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Complicated water orientations in the minor groove of the B-DNA decamer d(CCATTAATGG)$_2$ observed by neutron diffraction measurements

Shigeki Arai, Toshiyuki Chatake, Takashi Ohhara, Kazuo Kurihara, Ichiro Tanaka, Nobuhiro Suzuki[1,2], Zui Fujimoto[1], Hiroshi Mizuno[1] and Nobuo Niimura*

Neutron Science Research Center, Japan Atomic Energy Research Institute, 2-4 Shirakatashirane, Tokai, Ibaraki 319-1195, Japan, [1]Department of Biochemistry, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan and [2]Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

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ABSTRACT

It has long been suspected that the structure and function of a DNA duplex can be strongly dependent on its degree of hydration. By neutron diffraction experiments, we have succeeded in determining most of the hydrogen (H) and deuterium (D) atomic positions in the decameric d(CCATTAATGG)$_2$ duplex. Moreover, the D positions in 27 D_2O molecules have been determined. In particular, the complex water network in the minor groove has been observed in detail. By a combined structural analysis using 2.0 Å resolution X-ray and 3.0 Å resolution neutron data, it is clear that the spine of hydration is built up, not only by a simple hexagonal hydration pattern (as reported in earlier X-ray studies), but also by many other water bridges hydrogen-bonded to the DNA strands. The complexity of the hydration pattern in the minor groove is derived from an extraordinary variety of orientations displayed by the water molecules.

INTRODUCTION

The arrangement of water molecules acts as one of the important determinants of the stability (1), polymorphism (2) and flexibility (3,4) of the DNA duplex. Moreover, it is well known that a 'spine of hydration' exists in the narrow minor groove of the B-form of DNA. This spine of hydration was first observed in the X-ray crystallographic analysis of the dodecameric duplex d(CGCGAATTCGCG)$_2$ (5). More recent high-resolution X-ray crystallographic analyses on the same dodecamer (6–9) have shown that a series of hydrogen bonds, in a hexagonal pattern, is built up on this spine of hydration. In those studies, the observed hydration structures were deduced from a network consisting only of the O atoms of water molecules, because it is difficult in an X-ray crystallographic analysis to identify the H atoms. The highest resolution study of a B-DNA oligomer published thus far is the very recent 0.74 Å resolution X-ray analysis of d(CCAGTACTGG)$_2$ (PDB code 1D8G) by Kielkopf *et al*. (10). Despite the fact that they have included a discussion of the H atoms determined from their X-ray structure, the H positions of the water molecules have not been included in the PDB data file (1D8G) thus far. This suggests that the determination of H positions had not been easy, even though extremely high-resolution X-ray diffraction data were available. However, previous molecular dynamics (MD) simulations have included H atoms in their discussion of several specific hydration patterns. For example, Westhof *et al*. (11) have shown that certain RNA helices are more rigid than DNA helices because of the difference between the hydration structure of DNA and RNA. Moreover, by MD simulation calculations of various DNA oligomers, it has been observed that long-lived hydration patterns are sequence-dependent and mostly located in the minor groove (12). For example, water bridges between pyrimidine O_2 atoms have been observed by MD simulations of B-DNA (13).

*To whom correspondence should be addressed. Tel: +81 294 38 5254; Fax: +81 294 38 5272; Email: niimura@mx.ibaraki.ac.jp
Present addresses:
Toshiyuki Chatake, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 3 Shiomi-cho, Choshi, Chiba 288-0025, Japan
Ichiro Tanaka, Department of Biomolecular Functional Engineering, the College of Engineering, Ibaraki University, 4-12-1 Naka-Narusawa, Hitachi, Ibaraki 316-8511, Japan
Nobuo Niimura, Institute of Applied Beam Science, Graduate School of Science and Engineering, Ibaraki University, 4-12-1 Naka-Narusawa, Hitachi, Ibaraki 316-8511, Japan

To support the results of those pioneering studies, it is necessary to determine actual positions of the H atoms.

Neutron diffraction is a very powerful method for determining the detailed positions of H and D atoms of hydration. Recently, the neutron structural analyses of many kinds of proteins, such as hen egg white lysozyme (14), human lysozyme, sperm whale myoglobin (15,16), wild-type rubredoxin (15,17), a mutant rubredoxin (18), insulin (19) and dissimilatory sulfite reductase D (20), have been carried out, and most of the hydrogen and deuterium atomic positions in those crystal structures have been determined (21).

