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Water Molecules in DNA Recognition I: Hydration Lifetimes of *trp* Operator DNA in Solution Measured by NMR Spectroscopy

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The present NMR study investigates the residence times of the hydration water molecules associated with uncomplexed *trp* operator DNA in solution by measuring intermolecular nuclear Overhauser effects (NOE) between water and DNA protons, and the nuclear magnetic relaxation dispersion (NMRD) of the water ^{2}H and ^{17}O resonances. Both methods indicate that the hydration water molecules exchange with bulk water on the sub-nanosecond time scale at 4°C. No evidence was obtained for water molecules bound with longer residence times. In particular, the water molecules at the sites of interfacial hydration in the *trp* repressor/operator complex do not seem kinetically stabilized in the uncomplexed DNA. Analysis of the crystal structures of two different *trp* repressor/operator complexes shows very similar structural environments for the water molecules mediating specific contacts between the protein and the DNA, whereas much larger variations are observed for the location of corresponding water molecules detected in the crystal structure of an uncomplexed *trp* operator DNA duplex. Therefore, it appears unlikely that the hydration characteristics of the uncomplexed DNA target would be a major determinant of *trp* repressor/operator recognition.

Keywords: *trp* operator; DNA hydration; NMR spectroscopy; protein/DNA recognition

Introduction

Hydration is of fundamental importance for DNA structure, as was early recognized from fiber-diffraction X-ray studies of the water-dependence of the *A* to *B*-DNA conversion (Franklin & Gosling, 1953). Interfacial water molecules are also retained in many specific complexes (for example, Otwinowski *et al.*, 1988; Hedge *et al.*, 1992; Newman *et al.*, 1995), where they mediate contacts between functional groups of the protein and the DNA.

The *trp* repressor/DNA complex provided an early example of a specific protein/DNA complex in which nearly all contacts between the protein and DNA bases are water-mediated (Otwinowski *et al.*, 1988). This observation stimulated further studies by biochemical and genetic methods (for reviews see Youderian & Arvidson, 1994; Lavoie & Carey, 1994), crystallography (Lawson & Sigler, 1988; Lawson & Carey, 1993) and NMR (Zhao *et al.*, 1993; Zhang *et al.*, 1994). Interfacial hydration water molecules are present at equivalent major groove positions (Figure 1) in two different crystal forms of the *trp* repressor/operator complex despite a difference in protein:DNA stoichiometry (Otwinowski *et al.*, 1988; Lawson & Carey, 1993). Mutation as well as chemical modification of *trp* operator DNA sequences aimed at removing the water-mediated interactions resulted in severely reduced affinities for *trp* repressor (Joachimiak *et al.*,

Abbreviations used: NOE, nuclear Overhauser effect; NMRD, nuclear magnetic relaxation dispersion; NOESY, NOE spectroscopy; ROESY, NOE spectroscopy in the rotating frame.

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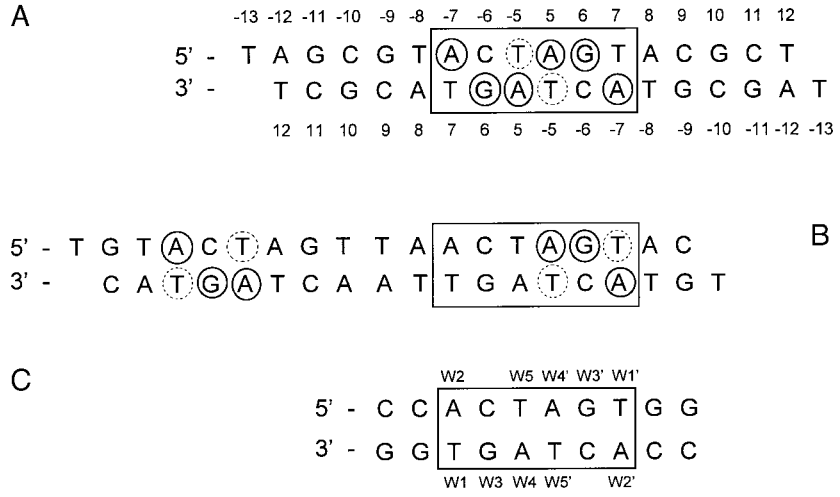

Figure 1. DNA sequences used for studies of uncomplexed and complexed *trp* operator. A, *trp*O 17-mer used in this work and for the crystal structure of the 2:1 *trp* repressor/operator complex by Lawson & Carey (1993). B, *trp*O 19-mer of the 1:1 complex crystal structure (Otwinowski *et al*., 1988). C, *trp*O decamer of the crystal structure of uncomplexed *trp* operator DNA (Shakked *et al*., 1994). The sequences are aligned by their common d-$(ACTAGT)_2$ segments (boxed). The nucleotides of the *trp*O 17-mer are numbered for the eight central base-pairs according to Lawson & Carey (1993) which coincides with the numbering of Otwinowski *et al*. (1988) for the d-(ACT)·d-(AGT) halfsites contacting the repressor in the 1:1 complex. The numbering of the *trp*O 17-mer is also used for the d-$(ACTAGT)_2$ segments of the *trp*O 19-mer and *trp*O decamer. The rest of the DNA is numbered so that the numbering reflects the *C*2 symmetry. Nucleotides for which water-mediated protein-DNA contacts with the bases were observed in the co-crystal structures are encircled with a continuous line. Dotted circles identify the nucleotides of the d-ACTAGT sequence, where water molecules hydrate bases of the DNA without contacting the protein. Water molecules suggested to be conserved between complexed and uncomplexed *trp* operator DNA, are indicated on the *trp*O decamer sequence, using the nomenclature of Shakked *et al*. (1994) for the respective water molecules.

1994; Smith *et al*., 1994). Water molecules located at corresponding positions on the surface of an uncomplexed *trp* operator DNA duplex (Figure 1) have been suggested to represent non-covalent extensions of the bases which may be used as recognition elements of the DNA target sequence (Shakked *et al*., 1994). These results highlight the importance of these water molecules, and raise the possibility that they might be characterized by prolonged residence times in uncomplexed *trp* operator DNA.

