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LETTERS TO THE EDITOR

Conformation of the DNA Undecamer 5′d(A-A-G-T-G-T-G-A-T-A-T) Bound to the Single-stranded DNA Binding Protein of *Escherichia coli*

A Time-dependent Transferred Nuclear Overhauser Enhancement Study

A time-dependent transferred nuclear Overhauser enhancement study of the conformation of the single-stranded DNA 11mer 5′d(A-A-G-T-G-T-G-A-T-A-T) bound to the single-stranded DNA binding protein of *Escherichia coli* (SSB) is presented. It is shown that the conformation of the bound 11mer is that of a right-handed *B*-type helix similar to that of the free 11mer. The observation of internucleotide transferred nuclear Overhauser enhancements for every base step excludes the possibility of intercalation by aromatic protein residues. In addition, it is shown that the effective correlation time of the bases (80 ns) corresponds to that of a complex of molecular weight ~ 170,000, containing two SSB tetramers. The sugars, on the other hand, exhibit a shorter effective correlation time (40 ns), indicating the presence of internal motion. This suggests that the bases are anchored to the protein surface, possibly by hydrophobic interactions, whereas the sugar-phosphate groups are directed outwards towards the solvent.

The single-stranded DNA binding protein of *Escherichia coli*, SSB, is a tetramer of identical subunits (overall M_r ~ 75,000), which has been shown to be essential for replication and which is also involved in recombination and repair processes (Meyer *et al.*, 1979; Glassberg *et al.*, 1979). There are no structural data available for either SSB or SSB–DNA complexes. Therefore, we decided to investigate the conformation of the single-stranded DNA oligonucleotide 5′d(A-A-G-T-G-T-G-A-T-A-T) bound to SSB using transferred nuclear Overhauser enhancement measurements to demonstrate the proximity of bound ligand protons in space (Clore & Gronenborn, 1982, 1983). This particular oligonucleotide was chosen for two reasons. First, physical studies had shown that short oligonucleotides bind weakly and non-co-operatively to SSB ($K = 10^3$ to 10^5 M^{-1}), in contrast to polynucleotides, which bind co-operatively with an apparent equilibrium constant $> 10^9$ M^{-1} (Krauss *et al.*, 1981). These weak binding conditions are ideally suited to TRNOE† measurements, which are based on the use of chemical exchange to transfer information concerning cross-relaxation between bound ligand protons from the bound state to the free state, where effects are easy to observe. Second, this 11mer had been investigated in detail by n.m.r. using NOE methods, complete resonance assignments had been obtained, and the chemical shift dispersion of its proton resonances is good (Clore & Gronenborn, 1984*a*).

As a preliminary step before carrying out the TRNOE measurements, the binding properties of the 11mer to SSB were assessed by monitoring the fluorescence quenching of SSB upon DNA binding. These experiments yielded an equilibrium constant of $5(\pm 2) \times 10^4$ M^{-1} at an ionic strength of 0·5 M. This is comparable to the value of 2×10^4 to 4×10^4 obtained for d(pT)$_8$ (Krauss *et al.*, 1981). Assuming that the association rate constant lies in the same range as that for all other oligo- and polynucleotides previously investigated, namely 5×10^7 to 5×10^8 M^{-1} s^{-1}, then the dissociation rate constant for the 11mer is of the order 10^3 to 10^4 s^{-1}.

The theory of time-dependent TRNOE has been discussed in great detail (Clore & Gronenborn, 1983), so that only the pertinent points need be summarized here. The experiment is easiest to apply when chemical exchange between bound and free ligand protons is fast on the chemical shift scale, so that only a single set of average ligand resonances is observed. This condition is satisfied in the case of the 11mer. The initial slope of the time development of the TRNOE, $N_{ij}(t)$, observed on the resonance of proton i following irradiation of the resonance of proton j for a time t is simply given by:

$$\left.\frac{dN_{ij}}{dt}\right|_{t=0} = \sigma_{ij}^{obs} = a\sigma_{ij}^{FF} + (1-a)\sigma_{ij}^{BB}, \quad (1)$$

† Abbreviations used: TRNOE, transferred nuclear Overhauser enhancement effect; NOE, nuclear Overhauser enhancement effect; n.m.r., nuclear magnetic resonance spectroscopy; p.p.m., parts per million.

