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Kinetics of DNA Hydration

Vladimir P. Denisov[1]*, Göran Carlström[2], Kandadai Venu[1] and Bertil Halle[1]

[1]*Condensed Matter Magnetic Resonance Group and*
[2]*Division of Physical Chemistry 2 Department of Chemistry Lund University, P.O. Box 124 S-22100, Lund, Sweden*

**Corresponding author*

The hydration of the d(CGCGAATTCGCG) *B*-DNA duplex in solution was studied by nuclear magnetic relaxation dispersion (NMRD) of the water nuclei ^{1}H, ^{2}H, and ^{17}O, and by nuclear Overhauser effects (NOEs) in high-resolution two-dimensional ^{1}H NMR spectra. By comparing results from the free duplex with those from its complex with netropsin, water molecules in the ''spine of hydration'' in the AATT region of the minor groove could be distinguished from hydration water elsewhere in the duplex. The ^{2}H and ^{17}O relaxation dispersions yield a model-independent residence time of 0.9(±0.1) ns at 4°C for five highly ordered water molecules in the spine. When corrected for frequency offset effects, the NOE data yield the same residence time as the NMRD data, giving credence to both methods. At 27°C, the residence time is estimated to 0.2 ns, a factor of 40 shorter than the tumbling time of the duplex. The NMRD data show that all water molecules associated with the duplex, except the five molecules in the spine, have residence times significantly shorter than 1 ns at 4°C. There is thus no long-lived hydration structure associated with the phosphate backbone. In contrast to ^{2}H and ^{17}O, the ^{1}H relaxation dispersion is dominated by labile DNA protons and therefore provides little information about DNA hydration.

Keywords: *B*-DNA hydration; water residence time; netropsin; oxygen-17 relaxation dispersion; NMR spectroscopy

Introduction

Water has long been recognized as an important determinant of nucleic acid structure (Franklin & Gosling, 1953); the stability, polymorphism and flexibility of the DNA double helix are all hydration-dependent (Saenger, 1987; Westhof, 1988, 1993; Beveridge *et al.*, 1993; Berman, 1994; Kochoyan & Leroy, 1995). Moreover, specific hydration patterns seem to play a role in nucleotide sequence recognition by proteins (Otwinowski *et al.*, 1988; Robinson & Sligar, 1993; Qian *et al.*, 1993; Shakked *et al.*, 1994; Clore *et al.*, 1994; Eisenstein & Shakked, 1995) and drugs (Kopka *et al.*, 1985a; Chalikian *et al.*, 1994).

The most prominent feature of the hydration of *B*-DNA duplexes is a chain of water molecules penetrating deeply into the minor groove. This spine of hydration was first observed in the crystal structure of the *B*-DNA duplex of the self-complementary dodecamer d(CGCGAATTCGCG) (Drew & Dickerson, 1981; Kopka *et al.*, 1983; Westhof, 1987), the central portion of which constitutes the binding site for the restriction endonuclease *Eco*RI. Subsequently, a similar spine of hydration has been found in narrow minor groove regions in a variety of oligonucleotide duplexes (Privé *et al.*, 1987; Narayana *et al.*, 1991; Quintana *et al.*, 1992; Lipanov *et al.*, 1993; Berman, 1994). While information about the hydration structure of DNA has come mainly from crystallography, several recent studies by high-resolution ^{1}H NMR spectroscopy have confirmed that a spine of hydration exists also in solution (Kubinec & Wemmer, 1992; Liepinsh *et al.*, 1992; Maltseva *et al.*, 1993; Fawthrop *et al.*, 1993; Liepinsh *et al.*, 1994; Wang & Patel, 1994; Jacobson *et al.*, 1996).

It is frequently stated that water is an integral part of nucleic acid structure (Westhof, 1988). In a thermodynamic sense, the spine of hydration in *B*-DNA is probably analogous to the internal hydration of globular proteins (Baker, 1995). In both

Present address: K. Venu, School of Physics, University of Hyderabad, Hyderabad-500046, India.

Abbreviations used: NMRD, nuclear magnetic relaxation dispersion; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy in the laboratory frame; ROESY, two-dimensional NOE spectroscopy in the rotating frame; BPTI, bovine pancreatic trypsin inhibitor.

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cases, the water molecules seem to be an essential component of the native macromolecular structure. In a kinetic sense, however, the spine of hydration in *B*-DNA is expected to be less ''integral'' than water molecules buried inside proteins. Unlike internal water molecules in proteins, the water molecules in the minor groove of DNA are exposed to the external solvent medium and should therefore exchange faster with bulk water. A quantitative characterization of the kinetics of DNA hydration is important for at least two reasons. First, if sufficiently slow, water exchange may become the rate-limiting step in the binding of drugs and proteins to DNA. Second, as in proteins (Denisov *et al*., 1996), water exchange may be coupled to DNA fluctuations and may then provide information about sparsely populated conformational substates of relevance for the replication and transcription events of double-stranded DNA.

Whereas time-averaged crystal structures provide no direct dynamic information, the kinetics of macromolecular hydration in solution can be studied by two complementary NMR techniques: two-dimensional nuclear Overhauser effect (NOE) spectroscopy (Otting & Wüthrich, 1989; Otting & Liepinsh, 1995) and nuclear magnetic relaxation dispersion (NMRD; Denisov & Halle, 1995a, 1996). The NOE method can provide an upper or lower bound on the residence time (inverse exchange rate) of water molecules in localized hydration sites. The NMRD method provides upper and lower bounds on the residence time that are generally much more restrictive than those derived from NOEs. To localize hydration water molecules, however, it is necessary to perform a difference-NMRD experiment involving a locally modified macromolecule, such as a mutant form or a macromolecule-ligand complex (Denisov *et al*., 1995, 1996; Denisov & Halle, 1995c). If hydrogen exchange contributions are negligible or under experimental control, a comparison of relaxation data from the three magnetic water isotopes, ^{1}H, ^{2}H and ^{17}O, can significantly enhance the information content of the NMRD experiment (Denisov & Halle, 1995c, 1996).

The principal objective of the present study is to determine the residence time of the water molecules in the spine of hydration of the dodecamer duplex d(CGCGAATTCGCG) in aqueous solution. From previous NOE studies of this dodecamer, lower bounds on the residence time in the range 1 to 10 ns have been reported (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992). An upper bound of order 10 ms is also implicit from the rate of base-pair opening (Guéron & Leroy, 1995).

We report here the water ^{1}H, ^{2}H and ^{17}O relaxation dispersion profiles from an aqueous solution of the dodecamer duplex at 4°C, the first application of multinuclear NMRD to nucleic acids. By measuring the same dispersion profiles after addition of the minor groove binding drug netropsin, we could use the difference-NMRD approach to isolate the contribution from the spine of hydration.

In addition, we recorded NOESY and ROESY spectra from the same DNA preparations, with and without netropsin. Usually, the NOE and NMRD methods can only provide bounds on the residence time. The dynamic parameters of the investigated DNA solution, however, are in a range where the residence time can actually be pinpointed by either method. We are thus in the fortunate situation where the two methods can be directly compared in a quantitative way. Reassuringly, we find that the NOESY and ROESY cross-peak intensities are consistent with the residence time, 0.9(±0.1)ns at 4°C, deduced from the ^{2}H and ^{17}O difference-NMRD profiles. This residence time is an order of magnitude shorter than the tumbling time of the duplex and, at room temperature, it is reduced to a few hundred picoseconds. Taking into account the temperature dependence of the residence time, our result is not entirely consistent with the previously reported lower bounds (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992).

In contrast to the ^{2}H and ^{17}O NMRD profiles, the ^{1}H profile exhibits a dominant low-frequency dispersion, not affected by netropsin. This we ascribe, not to hydration water, but to rapidly exchanging DNA protons. In addition, we observe a high-frequency ^{1}H dispersion in a range (>100 MHz) not accessible with ^{2}H or ^{17}O. This dispersion appears to be due to internal motion at DNA sites containing labile protons. The ^{1}H dispersion therefore provides very little information about DNA hydration, despite recent claims to the contrary (Zhou & Bryant, 1996).

Results and Discussion

Structural reference data

As a reference point for the discussion of our NMR data, it is helpful to briefly recall the crystal structures of the hydrated d(CGCGAATTCGCG) duplex (Drew & Dickerson, 1981; Kopka *et al*., 1983; Westhof, 1987) and its complex with netropsin (Kopka *et al*., 1985a,b; Sriram *et al*., 1992; Goodsell *et al*., 1995). While the solution structure is surely not identical with the crystal structure, there appears to be no significant differences in the narrow AATT region of the minor groove (Nerdal *et al*., 1989).

Figures 1 and 2 show the five water molecules in the spine of hydration, located in the narrow AATT region of the minor groove. Each of these water molecules donates two hydrogen bonds to thymine 2O or adenine 3N atoms, and accepts two weaker hydrogen bonds from a less structured secondary hydration chain on top of the spine. Figure 2 also shows the complex with the antitumor antibiotic netropsin. The netropsin molecule fits snugly into the central part of the minor groove, covering nearly six base-pairs and displacing the spine of hydration. The three amide nitrogen atoms of netropsin replace the oxygen atoms of the three central water molecules of the spine,

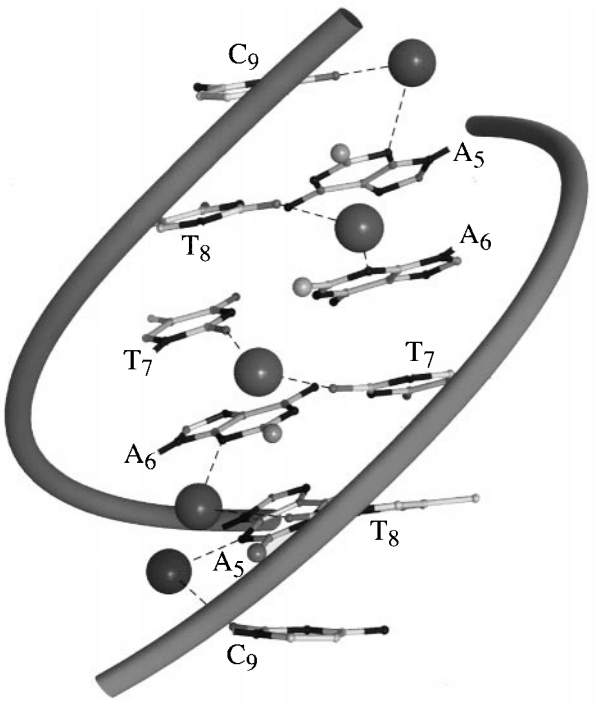

Figure 1. The spine of hydration in the central part of the minor groove of the d(CGCGAATTCGCG) duplex, displayed with GRASP (Nicholls *et al.*, 1991). For clarity, only the five water molecules (large dark spheres), the nucleotide bases, and the phosphate backbone (tubes) are shown. The broken lines represent hydrogen bonds between water molecules and the 2O and 3N atoms of the bases. The coordinates of the four adenine 2H protons (small grey spheres) were added to the PDB file 4BNA.

