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Catalytic properties and crystal structure of quinoprotein aldose sugar dehydrogenase from hyperthermophilic archaeon *Pyrobaculum aerophilum*

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ABSTRACT

We identified a gene encoding a soluble quinoprotein glucose dehydrogenase homologue in the hyperthermophilic archaeon *Pyrobaculum aerophilum*. The gene was overexpressed in *Escherichia coli*, after which its product was purified and characterized. The enzyme was extremely thermostable, and the activity of the pyrroloquinoline quinone (PQQ)-bound holoenzyme was not lost after incubation at 100 °C for 10 min. The crystal structure of the enzyme was determined in both the apoform and as the PQQ-bound holoenzyme. The overall fold of the *P. aerophilum* enzyme showed significant similarity to that of soluble quinoprotein aldose sugar dehydrogenase (Asd) from *E. coli*. However, clear topological differences were observed in the two long loops around the PQQ-binding sites of the two enzymes. Structural comparison revealed that the hyperthermostability of the *P. aerophilum* enzyme is likely attributable to the presence of an extensive aromatic pair network located around a β-sheet involving N- and C-terminal β-strands.

Introduction

The quinoprotein glucose dehydrogenase (Gdh)[2] uses pyrroloquinoline quinone (PQQ) as a prosthetic group. To date, two types of PQQ-dependent Gdhs have been identified: a membrane-bound form, which is widespread in Gram-negative bacteria, and a soluble form (sGdh), which has been observed exclusively in the periplasmic space of the bacterium *Acinetbacter calcoaceticus* [1,2]. The two types of Gdh have no sequence homology [3] and are kinetically and immunologically distinct [2]. The *A. calcoaceticus* sGdh, which has been extensively characterized and structurally defined, has a β-propeller fold comprised of six four-stranded, antiparallel β-sheets [4]. Analysis of PQQ-bound structures in complex with substrate glucose and a competitive inhibitor, methylhydrazine, has resolved the substrate-binding site and led to elucidation of the catalytic mechanism of the substrate oxidation process [5,6]. On the basis of genome information, putative homologues of the sGdh have been identified in phylogenetically diverse prokaryotic genera spanning Bacteria and Archaea [7].

Recently, a novel soluble quinoprotein sugar dehydrogenase (*ylil* gene product) was identified in *Escherichia coli* [8]. The amino acid sequence of that enzyme shows a relatively low identity (26%) with *A. calcoaceticus* sGdh, and the native state of the enzyme is a monomer, whereas that of *A. calcoaceticus* sGdh is a dimer. In addition, this enzyme has a low affinity for both glucose and maltose, but has a generic promiscuity toward mono-, di- and trisaccharide aldose sugars. Structural examination of the *E. coli* enzyme revealed that the PQQ-binding site lies in a distinctive shallow, solvent-exposed cleft, though the β-propeller fold of *A. calcoaceticus* sGdh is conserved [8]. Given the significant catalytic and structural differences between the *A. calcoaceticus* and *E. coli* enzymes, it has been proposed that the *ylil* gene product represents a new subgroup of PQQ-dependent soluble dehydrogenases that is distinguishable from *A. calcoaceticus* sGdh and has been named soluble aldose sugar dehydrogenase (Asd) [8].

Up to now, PQQ-dependent enzymes have not been characterized in archaea, the third domain of life, or in hyperthermophiles. Although it has been proposed, based on genomic information, that the hyperthermophilic archaeon *Pyrobaculum aerophilum* expresses a sGdh homologue [7], the structure and function of this protein have yet to be reported. The hyperthermophilic archaea are located in a relatively deep branch and are considered to be phylogenetically ancient organisms. Structural analysis of the *P. aerophilum* sGdh homologue may thus provide important new

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[2] *Abbreviations used:* Gdh, glucose dehydrogenase; PQQ, pyrroloquinoline quinone; Asd, soluble aldose sugar dehydrogenase; Cl2Ind, 2,6-dichloroindophenol; RMSD, root mean square deviation; ASA, solvent-accessible surface area.

