchange with the solvent at this temperature. [c] Tentative assignment based solely on a $d_\alpha(1';6)$ NOE between A22 and C23. [d] Italicized drug resonances exhibit a chemical shift difference of >|0.1| ppm relative to the free drug. The exchangeable protons of the free drug are not observed at neutral pH due to rapid exchange with solvent. [e] Resonance identified only at 276 K.

key remote peaks at ω_1 = 2′H + 2″H, ω_2 = 1′H is shown in Figure S4. Most of the resonances for the other sugar protons (3′H, 4′H, 5′H, 5″H) reported in Table I were also identified in the 2Q spectrum. The remainder of the resonances were identified in the course of complete analysis of the NOESY spectrum. The sequential assignment of these resonances (Table I) was made from NOESY spectra in the usual manner. It is important to note that some of the characteristic intraresidue and sequential NOEs were observed only barely above the thermal noise due to exchange broadening of resonances from residues that are drastically affected by the binding of the drug.

The assignment of the ^1H resonances of SN-6999 was also made from connectivities observed in the 2Q and NOESY spectra. The scalar couplings between drug proton resonances, except for the pyridinium ring, were identified in the 2Q spectrum. A firm assignment for the P-3,5H protons could only be obtained from the NOESY spectrum at 276 K due to exchange broadening. The assignment of the drug resonances to a specific position on one of the aromatic rings was based on the pattern of scalar connectivities and the proximity relationships derived from NOEs. The labile amino (Q/BQ-NH, P/BP-NH) and the amide proton (BQ/BP) resonances were assigned from the intramolecular NOEs to adjacent aromatic ring protons. The complete assignments for the proton resonances of the bound drug are given in Table I. A comparison of the ^1H chemical shifts of SN-6999 in the 1:1 complexes of $d(GCATTAATGC)_2$ [Table I in Leupin et al. (1986)] and d(GGTTAATGCGGT)·d(ACCGCATTAACC) reveals a close similarity for all resonances except P-3,5H, which was incorrectly assigned in the decamer complex due to the severe spectral overlap. The ambiguity in this assignment has been overcome in this study by the use of 2Q spectroscopy and analysis of NOESY spectra at three different temperatures. We note that the revised assignment has no effect on the conclusions drawn in the earlier study.

Intermolecular Contacts in the Complex. With the availability of sequence-specific resonance assignments for both components of the complex, it becomes possible to identify the regions of intermolecular contact between d(GGTTAATGCGGT)·d(ACCGCATTAACC) and SN-6999 by examining changes in the ^1H chemical shifts and identifying NOEs between drug and DNA protons. The changes in the chemical shifts of DNA protons induced by drug binding are plotted versus the location in the sequence for the adenine 2H, imino, base (6H or 8H), and deoxyribose (1′H, 2′H, 2″H, 3′H) resonances in Figure 2. These data clearly demonstrate that chemical shifts of protons of the nucleotides T_3 to G_8 in the (+) strand and A_{18} to C_{23} in the (−) strand are strongly influenced by the binding of the drug, with much weaker (induced) effects occasionally extending one base pair beyond these residues. The 2H and imino proton resonances from residues in the binding site are shifted to lower field upon complexation, whereas other DNA proton resonances shift upfield, indicating drug binding in the minor groove of DNA (Feigon et al., 1984; Leupin et al., 1985, 1986). Taken together, the profiles of perturbations of chemical shifts indicate that the effects of drug binding are predominantly felt in the AT-rich segment of the duplex as expected but surprisingly that the perturbations are extended one base (but not base pair) in the 3′ direction to G_8 on the (+) strand and to C_{23} on the (−) strand.