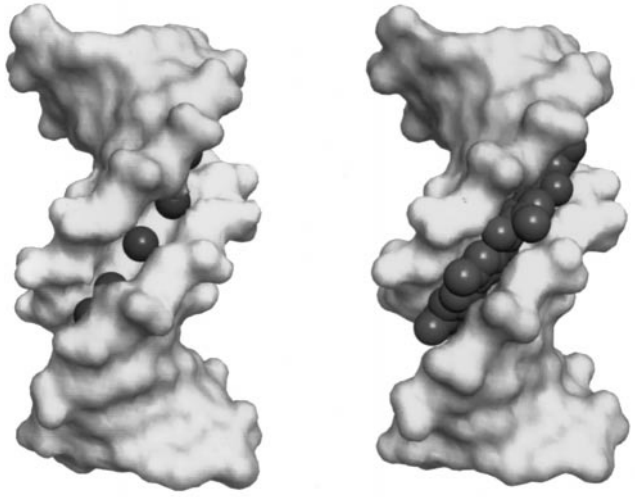

Figure 2. Crystal structures of the d(CGCGAATTCGCG) duplex (left) and its complex with netropsin (right), displayed with GRASP (Nicholls *et al.*, 1991) using atomic coordinates from the PDB files 4BNA and 101D, respectively. The oxygen atoms of the five water molecules in the spine of hydration and the netropsin molecule are shown in dark. In the duplex, AT base-pairs have a lighter shading than CG pairs.

forming bifurcated hydrogen bonds with thymine 2O and adenine 3N atoms.

In solution, netropsin binds with an association constant of ca 10^7 M^{-1} to *B*-DNA duplexes with at least four consecutive AT base-pairs (Rentzeperis & Marky, 1993). The mode of binding appears to be the same in solution (Patel, 1982) as in the crystal (Kopka *et al.*, 1985a,b; Sriram *et al.*, 1992; Goodsell *et al.*, 1995).

NOEs between DNA and water. Methodological background

As first shown by Otting & Wüthrich (1989), localized hydration sites on macromolecules in solution can under favorable conditions be indirectly detected *via* a relaxation-induced magnetization transfer between nearby dipole-coupled macromolecular and water protons. This intermolecular magnetization transfer, or nuclear Overhauser effect (NOE), is manifested as cross-peaks in two-dimensional ^{1}H NOE spectra. The NOE method has been aplied to the d(CGCGAATTCGCG) duplex in two previous studies (Kubinec & Wemmer, 1992; Liepinsh *et al.*, 1992). To enable a direct comparison with the NMRD results reported here, we have repeated the NOE experiments on our samples. The same NOE experiments were performed on the netropsin complex, to ensure that the drug displaces the spine of hydration in our solutions. These experiments also provide information about exchange rates of labile DNA protons, required for the analysis of the ^{1}H NMRD results.

Briefly, the analysis of NOE experiments is based on the following considerations (Macura & Ernst, 1980; van de Ven *et al.*, 1988; Otting & Liepinsh, 1995). Cross-peaks at the bulk water chemical shift are either intermolecular, due to hydration water or labile DNA protons exchanging with bulk water protons, or intramolecular, due to a pair of DNA protons one of which is accidentally degenerate with the bulk water resonance. The magnetization transfer responsible for an intermolecular cross-peak is either induced by direct exchange of a labile DNA proton or by dipolar cross-relaxation between a non-labile DNA proton and an exchanging DNA or water proton.

Direct exchange peaks can be distinguished from exchange-relayed NOE peaks by their relative signs in NOESY and ROESY spectra: exchange peaks are always positive (as the diagonal peaks), while NOE peaks are negative in ROESY. The sign of NOE peaks in NOESY spectra depends on the rate of fluctuation of the magnetic dipole coupling (Macura & Ernst, 1980). If the time correlation function of the intermolecular dipole-dipole coupling decays exponentially, the cross-peak intesity changes sign from negative to positive when the correlation time exceeds $\sqrt{5}/(4\pi\nu_0)$, i.e. 0.3 ns for

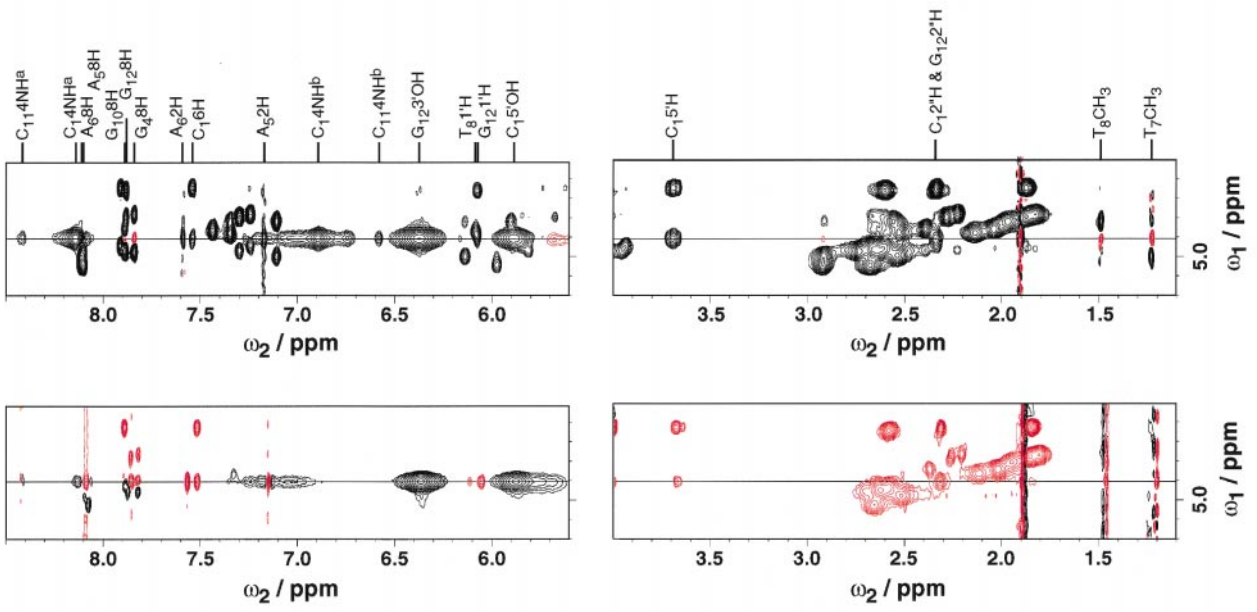

Figure 3. Identical strips from the two-dimensional ^{1}H NOESY (top) and ROESY (bottom) spectra recorded at 10°C from a 4.7 mM aqueous solution (10% ^2H_2O) of d(CGCGAATTCGCG) duplex at pH 7.0. The mixing time was 60 ms in NOESY and 30 ms in ROESY. The strips are centered around the bulk water chemical shift, 4.91 ppm, in the indirect (ω_1) dimension. Positive and negative peak amplitudes are shown in black and red, respectively. Resonance assignments are indicated with one-letter nucleotide symbols with the sequence position as subscript. The same enumeration is used for both strands in the symmetric duplex.

$\nu_0 = 600$ MHz. The quantitative interpretation of cross-peak intensities may be complicated by local motions (Otting *et al.*, 1991b; Brüschweiler & Wright, 1994), spin diffusion (Macura & Ernst, 1980; Borgias *et al.*, 1990; Withka *et al.*, 1991), and frequency offsets (Griesinger & Ernst, 1987). Finally, we recall that exchange-relayed NOE peaks from hydration water and from labile DNA protons cannot in general be distinguished without independent information about the three-dimen-

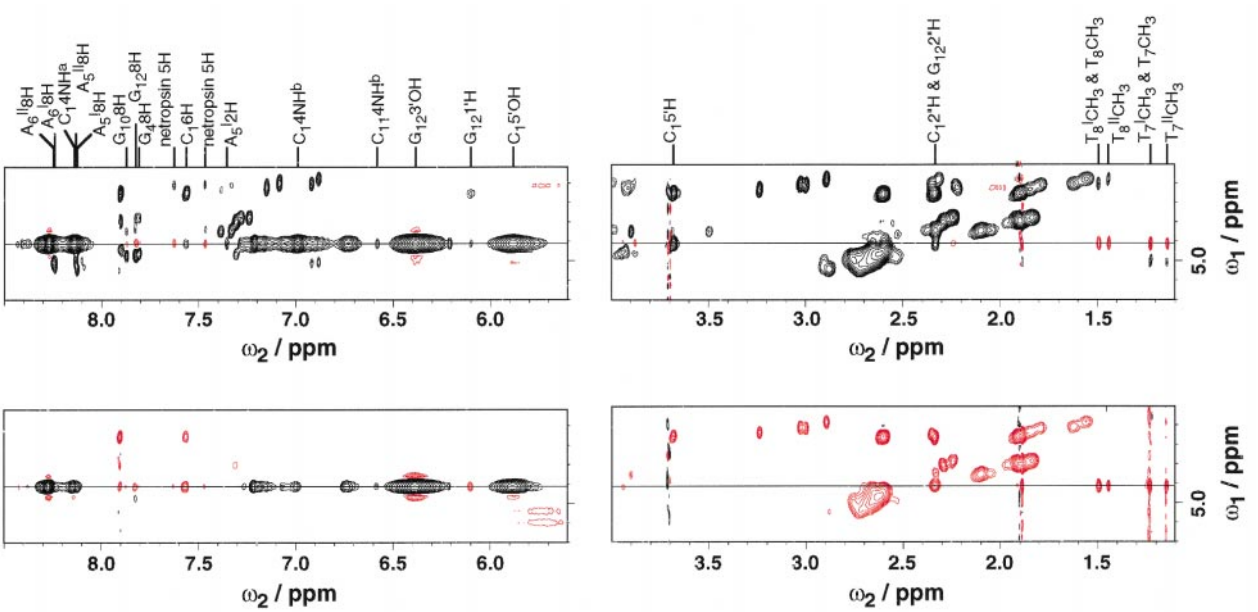

Figure 4. Identical strips from the two-dimensional ^{1}H NOESY (top) and ROESY (bottom) spectra recorded at 10°C from a 3.3 mM aqueous solution (7% ^2H_2O) of d(CGCGAATTCGCG) duplex at pH 7.0 after addition of ca one equivalent of netropsin. The mixing time was 60 ms in NOESY and 30 ms in ROESY. The strips are centered around the bulk water chemical shift, 4.91 ppm, in the indirect (ω_1) dimension. Positive and negative peak amplitudes are shown in black and red, respectively. Resonance assignments are indicated with one-letter nucleotide symbols with the sequence position as subscript. The two non-equivalent strands of the duplex are distinguished by superscripts I and II.

sional structure of the DNA duplex and/or about exchange rates (van de Ven *et al*., 1988).

NOE results for the free duplex

The spectral regions around the bulk water chemical shift ($\omega_1 = 4.91$ ppm) of the two-dimensional NOESY and ROESY spectra from solutions of the d(CGCGAATTCGCG) duplex and its complex with netropsin are shown in Figures 3 and 4, respectively. The full NOESY spectra (not shown) were used for the sequential assignments of the DNA protons in the free duplex and in the complex, following standard assignment procedures (Wijmenga *et al*., 1993). For the free duplex, there is a good overall agreement with the spectra recorded by Liepinsh *et al*. (1992) under near-identical instrumental conditions but a fourfold lower DNA concentration. Since these authors presented a rather detailed analysis of the relevant cross-peaks, we focus on the most important features and the few differences.