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information for phylogenetic analysis of the PQQ-dependent enzymes. In addition, a hyperthermostable sGdh is a potentially useful target for the development of novel biosensors and biofuel cells.

In the present study, we expressed the gene encoding the *P. aerophilum* sGdh homologue in *E. coli* and characterized the enzyme produced. We found that the enzyme is an extremely thermostable Asd. We also determined the crystal structures of the apoform of the enzyme (at 2.4 Å resolution) and the PQQ-bound holoenzyme (at 2.5 Å resolution). These structures provide insight into the molecular basis of its hyperthermostability.

Material and methods

Protein expression and purification

We initially carried out PCR to amplify a *P. aerophilum* enzyme gene fragment (without the first 17 codons encoding the original peptide) using the following primer pair: 5′-**CATATG**AGTCTGGGC TTGTTGACC-3′, containing a unique *Nde*I restriction site (bold) overlapping the 5′ initial codon, and 5′-A**GGATCC**TTAAGTGTGTT GCTCAGATACG-3′, containing a unique *Bam*HI restriction site (bold) proximal to the 3′ end of the termination codon. Chromosomal DNA from *P. aerophilum* was isolated as previously described [9] and used as the template. The amplified 1.0-kb fragment was confirmed from its sequence, digested with *Nde*I and *Bam*HI and ligated into pET11a expression vector (Novagen) linearized with *Nde*I and *Bam*HI, yielding pET/PaeAsd. *E. coli* strain BL21(DE3) codon plus RIL (Stratagene) was transformed with pET/PaeAsd, after which the transformants were cultivated at 37 °C in 1.0 L medium containing 12 g of tryptone, 24 g of yeast extract, 5 ml of glycerol, 12.5 g of K_2HPO_4, 3.8 g of KH_2PO_4, and 50 μg/ml ampicillin until the optical density at 600 nm reached 0.6. Expression was then induced by adding 0.5 mM isopropyl-β-D-thiogalacto-pyranoside to the medium, and cultivation was continued for an additional 3 h. The cells were then harvested by centrifugation, suspended in buffer (10 mM potassium phosphate (pH 7.0), 0.2 M NaCl, 1 mg/ml lysozyme from egg white, and 0.1 mg/ml DNase I from bovine pancreas), incubated for 10 min at 37 °C and lysed by sonication. The resultant lysate was centrifuged at 15,000 *g* for 20 min, after which the supernatant was collected, heated for 10 min at 80 °C, and clarified by centrifugation. This supernatant was then loaded onto a HiPrep 16/10 SP XL column (GE Healthcare) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). After washing the column with the same buffer, the enzyme was eluted with a linear gradient of 0–0.2 M NaCl in the same buffer. The active fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). The entire procedure was carried out at room temperature (~25 °C).

Enzyme reconstitution with PQQ

Reconstitution of the apoenzyme with PQQ was carried out using a method similar to that described by Southall et al. [8]. Briefly, purified protein (1.1 mg/ml) was incubated for 16 h at 4 °C with a 10-fold molar excess of PQQ in buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl and 1 mM $CaCl_2$. Unbound PQQ was removed by passing the mixture over a Sephadex G-25 column (PD-10; GE Healthcare). This step was also used to change the buffer for the protein to 10 mM potassium phosphate (pH 7.0).

Biophysical and biochemical characteristics

The enzyme activity was assayed by measuring the reduction rate of Cl2Ind. The standard reaction mixture contained 100 mM D-glucose, 0.1 mM Cl2Ind, 50 mM Bis–Tris propane (pH 8.0) and the enzyme (10 μg) in a total volume of 1 ml. To determine the activity of the apoenzyme, 1.2 μM PQQ was added to the reaction mixture. The mixture without the substrate (D-glucose) was previously incubated at 50 °C for about 3 min in a cuvette with a 0.4-cm light path length, and then the reaction was started by the addition of D-glucose. The initial decrease in the absorbance at 600 nm was measured continuously with a Shimadzu UV-120-02 spectrophotometer (Kyoto, Japan) equipped with a thermostat. One enzyme unit was defined as the amount catalyzing the reduction of 1 μmol of Cl2Ind per min at 50 °C. A millimolar absorption coefficient (ε mM) of 19.1 $mM^{-1}\,cm^{-1}$ at 600 nm was used for Cl2Ind. Protein levels were determined using the method of Bradford [10], with bovine serum albumin serving as the standard. The specific activities were calculated on the basis of protein concentration.

