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Hydrogen bonds of DsrD protein revealed by neutron crystallography

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The features of hydrogen bonds in DsrD protein from sulfate-reducing bacteria have been investigated by neutron protein crystallography. The function of DsrD has not yet been elucidated clearly, but its X-ray crystal structure revealed that it comprises a winged-helix motif and shows the highest structural homology to the DNA-binding proteins. Since any neutron structure of a DNA recognition protein has not yet been obtained, here detailed information on the hydrogen bonds in the winged-helix-motif protein is given and the following features found. (i) The number of hydrogen bonds per amino acid of DsrD is relatively fewer than for other proteins for which neutron structures were determined previously. (ii) Hydrogen bonds are localized between main-chain and main-chain atoms; there are few hydrogen bonds between main-chain and side-chain atoms and between side-chain and side-chain atoms. (iii) Hydrogen bonds inducted by protonation of specific amino acid residues (Glu50) seem to play an essential role in the dimerization of DsrD. The former two points are related to the function of the DNA-binding protein; the three-dimensional structure was mainly constructed by hydrogen bonds in main chains, while the side chains appeared to be used for another role. The latter point would be expected to contribute to the crystal growth of DsrD.

Keywords: DsrD; DNA-binding protein; hydrogen bond; neutron crystallography.

1. Introduction

Hydrogen bonding is an important interaction for the folding and function of protein molecules. In order to understand the role of the hydrogen bond clearly, the position of the H atom in the hydrogen bond must be determined unambiguously by an ultra-high-resolution X-ray analysis (higher than 1.0 Å resolution) or neutron analysis (Helliwell, 1997; Niimura, 1999; Tsyba & Bau, 2002; Schoenborn & Langan, 2004; Niimura *et al.*, 2006). In the present study we have solved the hydrogen structure of a dissimilatory sulfite reductase D (DsrD) from sulfate-reducing bacterium *Desulfovirio vulgaris* by neutron diffraction. Since the *dsrD* gene is located immediately downstream of the *dsrA* and *dsrB* genes, which code the subunits of dissimilatory sulfite reductase DsrAB, then DsrD was thought to be a cofactor of DsrAB. DsrD, however, does not possess high-affinity binding for sulfate, sulfite or sulfide (Hittel & Voordouw, 2000), but has a B- and Z-DNA-binding motif (Mizuno *et al.*, 2003) in its three-dimensional structure. Therefore, it is expected that DsrD would act as a transcription regulator for DsrAB. Since any neutron structure of DNA-binding protein has not yet been determined, we have determined the first neutron structure of DsrD at 2.4 Å resolution with an *R*-factor (R_{free}) value of 0.229 (0.289).

Hydrogen bonds in the DsrD structure were statistically analysed in the Hydrogen and Hydration Database for Biomacromolecules (HHDB), which was developed at the Japan Atomic Energy Agency and Japan Science and Technology Agency. HHDB is the first database that contains the stereochemical details of hydrogen and hydration structure within biomacromolecules determined by neutron crystallography. In addition, it is equipped with functions for statistical analysis and a three-dimensional viewer. The specific features of hydrogen bonds in DsrD were investigated by comparison with those of other proteins.

2. Materials and methods

2.1. Refinement of the DsrD structure

The preliminary neutron structure (*R*-factor and R_{free} were 0.287 and 0.308, respectively) reported earlier (Chatake *et al.*, 2004) was further refined for the present hydrogen-bond analysis. The refinement was carried out by the combination of model building using program *CNS* (Brunger *et al.*, 1998) and optimization of atomic parameters using the program *XtalView* (McRee, 1999). The refined structure includes 681 H atoms, 274 D atoms, 99 D_2O molecules and one SO_4^{2-} ion. Final *R*-factor and R_{free} values are 0.229 (0.289).

2.2. Analysis of the hydrogen bond

Statistical analysis of hydrogen bonds of DsrD was carried out on the HHDB web site (http://hhdb01.tokai-sc.jaea.go.jp/HHDB/). Hydrogen bonds in DsrD were searched using the following definitions (default in HHDB):

N—D$\cdots$*Y*: $d_{D\cdots Y} < 2.7$ Å, $\angle$N—D$\cdots Y > 90^\circ$,

O—D$\cdots$*Y*: $d_{D\cdots Y} < 2.6$ Å, $\angle$O—D$\cdots Y > 90^\circ$,

S—D$\cdots$*Y*: $d_{D\cdots Y} < 3.1$ Å, $\angle$S—D$\cdots Y > 90^\circ$,

where *Y* is an acceptor atom.

