Low temperature structures of dCpG-proflavine

Conformational and hydration effects

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ABSTRACT The structure of the complex of dCpG with proflavine was determined using x-ray data taken at −130°C (low temperature) and at −2°C (cold temperature) and compared with the structure of the complex determined previously at room temperature (Shieh, H. S., H. M. Berman, M. Dabrow, and S. Neidle. 1980. *Nucleic Acids Res.* 8:85–97). Low temperature was refined with 5,125 reflections between 8.0 and 0.93 Å. Anisotropically modeled temperature factors were used for DNA/drug atoms and isotropic ones for water oxygens to R factor of 12.2% in $P2_12_12$; a = 32.853, b = 21.760, c = 13.296 Å. Cold temperature was refined isotropically with 2,846 reflections 8.0–0.89 Å to R = 15.1% in $P2_12_12$; a = 32.867, b = 22.356, c = 13.461 Å. Both structures are very similar to the room temperature one, though some important differences were observed: one guanine sugar moiety is disordered and additional water molecules have been located that give rise to infinite polyhedral hydration networks.

INTRODUCTION

The structure of the intercalated dinucleoside phosphate, dCpG-proflavine, was first determined in 1980 (Shieh et al., 1980). Analysis of the results confirmed that the intercalation geometry observed in ribodinucleoside phosphates is maintained in the deoxy model compounds and that the conformation of the 3′ sugar can assume either the 3′ endo or 2′ endo conformation. It also showed the surprising result that water molecules could be arranged in an extensive array of edge-linked pentagons (Neidle et al., 1980). This crystal formed the basis for several theoretical studies whose object was to model this elegant water structure and improve the force field used to model nucleic acids (Kim et al., 1983; Mezei et al., 1983; Swaminathan et al., 1990).

Among the questions that arose as a result of this structural analysis were: Are all of the electron density peaks that were refined as waters real or are some a result of errors? Is it possible to locate more crystallographically ordered water molecules in the crystal cavities?

Preliminary diffraction studies of crystals of dCpG-proflavine measured at −2°C showed that the structure exhibits polymorphism with respect to the arrangement of water molecules in the room temperature (RT) study (Berman and Ginell, 1986). In these determinations, one of the 3′ sugars is disordered and there are more water molecules in the asymmetric unit. Given the continued interest in hydration of biological molecules and the interrelationship between the water and molecular structures, we decided to flash freeze a crystal of dCpG-proflavine and use a high intensity source of x-rays to define the structure more rigorously. The results of that analysis, as well as the refined results of the preliminary determination of the dCpG-proflavine structure measured at −2°C, are presented.

EXPERIMENTAL

A summary of the data collection and refinement statistics for the three crystals of dCpG-proflavine is reported in Table 1. The diffraction data of these crystals were taken at different temperatures, and the crystals are hereafter reported as RT for the room temperature crystal (Neidle et al., 1980; Shieh et al., 1980), CT for the cold temperature crystal (collected at −2°C) (Berman and Ginell, 1986), and LT for the low temperature crystal (collected at −130°C).

Room temperature

The full description of this data collection is described elsewhere (Shieh et al., 1980) and summarized in Table 1.

Cold temperature

Lyophilized d(CpG) was dissolved in deionized water and mixed with proflavine hemisulphate (Sigma Chemical, St. Louis, MO) to form a 2:2 complex of d(CpG)-proflavine. Crystals were grown by the vapor diffusion method at 4°C where the drops contained d(CpG), proflavine, ammonium sulfate, and water against a well solution of ammonium sulfate and water. After seed crystals were grown, they were transferred to a drop containing lower concentrations of d(CpG) and proflavine for further growth. Additional transfers to new growth baths every other day was continued until a crystal large enough for data collection was grown.

