Crystal Structure of the NAD Biosynthetic Enzyme Quinolinate Synthase* [S]

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A gene encoding a quinolinate synthase has been identified in the hyperthermophilic archaeon Pyrococcus horikoshii via genome sequencing. The gene was overexpressed in Escherichia coli, and the crystal structure of the produced enzyme was determined to 2.0 Å resolution in the presence of malate, a substrate analogue. The overall structure exhibits a unique triangular architecture composed of a 3-fold repeat of three-layer (αβα) sandwich folding. Although some aspects of the fold homologous to the each domain have been observed previously, the overall structure of quinolinate synthase shows no similarity to any known protein structure. The three analogous domains are related to a pseudo-3-fold symmetry. The active site is located at the interface of the three domains and is centered on the pseudo-3-fold axis. The malate molecule is tightly held near the bottom of the active site cavity. The model of the catalytic state during the first condensation step of the quinolinate synthase reaction indicates that the elimination of inorganic phosphate from dihydroxyacetone phosphate may precede the condensation reaction.

NAD is an essential and ubiquitous coenzyme that plays a fundamental role in cellular metabolism. Besides its direct action on redox equilibrium, it was recently shown to play important roles in DNA repair, calcium-dependent signaling, and life span extension (1–3). NAD biosynthesis is accomplished through either de novo or salvage pathways that differ substantially in prokaryotes and eukaryotes (4–6). In prokaryotes, the de novo pathway proceeds through a condensation reaction between L-aspartate and dihydroxyacetone phosphate (DHAP) that is catalyzed by two enzymes, L-aspartate oxidase (LAO; NAD proton donor product), and quinolinate synthase (QS; NAD2 gene product). LAO catalyzes the oxidation of L-aspartate to iminoaspartate, while QS catalyzes the condensation of iminoaspartate with DHAP to produce quinolinate (QA), which is then converted to NAD via a metabolic sequence common to all organisms. Until now, efforts to understand the catalytic mechanism of QS have been hampered by the chemical instability of iminoaspartate (t1/2 = 140 s at pH 8.0 and 25 °C) (7). It may be that iminoaspartate is directly transferred from LAO to the QS active site (6), but there is no evidence that QS and LAO function as a multienzyme complex. Moreover, nothing is currently known about the structure of QS.

Recently, we observed the presence of LAO in Pyrococcus horikoshii, an anaerobic hyperthermophilic archaeon (8). This is the first example of the occurrence of LAO either in the Archaea or in obligate anaerobic organisms. In addition to the oxidase reaction, the enzyme catalyzed L-aspartate dehydrogenation in the presence of fumarate. The genes that encode the homologue of all other enzymes involved in the de novo NAD biosynthesis were identified in the P. horikoshii genome. Thus, we proposed that the de novo pathway functions in the organism under anaerobic conditions. To elucidate the entire aspect of the pathway in P. horikoshii, we have undertaken the structural and functional analysis of these gene products (9).

In this study, the crystal structure of QS from P. horikoshii was determined in the presence of malate, a substrate analogue of iminoaspartate. Knowledge of the structure of QS should yield information about the catalytic mechanism of the enzyme.

EXPERIMENTAL PROCEDURES

Cloning, Protein Expression, and Purification—The gene encoding QS (open reading frame ID: PH0013), the gene information is available at www.bio.nite.go.jp/dogan/Top) was amplified by PCR. The following set of oligonucleotide primers was used to amplify the QS gene fragment: one is the primer (5'-GGATTTCTATGGATTTAGTTGAA-GAAATTTGAGG-3') containing a unique NdeI restriction site overlapping the 5' initiation codon, and the other (5'-CCGATCCCTTTATGGATTTAGTTGAA-GAAATTTGAGG-3') contained a unique BamHI restriction site proximal to the 3'-end of the termination codon. The chromosomal P. horikoshii DNA was isolated as described (10) and used as the template. The amplified 0.9-kb fragment was digested with NdeI and BamHI and ligated with the expression vector pET11a (Novagen) linearized with NdeI and BamHI to generate pEQS. The E. coli strain BL21(DE3) codon plus RIL (Stratagen) was transformed with pEQS. The transformants were cultivated at 37 °C in 500 ml of a nutritionally rich medium as described previously (11) until the optical density at 600 nm reached 0.6. The induction was carried out by the addition of 0.4 mM isopropyl β-D-thiogalactosidase to the medium, and cultivation was continued for 3 h. Cells were harvested by centrifugation, suspended in buffer (10 mM potassium phosphate, pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml lysozyme from egg white, and 1 mg/ml DNase I from bovine pancreas), incubated at 37 °C for 10 min, and lysed by sonication. The crude extract was heated at 90 °C for 15 min in the presence of 0.2 mM Na2SO4 and clarified by centrifugation. The protein was subjected to gel filtration on a Superdex 200 26/60 column (Amersham Biosciences) equilibrated with 10 mM potassium phosphate.

The abbreviations used are: DHAP, dihydroxyacetone phosphate; LAO, L-aspartate oxidase; QS, quinolinate synthase; QA, quinolinate; MIRAS, multiple isomorphous replacement with an anomalous scattering; NA, nicotinic acid; NAPRTase, nicotinic acid phosphoribosyltransferase.