A direct indication of the interaction between drug and DNA is available from intermolecular proton–proton contacts

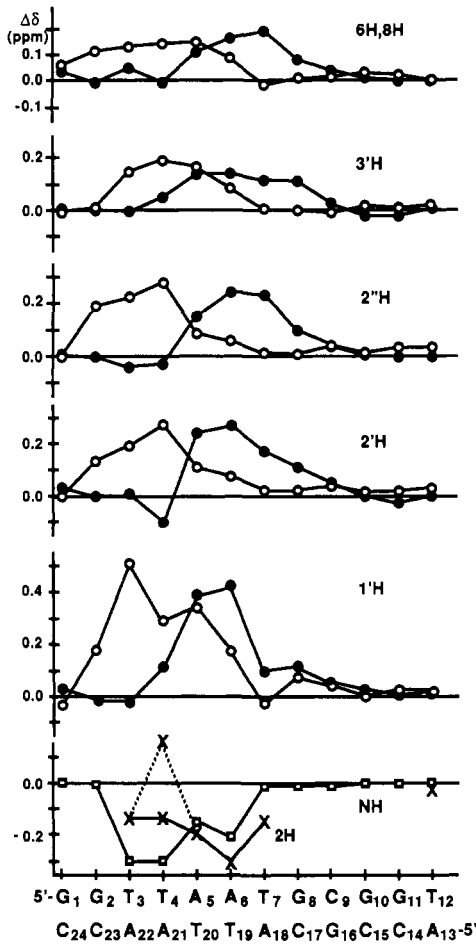

FIGURE 2: Drug binding induced changes in chemical shifts of selected protons in the DNA duplex. Positive values indicate that the resonances in the complex are at higher field than in the free duplex. The (+) and (−) strands of the DNA are identified by the filled circles and open boxes, respectively. In the bottom panel, the imino proton data are marked with open boxes and the adenine 2H data with crosses. The two resonance frequencies corresponding to the two orientations of the drug have been identified for A_{21} 2H (see text).

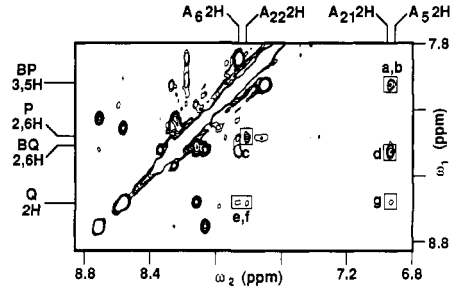

FIGURE 3: Region of the 500-MHz NOESY spectrum (τ_m = 150 ms) of the 1:1 complex d(GGTTAATGCGGT)·d(ACCGCATTAACC)–SN-6999 containing some of the intermolecular NOEs between the DNA duplex and the drug. The experiment was acquired at 310 K from a ^2H_2O solution containing 1 mM complex, 50 mM sodium phosphate buffer at pH 7.0, 100 mM NaCl, and 0.1% NaN_3. A selected number of the interresidue NOEs are identified in this plot: (a) BP-3,5H/$A_5$2H; (b) BP-3,5H/A_{21}2H; (c) P-2,6H/A_{22}2H; (d) BQ-2,6H/$A_5$2H; (e) Q2H/$A_6$2H; (f) Q2H/A_{22}2H; (g) Q2H/$A_5$2H.

that are identified from NOEs between SN-6999 and DNA resonances. A selected number of these are shown in Figure 3, a region from the NOESY spectrum of the complex acquired at 301 K with a 250-ms mixing time. Some of the unambiguously assigned cross peaks are identified in the caption to the figure; a complete list of all unambiguously assigned intermolecular NOEs is given in Table II. There

Table II: Unambiguously Assigned Intermolecular NOEs between d(GGTTAATGCGGT)·d(ACCGCATTAACC) and SN-6999[a]

drug	DNA
Q	
CH_3	A_{22}2H (A)
2H	$A_5$2H; A_{18}2H (B); A_{22}2H (A); C_{23}1′H (A)
3H	A_{22}2″H (A); A_{22}2H (A)
BQ	
3,5H	$A_5$2H; A_{21}1′H (A)
2,6H	$T_4$1′H (A); $A_5$1′H; $A_5$2H; T_{20}N3H; T_{20}1′H; A_{21}2H (A)
amide-NH	$A_5$2H; T_{20}1′H
BP	
3,5H	$T_4$1′H (B); $A_5$2H; A_{21}2H (B)
2,6H	$A_5$2′H; T_{19}1′H (A)
P	
2,6H	$G_8$1′H (A); A_{18}1′H (A); A_{18}2H (A); A_{22}2H (B)

[a] The intermolecular interproton contacts listed are the cumulative results from the analysis of the three NOESY spectra acquired for the complex. For some NOEs, it is possible to assign the intermolecular contact to a specific orientation of the drug on the DNA, as indicated by an A or B in parentheses. The two orientations are identified in Figure 4.