Cross-peaks that are negative in NOESY and ROESY are seen for the methyl protons of T7 and T8, the 8H proton of A6, and several guanine 8H protons, indicating short-lived hydration sites in the major groove (Liepinsh *et al*., 1992). Cross-peaks that are positive in NOESY and negative in ROESY are obtained with the adenine 2H protons of A5 and A6 and with the 6H proton of C1. Whereas the adenine 2H cross-peaks were seen in both previous studies and ascribed to long-lived (>0.3 ns) hydration sites in the minor groove (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992), the C1 6H cross-peak was observed only by Kubinec & Wemmer (1992), who ascribed it to an additional long-lived hydration site. We feel that this is rather unlikely, since stable, narrow water pockets are not expected at the frayed ends of the duplex. As an alternative interpretation, we suggest an exchange-relayed NOE *via* the terminal hydroxyl proton C1 5′OH. An exchange-relayed NOE with a terminal hydroxyl proton was also proposed as the mechanism for the cross-peak with G12 2″H and/or C1 2″H (Liepinsh *et al*., 1992), evident in Figure 3.

As did Liepinsh *et al*. (1992), we observed several direct exchange peaks due to labile DNA protons that exchange with bulk water on the timescale of the mixing time τ_m (60 ms in NOESY, 30 ms in ROESY). The strongest exchange peaks, corresponding to the highest exchange rates, are due to the four terminal hydroxyl protons, C1 5′OH and G12 3′OH, and to the four outermost guanine imino protons, G12 1NH and G2 1NH (not shown in Figure 3). Weaker exchange peaks are observed from the eight cytosine amino protons, C1 $4NH_2$ and C11 $4NH_2$, and from several imino protons (not shown). Exchange peaks from guanine amino protons are absent due to strong line broadening from 180° flips around the 2C-N bond (Liepinsh *et al*., 1992). We conclude, therefore, that roughly half of the 56 labile protons in the duplex exchange on the timescale 1 to 100 ms at 10°C. The exchange times can be estimated more accurately from the lifetime broadening (Abragam, 1961) of the exchange peaks in the ω_2 dimension. The observed linewidths are in the range 30 to 60 Hz. Making a relatively small correction for the natural linewidth, estimated from interproton distances in the crystal structure 1BNA (with hydrogens added), we thus obtain exchange times of ca 5 ms for the hydroxyl protons and ca 10 ms for the imino and amino protons.

Residence time for spine of hydration from NOE data

Whether a long residence time should be ascribed to all or only to some of the five water molecules in the spine of hydration is not immediately clear from the NOE data. The symmetry of the duplex, however, implies that there must be two statistically equivalent pairs among the five water molecules in the spine. Moreover, the duplex structure suggests that the central water molecule should have the longest residence time. In the crystal structure 1BNA (Drew & Dickerson, 1981), each of the four adenine 2H protons is within 2.5 to 3.5 Å of four water protons belonging to two of the five water molecules in the spine (Figure 1). Since the central water molecule has NOE contacts only with A6 (and its symmetry-related partner), the observation of a cross-peak with A5 shows that there must be three or five long-lived water molecules.

An effective residence time for these water molecules may be estimated from the ratio, $\rho = I_{NOESY}/I_{ROESY}$, of the integrated intensities (volumes) of the 2D cross-peaks in the NOESY and ROESY spectra. In the initial rate approximaion (*vide infra*), and with the carrier frequency on the water resonance, the intensity ratio is given by (Macura & Ernst, 1980; Bothner-By *et al*., 1984; Ernst *et al*., 1987):

$$\rho = \kappa_{off} \frac{\tau_m^{NOESY}}{\tau_m^{ROESY}} \frac{6j_D(2\omega_0) - j_D(0)}{2j_D(0) + 3j_D(\omega_0)} \qquad (1)$$

where the factor κ_{off} corrects for frequency offset effects on the ROESY cross-peak intensity (see Materials and Methods). Further, $j_D(\omega)$ is the reduced spectral density function for the intermolecular magnetic dipole coupling between water and DNA protons (Abragam, 1961). Unless otherwise noted we assume that the dipolar time correlation function decays exponentially, so that the spectral density function takes the simple Lorentzian form $j_D(\omega) = \tau_C/(1 + \omega^2\tau_C^2)$. Furthermore, we assume that the correlation time τ_C is related to the water residence time τ_W and the effective rotational correlation time τ_R of the DNA duplex as:

$$1/\tau_C = 1/\tau_W + 1/\tau_R \qquad (2)$$

The use of a Lorentzian spectral density function with a correlation time given by equation (2) is based on two simplifying assumptions. First, the

dynamic effects of any local rotational and translational motions are neglected. (The static effect of local motional averaging of the dipole coupling, however, can be incorporated through a generalized order parameter.) The (averaged) dipole coupling is thus treated as effectively intramolecular, being modulated only by the rotational diffusion of the DNA duplex and by the exchange event. The latter is modeled as a Markovian jump process between two discrete states (a hydration site and a bulk region). While reasonable for the highly ordered and relatively long-lived water molecules in the spine, the neglect of local motions is probably not justified for the more mobile water molecules in the major groove and elsewhere on the DNA surface (*vide infra*).

The second simplification is the neglect of the anisotropy in the rotational diffusion of the DNA duplex. This motion is well described by hydrodynamic theory (Tirado & Garcia de la Torre, 1980), modeling the duplex as a rigid cylinder of radius 10 Å and length 12 × 3.4 Å (Nuutero *et al*., 1994). Using the viscosity of H_2O at 10°C, we obtain for the three second-rank rotational correlation times of a symmetric top (Woessner, 1962): 8.1, 6.7 and 4.4 ns. Since these differ by a factor of less than 2 and since each cross-peak is due to several intermolecular NOEs (four in the case of A5 and A6 2H) with differently oriented H-H vectors, little error is introduced by using a Lorentzian spectral density function and taking the rotational correlation time in equation (2) as the average of the three symmetric-top correlation times, i.e. $\tau_R = 6.4$ ns. This value applies to infinite dilution, however. At a concentration similar to ours, but in the presence of 0.2 M NaCl and at pH 8.4, direct and hydrodynamic (to a lesser extent) interactions retard rotational diffusion by ca 40% (Nuutero *et al*., 1994). We thus arrive at a best estimate of $\tau_R = 9$ ns for the effective rotational correlation time at 10°C in the sample used for NOE experiments on the free duplex.

We focus now on the most accurately defined adenine 2H cross-peak, that with A6 2H, which reflects NOEs with the three central water molecules of the spine (Figure 1). Integrating the NOESY and ROESY 2D peaks, we obtain $\rho = -0.87 \pm 0.05$. The offset factor for the compensated ROESY sequence used here, calculated as described in Materials and Methods with a resonance offset for A6 2H of 2.7 ppm (Figure 3), is $\kappa_{off} = 1.48$. Figure 5 shows the variation of the intensity ratio ρ with the correlation time τ_C, as predicted by equation (1) for the mixing times used here. If local motions are neglected, the experimental ρ value corresponds to $\tau_C = 0.61(\pm 0.04)$ns. Since this is an order of magnitude shorter than τ_R, equation (2) shows that τ_C can be identified with the water residence time τ_W.

If we allow for fast local motion with effective correlation time τ_{loc} and orientational order parameter A_D, the spectral density function takes the form of a Lorentzian plus a constant (Halle & Wennerström, 1981; Lipari & Szabo, 1982):

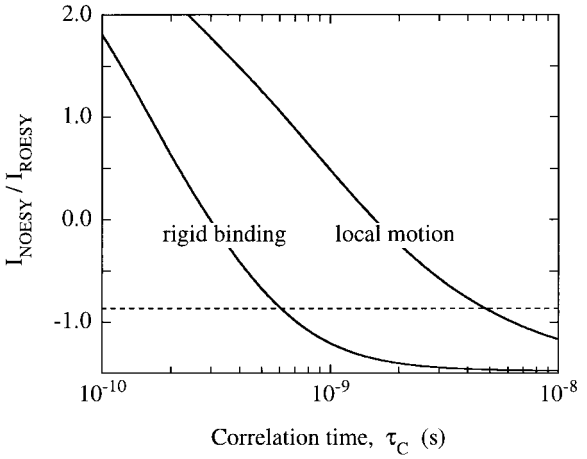

Figure 5. Ratio of NOESY and ROESY cross-peak intensities *versus* the correlation time τ_C, calculated from equation (1) with $\tau_m^{NOESY} = 2\tau_m^{ROESY}$ and $\kappa_{off} = 1.48$. The two curves correspond to rigid binding ($A_D = 1$) and to a local motion with $A_D = 0.5$ and $\tau_{loc} = 100$ ps. The horizontal line represents the experimental intensity ratio for the A6 2H protons.

$j_D(\omega) = (1 - A_D^2)\tau_{loc} + A_D^2\tau_C/(1 + \omega^2\tau_C^2)$. The upper curve in Figure 5, obtained by inserting this spectral density function in equation (1), shows that neglect of local motions can lead to an order-of-magnitude underestimation of the residence time. It is evident from Figure 5 that for residence times longer than 1 to 2 ns (with local motions neglected), only a lower bound on τ_W can be obtained from NOE data.

Neglect of spin diffusion in the analysis of NOESY cross-peak intensities can lead to serious errors in derived DNA solution structures (Borgias *et al*., 1990; Withka *et al*., 1991). We therefore checked the accuracy of the initial rate approximation, used to obtain equation (1), for the cross-peaks of interest here. Using interproton distances from the crystal structure 1BNA (with hydrogen atoms added), the NOESY and ROESY cross-peak intensities were calculated from the full relaxation matrix (Macura & Ernst, 1980; Borgias *et al*., 1990; Withka *et al*., 1991) for the proton cluster comprising A6 2H, A6 $6NH_2$, T7 3NH, and two protons belonging to a water molecule in the spine. The correlation time for dipole couplings involving at least one water proton is given by equation (2) with $\tau_R = 9$ ns and $\tau_W = 0.6$ ns, whereas dipole couplings between two DNA protons are modulated only by DNA tumbling ($\tau_C = 9$ ns). The calculations show that although the individual NOESY and ROESY cross-peak intensities are overestimated by ca 20% in the initial rate approximation, this error nearly cancels out in the intensity ratio, ρ, which is accurate to better than 1%. The errors introduced by the initial rate approximation are due mainly to auto-relaxation of the adenine 2H and water protons, rather than to sequential magnetization transfer (''spin diffusion''). The surprisingly high level of accuracy

of the initial rate approximation results from two fortunate circumstances. First, with $\tau_W \ll \tau_R$ the auto-relaxation and cross-relaxation times involving water protons are much longer than the mixing times used here. In contrast, for $\tau_W \gg \tau_R$ (as for buried water molecules in proteins), the cross-peak intensities would be overestimated by a factor of 3. Second, the modest errors in the cross-peak intensities nearly cancel out in the ρ ratio, since the two-fold shorter mixing time in ROESY is nearly compensated by the larger (transverse) auto-relaxation rate in ROESY. (In the limit $\omega_0\tau_C \gg 1$, the ROESY rate exceeds the NOESY rate by a factor of 5/2.) Although the error increases somewhat if the spin cluster is enlarged, it seems safe to conclude that the initial rate approximation underestimates the magnitude of ρ by at most 10%. If local motions are neglected, the best estimate of the residence time from the NOE data is then 0.6 (±0.1)ns at 10°C (Figure 5).