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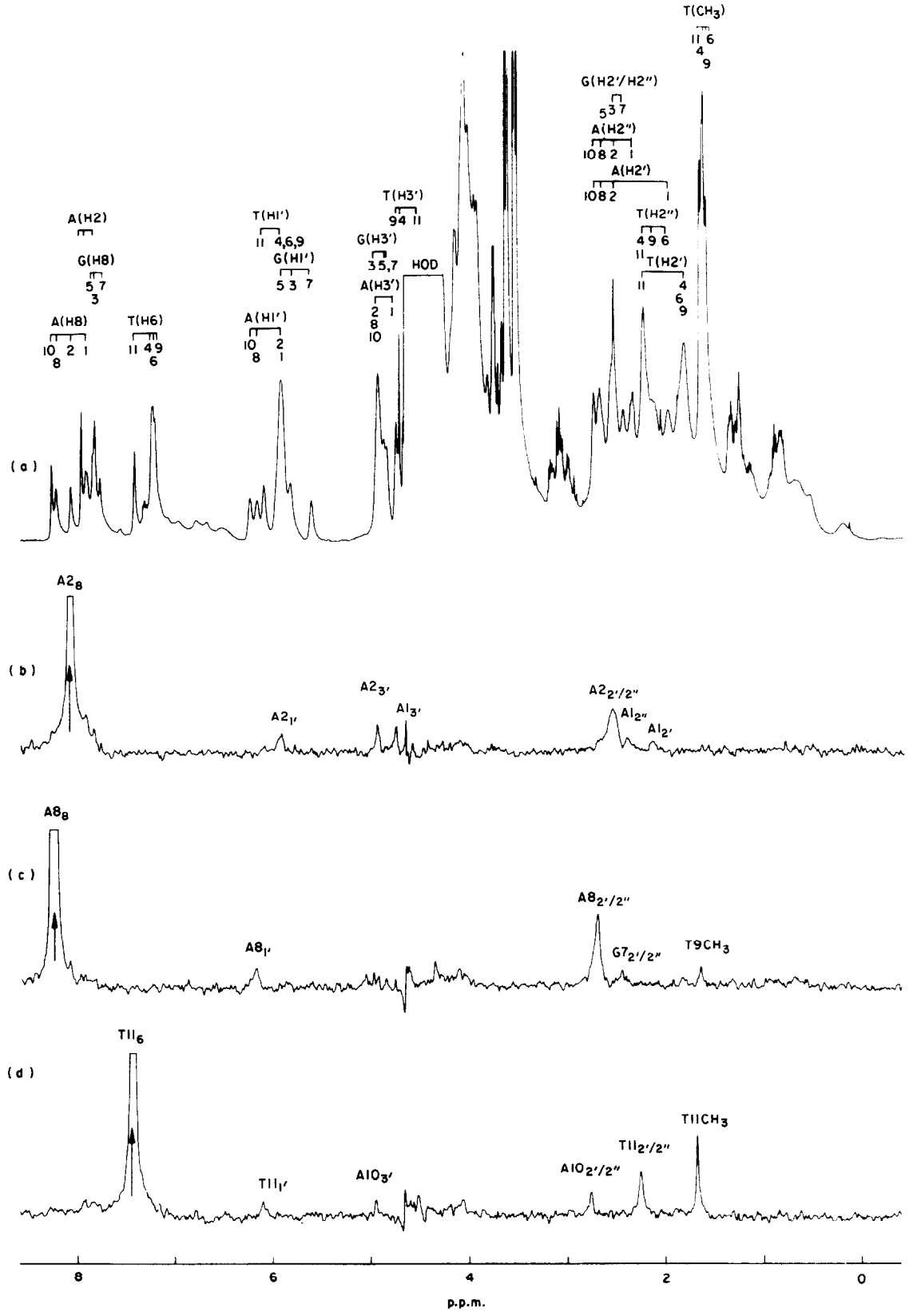