A crystal was mounted in a capillary. X-ray intensity data were collected using $\omega/2\theta$ scans at −2°C on a CAD4 diffractometer using a sealed tube x-ray generator. The intensity data were corrected for Lorentz, polarization, decay, and anisotropic absorption of x rays. There were 2,861 independent reflections stronger than $2\sigma(I)$ to a resolution of 0.89 Å. This represents 36% of the unique data possible at that resolution.

Since the cell dimensions of the CT structure are very close to the cell dimensions of the RT structure, the coor-

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dinates of the dCpG and proflavine from the RT structure were used for refinement with the SHELX76 program (Sheldrick, 1976). Throughout the refinement, all bond lengths were restrained to their expected values (Allen et al., 1987).

The positions of water molecules were located in Fo-Fc electron density maps using the peak search in SHELX76 and by graphical fitting of Fo-Fc maps calculated by the PROTEIN program (Steigemann, 1985) and displayed on the Evans and Sutherland graphics computer using FRODO (Jones, 1978). Water molecules were fit into peaks above 3σ level in Fo-Fc maps. Only those water molecules whose temperature factors remained below 50 after refinement were retained. The same procedure was employed for the LT structure.

The first Fo-Fc map revealed 21 water molecules, and the R factor decreased to 16.5%. The next refinement cycles revealed positions of the rest of the water molecules. The subsequent Fo-Fc map indicated that the G2 sugar ring is disordered. Atoms C3′, O3′, and C2′ were modeled in both C3′ endo and C2′ endo conformations. Occupancies of the three disordered atoms were refined, and each set was found to contribute 50% to the total electron density. The occupancies, bond angles, and lengths were restrained for the disordered atoms. The remaining water positions were located in difference Fourier maps. There are a total of 32 water positions, of which a few are disordered so that the total number of stoichiometric waters is 30.

The refinement of all atoms with isotropic temperature factors converged at an R value of 15.1% for 2,846 reflections between 8.0 and 0.89 Å. The highest peaks in the difference map were found in the solvent region and around the stacked proflavine molecules. The final values of refinement parameters are summarized in Table 1.

Low Temperature

Lyophilized d(CpG) was dissolved in deionized water and mixed with proflavine hemisulphate (Sigma) to form a 2:2 complex of d(CpG)-proflavine. Crystals were grown by the vapor diffusion method at 4°C where the drops contained d(CpG), proflavine, ammonium sulfate, and water against a well solution of ammonium sulfate and water. Large crystals were grown in the same way as described for the CT crystal.

A crystal was mounted following the procedure described by Hope (1988). The crystal was placed in an oil drop (50% Paratone-N and 50% mineral oil) where precipitate, satellites, and mother liquor were removed from the crystal surface. The crystal was then scooped from the oil drop on the end of a glass fiber and placed on a goniometer in the path of a −130°C nitrogen stream that was momentarily blocked off.

X-ray intensity data were collected using $\omega/2\theta$ scans with a CAD4 diffractometer on a rotating anode x-ray generator (model 571; Enraf-Nonius, Bohemia, NY) equipped with a graphite monochromator and a cryo crystal cooler (model FR558; Enraf-Nonius). The temperature of the crystal during the data collection was −130°C. Intensity measurements were corrected for Lorentz and polarization factors, the anisotropic absorption of x-rays, and crystal decay as a function of time using the program package MOLEN (Fair, 1990). Unique reflections numbering 6,688 were collected to a resolution of 0.93 Å, 5,140 of which had intensities greater than $2\sigma(I)$.

The refinement was started with coordinates of the dCpG and proflavine atoms of the RT structure using the SHELX76 program (Sheldrick, 1976). All bond lengths were restrained to their expected values (Allen et al., 1987). The positions of water molecules were independently searched from Fo-Fc electron density maps using the peak search in SHELX76 (Sheldrick, 1976) and by graphical fitting of Fo-Fc maps in FRODO (Jones, 1978) as in the case of the CT form.