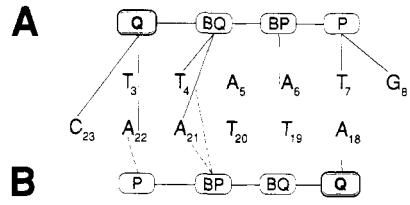

FIGURE 4: Schematic diagram of the interaction between SN-6999 and d(GGTTAATGCGGT)·d(ACCGCATTAACC) based on the combined information from exchange-based line broadening effects, changes in the chemical shifts induced by binding of the drug, and the intermolecular proton–proton contacts. The two orientations of the drug are shown relative to a single DNA duplex and are arbitrarily assigned as A and B. Intermolecular NOEs that are specifically assigned to only one of the two orientations are indicated by lines between the drug moiety and the residue on the DNA duplex.

are a number of additional intermolecular NOEs that are ambiguous, in particular to 1′H and 4′H of the DNA, because the protons resonate in severely crowded regions of the ^1H NMR spectrum. The intermolecular NOE connectivities involve protons situated in the minor groove of the DNA, thereby directly establishing that the drug binds in the minor groove of the DNA.

The NOE data are fully consistent with the analysis of drug-induced chemical shift changes given above, indicating that the drug is bound primarily in the AT-rich region of the duplex and that interactions extend one base in the 3′ direction along each strand to G_8 and C_{23}. The observation of intermolecular contacts to residues adjacent to the AT-rich region demonstrates that the stabilization of drug binding for the bis(quaternary ammonium) heterocycle family of AT-specific, minor groove binding drugs is not exclusively based on interactions with AT base pairs. Similar observations have been made for Hoechst 33258 (Harshman & Dervan, 1985; de C.T. Carrondo et al., 1989; Fede et al., 1991; Quintana et al., 1991a). Molecular mechanics/dynamics simulations are in progress to establish the molecular determinants leading to the favorable interactions of the drug with the guanosine and cytosine residues adjacent to the AT-rich region.

An inspection of the NOEs in Table II indicates that the asymmetric SN-6999 binds in the minor groove of d-($T_3T_4A_5A_6T_7$[G_8])·d($A_{18}T_{19}T_{20}A_{21}A_{22}$[$C_{23}$]) in two orientations relative to the helix axis and with no preference for one orientation over the other. For example, the 3H resonance of the quinolinium ring of the drug shows NOEs to both A_{18} and C_{23} at opposite ends of the binding pocket. The inter-

molecular contacts identified by NOEs can be used to align the drug along the duplex in the two orientations (arbitrarily labeled A and B) as shown in schematic form in Figure 4. This diagram helps to visualize how the various NOEs in Table II can be broken down into proton–proton contacts arising from only one of the orientations of the drug. These results, in combination with the observations of only one set of NMR resonances and of specific resonances that are exchange broadened, leads to the conclusion that, on the NMR time scale, the asymmetric drug exchanges rapidly between its two possible orientations in the binding pocket of the DNA duplex.