Residence times of hydration water can be measured by two complementary NMR methods. One method measures intermolecular nuclear Overhauser effects (NOEs) between water protons and biomolecule protons (Otting & Wüthrich, 1989; Otting *et al*., 1991a; Otting, 1997), while the other records the nuclear magnetic relaxation dispersion (NMRD) of the quadrupolar water nuclei ^{17}O and ^{2}H (Denisov & Halle, 1995a, 1996; Halle *et al*., 1998). The NOE experiment probes hydration water near specific DNA protons due to the short-range nature of the NOE. Water molecules without nearby DNA protons, such as those associated with the phosphodiester backbone, or water molecules located close to rapidly exchanging DNA protons, which provide alternative routes of magnetization transfer, cannot be studied by this method. The sign of the intermolecular NOE permits distinction between hydration water molecules with residence times longer or shorter than about 0.5 ns. NMRD, in contrast, determines bound-water residence times from about 1 ns up to the rotational correlation time of the biomolecule directly from the dispersion frequency (Denisov *et al*., 1997a; Jóhannesson & Halle, 1998). The amplitude of the dispersion step is proportional to the number of water molecules bound with residence times between 1 ns and 10^{-6} seconds (^{17}O) or 10^{-4} seconds (^{2}H), respectively. Residence times in the microsecond to millisecond range can be determined from the attenuation of the dispersion amplitude as the exchange rate becomes comparable to the intrinsic relaxation rate (Denisov *et al*., 1996). Thus, the NMRD method probes residence times of hydration water molecules over large time scales, albeit without identification of the hydration sites.

In view of the suggested importance of hydration in the *trp* repressor/operator system, we have carried out a joint NOE and NMRD study of the hydration kinetics in the DNA duplex d-$(TAGCGTACTAGTACGCT)_2$. This symmetric DNA fragment (the ''*trp*O 17-mer'') is identical to that used in the crystal structure of the 2:1 *trp* repressor/DNA complex (Lawson & Carey, 1993; Figure 1). In the 1.95 Å resolution crystal structure of uncomplexed d-$(CCACTAGTGG)_2$ (the ''*trp*O decamer''), a deep major groove is formed by the d-$(ACTAGT)_2$ duplex sequence, with each symmetric half housing three water molecules suggested to correspond to the water molecules mediating hydrogen bonds between *trp* repressor and DNA bases A−7, A5 and G6 in the *trp* repressor/operator complexes (Figures 1, 2; Shakked *et al*., 1994). In addition, three water molecules penetrate deeply into the minor groove of the d-$(ACTAGT)_2$ segment (Figure 2). The residence times of all these water molecules can be assessed by the NOE and NMRD methods. The results of the NOE and NMRD study of the *trp*O 17-mer are compared with water residence times determined

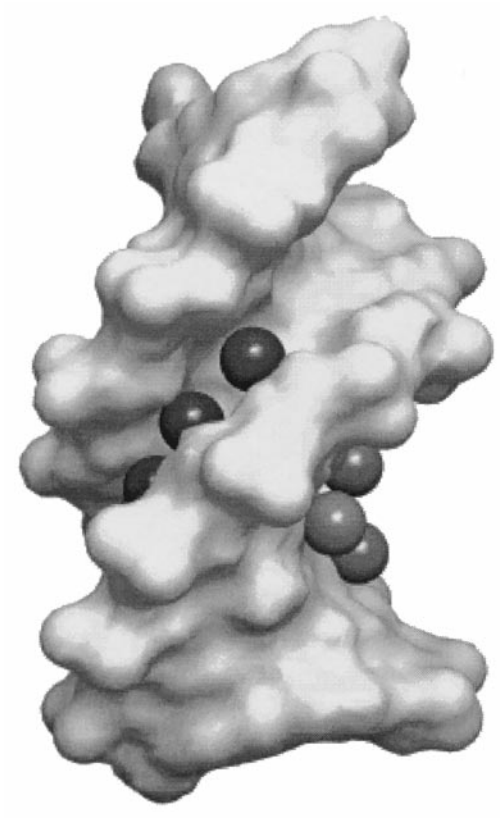

Figure 2. Surface of the *trp*O decamer in the crystal structure (Shakked *et al*., 1994) represented by GRASP (Nicholls *et al*., 1991). Spheres with a radius of 1.4 Å indicate three of the water molecules in the major groove hydrating the bases of A−7 (W2′), A5 (W4′), and G6 (W3′) (average *B*-factor 16.9 Å^2) and three water molecules in the minor groove (average *B* factor 13.3 Å^2).

for the *B*-DNA duplex d-(CGCGAATTCGCG)$_2$ containing the *Eco*RI recognition site (the ''*Eco*RI dodecamer''), the hydration of which has been studied extensively by X-ray crystallography (Drew & Dickerson, 1981; Westhof, 1987) and NMR spectroscopy (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992; Denisov *et al*., 1997a; Jóhannesson & Halle, 1998). An analysis of the location of hydration water molecules in the crystal structures of uncomplexed and complexed *trp*O DNA sequences was prompted by the results of this investigation, and is presented in the Appendix.

Results

Intermolecular water-DNA NOEs

Nearly complete ^{1}H resonance assignments for the uncomplexed *trp*O 17-mer were obtained at 4°C, pH 7.0, using conventional assignment strategies based on TOCSY, NOESY, and ROESY experiments (Table 1). The sequence of the *trp*O 17-mer is symmetric around the dyad center, leading to a symmetric structure and identical chemical shifts for the two halves of the DNA sequence. The ^{1}H NMR resonance assignments of the DNA were used to assign the water-DNA cross-peaks observed in the NOE-NOESY and ROE-NOESY spectra (Figure 3).

Water-DNA cross-peaks arise from magnetization transfer between water and DNA protons which can involve different mechanisms (Otting & Wüthrich, 1989). Intermolecular NOEs are manifested by negative ROESY cross-peaks which increase in intensity for longer water residence times, as long as the residence times are shorter than the rotational correlation time of the DNA. In

Table 1. Assignment of non-exchangeable protons of the *trp* operator, 5′-d-TAGCGTACTAGTACGCT-3′

Base	1′H	2′, 2″H		3′H	4′H	C5H	TCH$_3$	6H	8H	A2H	C4NH$_2$	G1NH/ T3NH
T−13	5.53			4.50	3.93[a]		1.57[a]	7.16				
A−12	5.82[a]		2.87	4.96	4.33[a]				8.29[a]	7.89		
G−11	5.79[b]	2.57	2.62	4.94	4.38[b]				7.78[a]			12.82
C−10	5.77[b]	2.03	2.43	4.84	4.22[b]	5.27		7.30			8.21[a] 6.38	
G−9	5.94[a]	2.60	2.77	4.96	4.37[b]				7.90[b]			
T−8	5.69[b]	2.05	2.45	4.85	4.17[b]		1.47[a]	7.19				13.51
A−7	6.15[b]	2.67	2.83	4.84	4.41				8.27[a]	7.27[a]		
C−6	5.74	1.92	2.42	4.62	4.20[a]	5.15		7.29[a]			7.97 6.70	
T−5	5.66	2.08	2.48	4.84	4.11[a]		1.53[a]	7.33				13.61
A5	6.02[a]	2.69	2.85	4.97	4.39[b]				8.19[a]	7.06[a]		
G6	5.70[b]	2.41	2.67	5.00	4.39[b]				7.55[a]			12.68
T7	5.67	2.04	2.45	4.84	4.16		1.25[a]	7.16[b]				13.41
A8	6.16[b]	2.63	2.84	4.99	4.39[b]				8.24	7.39[a]		
C9	5.54	1.98	2.32	4.80	4.15[b]	5.19		7.25			8.10 6.53	
G10	5.91[a]	2.63	2.72	4.97	4.36[a]				7.85[a]			12.90
C11	5.98	2.04	2.47	4.76	4.16[b]	5.38		7.42			8.26 6.70	
T12	6.35[a]	2.35	2.36	4.64	4.05	1.73[a]		7.57				13.91

Underlined shifts correspond to hydrogen atoms within 4 Å of water molecules conserved in crystal structures of isolated and complexed *trp* operator (Shakked *et al*., 1994, Otwinowski *et al*., 1988).