The first Fo-Fc map revealed 17 water molecules and the second one 12 additional water molecules. The value of R decreased to 17.1%. The temperature factors of the dCpG atoms were converted to anisotropic parameters, and temperature factors of both proflavine molecules were converted to anisotropic group parameters. At this stage, the R value for data between 8.0 and 0.93 Å was 12.6%. The Fo-Fc map indicated that the sugar ring belonging to the G2 residue is disordered. The positions of the disordered atoms, C3′, O3′, and C2′, were refined isotropically and their bond lengths and angles were restrained. The occupancies of these atoms refined to 80% for the C3′ endo sugar and 20% for the C2′ endo sugar. Further difference Fourier maps showed several more water molecules, some of which are disordered. There are 34 water positions for a total of 28.5 stoichiometric waters. All water molecules were refined isotropically. In the final cycles of refinement, bond length restraints were removed from all but 16 bonds in the complex; without restraints these deviate >0.1 Å from the targeted values (Allen et al., 1987). Ten of these bonds are in the proflavine molecules.

The refinement converged at an R value of 12.2% for 5,125 reflections between 8.0 and 0.93 Å. The highest peaks in the Fo-Fc map were found in the solvent region around the disordered waters.

The coordinates of the RT, CT, and LT structures are in the Nucleic Acid Database (Berman et al., 1992). The coordinates of the LT and CT forms will be deposited with the Cambridge Structure Database (Allen et al., 1979).

RESULTS AND DISCUSSION

Crystal and molecular comparisons

The different conditions for data collection of the three structures lead to overall changes in the unit cell dimen-

TABLE 1 Crystallographic information for the three dCpG-proflavine crystals

	RT	CT	LT
Unit cell			
a (Å)	32.991	32.867 (4)	32.853 (5)
b (Å)	21.995	22.356 (5)	21.760 (4)
c (Å)	13.509	13.461 (2)	13.296 (2)
$\alpha = \beta = \gamma = 90°$			
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Volume ($Å^3$)	9,802	9,891	9,505
Data collection			
Crystal size (mm)	0.3 × 0.3 × 0.05	0.5 × 0.4 × 0.15	0.3 × 0.2 × 0.1
Temperature	Room	−2°C	−130°C
Crystal mounting	Capillary	Capillary	Fiber
Data collection device	Syntex P 1	Enraf-Nonius CAD4	Enraf-Nonius CAD4
Radiation	CuKα	CuKα	CuKα/rotating anode
Unique data collected	8,571	8,046	6,688
Reflections $I > 2\sigma(I)$	2,654	2,861	5,140
Resolution limit (Å)	0.83	0.89	0.93
Refinement			
Resolution (Å)	33.0–0.83	8.0–0.89	8.0–0.93
Reflections > 4σ (Fo)	2,654	2,846	5,125
No. stoichiometric waters	25	30	28.5
No. water positions	26	32	34
R value[*]	15.0	15.1	12.2
wR value[‡]		18.6[§]	14.3[‖]
Average value of temperature factor *B*			
Base atom	4.3	4.2	2.9
Backbone atoms	5.3	4.6	3.7
Proflavine atoms	7.5	7.2	5.9
Water oxygens	16	16	9
Residual electron densities ($e^-/Å^3$)[¶]		+0.78; −0.60	+0.80; −0.73

[*] $R = \Sigma(Fo - Fc)/\Sigma(Fo)$.

[‡] $wR = (\Sigma w(Fo - Fc)^2/\Sigma w(Fo)^2)^{1/2}$.

[§] $w = 0.254/(\sigma(Fo)^2 + 0.0009Fo^2)$.

[‖] $w = 3.903/(\alpha(Fo)^2 + 0.0009Fo^2)$.

[¶] Number of electrons in the asymmetric unit taken as that of refined atoms.

sions, the conformation of the dCpG molecules, and the interactions of the water molecules with the complex and with one another. We will describe each aspect of the structures in turn.