In previous neutron studies of DNA duplexes, the technique used was neutron fiber diffraction and hydration structures have been reported (22). In those investigations, a clear network of water molecules running along the inside edge of the minor groove was observed. However, the structural information from those studies was limited, and it was difficult to discuss the finer details, such as the orientations of the hydration water molecules. Thus, single-crystal neutron diffraction is an indispensable technique to determine the precise positions of the H atoms in DNA strands and the orientations of the water molecules around the DNA duplex. By using a crystal of the B-form DNA decamer d(CCATTAATGG)$_2$, we have carried out such a study. In order to obtain the initial model of the DNA structure, we have also carried out a synchrotron X-ray data collection on the sample. Goodsell *et al*. (23) had earlier discussed the 2.3 Å resolution X-ray structure of this particular oligomer, the d(CCATTAATGG)$_2$ duplex, had discovered a bending of B-DNA at T–A steps and had discussed the implications of that bend. In the molecular structure determined by their study (PDB code 167D), only the O positions of 44 water molecules were observed, and hydrogen bonds were defined using the criteria of O···O or O···N distances <3.5 Å (23). In order to get more information about the orientations of water molecules, we have carried out the X-ray and neutron structural analyses of this sample as described in this paper.

MATERIALS AND METHODS

Crystallization

The DNA decamer d(CCATTAATGG) was synthesized with a DNA synthesizer. The purity of the DNA was analyzed with high-performance liquid chromatography and impurities arising from other macromolecules or species were <3.9%. Salt impurities were removed by dialysis and the final electrical conductivity of the outer solution of the dialysis was 2.1 μS cm^{-1}, corresponding to a concentration of 0.08 mM NaCl, which was negligibly small compared with the precipitant concentration used in the crystallization experiments.

In order to carry out a high-resolution neutron diffraction experiment, a single crystal having a volume larger than 1 mm^3 is necessary (14). This particular DNA oligomer, d(CCATTAATGG), was selected as the target sample because we had earlier succeeded in determining the best conditions for crystal growth by using a crystallization phase diagram technique (24). Moreover, we had also assessed the quality of the resulting crystals by using a 'relative Wilson plot' method (25), and we have found that the quality of DNA crystal improves, with a concomitant increase in crystal size, near the DNA solubility minimum point on the crystallization phase diagram. From those results, good-quality and large single crystals were grown under the following conditions: 2.0 ml of 2.0 mM D_2O solutions of DNA (In a neutron diffraction experiment, in order to avoid the high background coming from the incoherent neutron scattering of H atoms, crystallization in D_2O solution instead of H_2O is generally conducted.), $MgCl_2$ (100 mM), 30% (v/v) 2-methyl-2,4-pentanediol and pD 6.6 at 279K. Subsequently, using a batch method, we succeeded in increasing the crystal size of this B-DNA dodecamer to 2.8 mm^3, which is the largest single crystal of an oligomeric B-DNA reported thus far (26).

Data collection

Initial X-ray analysis: the previous X-ray work of d(CCATTAATGG)$_2$ duplex (23) was carried out with a crystal grown in H_2O solution while our crystal was grown in D_2O solution. Moreover, the crystallization conditions between our study and the previous one were slightly different. We thought that these different conditions might cause some small but significant differences of the atomic positions in crystals between our study and the previous one (PDB code 167D), especially in view of the fact that the lattice constants between two crystals were slightly different. Therefore, prior to the neutron diffraction experiment, we carried out the X-ray diffraction analysis of the crystal grown in D_2O using the BL-41XU diffractometer installed at the Spring8 synchrotron radiation facility. The wavelength of the incident X-ray beam was 0.71 Å, and an oscillation method was used for the X-ray data collection. The total oscillation range was 180° in steps of 1.0°. A Cryo-systems Cryostream was used to cool the DNA crystals at 93K under a nitrogen vapor stream.

The subsequent neutron diffraction experiments were carried out using the BIX-4 neutron diffractometer installed at the JRR-3M reactor at JAERI (27). The wavelength of the incident neutron beam was 2.6 Å, obtained by using an elastically bent perfect-Si crystal monochromator (28). Bragg reflections were detected using neutron imaging plates surrounding the sample. A step scanning method ($\Delta\phi = 0.3°$) was used for data collection. The exposure time of each frame varied from 3 to 6 h and the total time for data collection was 31 days. The crystal was sealed in a quartz capillary for the measurements. In this diffraction experiment, the quartz capillary was slightly cooled to 279K, which was the temperature used in the crystal growth experiments. We used two crystals to collect the entire neutron dataset because the first DNA crystal was damaged by drying, which accidentally occurred when the temperature increased unexpectedly inside the quartz capillary. Those two crystals, whose volumes were 2.8 and 1.6 mm^3, respectively, were obtained from the same crystallization solution.

The diffraction images obtained from X-ray and neutron diffraction experiments were processed and scaled using the program DENZO and SCALEPACK (29). Data collection and refinement statistics of X-ray and neutron diffraction are given in Tables 1 and 2, respectively.