[a] Hydrogen atoms showing unambiguous small negative peaks in both NOE-NOESY and ROE-NOESY.

[b] Hydrogen atoms that can be tentatively assigned to small negative cross-peaks in the NOE-NOESY spectrum, however, the shifts overlap with those of other hydrogen atoms and thus the assignment is not unambiguous.

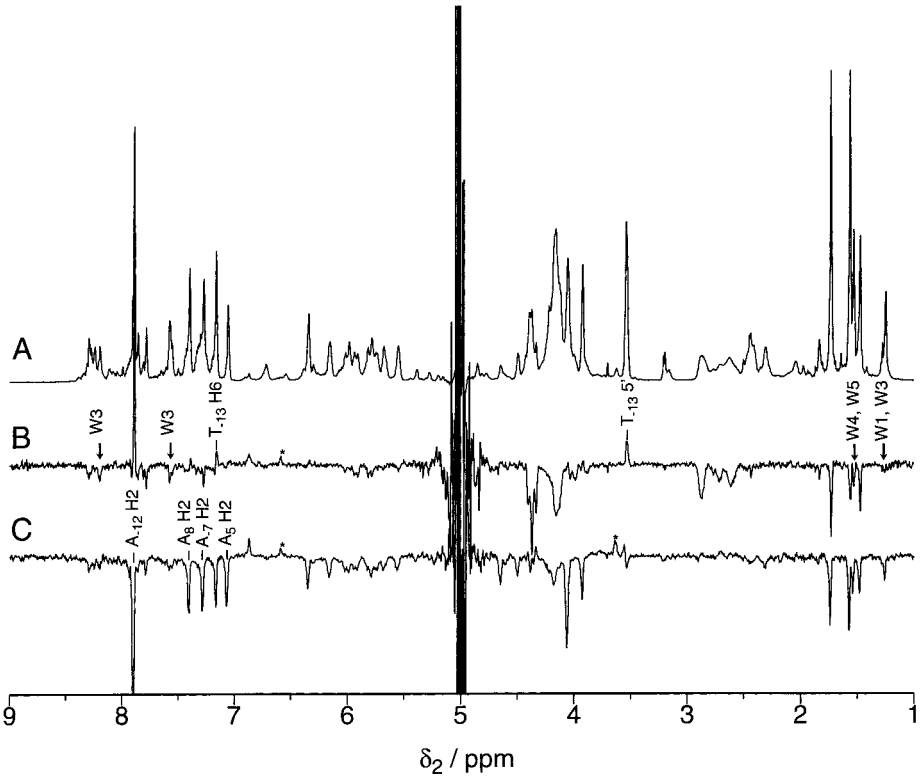

Figure 3. Diagonal cross sections through (A) NOESY, (B) NOE-NOESY and (C) ROE-NOESY spectra of the *trp*O 17-mer. Peaks labelled with nucleotide numbers and atom names are discussed in the text. Peaks labelled W1 to W5 identify the chemical shifts of DNA protons which are less than 4.0 Å from any of the water molecules W1 to W5 or W1′ to W5′ in the uncomplexed *trp*O decamer (Figure 1). The same DNA protons are within 4.0 Å of water molecules located in the protein-DNA interface of 1TRO and 1TRR (Table 1). The cross-peak of T7 CH_3 at 1.5 ppm appears less intense in the NOE-NOESY than in the ROE-NOESY spectrum due to interference with a noise band. The intensity of the corresponding cross-peak in the cross section taken along the δ_2 axis at the chemical shift of the water resonance (not shown) is comparable to that of the other methyl resonances. Asterisks identify peaks from impurities.

contrast, the sign of the corresponding NOESY cross-peaks is sensitive to water exchange rates on the 0.1 to 1 ns time scale. If local motions of the water molecules during their residence times are neglected, positive and negative NOESY cross-peaks observed at a ^1H NMR frequency of 600 MHz identify water molecules with residence times longer or shorter than 0.3 ns, respectively (Otting *et al*., 1991a; Otting, 1997). In the presence of local motions, the sign change occurs at longer residence times, e.g. at about 1 ns for an order parameter of 0.5 and a local correlation time of 50 ps (Denisov *et al*., 1997a). Cross-peaks due to chemical exchange of labile DNA protons with water can be distinguished from water-DNA NOEs by their positive sign in the ROESY spectrum. They are strongly suppressed on the diagonal of the NOE-NOESY and ROE-NOESY experiments employed here (Jacobson *et al*., 1996). Exchange-relayed NOEs arise from intramolecular NOEs involving DNA protons which rapidly exchange with water. They are manifested by positive and negative cross-peaks in the NOESY and ROESY spectra, respectively. The exchange-relayed mechanism can be excluded, if the DNA proton involved in a water-DNA NOE is located at least 4.5 Å from any of the labile DNA protons.

For all but three of the DNA nucleotides in the *trp*O 17-mer, at least one direct water-DNA NOE could be unambiguously assigned that was manifested by negative cross-peaks in the ROE-NOESY as well as in the NOE-NOESY spectrum (Table 1). The H2 protons on the minor groove side of residues A5, A8, and A−7 all show quite intense negative cross-peaks in the ROE-NOESY spectrum, but only very little cross-peak intensity in the NOE-NOESY spectrum suggesting that residence times of the water molecules near those protons are in the regime where the NOE changes its sign. The few positive NOE cross-peaks observed in the NOE-NOESY spectrum were all with protons which are expected to be involved in exchange-relayed NOEs. For example, a large positive cross-peak is observed for A−12 H2, but this proton is expected to be close to the rapidly exchanging amino proton of T12 at the 5′ end of the second strand of the duplex. Similarly, positive cross-peaks with T−13 H6 and T−13 H5′ were observed which most likely represent NOEs with the rapidly exchanging imino proton of the unpaired T−13

nucleotide. Negative cross-peaks appearing in the spectral region of the 2′ and 2″ protons of the NOE-NOESY but not the ROE-NOESY spectrum represent NOEs with 3′ protons with chemical shifts close to that of the water resonance (residues A−12, G−9, A5, G10, and T12; see Table 1). These H3′ resonances may have been excited by a negative sidelobe of the 90° Gaussian excitation pulse (Bauer *et al.*, 1984) used to selectively excite the water resonance. A small temperature difference between the NOE-NOESY and the ROE-NOESY experiment would have shifted the water resonance and therefore altered the excitation efficiency of the 3′ protons.