Figure 1. (a) 500 MHz ^{1}H n.m.r. spectrum of 1·45 mM-5'd(A-A-G-T-G-T-G-A-T-A-T) in the presence of 16·4 μM-SSB. TRNOE difference spectra obtained upon irradiation of (b) the A2 (H8), (c) A8 (H8) and (d) T11 (H6) resonances for 0·2 s. The experimental conditions were as follows: 1·45 mM-11mer and 16·4 μM-SSB in ^2H_2O buffer containing 20 mM potassium phosphate (pH 7·8), 500 mM-NaCl, 0·1 mM-EDTA. Temperature, 30°C. The TRNOEs were observed by directly collecting the difference free induction decay by interleaving 8 transients after saturation for a time t of a given resonance with 8 transients of off-resonance saturation applied for the same length of time. The spectra were recorded with a 90° observation pulse, a 0·5 s acquisition time and a 1 s relaxation delay. The irradiation power used was sufficient to be in the high power limit, ensuring that saturation is effectively instantaneous whilst preserving

where a is the mole fraction of free ligand, σ_{ij}^{FF} and σ_{ij}^{BB} are the cross-relaxation rates between protons i and j in the free and bound states, respectively, and σ_{ij}^{obs} is the observed cross-relaxation rate. The cross-relaxation rate σ_{ij} is given by:

$$\sigma_{ij} = \frac{\hbar^2\gamma^4}{r_{ij}^6}\left(\frac{6\tau_{eff}(ij)}{1+4\omega^2\tau_{eff}(ij)^2} - \tau_{eff}(ij)\right), \qquad (2)$$

where γ is the gyromagnetic ratio of the proton, $\hbar$ is Planck's constant divided by 2π, ω is the spectrometer frequency, r_{ij} is the distance between protons i and j, and $\tau_{eff}(ij)$ is the effective correlation time for the i–j interproton vector. It follows from equation (2) that distance ratios or distances, if one distance is known, can be obtained from the simple relationship $r_{kl}/r_{ij} = (\sigma_{ij}/\sigma_{kl})^{1/6}$, provided that the effective correlation time for the two interproton vectors is the same. It also follows from equation (2) that the sign of σ_{ij} depends on the value of the effective correlation time $\tau_{eff}(ij)$. For $\omega^2\tau_{eff}(ij)^2 < 1{\cdot}12$, σ_{ij} and the corresponding NOE terms are positive, whereas for $\omega^2\tau_{eff}(ij)^2 > 1{\cdot}12$, they are negative. In all previous applications of the TRNOE, the steady state NOEs for the free ligand were always positive, whereas pre-steady state TRNOEs are always negative, as these arise from the protein–ligand complex for which $\omega\tau_{eff} \gg 1$ (Clore & Gronenborn, 1982, 1983; Clore *et al.*, 1984; Gronenborn & Clore, 1982*a*,*b*; Gronenborn *et al.*, 1984*a*,*b*,*c*,*d*). Moreover, as the build-up rate of the free NOE for small ligands is very slow, it is a simple matter to choose an appropriate range of irradiation times at which no detectable free NOEs can be observed. In the case of the 11mer, the situation is slightly more complex, as negative NOEs are observed in the free state. Fortunately, on account of the intrinsic flexibility of single-stranded oligonucleotides, the negative NOEs for the free 11mer are very small and their time development is slow, such that for an irradiation time of 0·2 s only a handful of very small NOEs are still observable.