Initial model from the X-ray analysis

The crystallographic refinement was performed using the program CNS (30). The structure of B-DNA duplex d(CCATTAATGG)$_2$ (PDB code 167D) was used for the initial phasing of the dataset. The space group is $P3_221$, with unit cell

Table 1. Data statistics of X-ray analysis

Data collection statistics	
Space group	$P3_221$
a	32.9 Å
b	32.9 Å
c	96.1 Å
Resolution range	50.0–1.6 Å
Completeness (overall)	99.1%
Completeness (final resolution shell)	97.9% (1.66–1.60 Å)
R-merge (overall)	2.5%
R-merge (final resolution shell)	10.8% (1.66–1.60 Å)
Refinement statistics	
Resolution range of refinement	50.0–2.0 Å
R-factor	23.7%
R-free	28.4%
No. of reflections, working set	3887 (87.1%)
No. of reflections, free set	474 (10.6%)
Highest resolution shell	2.09–2.00 Å
No. of reflections in the highest resolution shell, working set	487 (89.5%)
R-factor in the highest resolution shell	28.8%
R-free in the highest resolution shell	33.8%
No. of reflections in the highest resolution shell, free set	49 (9.1%)

PDB ID code 1WQY.

Table 2. Data statistics of neutron analysis

Data collection statistics	
Space group	$P3_221$
a	32.9 Å
b	32.9 Å
c	96.1 Å
Resolution range	100.0–3.0 Å
Completeness (overall)	63.1%
Completeness (final resolution shell)	62.4% (3.18–3.00 Å)
R-merge (overall)	23.3%
R-merge (final resolution shell)	24.5% (3.18–3.00 Å)
Refinement statistics	
Resolution range of refinement	30.0–3.0 Å
R-factor	25.2%
R-free	27.4%
No. of reflections, working set	800 (57.7%)
No. of reflections, free set	79 (5.7%)

PDB ID code 1WQZ.

dimensions $a = b = 32.9$ Å and $c = 96.1$ Å. The program XtalView (31) was used for viewing electron-density maps and model building. The limiting resolution estimated from data collection statistics was 1.6 Å. However, the structural refinement processing was computed by using reflections from 50.0 to 2.0 Å resolution, because the *R*-factor and *R*-free values for reflections having resolutions >2.0 Å were rather poor (Table 1).

Neutron analysis

We used our refined result of X-ray structural analysis as the initial model of DNA structure for neutron analysis, because the unit cell parameters and the space group ($P3_221$) of the X-ray dataset and the neutron dataset were almost the same. The structure refinement was carried out using the program CNS, whose topology and parameter files had to be modified for neutron protein crystallography to include H and D atoms. It was initially assumed that H atoms bound to C atoms were not replaced by D atoms, whereas those bonded to N and O atoms were deuterated.

The limiting resolution estimated from the data collection statistics was 3.0 Å. However, the use of even higher-resolution data was found to yield a better model, even though the completeness of the dataset was not high (19,32). In our neutron structural analysis of oligomeric DNA, we included reflection data from 30.0 to 2.5 Å resolution in the neutron map calculation. The final values of *R*-factor and *R*-free for the (30.0–3.0 Å) dataset were 25.2 and 27.4%, respectively. However, the final values of *R*-factor and *R*-free for the (30.0–2.5 Å) dataset were 28.4 and 32.6%, respectively.

In the refinement processing, the positions of exchangeable H atoms of the DNA duplex were identified using the (2|Fo| − |Fc|) and (|Fo| − |Fc|) neutron Fourier maps. H and D atoms could be identified as negative and positive peaks in the 2|Fo| − |Fc| map, respectively.

Determination of the orientation of the hydration molecules and hydrogen bond lengths

In a neutron analysis, neutron Fourier maps of hydration water molecules can be categorized as having triangular, ellipsoidal and spherical shape (33). This classification conveniently reflects the degree of disorder and/or dynamic behavior of water molecule. In this paper, we classify all water molecules as having only the triangular shape because it was difficult to determine the accurate shape of the water contours from the 3.0 Å resolution neutron data.

To identify D_2O molecules, first the O positions of D_2O as determined from the X-ray analysis were used. Then, the D atoms of D_2O molecules were located by considering the direction of the hydrogen bond acceptor (N or O) atoms. The shape of 2|Fo| − |Fc| neutron Fourier map was very helpful in determining the orientations of water molecules. Finally, 27 D_2O molecules including D positions were determined in the present neutron structural analysis.