The three labile proton resonances of the Q/BQ amino, P/BP amino, and the amide groups of SN-6999 are detectable in aqueous solution when bound to the DNA but not when the drug is free in solution, indicating that the rate of exchange with the solvent is greatly decreased in the complex. This observation is consistent with the formation of specific hydrogen bonds between these NH donors and hydrogen-bond acceptors of the DNA duplex, e.g., the N3 of adenine or the 2-keto group of thymine in the minor groove of B-type DNA (Kopka et al., 1985; Fede et al., 1991; Quintana et al., 1991a). Although the direct determination of specific hydrogen bonds by ^{1}H NMR is not possible, the observation of slowed exchange rates and certain characteristic NOEs serves to indirectly identify hydrogen-bonding interactions. For example, the NOEs from the amide proton of SN-6999 to the 2H of A_5 and to the 1′H of T_{20} are highly indicative of a hydrogen bond between the amide proton and the 2-keto group of T_{20}. Specific hydrogen-bonding interactions could not be identified for the two amino groups of SN-6999, due to the inability to identify proton–proton contacts involving these protons because their NOEs are quenched by the high rate of exchange with solvent.

The ^{13}C isotope labeling of the methyl groups in SN-6999 was carried out in order to simplify the ^{1}H NOESY spectra via heteronuclear editing, as demonstrated for the 1:1 complex of SN-6999 and d(GCATTAATGC)$_2$ (Leupin et al., 1990). The expected intramolecular NOEs were observed from the methyl protons to the adjacent ring protons, but, surprisingly, only one intermolecular NOE could be identified, between the Q-methyl and A_{22}2H. A few additional intermolecular NOEs were observed to 4′H resonances, but these could not be assigned due to the extreme crowding in this region of the spectrum. The observation of only one NOE to 2H and 1′H protons of the DNA from the Q- and P-methyl groups contrasts sharply with the eight NOEs that were observed in the SN-6999–d(GCATTAATGC)$_2$ complex (Leupin et al., 1990).

Although we have no unequivocal evidence at this time, there are two plausible explanations, either quenching of the NOEs due to an increase in the drug exchange kinetics or differences in the local structure of the binding site. The replacement of one of the six consecutive AT base pairs in the decanucleotide by a GC base pair in the dodecanucleotide changes the local chemical structure of the minor groove because there is an extra amino group protruding into the minor groove at the C2 position of the guanine base. This structural change could effectively destabilize interactions of the drug's methyl groups at the outer edge of the binding site or cause changes in the molecular details of the interaction between SN-6999 and the DNA. Such a change is strongly implicated by the observation of intermolecular NOEs to protons of residues G_8 and C_{23} in the present study; no NOEs are observed to protons of the guanosine or cytosine residues in the SN-6999 complex with d(GCATTAATGC)$_2$.

Dynamic Properties of the Complex. As alluded to above, the line widths of certain resonances in the ^{1}H NMR spectrum of the complex exhibit a strong temperature dependence. It is not possible to freeze out two sets of signals for the well-resolved methyl resonances in 1D spectra, as observed for the previously studied complex (Leupin et al., 1986). However, a strong temperature dependence of the line widths of the C2 proton of A_{21} is observed, and two sets of signals are found in the NOESY spectrum at 276 K (Figure S5). The 1D and 2D NOESY spectra acquired at various temperatures have been carefully examined to determine coalescence temperatures. The estimated coalescence temperature for A_{21} is 315 K. The limiting chemical shift difference of 139 Hz (determined at 276 K) can be used to calculate the exchange rate at coalescence (Sutherland, 1971). The rate of exchange between the two binding sites is found to be $>3 \times 10^2$ s^{-1} at 315 K. This corresponds to a lifetime of the drug–DNA complex of <3 ms at 315 K and an estimated activation energy $\Delta G^{\ddagger}$ of 62 ± 6 kJ mol^{-1} at the coalescence temperature for the observed exchange process (Günther, 1980). This value is very similar to the 60 ± 5 kJ mol^{-1} found previously for the activation energy of the observed exchange process in d-(GCATTAATCG)$_2$–SN-6999 complex and suggests that these exchange processes are due to the same physical phenomenon, e.g., flipping of the SN-6999 molecule relative to the DNA duplex (Leupin et al., 1986). Such a flip-flop mechanism has been observed for the exchange of other minor groove binding drugs between two symmetrically related or similar DNA binding sites [netropsin (Patel & Shapiro, 1985, 1986); distamycin A (Klevit et al., 1986; Pelton & Wemmer, 1989, 1990b); lexitropsins (Lee et al., 1988; Kumar et al., 1990, 1991); (4S)-(+)- and (4R)-(−)-anthelvencin A (Lee et al., 1989); Hoechst 33258 (Parkinson et al., 1990; Fede et al., 1991)]. The flip-flop process would therefore seem to represent a property inherent to complexes between minor groove binding ligands and duplex DNA. It is interesting to note that the activation energies of these processes all lie within the range 55–75 kJ mol^{-1}, yet the lifetimes determined for these complexes vary from the millisecond to the second time scale. Although the temperatures and solvent conditions of these studies are not the same, the differences in experimental conditions appear to be insufficient to explain this large variation in lifetimes.