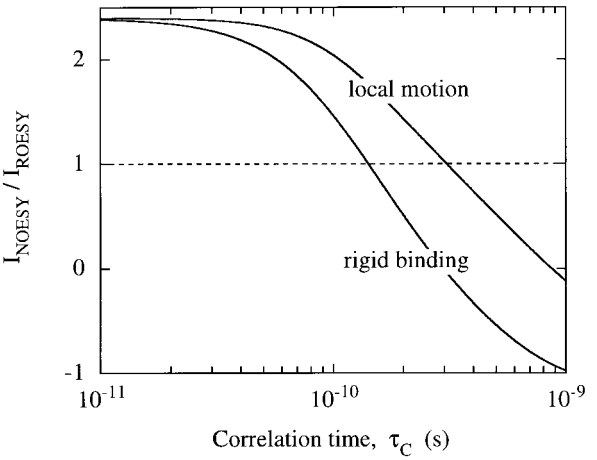

Figure 6. Ratio of NOESY and ROESY cross-peak intensities *versus* the correlation time τ_C, calculated from equation (1) with $\tau_m^{NOESY} = 2\tau_m^{ROESY}$ and $\kappa_{off} = 1.20$. The two curves correspond to rigid binding ($A_D = 1$) and to a local motion with $A_D = 0.5$ and $\tau_{loc} = 50$ ps. The horizontal line represents the experimental intensity ratio for the T7 and T8 methyl protons.

Residence time for major groove hydration from NOE data

The preceding analysis, leading to an estimate of the residence time for minor groove water, can also be carried out for some water molecules in the major groove. The cross-peaks with the methyl protons of T7 and T8 (Figure 2) yield essentialy the same integrated intensity ratio, $\rho = 1.0 \pm 0.1$. With a resonance offset of 3.6 ppm, equation (1) yields the curves shown in Figure 6. If the water molecules responsible for these NOEs are rigidly bound during their residence time ($A_D = 1$), we obtain $\tau_W = 140$ ps. Since water molecules in the major groove are not strongly confined, however, we expect local motions of substantial amplitude. To obtain a more realistic estimate of τ_W, we take, somewhat arbitrarily, $A_D = 0.5$ and $\tau_{loc} = 50$ ps, whereby $\tau_W = 310$ ps. Since the actual values of A_D and τ_{loc} are not known, the uncertainty in this result is large. In addition, the accuracy of the initial rate approximation was not checked for these cross-peaks. A reasonable range for τ_W might be 200 to 500 ps. It should be noted that the frequently invoked upper bound, $\tau_W < 0.3$ ns (*vide supra*), for hydration water giving negative cross-peaks in NOESY is valid only under the unrealistic assumption of rigid binding (no local motion).

NOE results for the netropsin complex

Turning now to the spectra from the DNA-netropsin complex (Figure 4), we note first that binding of the asymmetric netropsin molecule breaks the 2-fold symmetry of the self-complementary duplex, resulting in a splitting of the degenerate resonances from symmetry-related protons in the two strands (Patel, 1982). We distinguish the two inequivalent strands in the complex by superscripts I and II.

In addition to the expected doubling of resonances, a third set of resonances was identified in the NOESY spectrum, with the same chemical shifts as the free duplex. On the basis of integrated cross-peak intensities, we thus estimate that ca 20% of the DNA is free from netropsin although the sample was prepared with a nominal netropsin/DNA ratio of 1.1. We tentatively ascribe this minor discrepancy to a systematic error in the spectrophotometric DNA concentration determination (see Materials and Methods).

Comparing the spectra in Figures 3 and 4, we note that while the thymine CH_3 and guanine 8H cross-peaks from short-lived water molecules in the major groove are still present, the cross-peaks between adenine 2H protons and long-lived water molecules in the minor groove are absent in the ROESY spectrum from the netropsin complex.

A careful search at the chemical shifts of the four adenine 2H protons in the complex, 7.35(I)/7.42(II) ppm for A5 and 8.08(I)/8.07(II) ppm for A6, revealed only a weakly positive NOESY cross-peak for $A5^I$ 2H (but no corresponding cross-peak in ROESY). We attribute this cross-peak to a NOE between $A5^I$ 2H and an exchanging NH_2 proton in netropsin. The suppression of the corresponding ROESY cross-peak might then be an off-resonance effect (the netropsin NH_2 protons resonate at 9.18 pm, far from the carrier frequency at 4.91 ppm). This explanation is supported by the short N-N distance in the crystal structure of the complex (2.6 Å in the original structure 6BNA, 3.1 Å in the re-refined structure 101D), and by the observation of a direct NOE between $A5^I$ 2H and a netropsin NH_2 proton in both NOESY and ROESY spectra.

The analysis of A6 2H is somewhat complicated by the strong direct exchange cross-peak from C1 $4NH^a$, but a negative cross-peak from A6 2H of

reasonable intensity would still have been readily identified in the ROESY spectrum. The pair of negative cross-peaks at 7.47 and 7.63 ppm correspond to NOEs between the pyrrole 5H protons in netropsin (projecting out from the minor groove) and (short-lived) hydration water (Patel, 1982).

In conclusion, the NOE data demonstrate that in solution, as in the crystal (Kopka *et al.*, 1985a,b; Sriram *et al.*, 1992; Goodsell *et al.*, 1995), netropsin displaces the long-lived water molecules in the spine of hydration. This is, of course, also indicated by the sizeable shifts (0.2 to 0.5 ppm) of the adenine 2H resonances on netropsin binding.

Water ^{1}H, ^{2}H and ^{17}O NMRD. Methodological background

Recent water ^{2}H and ^{17}O NMRD studies of several small proteins have unequivocally demonstrated that the dispersion in the 1 to 100 MHz range of the longitudinal relaxation rate is due to a small number of long-lived water molecules that can be identified in high-resolution crystal structures (Denisov & Halle, 1995a,b,c, 1996; Denisov *et al.*, 1995; 1996). The intrinsic relaxation time of such long-lived water molecules depends on the resonance frequency, ν_0, and on the rotational correlation time, τ_R, and is much shorter than that of bulk water. If they exchange with bulk water on a timescale short compared to the intrinsic relaxation time, the few long-lived water molecules act as strong relaxation sinks, giving rise to the dispersion of the observed bulk water resonance.

The dispersion of the longitudinal relaxation rate, R_1, of the water nuclei ^{2}H and ^{17}O in a macromolecular solution can usually be accurately described by the simple expression (Denisov & Halle, 1995a,b):

$$R_1 = R_{\text{bulk}} + \alpha + \beta[0.2\, j_Q(\omega_0) + 0.8\, j_Q(2\omega_0)] \qquad (3)$$

with two amplitude parameters, α and β, and a reduced (quadrupolar) spectral density function $j_Q(\omega)$. The β term is the contribution from a small number, N_β, of ''long-lived'' water molecules with residence time τ_W. In the DNA solutions investigated here, τ_W is short compared to the zero-frequency intrinsic spin relaxation time of these water molecules, i.e. we are in the fast-exchange regime with respect to relaxation. The spectral density function is then of the same form as in the dipolar case (*vide supra*), $j_Q(\omega) = \tau_C/(1 + \omega^2\tau_C^2)$, with the correlation time τ_C related to τ_W and τ_R as in equation (2). The amplitude of the β term is given by:

$$\beta = (N_\beta/N_T)\,(\omega_Q A_Q)^2 \qquad (4)$$

where N_T is the total number of water molecules per macromolecule in the solution, ω_Q is the rigid-lattice nuclear quadrupole frequency, and A_Q is the generalized orientational order parameter for the observed nucleus (^{2}H or ^{17}O) in the N_β water molecules (Denisov & Halle, 1995c). Fast local motion of long-lived water molecules is manifested through the order parameter A_Q and a (usually negligible) contribution to the frequency-independent α term (Halle & Wennerström, 1981). This α term represents the relaxation rate enhancement, above the bulk solvent value R_{bulk}, due to a large number, N_α, of water molecules at the macromolecular surface with average intrinsic spin relaxation rate $\langle R_\alpha \rangle$ and with residence times much shorter than τ_C. Consequently:

$$\alpha = (N_\alpha/N_T)\,(\langle R_\alpha \rangle - R_{\text{bulk}}) \qquad (5)$$

Strictly speaking, the separation of the relaxation rate into α and β contributions is phenomenological; the dispersion of the α contribution occurs above the highest accessible frequency. By performing a difference NMRD experiment, however, the α/β separation can be given an unambiguous microscopic interpretation.

Most macromolecules contain several long-lived water molecules with different residence times. Unless they all happen to be much shorter than the intrinsic relaxation time and much longer than the rotational correlation time, so that $\tau_C = \tau_R$, the β term in equation (3) should really be replaced by a sum over water molecules. If the data are accurately described by equation (3), however, additional parameters cannot be justified. The parameters β and τ_C should then be regarded as (weighted) averages over the population of long-lived water molecules. A more detailed dynamic characterization is possible if some hydration water molecules can be displaced in a controlled way. A difference NMRD experiment can then yield the residence time of the displaced water molecule(s) (Denisov & Halle, 1995c; Denisov *et al.*, 1995, 1996).

Whereas the ^{17}O isotope always reports on water dynamics, the ^{1}H and ^{2}H relaxation rates generally include a contribution from labile macromolecular hydrogen atoms that exchange rapidly (compared to their intrinsic relaxation rates) with bulk water (Denisov & Halle, 1995b; Venu *et al.*, 1997). Because protons relax at least an order of magnitude more slowly than deuterons, the ^{1}H rate is more susceptible to such exchange contributions. For the DNA solutions investigated here, we estimate intrinsic relaxation times (at zero frequency) for labile hydrogen atoms of ca 0.2 ms for ^{2}H and 3 to 50 ms for ^{1}H. Since the NOE data indicate that about half of the 56 labile protons of the DNA duplex have exchange times in the range 1 to 100 ms (*vide supra*), we anticipate a sizeable exchange contribution to the ^{1}H rate, whereas the ^{2}H rate should hardly be affected at all. These expectations are borne out by the NMRD data (*vide infra*).

The analysis of water ^{1}H NMRD data may be further complicated by intermolecular dipole couplings between water and DNA protons. These are the dipole couplings responsible for the intermolecular NOEs that give positive cross-peaks in the NOESY spectrum. Inspection of the crystal structure of the d(CGCGAATTCGCG) duplex (with hy-

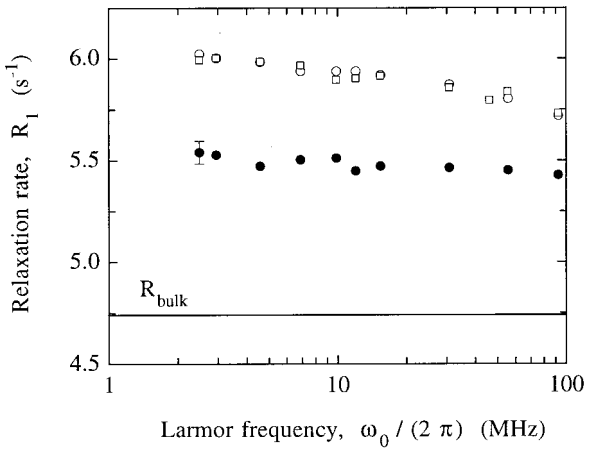

Figure 7. Dispersion of the water ^2H longitudinal relaxation rate measured at 4°C on a 8.0 mM aqueous solution (^{17}O-enriched ^2H_2O) of d(CGCGAATTCGCG) duplex at pD 7.4. Data points represented by open and filled symbols were obtained before and after, respectively, addition of 1.1 equivalent of netropsin to the DNA solution. Open circles and squares refer to measurements on two different samples. The horizontal line represents the (frequency independent) bulk water ^2H relaxation rate at the same temperature and isotopic composition as in the DNA solution. An error bar of ±1% is indicated for one datum point.