In the book by Desiraju (34), hydrogen bonds are classified as follows:

(i) Very strong hydrogen bonds: 1.2 Å < d[H···A] < 1.5 Å, 175° angle [X-H···A] < 180°.
(ii) Strong hydrogen bonds: 1.5 Å < d[H···A] < 2.2 Å, 130° angle [X-H···A] < 180°.
(iii) Weak hydrogen bonds: 2.0 Å < d[H···A] < 3.0 Å, 90° angle [X-H···A] < 180°.

The van der Waals radius of atom 'A' relates to the hydrogen bond length. However, in our study, it was difficult to classify the hydrogen bonds into the above types, because the limiting resolution of our neutron dataset was not good enough and the peak contours in our observed neutron Fourier maps were not clear enough. In principle, we defined hydrogen bonds as d[H···A] < 3.0–3.1 Å, 90° angle [X-H···A] < 180° based on the description in the book by Desiraju (34). Therefore, hydrogen bond distances described in this paper are approximate values determined by a medium resolution neutron analysis.

RESULTS AND DISCUSSION

Water bridges

From early X-ray studies, it has been well known that water molecules form hydrogen bonds to the anionic oxygens of the phosphate groups, to the ester oxygens of the phosphodiester

linkages, to the O4′ oxygens of the furanose rings and to the electronegative atoms of the base pairs (13). Moreover, water bridges, which are made by binding waters between polar atoms belonging to the same nucleotide or to neighboring ones, have frequently been observed. However, those water bridges have been assumed to exist by connecting only O positions of water molecules. Thus far, only MD studies have included H positions in the discussions of water bridges (13,35).

On the other hand, we have succeeded in determining the orientations of water molecules in many water bridges. For example, Figure 1 shows a D_2O molecule, with D atoms in light blue, near the Cyt1•Gua20 base pair in the minor groove. As shown in this figure, an inter-strand water bridge is formed between the O_2 atom of Cyt1 and the 2D2 atom of Gua20 and thus it seems that in this case the water molecule is helping to support the Watson–Crick hydrogen bonds of this base pair.

Figure 2 shows an example of a cross-strand water bridge [here, the cross-strand water bridge in the minor groove is a special inter-strand water bridge, which links bases that are neighboring in the 3′ direction; for example, see Figure 12f in (36)], connecting O2 of Thy5 with N3 of Ade17. Thus, Figures 1 and 2 illustrate the fact that a water molecule is a versatile connector, which can serve as both a hydrogen bond donor and acceptor. These water bridges contribute to the overall stability of helical conformations of DNA (13,35). A spine of hydration is built by a combination of many water bridges, such as those shown in Figures 1 and 2.

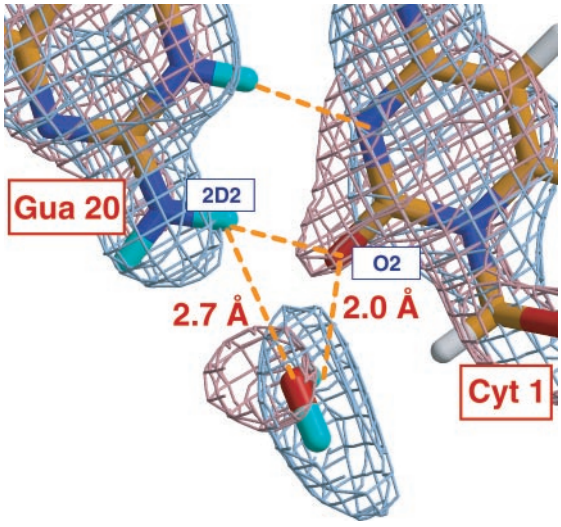

Figure 1. A D_2O molecule hydrogen-bonded to a Cyt1•Gua20 base pair in the minor groove. The purple and blue contours indicate 2|Fo| − |Fc| X-ray map (3.0 σ level) and neutron Fourier map (2.5 σ level), respectively.

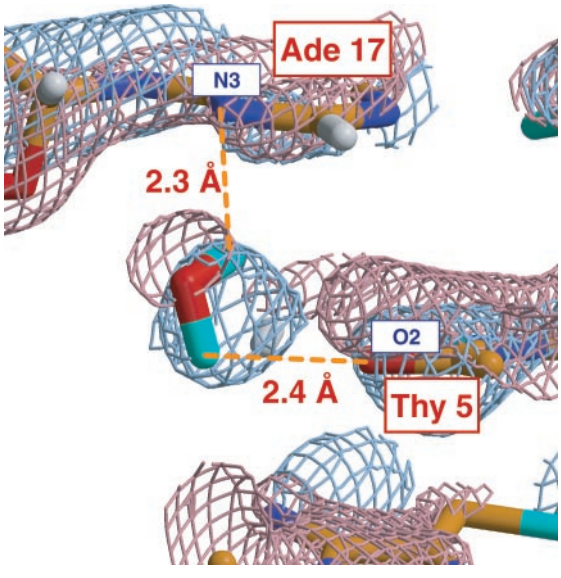

Figure 2. A cross-strand water bridge in the minor groove. The purple and blue contours indicate 2|Fo| − |Fc| X-ray map and neutron Fourier map, respectively. Both maps contoured at 4.0 σ level.