A comparison of the unit cell dimensions (Table 1) of the three structures shows that the *b* and *c* directions are most affected by changes in the conditions of data collection. Specifically, the *c* axis shrinks on freezing so that the average stacking distance between bases and proflavines in that crystal decreases from 3.37 to 3.32 Å. The *b* axis is also decreased, which leads to the shrinking of the major and minor groove cavities. In the CT crystal, the *b* axis is larger than at RT, causing the volume of these cavities to be larger.

The overall conformation of the dCpG molecule is quite similar in the three structures (Table 2 and Fig. 1). The root mean square (rms) deviation per atom between the dCpG molecules in the RT and LT structures is 0.27 Å. For the CT and LT structures, the deviation is 0.11 Å. There are, however, some significant differences in the conformation of one of the chains. At RT, one dCpG chain has a C3′ endo sugar conformation at the 5′ end of the dinucleotide chain and a C2′ endo sugar at the 3′ end. The other chain has two C3′ endo sugars. At −2°C, the sugar at the 3′ end of the chain is disordered so that half the time it is C3′ endo and the other it is C2′ endo. At low temperature, the same sugar is disordered with a 80:20 ratio between the two forms. An examination of the RT results shows that the temperature factor of O3′ is significantly higher than any of the other main chain atoms.

Another less dramatic change is seen in the conformation of the phosphodiester linkage of the residue G4. The C5′4 atom is at a different position in the RT than in the LT and CT structures that affects torsion angles on both the O5′4-C5′4 and C5′4-C4′4 bonds (Table 2). This change appears to be related to the compression between the planes of the bases and the proflavines in the lower temperature forms. It must be noted that this explanation became most apparent after examination of a Kinemage animation of the structures (Richardson and Richardson, 1992).

The $d(CpG)_2$ molecule forms only three intermolecular hydrogen bonds with other symmetry related DNA molecules. These bonds are maintained in all three

TABLE 2 Comparison of backbone torsion angles in the three structures of dCpG-proflavine

Atoms	RT	CT	LT
Strand 1			
O5′1-C5′1-C4′1-C3′1	55	53 (2)	56 (1)
C5′1-C4′1-C3′1-O3′1	84	85 (1)	82 (1)
C4′1-C3′1-O3′1-P2	210	$\underline{197}$ (1)	202 (1)
C3′1-O3′1-P2-O5′2	290	290 (1)	294 (1)
O3′1-P2-O5′2-C5′2	290	298 (1)	288 (1)
P2-O5′2-C5′2-C4′2	219	217 (2)	217 (1)
O5′2-C5′2-C4′2-C3′2 (C3′ endo)	46	51 (3)	46 (1)
(C2′ endo)	—	$\underline{67}$ (3)	$\underline{63}$ (2)
C5′2-C4′2-C3′2-O3′2 (C3′ endo)	95	86 (3)	88 (2)
(C2′ endo)	—	$\underline{130}$ (4)	$\underline{130}$ (3)
O4′1-C1′-N1 1-C6 1	16	18 (2)	23 (1)
O4′2-C1′2-N9 2-C8 2	80	$\underline{93}$ (3)	81 (1)
Strand 2			
O5′3-C5′3-C4′3-C3′3	53	$\underline{70}$ (2)	60 (1)
C5′3-C4′3-C3′3-O3′3	79	82 (2)	80 (1)
C4′3-C3′3-O3′3-P4	203	203 (2)	202 (1)
C3′3-O3′3-P4-O5′4	300	294 (1)	297 (1)
O3′3-P4-O5′4-C5′4	287	291 (1)	292 (1)
P4-O5′4-C5′4-C4′4	218	$\underline{228}$ (2)	$\underline{228}$ (1)
O5′4-C5′4-C4′4-C3′4	73	$\underline{59}$ (2)	$\underline{59}$ (1)
C5′4-C4′4-C3′4-O3′4	150	155 (2)	154 (1)
O4′3-C1′3-N1 3-C6 3	10	13 (2)	14 (1)
O4′4-C1′4-N9 4-C8 4	113	111 (2)	113 (1)