In summary, the water-DNA NOEs indicate that the residence times of water molecules associated with the uncomplexed *trp*O 17-mer in solution are short at 4°C. In particular, all of the DNA protons within 4 Å of water molecules W1, W3, W4, W5 and W1′, W3′, W4′, W5′ in the crystal structure of the *trp*O decamer (Figure 1; Shakked *et al.*, 1994), clearly show weak negative NOE cross-peaks both in NOESY and ROESY experiments, indicating similarly short water residence times at these hydration sites as for sites near the corresponding base protons of the other nucleotides (Table 1; Figure 3). If the residence times of these water molecules were significantly longer than 1 ns, a positive water-DNA cross-peak should have been observed in the NOE-NOESY spectrum together with a sizeable cross-peak in the ROE-NOESY spectrum. Hence, no water molecules associate with these sites on the DNA for measurably longer time periods than with any of the other hydration sites. Since no non-exchangeable DNA proton is close to the water molecules W2 or W2′ in the crystal structure of the *trp*O decamer (Shakked *et al.*, 1994), water residence times at those sites could not be assessed by the NOE method.

Residence time analysis by NMRD

Figure 4 shows the relaxation dispersions of the water ^{17}O and ^{2}H nuclei in an aqueous ($^{2}H_2{}^{17}O$) solution of uncomplexed *trp*O 17-mer. For comparison, the corresponding dispersions for the *Eco*RI dodecamer with and without netropsin (Denisov *et al.*, 1997a) are also shown. The water relaxation dispersion from the uncomplexed *Eco*RI dodecamer has previously been ascribed to five water molecules of the minor-groove spine (Denisov *et al.*, 1997a), by using a difference-NMRD experiment in which these sites were occupied by the high-affinity, minor groove-binding ligand netropsin. Only two more long-lived hydration water molecules were detected in a corresponding study at −20°C, where the complete dispersion curve could be measured (Jóhannesson & Halle, 1998). The dispersion frequency at 4°C yields a mean residence time of 0.9 ns at 4°C for the long-lived hydration water, while the relaxation rates indicate a high degree of orientational order (Denisov *et al.*, 1997a; Jóhannesson & Halle, 1998). Since the DNA-netropsin complex exhibits no significant dispersion, there are no other similarly long-lived water molecules interacting with the *Eco*RI dodecamer. The ^{17}O relaxation rates measured for the *trp*O 17-mer are virtually identical to those obtained with the *Eco*RI dodecamer-netropsin complex (scaled to the same nucleotide concentration), while significantly faster relaxation was observed for the uncomplexed *Eco*RI dodecamer (Figure 4A). Similarly, the ^{2}H relaxation rates for *trp*O 17-mer are closer to those for the *Eco*RI dodecamer-netropsin complex than to those of the uncomplexed *Eco*RI dodecamer. Neither the ^{17}O nor the ^{2}H relaxation rates for the *trp*O 17-mer show a significant dispersion in the frequency

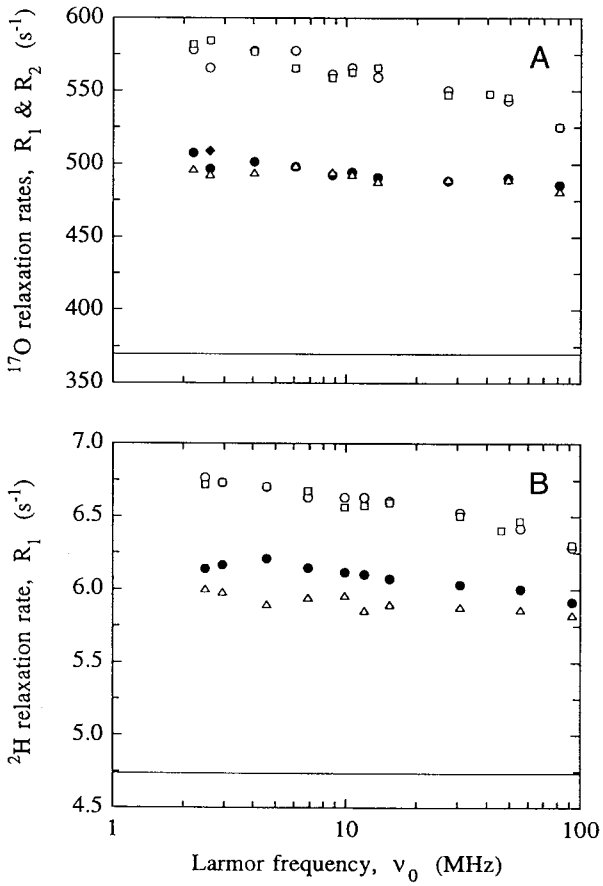

Figure 4. Frequency dependence of the longitudinal relaxation rates R_1 of (A) ^{17}O and (B) ^{2}H of the water in an 8.6 mM solution of the *trp*O 17-mer. The horizontal line in each of the Figures indicates the relaxation of the bulk solvent. Filled circles: *trp*O 17-mer; open circles and squares: two different samples of uncomplexed *Eco*RI dodecamer (Denisov *et al.*, 1997a); triangles: *Eco*RI dodecamer in complex with netropsin. A filled diamond in A identifies a single measurement of the transverse ^{17}O relaxation rate R_2 of the *trp*O 17-mer. The data for the *Eco*RI dodecamer were scaled to the same nucleotide concentration as the *trp* operator DNA using the fact that $R_1 - R_{bulk}$ is proportional to $1/N_T$.

range investigated, in contrast to the uncomplexed *Eco*RI dodecamer. The coincidence of the ^{17}O transverse relaxation rate R_2 (measured at 2.6 MHz) and the ^{17}O longitudinal rate (R_1) shows that the extreme motional narrowing limit is reached at this frequency (Figure 4A) and, hence, that there cannot be a dispersion step at lower frequencies (Halle *et al*., 1998). Within the accuracy of the NMRD data, a single ordered water molecule bound with a residence time of about 1 ns may have escaped detection, but two or more such water molecules would have produced a detectable dispersion.