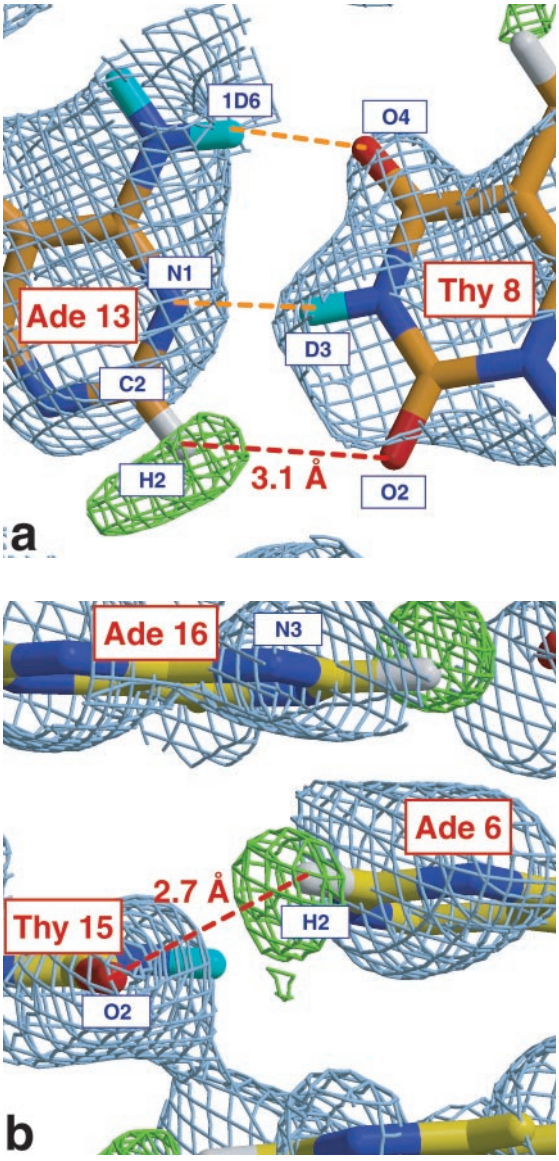

Figure 3. Examples of C–H···O hydrogen bonds (indicated by red broken lines) in Ade•Thy base pairs. The 2|Fo| − |Fc| neutron Fourier maps are contoured at 3 σ level (blue) and −5 σ level (green). H atoms appear as negative contours due to their negative scattering lengths. (**a**) A horizontal sectional view of the Thy8•Ade13 base pair. (**b**) A vertical sectional view of the Ade6•Thy15 base pair.

C–H···O hydrogen bonds

As shown in Figures 1 and 3a, the H atoms of the base pairs that participate in the formation of hydrogen bonds were all replaced by D atoms [i.e. D1(G), 2D2(G), 2D4(C), D3(T) and 1D6(A)], and some of them were solvated by D_2O molecules. These results indicate that water molecules can easily access even the center regions of base pairs. In our case, the H atoms bonded to N and O atoms on the entire oligomeric DNA duplex are exchangeable for D atoms of solvents. In addition to that, we have also succeeded in directly observing the H atoms bound to C atoms in Watson–Crick base pairs (Figure 3a and b).

It has been postulated that C–H···O hydrogen bonds also contribute to the structural rigidity and stability of nucleic acids (34,37). For example, the electropositive C_2–H_2 group of adenine is in very close proximity to the keto O atoms of both pyrimidine bases in the antiparallel strand of the duplex structure (38). In our neutron diffraction experiment, we observed several examples of inter-base pair C–H···O hydrogen bonds in the minor groove, one of which is shown in Figure 3b.

Figure 4 shows an example of a water molecule hydrogen-bonded to a phosphate group. Previous X-ray studies suggested that each charged oxygen of a phosphate group can be surrounded by up to three water molecules, which can be arranged in a cone of hydration (35,39). In addition, it has been observed that several water molecules can be in contact with the O3′ and O5′ ester oxygen atoms, and a few with the sugar O4′ atoms (40). In our neutron case, it has also been observed that water molecules connect O1P or O2P with various H atoms of the 2′-deoxyribose group, such as the one shown in Figure 4. Perhaps such hydrogen bonds are important in maintaining a helical conformation: in fact, it has been pointed out that there is an intimate relationship between the hydration around the phosphate backbone and the structure of DNA duplex (35). In summary, we have succeeded in observing a significant number of weak C–H···O hydrogen bonds, as illustrated in Figures 3b and 4.