Values of torsion angles of CT and LT that differ by >10° from RT are underlined. Estimated standard deviations of the torsion angles are given in parentheses for the CT and LT structures.

structures. No hydrogen-bonding interactions were observed between the proflavines or proflavines and DNA (Fig. 2). It is important to note that none of the oxygen atoms in the disordered G2 deoxyribose is involved in these intermolecular hydrogen bonds. Thus, this deoxyribose is potentially quite mobile.

The average temperature factors for the base, backbone, proflavine, and water atoms in the three structures are in Table 1. Their values are highest for RT and lowest for LT as is expected. The difference between temperature factors of RT and CT is small; only LT has significantly lower values of temperature factors.

Water assemblies

All of the water molecules seen in the RT structure are also observed in the cold and low temperature structures. The average rms deviation of the positions of these 25 waters between the RT and LT structures is 0.06 Å and the corresponding value for the CT and LT structures is 0.43 Å.

Some doubts were raised about the positions and proposed hydrogen bonding of some of the water molecules in subsequent analyses of the RT structure (Savage, 1986). The LT study has validated the positions of these molecules, although the standard errors in their positions may have been underestimated in the original study. For example, in RT, the water molecules W23 and W15 had a short contact of 2.4 Å, which called into question the positions of both of these oxygen atoms. In the LT structure, this contact is 2.6 Å and is consistent with good hydrogen bonding geometry. It is apparent that lowering of the temperature improved the data sufficiently to correct the positions of these atoms.

All the gaps seen in the RT structure are filled with crystallographically ordered water molecules in the LT structure, giving a total of 28.5 stoichiometric water molecules in the asymmetric unit. Thus, although the volume of the LT unit cell is smaller than that of the RT form, there are more waters observed. For the purpose of molecular dynamic simulation of the dCpG-proflavine structure, it was estimated that there were 27 waters in the asymmetric unit (Swaminathan et al., 1990). If it is assumed that the difference in volume of the RT and LT crystals is caused only by the fact that atoms take up less