Figure 1(a) shows the spectrum of 1·45 mM-11mer in the presence of 16·4 μM-SSB, corresponding to a ratio of free to bound 11mer of 21, assuming that four 11mers are bound per SSB tetramer. The latter is perfectly reasonable, as previous studies have shown that ~ 50 nucleotides are bound per SSB tetramer at an ionic strength of ~ 0·5 M (Lohmann & Overman, 1985), and that the stoichiometry of binding for the similar-sized oligonucleotide $d(pT)_8$ is 4 to 1 (Krauss *et al.*, 1981). Because exchange is fast on the chemical shift scale, only a single set of exchange broadened average ligand resonances is observed, with chemical shifts approximately the same as those for the free 11mer. Examples of TRNOE difference spectra are shown in Figure 1(b) to (d). Thus, for example, irradiation of the A_8(H8) resonance results in intranucleotide TRNOEs on the A_8(H1′) (−7%) and A_8(H2′/H2″) (−21%) resonances and in internucleotide TRNOEs on the G_7(H2′/H2″) (−2%) and $T_9(CH_3)$ (−3%) resonances. Note that the build-up rates of these TRNOEs as well as all others measured are a factor of 5 or more greater than in the free state, so that the dominant contribution to the observed TRNOEs arises from cross-relaxation in the bound state. This is easily appreciated from a comparison of the time-dependence of the TRNOEs observed upon irradiation of the T_{11}(H6) resonance with that of the corresponding NOEs for the free 11mer under identical experimental conditions (Fig. 2). A summary of the bound cross-relaxation rates derived from the TRNOE measurements is given in Table 1.

Because the cross-relaxation rate σ_{ij}^{BB} is dependent on only two variables, namely the distance r_{ij} and the effective correlation time $\tau_{eff}(ij)$, the effective correlation times for vectors of fixed distance can be determined. The average cross-relaxation rates for the measured T(H6)–$T(CH_3)$ base vectors and the H2′–H2″ and H1′–H2″ sugar vectors are 10 s^{-1}, 60 s^{-1} and 16 s^{-1}, respectively. The $(\langle r^{-6}\rangle)^{-1/6}$ mean distance between the H6 and methyl protons of a thymidine residue is 2·7 Å (assuming rapid rotation of the methyl group), the distance between the H2′ and H2″ sugar protons is 1·8 Å, and the distance between the H1′ and H2″ sugar protons lies in the range 2·3±0·1 Å irrespective of sugar pucker. From these data we calculate an effective correlation time of 80 ns for the base vectors and 40 ns for the sugar vectors. The value of 80 ns corresponds to a molecular weight of ~ 170,000; that is to say, a dimer of two SSB tetramers. The smaller effective correlation time for the sugar vectors implies a degree of internal flexibility for the sugar moieties, a feature which has been observed in DNA fragments (Clore & Gronenborn, 1984*b*). These data also allow an estimation of an upper limit for distances detectable by the TRNOE measurements. The smallest TRNOE that can be observed is around −1% at an irradiation time of 0·2 s, which corresponds to a cross-relaxation rate in the bound state of ~ 1 s^{-1}. Thus, for an interproton vector with the same effective correlation time as that of the bases, the upper limit is ~ 4 Å, whereas it is ~ 3·6 Å for one with the same effective correlation time as that of the sugars.

selectivity, so that only a single resonance at a time was saturated (Dobson *et al.*, 1982; Clore & Gronenborn, 1983). In all, 1000 transients were collected for the reference spectrum and 6400 transients for each difference spectrum. The 11mer was prepared and purified as described (Clore & Gronenborn, 1984*a*). SSB protein was purified from an overproducing strain of *E. coli* carrying the *ssb*A^+ gene (Chase *et al.*, 1980) as described by Krauss *et al.* (1981). The concentration of SSB tetramer was determined by ultraviolet light spectroscopy using an extinction coefficient of 94800 M^{-1} cm^{-1} per tetramer at 280 nm (Krauss *et al.*, 1981). The resonance assignments were taken from Clore & Gronenborn (1984*a*), and the chemical shifts are given relative to 4,4-dimethylsilapentane-1-sulphonate.

