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Structure of a UDP-glucose dehydrogenase from the hyperthermophilic archaeon *Pyrobaculum islandicum*

The crystal structure of an extremely thermostable UDP-glucose dehydrogenase (UDP-GDH) from the hyperthermophilic archaeon *Pyrobaculum islandicum* was determined at a resolution of 2.0 Å. The overall fold was comprised of an N-terminal NAD⁺ dinucleotide binding domain and a C-terminal UDP-sugar binding domain connected by a long α -helix, and the main-chain coordinates of the enzyme were similar to those of previously studied UDP-GDHs, including the enzymes from *Burkholderia cepacia*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*. However, the sizes of several surface loops in *P. islandicum* UDP-GDH were much smaller than the corresponding loops in *B. cepacia* UDP-GDH but were comparable to those of the *S. pyogenes* and *K. pneumoniae* enzymes. Structural comparison revealed that the presence of extensive intersubunit hydrophobic interactions, as well as the formation of an intersubunit aromatic pair network, is likely to be the main factor contributing to the hyperthermostability of *P. islandicum* UDP-GDH.

1. Introduction

UDP-glucose dehydrogenase (UDP-GDH; EC 1.1.1.22) catalyzes a two-step NAD-dependent oxidation of UDP-glucose (UDP-Glc) to produce UDP-glucuronic acid (UDP-GlcA). UDP-GDH is widely distributed in Bacteria and Eukarya, and the physiological functions of the enzyme have been investigated in a number of organisms. These studies have demonstrated its importance in polysaccharide biosynthesis, detoxification and embryonic development (Egger *et al.*, 2010; Princivalle & de Agostini, 2002). In mammals, for example, the UDP-GlcA provided by UDP-GDH is essential for the biosynthesis of hyaluronic acid and of various glycosaminoglycans such as chondroitin sulfate and heparin sulfate, as well as for the formation of glucuronide conjugates with various substances such as bilirubin (Tukey & Strassburg, 2000). In plants, several UDP-GDH isoforms play an important regulatory role in carbon partitioning between cell-wall formation and sucrose synthesis (Klinghammer & Tenhaken, 2007). In many strains of pathogenic bacteria, UDP-GlcA is known to be necessary for construction of the antiphagocytic capsular polysaccharide (Arrecubieta *et al.*, 1994; Campbell *et al.*, 1997).

We recently demonstrated the presence of UDP-GDH in Archaea, the third domain of life, for the first time. On the basis of genome information, we identified a gene (Pisl_1505) encoding a UDP-GDH homologue in the anaerobic hyperthermophilic archaeon *Pyrobaculum islandicum* and confirmed that the gene product exhibits a high level of UDP-GDH activity (Satomura *et al.*, 2011). The physiological function of the enzyme in *P. islandicum* is currently unknown, but the enzyme was found to be the most thermostable UDP-GDH described to date, with a half-life of 10 min at 363 K. The enzyme retained full activity after incubation in the pH range 5.0–10.0 for 10 min at 353 K. To date, crystal structures of UDP-GDHs from *Streptococcus pyogenes* (Campbell *et al.*, 2000), human (Egger *et al.*, 2011), *Klebsiella pneumoniae* (Chen *et al.*, 2011), *Burkholderia cepacia* (Rocha *et al.*, 2011), *Caenorhabditis elegans* (PDB entry 2o3j; New York SGX Research Center for Structural Genomics, unpublished work) and *Porphyromonas gingivalis* (PDB entry 3gg2; New York SGX Research Center for Structural Genomics, unpublished work) have been determined. However, there is no information about

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution data shell. R.m.s.d., root-mean-square deviation.

Data collection	
Space group	C2
Unit-cell parameters (Å, °)	$a = 117.7$, $b = 76.7$, $c = 75.6$, $\beta = 125.8$
Wavelength (Å)	1.0
Maximum resolution (Å)	2.0 (2.03–2.00)
Total No. of reflections	274635
No. of unique reflections	36575
Multiplicity	7.5 (7.3)
Completeness (%)	99.1 (99.0)
$R_{\text{merge}}^{\dagger}$ (%)	3.9 (19.9)
$\langle I/\sigma(I) \rangle$	26.5 (14.8)
Refinement	
Resolution range (Å)	50–2.0
$R/R_{\text{free}}^{\ddagger}$ (%)	17.7/21.6 (20.6/26.2)
No. of protein atoms	3287
No. of water molecules	285
R.m.s.d.	
Bond lengths (Å)	0.023
Bond angles (°)	2.1
Average B factor (Å ²)	29.8
Ramachandran statistics (%)	
Favoured	98.4
Allowed	1.6
Outliers	0