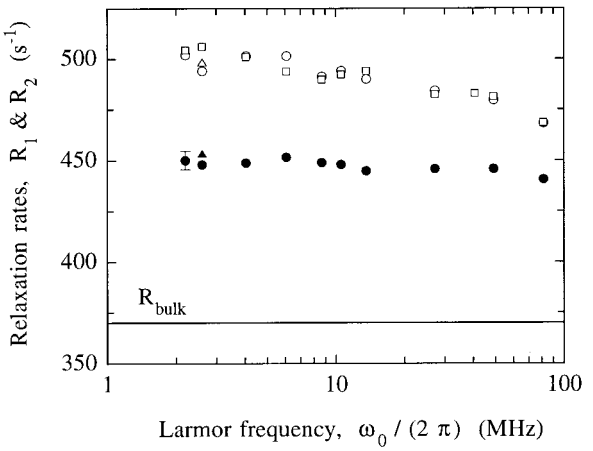

Figure 8. Dispersion of the water ^{17}O longitudinal relaxation rate measured at 4°C on a 8.0 mM aqueous solution (^{17}O-enriched ^2H_2O) of d(CGCGAATTCGCG) duplex at pD 7.4. Data points represented by open and filled symbols were obtained before and after, respectively, addition of 1.1 equivalent of netropsin to the DNA solution. Open circles and squares refer to measurements on two different samples. At 2.6 MHz, the ^{17}O transverse relaxation rate R_2 is also shown (triangles). The horizontal line represents the (frequency independent) bulk water ^{17}O relaxation rate at the same temperature and isotopic composition as in the DNA solution. An error bar of ±1% is indicated for one datum point.

drogen atoms added) shows that only adenine 2H and deoxyribose 1′H protons are sufficiently close to minor groove water protons to contribute significantly to water ^1H relaxation. (In conventional NOESY and ROESY spectra, cross-peaks due to intermolecular NOEs with 1′H protons are difficult to observe due to overlap with the intense exchange peaks from the terminal hydroxyl protons (see, however, Kubinec & Wemmer, 1992). Using NOE-NOESY and ROE-NOESY experiments to filter out exchange peaks, Jacobson *et al*. (1996) have recently observed intermolecular NOEs with 1′H protons in other duplexes.) Using the crystal geometry, we have calculated the intermolecular contributions to the ^1H rate (or to the β parameter in equation (3)) for the five water molecules in the spine of hydration. On average, this contribution is merely 3% of the intramolecular contribution due to the dipole coupling between the two protons in the same water molecule and can therefore be neglected. The ^1H NMRD data can then be analyzed within the same framework as the ^2H and ^{17}O data, equations (3) to (5), except that the rigid-lattice quadrupole frequency ω_Q is replaced by the corresponding (intramolecular) dipole frequency ω_D.

Residence time for spine of hydration from ^2H and ^{17}O NMRD data

Figures 7 and 8 show the ^2H and ^{17}O relaxation dispersions from the same 8.0 mM ^2H_2O solution of the d(CGCGAATTCGCG) duplex at 4°C, recorded before and after addition of netropsin. The results for the two nuclei are very similar, as expected in the absence of hydrogen exchange contributions to the ^2H rate (*vide supra*). The spine of hydration displaced by netropsin contributes nearly as much as all remaining hydration water to R_1, indicating substantially slower exchange and/or higher order for the spine water. More decisively, the free duplex exhibits a dispersion near 100 MHz, whereas R_1 for the complex is essentially constant in the investigated frequency range. Although only a part of the dispersion is observed in our field range (up to 14.1 T), the correlation time τ_C can nevertheless be accurately estimated if a Lorentzian spectral density function is assumed.

Using the viscosity of our ^{17}O-enriched ^2H_2O solvent at 4°C, we estimate (*vide supra*) an effective rotational correlation time, $\tau_R = 15$ ns, for the duplex. If the residence time of spine water was long compared to τ_R, as has been suggested (Kubinec & Wemmer, 1992), we would have $\tau_C = \tau_R$ and a large dispersion around 6 MHz. From the data in Figures 7 and 8, however, it is evident that the dispersion is centered at a much higher frequency, implying that the residence time is an order of magnitude shorter than τ_R. This assertion is quantitatively illustrated in Figure 9, where we have used equations (2) to (4) to calculate the R_1 dispersion from the free duplex expected for different values of the water residence time τ_W. If $\tau_W = \tau_R$

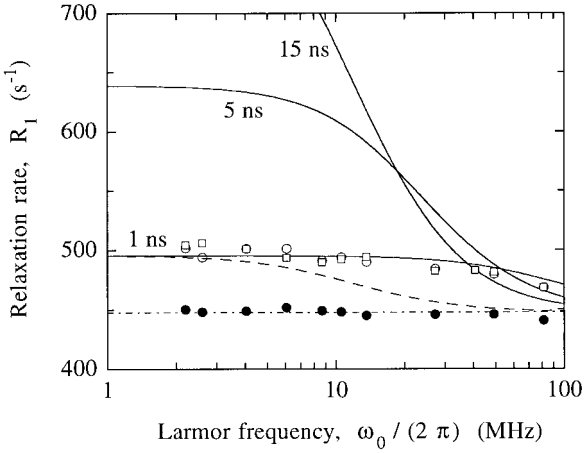

Figure 9. Dispersion of the water ^{17}O longitudinal relaxation rate calculated from equation (3) with $R_{bulk} + \alpha$ equal to the average R_1 for the netropsin complex (horizontal line), β as in equation (4) with $N_\beta = 6.4$, $N_T = 6460$, and $\omega_Q A_Q = 7.2 \times 10^6\ s^{-1}$, and τ_C as in equation (2) with $\tau_R = 15$ ns and the indicated water residence time τ_W. To highlight the frequency shift of the dispersion, the 15 ns curve has also been drawn normalized (broken curve) to the same dispersion amplitude as the 1 ns cuve. The experimental data from Figure 8 are included.

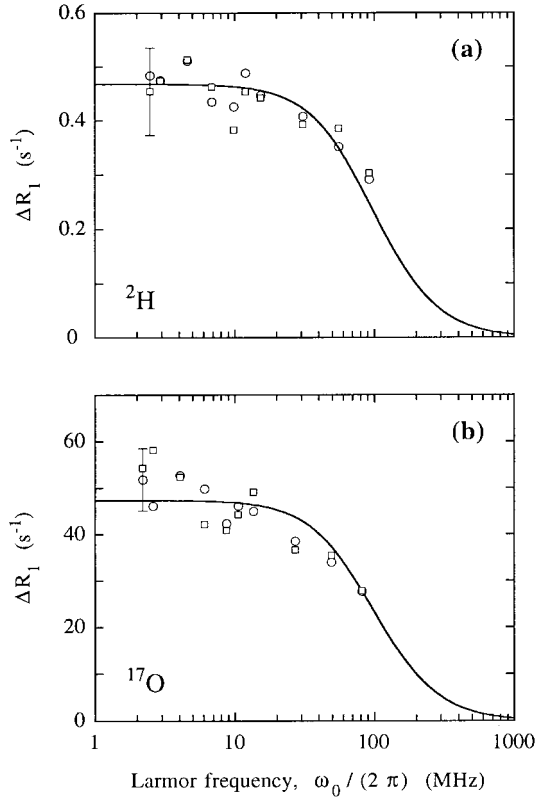

Figure 10. The difference induced by netropsin binding in the water (a) ^2H and (b) ^{17}O longitudinal relaxation rates, as shown separately in Figures 6 and 7. The two curves resulted from a three-parameter fit, according to equation (3), to the combined ^2H and ^{17}O data. An error bar corresponding to $\pm 1\%$ of R_1 is indicated for one datum point.

(15 ns), for example, the dispersion should appear at 12 MHz (the broken curve) rather than at ca 100 MHz as observed. Moreover, if netropsin displaces five highly ordered water molecules, the R_1 difference between free and complexed DNA at low frequencies would be a factor of 7 larger than observed.

Water molecules with residence times $\tau_W > \tau_R$ might escape detection if τ_R was much longer than our 15 ns estimate (e.g. due to DNA association), whereby the associated R_1 dispersion step would occur below our low-frequency limit of 2 MHz. Since the transverse relaxation rate, R_2, probes the spectral density at zero frequency (Abragam, 1961), it should then be substantially larger than R_1 even at the lowest frequencies examined. We therefore measured R_2 (^{17}O) at 2.6 MHz (Figure 8). The finding that $R_2 = R_1$ within experimental error excludes the possibility of a dispersion step below 2 MHz. (In bulk ^2H_2O at pD 7.4, a difference $R_2 - R_1$ of the order of $20\ s^{-1}$ is expected from exchange modulation of the scalar J coupling between ^{17}O and ^2H (Halle & Karlström, 1983). Due to DNA-promoted exchange catalysis, however, such a contribution is not observed in the DNA solutions.)

To quantitatively determine the residence time of water molecules in the spine of hydration, we fit the β term of equation (1) to the difference of the relaxation rates R_1 measured for the free duplex and for the netropsin complex. The fits are shown in Figure 10 on an extended scale to display the full dispersion. A joint three-parameter fit to the combined ^2H and ^{17}O data yields $\beta(^2H) = 5.0(\pm 0.7) \times 10^8\ s^{-2}$, $\beta(^{17}O) = 5.1(\pm 0.7) \times 10^{10}\ s^{-2}$, and $\tau_C = 0.9(\pm 0.1)$ns. (The quoted uncertainties are based on 1% accuracy in the individual R_1 measurements.) Since $\tau_C \ll \tau_R$, the correlation time can be directly identified with the residence time τ_W.

According to equation (4), the amplitude parameter β contains information about the number N_β of water molecules giving rise to the dispersion, as well as about the amplitude of their local motion (*via* the generalized order parameter A_Q). Neglecting any local motion, whereby $A_Q(^2H) = 1.00$ and $A_Q(^{17}O) = 1.135$ (Denisov & Halle, 1996), adopting the standard ice I*h* values for the quadrupole frequencies, $\omega_Q(^2H) = 8.2 \times 10^5\ s^{-1}$ and $\omega_Q(^{17}O) = 6.3 \times 10^6\ s^{-1}$ (Denisov & Halle, 1996), and using $N_T = 6460$ (as given by the spectrophotometrically determined DNA concentration and molecular volumes), we obtain with equation (4) $N_\beta(^2H) = 4.8 \pm 0.6$ and $N_\beta(^{17}O) = 6.4 \pm 0.8$. Considering the quoted propagated experimental errors and allowing for some uncertainty in the rigid-lattice quadrupole frequencies, these results are consistent with five water molecules being displaced by netropsin, as expected from crystallographic data (Kopka *et al.*, 1985a,b; Sriram *et al.*,

1992; Goodsell *et al*., 1995) as well as from our NOE results (*vide supra*). Furthermore, this agreement indicates that there is very little internal motion in the spine of hydration during the residence time. If the spine of hydration is kinetically heterogeneous, three distinct residence times would be needed to characterize the five water molecules in the spine (taking the 2-fold symmetry into account). If these residence times are not very different, the effect would be to stretch out the dispersion profile. A systematic tendency in this direction can be discerned in the difference dispersions of both nuclei (Figure 10).

