Biochemical and Biophysical Research Communications 432 (2013) 350–354

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure of type I 3-dehydroquinate dehydratase of *Aquifex aeolicus* suggests closing of active site flap is not essential for enzyme action

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ARTICLE INFO

Article history:
Received 16 January 2013
Available online 8 February 2013

Keywords:
Shikimate pathway
Type I 3-dehydroquinate dehydratase
Crystal structure
Aquifex aeolicus
Thermostability
Antimicrobial

ABSTRACT

Structural analyses of enzymes involved in biosynthetic pathways that are present in micro-organisms, but absent from mammals (for example Shikimate pathway) are important in developing anti-microbial drugs. Crystal structure of the Shikimate pathway enzyme, type I 3-dehydroquinate dehydratase (3-DHQase) from the hyperthermophilic bacterium *Aquifex aeolicus* was solved both as an apo form and in complex with a ligand. The complex structure revealed an interesting structural difference when compared to other ligand-bound type I 3-DHQases suggesting that closure of the active site loop is not essential for catalysis. This provides new insights into the catalytic mechanism of type I 3-DHQases.

1. Introduction

Infections due to pathogenic bacteria are rapidly increasing and new therapeutic agents alternative to antibiotics are required to suppress or eliminate them. As a method of choice, essential biosynthetic pathways present in micro-organisms, but absent from mammals are targeted to develop new antimicrobial agents and herbicides to selectively act against micro-organisms and weeds. Structural analyses of the enzymes involved in such pathways are therefore vital in the design of potential inhibitors.

The Shikimate pathway found in plants, algae, fungi and bacteria has seven enzymes catalyzing a series of sequential reactions to generate chorismate, a precursor for the synthesis of aromatic amino acids and other secondary metabolites [1]. The third step in this pathway is the reversible dehydration of 3-dehydroquinate to 3-dehydroshikimate catalyzed by the enzyme 3-dehydroquinate dehydratase or 3-dehydroquinase or 3-DHQase (EC 4.2.1.10). 3-DHQases are divided into two classes. Type I class is a homodimer with subunit of ~27,000 Da and catalyzes a cis-dehydration of 3-dehydroquinate via a covalent imine intermediate [2,3]. The type II is a dodecamer of identical subunits of ~16,000 Da [3] and catalyzes a trans-dehydration reaction via an enolate intermediate [2,3]. Although the two classes are different in their structure and mechanism of action they ultimately catalyze the same overall reaction.

The structure of type I 3-DHQase has been solved from a few mesophilic bacteria such as *Staphylococcus aureus, Salmonella typhi, Salmonella enterica, Streptococcus pyogenes* and *Clostridium difficile;* the hyperthermophilic archael species *Archaeoglobus fulgidus* and the non-archael thermophilic bacterium *Geobacillus kaustophilus* of which structural characterization of the enzyme from the non-archaeal thermophilic bacterium has not yet been published. We have here presented a report on the structural characterization of the enzyme from the non-archaeal hyperthermophilic bacterium, *Aquifex aeolicus*.

In *A. aeolicus*, the open reading frame Aq021 corresponds to the aroD gene that codes for the Shikimate pathway enzyme type I 3-DHQase. The crystal structure of Aq021 revealed similar fold as the type I 3-DHQases from other sources mentioned above. Interestingly a long flexible loop near the active site that normally swings to close in the presence of a substrate was seen to be uninfluenced by the ligand binding as the liganded structure of Aq021 showed no loop swinging. This observation provides new insights into the catalytic mechanism of type I 3-DHQases. Structural analysis of Aq021 in its apo and complex forms suggesting the possible

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http://dx.doi.org/10.1016/j.bbrc.2013.01.099

reason that hinders this loop closure and some other structural aspects that shows its thermophily are discussed here.

2. Materials and methods

2.1. Cloning, expression and purification of Aq021

The cloning, expression and purification of Aq021 are described in detail in the supplementary data. Briefly, the aroD gene encoding the enzyme type I 3-DHQase from *A. aeolicus* VF5 was cloned into the expression vector pET-21a and expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene) strain. The harvested cells were resuspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 5 mM βME, 1 mM PMSF) followed by sonication and heat-treatment to denature most of the unwanted contaminant proteins. The protein was purified using SuperQ Toyopearl 650, ResourceQ, Hydroxylapatite and Superdex 200 columns as described in the supplementary data. The purified protein was concentrated to 15.4 mg/ml for crystallization studies.

2.2. Crystallization and data collection

Aq021 was co-crystallized with the ligand TLA($C_4H_6O_6$) or L(+)-Tartaric acid which is 53% similar to the substrate (3-dehydroquinic acid). Crystallization was carried out at 20 °C by sitting-drop vapour diffusion method by adding 1 μl of protein solution to 1 μl of well solution containing 30% PEG (polyethylene glycol) 4000, 0.2 M ammonium acetate and 0.1 M citrate, pH 5.6. Suitable crystals grew within a week. Complete MAD data sets were collected at Structural Genomics Beamline II, BL26B2, at the SPring-8, Hyogo, Japan. Native Aq021 was also crystallized by the sitting-drop vapour diffusion method by adding 1 μl of protein solution to 1 μl of well solution containing 44% MPD (2-methyl-2,4-pentanediol), 0.1 M acetate NaOH and 0.05 M $MgCl_2$, pH 4.8. A complete data set was collected at beamline 22-BM, APS, Argonne. All the data sets were processed using the HKL 2000 suite [4]. The data collection statistics are given in Supplementary Table 1.