The spine of hydration in the minor groove

The most prominent feature, in most of the previous discussions of DNA hydration, has been the spine of water molecules along the minor groove of the duplex, and this is where we present the most important part of our results. Figure 5a shows the spine of hydration in the minor groove determined initially by our X-ray structural analysis. This X-ray analysis basically confirmed the results of the previous (23) X-ray structure determination (PDB code 167D). In the previous X-ray

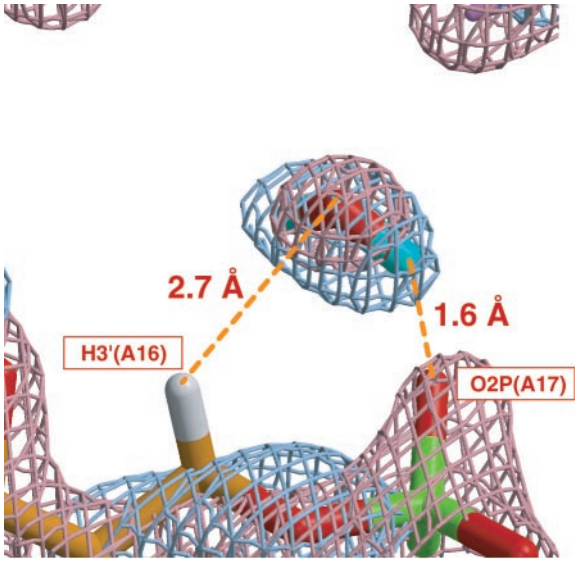

Figure 4. An example of a water molecule hydrogen-bonded to a phosphate group. The red and blue contours indicate 2|Fo| − |Fc| X-ray map (4 σ level) and neutron Fourier map (4 σ level), respectively. This water connects a phosphate oxygen, O2P(A17), with the C(3′)–H bond of a neighboring ribose sugar, H3′ (A16).

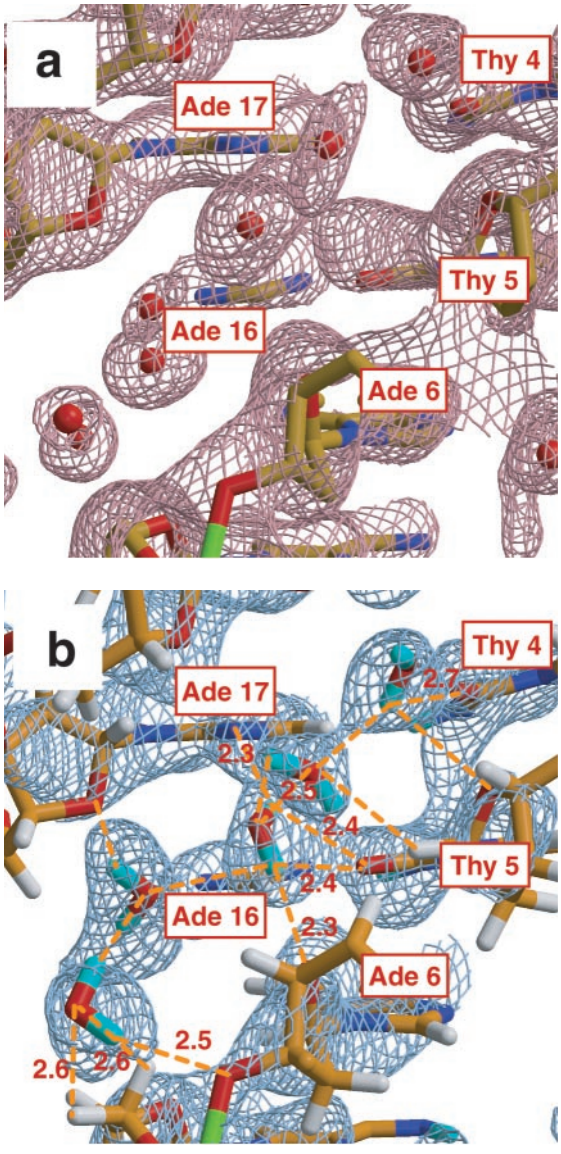

Figure 5. The spine of hydration in the minor groove. (**a**) The structure as determined by our X-ray diffraction analysis. Purple contours indicate peaks in the 2|Fo| − |Fc| X-ray map (1.5 σ level). Red balls show the O atoms of D_2O molecules. (**b**) The structure as determined by our neutron diffraction analysis. Blue contours indicate peaks in the 2|Fo| − |Fc| neutron map (3.0 σ level).