Concluding Remarks

The results obtained on the 1:1 complex formed between SN-6999 and d(GGTTAATGCGGT)·d(ACCGCATTAACC) are fully consistent with the excluded-site size of five base pairs (Braithwaite & Baguely, 1980) and the overall length of the molecule, 18.5 Å. SN-6999 interacts in the minor groove of the duplex, primarily with the AT-rich region d-($T_3T_4A_5A_6T_7$)·d($A_{18}T_{19}T_{20}A_{21}A_{22}$), but also with G_8 and C_{23}. The contact with one additional residue in the 3′ direction along each strand is unique and suggests that this complex is stablized by interactions with the guanosine or cytosine residue adjacent to the AT-rich region. This was not observed for the 1:1 complex with d(GCATTAATGC)$_2$ (Leupin et al., 1986). Among the family of "AT-specific" minor groove binding drugs, only Hoechst 33258 has exhibited a similar property; in studies of complexes of Hoechst 33258 with two different DNA duplexes, the interaction of the terminal heterocyclic ring of the drug extends beyond the AT-rich region to the first GC basepair (de C. T. Carrondo et al., 1989; Fede et al., 1991).

The d(GGTTAATGCGGT)·d(ACCGCATTAACC) duplex has been designed to preclude sliding of the ligand along

the minor groove, using the guanine C2 amino group that protrudes into the minor groove as a structural constraint. The experimentally observed evidence of chemical exchange can therefore be attributed to rapid exchange of the drug between its two possible orientations in the binding pocket. The overall conformational features of the dodecanucleotide (right-handed B-form helix with predominantly C2′-endo/S-type sugar pucker) do not change upon binding of the drug (within the limitations of NMR for determining certain structural features). However, the drug-induced broadening of the NMR resonances of protons in the binding site precludes an in-depth analysis of small changes in the *local* conformation of some of the nucleotides in the binding site. Computational methods are now being applied to search for the molecular determinants of the intermolecular interactions in this complex. This key information will be used to refine the design of the DNA sequence and the drug to increase the complementarity between the ligand and its binding sites in the minor groove of the DNA duplex and provide insights into the factors that determine the relative DNA affinity and specificity of this family of minor groove binding antitumor agents.

ACKNOWLEDGMENTS

We thank Beth Larson for her help with typing the manuscript.

SUPPLEMENTARY MATERIAL AVAILABLE

A detailed description of the assignment and characterization of the free DNA duplex, two tables listing ^1H resonance assignments and scalar coupling constants for the DNA duplex, and five figures showing sections from 2D ^1H NMR spectra of the free duplex and the complex (11 pages). Ordering information is given on any current masthead page.