An indication of the temperature dependence of the residence time may be obtained from ^{2}H and ^{17}O R_1 data at 55.3 and 49.0 MHz, respectively, recorded on the same sample (with and without netropsin) but at 27°C. Since β is independent of temperature (at least in the absence of local motion), equation (3) shows that the ratio of ΔR_1 at two temperatures is fully determined by the corresponding ratio of residence times. In this way, we obtain $\tau_W = 0.2$ ns at 27°C, a factor of 37 shorter than the DNA tumbling time τ_R (7 ns at this temperature). From these two temperatures, we can estimate an apparent activation enthalpy for water exchange of ca 45 kJ mol^{-1}. With this activation enthalpy, the NMRD result at 4°C, $\tau_W = 0.9$ ns, translates into $\tau_W = 0.6$ ns at 10°C, in quantitative agreement with the NOE result at this temperature.

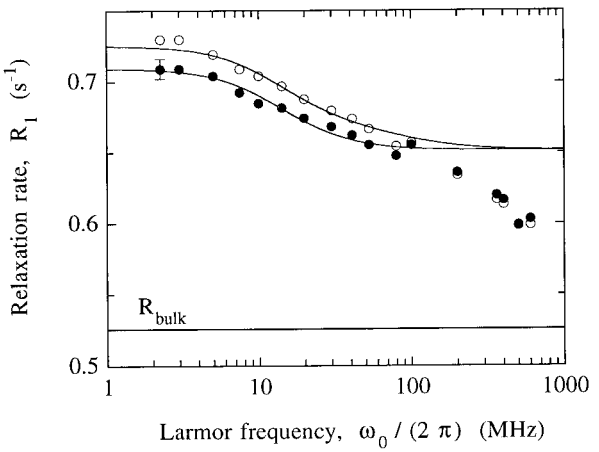

Figure 11. Dispersion of the water ^{1}H longitudinal relaxation rate measured at 4°C on a 7.2 mM aqueous solution (H_2O) of d(CGCGAATTCGCG) duplex at pH 7.0. Data points represented by open and filled symbols were obtained before and after, respectively, addition of 1.0 equivalent of netropsin to the DNA solution.The horizontal line represents the (frequency independent) bulk water ^{1}H relaxation rate at 4°C. An error bar of ±1% is indicated for one datum point. The lower curve resulted from a fit of equation (3) to the low-frequency (<100 MHz) ^{1}H data for the netropsin complex (see the text). The result of the joint fit to the ^{1}H, ^{2}H, and ^{17}O difference dispersions corresponds to the difference of the two curves.

Dynamics of surface hydration from ^{2}H and ^{17}O NMRD data

The excess relaxation rate, $R_1 - R_{bulk}$, from the DNA-netropsin complex can give a global view of the local dynamics of the traditional hydration layer surrounding the duplex, including water molecules associated with the major groove and the phosphate backbone. Since it is independent of frequency, this is an α contribution. By means of equation (5) and the measured R_{bulk}, we can then estimate the quantity $\langle R_\alpha \rangle / R_{bulk}$, which may be identified as the ratio $\langle \tau_\alpha \rangle / \tau_{bulk}$ of the average effective correlation time of surface water to that of bulk water (Denisov & Halle, 1995a, 1996). With $N_T = 6460$ (from the DNA concentration) and taking $N_\alpha = 12 \times 20 = 240$ (Saenger, 1987; Schneider & Berman, 1995), we thus obtain $\langle \tau_\alpha \rangle / \tau_{bulk} = 5.2$ and 6.6 from the ^{2}H and ^{17}O data, respectively. This may be compared to $\langle \tau_\alpha \rangle / \tau_{bulk} = 6.2$ for BPTI at pD 4.7 and 27°C (Denisov & Halle, 1995a), showing that, on average, water molecules in contact with the surface of the DNA duplex are dynamically similar to water at protein surfaces. From the 27°C data (*vide supra*), we similarly obtain $\langle \tau_\alpha \rangle / \tau_{bulk} = 5.4$ and 5.9.

Analysis of ^{1}H NMRD data

Figure 11 shows the water ^{1}H relaxation dispersions from a 7.2 mM solution of the d(CGCGAATTCGCG) duplex in H_2O at 4°C, recorded before and after addition of netropsin. There are three points to be noted here. First, as for the ^{2}H and ^{17}O dispersions, netropsin binding reduces the ^{1}H rate, but to a lesser (relative) extent. Second, even in the presence of netropsin, there is a strong low-frequency (<100 MHz) dispersion, not seen in the ^{2}H and ^{17}O dispersions. Third, at high frequencies (>100 MHz), where ^{2}H and ^{17}O data cannot be obtained, there is also a dispersion.

As seen from Figure 11, the low-frequency ^{1}H dispersion for the free duplex is also present for the netropsin complex. This dispersion must therefore be due to exchanging DNA protons. If these protons exchanged rapidly compared to their intrinsic relaxation rates, however, we would expect a much larger dispersion amplitude β and a lower dispersion frequency (longer τ_C). The estimated DNA tumbling time τ_R in H_2O at 4°C s 11 ns (*vide supra*), corresponding to a dispersion frequency of 8.4 MHz. Both of these discrepancies can be resolved if the labile protons are in the intermediate exchange regime. Under these coditions, equation (3) is still valid to an excellent approximation but β and $\tau_C = \tau_R$ should both be multiplied by a factor $(1 + \tau_L / T_L^0)^{-1/2}$, where T_L^0 is the intrinsic relaxation time at zero frequency of the labile proton and τ_L its residence time (Denisov & Halle, 1996).

The lower curve in Figure 11 resulted from a fit, according to equation (3), to the low-frequency dispersion data for the netropsin complex, yielding an

apparent correlation time $\tau_R(1 + \tau_L/T_L^0)^{-1/2} = 6(\pm 2)$ns and an apparent amplitude parameter $\beta(1 + \tau_L/T_L^0)^{-1/2} = 9(\pm 2) \times 10^6\ s^{-2}$. With $N_T = 7230$ and $\tau_R = 11$ ns, these parameters can be quantitatively accounted for by (1) eight amino protons with $\tau_L \approx 10$ ms, obtained from linewidths of NOESY exchange peaks (*vide supra*) and $T_L^0 \approx 4$ ms, estimated from the crystal structure, and (2) four hydroxyl and four imino protons, with $\tau_L \approx 5$ and 10 ms, respectively, and $T_L^0 \approx 10$ to 15 ms. The amino protons account for more than half of the dispersion step, with an effective correlation time of 6 ns. The slightly longer effective correlation times for the imino and hydroxyl protons (8 to 10 ns) may account for the slight stretching of the dispersion seen in Figure 11.

In spite of a slightly larger scatter above 100 MHz (all the data at lower frequencies were acquired on a single NMR spectrometer), the ^{1}H difference dispersion is consistent with the ^{2}H and ^{17}O difference dispersions in Figure 10, i.e. if the data from all three nuclei are fitted together the resulting parameters τ_C, $\beta(^2H)$ and $\beta(^{17}O)$ are the same as when only ^{2}H and ^{17}O data are included. The result of the joint fit corresponds to the difference of the two curves in Figure 11. The ^{1}H amplitude parameter $\beta(^1H)$, however, is only half of the expected value; with the rigid-lattice dipole frequency, $\omega_D = 2.2 \times 10^5\ s^{-1}$, for ice I*h*, it yields $N_\beta(^1H) = 2.6 \pm 0.6$. This discrepancy cannot be resolved by librational averaging (Denisov & Halle, 1996), since this would reduce the ^{2}H and ^{17}O N_β values even more. A distortion of the intramolecular water geometry in the spine that reduced ω_D by a factor of $\sqrt{2}$ below the ice I*h* value can also be ruled out, since this would require an unreasonably large 0.2 Å perturbation and, besides, would also reduce the ^{2}H and ^{17}O quadrupole frequencies.

A more likely explanation of the small β value of the ^{1}H difference dispersion is an exchange contribution from the eight terminal NH_2 protons in the netropsin molecule. On the basis of the intense exchange cross-peak at 9.18 ppm from these protons (not shown in Figure 4) and exchange studies on the related model compound acetyl-arginine (Liepinsh & Otting, 1996), we estimate an exchange time of $\tau_L \approx 50$ ms. Since $T_L^0 \ll \tau_L$, it follows that the eight NH_2 protons can produce a dispersion step comparable with that observed. In other words, in the absence of this exchange contribution, $N_\beta(^1H)$ would be close to 5. Moreover, the effective correlation time for the exchange contribution should be a few nanoseconds (rather than $\tau_R = 11$ ns), since the slow exchange ($\tau_L \gg T_L^0$) shifts the dispersion to higher frequencies (Denisov & Halle, 1996). The presence of some uncomplexed duplex in the H_2O sample used for the ^{1}H NMRD and NOE studies, as indicated by the NOE data (*vide supra*), would also reduce $\beta(^1H)$, but not to the required extent.

Finally, we consider the high-frequency (>100 MHz) ^{1}H dispersion seen for the free duplex as well as for the netropsin complex (Figure 11). Since no labile DNA proton can exchange on a subnanosecond timescale, another dynamic process must be responsible for this dispersion. We recall that the analysis of NOE cross-peaks with water molecules in the major groove indicates residence times in the range 200 to 500 ps. Such water molecules would exhibit dispersion frequencies in the range 200 to 500 MHz, where the high-frequency ^{1}H dispersion is observed. To account for the magnitude of the dispersion, however, several dozen water molecules with residence times in this range would be required. Although such water molecules might be associated with the phosphate backbone, in which case they would escape detection by the NOE method, the consistent finding of much faster water dynamics even at highly charged interfaces (Carlström & Halle, 1988) makes this hypothesis unlikely. Furthermore, for the protein BPTI, no such high-frequency ^{1}H dispersion is seen (unpublished results).

In the absence of labile proton contributions, the high-frequency relaxation enhancement α, due to highly mobile surface water, is expected to be the same for all three water nuclei when scaled by the respective bulk water relaxation rate, R_{bulk} (Venu *et al.*, 1997). Using the ^{2}H and ^{17}O data for the netropsin complex, and accounting for the small difference in DNA concentration (N_T), we can thus predict a high-frequency plateau in $R_1(^1H)$ of $0.61\ s^{-1}$. This is very close to the R_1 value measured at the highest frequency (Figure 11), suggesting that the >100 MHz dispersion is not predominantly due to water protons. We therefore ascribe this dispersion to internal motions, in the 200 to 500 ps range, at DNA sites containing labile protons. The dominant contribution probably comes from restricted rotational motions at the frayed ends of the duplex.

Concluding Discussion

NMRD *versus* NOE

The study of DNA hydration by nuclear magnetic resonance has a long history (Jacobson *et al.*, 1954). Currently, the two most powerful NMR techniques for probing biomolecular hydration are the NMRD and NOE methods. Here we have combined these two methods in a study of the kinetics of hydration of a DNA duplex in solution. Since this study represents the first direct and quantitative comparison of the two methods, it seems appropriate to briefly comment on their relative strengths and weaknesses.