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the scaled intensity of the i th observation of reflection hkl , $\langle I(hkl) \rangle$ is the mean value and the summation is over all measurements. $\ddagger R_{\text{free}}$ was calculated using randomly selected reflections (5%).

either the structure of archaeal UDP-GDH or the structural features that underlie the thermostability of hyperthermophilic UDP-GDH. In the present study, we succeeded in determining the crystal structure of *P. islandicum* UDP-GDH. Through comparison with the structures of mesophilic UDP-GDHs, we evaluated the structural features that are responsible for the high thermostability of *P. islandicum* UDP-GDH. Here, we present the first crystal structure of a UDP-GDH from a hyperthermophilic archaeon.

2. Materials and methods

2.1. Overexpression and purification of recombinant protein

The expression and purification of *P. islandicum* UDP-GDH was carried out as described previously (Satomura *et al.*, 2011), except that gel-filtration column chromatography was employed as the final purification step. Briefly, *Escherichia coli* strain BL21 (DE3) CodonPlus RIL cells (Stratagene, La Jolla, California, USA) harbouring the recombinant plasmid pUDPGDH, which carries the *PisI_1505* gene preceded by a sequence coding for six histidine residues, were cultivated at 310 K in 1 l SB medium (1.2% tryptone peptone, 2.4% yeast extract, 1.25% K₂HPO₄, 0.38% KH₂PO₄, 0.5% glycerol) containing 50 µg ml^{−1} ampicillin until the optical density at 600 nm reached 0.4. Expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside to the medium and cultivation was continued for an additional 3 h. The cells were then harvested by centrifugation and washed twice with 0.85% NaCl solution. The washed cells were suspended in LEW buffer (50 mM NaH₂PO₄, 300 mM NaCl pH 8.0) and disrupted by ultrasonication. The crude extract was heated at 353 K for 10 min and the denatured protein was removed by centrifugation (10 000g for 10 min). The supernatant was loaded onto a Protino Ni-IDA column (Macherey-Nagel, Germany) equilibrated with LEW buffer and the column was washed with ten bed volumes of the same buffer. The enzyme was eluted with a ten-

bed-volume linear gradient of 0–250 mM imidazole in LEW buffer. The active fractions were then pooled, concentrated and loaded onto a HiLoad 26/60 Superdex 200 gel-filtration column (GE Healthcare) equilibrated with 20 mM potassium phosphate buffer pH 7.0. After elution, the active fractions of the eluate were pooled and used as the purified enzyme preparation. UDP-GDH activity and protein levels were determined as described previously (Satomura *et al.*, 2011).

2.2. Crystallization and structure determination

The purified enzyme was concentrated to 10 mg ml^{−1} by ultrafiltration and the resultant solution was complemented with 5 mM UDP-Glc for crystallization trials. Initial screening was carried out using Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA). Crystallization was accomplished at 293 K using the sitting-drop vapour-diffusion method, in which 1 µl drops of protein solution were mixed with an equal volume of reservoir solution and equilibrated against 0.1 ml reservoir solution using CompactClover crystallization plates (Emerald BioSystems). Initially, microcrystals were grown using reagent No. 36 of Crystal Screen 2: 4.3 M NaCl, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.5. After optimization of the precipitant concentration and crystallization temperature, diffraction-quality crystals (maximum dimensions of 0.1 × 0.1 × 0.1 mm) were obtained within one week at 298 K using reservoir solution composed of 4.0 M NaCl, 100 mM HEPES pH 7.5. The crystals belonged to the monoclinic space group C2, with unit-cell parameters $a = 117.7$, $b = 76.7$, $c = 75.6$ Å, $\beta = 125.8^\circ$.

The *P. islandicum* UDP-GDH crystals were flash-cooled in liquid nitrogen at 100 K. The crystal was cryoprotected with reservoir solution supplemented with 30% (v/v) ethylene glycol, replacing the water in the buffer with cryoprotectant. Diffraction data were collected to 2.0 Å resolution using monochromated radiation at $\lambda = 1.0$ Å and an ADSC CCD detector system on the BL5A beamline at the Photon Factory, Tsukuba, Japan. The data were processed using HKL-2000 (Otwinowski & Minor, 1997).

