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Biochemical and structural characterization of quinoprotein aldose sugar dehydrogenase from *Thermus thermophilus* HJ6: Mutational analysis of Tyr156 in the substrate-binding site

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ABSTRACT

The gene encoding a quinoprotein aldose sugar dehydrogenase (ASD) from *Thermus thermophilus* HJ6 (Tt_ASD) was cloned and sequenced; it comprised 1059 nucleotides encoding a protein containing 352 amino acids that had a predicted molecular mass of 38.9 kDa. The deduced amino acid sequence showed 42.9% and 33.9% identities to the ASD proteins from *Pyrobaculum aerophilum* and *Escherichia coli*, respectively. The biochemical properties of Tt_ASD were characterized. The optimum pH for the oxidation of glucose was 7.0–7.5 and the optimum temperature was 70 °C. The half-life of heat inactivation for the apoenzyme was about 25 min at 85 °C. The enzyme was highly thermostable, and the activity of the pyrroloquinoline quinone-bound holoenzyme was not lost after incubation at 85 °C for 100 min. Tt_ASD could oxidize various sugars, including hexoses, pentoses, disaccharides, and polysaccharides, in addition to alcohols. Structural analysis suggested that Tyr156 would be the substrate-binding residue. Two mutants, Y156A and Y156K, had impaired activities and affinities for all substrates and completely lost their activities for alcohols. This structural and mutational analysis of Tt_ASD demonstrates the crucial role of Tyr156 in determining substrate specificity.

1. Introduction

PQQ-dependent dehydrogenases are the largest enzyme group within the quinoprotein family [1]. PQQ is synthesized exogenously and then associates with an apoprotein to form the active holoenzyme. The PQQ enzymes are broadly classified as quinoprotein alcohol dehydrogenases (ADH) and quinoprotein glucose dehydrogenases (GDH). Quinoprotein ADH, which catalyzes the oxidation of alcohols into the corresponding aldehydes, is classified into three types. Type I comprises methanol dehydrogenase (MDH) and ethanol dehydrogenase. Type II ADH is a soluble quinohemoprotein having a C-terminal extension containing heme C. Type III ADH, a membrane-bound quinohemoprotein, is found only in acetic acid bacteria [2,3]. Quinoprotein GDH catalyzes the oxidation of glucose and other aldoses to their corresponding aldono-δ-lactones with concomitant reduction of its cofactor, PQQ. Two kinds of PQQ-GDHs are known; one is a soluble glucose dehydrogenase (sGDH) that has only been found in the periplasm of *Acinetobacter calcoaceticus* [4] and the other is a membrane-bound form (mGDH) that is widespread in gram-negative bacteria. Of these, mGDH has high glucose selectivity but requires suitable detergents for solubilization and purification [5], while sGDH exhibits low substrate specificity and lacks thermal stability [6,7].

A. calcoaceticus sGDH (Ac_sGDH), which has been extensively characterized and structurally defined, has a β-propeller fold comprising six four-stranded, antiparallel β-sheets [8]. This enzyme is homodimeric, consisting of identical subunits of 50 kDa, and contains one PQQ per subunit. Ca^{2+} has a dual role in sGDH, being

Abbreviations: Ac, *Acinetobacter calcoaceticus*; ASD, aldose sugar dehydrogenase; Ec, *Escherichia coli*; Pa, *Pyrobaculum aerophilum*; PQQ, pyrroloquinoline quinone; sGDH, soluble glucose dehydrogenase; ADH, alcohol dehydrogenase; MDH, methanol dehydrogenase; Tt, *Thermus thermophilus*.

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required for dimerization and for binding of PQQ [9]. The enzyme oxidizes a wide variety of pentose and hexose sugars, both mono- and di-saccharides, into their corresponding lactones [10]. sGDH is able to donate electrons to several neutral or cationic artificial electron acceptors, including *N*-methylphenazonium methyl sulfate [11] and electroconducting polymers [12]. The enzyme has a high turnover number and electrochemical regeneration of the PQQ cofactor can be easily achieved without negative effects of O_2 tension and product inhibition. Therefore, sGDH has been utilized for glucose-based biosensors [9] and biofuel cells [13–15].

Recently, a novel soluble quinoprotein sugar dehydrogenase (Ec_ASD) was identified in *Escherichia coli* [16]. The amino acid sequence of Ec_ASD shows a relatively low identity (18%) with Ac_sGDH, and the native state of enzyme is a monomer, whereas that of Ac_sGDH is a dimer. In addition, this enzyme has a low affinity for both glucose and maltose, but converts a wide range of mono-, di-, and tri-saccharide aldose sugars into their corresponding lactones. The crystal structure of Ec_ASD revealed major structural differences in the loop and surface-exposed regions, compared with Ac_sGDH, although the β-propeller fold is conserved [16]. Based on catalytic and structural differences between Ac_sGDH and Ec_ASD, it has been proposed that Ec_ASD represents a new subgroup of PQQ-dependent soluble dehydrogenases that is distinguishable from Ac_sGDH and has been named the soluble aldose sugar dehydrogenase (ASD) group [16]. In Archaea, an ASD from *Pyrobaculum aerophilum* (Pa_ASD) has been identified and structurally defined. Catalytic and structural analysis of this protein showed that it has significant similarity to Ec_ASD [17].