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**RESULTS AND DISCUSSION**

**Enzyme Activity**—The predicted amino acids sequence of the *P. horikoshii* QS showed 35% identity to that reported for the *E. coli* QS (supplemental Fig. 1) (12). The formation of QA from L-aspartate, O2, and DHAP in the presence of the *E. coli* QS and LAO has been demonstrated (12). In the present study, we performed the determination of QA formed by the *P. horikoshii* QS and LAO and found that QA is produced in a time-dependent manner within 30 min under the used conditions. The specific activity was estimated to be 2.2 μmol/min/mg. This provides direct proof that the PH0013 gene product is actually QS. Detailed characteristics of the enzyme will be described elsewhere.

**Overall Structure**—QS from *P. horikoshii* is a monomer comprised of 300 amino acid residues. We found that its overall structure includes a central region plus N- and C-terminal helical segments (a1 and a14, respectively) (Fig. 1a). The central region forms a unique triangular convexo-concave structure that is ~50 Å along each edge and 20 Å thick and is composed of three analogous domains, which relate to a pseudo-3-fold symmetry and exhibit similar folds (Fig. 2a, Domains 1, 2, and 3); a four-stranded parallel β sheet flanked by two α helices on either side, forming a three-layer (αβα) sandwich. Domain 2 contains two right-handed αββ motifs; the first is formed by α5-β5-α6-β6 and the second by α7-β7-α8-β8. The strand order of the β sheet is 6-5-7-8. Domain 3 is formed by α9-β9-α10-β10 and α11-β11-α12-β12 in the same manner as in Domain 2. Domain 1, by contrast, is formed by α13, β1-α2-β2, and α3-β3-α4-β4. In our present model, three surface loops (amino acids 79–90, 165–175, and 257–260), which separate the three domains, plus two residues at the C terminus (amino acids 299 and 300) are disordered and are not visible in electron density maps.

**Correlation among Three Domains**—We also detected a 3-fold repeat of about 85 amino acid residues in the amino acid sequence of QS that corresponds to the three domains. This suggests QS evolved through a process involving two gene duplications. Fig. 2c shows the amino acid alignment of the three repeats, which are related by 20–30% sequence identity. The folds of the three repeats are consistent with one another, except that helix a1 of repeat 1 extends away from the β sheet in Domain 1 (Fig. 2a), and helix a13 presents in an arm extending from Domain 3 to helix a14 at the C terminus to complete the three-layer αβα sandwich of this domain (Fig. 2b). Superposition of the three domains yields root mean square deviations of less than 1.5 Å for 47 equivalent Cα atoms (residues 15–43/53–70 in Domain 1, 103–131/141–158 in Domain 2, and 190–218/226–243 in Domain 3) (Fig. 2b). At the interfaces between adjacent domains, we detected only one to four pairwise interactions based on hydrogen bonding. On the other hand, we found striking hydrophobic interactions around helices a13 and a14: Ile262, Leu264, Ile267, Leu271, and Met274 in helix a13 and its vicinity were buried in a large hydrophobic cluster formed by Ile6 (α1), Leu13 (α1), Ala16 (β1), Ile18 (β1), Val28 (α2), Ile31 (α2), Val52 (β3), Val54 (β3), Ala66 (β3), Val73 (β4), Leu75 (β4), Ile76 (loop), and Pro77 (loop) of Domain 1; and Val280, Ile284, Ala285, Ala288, and Ile292 in helix a14 and its vicinity formed a hydrophobic core with Ile37 (α4) and Leu68 (α4) of Domain 1 and Ala314 (α6) and Ala318 (loop) of Domain 2. With these interactions, the arm extending from Domain 3 to the C terminus appears to fix the three domains and could be essential for formation of the tripartite structure of QS.
Conserved residues are highlighted in cyan. The active site cavity is indicated with stars.

FIG. 2. The 3-fold repeat of QS. a, structure comparison of the three domains presented with similar orientations. b, superimposed Ca traces of the three domains. Domains 2 (cyan) and 3 (green) are superimposed on Domain 1. The arm extending from Domain 3 to the C terminus is shown in red. A disordered loop between Domains 1 and 3 is indicated by red dots. c, sequence alignment of the three repeats. Conserved residues are highlighted in cyan. The residues comprising the active site cavity are indicated with stars. α-Helices (α1–α14; green) and β-strands (β1–β12; yellow) are indicated.

Structural Homologues—When we searched the structural databases using DALI (18), we found that the structure of the Domain 1 is most closely related to that of the N5-carboxyaminoimidazole ribonucleotide mutase (PurE) monomer (Protein Data Bank entry 1Q5Z) (19), having a Z-score of 6.6 and an root mean square deviation of 2.2 Å over 70 Ca positions. The central domain of PurE consists of a five-stranded parallel β sheet flanked by three α-helices on one side and two on the other and adopts a fold akin to the dinucleotide-binding domain found in many nucleotide-binding enzymes (20). Domains 2 and 3 are most similar to sulfotransferase (GlpE; Protein Data Bank entry 1GN0) (21), which adopts a fold typical of a single α/β rhodanese domain. Still, the overall structure of QS shows no similarity to any known protein structures. By contrast, the degree of sequence conservation among QS homologues is extremely high. Apparently, the unique overall structure of QS is a common feature of the QS family.