The initial phases for the *P. islandicum* UDP-GDH structure were determined by molecular replacement using the program *MOLREP* in the CCP4 program suite (Vagin & Teplyakov, 2010; Winn *et al.*, 2011); the structure of chain A of *P. gingivalis* UDP-GDH (PDB entry 3gg2) served as the search model. Further model building was performed using the program *Coot* (Emsley *et al.*, 2010) and refinement to a resolution of 2.0 Å was carried out using *REFMAC5* (Murshudov *et al.*, 2011) and *CNS* (Brünger *et al.*, 1998). The UDP-Glc and ethylene glycol molecules were clearly visible in both the σ_A -weighted $2F_o - F_c$ and $F_o - F_c$ density maps and were included in the latter part of the refinement. Water molecules were incorporated using *Coot* (Emsley *et al.*, 2010). Model geometry was analyzed using *RAMPAGE* (Lovell *et al.*, 2003). The data-collection and refinement statistics are listed in Table 1.

Ion pairs (with a cutoff distance of 4.0 Å) and hydrophobic interactions were identified using the *WHAT IF* web server (Rodriguez *et al.*, 1998). To determine the number of hydrophobic interactions, the interatomic contacts between atoms from hydrophobic side chains were calculated. A contact is defined as two atoms for which the distance between the van der Waals surfaces is less than 1.0 Å. The aromatic interactions were identified using a cutoff distance between aromatic ring centres of 7.0 Å. The accessible surface area (ASA; the radius of the probe solvent molecule was 1.4 Å) was calculated using *AREAIMOL* in the CCP4 program suite (Winn *et al.*, 2011). The protein volumes were calculated using *Swiss PDB Viewer* (<http://www.expasy.org/spdbv/>; Guex & Peitsch, 1997). A drawing quality of

6 (grid size = 0.47 Å) was used in the calculation. Molecular-graphics figures were created using *PyMOL* (<http://www.pymol.org/>).

3. Results and discussion

3.1. Overall structure and substrate-binding site

The structure of *P. islandicum* UDP-GDH was determined at a resolution of 2.0 Å. The *R*-factor and *R*_{free} values for the final model were 17.7% and 21.6%, respectively (Table 1). The asymmetric unit contained one protein molecule with a solvent content of 57.3%, which corresponds to a Matthews coefficient (Matthews, 1968) of 2.9 Å³ Da⁻¹. In the present model, the amino acids extending from Met-19 to Ser-8 were disordered and not visible in the electron-

density map. These residues, which include the His-tag sequence, are derived from the pET15b expression vector. The final structure showed good geometry without Ramachandran outliers and consisted of 432 amino-acid residues, one UDP-Glc molecule, one ethylene glycol molecule and 185 water molecules.

The molecular mass of native *P. islandicum* UDP-GDH has previously been estimated to be about 44 kDa by gel-filtration chromatography (Satomura *et al.*, 2011). Since the subunit molecular mass was calculated to be about 50 kDa by SDS-PAGE, the enzyme was proposed to have a monomeric structure (Satomura *et al.*, 2011) similar to that of *S. pyogenes* UDP-GDH (Campbell *et al.*, 1997). However, the crystal structure indicated that the quaternary structure of *P. islandicum* UDP-GDH was a dimer generated by a crystallographic twofold axis (Fig. 1*a*), as in the case of *S. pyogenes* UDP-GDH (Campbell *et al.*, 2000). Oligomerization-state analysis using *PISA* (Krissinel & Henrick, 2007) also confirmed the dimeric arrangement of *P. islandicum* UDP-GDH, with an interface ASA of 2900 Å², which corresponds to 15% ASA per monomer.