The somewhat higher (concentration normalized) ^2H relaxation rates obtained with the *trp*O 17-mer as compared to the *Eco*RI dodecamer-netropsin complex indicates a small contribution to the ^2H relaxation from labile DNA deuterons. Indeed, no exchange cross-peaks between water and the four terminal hydroxyl protons were observed in the NOESY and ROESY spectra, indicating that these protons exchange unusually fast (Liepinsh *et al*., 1992; Jacobson *et al*., 1996). Chemical exchange is expected to quench exchange peaks when the ^1H NMR line widths are larger than about 300 Hz, corresponding to exchange life times <1 ms. The labile-proton contribution to the ^2H relaxation dispersion can be accounted for by, for example, four highly ordered hydroxyl protons with lifetimes of 1.2 ms (Figure 4B and Materials and Methods). Also the imino protons from the unpaired thymidine nucleotides present at both ends of the *trp*O 17-mer exchange rapidly. A water-DNA exchange peak at 10.8 ppm was tentatively assigned to these imino protons. The signal was about 60 Hz wide, corresponding to an exchange life time of about 5 ms.

Thus, the near coincidence of the ^{17}O relaxation rates of the *trp*O 17-mer and the *Eco*RI dodecamer-netropsin complex, and the absence of a relaxation dispersion, demonstrate that hydration of the *trp*O 17-mer does not include long-lived water molecules. This conclusion is consistent with the present NOE data, and holds for the entire DNA surface, including those sites which cannot be studied by water-DNA NOEs.

Discussion

The present results indicate that, in solution, all hydration water molecules associated with the *trp*O 17-mer have short (<1 ns) residence times at 4°C. Even shorter residence times would be expected at higher temperatures. Intermolecular water-DNA NOEs show that the residence times are short not only in the major groove and close to the sugar backbone, consistent with earlier studies on other DNA sequences (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992, 1994; Fawthrop *et al*., 1993; Denisov *et al*., 1997a), but also in the minor groove, where longer residence times would have been expected due to limited access to bulk water (Otting *et al*., 1991a, 1997; Meiering & Wagner, 1995; Denisov & Halle, 1996; Denisov *et al*., 1997a). The NMRD data confirm this picture of kinetically labile hydration and demonstrate further that water molecules not observable by the NOE method have similarly short residence times.

The minor groove of the d-$(ACTAGT)_2$ segment of the *trp*O decamer is equally narrow as the minor groove of the central d-$(AATT)_2$ segment of the *Eco*RI dodecamer, when measuring the shortest atom-atom distances between the sugar/phosphate backbone across the minor groove in the crystal structures (Drew & Dickerson, 1981; Shakked *et al*., 1994). However, in contrast to the *trp*O decamer, the NOESY cross-peaks between water molecules and adenine H2 protons in the minor groove of the *Eco*RI dodecamer are clearly positive and of similar intensity in NOESY and ROESY experiments (Kubinec & Wemmer, 1992; Liepinsh *et al*., 1992; Denisov *et al*., 1997a), and residence times of about 0.9 ns have been estimated for these water molecules at 4°C from difference-NMRD experiments (Denisov *et al*., 1997a). The absence of positive water-DNA NOESY cross-peaks and $^{17}O/^2H$ relaxation dispersion for the *trp*O 17-mer could be explained by about twofold shorter residence times and/or significantly increased local motion of the bound water molecules. It is possible that the minor groove of the d-$(ACTAGT)_2$ segment of the *trp*O 17-mer is wider in solution, facilitating exchange with bulk water, as the minor groove is considerably widened in the co-crystals with *trp* repressor (Otwinowski *et al*., 1988; Lawson & Carey, 1993). In fact, the crystal structure of a different dodecamer containing the d-$(CTAG)_2$ segment displays a wide minor groove (Urpí *et al*., 1996), in agreement with a very small dispersion step observed in NMRD measurements at −20°C (Jóhannesson & Halle, 1998). Unfortunately, it is impossible to determine the width of the minor groove with good accuracy by high-resolution NMR experiments (Leijon *et al*., 1995). A wider minor groove is observed within the d-$(ACTAGT)_2$ segment in a molecular dynamics (MD) simulation in water (Bonvin *et al*., 1998). Alternatively, conformational fluctuations on the sub-nanosecond time scale could accelerate the water exchange and/or increase the local motions of the hydration water molecules.

Upon first thought, one might think that the localized water molecules with low thermal *B* factors in the *trp*O decamer crystal structure would be characterized by prolonged residence times in solution. However, localized water molecules and short residence times in solution could in principle be reconciled in a model of water molecules ''hopping'' in and out of DNA hydration sites, where water molecules reside on average a little longer than in the immediate vincinity, for example because of the possibility to form a hydrogen bond to the DNA. This would allow for both rapid exchange and a high degree of localization. A similar model has been suggested previously for protein surfaces (Teeter, 1991; Gu & Schoenborn,

1995), where surface water residence times of 20 ps have been observed (Denisov & Halle, 1995a), i.e. only six times longer than the bulk water rotational correlation time in water (Hindman, 1974; Lankhorst *et al*., 1982).

It is important to note that the water exchange rates presented here do not allow any conclusions about the contribution of the water molecules to the thermodynamics of protein-DNA complex formation. In general, configurational fluctuations (of water molecules or internal degrees of freedom) have a static aspect (fluctuation amplitude) and a dynamic aspect (fluctuation rate). Only the static aspect is relevant for thermodynamic properties (Hill, 1986). Thus, short residence times of water at a protein-DNA interface imply neither the absence of entropic destabilization (Billeter *et al*., 1996; Schwabe, 1997) nor a reduced heat capacity (Ladbury *et al*., 1994) of the complex. A favorable entropic contribution to the free energy of complex formation is often invoked on the assumption that the hydration water that is displaced at the protein-DNA interface is more highly ordered than bulk water (e.g. Härd & Lundbäck, 1996; Schwabe, 1997). While this may sometimes be true, the generality of this notion is challenged by the finding in a recent NMRD study that even extensively hydrogen-bonded water molecules buried in protein cavities can have entropies comparable to that of bulk water (Denisov *et al*., 1997b).

Water molecules detected in the single crystal structure of the uncomplexed *trp*O decamer have been suggested to be conserved in the interface of the protein-DNA complex, implying a role in recognition (Shakked *et al*., 1994). The present NMR results show that the residence times of the hydration water molecules at these sites are short in solution, and cannot be distinguished from residence times at other bases. This result is supported by molecular dynamics simulation of the duplex part of the *trp*O 17-mer, which also shows short residence times near all bases (Bonvin *et al*., 1998). Analysis of the single crystal structures of uncomplexed and complexed *trp* operator DNA duplexes shows significant differences in location and hydrogen bonding between the contact-mediating water molecules in the co-crystals and the corresponding hydration water molecules associated with the uncomplexed DNA (Appendix). Thus, the hydration of uncomplexed *trp*O DNA sequences does not seem structurally pre-organized or kinetically stabilized to match the hydration in the *trp* repressor/operator complex. Rather, the present results indicate that the demonstrated importance of water molecules at the interface of the *trp* repressor/operator complex (Joachimiak *et al*., 1994) is more adequately attributed to structural complementarity of the protein with the DNA, providing well-defined spaces for water molecules at the interface.

