Kalnik, M. W., Kouchakdjian, M., Li, B. F. L., Swann, P. F., & Patel, D. J. (1988) *Biochemistry* (preceding paper in this issue).
Keepers, J. W., Schmidt, P., James, T. L., & Kollman, P. A. (1984) *Biopolymers 23*, 2901–2929.
Kneale, G., Brown, T., Kennard, O., & Rabinovich, D. (1985) *J. Mol. Biol. 186*, 805–814.
Li, B. F., Resse, C. B., & Swann, P. F. (1987) *Biochemistry 26*, 1086–1093.
Loeb, L. A., & Kunkel, T. A. (1982) *Annu. Rev. Biochem. 51*, 429–457.
Loeb, L. A., Liu, P. K., & Fry, M. (1986) *Prog. Nucleic Acid Res. Mol. Biol. 33*, 57–110.
Ollis, D. L., Buch, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature (London) 313*, 762–766.
Patel, D. J., Kozlowski, S. A., Marky, L. A., Rice, J. A., Broka, C., Dallas, J., Itakura, K., & Breslauer, K. J. (1982) *Biochemistry 21*, 437–444.
Preston, B. D., Singer, B., & Loeb, L. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8501–8505.
Singer, B. (1986) *Cancer Res. 46*, 4879–4885.
Singer, B., Kroger, M., & Carrano, M. (1978) *Biochemistry 17*, 1246–1250.
Singer, B., Sagi, J., & Kusmierek, J. T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4884–4888.
Singer, B., Spengler, S. J., Fraenkel-Conrat, H., & Kusmierek, J. T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 28–32.
Swenberg, J. A., Dryoff, M. C., Bedell, M. A., Popp, J. A., Huh, N., Kirstein, U., & Rajewsky, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1692–1685.

Selective Reversible Deuteriation of Oligodeoxynucleotides: Simplification of Two-Dimensional Nuclear Overhauser Effect NMR Spectral Assignment of a Non-Self-Complementary Dodecamer Duplex†

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ABSTRACT: Oligodeoxynucleotides are reversibly deuteriated at the purine C8 and cytosine C5 positions with deuterioammonium bisulfite at pD 7.8. The exchange reaction is complete after 48 h at 65 °C. When an oligomer deuteriated under these conditions is analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, the purine H8 and cytosine H5 proton signals are selectively removed from the spectrum. A non-self-complementary oligodeoxynucleotide that has been deuteriated in this manner may be annealed with its complement and the resulting heteroduplex analyzed by two-dimensional nuclear Overhauser enhancement (NOESY) spectroscopy. NOE cross-peaks arising from pyrimidine H6–deoxyribose H1′ dipolar interactions in both strands are observed, but purine H8–deoxyribose H1′ and purine H8–deoxyribose H2′,H2″ dipolar interactions are only observed for the nondeuteriated strand. The intense cytosine H5–H6 cross-peaks are also removed from the spectrum of the deuteriated strand, which further simplifies interpretation since these strong cross-peaks often interfere with less intense NOE cross-peaks arising from dipolar coupling between purine H8 or pyrimidine H6 and deoxyribose anomeric protons. The resulting spectral simplification allows unambiguous assignments to be made on NOEs that otherwise may be difficult to distinguish. The deuteriation procedure is demonstrated with the sequence d(CGTTATAATGCG)·d(CGCATTATAACG), which has previously been assigned by traditional NOESY methods [Wemmer, D. E., Chou, S.-H., Hare, D. R., & Reid, B. R. (1984) *Biochemistry 23*, 2262–2268]. Although the assignment of this dodecadeoxynucleotide may be completed without deuteriation, several NOEs must be assigned indirectly because of degeneracies in the chemical shift of the purine H8 protons. This methodology should have wide applicability to NMR spectral interpretation of oligodeoxynucleotides, particularly to oligonucleotides of 12 bases or longer.

Because of recent improvements in the chemical synthesis of oligodeoxynucleotides [Dorman et al., 1984; reviewed in the text edited by Gait (1984)], relatively large quantities of DNA fragments having defined sequences are accessible for study by physical methods. In particular, there is great interest in the use of high-field nuclear magnetic resonance spectroscopy (NMR)[1] to probe the solution structure of oligodeoxynucleotides and their interactions with various ligands, including drug and carcinogen molecules (Patel et al., 1981, 1986; Pardi et al., 1983; Chandrasekaran et al., 1984; Feigon

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[1] Abbreviations: NOE, nuclear Overhauser effect; HPLC, high-performance liquid chromatography; SAX, strong anion-exchange resin; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; TPPI, time-proportional phase increments; 1D, one dimensional; 2D, two dimensional; tRNA, transfer RNA; DMSO, dimethyl sulfoxide; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate. None of the oligodeoxynucleotides discussed in this paper have terminal phosphates. We abbreviate the notation for oligomers by leaving out the phosphodiester linkage. A, C, G, and T refer to mononucleotide units. A right superscript refers to position in the oligodeoxynucleotide sequence starting from the 5′-terminus of chain A and proceeding to the 3′-terminus of chain A and then from the 5′-terminus of chain B to the 3′-terminus of chain B. C2, C5, C6, C8, C1′, C2′, and C2″ represent specific carbon nuclei in nucleotides. H2, H5, H6, H8, H1′, H2′, H2″, etc., represent the protons attached to these carbons.

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et al., 1984; Graves et al., 1984, 1985; Klevit et al., 1986; Keniry et al., 1987). Central to the strategy of structure determination by NMR is the use of two-dimensional nuclear Overhauser effect (NOESY) spectroscopy to aid in the measurement of interproton distances and thereby to deduce the three-dimensional configuration (Weiss et al., 1984; Hare et al., 1983; Borah et al., 1985; Gronenborn & Clore, 1985; Broido et al., 1985; Wuthrich, 1986). For effective use of this strategy, the assignment of the resonances in the proton spectrum must be as complete as possible.

In recent years there have been substantial advances in the spectral assignment of biological macromolecules; in 1984 the proton spectrum assignment of all but the 4′, 5′, and 5″ resonances of the non-self-complementary dodecamer duplex d(CGTTATAATGCG)·d(CGCATTATAACG) was achieved with two-dimensional NMR (Wemmer et al., 1984). In general, the protons of each deoxyribose spin system can be grouped by two-dimensional autocorrelated spectroscopy (COSY), but it is not possible to determine to which nucleotide in the sequence each sugar belongs. Thus, the critical factor in the assignment procedure is the ability to link a particular base to the corresponding deoxyribose unit in the sequence. Although there is no scalar coupling between the base and sugar protons, dipolar coupling may be observed, provided that the DNA sequence is in an ordered conformation. Furthermore, the base protons show specific dipolar coupling to adjacent bases and sugars. Thus, for a right-handed helix (B-form DNA), NOEs may be observed between each base and corresponding deoxyribose and also between each base and its 5′-neighboring sugar; in principle, the entire strand may be connected and each proton resonance assigned.