A total of 114 hydrogen bonds found by HHDB were categorized into three groups by donor and acceptor: (i) both a donor and an acceptor belong to the main chain (mc–mc interaction); (ii) either a donor or an acceptor belongs to the main chain, and its counterpart belongs to a side chain (mc–sc interaction); and (iii) both a donor and an acceptor belong to a side chain (sc–sc interaction). For the object of comparison, neutron and high-resolution ($d_{min} < 0.9$ Å) X-ray structures of wild-type protein, which are larger than 50 amino acids, were selected. Nine neutron structures {rubredoxin [Protein Data Bank (PDB) id: 1VCX], rebonulcease A (6RSA), myoglobin (1L2K), concanavalin A (1XQN), trypsin (1NPT), dihydrofolate reductase (2INQ), insulin (3INS), lysozyme (1IO5), endothiapepsin (1GKT)} and seven X-ray structures [trypsin inhibitor (5PTI), crambin (1EJG), subtilisin (1GCI), anti-freezing protein (1UCS), xylose isomerase (1MUW), endo-1,4-beta-xylanase (1I1W) and photoactive yellow protein (3PYP)] were calculated in the same procedure on HHDB. They are all proteins deposited in the HHDB, without their variant. In the present analysis, C—H$\cdots$*Y* is excluded because this kind of hydrogen bond is so weak as to be negligible in counting hydrogen bonds.

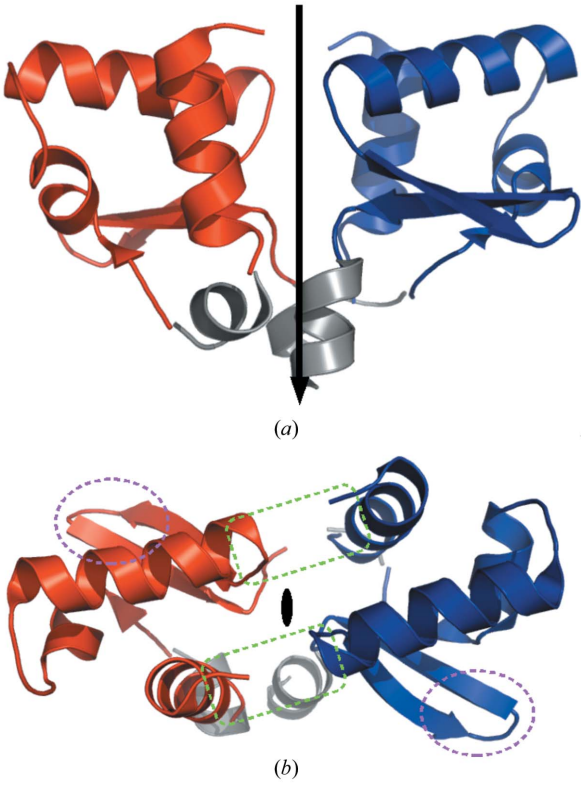

Figure 1
Ribbon models of DsrD in an asymmetric unit from different viewpoints. Molecule A (red) and molecule B (blue) are related by non-crystallographic twofold symmetry. Grey residues are missing in the present analysis. In (*b*), residues in the purple dashed ellipses are highly conserved in the DsrD family. Residues in the green dashed square are the interface between molecules A and B.

Table 1
Statistics of hydrogen bonds in proteins.

mc: main chain; sc: side chain; aa: amino acid; ×2: two molecules in asymmetric unit. Values in parenthesis are standard deviations.

	DsrD	Other 16 proteins
Number of hydrogen bonds		
mc–mc	92	
mc–sc	15	
sc–sc	4	
Ratio of hydrogen bonds (%)		
mc–mc	82.9	68.3 (6.9)
mc–sc	13.5	23.5 (6.1)
sc–sc	3.6	8.2 (4.1)
Hydrogen bonds per aa	0.84†	1.05 (0.15)

† 25 residues missing in the neutron structure were excluded from the calculation.