Although NMRD and NOE data have little superficial resemblance (compare Figures 3 and 7), the two methods have a common physical basis. In both methods, information about hydration kinetics is deduced indirectly by measuring spin relaxation rates involving water nuclei. The two essential differences between the NMRD and NOE methods are consequences of the different strat-

egies used for probing spin relaxation: (1) while NMRD maps out the frequency dependence of the relaxation rate, the NOE method, as so far implemented, probes relaxation at a single frequency, and (2) while the NMRD dispersion profile reflects all rapidly exchanging water molecules, the chemical shift labeling of NOE cross-peaks can establish the proximity of some water molecules to non-labile macromolecular protons that are remote from any labile protons.

Quite generally, a nuclear spin relaxation rate can be expressed as a product of an amplitude factor (involving the square of a nuclear coupling constant) and a linear combination of reduced spectral densities, $j(k\omega_0)$, that contain all the accessible information about water dynamics. Being a single-frequency experiment, the NOE method cannot separate these factors. Furthermore, the relaxation rate is measured indirectly *via* a cross-peak intensity and is therefore susceptible to a variety of factors such as spin diffusion and imperfect spin locking, which, if not under full control, can introduce systematic error. Some of these complications are partly avoided by focusing on the *ratio* of cross-peak intensities in NOESY and ROESY spectra. The intrinsic amplitude factor thereby cancels out, leaving a combination of spectral densities (see equation (1)). Care has to be taken, however, to properly compensate for other factors that affect cross-peak intensities differently in NOESY and ROESY and, hence, do not cancel out in the ratio. Even when the desired combination of spectral densities can be accurately determined, one has only a single number that is to be transformed into a residence time. This transformation step is model-dependent and usually involves the tacit neglect of local motions, a simplification that is particularly questionable for the relatively unrestricted water molecules at protein surfaces or in the major groove of DNA duplexes. While the local motion problem has been addressed in terms of specific, somewhat idealized models (Otting *et al*., 1991b; Brüschweiler & Wright, 1994), we prefer to use the more general order parameter formalism (Halle & Wennerström, 1981; Lipari & Szabo, 1982). In general, the intermolecular spectral density, $j_D(\omega)$, governing the NOE reflects translational as well as rotational motions and is therefore not so easily modeled as the purely rotational, intramolecular spectral density, $j_Q(\omega)$, determining the ^{2}H and ^{17}O NMRD.

All the foregoing problems are avoided in the NMRD method, where, by directly measuring the relaxation rate over a wide frequency range, one effectively maps out the shape of the spectral density function. In cases, such as the present study, where water exchange is faster than macromolecular tumbling, the dispersion frequency directly yields the residence time in an essentially model-independent way. Moreover, the dispersion amplitude (β parameter) can yield the number of water molecules associated with this residence time. In contrast, the NOE method cannot determine the number of water molecules contributing to a cross-peak.

Although the water relaxation rate measured in an NMRD experiment reflects all rapidly exchanging water molecules in the system, the frequency dependence separates the contributions from short-lived and long-lived water molecules. To localize long-lived water molecules in the macromolecular structure, a difference-NMRD experiment is required, as illustrated here with the netropsin complex. This strategy relies, of course, on independent structural information. Also in the NOE method, independent structural information is needed to convert spectral assignments into spatial locations, and to distinguish water NOEs from exchange-relayed NOEs with labile macromolecular protons. Although the spatial resolution afforded by chemical shift labeling is a definite advantage of the NOE method, the more global view provided by NMRD can also be useful. Thus, whereas some short-lived water molecules in the major groove could be identified through negative NOESY cross-peaks, the absence of a dispersion in the (^{2}H and ^{17}O) NMRD data from the netropsin complex tells us directly that all water molecules not in the AATT region of the minor groove have residence times considerably shorter than 1 ns and/or are highly disordered (in which case a long residence time is unlikely).

Comparison with previous studies

The present NOE results differ somewhat from the two previously reported NOE studies of the same dodecamer (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992), both with regard to the primary data and their interpretation. In the brief report by Kubinec & Wemmer (1992), the interpretation is based solely on the sign of the NOESY/ROESY cross-peak intensity ratio ρ. From the observation of negative ρ values, these authors conclude that water molecules in the minor groove ''remain bound for a time longer than required for molecular tumbling''. This is claimed to be the case at 10°C as well as at 25°C. Moreover, from the observation of a negative ρ for the cross-peak with C1 6H, they infer the presence of long-lived water at the helix terminal. Our combined NOE and NMRD results are at variance with each of these three conclusions. At 10°C, we find $\tau_W = 0.6$ ns, more than an order of magnitude shorter than the estimated tumbling time, $\tau_R = 9$ ns. At 27°C, our (NMRD) data indicate an even larger difference, with $\tau_W \approx 0.2$ ns and $\tau_R = 7$ ns. The observation of a negative ρ does not imply that $\tau_W > \tau_R$ (Kubinec & Wemmer, 1992), but rather that $\tau_C = (1/\tau_W + 1/\tau_R)^{-1} > \sqrt{5}/(4\pi\nu_0)$ (if internal motions can be neglected). If $\tau_W \ll \tau_R$, as is the case here, we thus expect $\rho < 0$ if $\tau_W > 0.3$ ns (for $\nu_0 = 600$ MHz). On the basis of our results, one would thus not expect $\rho < 0$ for minor groove water at 25°C. The observation by Kubinec & Wemmer (1992) of $\rho < 0$ for A6 2H at 25°C and for C1 6H is probably attribu-

table to exchange-relayed NOEs involving labile DNA protons (*vide supra*). We note also that the initial-rate approximation fails badly (mainly due to auto-relaxation effects) for the mixing times ($\tau_m^{NOESY} = 200$ ms and $\tau_m^{ROESY} = 50$ ms) used by Kubinec & Wemmer (1992).

The results of Liepinsh *et al*. (1992) are more in line with the present work, although quantitative differences exist. Since the A6 2H resonance is at a maximum of the (NOESY) excitation profile, our slight modification of the ROESY pulse sequence used by Liepinsh *et al*. (1992) should have no effect on the cross-peak ratio ρ for this resonance (Materials and Methods). From the reported spectra (Liepinsh *et al*., 1992), one obtains for A6 2H $\rho = -0.5$ at 10°C, to be compared with our value $\rho = -0.81$, derived from peak amplitudes in the 1D cross-section at $\omega_1 = 4.91$ ppm. (In the preceding analysis, we used the more accurate value, $\rho = -0.87$, derived from the integrated 2D peaks.) This difference may be due partly to different ROESY spin-lock field strengths in the two studies and partly to inferior signal-to-noise ratio in the spectra obtained at the fourfold lower DNA concentration of the earlier study. On the basis of the sign of ρ, Liepinsh *et al*. (1992) conclude that water molecules in the minor groove have residence times ''significantly longer than 1 ns'', but they also mention that the small magnitude of ρ indicates either internal motion or a residence time shorter than 1 ns. Our result, $\tau_W = 0.6$ ns at 10°C, suggests that the latter explanation is the correct one.

Finally, we comment on a ^{1}H NMRD and NOESY study, of the same dodecamer as studied here, that appeared in print after the completion of this work (Zhou & Bryant, 1996). The ^{1}H NMRD profile reported by these authors extends up to 30 MHz, as compared to 600 MHz here. Within the overlapping range, 1 to 30 MHz, both studies reveal a dispersion with an amplitude much smaller than usually found for proteins. In contrast to Zhou & Bryant (1996), we argue that this dispersion is due to labile DNA protons rather than to long-lived water molecules. In particular, our finding that netropsin binding does not eliminate this dispersion (Figure 11), conclusively demonstrates that it cannot be due to water molecules in the minor groove. Although temperature, pH and DNA concentration are virtually the same in the two studies, Zhou & Bryant (1996) find a nearly fourfold larger excess relaxation rate, $R_1 - R_{bulk}$, at low frequencies. We attribute this large difference to the presence, in the sample investigated by Zhou & Bryant (1996), of 20 mM phosphate buffer, which catalyzes proton exchange (Liepinsh & Otting, 1996) and thus enhances the labile proton contribution to R_1. This effect also explains the longer correlation time deduced by Zhou & Bryant (1996; *vide supra*).

In analyzing their data, Zhou & Bryant (1996) neglect the contribution from labile DNA protons. They then proceed to estimate the number and residence time of long-lived water molecules in two limiting cases. One of these limits, $\tau_W \gg \tau_R$, is clearly inconsistent with our results. For the other limiting case, $\tau_W \ll \tau_R$, Zhou & Bryant (1996) claim that their data imply an average residence time of 1 ns at 15°C for an assumed 65 bound water molecules (this is the number of water molecules associated with the phosphate backbone in the crystal (Kopka *et al*., 1983)). In this case, however, it remains to explain the origin of the primary observation, the relaxation dispersion. If this deduction were correct, the ^{2}H and ^{17}O dispersion amplitides would be more than an order of magnitide larger than observed here. Conversely, our results imply that the contribution from long-lived water molecules to the 5°C ^{1}H dispersion reported by Zhou & Bryant (1996) is ca 0.03 s^{-1}, a factor of 16 smaller than the dispersion step observed by these authors.

A dynamic view of DNA hydration in solution

While the spine of hydration may be considered as an integral part of the d(CGCGAATTCGCG) duplex in a structural and thermodynamic sense, being crystallographically well-defined and involving a subtantial interaction enthalpy, the present study demonstrates that it is kinetically labile. For the five water molecules in the AATT region of the minor groove that are displaced by netropsin, the ^{2}H and ^{17}O NMRD profiles directly yield a residence time of 0.9(±0.1)ns at 4°C, while the single-field relaxation data at 27°C suggest $\tau_W \approx 0.2$ ns. This agrees quantitatively with the NOE result, giving credence to both methods. The results also indicate a high degree of orientational order, with little local reorientation during the residence time.

Based on a comparison of ^{2}H and ^{17}O NMRD data with high-resolution crystal structures for a series of globular proteins, we have proposed structural criteria for long-lived water molecules (>ns at room temperature) in proteins (Denisov & Halle, 1996). Apart from completely buried water molecules, with zero solvent-accessible surface area (A_S), we identified several cases of ''cleft water'' residing in narrow pockets with $A_S < 10$ Å^2, a thermal B factor < 20 Å^2, and at least two strong hydrogen bonds to the protein backbone. While the B factors are not comparable between protein and DNA crystals (due to vibrations and lattice disorder in the latter), the other two criteria are satisfied by the water molecules in the spine of hydration. While the cleft water molecules identifed in proteins occur as isolated molecules, the minor groove spine is a linear chain of five water molecules, indirectly hydrogen bonded *via* a secondary water chain. The consequent possibility of a cooperative exchange mechanism, involving several water molecules in a concerted fashion, may account for the somewhat shorter residence time in the spine. The question then arises of whether water exchange is coupled to conformational fluctuations in the DNA duplex. The large-scale

''breathing'' motion of the duplex, involving base-pair opening, is clearly too slow. With base-pair lifetimes of 8 ms and 30 ms for A5 and A6 at 15°C (Guéron & Leroy, 1995), the spine would exchange some 10^4 times before base-pairing is disrupted. The librational motion of the nucleotide bases, on the other hand, appears to be too fast and of too small amplitude (<10°) to be relevant (Kintanar *et al.*, 1989; Robinson & Drobny, 1995). The somewhat slower and larger amplitude motion in the furanose rings (Borer *et al.*, 1994; Robinson & Drobny, 1995) may be more relevant. Apart from such localized motions, it is conceivable that a collective twist motion could lead to a widening of the minor groove, thus facilitating water exchange.