A potential pitfall to this method arises when two or more resonances overlap, rendering determination of the connectivities difficult or impossible. In a dodecamer duplex in which the critical NOEs arise from 36 base and 24 anomeric protons, this occurrence is not unlikely. The problems arising from these occasional overlaps may be overcome by correlations from other NOEs, but in cases where multiple overlaps exist or other correlations are not possible, an alternative solution is necessary. We have developed a method for non-self-complementary oligomers in which the spectrum in the aromatic region is simplified by the incorporation of deuterium at the C8 position of purines and C5 position of cytosine by reversible exchange in deuterioammonium bisulfite/D_2O [Maeda et al., 1971; Kai et al., 1971; Hayatsu, 1976; Gautam-Basak et al. (1985) and references cited therein]. Deuteriation of one strand and formation of the duplex with the all-proton complementary strand result in a heteroduplex whose NOESY spectrum lacks a cross-peak wherever a deuterium atom has been incorporated. Similarly, deuteriation of the second strand and duplex formation with its all-proton complement yield a heteroduplex whose NOESY spectrum has the other set of cross-peaks suppressed. The resulting spectra are simplified, and unequivocal assignments can be made. The use of such a strategy to simplify spectral assignment is demonstrated on the dodecamer duplex d(CGTTATAATGCG)·d(CGCATTATAACG), originally assigned by Wemmer et al. (1984). The deuteriation procedures demonstrated in this work should be particularly useful in the study of longer oligonucleotides (>12 bases), where spectral interpretation becomes increasingly complex as the length of the oligonucleotide is increased.

Materials and Methods

Synthesis and Purification. Dodecanucleotides d(CGTTATAATGCG) (strand A) and d(CGCATTATAACG) (strand B) were synthesized on a solid support with β-cyanoethyl phosphoramidite chemistry. After deprotection with concentrated ammonia, an ion-exchange HPLC profile for each strand was obtained on an analytical scale [SAX column; gradient, 100% buffer A (0.01 M ammonium acetate/20% ethanol) to 100% buffer B (2.5 M ammonium acetate/20% ethanol) over 30 min]. Each oligomer was then purified on a preparative scale on a 2.5 × 50 cm column of (diethylaminoethyl)cellulose with a gradient of 0.05–1 M triethylammonium bicarbonate (600 mL). Peak detection was done by UV monitoring at 254 nm. The appropriate fractions were pooled and evaporated. The oligomers were further purified on a 9 × 250 mm preparative C-18 HPLC column with an isocratic system of 11% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0.

The pooled fractions were lyophilized, and the oligomers were obtained as the triethylammonium salts. Conversion to the sodium salt was done by dissolving the DNA in 2 mL of 0.2 M sodium phosphate buffer, pH 7.4, containing 0.01 M Na_2EDTA. The DNA was adsorbed onto a C-18 cartridge and was washed with 20 mL of 0.2 M sodium phosphate buffer, pH 7.4. It was eluted with 20% acetonitrile in water. The NMR spectra of the single-stranded oligomers were obtained at 60 °C. Purified yields were in the range of 30–35%. A_{260}/A_{280} for strand A = 1.80 (calcd 1.85) and for strand B = 1.92 (calcd 2.02). The extinction coefficients (ambient temperature) used were strand A = 1.172×10^5 L M^{-1} cm^{-1} and strand B = 1.180×10^5 L M^{-1} cm^{-1}. The calculation utilized to yield these extinction coefficients (Borer, 1975) is estimated to be accurate to within 10%. These calculated values proved adequate for the experiments that we describe; a more quantitative evaluation of the extinction coefficients was not performed.

Deuteriation of the Oligomers. Deuterioammonium sulfite was prepared by lyophilizing ammonium sulfite twice in D_2O. The $(ND_4)_2SO_3$ (2.65 g) was made up to 10 mL in D_2O to give a 2.5 M solution. The pD was adjusted to 7.8 on a pH meter with sodium deuteriobisulfite (from sodium metabisulfite dissolved in D_2O) and the solution stored in a sealed polypropylene tube at 5 °C until use. The pD was readjusted at the time of use with sodium deuteriobisulfite to 7.8. The pD is critical; under more acidic conditions, deamination of cytosine to form uracil will occur (Hayatsu, 1976). Approximately 250 A_{260} units (2.1 μmol) of dodecamer was dissolved in 3 mL of the deuteriobisulfite, and the reaction mixture was placed in a tightly sealed screw-cap polypropylene vial. The solution was heated at 65 °C for 48 h. The progress of the deuteriation was followed by removal of 0.4 mL of the reaction mixture and examination of the NMR spectrum. Partially reacted aliquots were returned to the reaction mixture and heated further. The reaction mixtures were combined and diluted 10-fold with 0.1 M phosphate buffer, pH 7.4, and desalted on a C-18 cartridge as described above. The NMR sample was prepared and the spectrum recorded as described above. Yields of the deuteriated oligomers were in the 90–99% range with deuterium incorporation of 90 to >95%. A_{260}/A_{280} for the deuteriated A strand was 1.87, and for the deuteriated B strand A_{260}/A_{280} = 1.92.

Nuclear Magnetic Resonance. NMR spectroscopy was performed on a Bruker AM-400 spectrometer. NMR samples were prepared by adding 0.4 mL of NMR buffer (0.01 M sodium phosphate, pH 7.4, 5×10^{-5} M EDTA, 0.1 M NaCl) to the desired quantity of purified oligodeoxynucleotide, followed by lyophilization with D_2O 3 times; samples were prepared with 0.4 mL of 99.96% D_2O. The spectra of the single-stranded oligomers were obtained at 60 °C. For the