3. Results and discussions

3.1. Overall structure

Two DsrD molecules in an asymmetric unit are related by a non-crystallographic twofold axis (Fig. 1). The root-mean-square distance of all non-H atoms between the two molecules is 0.99 Å. Since there is no indication that DsrD forms a dimer in solution, the dimeric structure could result from crystal packing. In addition, the relative disposition of the second helices of the HTH motif in DsrD in the dimeric form is different from that found in the crystal structures of the DNA-binding proteins in the dimeric active form (Mizuno *et al.*, 2003). There are a few interactions between the dimeric DsrD molecules in the crystal. Direct hydrogen bonds could be observed between Gln6 and Glu50, which are indicated by the green dashed square in Fig. 1(*b*). The sequence Tyr54–Trp–Ser–Ser–Gly Ser–Thr–Thr61 (purple dashed ellipses in Fig. 1*b*) is highly conserved in the DsrD family. It has a β-hairpin structure, and would be expected to participate in DNA binding (Mizuno *et al.*, 2003). DsrD has a structural similarity with Z-DNA-binding protein ADAR1. The regions of DsrD which correspond to the potential DNA-binding site of ADAR1 are Gln36, Arg37, Lys40, Lys41, Thr44, Thr60, Thr61 and Tyr63. The residues LysA15–LysA19, GlnA71–AspA78, SerB16–GlyB17 and GlyB69–AspB78 (coloured in grey in Fig. 1) could not be observed in the present neutron structure. In Fig. 1, the structures of these regions (in grey), which have high temperature factors, are supplemented from the X-ray structure (PDB id: 1UCR).

3.2. Hydrogen bonds in DsrD

Table 1 shows the number of hydrogen bonds calculated using HHDB. The number of total hydrogen bonds per amino acid is also included. Two striking features are found in this table.

The first point is that the number of hydrogen bonds per amino acid (0.84) is relatively smaller than that of the other proteins. The averaged value and standard deviation of the other 16 proteins are 1.05 and 0.15, respectively. Assuming that DsrD is the DNA-binding protein, this feature may be explained. DNA is a relatively larger substrate than that of enzymatic proteins. DNA-binding proteins have a large DNA-binding site and alter the shape of the binding site when they bind DNA. Some of them take an induced-fit mechanism to recognize DNA; others change their structural conformation to transmit DNA information. In order to alter the shape, the protein should be more flexible than the other kinds of proteins. For this purpose the number of hydrogen bonds may be fewer than for the other kinds of proteins. The number of hydrogen bonds per amino acid of trypsin inhibitor (0.81) is comparable with that found in DsrD.

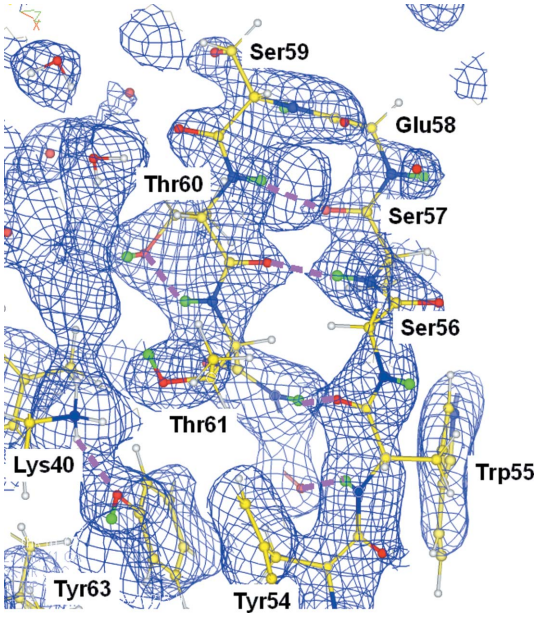

Figure 2
$2|F_o| - |F_c|$ neutron Fourier map around the conserved residues Tyr54–Trp–Ser–Ser–Gly Ser–Thr–Thr61 in molecule A. Blue contours indicate $+1.5\sigma$ neutron densities. Purple dashed lines are hydrogen bonds.

This is because trypsin inhibitor has a large substrate binding site as well as DNA-binding protein (Marquart *et al.*, 1983).

The second point is that almost all hydrogen bonds in DsrD are categorized by mc–mc interactions (mc–mc, mc–sc and sc–sc interactions are 92, 15 and 4, respectively). The sum of mc–sc and sc–sc hydrogen bonds amounts to 18% of the total hydrogen bonds. It is the smallest value among the 17 proteins, and much smaller than the average value (31.7%). Moreover, all of four sc–sc hydrogen bonds are used for DsrD dimerization in an asymmetric unit (see §3.3). We suppose that these sc–sc interactions would be kept for DNA binding. A typical example is given in Fig. 2, which shows a neutron density map around the residues Tyr54–Trp–Ser–Ser–Gly Ser–Thr–Thr61 conserved in the DsrD family. They include the possible DNA binding site (Lys40, Thr60, Thr61 and Tyr63). The β-hairpin structure