Materials and Methods

NMR sample preparation

The self-complementary *trp*O 17-mer DNA sequence 5′-d-(TAGCGTACTAGTACGCT)-3′ (*trp*O 17-mer) was the same as that used by Lawson & Carey (1993) in the 2:1 co-crystal structure. The DNA was synthesized on an Applied Biosystems Automated DNA synthesizer and purified by reversed-phase HPLC at the synthesis facility in the Molecular Biology department, Princeton University. The DNA sample was extensively dialyzed to remove excess salts which could catalyze proton exchange, and in a final step ultrafiltrated with a 10 mM NaCl solution to provide some stabilizing counter-ions for the DNA. Before the final ultrafiltration step, the pH was adjusted to 7.0 and checked again after the ultrafiltration. The final concentration of the sample was 3 mM duplex DNA, and the volume 400 μl.

After the ^{1}H NMR measurements, the sample was lyophilized and redissolved in 137 μl ^{17}O-enriched ^2H_2O for ^{17}O and ^{2}H relaxation dispersion measurements. The measured pH (uncorrected for the isotope effect) was 6.9. The concentration was determined from the weighed amounts of the water and the DNA (163 mg and 15.7 mg, respectively), using molar masses of 21.5 for the water and 11,075 for the duplex and assuming the presence of ten water molecules per base-pair in the lyophilized DNA as found in a previous study (Denisov *et al*., 1997a). The concentration obtained in this way was 8.6 mM in duplex corresponding to a total number of water molecules per duplex of $N_T = 5800$.

NMR measurements

The spectra used for ^{1}H resonance assignment and the detection of intermolecular water-DNA NOEs were recorded at 4°C on a Bruker DMX-600 NMR spectrometer equipped with a Q-switch probe head. The water signal in the NOESY and ROESY experiments was suppressed before the acquisition time using a spin-lock pulse (Otting *et al*., 1991b, 1992). The NOE-NOESY and ROE-NOESY experiments (Otting & Liepinsh, 1995) were performed as described previously (Jacobson *et al*., 1996). Mixing times of 50 ms were used for the first mixing times of the NOE-NOESY and ROE-NOESY experiments for the buildup of the intermolecular water-DNA NOEs. The second mixing time was set to 200 ms in both experiments. Acquisition parameters were $t_{1max} = 55$ ms and $t_{2max} = 373$ ms, with a total experimental time of 16 hours per spectrum.

Water ^{17}O and ^{2}H NMRD data were measured at 4°C in parallel with a related hydration study of the *Eco*RI dodecamer d-(CGCGAATTCGCG)$_2$ using identical procedures (Denisov *et al*., 1997a). An estimate of the exchange rates of the labile DNA protons in the *trp*O 17-mer was obtained from the labile-proton contribution to the ^{2}H relaxation dispersion (Figure 4B). The difference between the relaxation rates for the *trp*O 17-mer and the *Eco*RI dodecamer-netropsin complex is about 0.25 s^{-1} at the lower frequencies; it can be expressed as (Denisov & Halle, 1995b):

$$\Delta R_1 = N_L/[2N_T(T_L + \tau_L)]$$

where N_L and N_T are the numbers of exchanging deuterons and water molecules per duplex, respectively. Further, T_L and τ_L are the intrinsic zero-frequency relaxation time and the residence time of the labile deuteron.

The intrinsic relaxation time can be estimated from:

$$1/T_L = (3\pi^2/2)A^2\chi^2\tau_R$$

where A is the order parameter, χ the quadrupole coupling constant (about 200 kHz), and τ_R the rotational correlation time of the DNA duplex. With the viscosity of our ^{17}O-enriched ^2H_2O and at our high DNA concentration we estimate $\tau_R \approx 30$ ns. This gives $T_L \approx 56/A^2$ μs. With $\Delta R_1 \approx 0.25$ s^{-1} (Figure 4B) and $N_T = 5800$, we have $(T_L + \tau_L)/N_L \approx 0.34$ ms which could, for example, be accounted for by four highly ordered ($A = 1$) hydroxyl protons with exchange life times τ_L of 1.2 ms.

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Appendix

Comparison of hydration sites in crystal structures of complexed and uncomplexed *trp* operator DNA

Maria Sunnerhagen, Alexandre M. J. J. Bonvin Bertil Halle and Gottfried Otting

Three crystal structures of DNA duplexes containing parts of the *trp* operator have been determined to high resolution: uncomplexed *trp*O decamer d-$(CCACTAGTGG)_2$ (1.95 Å; NDB identification code BDJ061; Shakked *et al*., 1994), d-$(TGTACTAGTTAACTAGTA)_2$ complexed with one *trp* repressor dimer (1.9 Å; PDB accession code 1TRO; Otwinowski *et al*., 1988), and d-$(TAGCGTACTAGTACGCT)_2$ complexed with two *trp* repressor dimers (2.4 Å PDB accession code 1TRR; Lawson & Carey, 1993). Water molecules are associated with the bases of the d-$(ACTAGT)_2$ segment in the crystal structures of both complexed and uncomplexed DNA (Shakked *et al*., 1994), and mediate interactions between protein and the bases of nucleotides A−7, A5 and G6 in the co-crystal structures 1TRO and 1TRR (Figure 1; Otwinowski *et al*., 1988; Lawson & Carey, 1993).

The hydration sites of complexed and uncomplexed *trp* operator DNA were analyzed in two ways. First, using a simple distance criterion, possible hydrogen bonding partners were identified for the hydration water molecules most closely associated with the nucleotide bases in the major groove of the d-(ACT)·d-(AGT) segments (Figure A1). Second, the bases of the d-(ACT)·d-(AGT) segments were superimposed for the different crystal structures in order to compare the three-dimensional location of the hydration sites in complexed and uncomplexed *trp* operator DNA sequences (Figure A2). Only the d-(ACT)·d-(AGT) segments contacted by protein were included in the comparison for the complex structures, whereas both d-(ACT)·d-(AGT) segments were included for the uncomplexed *trp*O decamer. The bases of all segments superimpose with an average heavy atom r.m.s. deviation of 0.32 Å to the mean structure.