In the genome databases of the thermophilic bacterium *Thermus thermophilus* strains HB8, HB27, JL-18, and SG0.5JP17-16, we found putative homologues of the sGDH gene. *T. thermophilus* HJ6 is an aerobic chemoorganotroph, gram-negative, rod-shaped, and extremely thermophilic bacterium that grows between 80 and 95 °C and optimally at 80 °C [18]. In this study, we cloned and expressed the gene encoding a sGDH homolog from *T. thermophilus* HJ6 in *E. coli*. We describe the structural analysis and biochemical characterization of the recombinant protein and also report a mutational analysis of the residue Tyr156 located in the substrate-binding site.

2. Materials and methods

2.1. Chemicals, strains, and plasmids

DNA primers and substrates were prepared by Bioneer (Daejeon, Korea). Taq DNA polymerase was purchased from Takara (Tokyo). Chemicals used for the determination of laccase activity were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of reagent grade. *E. coli* DH5α and BL21 Codon-Plus (DE3) (Novagen, Inc., San Diego, CA, USA) were used as the cloning and expression host cells, respectively. The plasmid pET-21a was purchased from Novagen as the expression vector for the enzyme protein.

2.2. Cloning and DNA sequencing

Chromosomal DNA of *T. thermophilus HJ6* was prepared using the GeneAll® GENEx Genomic kit (GeneAll Biotechnology, Seoul, Korea). To obtain the ASD gene from HJ6, PCR was performed using two primers (F1, 5′-GTCGGGGAAGGGGGTGGCTG-3′ and R1, 5′-AGGTCCGCTTCCGCAAGAGG-3′) which were designed based on the upstream sequence of the initiation codon (GTG) and the downstream sequence of the termination codon (TAG), for sGDH gene from the genome sequence of *T. thermophilus* HB8, HB27, or JL-18. DNA amplification was carried out using Taq DNA polymerase for 30 cycles at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s. To prepare templates for sequencing, the amplified 1.11-kbp fragment was cloned into pGEM®-T as an asd candidate and the construct was designated as pGEM-asd. DNA sequencing analysis was performed by an ABI Prism 3700 genetic analyzer (Perkin–Elmer Applied Biosystems, Foster City, CA, USA).

2.3. Construction of the wild-type and mutant enzymes

The gene was amplified by PCR using the pGEM-asd plasmid DNA as a template, and two primers: F2, 5′-GGGGTGGCATATGGACCGGAGGCGCTTTCT-3′ (the *Nde* I site is underlined); and R2, 5′-CCGAAGCTTAAGGAGGCGTAGCACCCGG-3′ (the *Hin*d III site is underlined). PCR amplification was conducted for 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 74 °C for 1 min for 30 cycles using Pfu DNA polymerase (INTRON Biotech., Korea). An amplified 1.07-kbp DNA fragment was digested with the restriction enzymes *Nde* I and *Hin*d III, and inserted into the pET-21a that contains a region coding for His-tag sequence. The resulting plasmid was designated as pET-asd.

The Y156A mutant construct was generated by introducing a point mutation into the wild-type ASD vector, pET-asd using a site-directed mutagenesis kit (Muta-DirectTM, iNtRon). The primers were F3: 5′-GGGGAGGTCGCCGAGCGGGAG-3′, R3: 5′-CTCCCGCTCGGCGACCTCCCC-3′. The Y156K mutation was then introduced into pET-asd using the same technique. The primers were F4: 5′-GGGGAGGTCAAAGAGCGGGAG-3′, R4: 5′-CTCCCGCTCTTTGACCTCCCC-3′. The mutated nucleotides are underlined. All mutations were verified by nucleotide sequencing.

2.4. Expression and purification of His-tagged ASD and mutants

His-tagged ASD and mutants were expressed independently as follows. *E. coli* BL21 Codon-Plus (DE3) cells harboring constructed pET-asd plasmid to an OD_{600} of approximately 0.5 and then inducing expression with 0.3 mM IPTG for 16 h at 25 °C. The cells were centrifuged and the pellet was washed with resuspension buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). The cells were then disrupted by sonication and supernatant fraction was recovered by centrifugation at 27,000×*g* for 30 min. The supernatant fraction was heat-treated at 75 °C for 20 min followed by recentrifugation. The supernatant was applied to HisTrap HP column (GE Healthcare) and washed with at least 10 column volumes (CV) of the resuspension buffer, and the ASD-His_6 fusion protein was finally eluted at a flow rate of 1 ml/min with a linear gradient of 0–500 mM imidazole in resuspension buffer using ÄKTAprime (GE Healthcare). The purity of the fusion protein was confirmed by SDS-PAGE. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

2.5. Enzyme reconstitution with PQQ

Reconstitution of the apoenzyme with PQQ was carried out using a method similar to that described by Southall et al. [16]. The purified protein (1.7 mg/ml) was incubated for 16 h min at 25 °C with a 10-fold molar excess of PQQ in buffer containing 20 mM Tris-HCl (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).

2.6. Enzyme assay and kinetic calculations

Enzyme activity was determined spectrophotometrically at 70 °C by following the reduction of 2,6-dichlorophenol-indophenol