Similar to other UDP-GDH structures, the *P. islandicum* UDP-GDH monomer consists of two discrete α/β domains, each of which contains a parallel β -sheet core flanked by α -helices on both sides (Fig. 1*b*). These two domains are connected by a long central α -helix (α 9; residues 201–232) that also contributes to the homodimerization of the enzyme. The core of the N-terminal NAD⁺ dinucleotide binding domain (residues 1–200) is a six-stranded parallel β -sheet (β 1– β 6), and an additional $\beta\alpha\beta$ unit (β 7, α 8 and β 8) is situated close to the central β -sheet. The core of the C-terminal UDP-sugar binding domain (residues 233–424) consists of a six-stranded parallel β -sheet (β 9– β 14). The UDP-Glc binding site in *P. islandicum* UDP-GDH had the typical geometry found for this site in other UDP-GDHs. The UDP moiety is flanked by a coiled stretch comprising residues 253–261, with the uracil and Tyr253 rings showing a π -edge stacking similar to that observed for the homologous Phe259 and Tyr249 in the *B. cepacia* and *S. pyogenes* UDP-GDHs, respectively. Arg248 from one monomer extends its side chain into the active site of the partner monomer; NH1 and NH2 of Arg248 form hydrogen bonds to the O2' and O3' hydroxyls of the sugar.

3.2. Structural comparison with other UDP-GDHs

As mentioned above, the crystal structures of UDP-GDHs from six organisms have been determined to date. The quaternary structures of these enzymes have been estimated to be a hexamer for the human and *C. elegans* enzymes, a tetramer for the *P. gingivalis* enzyme and a dimer for the *S. pyogenes*, *K. pneumoniae* and *B. cepacia* enzymes. Therefore, we compared the structure of *P. islandicum* UDP-GDH with those of the dimeric *S. pyogenes* (PDB entry 1dli; Campbell *et al.*, 2000), *B. cepacia* (PDB entry 2y0e; Rocha *et al.*, 2011) and *K. pneumoniae* (PDB entry 3pln; Chen *et al.*, 2011) enzymes. For the superposition of these structures, secondary-structure matching (Krissinel & Henrick, 2004) was performed using *Coot* (Emsley *et al.*, 2010). The main-chain coordinates of the *P. islandicum* UDP-GDH monomer were essentially the same as those of the other three enzymes, as was the dimeric assembly. However, we found that several surface loops in *P. islandicum* UDP-GDH were markedly smaller than the corresponding loops in *B. cepacia* UDP-GDH. Residues 196–201 (L1), 372–385 (L2), 416–425 (L3) and the C-terminal residues 452–459 (L4) in the *B. cepacia* enzyme were absent from the loop regions of *P. islandicum* UDP-GDH (Fig. 2). On the other hand, the sizes of these loops are comparable to those in the *S. pyogenes* and *K. pneumoniae* enzymes. The calculated ASAs of dimeric and monomeric *B. cepacia* UDP-GDH were larger than those

Figure 1
Dimer (*a*) and monomer (*b*) structures of *P. islandicum* UDP-GDH. The N-terminal NAD⁺ dinucleotide binding and C-terminal UDP-sugar binding domains are shown in green and cyan, respectively. A long central α -helix (α 9) connecting these two domains is shown in magenta. UDP-Glc (yellow) is shown as a stick model. O and N atoms are shown in red and blue, respectively.

of the *S. pyogenes*, *K. pneumoniae* and *P. islandicum* enzymes, as were the overall volumes (Table 2). This may reflect the larger size of the surface loops in *B. cepacia* UDP-GDH, as judged from a comparison with the corresponding loops in other enzymes.

3.3. Structural features underlying thermostability

P. islandicum UDP-GDH was found to be the most thermostable UDP-GDH yet described; the enzyme retained full activity after heating to 353 K for 10 min (Satomura *et al.*, 2011). In contrast, the activity of *B. cepacia* UDP-GDH was shown to decrease significantly at assay temperatures of above 318 K (Rocha *et al.*, 2011). In the case of *S. pyogenes* UDP-GDH, it has been reported that incubation for 1.6 h at 303 K resulted in a loss of 40% of the activity (Campbell *et al.*, 1997). Unfortunately, there is no information about the thermostability of the mesophilic *K. pneumoniae* UDP-GDH. However, measurement of *K. pneumoniae* UDP-GDH activity has reportedly been carried out at 298 K (Chen *et al.*, 2011) and this suggests that the enzyme exhibits a much lower thermostability than *P. islandicum* UDP-GDH.