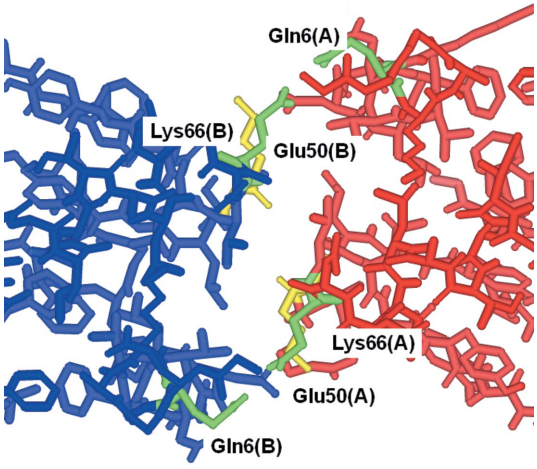

Figure 3
Schematic diagram showing the locations of interaction between two DsrD molecules in an asymmetric unit. Red and blue sticks indicate molecules A and B, respectively. Green sticks are Gln6 and Glu50, which form direct hydrogen bonds. Yellow sticks are Lys66, where an indirect hydrogen-bonding network is mediated by a D_2O molecule.

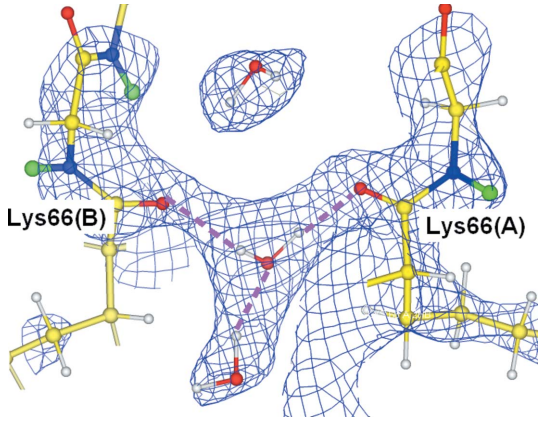

Figure 4
$2|F_o| - |F_c|$ neutron Fourier map around two Lys66. White, yellow, blue, red and green spheres indicate H and D, C, N, O and D, respectively, in the main chain. Carbonyl O atoms in the main chain interact by indirect hydrogen bonds (dashed purple line) mediated by a D_2O molecule. It is located at the centre of this figure on the twofold axis.

(Tyr54–Thr61) was formed by four mc–mc and only one mc–sc ($O_{\gamma 1}$ in Thr60, D in Thr61) hydrogen bonds. In the previous paragraph, we mentioned that DsrD would keep hydrogen bonds for DNA binding. It is thought that the reserved hydrogen bonds correspond to some mc–sc or sc–sc hydrogen bonds. Consequently, as for DsrD, the three-dimensional structure was mainly constructed of mc–mc interactions, and it is possible that sc interactions were used for another role, for example the interaction between DsrD and DNA.

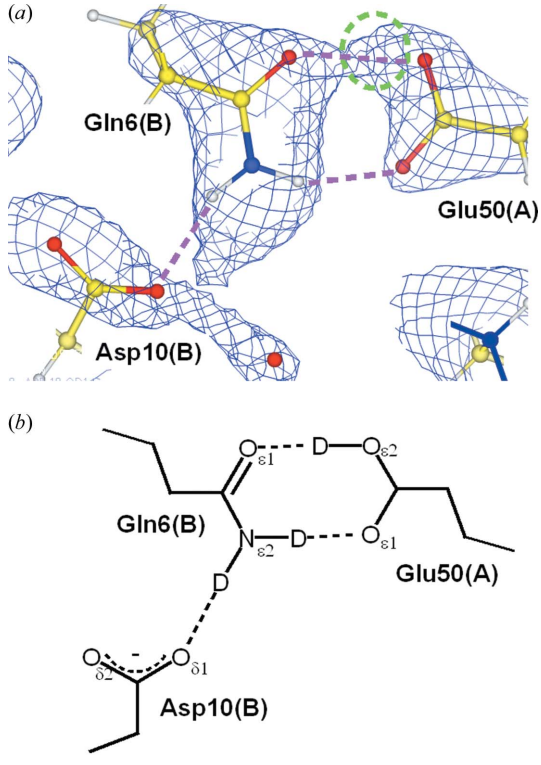

Figure 5
Direct interaction between two DsrDs. (*a*) $2|F_o| - |F_c|$ neutron Fourier map around Glu50 in molecule A and Gln6 and Asp10 in molecule B. Hydrogen bonds are shown as dashed purple lines. (*b*) Postulated chemical structures of the three residues. Note that the carboxyl group of Glu50 is protonated, while that of Asp10 is deprotonated.