Active Site—The active site of QS is located at the interface of the three domains on the concave side of the molecule and is centered on the pseudo-3-fold axis. The active site cavity is 10 Å deep with a narrow opening and a wide inner space. The electrostatic potential surface shown in Fig. 1b highlights the negative patches at the bottom of the cavity, which is lined by amino acids from loop 1 (His21, Tyr23), loop 2 (Asp37, Ser38), loop 3 (Tyr109, Asn111), loop 4 (Thr125, Ser126), loop 5 (His196, Glu198), and loop 6 (Ser212, Thr213) (loop numbers in Fig. 2a were used). These residues are completely conserved in all QS homologues listed in the database, as well as in the E. coli QS (supplemental Fig. 1). Our initial experimental electron density map showed extra density within the cavity, and after construction and refinement of the peptide chain, a malate molecule could be modeled unambiguously into that density. The map clearly defined the precise orientation of the malate (Fig. 3a): two oxygen atoms from the C-1 carboxylate of malate form hydrogen bonds with the side chains of His196, His198, and Thr213 and the main chain amide proton of Thr213; the oxygen atoms of the C-4 carboxylate are within hydrogen bonding distance of the side chains of Tyr109 and Ser126, the backbone amide protons of Ser38 and Ser126, and a water molecule (W1); and the oxygen atom of the C-2 hydroxyl group is hydrogen-bonded to the side chains of Tyr109 and His196.

Insight into the QS Reaction—The formation of QA from iminoaspartate and DHAP involves the removal of an inorganic phosphate and two water molecules (7). Labeling studies previously showed that the phosphate-bearing carbon of DHAP is incorporated into C-4 of QA (22) and that in the presence of both QS and L4O the aspartate nitrogen and carbon are incorporated directly into the pyridine ring of QA (23). Those results provide definitive information on the direction of approach and orientation of DHAP in the catalytic site. With that information, we were able to estimate the positions of C-1 and C-2 of the bound DHAP and the pyridine ring of QA from the orientation of malate. But we were unable to find a space to accommodate the DHAP phosphate group in our structure nor were we able to model both DHAP and malate bound to a single QS active site. For that reason, we built our model of the catalytic
state during the first condensation step of the QS reaction without phosphate (Fig. 3b).

Nasu et al. (7) proposed the following reaction mechanism for QS based on the aforementioned labeling studies (Fig. 4, Pathway 1): 1) the electron-withdrawing groups of iminoaspartate facilitate removal of a proton from C-3, and the resulting molecule carries out a nucleophilic attack on C-3 of DHAP accompanied by the elimination of inorganic phosphate; 2) the condensation product formed undergoes a keto-aldol isomerization; and 3) the loss of a proton from the resulting molecule produces an amino aldehyde that undergoes a Schiff-base formation followed by dehydration to produce QA. This mechanism differs substantially from the pathway recently proposed by Begley et al. (4) (Fig. 4, Pathway 2): 1) initially DHAP is isomerized to glyceraldehyde 3-phosphate in a reaction analogous to one catalyzed by triose phosphate isomerase; 2) subsequent imine formation precedes the elimination of phosphate; and 3) electrocyclic ring closure of the resulting molecule followed by tautomerization and dehydration yields QA. The second mechanism certainly requires formation of a condensation product with a phosphate group; thus our binding model without phosphate suggests the first mechanism is more feasible. That mechanism is also in good agreement with the observation that QS does not utilize glyceraldehyde 3-phosphate as a three-carbon precursor (24). That said, when we placed QS crystals in a reservoir solution containing DHAP, x-ray diffraction quality quickly deteriorated, which could mean that DHAP binding causes a large conformational change in the structure of the enzyme-malate complex. The structure of the tertiary complex with DHAP bound will be the focus of further investigation.

It has been suggested that QS is one of the potential targets for the development of novel antibacterial agents (25), as well as LAO (26), because the two-step conversion of aspartate to quinolinate is not present in humans, in whom de novo NAD biosynthesis occurs via degradation of tryptophan (5). Besides de novo synthesis of NAD, a salvage pathway exists that enables NAD to be recycled. This pathway usually proceeds via degradation of NAD to nicotinic acid (NA), followed by conversion of NA to nicotinic acid mononucleotide by nicotinic acid phosphoribosyltransferase (NAPRTase). Unlike most organisms, Mycobacterium tuberculosis, a tubercular pathogen, lacks NAPRTase activity and cannot recycle NA to NAD (27). Similarly, analysis of the genome of Helicobacter pylori, a major cause of gastroduodenal disease, showed it to lack NA salvage genes, including that encoding NAPRTase (25, 28). This makes QS an excellent target for the design of drugs against these organisms, and our results may provide critical information facilitating the design of such antibacterial agents.

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