REFERENCES

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.
Braithwaite, A. W., & Bagueley, B. C. (1980) *Biochemistry 19*, 1101–1106.
Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521–528.
Braunschweiler, L., Bodenhausen, G., & Ernst, R. R. (1983) *Mol. Phys. 48*, 535–560.
Cain, B. F., Atwell, G. J., & Seelye, R. N. (1969) *J. Med. Chem. 12*, 199–206.
de C. T. Carrondo, M. A. A. F., Coll, M., Aymami, J., Wang, A. H.-J., van der Marel, G. A., van Boom, J. H., & Rich, A. (1989) *Biochemistry 28*, 7849–7859.
Celda, B., Widmer, H., Leupin, W., Chazin, W. J., Denny, W. A., & Wüthrich, K. (1989) *Biochemistry 28*, 1462–1471.
Chazin, W. J., Wüthrich, K., Hyberts, S., Rance, M., Denny, W. A., & Leupin, W. (1986) *J. Mol. Biol. 190*, 439–453.
Chazin, W. J., Rance, M., Chollet, A., & Leupin, W. (1991) *Nucleic Acids Res. 19*, 5507–5513.
Coll, M., Frederick, C. A., Wang, A. H.-J., & Rich, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8385–8389.
Coll, M., Aymami, J., van der Marel, G. A., van Boom, J. H., Rich, A. & Wang, A. H.-J. (1989) *Biochemistry 28*, 310–320.
Davis, D. G. (1989) *J. Magn. Reson. 81*, 603–607.
Fede, A., Labhardt, A., Bannwarth, W., & Leupin, W. (1991) *Biochemistry 30*, 11377–11388.
Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1984) *J. Med. Chem. 27*, 450–465.
Griesinger, C., Sørensen, O. W., & Ernst, R. R. (1987) *J. Magn. Reson. 75*, 474–492.
Günther, H. (1980) *NMR Spectroscopy*, pp 234–280, Wiley, New York.
Harshman, K. D., & Dervan, P. (1985) *Nucleic Acids Res. 13*, 4825–4835.
Klevit, R. E., Wemmer, D. E., & Reid, B. R. (1986) *Biochemistry 25*, 3296–3303.
Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376–1380.
Kumar, S., Yadagiri, B., Zimmermann, J., Pon, R. T., & Lown, J. W. (1990) *J. Biomol. Struct. Dyn. 8*, 331–357.
Kumar, S., Bathini, Y., Joseph, T., Pon, R. T., & Lown, J. W. (1991) *J. Biomol. Struct. Dyn. 9*, 1–21.
Lambert, B., & Lepecq, J.-B. (1987) in *DNA–Ligand Interactions—From Drugs to Proteins* (Guschlbauer, W., & Saenger, W., Eds.) pp 141–157, Plenum Press, New York.
Lee, M., Chang, D.-K., Pon, R. T., Krowocki, K., & Lown, J. W. (1988) *Biochemistry 27*, 445–455.
Lee, M., Shea, R. G., Hartley, S. A., Kissinger, K., Pon, R. T., Vesnaver, G., Breslauer, K. J., Dabrowiak, J. C., & Lown, J. W. (1989) *J. Am. Chem. Soc. 111*, 345–354.
Leupin, W., Feigon, J., Denny, W. A., & Kearns, D. R. (1985) *Biophys. Chem. 22*, 299–305.
Leupin, W., Chazin, W. J., Hyberts, S., Denny, W. A., & Wüthrich, K. (1986) *Biochemistry 25*, 5902–5910.
Leupin, W., Otting, G., Amacker, H., & Wüthrich, K. (1990) *FEBS Lett. 263*, 313–316.
Levitt, M., & Freeman, R. (1979) *J. Magn. Reson. 33*, 473–476.
Macura, S., & Ernst, R. R. (1980) *Mol. Phys. 41*, 95–117.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–974.
Otting, G., Senn, H., Wagner, G., & Wüthrich, K. (1986) *J. Magn. Reson. 70*, 500–505.
Parkinson, J. A., Barber, J., Douglas, K. T., Rosamond, J., & Sharples, D. (1989) *J. Chem. Soc., Chem. Commun.*, 1023–1025.
Parkinson, J. A., Barber, J., Douglas, K. T., Rosamond, J., & Sharples, D. (1990) *Biochemistry 29*, 10181–10190.
Patel, D. J., & Shapiro, L. (1985) *Biochimie 67*, 887–915.
Patel, D. J., & Shapiro, L. (1986a) *Biopolymers 25*, 707–727.
Patel, D. J., & Shapiro, L. (1986b) *J. Biol. Chem. 261*, 1230–1240.
Patel, D. J., Shapiro, L., & Hare, D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 423–454.
Pelton, J. G., & Wemmer, D. E. (1988) *Biochemistry 27*, 8088–8096.
Pelton, J. G., & Wemmer, D. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5723–5727.
Pelton, J. G., & Wemmer, D. E. (1990a) *J. Biomol. Struct. Dyn. 8*, 81–97.
Pelton, J. G., & Wemmer, D. E. (1990b) *J. Am. Chem. Soc. 112*, 1393–1399.
Pjura, P. E., Grzeskowiak, K., & Dickerson, R. E. (1987) *J. Mol. Biol. 197*, 257–271.
Plateau, P., & Guêron, M. J. (1982) *J. Am. Chem. Soc. 104*, 7310–7311.
Quintana, J. R., Lipanov, A. A., & Dickerson, R. E. (1991a) *Biochemistry 30*, 10294–10306.
Quintana, J. R., Orzeskowiak, K., Yanagi, K., & Dickerson, R. E. (1991b) *Biophys. J. 59*, 490a.
Rance, M. (1987) *J. Magn. Reson. 74*, 557–564.
Rance, M., & Wright, P. E. (1986) *J. Magn. Reson. 66*, 372–378.

Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.
Rance, M., Wright, P. E., Messerle, B. A., & Field, L. D. (1987) *J. Am. Chem. Soc. 109*, 1591–1593.
Reid, B. R. (1987) *Q. Rev. Biophys. 20*, 1–34.
Searle, M. S., & Embrey, K. J. (1990) *Nucleic Acids Res. 18*, 3753–3762.
Shaka, A. J., Keeler, J., Frenkiel, T., & Freeman, R. (1983) *J. Magn. Reson. 52*, 335–338.
Sutherland, I. O. (1971) *Annu. Rep. NMR Spectrosc. 4*, 71–225.
Teng, M.-K., Usman, N., Frederick, C. A., & Wang, A. H.-J. (1988) *Nucleic Acids Res. 16*, 2671–2690.
van de Ven, F. J. M., & Hilbers, C. W. (1988) *Eur. J. Biochem. 178*, 1–38.
Wemmer, D. E., & Reid, B. R. (1985) *Annu. Rev. Phys. Chem. 36*, 105–137.
Widmer, H., & Wüthrich, K. (1986) *J. Magn. Reson. 70*, 270–279.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Zimmer, C., & Wähnert, U. (1986) *Prog. Biophys. Mol. Biol. 39*, 31–112.

Assignment of the Aliphatic ^1H and ^{13}C Resonances of the *Bacillus subtilis* Glucose Permease IIA Domain Using Double- and Triple-Resonance Heteronuclear Three-Dimensional NMR Spectroscopy†

Wayne J. Fairbrother,‡,§ Arthur G. Palmer, III,‡,∥ Mark Rance,‡ Jonathan Reizer,⊥ Milton H. Saier, Jr.,⊥ and Peter E. Wright*,‡

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, and Department of Biology, University of California at San Diego, La Jolla, California 92093-0116