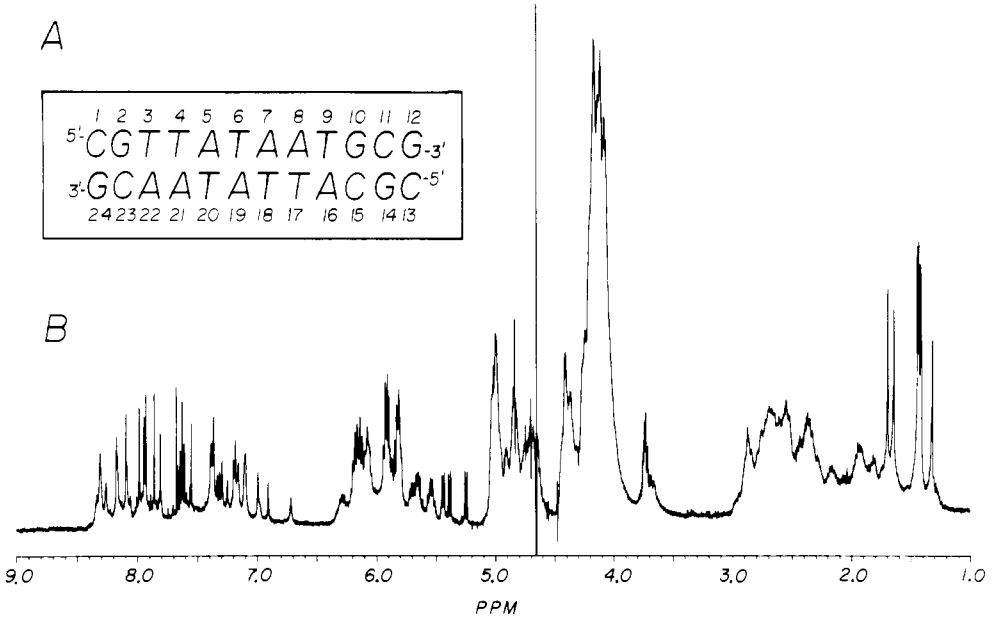

FIGURE 1: (A) Numbering scheme for the non-self-complementary dodecanucleotide d(CGTTATAATGCG)·d(CGCATTATAACG). Numbering proceeds from the 5′ to the 3′ terminus of strand A (dCGTT...) and continues from the 5′ to the 3′ terminus of strand B (dCGCA...). (B) The 400-MHz ^{1}H NMR spectrum of d(CGTTATAATGCG)·d(CGCATTATAACG), 7.1 mM, 37 °C, prepared in D_2O as described in the text. The T_m of this sample was not measured precisely but was estimated to be ~50–60 °C from inspection of chemical shift data as a function of temperature. The 2D NOESY experiments described in this paper were performed at 30 or 37 °C, conditions under which the two strands are predominantly in the duplex state.

NOESY experiments, each deuteriated strand was combined with 1 equiv of nondeuteriated complement in D_2O containing 0.4 mL of the NMR buffer. The sample was lyophilized 3 times in D_2O and dissolved in enough 99.96% D_2O to make the solution up to 0.4 mL. The NOESY experiment was performed in the phase sensitive TPPI mode (Bodenhausen et al., 1984) at 30 or 37 °C, using quadrature detection. A mixing time of 0.3 s was used (Macura et al., 1981) with 80 or 160 scans per experiment, 256 experiments, zero filling in F_1, 1K data points in F_2, 90° phase-shifted sine-bell apodization in F_2, sine-bell apodization in F_1, and symmetrization about the diagonal. Chemical shifts were referenced internally to DSS. The concentration of the sample [A(deuteriated)·B] was 5.1 mM. Sample [A·B(deuteriated)] was 4.4 mM. After the NMR experiments on the heteroduplexes were completed, the two samples were lyophilized, combined, redissolved in 3 mL of 2.5 M NH_4HSO_3, pH 7.8, and treated as above to yield the all-proton DNA duplex. The sample for the all-proton NOESY experiment was 7.1 mM, a 74% recovery.

Separation of the Oligodeoxynucleotides. A sample of the all-proton duplex was applied to a C-18 HPLC column (PRP-1, Hamilton) and was eluted with a gradient of 1–10% acetonitrile in 0.15 M sodium phosphate, pH 12, over a 15-min period. The duplex was separated into its components, with retention times of 14.0 min for the B strand and 15.4 min for the A strand.

RESULTS

The NOESY Experiment. Figure 1 shows the numbering scheme utilized in the sequence assignment of the dodecamer and the one-dimensional 400-MHz ^{1}H NMR spectrum of the dodecanucleotide duplex dissolved in D_2O. The 1D spectrum is complex and difficult to interpret; the NMR assignment procedures require the use of 2D NMR. Figure 2 shows the phase-sensitive (TPPI) NOESY spectrum of the dodecamer sequence, from which detailed sequential assignments may be made. This spectrum essentially reproduces the work of Reid and co-workers (Wemmer et al., 1984), to which the reader is referred for a detailed description of the NOESY experiment and spectral assignment. Briefly, cross-peaks arising in the NOESY experiment shown in Figure 2 result from NOE effects between proximate protons. Since the NOE is a short-range effect, varying as a function of the inverse sixth power of the distance between two protons, only protons ≤5 Å apart will exhibit first-order NOEs. Thus the presence of an NOE is important in establishing spatial proximity. The NOEs that are important in establishing the connectivities between nucleotides are detailed in Table I.

Deuteriation of Specific Strands as an Aid to NOESY Interpretation. The 400-MHz ^{1}H NMR spectra of the aromatic and anomeric regions of d(CGCATTATAACG) before and after deuteriation are shown in panels A and B of Figure 3. In the downfield portion of the aromatic region, the resonances for the C8 position protons of adenine and guanine are missing from the spectrum of the deuteriated oligomer. Furthermore, the cytosine C6 protons (~7.7 ppm) have been converted to singlets because of deuteriation of the C5 position, whose resonances are missing from the upfield end of the anomeric region. The 1D spectrum of d(CGTTATAATGCG) is also simplified by the deuteriation procedure (not shown). The effect of deuteriation in simplifying 2D spectral interpretation is detailed in Figures 4 and 5, which represent expansions of the NOESY spectrum shown in Figure 2. The expanded regions (representing boxes I and II from Figure 2) are significant to establishment of the connectivities between nucleotides. Each NOE observed in Figure 4 is identified in Table I by reference to a number next to the NOE cross-peak.

It may be seen that several overlapping or closely spaced cross-peaks complicate the NOESY peaks in Figure 4A. In particular, the NOESY plot is complicated by the presence of large cross-peaks arising primarily from cross-relaxation (there is also a contribution from scalar coupling) between the H5 and H6 protons of cytosine resonances, of which there are five in this duplex. This may be observed by inspection of box I, Figure 4A, consisting of the cytosine H5–H6 couplings from C^1 and C^{13} and the H6–H1′ intranucleotide NOEs of C^1 and C^{13}. This pattern is difficult to interpret due to the intensity of the coupling at C^{13}. Cross-peaks numbered 2 and 28 are