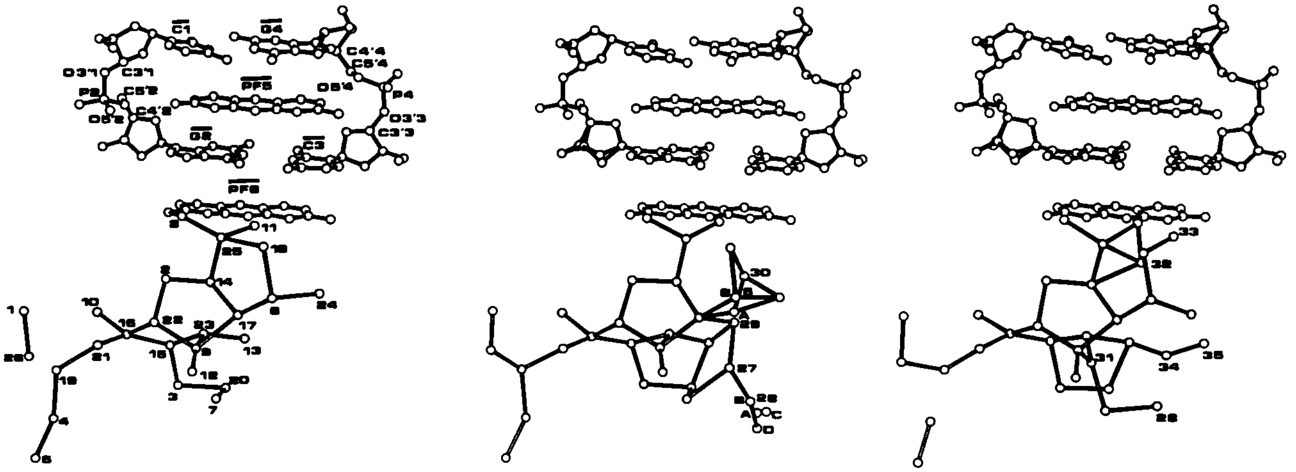

FIGURE 1 Comparison of the three structures of the dCpG-proflavine complex. The RT structure (Shieh et al., 1980) is at the left with the key dCpG atoms and all water molecules labeled. Residues are labeled C1, G2, C3, G4, PF5, PF6. In the LT (*center*) and CT (*right*) structures, only water molecules not present in the RT structure are labeled.

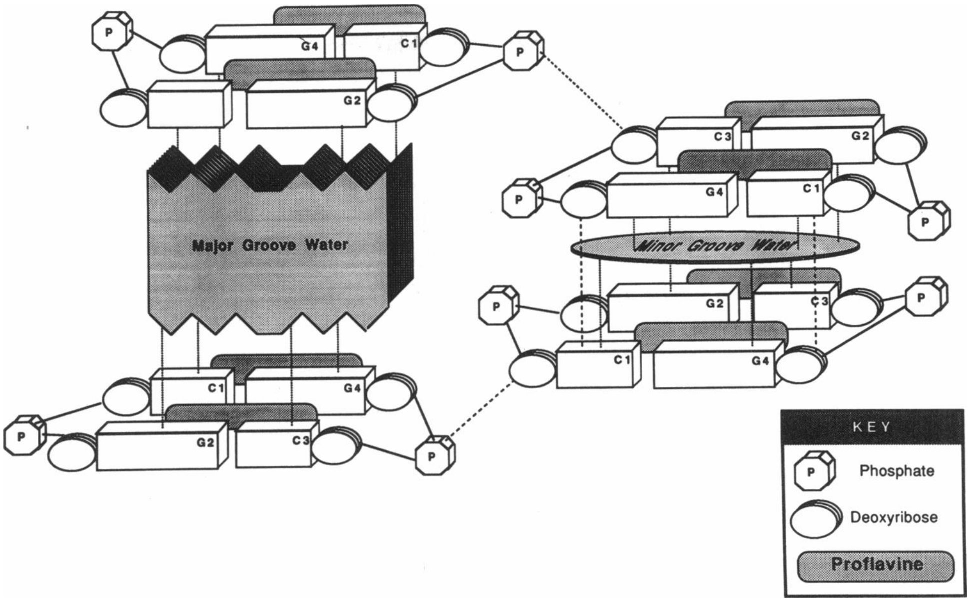

FIGURE 2 Packing schematic of the dCpG-proflavine complex. Dashed lines represent DNA-DNA hydrogen bonds and dotted lines illustrate interactions between dCpG-proflavine and water molecules.

space at low temperatures, then this is only a slight underestimate.

The water molecules in the LT form are involved in an even more complex hydrogen bonding network than was observed in the RT structure. If one counts all nonbonding contacts shorter than 3.20 Å as potential hydrogen bonding interactions, there are twice as many water–water contacts in LT than in RT. Nine of the water molecules hydrogen bond only with other waters and not with the dCpG or proflavine molecules.

The new water molecules allow for the formation of new pentagons in the LT asymmetric unit (Fig. 1). Their positions are such that they are hydrogen bonded to symmetry-related waters forming new polyhedrons of connected water molecules. Hydrogen-bonded water molecules form the infinite three-dimensional network spreading throughout the whole LT crystal (Fig. 3).

The CT structure has the largest number of crystallographically determined water molecules, with 10 of the water molecules in the second hydration shell. These additional water molecules are not all the same as the new ones observed in the LT structure. The number of water–water contacts is intermediate between those observed in the LT and RT structures. The patterns of connections are also slightly different from either LT or RT. In the LT and RT structures, waters W16, W15, W23, W9, and W22 form a pentagon, but a hexagonal unit is formed by waters W16, W15, W23, W31, W9, and W22 in the CT structure. The average temperature factor of the water molecules in CT is as high as in RT, which might be caused by the high number of second shell water molecules in CT.