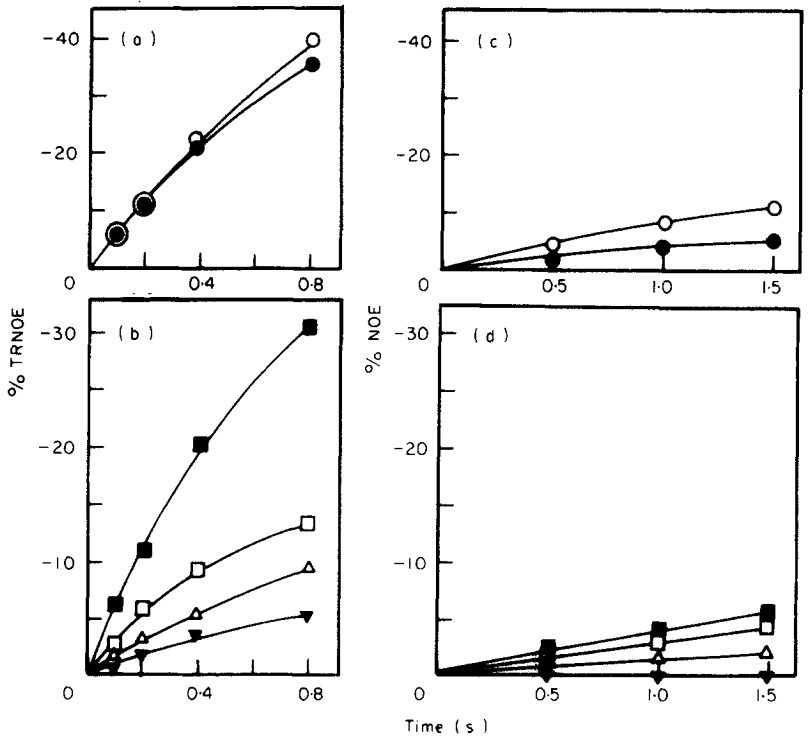

Figure 2. Time-dependence of the TRNOEs observed upon irradiation of the T11 (H6) resonance of the 11mer at a ratio of free to bound ligand of 21 ((a) and (b)) together with the time-courses for the corresponding NOEs of the free 11mer (i.e. in the absence of SSB: ((c) and (d)). The time-dependences of the TRNOEs and corresponding free NOEs between the H6 and CH_3 protons of residues T4, T6 and T9 are shown in (a) and (c), respectively. As a result of exchange broadening and limited chemical shift dispersion, the TRNOEs between the H6 and CH_3 protons of T4, T6 and T9 could not be quantified individually, so that the plotted time-dependence represents the average for the 3 TRNOEs. Nevertheless, it was obvious from the TRNOE difference spectra that the TRNOEs between the H6 and CH_3 protons of these 3 residues were approximately equal. The experimental conditions are the same as those for Fig. 1. (●) T11 (H6)–T11(CH_3) TRNOE and free NOE; (○) average TRNOE for the 3 TRNOEs involving the H6 and CH_3 protons of residues T4, T6 and T9; (○) T4(H6)–T4(CH_3), T6(H6)–T6(CH_3) and T9(H6)–T9(CH_3) free NOEs (note that the time-dependence of these 3 NOEs is identical within experimental error, so that only 1 symbol is shown for all 3); (△) T11(H6)–T11(H1′) TRNOE and free NOE; (■) T11(H6)–T11(H2′/H2″) TRNOE and free NOE; (▼) T11(H6)–A10(H3′) TRNOE and free NOE; (□) T11(H6)–A10(H2′/H2″) TRNOE and free NOE. Note the different time scales in (a) and (b) on the one hand, and (c) and (d) on the other.

Because of exchange broadening and resonance overlap, not all TRNOEs could be assigned to individual proton pairs. Nevertheless, a picture of the $(\langle r^{-6}\rangle)^{-1/6}$ average structure of the bound 11mer can be deduced that is rather similar to that of the free 11mer (Clore & Gronenborn, 1984*a*). With the exception of residue A1, the pattern of intranucleotide cross-relaxation rates

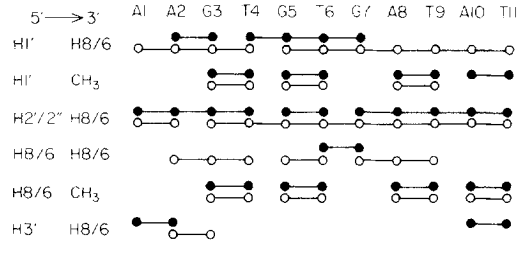

Figure 3. Summary of the internucleotide TRNOEs observed for the 11mer in the presence of SSB (●). The internucleotide NOEs observed for the free 11mer (i.e. in the absence of SSB) are shown for comparison (○).