studies, hydrogen bond lengths have been assigned based on the distance between O atoms of water molecules; however, several hydrogen bonds assigned in this earlier work had unrealistically long O···O distances, such as 4.9 Å (41). On the other hand, in our neutron case (Figure 5b), we have found that hydrogen bond lengths between water molecules have relatively reasonable values, because the positions of the D atoms are observable and thus can be used to determine the hydrogen bond lengths reliably, even though the neutron experiments were carried out only at medium resolution (3.0 Å). The resulting orientations of those water molecules are represented by the complicated network of hydrogen bonds as shown in Figure 5b (for more details see below).

Figure 6 shows a schematic drawing of the spine of hydration in the minor groove of d(CCATTAATGG)$_2$, as observed by a combination of our X-ray and neutron diffraction measurements. In this figure, the O atoms of D_2O determined by the X-ray analysis are colored in blue and are superimposed over the results obtained by the neutron structural analysis. Such a spine of hydration in the minor groove of A•T-tract was formed by four layers of hydration shells (8,41). In our case, it was difficult to observe the water molecules in the third and fourth hydration shells by neutron diffraction, because the orientation of those water molecules are highly disordered and the maximum resolution of our neutron study was limited to 3.0 Å. However, the orientations of some D_2O molecules in the first and second hydration shells are relatively easily observed because some of those hydrogen bonds were clearly visible. Previous MD studies have shown that water molecules with long residence times (i.e. those that are held more tightly to the duplex) are found mainly around the DNA phosphate groups and in DNA minor groove (11). In particular, it has been known that, especially in the shallow/minor grooves, the residence times are much longer in the vicinity of the pyrimidine O2 and the purine N3 atoms. Moreover, it has been frequently observed in earlier X-ray studies that many water molecules bind to those pyrimidine O2 and/or purine N3 atoms (41). Our neutron results support those previous studies.

Figure 6 shows the detailed connectivity of hydrogen bonds between O atoms of D_2O and H or D atoms of DNA strands.