Four of the water molecules hydrogen bonded to the bases of the d-(ACT)·d-(AGT) segments in the

complex structures show remarkably similar positions (Figure A2, Table A1) and sets of hydrogen bonding partners (Figure A1A, B) in both co-crystal structures in spite of the different protein:DNA stochiometry and minor differences in protein contacts. These water molecules correspond to those numbered W2 to W5 and W2′ to W5′ for the symmetry-related half-sites of the uncomplexed *trp*O decamer (Figure A1C and D) and include those bound to the bases of A−7, A5, and G6 which

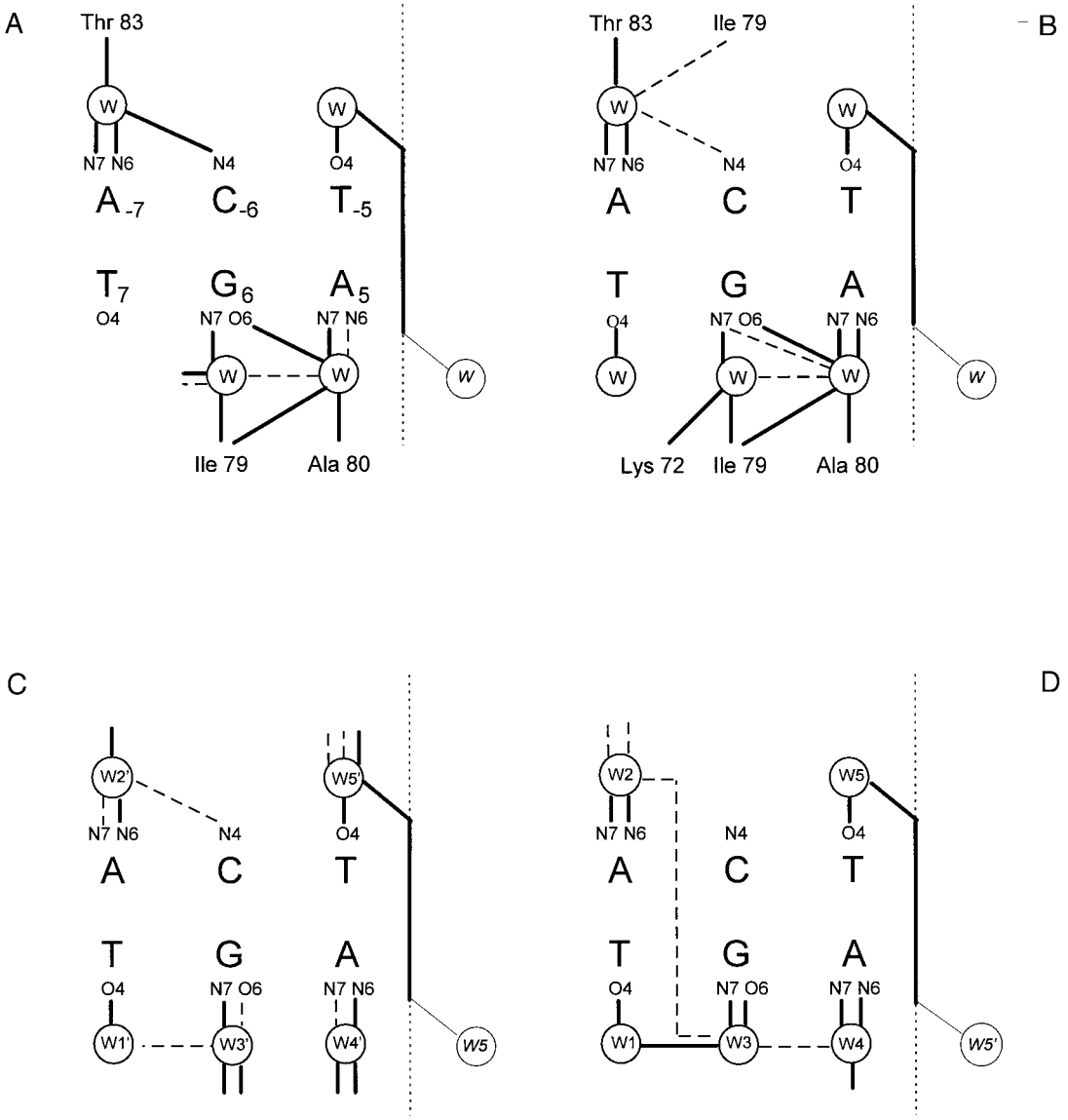

Figure A1. Hydrogen bonds of water molecules associated with the bases of the d-(ACT)·(AGT) segments in complexed and uncomplexed *trp* operator sequences. Hydrogen bonds were identified from heavy atom - heavy atom distances between water oxygen atoms and donor/acceptor atoms of DNA and/or protein molecules in the single crystal structures 1TRR, 1TRO and the uncomplexed *trp*O decamer (Figure 1). Continuous lines: distances $\leqslant$3.3 Å, indicating probable hydrogen bonds; broken lines: distances $\leqslant$4.0 Å, indicating hydrogen bonds plausible within the resolution of the crystal structures. Open-ended lines connect to water molecules not displayed in the Figure. The dyad axis of the d-$(ACTAGT)_2$ sequence is identified by a dotted line. A and B, Hydrogen bonding pattern in the protein-bound d-(ACT)·d-(AGT) segments in 1TRR (A) and 1TRO (B). Each of the PDB entries provides the coordinates of four such segments, which are crystallographically independent. The hydrogen bonding pattern within each set of four segments is very similar with an average rms deviation of the donor-acceptor distances of <0.2 Å. The nucleotides are numbered according to Figure 1. C and D, Hydrogen bonding pattern in the two d-(ACT)·d-(AGT) segments of the uncomplexed *trp*O decamer, showing water molecules W1 to W5 and W1′ to W5′ suggested to correspond to the interfacial water molecules in the complex structures (Shakked *et al.*, 1994). The two segments show distinct hydrogen bonding patterns and are therefore shown separately. Additional water molecules hydrogen bonding to base atoms are W3″ and W4″ (Shakked *et al.*, 1994; not shown in the Figure). W3″ forms probable hydrogen bonds to W2′, G6 O6 and W4′, and plausible hydrogen bonds to W3′ and W4″. W4″ forms probable hydrogen bonds to W4′ and A5 N7, to W4 and T−5 N1 across the dyad axis, and a plausible hydrogen bond to W3″.