($\sigma_{H2'/H2'' H8/H6} > \sigma_{H1' H8/H6} \sim \sigma_{H3' H8/H6}$) is indicative of an *anti* conformation about the glycosidic bond with χ in the range $-60°$ to $-100°$ and a C-5′–C-4′–C-3′–O-3′ bond torsion angle δ in the range 110° to 140°. In the case of residue A1, only a single intranucleotide sugar-base TRNOE between the H1′ and H8 protons was observed, indicative of a *syn* conformation about the glycosidic bond. At least one internucleotide TRNOE was observed for every base step and, with the exception of TRNOEs between adjacent H8/H6 base protons, all internucleotide TRNOEs exhibited directional specificity (see Fig. 3). In particular, TRNOEs were observed between the sugar protons (H1′, H2′/H2″, H3′) of a given residue and the base protons (H8/H6, CH_3) of the adjacent 3′ but not 5′ residue, and between the T(CH_3) protons of a given residue and the base protons (H8) of the adjacent 5′ but not 3′ residue. Moreover, in all cases, the intranucleotide H2′/H2″(i)–H8/H6(i) sugar-base TRNOE was larger than the corresponding internucleotide H2′/H2″($i-1$)–H8/H6(i) TRNOE. This pattern of internucleotide TRNOEs is indicative of a right-

Table 1

Cross-relaxation rates (σ_{ij}^{BB}) for the 11mer bound to SSB determined from time-dependent TRNOE measurements

A. *Intranucleotide*

Proton pair	σ_{ij}^{BB} (s^{-1}) A1	A2	G3	T4	G5	T6	G7	A8	T9	A10	T11
Sugar–sugar											
H1′–H2′	[c]		33	[a]		[a]	23	18	[a]	19	18
H1′–H2″	15			[b]		[d]			[b]		
H2′–H2″	60			[c]		60			[c]		
Sugar–base											
H1′–H8/H6	9	7	5		[d]		2	7	3	3	3
H2′–H8/H6	nd	27	[e]	[g]	[e]	[g]	13	24	[g]	19	11
H2″–H8/H6	nd			lag	lag	lag			lag		
H3′–H8/H6	nd	7	8	lag	nd	lag	3		lag	3	nd
Base–base											
H6–CH_3				10		10			10		11

B. *Internucleotide*

Proton of 5′ nucleotide	Proton of 3′ nucleotide	σ_{ij}^{BB} (s^{-1}) A1pA2	A2pG3	G3pT4	T4pG5	G5pT6	T6pG7	G7pA8	A8pT9	T9pA10	A10pT11
H1′	H8/H6	nd	[d]	nd	[d]	[f]	1	nd	lag	lag	lag
H1′	CH_3			4		3			2		1
H2′	H8/H6	4	[e]	[i]	nd	[i]	nd	3	[h]	nd	6
H2″	H8/H6	4			nd	[h]	nd			1	
H8/H6	H8/H6	nd	nd	lag	lag	lag	1	nd	nd	nd	nd
H8/H6	CH_3			6		4			6		2
H3′	H8/H6	5									2

In calculating the bound cross-relaxation rates from the observed cross-relaxation rates using eqn (1), the contribution arising from cross-relaxation in the free state was determined from the data given by Clore & Gronenborn (1984*a*). The ratio of free to bound ligand used in the TRNOE experiments was 21, and the experimental conditions are given in the legend to Fig. 1. nd, not detectable.

[a] The H1′ and H2′ resonances of T4, T6 and T9 are superimposed at ~ 5·9 and ~ 1·8 p.p.m., respectively. The observed cross-relaxation rate between these 2 sets of resonances is 1·2 s^{-1}, corresponding to an average of 8 s^{-1} per H1′–H2′ vector in the bound state.