The molecular mass of the enzyme was determined by Superose 6 10/300 GL gel filtration chromatography (GE Healthcare) using 10 mM potassium phosphate buffer (pH 7.3) containing 0.2 M NaCl as the elution buffer. Gel filtration calibration kits (GE Healthcare) were used for the molecular mass standards. The molecular mass of the native enzyme was also examined by measuring dynamic light scattering using a DynaPro-MS/X (Protein Solutions). The subunit molecular mass was determined by SDS–PAGE [11] using eight marker proteins (6–175 kDa) (New England Biolabs Inc.), and the N-terminal amino acid sequence of the enzyme was analyzed using an Edman degradation protein sequencer. The phenylthiohydantatoin derivatives were separated and identified using a PPSQ-21A protein sequencer (Shimadzu).

The thermostability of the apo- and holoenzymes was determined by incubating them (1.1 mg/ml in 10 mM potassium phosphate buffer, pH 7.0) for 10 min at various temperatures and then detecting the remaining activity by running the standard assay at 50 °C. To determine pH stability, the apo- and holoenzymes (0.5 mg/ml) were incubated for 30 min at 70 °C in buffers of various pHs, after which the residual activity was assayed by using the standard assay at 50 °C. The buffers (0.1 M) used were sodium citrate (pH 3.0–6.0), HEPES/NaOH buffer (pH 7.0–8.0), glycine/NaOH buffer (pH 9.0–11.0) and Na_2HPO_4/NaOH buffer (pH 8.0–11.0). In the thermostability and pH stability experiments, the remaining activity of the apoenzyme was determined in the presence of 1.2 μM PQQ. The optimal temperature of the reaction was determined using the standard assay at temperatures ranging from 50 to 75 °C. The effect of pH on the enzymatic activity was determined using the standard assay at 50 °C in 50 mM potassium phosphate buffer (pH 6.5–7.5), HEPES/NaOH buffer (pH 7.0–8.5), Tris/HCl buffer (pH 7.7–9.0), Bis–Tris propane buffer (pH 8.0–9.0) and glycine/NaOH buffer (pH 9.0–9.5). To evaluate substrate specificity, D-glucose in the standard reaction mixture was replaced by several sugars and sugar alcohols. The initial substrate concentration was 100 mM in all cases. For the kinetic analyses, various concentrations of D-glucose (0.1–1.5 M), D-galactose (0.1–1.25 M), D-fructose (0.1–0.75 M), L-arabinose (0.1–0.6 M), D-mannose (0.1–2.0 M), D-xylose (0.1–2.0 M), D-ribose (0.1–0.75 M), maltose (0.1–1.0 M) and D-cellobiose (0.1–0.4 M) were used. Kinetic constants were estimated by a nonlinear least squares method using SigamPlot (Hulinks Inc., Tokyo, Japan). Kms for D-fructose, L-arabinose, and D-ribose were too high to determine the correct values and that for D-cellobiose was not determined due to the limited solubility of this sugar.

Crystallization and data collection

Crystals of the apoenzyme were obtained using the sitting-drop vapor diffusion method, in which 1-μl drops of 12.8 mg/ml protein solution were mixed with an equal volume of mother liquor, which was comprised of 1.5 M ammonium sulfate and 0.1 M Tris/HCl buffer (pH 8.4). The crystals were grown at 20 °C for 6 days. When we placed apoenzyme crystals in mother liquor containing 1 mM