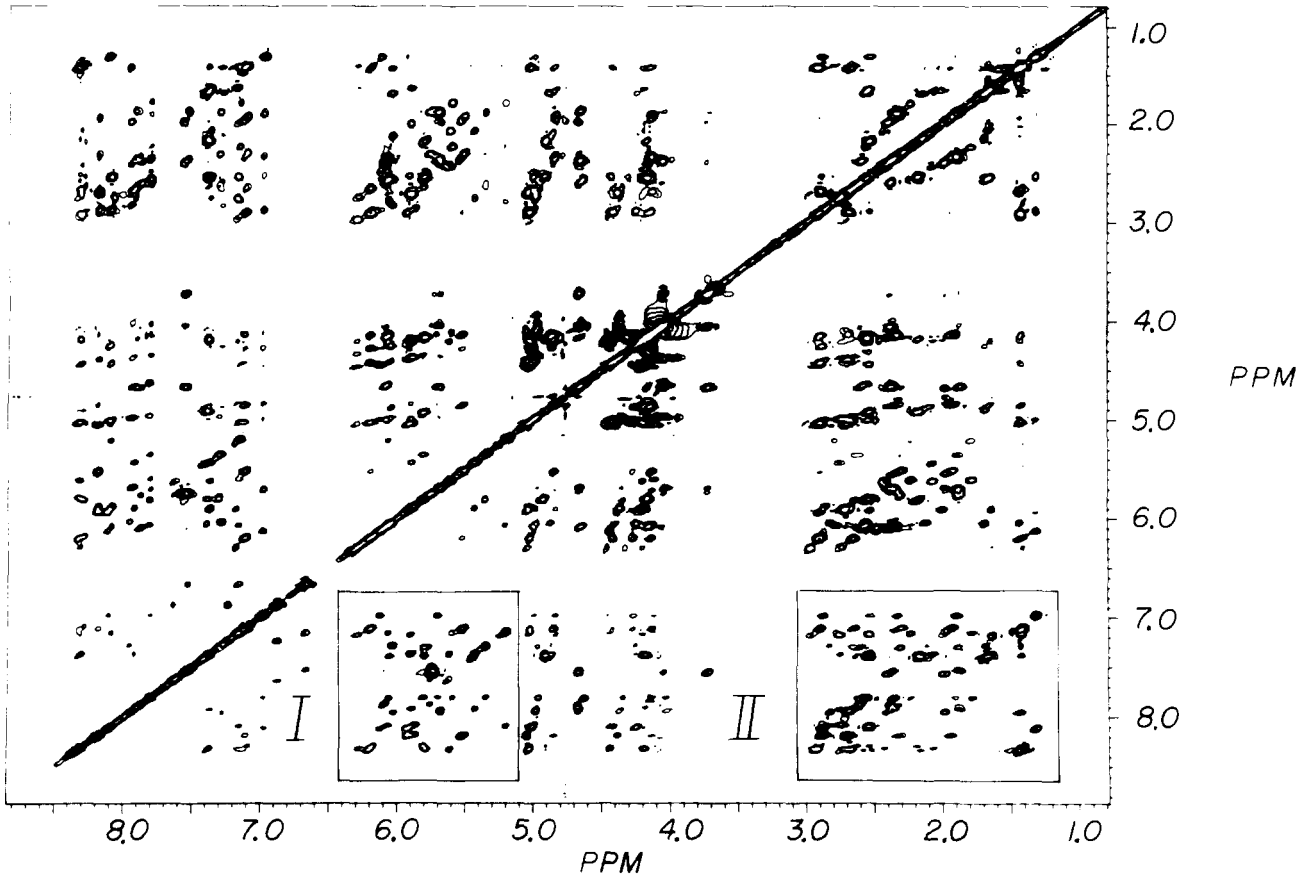

FIGURE 2: Phase-sensitive (TPPI) NOESY spectrum of d(CGTTATAATGCG)-d(CGCATTATAACG) shown in the contour plot representation; spectral conditions were the same as the 1D spectrum shown in Figure 1. Cross-peaks arising from dipolar interactions between the purine H8 and pyrimidine H6 protons and the deoxyribose anomeric protons are located in box I, as are the scalar coupling interactions between cytosine H5 and H6 protons. Cross-peaks arising from dipolar interactions between the purine H8 and pyrimidine H6 protons and the deoxyribose H2′ and H2″ protons are located in box II.

difficult to observe in the spectrum. Both of these represent NOE cross-peaks from terminal cytosine residues. The low intensity of these two cross-peaks was not investigated further. Oligodeoxynucleotides are susceptible to various "end effects", such as "fraying" of the strands or other alteration in helical structure at the ends of the duplex. Such effects could account for the observed lower intensity of these cross-peaks. In principle, the intensity of scalar coupling interactions may be reduced by the use of a variable mixing time in the NOESY experiment. This is accomplished directly by deuteriation, as may be observed in box I, Figure 4B, where the C^1 H5–H6 coupling (cross-peak 1; arising from both scalar coupling and cross-relaxation contributions) is eliminated because of the deuteriation of C^1 H5, improving the resolution in that area. Figure 4C shows a similar effect when C^{13} H5 is deuteriated, removing cross-peak 27. In Figure 4A the cross-peak in box II is a direct overlap of the internucleotide NOEs of T^6 H1′–A^7 H8 and T^{20} H1′–A^{21} H8 (cross-peaks 13 and 43). In Figure 4B, since A^7 H8 has been exchanged for deuterium, only the T^{20}–A^{21} NOE (cross-peak 43) remains. Figure 4C shows the T^6–A^7 NOE after deuteriation of A^{21} H8 to remove cross-peak 13. The very similar chemical shifts of A^7 H8 and A^{21} H8 also cause a problem in box III (cross-peaks 14 and 44). In addition, the C8 protons of A^8 and A^{22} have almost identical chemical shifts and are also in this region (cross-peaks 15, 16, 45, and 46), making the assignment of the NOEs difficult. Panels B and C of Figure 4, in which one of each pair of overlapped resonances is suppressed by deuteriation, show clearly the correct assignments. Other cross-peaks that are close enough to partially overlap but are resolved by deuteriation are in boxes IV and V. In box IV, cross-peak 10, arising from A^5 H8, may be separated from cross-peaks 34 and 40, arising from A^{16} and A^{19} H8. In box V, cross-peaks 3, 19, 20, and 23, arising from G^2, G^{10}, and G^{12} H8 protons, are selectively removed upon deuteriation (Figure 4B); deuteriation of the opposite strand removes cross-peaks 29 and 49, which arise from G^{14} and G^{24} (Figure 4C). In Figure 4C, box V, cross-peak 3 is very weak, and cross-peak 23 is not observed at the contour level plotted.

In the region of the NOEs between the aromatic C6 and C8 protons and the C2′, C2″, and thymine methyl protons, simplification of the 2D spectrum is also observed. The NOESY contour plot of the all-proton duplex is shown in Figure 5A, the NOESY of the duplex with the A strand deuteriated in Figure 5B, and that with the B chain deuteriated in Figure 5C. The cross-peaks in Figure 5 have not been numbered but have been previously assigned (Wemmer et al., 1984); they reproduce the work of those authors. In Figure 5A, box I, there is a group of cross-peaks, closely spaced, which arise from the inter- and intranucleotide NOEs between the five guanine H8 protons in the duplex and deoxyribose H2′,H2″ protons. The strong cross-peaks at lower field represent short-range intranucleotide NOEs to the guanine H2′ protons; the weaker NOEs are longer range internucleotide NOEs. Comparison of box V, Figure 4, shows that G^{10} and G^{24} H8 and G^2 and G^{14} H8 overlap. The effect of selective strand deuteriation is nicely illustrated in Figure 5B,C, box I. In Figure 5B, two sets of guanine cross-peaks, arising from G^{14} and G^{24}, remain, whereas in Figure 5C there are three sets of cross-peaks, arising from G^2, G^{10}, and G^{12}. A similar effect is noted in the adenine H8 region of the spectrum, Figure 5A–C, box II. The seven adenine H8 protons in the duplex