[b] The H1′ and H2″ resonances of T4 and T9 are superimposed at ~ 5·9 p.p.m. and ~ 2·3 p.p.m. The observed cross-relaxation rate between these 2 sets of resonances is 1·5 s^{-1}, corresponding to an average of 15 s^{-1} per H1′–H2″ vector in the bound state.

[c] The A1(H2″) and T6(H2″) resonances are superimposed at ~ 2·2 p.p.m. and the A1 (H1′) and T6 (H1′) resonances at ~ 6·0 p.p.m. The observed cross-relaxation rate between these 2 sets of resonances is 1·9 s^{-1}.

[d] The H8 resonances of G3 and G5 are superimposed at ~ 7·9 p.p.m. and the A2 (H′), T4 (H1′) and G5 (H1′) resonance at ~ 5·9 p.p.m. The observed cross-relaxation rate between these 2 sets of resonances is 0·45 s^{-1}.

[e] The A2 (H2′), G3 (H2′), G3 (H2″) and G5 (H2′) resonances are superimposed at ~ 2·6 p.p.m. The observed cross-relaxation between this set of resonances and the G3(H8)/G5(H8) resonances is 1·3 s^{-1}.

[f] The H6 resonances of T4 and T6 are superimposed at ~ 7·2 p.p.m., and the H1′ resonances of T4, G5 and T6 at ~ 6·0 p.p.m. The observed cross-relaxation rate between these 2 sets of resonances is 0·4 s^{-1}.

[g] The H6 resonance of T9 is superimposed on those of T4(H6) and T6(H6). The H2′ resonances of T4, T6 and T9 are superimposed at ~ 1·8 p.p.m. The observed cross-relaxation between these 2 sets of resonances is 1·3 s^{-1}.

[h] The G5 (H2″), A8 (H2′) and A8(H2″) resonances are superimposed at ~ 2·7 p.p.m. The observed cross-relaxation rate between this set of resonances and the T6 (H6)/T9 (H6) resonance is 0·2 s^{-1}.

[i] The G3 (H2′), G3 (H2″) and G5 (H2′) resonances are superimposed at ~ 2·6 p.p.m. The observed cross-relaxation rate between this set of resonances and the T4 (H6)/T6 (H6) resonances is 0·2 s^{-1}.

handed *B*-type helix with stacked bases (Gronenborn *et al.*, 1984*e*; Clore & Gronenborn, 1985; Gronenborn & Clore, 1985).

What do our TRNOE results imply for the structure of the SSB–11mer complex? First, intercalation of aromatic amino acids between the bases of the DNA can be excluded, as internucleotide NOEs are observed at every single-base step. Second, the finding that the mobility of the sugars is higher than that of the bases suggests that the bases are anchored to the protein by direct interaction with amino acid residues at the protein surface, whereas the sugar-phosphate groups are directed outwards towards the solvent. This configuration would imply that the complex is stabilized principally by hydrophobic interactions. Such a mechanism would account for the fact that the equilibrium constant for the binding of both oligo- and polynucleotides to SSB is only weakly dependent on ionic strength, ruling out a dominant role for electrostatic interactions (Krauss *et al.*, 1981). Hydrophobic interactions have been proposed to make the major contribution to the stability of two other single-stranded DNA binding

protein complexes. In particular, stacking of aromatic protein residues upon the adenyl five-membered ring in gene 5 protein–oligo(dA) complexes has been demonstrated directly by means of NOE measurements (Alma *et al.*, 1983) and has been implicated on the basis of chemical shift changes in the case of gene 32 protein (Prigodich *et al.*, 1984). Interestingly d(pA) oligonucleotides bind more tightly to gene 5 protein than do d(pT) oligonucleotides (Coleman *et al.*, 1976), whereas the reverse is true for SSB (Krauss *et al.*, 1981). This may suggest a different type of hydrophobic interaction in the case of SSB, perhaps one principally involving the interaction of methyl-group-containing side-chains rather than aromatic ones with the bases of the DNA. Moreover, such a layer of protein methyl groups may provide an ideal surface for the high-speed translocation of SSB along the single-stranded DNA template (Romer *et al.*, 1984).

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