2.3. Structure determination and refinement

The structure of Aq021-TLA was solved by MAD (Multi-wavelength anomalous dispersion) phasing method using the automatic structure determination software *SOLVE* [5]. Using this structure as the model, the native Aq021 structure was solved by molecular replacement method using *MOLREP* [6]. Refinement for both the structures was carried out using *CNS* [7]. The model was further improved using the graphics program *COOT* [8] through its real space fitting and interactive manual building. A blob of positive density was observed in Fo–Fc map at 3.0 σ level at the active site of the Aq021-TLA structure that could be interpreted as a TLA molecule. The refinement statistics are given in Supplementary Table 1.

3. Results and discussion

3.1. Overall structure of Aq021

The native form of Aq021 reveals a homotrimer, namely A, B and C, where molecule A with B and C with symmetry related molecule C associate to assemble as dimer (Supplementary Fig. S1). This packing arrangement thus satisfies the requirement of a homodimer as a biological unit as observed in other type I 3-DHQases. Each monomer has an overall topology of $(\alpha/\beta)_8$ or TIM Barrel and interact with each other through the exposed residues on the helices F, G and H to form a homodimer (Fig. 1A).

The two ends of the barrel are open as observed in the 3-DHQases of *S. aureus*[9], *S. pyogenes* and *A. fulgidus*[10] [Fig. 1B (I) & (II)]. However, in type I 3-DHQases from *C. difficile*, *S. typhi*, *S. enterica* and *G. kaustophilus*, two short antiparallel β-strands block the opening of the barrel at the N-terminal end [Fig. 1B (III) & (IV)]. Through the opening at the opposite end of N-terminal region, the substrate can reach the active site which is located at the entrance of the opening [Fig. 1B (II)]. The helix and β-strand numbering follows standard practice for TIM barrel domains.

Type I 3-DHQases have a long flexible hH loop at the one end of the barrel, near the active site, that adopts open/closed conformation depending on the absence/presence of the substrate and was suggested to be functionally important for enzyme catalysis [11] (Fig. 2). The hH loop in Aq021 is 13 residues long (192–204) and has five residues (Ala200, Pro201, Gly202, Gln203 and Ile204) which are strictly conserved when compared to other type I 3-DHQases (Supplementary Fig. S2). Similar to other apo forms of type I 3-DHQases, the hH loop of native Aq021 displayed an open conformation which is ordered. Surprisingly, in the ligand-bound Aq021 structure (Aq021-TLA) this loop adopts the same open conformation but is disordered. This is contrary to the closed and ordered loop behaviour observed in other ligand-bound structures (Fig. 2). In Aq021-TLA, the three residues Lys197, Ala198 and Phe199 in this loop displayed poor electron density and could not be modelled. Therefore, the hH loop of ligand-bound Aq021 showed a conformation that was not observed in any of the structures of ligand-bound type I 3-DHQases determined so far.

3.2. Sequence and structure comparison

Sequence homologues of Aq021 were identified using BLASTp search against non-redundant database from NCBI [12] which identified 100 sequences with identity in the range 32–65%. The top ten homologues were found to be type I 3-DHQases; however none of them has any structural information.

The A chain of Aq021 (2YSW A) was then subjected to structural similarity search using DALI [13]. The closest structural homologues were found to be type I 3-DHQases from *C. difficile* (PDB 3JS3), *S.typhi* (1GQN), *S. enterica* (3L2I), *G. kaustophilus* (2YR1), *A. fulgidus* (2OX1), *S. aureus* (1SFL), and *S. pyogenes* (2OCZ) with Z score ranging from 27.7 to 22.3. Aq021 displayed very low sequence homology with its structural homologues (26–31%), however the overall structure was found to be conserved with rmsd (root-mean-square deviation) in the range 1.73–2.05 Å for 176–212 common Cα atoms.

A structure-based sequence alignment using Expresso from T-coffee [14] showed that the residues that form the catalytic triad Glu65, His116 and Lys142 and the residue (Arg180) that recognizes the 1-carboxyl group of the substrate are strictly conserved in Aq021 (Supplementary Fig. S2).

Of the type I 3-DHQase structures determined so far, the enzyme from *C. difficile* and *S.typhi* were solved both as native and as a complex with the ligand 3-amino-4,5-dihydroxy-cyclohex-1-enecarboxylate (DHS), while the structures of the enzyme from *S. aureus* and *S. enterica* were solved in complex with 3-dehydroshikimate (DHK) and 1,3,4-trihydroxy-5-oxo-cyclohexanecarboxylic acid (DQA) respectively; hence structural comparison of Aq021 has been carried out with that from *C. difficile*, *S.typhi, S.aureus* and *S. enterica*.

The superposition of the complex structures of type I 3-DHQase from *A. aeolicus* (Aq021), *C. difficile*, *S. typhi*, *S. aureus* and *S. enterica* (Fig. 2) revealed an interesting structural feature in the hH loop of Aq021-TLA complex which adopts an open disordered conformation in spite of the presence of the ligand TLA unlike other ligand-bound structures. The superposition of Aq021 as an apo protein and in complex with TLA gave an rmsd of 0.5 Å for 433