Table I: Detailed Cross-Peak Assignments in the Two-Dimensional NOESY Spectra Shown in Figure 4[a]

strand A		strand B	
cross-peak	assignment	cross-peak	assignment
1	C^1 H5–H6	27	C^{13} H5–H6
2	C^1 H6–H1′	28	C^{13} H6–H1′
3	C^1 H1′–G^2 H8	29	C^{13} H1′–G^{14} H8
4	G^2 H8–G^2 H1′	30	G^{14} H8–H1′
5	G^2 H1′–T^3 H6	31	G^{14} H1′–C^{15} H6
6	T^3 H6–H1′	32	C^{15} H6–H1′
7	T^3 H1′–T^4 H6	33	C^{15} H1′–A^{16} H8
8	T^4 H6–H1′	34	A^{16} H8–H1′
9	T^4 H1′–A^5 H8	35	A^{16} H1′–T^{17} H6
10	A^5 H8–H1′	36	T^{17} H6–H1′
11	A^5 H1′–T^6 H6	37	T^{17} H1′–T^{18} H6
12	T^6 H6–H1′	38	T^{18} H6–H1′
13	T^6 H1′–A^7 H8	39	T^{18} H1′–A^{19} H8
14	A^7 H8–H1′	40	A^{19} H8–H1′
15	A^7 H1′–A^8 H8	41	A^{19} H1′–T^{20} H6
16	A^8 H8–H1′	42	T^{20} H6–H1′
17	A^8 H1′–T^9 H6	43	T^{20} H1′–A^{21} H8
18	T^9 H6–H1′	44	A^{21} H8–H1′
19	T^9 H1′–G^{10} H8	45	A^{21} H1′–A^{22} H8
20	G^{10} H8–H1′	46	A^{22} H8–H1′
21	G^{10} H1′–C^{11} H6	47	A^{22} H1′–C^{23} H6
22	C^{11} H6–H1′	48	C^{23} H6–H1′
23	C^{11} H1′–G^{12} H8	49	C^{23} H1′–G^{24} H8
24	G^{12} H8–H1′	50	G^{24} H8–H1′
25	C^{11} H5–H6	51	C^{15} H5–H6
26	C^{11} H5–G^{10} H8	52	C^{23} H5–H6
		53	C^{15} H5–G^{14} H8
		54	C^{23} H5–A^{22} H8

[a] The H8 protons of purines and the H6 protons of pyrimidines are positioned over the deoxyribose rings in their nucleotide units (anti conformation about the glycosyl bond in B-form DNA). A strong NOE exists between H8 or H6 and the H2′ proton on the same side of the deoxyribose ring. Weaker NOEs are observed between H6 or H8 and the H1′ and H2″ protons, which are on the opposite side of the deoxyribose ring. These are used to establish the connectivity between each base and the corresponding deoxyribose. Strong NOEs are also observed between H6 or H8 and the H1′ and H2″ of the neighboring deoxyribose in the 5′ direction. A weaker NOE is also seen to the H2′ of the 5′ neighbor. These are used to establish the connectivities between nucleotide units. There are additional NOEs among the deoxyribose protons and a few more to the base protons which can provide useful information to confirm assignments.

form a complex contour plot in box II, Figure 5A. Upon selective deuteriation of the strands, the four sets of cross-peaks are observed in Figure 5B, box II, and three sets are seen in Figure 5C, box II. Box III, Figure 5A–C, shows internucleotide NOEs between purine H8 protons and 3′-neighbor thymine methyl groups. Inspection of the dodecamer sequence shows that there are five such sequences, G^2-T^3, A^5-T^6, A^8-T^9, A^{16}-T^{17}, and A^{19}-T^{20}. Upon deuteriation of strand A, two (overlapping) cross-peaks remain in box III, Figure 5B. Likewise, three cross-peaks are seen in Figure 5C, box III.

Discussion

Utilization of Specific Deuteriation in Spectral Assignment. Although the use of deuteriation in the spectral assignments of oligonucleotides is not a new idea and has been demonstrated to be a powerful method for assignment of complex spectra, it has not been extensively utilized due to the difficulty and expense usually associated with the preparation of specifically labeled molecules. An early demonstration of the utility of deuteriation in the assignment process was the complete assignment of the anomeric and aromatic protons in the oligoribonucleotide AAA (Kondo et al., 1975). Labeled mononucleotides were extracted from blue-green algae grown in D_2O; a combination of chemical and enzymatic synthesis was used to obtain selectively labeled dimers and trimers. Kan

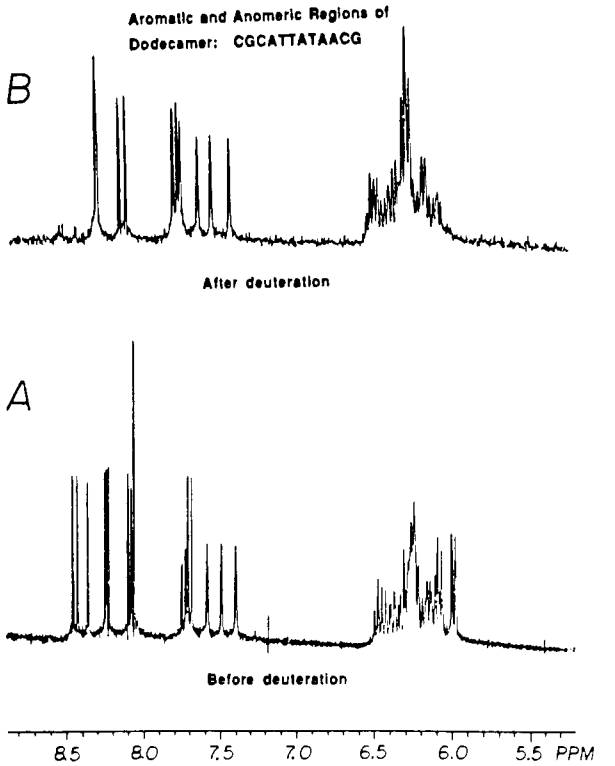

FIGURE 3: An expansion of the anomeric and aromatic regions (5.2–8.9 ppm) of the 400-MHz ^1H NMR spectrum of d(CGCATTATAACG) in D_2O before (A) and after (B) treatment with ND_4DSO_3, as described in the text. After deuteriation, the six purine H8 and three cytosine H5 protons are not observed in the spectrum. Note the collapse of the cytosine H6 doublets at ~7.7 ppm to singlets in the spectrum of the deuteriated material.

and co-workers (Chandrasegaran et al., 1985) have grown algae under $^{13}CO_2$ to obtain ^{13}C-enriched mononucleotides. Redfield and co-workers (Roy et al., 1982; Sanchez et al., 1980) have incorporated adenine deuteriated at the C2 or C8 position into tRNA molecules. This was accomplished by feeding C2-deuteriated hypoxanthine (Richter et al., 1960) or C8-deuteriated adenine to purine-requiring yeast cells. In contrast, the deuteriation experiments demonstrated here are relatively simple and inexpensive to perform and yet result in substantial simplification of the oligonucleotide assignment procedure. The incorporation of deuterium occurs with high yield and can easily be monitored by NMR. The deuteriation is reversible, and the oligonucleotide strands are recovered in high yield.