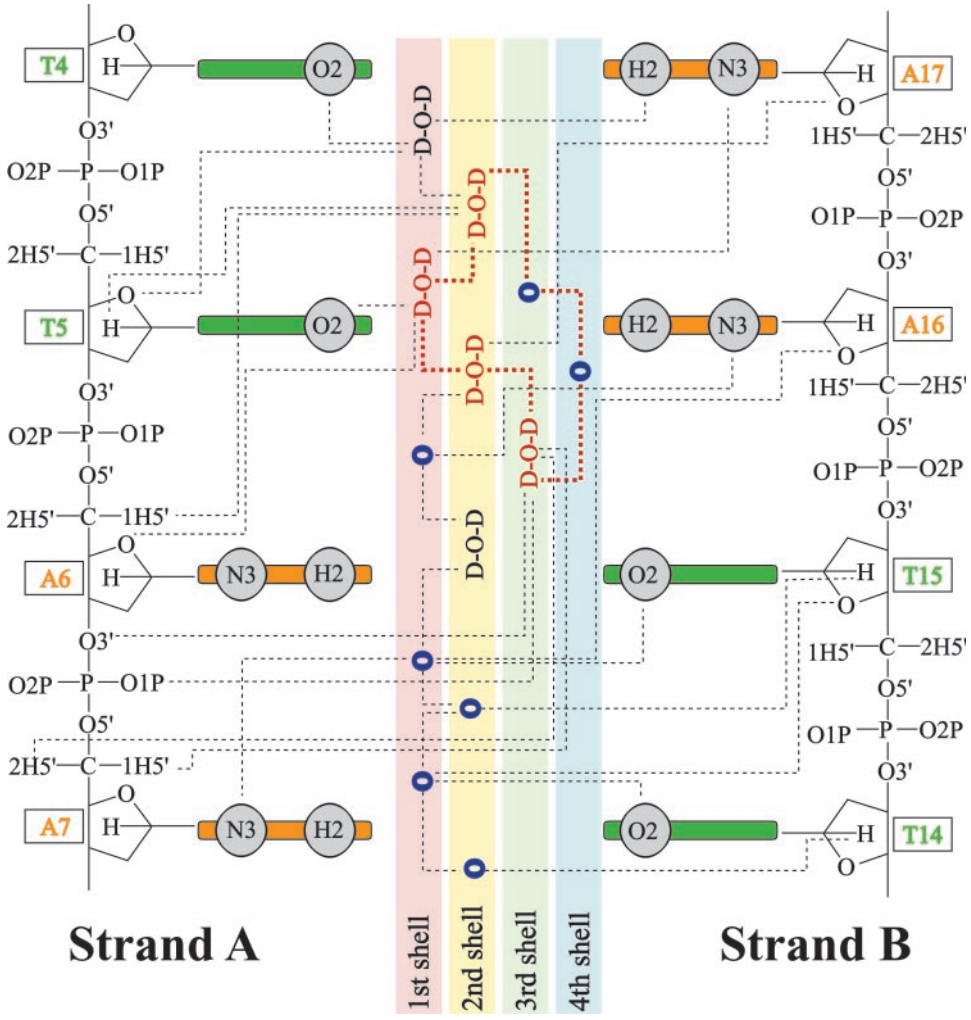

Figure 6. A schematic diagram showing the minor groove hydration network of d(CCATTAATGG)$_2$, with broken lines indicating the hydrogen bonding linkages. The four hydration shells are shown between strands A and B. The blue circles represent O atoms located in the X-ray analysis but whose D atoms were not observed in the neutron measurements. The hydrogen bonds shown as red broken lines indicate one of the hexagonal patterns frequently discussed in earlier publications of the hydration spine. Since the maximum resolution in this study was limited to 3.0 Å, several hydrogen bonds were observed to have slightly longer distances than expected.

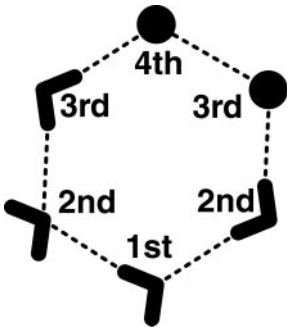

Figure 7. A schematic drawing of orientations of water molecules based on a hexagonal motif. This diagram depicts the rather complex and irregular hydration pattern that was actually observed in one of the hexagonal arrays in the present analysis of the $d(CCATTAATGG)_2$ decamer, as based on our neutron and X-ray measurements. The two balls marked '3rd' and '4th' indicate water molecules whose oxygens were observed by the X-ray diffraction measurement, but whose hydrogens were unobserved in the neutron maps.

These clearly contribute to the stability of the DNA duplex and the formation of the hydration network.

The hydration network based on a hexagonal motif

Previous X-ray studies have shown that the hydration pattern in the minor groove of A•T-tract can be represented by a simple hexagonal pattern of hydrogen bonds formed by connecting O atoms of water molecules (8,41). In the hexagonal hydration network observed in A•T-tract sequences, hydration water molecules in the first hydration shell make well-ordered cross-strand water bridges [for example, see Figure 8 in (41)]. Moreover, previous MD studies of B-DNA have concluded that the orientations of water molecules (including H atoms) in the cross-strand water bridges between two pyrimidine O2 atoms, or between the pyrimidine O2 and purine N3 atoms, are nearly perpendicular to the plane of base pairs, because the H positions of those water molecules can be easily directed toward those polar atoms of DNA (13). The results of those previous studies gave the impression that there is at least some orientational regularity of water molecules in the first hydration shell. On the other hand, from those previous studies, it is difficult to conclude that water molecules in the second, third and fourth hydration shells show some orientational regularity or not.

In Figure 6, the hydrogen bonds colored in red show a hexagonal pattern observed by our X-ray and neutron diffraction measurements. The observed orientations of water molecules in that particular hexagonal motif are schematically shown in Figure 7. Our neutron results suggest that it is difficult to predict an orientational regularity of water molecules in a single hexagonal motif since we have found an irregular and rather complex pattern as shown in Figure 7. This extraordinary variety of orientations of the observed water molecules causes the complexity of the hydrogen bonds network as shown in Figure 6.

CONCLUSION

Although many investigators have discussed the hydration network in the DNA grooves so far, the orientations of the water molecules have never been clarified because it was difficult to observe the H atoms of water molecules in DNA. The neutron crystallographic analysis of B-form DNA decamer $d(CCATTAATGG)_2$ reveals portions of the detailed structure, including the H positions of some of the hydration molecules, although the resolution was limited. By observing the H and D atoms in DNA and water molecules, we have succeeded in determining the orientations of those water molecules. A lot of inter-strand and intra-strand hydration bridges have also been observed. In addition, we have recently succeeded in determining the detailed hydration structure of a Z-form DNA oligomeric duplex, $d(CGCGCG)_2$, by neutron crystallographic analysis at a much higher resolution (1.8 Å) (T. Chatake, I. Tanaka, H. Umino, S. Arai and N. Niimura, manuscript in preparation). Hopefully, the structural information derived from our neutron studies will provide a scaffold on which to build new models of the hydration network structure of nucleic acids.

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Conflict of interest statement. None declared.

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