As in the LT form, there are contacts between symmetry-related water molecules that lead to the infinite three-dimensional network of connected water molecules (Fig. 3).

Water-complex interactions

In all of the structures, most of the observed water molecules are first shell and are hydrogen bonded to the par-

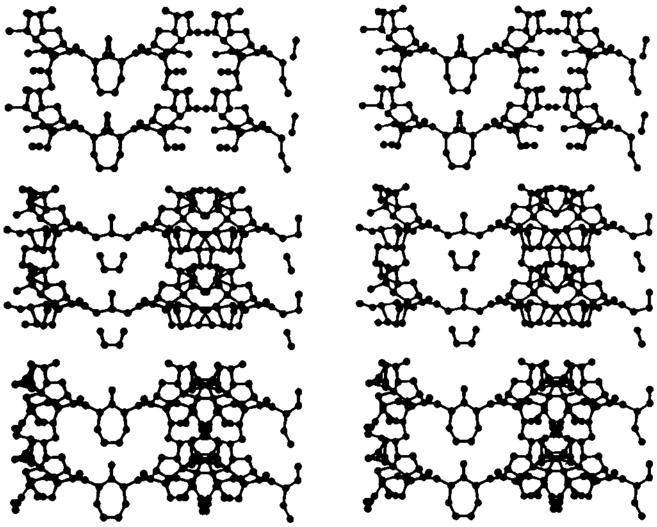

FIGURE 3 Water networks in the three structures of the dCpG-proflavine complex. Shown are approximately six asymmetric units without the dCpG-proflavine atoms. Water molecules within 3.20 Å of one another are shown as hydrogen bonded. The RT structure is on the top, CT in the middle, and LT at the bottom.

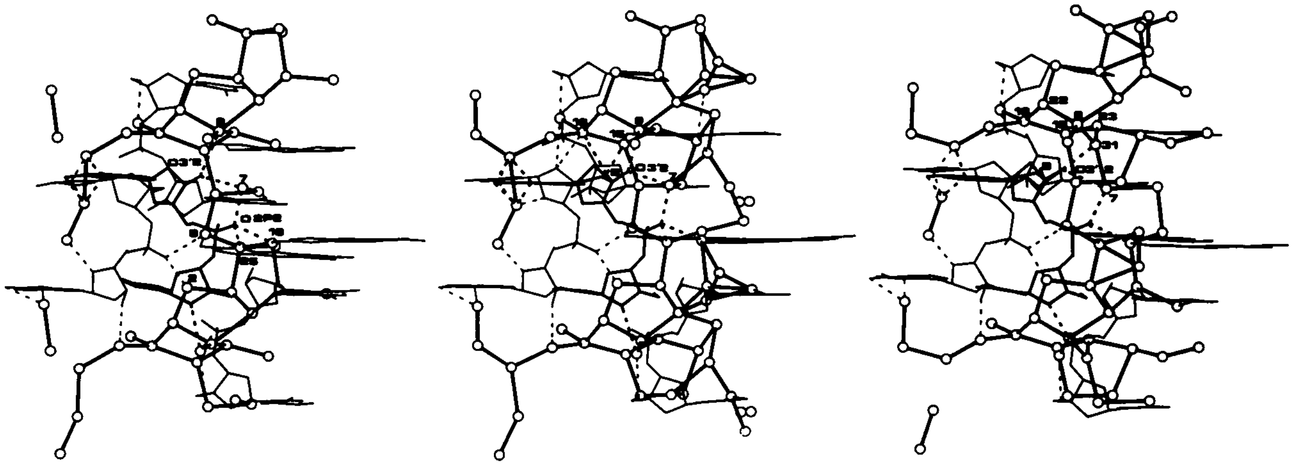

FIGURE 4 Surroundings of the disordered O3'2 atom in the three structures of the dCpG-proflavine complex. The molecules of dCpG and proflavine are drawn as stick figures. The water arrays are shown as ball and stick. The complex-water hydrogen bonds are indicated by dashed lines. The RT structure is at the left, LT in the middle, and CT at the right. The C2′ endo conformation of O3'2 is labeled *B* in the LT and CT structures.