Structural studies of hyperthermophilic proteins have suggested that larger numbers of hydrophobic interactions and ion pairs are responsible for their high thermostability (Bhuiya *et al.*, 2005; Vieille & Zeikus, 2001; Yip *et al.*, 1995). We therefore compared the numbers of hydrophobic interactions and ion pairs within the structures of the *B. cepacia*, *S. pyogenes*, *K. pneumoniae* and *P. islandicum* UDP-GDHs (Table 2). With regard to the number of ion pairs within the monomeric structure, we found that the numbers did not vary greatly among the *B. cepacia*, *K. pneumoniae* and *P. islandicum* enzymes (52–56), although the *S. pyogenes* enzyme actually has fewer ion pairs (40) than the other UDP-GDHs. In addition, we identified a total of four intersubunit ion pairs in the *P. islandicum* UDP-GDH, which is fewer than in the *K. pneumoniae* enzyme (six). These results suggest that ion-pair interactions do not contribute greatly to the higher thermostability of *P. islandicum* UDP-GDH.

In contrast, when we counted the hydrophobic interactions, we found that the number of intersubunit interactions in *P. islandicum*

Table 2
Comparison of the structural features of *P. islandicum* UDP-GDH with those of *S. pyogenes*, *B. cepacia* and *K. pneumoniae* UDP-GDHs.

UDP-GDH (PDB entry)	<i>P. islandicum</i> (3vtf)	<i>S. pyogenes</i> (1dli)	<i>B. cepacia</i> (2y0e)	<i>K. pneumoniae</i> (3pln)
Accessible surface area (Å ²)				
Dimer	32000	34000	35000	33000
Monomer	19000	20000	21000	19000
Interface	2900	2600	2700	2600
Volume (Å ³)				
Monomer	51000	50000	55000	46000
No. of ion pairs				
Monomer	54	40	56	52
Interface	4	0	0	6
No. of hydrophobic interactions				
Monomer	552	526	627	508
Interface	199	93	136	83

UDP-GDH was markedly larger than those in the other enzymes; the 199 interactions found in *P. islandicum* UDP-GDH was 1.5–2.4 times larger than the numbers in the UDP-GDHs from the other sources (Table 2). The intersubunit interaction was especially striking around the dimerization helix (α 9). The hydrophobic residues involved in the interaction are Pro128, Pro195, Leu197 (β 8), Leu206 (α 9), Val207 (α 9), Val213 (α 9), Phe214 (α 9), Leu215 (α 9), Leu217 (α 9), Phe221 (α 9), Val225 (α 9), Leu227 (α 9), Leu228 (α 9), Leu232, Val234, Val239 (α 10), Val243 (α 10), Leu245, Ile249, Phe254, Phe272 (α 11), Phe275 (α 11), Leu279, Leu281, Met283, Ala284 (α 12), Ile285 (α 12), Ala288 (α 12) and Val292 (α 12). Moreover, five of these residues (Phe214, Phe221, Phe254, Phe272 and Phe275) were found to form an intersubunit aromatic pair network (Fig. 3). A pair of aromatic interactions contributes between -2.5 and -5.4 kJ mol⁻¹ to protein stability (Serrano *et al.*, 1991). In the dimer interfaces of the *S. pyogenes* and *K. pneumoniae* UDP-GDHs, an aromatic pair network mainly formed by Tyr residues is also observed; the network is formed by Phe206, Tyr210, Tyr217, Tyr224 and Tyr272 in *S. pyogenes* UDP-GDH and by Phe199, Tyr203, Tyr210, Tyr217 and Tyr265 in the *K. pneumoniae* enzyme. However, the total numbers of intersubunit hydrophobic interactions in these two enzymes are

Figure 2
Stereo representation of the structure of *B. cepacia* UDP-GDH (red) superimposed on that of *P. islandicum* UDP-GDH (green). The surface loops (L1–L4) in the *B. cepacia* enzyme are shown in blue.

Figure 3

Stereographic representation of the intersubunit aromatic pair network in *P. islandicum* UDP-GDH. Residues involved in aromatic interaction are labelled. Among them, residues belonging to subunits *A* and *B* are shown in green and cyan, respectively.

significantly smaller than that in *P. islandicum* UDP-GDH (Table 2). In contrast, there were no aromatic pairs in the dimer interfaces of *B. cepacia* UDP-GDH, although the number of hydrophobic interactions within its monomeric structure was greater than those in the other three enzymes. Taken together, these observations suggest that the presence of extensive intersubunit hydrophobic interactions, as well as the formation of an intersubunit aromatic pair network, is likely to be the main factor contributing to the hyperthermostability of *P. islandicum* UDP-GDH.

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