For hydration water not in the minor groove, the ^{2}H and ^{17}O NMRD data yield an effective correlation time roughly six times longer than that of bulk water, i.e. ca 30 ps at 4°C and half of that at room temperature. This correlation time mainly reflects local reorientation and is not inconsistent with a relaxation time of ca 70 ps (interpolated to 4°C) deduced from Brillouin scattering from monolayer hydrated DNA films (Tao *et al.*, 1987; Tao, 1993). (Theoretically, a first-rank correlation time should be a factor of 3 longer than the second-rank correlation times measured by NMR.) Furthermore, the absence of a water dispersion at frequencies <100 MHz for the netropsin complex shows that there are no long-lived (>1 ns) water molecules that are not in the minor groove. Although the phosphate backbone is the most strongly hydrated part of the DNA duplex in a thermodynamic sense, the NMRD data thus show that these water molecules are kinetically labile with subnanosecond residence times even at 4°C. (These water molecules are not observable by the NOE method due to the lack of suitable NOE partners in DNA.) The NOE data indicate a few water molecules with residence times in the range 200 to 500 ps at 4°C, some of which are located in the major groove. Molecular dynamics simulations of hydrated DNA have so far focused more on structure than dynamics (Chuprina *et al.*, 1991; Beveridge *et al.*, 1993; Beveridge & Ravishanker, 1994; York *et al.*, 1995; Cheatham *et al.*, 1995; Elcock & McCammon, 1995), but might in the near future provide interesting details about the mechanism of water exchange from the minor groove. A preliminary analysis of water residence times from a 2.2 ns simulation of a crystal unit cell of the d(CGCGAATTCGCG) duplex is in excellent agreement with the results presented here (D. M. York, unpublished results).

Materials and Methods

DNA solutions

The sodium salt of the self-complementary DNA dodecamer d(CGCGAATTCGCG) (M 3888.4 g (mol strand)$^{-1}$) was purchased in two batches from Keystone Laboratories (Menlo Park, CA, USA) and the BM Unit at Lund University (Lund, Sweden). According to HPLC, the fraction of full-length oligomer in the two batches was >95% and >90%, respectively. After initial NMR experiments on each batch, all material was pooled, lyophilized and redissolved in pure water of the appropriate isotopic composition to yield the final NMR samples. No salt or buffer was added. Solution pH was adjusted by addition of small amounts of 0.1 M NaOH. For the ^2H_2O sample, we quote the value of pD = pH* + 0.41, with pH* the meter reading after calibration with standard H_2O buffers.

DNA concentrations were determined from the optical absorption at 260 nm after diluting the NMR sample (up to 1500-fold) with phosphate buffer (pH 7.0, 0.1 M KCl). Temperature scans in the range 10 to 95°C, recorded on GBC UV/VIS 920 and PE Lambda-14 spectrophotometers, exhibited the expected hairpin-melting transition at $t_m = 54$°C (Marky *et al.*, 1983). From a fit to the absorbance *versus* temperature data (Marky & Breslauer, 1987), using a calculated (nearest-neighbour) extinction coefficient of $\varepsilon = 1.14 \times 10^5$ M^{-1} cm^{-1} for single strands at 25°C (Gray *et al.*, 1995), we obtained $\varepsilon = 0.98 \times 10^4$ M^{-1} cm^{-1} for hairpins at 25°C. The latter value was used to determine the DNA concentrations.

The ^{1}H NMRD sample was 7.2 mM in duplex, pH 7.0, with doubly distilled H_2O as the solvent. The ^{2}H/^{17}O NMRD sample was 8.0 mM in duplex, pD 7.4, with ^{17}O-enriched ^2H_2O (21.9 atom% ^{17}O, 61.9 atom% ^{18}O, 99.95 atom% ^{2}H, M 21.5 g mol^{-1}) obtained from Ventron (Karlsruhe, Germany), as the solvent. The NOE experiments on uncomplexed DNA (as well as preliminary ^{2}H/^{17}O NMRD experiments) were performed on a sample with 4.7 mM duplex, pH 7.0, and an ^{17}O-enriched aqueous solvent containing 10% ^{2}H. The NOE experiments on the netropsin-DNA complex were performed on the ^{1}H NMRD sample after dilution to yield 3.3 mM duplex, pH 7.0, and 7% ^{2}H in the aqueous solvent.

Although the self-complementary dodecamer adopts a hairpin conformation at low concentrations (Marky *et al.*, 1983), the duplex form is expected to be stable at the high DNA concentrations of our NMR samples. This was verified by recording the temperature dependence of the ^{1}H chemical shifts of the thymine 6H and CH_3, adenine 2H and 8H, and cytosine 5H resonances at 360 MHz in a ^2H_2O solution, pD 6.9, 3.9 mM in duplex, exhibiting the characteristic duplex melting transition at ca 70°C (Patel *et al.*, 1982).

Netropsin (M 539.3 g mol^{-1}, purity > 98%) was obtained from Boehringer (Mannheim, Germany). To displace the spine of hydration, crystalline netropsin was added directly to the NMR samples. On the basis of the spectrophotometrically determined DNA concentrations, the mole ratio netropsin/duplex was 1.04±0.1 in the H_2O sample (also used for NOE experiments) and 1.09±0.1 in the ^2H_2O sample. The NOESY spectrum, however, indicated a mole ratio of ca 0.8 in the H_2O sample, suggesting a systematic error in the concentration determination. On the basis of the weighed amount of lyophilized DNA added to the samples, lower bounds on the mole ratio (assuming no residual water) of 0.74 (H_2O) and 0.84 (^2H_2O) were obtained. This uncertainty does not seriously affect the interpretation of the NMRD data. In particular, concentrations are not needed to determine the residence time.

Relaxation dispersion experiments

The longitudinal relaxation rate, R_1, of the water nuclei ^{1}H, ^{2}H and ^{17}O was measured with the inversion recovery method as described (Denisov & Halle, 1995a, b) at up to 17 magnetic field strengths using eight NMR spectrometers covering the field range 0.05 to 14.1 T. A few measurements of the transverse relaxation rate, R_2, were also made with the spin-echo method.

On all spectrometers, the sample temperature was adjusted with a copper-constantan thermocouple and was maintained to within ±0.1 deg. C with a thermostated air flow. The bulk of the reported relaxation data were obtained at 4.0°C, but measurements were also performed at 10 and 27°C.

The NMR samples had a volume of ca 0.2 ml and were contained in 5 mm tubes. Prior to relaxation measurements, the ^{1}H samples were gently bubbled by argon gas for ca two hours to remove dissolved oxygen. The NMR tubes were pretreated with HCl and EDTA to remove trace amounts of paramagnetic ions.

At the highest magnetic fields, the water ^{1}H relaxation may be influenced by radiation damping (Bloembergen & Pound, 1954; Mao *et al.*, 1994). This effect could be eliminated, however, by a combination of small filling factor, relatively large magnetic field inhomogeneity, and the use of decoupling coils for all ^{1}H measurements at 100 MHz and above. Within experimental uncertainty, the same R_1 value was measured with the saturation recovery and inversion recovery methods.

Relaxation rates for bulk water of the same isotopic composition as the DNA samples (but at pH* 3.1) were measured at 4.0°C over the entire field range: $R_1(^1\mathrm{H}) = 0.525(\pm 0.008)\mathrm{s}^{-1}$, $R_1(^2\mathrm{H}) = 4.74(\pm 0.07)\mathrm{s}^{-1}$, and $R_1(^{17}\mathrm{O}) = 370(\pm 4)\mathrm{s}^{-1}$. These reference measurements also served as a useful check of the sample temperature.

The accuracy of the reported relaxation rates is estimated to ca 1% for ^{2}H and ^{17}O, and ca 1.5% for ^{1}H at frequencies above 100 MHz.

Nuclear Overhauser effect experiments

Two-dimensional homonuclear ^{1}H NOESY and ROESY spectra were recorded at 10°C on a Varian UNITY*plus* NMR spectrometer operating at 600 MHz. Pulse sequences were taken from Otting *et al.* (1991a), with the following slight modifications. In the NOESY sequence, a weak (0.2 G cm^{-1}) z gradient was applied during the mixingz time, replacing the first spin-lock pulse, $SL_{\phi 4}$ (Otting *et al.*, 1992). This gradient pulse cancels water magnetization not aligned with the z axis at the end of the mixing time (Sklenar, 1995). In the ROESY sequence, the spin-lock pulse train was flanked by two $\pi/2$ pulses to compensate for frequency offset effects (Griesinger & Ernst, 1987). In addition, a weak (0.2 G cm^{-1}) bipolar z gradient was applied during the t_1 evolution period to reduce radiation damping effects (Sklenar, 1995).

The experimental parameters were essentially the same as in the previous NOE study of d(CGCGAATTCGCG) by Liepinsh *et al.* (1992). The mixing time τ_m was 60 ms in NOESY and 30 ms in ROESY. The water suppression spin-lock pulse, $SL_{\phi 5}$ in NOESY and $SL_{\phi 3}$ in ROESY, was of 2.0 ms duration and was preceded by a short delay, $\tau = 156$ μs. The pulse spacing in the ROESY spin-lock pulse train was ten times the $\pi/6$ pulse length, yielding an effective spin-lock field of 2.30 kHz. The time domain data size was 280×4096 complex points, yielding $t_{1\max} = 29$ ms and $t_{2\max} = 328$ ms. Quadrature detection in the indirectly detected dimension was achieved by the TPPI-States method (Marion *et al.*, 1989). To minimize baseline artifacts, polynomial corrections were applied in both dimensions after Fourier transformation. ^{1}H chemical shifts were referenced to the H_2O resonance at 4.91 ppm (Hartel *et al.*, 1982; Orbons *et al.*, 1987).

In the theoretical calculation of the ratio, $\rho = I_{NOESY}/I_{ROESY}$, of the water cross-peak intensities in the NOESY and ROESY spectra, a correction must be applied for the effect of resonance offset on the ROESY cross-peak intensity. This corection is represented by the factor κ_{off} in equation (1). When the carrier frequency is set to the water resonance:

$$\kappa_{off} = \frac{1}{\sin\theta}\,\frac{\sin(\Omega\tau)}{\sin(\Omega\tau + \theta - \pi/2)} \qquad (6)$$

where Ω is the resonance offset from the carrier, τ the delay before the final spin-lock pulse, and θ the angle between the spin-lock axis and the B_0 field. For a spin-lock field strength ω_1 (in angular frequency units) and a resonance offset Ω, $\theta = \arctan(\omega_1/\Omega)$. In the absence of offset, $\Omega = 0$ so that $\theta = \pi/2$ and $\kappa_{off} = 1$. The factor $1/\sin\theta$ in equation (6) is due to the mixing of laboratory-frame and rotating-frame cross-relaxation rates (Griesinger & Ernst, 1987). The second factor in equation (6) accounts for the different effective excitation profiles in NOESY and ROESY, when offset compensating $\pi/2$ pulses are used in the latter experiment together with a free evolution period τ and a water suppression spin-lock. If the resonance is at an extremum of the NOESY excitation profile, i.e. if $\Omega\tau = \pm(2n+1)\pi/2$, the second factor reduces to $1/\sin\theta$, so that $\kappa_{off} = 1/\sin^2\theta$ as for an uncompensated ROESY sequence (Griesinger & Ernst, 1987). In this special case, which applies to the A6 2H resonance in the present study, the intensity gain expected from the two $\pi/2$ pulses is thus precisely cancelled.

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