Experimental Strategy. Incorporation of deuterium in the C8 position of purines allows observation of the NOEs in the purine aromatic region (7.5–8.5 ppm) from only one strand of the duplex at a time. This simple deuteriation requires nothing more than heating the sample under neutral or basic conditions in the presence of D_2O. If the sequence is not rich in cytosine residues, this change alone may be sufficient to simplify NOESY interpretation. Deuteriation of the C5 position of cytosine does not directly simplify NOESY interpretation but is important because it eliminates the very intense H5–H6 cross-peaks arising from cross-relaxation and scalar coupling interactions. The need for deuteriation at the C5 position will depend on the sequence of interest, and the number of cytosine residues it contains. In the dodecamer sequence studied here, there are two cytosines in strand A and three in strand B. These overlap the smaller NOEs in the same region of the spectrum, especially (in this instance) those between H6 and H1′ on the same cytosine nucleotide. Deuteriation of the cytosine C5 position results in substantial

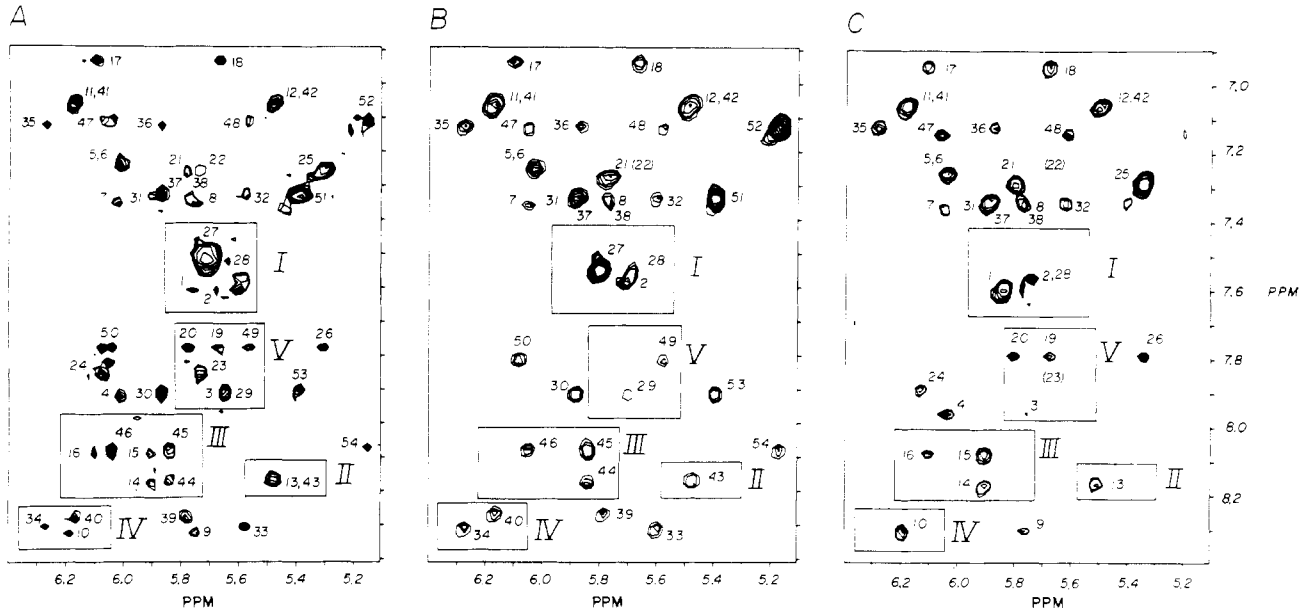

FIGURE 4: An expansion of the region enclosed by box I in the contour plot of the TPPI NOESY spectrum shown in Figure 2. NOE cross-peaks between purine H8 and deoxyribose anomeric protons in the fully protonated duplex (A), the duplex where strand A has been deuteriated (B), and the duplex where strand B has been deuteriated (C). Numbers next to the cross-peaks are used to identify specific NOE interactions, listed in Table I. Boxes I–V contain specific sets of NOE cross-peaks which are affected by deuteriation, as is discussed in the text.

spectral simplification and enables the chain-terminating NOESY cross-peaks (which in this instance are of low intensity) to be more easily observed in the spectrum.

A potentially more useful site for deuteriation would be the C6 position of pyrimidines. This would selectively remove the pyrimidine H6–H1′ NOEs (6.7–7.5 ppm) from each strand. Unfortunately, the present methodology for incorporation of deuterium at this position is not simple. One method reported in the literature for the deuteriation of the C6 position of cytosine requires extensive heating in D_2O in the presence of an equivalent weight of platinum. We found that this reaction works well as described for nucleosides (Maeda & Kawazoe, 1971; Kinoshita et al., 1982). Efforts to achieve the same result with an oligonucleotide failed with loss of the oligonucleotide, presumably due to decomposition of oligodeoxynucleotides from the harsh reaction conditions or to irreversible adsorption to the platinum. This line of experiments was not pursued further. The C6 position of pyrimidine nucleosides and deoxynucleosides has been deuteriated by exchange with strong bases, such as butyllithium (Pichat et al., 1973), or NaOD or $NaOCH_3$ in [$^2H_6$]DMSO at temperatures of 60–135 °C for 17–48 h (Rabi & Fox, 1973). These methods are clearly unsuitable for application to oligonucleotides. Thus, preparation of a single strand of DNA with 6-deuteriopyrimidines is possible, but would require synthesis of the oligomer from the 6-deuteriodeoxynucleoside precursors, a task requiring considerable chemical expertise and not readily amenable to routine spectral assignment problems. Nonetheless, we are exploring the utility of this approach and also an alternative method, the selective deuteriation of the anomeric proton resonances, as a means to the solution of unique assignment problems.

The reversibility of the deuteriation reactions and the high recovery of DNA make it practical to perform these experiments on relatively small amounts of DNA, through recycling of the strands. The limiting factor appears to be the ability to separate the two strands from each other chromatographically. With C-18 HPLC, this will depend primarily on the base composition of each strand. Assuming that the strands may be separated quantitatively, the following strategy may be employed: (1) strand A is deuteriated, it is annealed to B, and the NMR spectrum is acquired; (2) the strands are separated by HPLC, and A is converted to the all-proton form with NH_4HSO_3; (3) B is deuteriated, it is annealed to A, and the NMR spectrum is reacquired; (4) the duplex is converted to the all-proton form, and the NMR spectrum of the fully protonated duplex is acquired if desired.