3.3. Interaction between two DsrDs in an asymmetric unit

As shown in Fig. 3, there are a few contacts between the dimeric DsrDs in the crystal. Direct hydrogen bonds are formed only between Gln6 and Glu50 (green sticks in Fig. 3). This interaction is duplicated by a non-crystallographic symmetry operation (twofold axis is perpendicular to the plane of Fig. 3). An indirect hydrogen-bond network mediated by a D_2O molecule is formed around the centre of the interface of the dimer near the twofold axis. Carbonyl O atoms of Lys66 in dimeric DsrD are connected by indirect hydrogen bonds mediated by a D_2O molecule (Fig. 4). The major interaction between the two DsrDs is the direct hydrogen bond between Gln6 and Glu50. They are located at both ends of the interface, and there is no direct hydrogen bond elsewhere. This interaction seems to be the connecter for making the dimeric structure. As mentioned in the previous section, this is the only sc–sc hydrogen bond found in the crystal structure of the DsrD dimer. There are two hydrogen bonds in this interaction: $N_{\varepsilon 2}$ of Gln6 in molecule B to $O_{\varepsilon 1}$ of Asp10 in molecule B, and $N_{\varepsilon 2}$ of Gln6 in molecule B to $O_{\varepsilon 1}$ of Glu50 in molecule A (Fig. 5*a*). Interestingly, one possible hydrogen bond ($O_{\varepsilon 1}$ of Gln6 in molecule B and $O_{\varepsilon 2}$ of Glu50 in molecule A) is also found. The distances between the two O atoms are 2.52 Å for Gln6 (molecule A)–Glu50 (molecule B) and 2.67 Å for Gln6 (molecule B)–Glu50 (molecule A). These distances are comparable with those of O—D···O-type hydrogen bonds. Moreover, side chains of Gln6 and Glu50 take the ideal position for forming a hydrogen bond. In fact, there is a positive density peak between the two O atoms in the Fourier map even though any atomic species is incorporated in the calculation of the map, suggesting that $O_{\varepsilon 2}$ of Glu50 is protonated (see the green dashed circle in Fig. 5*a*). This protonation enables the hydrogen bond between $O_{\varepsilon 1}$ of Gln6 and $O_{\varepsilon 2}$ of Glu50. In other words, if it is not protonated, the ideal shape formed by Gln6 and Glu50 would be broken owing to the electrostatic repulsion by the negative charges of $O_{\varepsilon 1}$ of Gln6 and $O_{\varepsilon 2}$ of Glu50. Therefore, the protonation of $O_{\varepsilon 2}$ of Glu50 can be considered to have an essential role for DsrD dimerization.

The protonation of Glu50 would also contribute to the crystal growth of DsrD. DsrD crystals were grown under acidic conditions at pH 5.5. This pH is the lower limit of the condition in which single crystals of DsrD can be obtained. Though DsrD was precipitated in the crystallization solution within one day at pH 5.0, small single crystals appeared for three days, and grew up to the maximum size for two or three weeks at pH 5.5. On the other hand, the rate of crystal growth was much slower at higher pH (6.0–6.5), and only small crystals grew, even after two months. These results obtained from the crystallization experiments could be explained by the protonation of Glu50. In general, pK_a of the carboxyl group of glutamic acid is 4.4. In the present crystal obtained at pH 5.5, any carboxyl groups in DsrD were not protonated except Glu50. The reason for protonation of Glu50 in this crystal is not clear; it could be essential for dimerization and also for growth of the DsrD crystals.

4. Conclusion

Neutron diffraction has revealed details of hydrogen bonds in DsrD. Statistical analysis of hydrogen bonds has provided new information on protein folding and function. In DsrD the number of hydrogen bonds per amino acid is relatively smaller than for other proteins, and hydrogen bonds are localized in the main chain; the functional groups of side chains are kept for another role that may be DNA binding. Another finding is that protonation could control crystal growth. The carboxyl group of Glu50 is the connector for DsrD dimerization in the crystal. The protonated state of Glu50 is necessary for joining the two DsrDs by hydrogen bonds between Glu50 and Gln6 in the counterpart.

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