Received December 26, 1991; Revised Manuscript Received February 27, 1992

ABSTRACT: Nearly complete assignment of the aliphatic ^1H and ^{13}C resonances of the IIA^{glc} domain of *Bacillus subtilis* has been achieved using a combination of double- and triple-resonance three-dimensional (3D) NMR experiments. A constant-time 3D triple-resonance HCA(CO)N experiment, which correlates the $^1H^\alpha$ and $^{13}C^\alpha$ chemical shifts of one residue with the amide ^{15}N chemical shift of the following residue, was used to obtain sequence-specific assignments of the $^{13}C^\alpha$ resonances. The $^1H^\alpha$ and amide ^{15}N chemical shifts had been sequentially assigned previously using principally 3D ^1H–^{15}N NOESY-HMQC and TOCSY-HMQC experiments [Fairbrother, W. J., Cavanagh, J., Dyson, H. J., Palmer, A. G., III, Sutrina, S. L., Reizer, J., Saier, M. H., Jr., & Wright, P. E. (1991) *Biochemistry 30*, 6896–6907]. The side-chain spin systems were identified using 3D HCCH-COSY and HCCH-TOCSY spectra and were assigned sequentially on the basis of their $^1H^\alpha$ and $^{13}C^\alpha$ chemical shifts. The 3D HCCH and HCA(CO)N experiments rely on large heteronuclear one-bond J couplings for coherence transfers and therefore offer a considerable advantage over conventional ^1H–^1H correlation experiments that rely on ^1H–^1H ^3J couplings, which, for proteins the size of IIA^{glc} (17.4 kDa), may be significantly smaller than the ^1H line widths. The assignments reported herein are essential for the determination of the high-resolution solution structure of the IIA^{glc} domain of *B. subtilis* using 3D and 4D heteronuclear edited NOESY experiments; these assignments have been used to analyze 3D ^1H–^{15}N NOESY-HMQC and ^1H–^{13}C NOESY-HSQC spectra and calculate a low-resolution structure [Fairbrother, W. J., Gippert, G. P., Reizer, J., Saier, M. H., Jr., & Wright, P. E. (1992) *FEBS Lett. 296*, 148–152].

Glucose-specific enzyme IIA (IIA^{glc}; previously referred to as III^{glc})[1] is the central regulatory protein of the bacterial phosphoenolpyruvate/sugar phosphotransferase system (PTS). Transcriptional regulation by the PTS involves both catabolite repression and inducer exclusion [see Reizer et al. (1988), Saier (1989), and Meadow et al. (1990) for reviews]. In catabolite repression, the phosphorylated form of IIA^{glc} acts as an allosteric activator of adenylate cyclase; in inducer exclusion, the free (unphosphorylated) form functions as an allosteric

† This work was supported by grants from the National Institutes of Heatlh, GM-36643 (P.E.W.), RI-21702 (M.H.S.), and RI-14176 (M. H.S.), and from the National Science Foundation, DMB8903777 (M.R.). W.J.F. was supported by Damon Runyon–Walter Winchell Cancer Research Fund Fellowship DRG-1059. A.G.P. was supported by a National Science Foundation Postdoctoral Fellowship in Chemistry, under Grant CHE-8907510 awarded in 1989.

* To whom correspondence should be addressed.
‡ The Scripps Research Institute.
§ Present address: Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080.
∥ Present address: Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, 630 West 168th St., New York, NY 10032.
⊥ University of California, San Diego.

[1] Abbreviations: 3D, three dimensional; HCA(CO)N, 3D triple-resonance $^1H^\alpha$–$^{13}C^\alpha$–^{15}N correlation spectroscopy; HCCH-COSY, 3D ^1H–^{13}C–^{13}C–^1H correlation spectroscopy via $^1J_{CC}$ couplings; HCCH-TOCSY, 3D ^1H–^{13}C–^{13}C–^1H total correlation spectroscopy via isotropic mixing of ^{13}C magnetization; ^1H–^{13}C NOESY-HMQC, 3D heteronuclear ^1H nuclear Overhauser ^1H–^{13}C multiple-quantum coherence spectroscopy; ^1H–^{13}C NOESY-HSQC, 3D heteronuclear ^1H nuclear Overhauser ^1H–^{13}C single-quantum coherence spectroscopy; HNCA, 3D triple-resonance NH–^{15}N–$^{13}C^\alpha$ correlation spectroscopy; ^1H–^{15}N NOESY-HMQC, 3D heteronuclear ^1H nuclear Overhauser ^1H–^{15}N multiple-quantum coherence spectroscopy; ^1H–^{15}N TOCSY-HMQC, 3D heteronuclear ^1H total correlation ^1H–^{15}N multiple-quantum coherence spectroscopy; IIA^{glc}, glucose specific enzyme IIA; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PTS, phosphoenolpyruvate/sugar phosphotransferase system; rf, radiofrequency; TMS, tetramethylsilane; TPPI, time-proportional phase incrementation; TSP, 3-(trimethylsilyl)propionic acid; TSS, 3-(trimethylsilyl)propanesulfonic acid.

0006-2960/92/0431-4413$03.00/0