ent complex. In two cases, dCpG atoms form vertices of the hydrogen-bonded polygonal arrays. The O2P4 atom hydrogen bonds to W8 and W2, forming the fifth vertex of the pentagon (O2P4-W8-W25-W14-W2-O2P4). As was pointed out by Savage in his critical analysis of the RT structure (Savage, 1986), O2P2 is involved in the formation of a hexagonal network of hydrogen bonds containing W18 and W25, which connect to W6, W17 and W14. This water arrangement is observed in all three structures.

An important difference between the three structures is the environment around the G2 sugar (Fig. 4). In the RT and LT structures, O3'2, in the prevalent C3′ endo conformation, bridges two water polygons via W7 and W9. In the LT form, O3'2 in the C2′ endo conformation (*B* in Fig. 4) interacts with the water molecules W15 and W16.

In the CT form, the O3'2 atom in the C2′ endo conformation forms no hydrogen bonds. O3'2 in the C3′ endo conformation interacts with W7 and W31 and the water molecule W31 is a part of the hexagonal water network that is unique to the CT form. Therefore, the O3'2 environment in the CT form is different from that observed in the RT and LT forms.

Thus, in the LT and CT forms, the change in sugar conformation is associated with different water–DNA interactions and a different water conformation. In the LT form, there is no change in the water–DNA or water–water interactions of the C3′ endo conformation as compared with the parent RT structure. Rather, there are some additional interactions due to the disordered G2 sugar atoms and newly localized water molecules. In the CT form, the sugar disorder is detected but no water molecule(s) that could stabilize the C2′ endo conformation was located within a hydrogen bonding distance limit from O3'2.

CONCLUSIONS

The present analysis allows us to make some observations and conclusions. The experiments reported in this paper show unequivocally that all 25 water molecules observed in the RT study are an intrinsic part of the structure. These "core" water molecules are found in all three crystals. The newly found water molecules in the LT and CT structures fill the formerly empty cavity in the center of the major groove and connect with their symmetry-related counterparts to form elegant infinite networks throughout the crystal. The new waters observed in the LT and CT structures are no doubt present at room temperature but are disordered and cannot be detected on electron density maps. These new waters are, however, different to some extent in the LT and the CT structures.

The apparent rigidity of the core waters is not in contradiction with the molecular dynamic calculations (Swaminathan et al., 1990) that show that the water network in the dCpG-proflavine system is dynamic. In that molecular dynamic simulation, the force field is not constrained to maintain exact crystallographic symmetry. On average, however, the water structure is maintained. It is interesting to speculate that if it were possible to examine this crystal at very high resolution, the water molecules might not be identical in each unit cell and that, in fact, the symmetry is lower than is apparent at 1 Å resolution. Indeed, the failure of any of these structures to refine to lower R values might be attributed to this possibility.

It has been shown in a systematic analysis of the hydration of bases of nucleic acids (Schneider et al., 1992) that the possible positions of water molecules around bases are highly variable and depend on their crystallographic and chemical environments. That analysis emphasizes the flexible nature of water positions that can easily change whenever their environment changes. On the other hand, if the chemical environment is very similar—as in the crystals of dCpG-proflavine—the positions of water molecules are mostly conserved and should be considered an integral part of the structure. Slight variations in crystallization conditions and different temperatures of the data collections of the three studied forms of the dCpG-proflavine complex correlate with subtle changes in conformations of their sugar phosphate backbones and with equally subtle changes in the water structure.

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