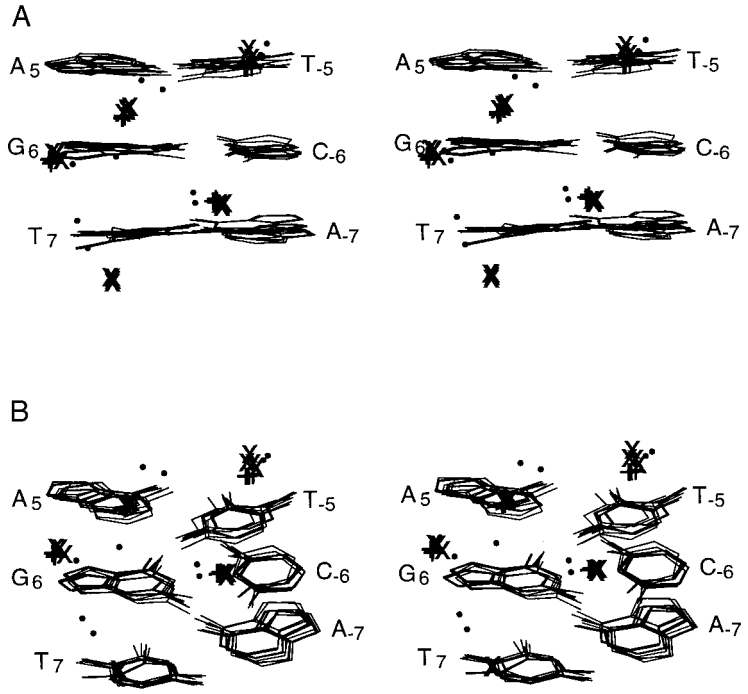

Figure A2. Stereo views of the bases of the d-(ACT)·d-(AGT) segments of 1TRR, 1TRO and uncomplexed *trp*O decamer (Shakked *et al*., 1994) with associated hydration water molecules. All eight sites contacted by protein in 1TRR and 1TRO are displayed, as well as both d-(ACT)·d-(AGT) sites in the uncomplexed *trp*O decamer. The heavy atoms of the bases were superimposed onto the mean structure and water molecules were not included in the superposition. The hydration water molecules of Figure A1 are identified by (+), (×) and (●) symbols for the crystal structures of 1TRR, 1TRO and the *trp*O decamer, respectively. The nucleotides were numbered according to the numbering of the *trp*O 17-mer (Figure 1). The views in A and B differ by a rotation by 22° around a horizontal axis. The Figure was prepared with MolScript (Kraulis, 1991).

mediate protein-DNA contacts. The presence or absence of a fifth water molecule contacting T7 O4 (W1 and W1′ in the *trp*O decamer) and differences in hydrogen bond lengths (Figure A1A, B) may be attributed to insufficient resolution of the 1TRR co-crystal structure.

The corresponding hydration water molecules associated with the d-(ACT)·d-(AGT) segments of the uncomplexed *trp*O decamer differ from those of the complexed segments in position (Figure A2) and hydrogen bonding partners (Figure A1). The average displacements are almost as large as those obtained for a comparison with randomly hydrated DNA (Table A1) and larger than the radius of a water molecule. In the three-dimensional lattice of ice (as a coarse approximation of water structure), a water molecule can be displaced by at most 1.4 to 2.8 Å (average 2.1 Å), before it is closer to the site of a neighboring water molecule than to its original site. The average displacement observed between corresponding water molecules of complexed and uncomplexed d-(ACT)·d-(AGT) segments is of comparable magnitude, indicating poor correlation between the water locations in the two states. Furthermore, variability in the hydration structure of uncomplexed DNA is indicated by differences in water positions and hydrogen bonding patterns between the symmetry-related halves of the uncomplexed *trp*O decamer (Figures A1C, D, and A2, Table A1).

Both co-crystal structures contain a bridging water molecule hydrogen bonding to A5 N7 and G6 O6 (Figures A1A, B and A2) which appears to be critical for high-affinity protein binding from biochemical mutation studies (Joachimiak *et al*., 1994; Smith *et al*., 1994). In contrast, the *trp*O decamer structure does not show any water molecule bridging between the bases of the d-$(ACTAGT)_2$ segment. Instead, the water molecules of the uncomplexed DNA lie approximately in the plane of the bases, close to the locations that would be predicted from the DNA hydration analysis compiled by Berman and co-workers (Table A1; Schneider *et al*., 1993; Schneider & Berman, 1995).

The asymmetries in the hydration of the two uncomplexed d-(ACT)·d-(AGT) segments may be

Table A1. Comparison of the locations of water molecules W1 to W5 and W1′ to W5′ in uncomplexed *trp*O DNA and water molecules at corresponding positions (Figure A1) in the protein/DNA complexes

Structures compared	Average distance (range)/Å
1TRR *versus* 1TRO[a]	0.4 (0.0–0.9)
*trp*O decamer *versus* 1TRO and 1TRR[a]	1.6 (0.4–2.9)
Random positions *versus* 1TRO[b]	2.0 (0.0–4.6)
W1 to W5 *versus* W1′ to W5′ in *trp*O decamer	1.1 (0.5–1.9)
*trp*O decamer *versus* prediction[c]	0.7 (0.3–1.2)

Superposition as in Figure A2.

[a] W1/W1′ were excluded from comparisons with 1TRR, which does not contain a corresponding water molecule.

[b] Random water positions were defined by a grid search for solvent accessible space around the donor or acceptor atoms defining the sites of W1′ to W5′ in 1TRO (Figure A1). Using a 0.1 Å distance between neighboring grid points, 5000 to 10,000 points were identified surrounding each of the donor or acceptor atoms, where a water molecule of 1.4 Å radius could be placed within hydrogen bonding distance (3.3 Å) without van der Waals clashes with the DNA. For each of the five hydration sites, the distances were calculated between the water coordinates and the grid points defining the corresponding random hydration space.

[c] Average hydration sites in *B*-DNA according to Schneider *et al*. (1993).

explained by crystal packing effects, although the water molecules shown in Figure A2 do not make direct contacts with neighboring DNA duplexes in the crystal lattice. Clearly, the unexpectedly few or long hydrogen bonds observed for the hydration water could be a consequence of averaging over different conformations, as single crystal structures represent time and ensemble averages over many molecules. This would be consistent with the picture emerging from the MD study of the hydration of the 16 base-pair duplex part of the uncomplexed *trp*O 17-mer, where water molecules hydrogen bonded to the DNA bases displayed a multitude of different hydrogen bonding interactions within 1 ns, yet with an average of about four good hydrogen bonds during the course of the simulation (Bonvin *et al*., 1998).

The high structural conservation of water molecules at the protein/DNA interface in the co-crystal structures suggests that these water molecules are important components of the *trp* repressor/operator complex. The hydration of the uncomplexed *trp*O decamer is not as well conserved and differs significantly from the hydration of the complexes. Together with the observation of rapidly interconverting hydration networks in the MD simulation (Bonvin *et al*., 1998), it appears unlikely that the hydration of the uncomplexed DNA target is a major structural determinant of *trp* repressor/operator recognition.

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