For the dodecamer sequence utilized in these experiments, the individual strands were of very similar base composition and sequence. Although separation of the two strands was possible by reverse-phase HPLC at pH 12 on an analytical scale, the difference in retention times did not appear to be large enough to warrant an attempt at a scale-up to a preparative column. We chose therefore to first deuteriate each strand individually, combine with nondeuteriated complement, and record the NOESY spectrum. Then each heteroduplex was treated with NH_4HSO_3 to recover the all-proton analogue for the reference NOESY spectrum. In this manner the problem of chromatographic separation was avoided, although a larger amount of oligodeoxynucleotide was required to perform the experiments.

The NOESY experiments on the deuteriated dodecamers were run under conditions closely paralleling those of Reid and co-workers (Wemmer et al., 1984) in order to facilitate comparison of the resulting spectra with the earlier work. There were, however, several differences that should be noted. The three NOESY samples [i.e., A(deuteriated)·B, A·B(deuteriated), and A·B] varied in concentration from 4.4 to 7.1 mM. Because the transition between single strands and duplex is concentration dependent, concentration differences could introduce complications in the interpretation of the experiment. Small chemical shift changes due to the concentration differences are observed in our results but do not interfere with the interpretation of the experiment. In practice, the effect of concentration may be minimized by working at strand concentration and temperature conditions which strongly favor duplex formation. A second difference warranting discussion is the change in magnetic field strength from 500 to 400 MHz. The net effect of the lower field strength is a reduction in the spectral resolution, both in the 1D and in the 2D experiment. This points to a potential major advantage of the deuteriation methodology presented here. By simplification of the spectra,

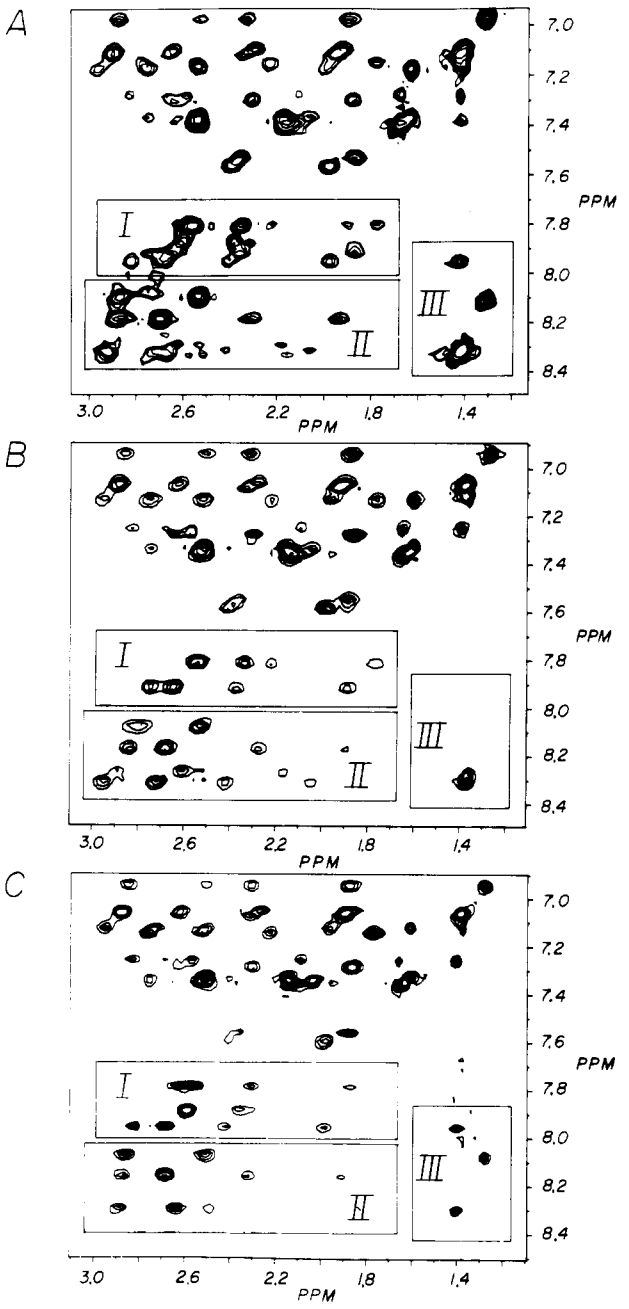

FIGURE 5: An expansion of the region enclosed by box II in the contour plot of the TPPI NOESY spectrum shown in Figure 2. NOE cross-peaks between purine H8 and deoxyribose H2′ and H2″ protons in the fully protonated duplex (A), the duplex where strand A has been deuteriated (B), and the duplex where strand B has been deuteriated (C). Boxes I–III contain specific sets of NOE cross-peaks which are affected by deuteriation, as is discussed in the text.

it may make spectral assignments of complex oligodeoxynucleotides more readily accessible to workers not having access to the highest field NMR instruments.

Deamination of Cytosine. It is essential that the bisulfite reaction be run under conditions in which deamination of cytosine to form uracil (Hayatsu, 1976) is negligible. Recovery of the desired product from a mixture of partially deaminated oligonucleotides would be difficult or impossible. Substantial deamination can occur if the pD of the reaction mixture drops below neutrality. In order to minimize this side reaction, the exchange was done at pD 7.8. It is important that the bisulfite reaction be carried out in a tightly sealed tube because in the presence of air sulfite is oxidized to sulfate, with a concomitant decrease in pD. Ammonium bisulfite was used rather than sodium; the ammonia present participates in an exchange amination reaction that effectively competes with the attack of water at C4, thus suppressing deamination (Kai et al., 1971). Although exchange proceeds more slowly (but reaches ~95%) under these slightly basic conditions, the extent of deamination to form uracil appears to be negligible after as long as 48 h at 65 °C. In principle, the presence of uracil should be detectable in the NMR spectrum and be observable in a HPLC chromatogram.

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REFERENCES

Bodenhausen, G., Kogler, H., & Ernst, R. R. (1984) *J. Magn. Reson. 58*, 370–388.

Borah, B., Cohen, J. S., & Bax, A. (1985) *Biopolymers 24*, 747–765.

Borer, P. N. (1975) in *Handbook of Biochemistry and Molecular Biology*, 3rd ed. (Fasman, G. D., Ed.) p 589, CRC Press, Cleveland, OH.

Borer, P. N., Kan, L.-S., & Ts'o, P. O. P. (1975) *Biochemistry 14*, 4847–4863.

Broido, M. S., James, T. L., Zon, G., & Keepers, J. W. (1985) *Eur. J. Biochem. 150*, 117–128.

Chandrasegaran, S., Kan, L-S., Sillerud, L. O., Skoglund, C., & Bothner-By, A. A. (1985) *Nucleic Acids* Res. *13*, 2097–2110.

Chandrasekaran, S., Krishnamoorthy, C. R., Jones, R. L., Smith, J. C., & Wilson, W. D. (1984) *Biochem. Biophys. Res. Commun. 122*, 804–809.

Dorman, M. A., Noble, S. A., McBride, L. J., & Caruthers, M. H. (1984) *Tetrahedron 40*, 95–102.

Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1984) *J. Med. Chem. 27*, 450–465.

Gait, M. J., Ed. (1984) *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, Oxford.

Gautam-Basak, M., Jacobson, D. G., & Sanders, E. G. (1985) *Bioorg. Chem. 13*, 312–322.

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

Graves, D. E., Stone, M. P., & Krugh, T. R. (1985) *Biochemistry 24*, 7573–7581.

Gronenborn, A. M., & Clore, G. M. (1985) *Prog. Nucl. Magn. Reson. Spectrosc. 17*, 1–32.

Hare, D. R., Wemmer, D. E., Chou, S.-H., Drobny, G., & Reid, B. R. (1983) *J. Mol. Biol. 171*, 319–336.

Hayatsu, H. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 16*, 75–124.

Kai, K., Wataya, Y., & Hayatsu, H. (1971) *J. Am. Chem. Soc. 93*, 2089–2090.

Keniry, M. A., Brown, S. C., Berman, E., & Shafer, R. H. (1987) *Biochemistry 26*, 1058–1067.

Kinoshita, T., Schram, K. H., & McCloskey, J. A. (1982) *J. Labelled Compd. Radiopharm. 19*, 525–534.

Klevit, R. E., Wemmer, D. E., & Reid, B. R. (1986) *Biochemistry 25*, 3296–3303.

Kondo, N. S., Ezra, F., & Danyluk, S. S. (1975) *FEBS Lett. 53*, 213–216.

Macura, S., Huang, Y., Suter, D., & Ernst, R. R. (1981) *J. Magn. Reson. 43*, 259–281.

Maeda, M., & Kawazoe, Y. (1975) *Tetrahedron Lett.*, 1643–1646.

Maeda, M., Saneyoshi, M., & Kawazoe, Y. (1971) *Chem. Pharm. Bull. 19*, 1641-1649.
Pardi, A., Morden, K. M., Patel, D. J., & Tinoco, I., Jr. (1983) *Biochemistry 22*, 1107-1113.
Patel, D. J., Kozlowski, S. A., Rice, J. A., Broka, C., & Itakura, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7281-84.
Patel, D. J., Shapiro, L., Kozlowski, S. A., Gaffney, B. L., & Jones, R. A. (1986) *Biochemistry 25*, 1027-1036.
Pichat, L., Godbillon, J., & Herbert, M. (1973) *Bull. Chim. Soc. Fr.*, 2715-2719.
Rabi, J. A., & Fox, J. J. (1973) *J. Am. Chem. Soc. 95*, 1628-1632.
Richter, E., Loeffler, J. E., & Taylor, E. C. (1960) *J. Am. Chem. Soc. 82*, 3144-3146.
Roy, S., Papaztavros, M. Z., & Redfield, A. G. (1982) *Nucleic Acids Res. 10*, 8341-8349.
Sanchez, V., Redfield, A., Johnston, P. D., & Tropp, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5659-5662.
Weiss, M. A., Patel, D. J., Sauer, R. T., & Karplus, M. (1984) *Nucleic Acids. Res. 12*, 4305-4047.
Wemmer, D. E., Chou, S.-H., Hare, D. R., & Reid, B. R. (1984) *Biochemistry 23*, 2262-2268.
Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.

Sequential ^{1}H NMR Assignments and Secondary Structure of Hen Egg White Lysozyme in Solution†

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ABSTRACT: Assignments for ^{1}H NMR resonances of 121 of the 129 residues of hen egg white lysozyme have been obtained by sequence-specific methods. Spin systems were identified with phase-sensitive two-dimensional (2-D) correlated spectroscopy and single and double relayed coherence transfer spectroscopy. For key types of amino acid residues, particularly alanine, threonine, valine, and glycine, complete spin systems were identified. For other residues a less complete definition of the spin system was found to be adequate for the purpose of sequential assignment. Sequence-specific assignments were achieved by phase-sensitive 2-D nuclear Overhauser enhancement spectroscopy (NOESY). Exploitation of the wide range of hydrogen exchange rates found in lysozyme was a useful approach to overcoming the problem of spectral overlap. The sequential assignment was built up from 21 peptide segments ranging in length from 2 to 13 residues. The NOESY spectra were also used to provide information about the secondary structure of the protein in solution. Three helical regions and two regions of β-sheet were identified from the NOESY data; these regions are identical with those found in the X-ray structure of hen lysozyme. Slowly exchanging amides are generally correlated with hydrogen bonding identified in the X-ray structure; a number of exceptions to this general trend were, however, found. The results presented in this paper indicate that highly detailed information can be obtained from 2-D NMR spectra of a protein that is significantly larger than those studied previously.

Hen egg white lysozyme, an enzyme containing 129 amino acid residues, was one of the first proteins to be studied by NMR[1] (Meadows et al., 1967; McDonald & Phillips, 1967; Sternlicht & Wilson, 1967; Cohen & Jardetsky, 1968). At an early stage it was found that many resonances in the complex ^{1}H NMR spectrum are strongly perturbed by magnetic interactions within the folded protein (McDonald & Phillips, 1967; Sternlicht & Wilson, 1967). This results in a number of individual resonances being sufficiently well resolved to be observed separately from the mass of overlapping signals. Assignments for these resolved resonances were proposed, initially on the basis of calculations of ring-current shifts associated with aromatic residues, with the assumption that the structure of the protein in solution was identical with that defined in the crystalline state by X-ray diffraction studies (Sternlicht & Wilson, 1967; McDonald & Phillips, 1970).

The number of assignments in the spectrum of lysozyme has increased steadily over the last 15 years as higher field spectrometers have become available and as new methods for resolving and identifying resonances have been developed. Assignments have been reported for protons of some 50 of the

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[1] Abbreviations: NMR, nuclear magnetic resonance; 2-D, two dimensional; lysozyme, hen egg white lysozyme; COSY, two-dimensional *J*-correlated spectroscopy; RELAY, two-dimensional relayed coherence transfer spectroscopy; TM, trapezoidal multiplication; DM, double exponential multiplication; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; type J, amino acid residue belonging to the group consisting of Trp, Tyr, Phe, His, Asp, Asn, Cys, and Ser; type U, amino acid residue belonging to the group consisting of Lys, Arg, Met, Gln, Glu, Leu, and Ile; type X, any of the 20 common amino acid residues; $d_{\alpha N}(i,j)$, NOE connectivity between the αCH proton on residue i and the NH proton on residue j; $d_{NN}(i,j)$, NOE connectivity between the NH proton on residue i and the NH proton on residue j; $d_{\beta N}(i,j)$, NOE connectivity between the βCH proton on residue i and the NH proton on residue j; $d_{\alpha N}$, $d_{\alpha N}(i,i+1)$; d_{NN}, $d_{NN}(i,i+1)$; $d_{\beta N}$, $d_{\beta N}(i,i+1)$; $d_{\alpha P\delta}$, NOE connectivity between the αCH proton on residue i and the δCH proton on proline residue $i+1$.

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