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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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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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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

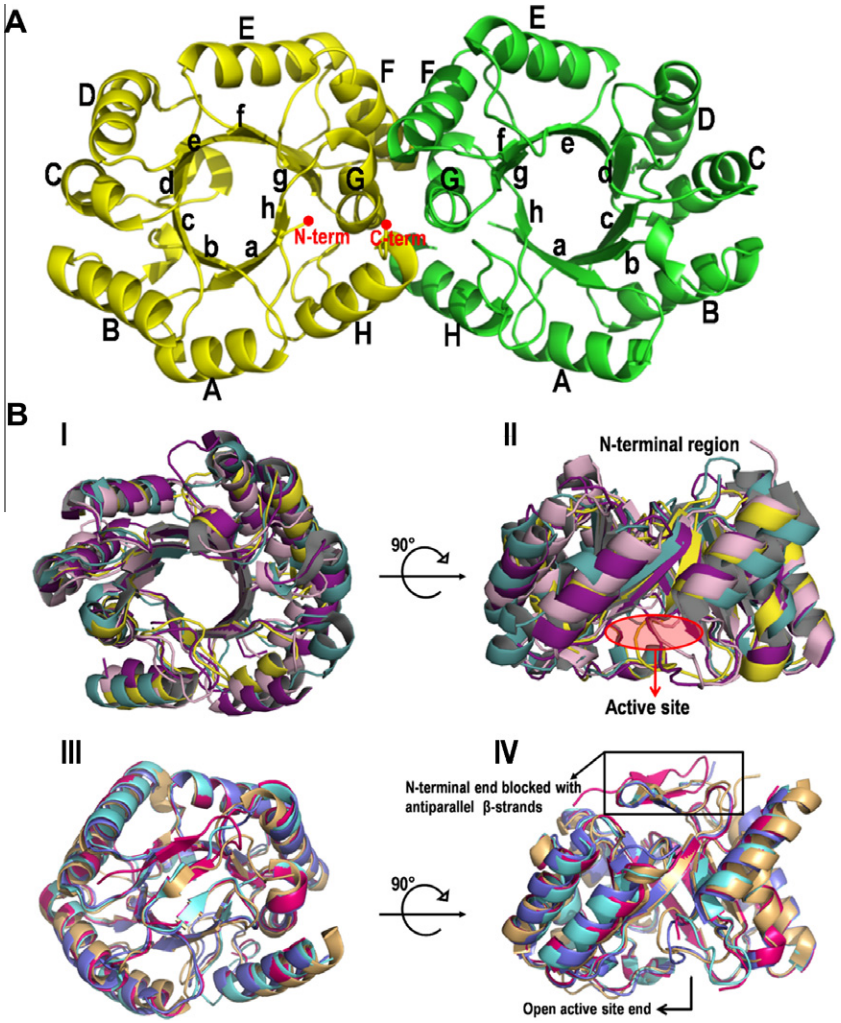

Fig. 1. Structure of Aq021 and its comparison with other 3-DHQases. (A) Cartoon representation of Aq021 homodimer formed by chains A (yellow) and B (green). The eight helices (A–H) surround the eight strands (A–H) to give an overall topology of a TIM Barrel. The dimer is stabilized by interaction between helices F, G and H of each monomer. (B) Open and closed barrel structures of 3-DHQases. (I) Top view and (II) side view of cartoon representation of the structural superpose of Aq021 (light pink) with the 3-DHQases of *S. aureus* (purple), *A. fulgidus* (yellow) and *S. pyogenes* (light teal). In all these structures the $(\alpha/\beta)_8$ barrel is open at both ends. (III) Top view and (IV) side view of structural superpose of 3-DHQases of *C. difficile* (blue), *S. typhi* (cyan), *S. enterica* (dark pink) and *G. kaustophilus* (light orange) showing the barrel closed at the N-terminal end with antiparallel β-strands. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cα atoms and revealed no major difference between them (Supplementary Fig. S3).

3.3. Aq021-TLA interactions

Previous studies have proposed the reaction mechanism of type I 3-DHQase suggesting the minute intricacies of the role played by the catalytic residues and subsequent conformational changes associated during the pre- and post-dehydration steps [11,15]. Briefly, this reaction undergoes a Schiff's base intermediate state formation initiated by the nucleophilic attack of a lysine residue, followed by a carbanion formation and the release of a water molecule (Supplementary Fig. S4).

TLA occupied 270 Å^2 of the active site region and its interactions with the conserved catalytic triad Glu65, His116 and Lys142 in Aq021 showed features of both the pre- and post-dehydration intermediate states of the enzyme. The Schiff's base forming residue Lys142 in Aq021 forms hydrogen bond with the O3 and O4 groups of TLA at a distance of 3.0 and 2.9 Å, respectively (Supplementary Fig. S5). The proton abstracting general base His116 also makes hydrogen bond at a distance of 2.9 Å to the O4 group of TLA.

In the pre- and post-dehydration complexes of type I 3-DHQases of *S. enterica* and *C. difficile*, respectively it was seen that His143 in the catalytic site adopted two different conformational states depending on the presence or absence of the substrate leaving group (1-hydroxyl group) (Fig. 3A). In the pre-dehydration form, NE2 group of His143 makes hydrogen bond with the leaving group at a distance of 2.8 Å, while in the absence of the leaving group, i.e., in the apo and post-dehydration forms, the side chain of His143 displaces away from the catalytic site and interacts with the conserved Glu86 favoured by a hydrogen bond (2.9 Å) between the ND1 and OE1 groups of the two residues, respectively [15]. In the pre-dehydration form His143 (ND1) and Glu86 (OE1) is 4.3 Å apart. A similar feature of the apo and post-dehydration conformation of histidine was also seen in the apo and complex forms of *S. aureus* (His133) and *S. typhi* (His143) type I 3-DHQases. In Aq021-TLA, His116 (NE2) makes hydrogen bond with the O4 group of TLA at a distance of 2.9 Å, a feature similar to that observed in the pre-dehydration form. In addition, His116 (ND1) was found to be at the same hydrogen bond distance (2.8 Å) from Glu65 (OE1) in both the apo and complex structures of Aq021 (Fig. 3B). However, the interaction of histidine with a conserved glutamic acid was

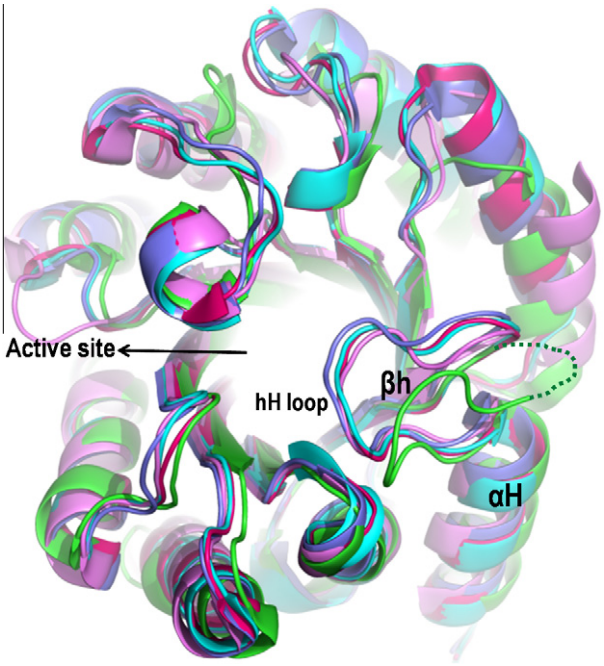

Fig. 2. Structural superpose of Aq021-TLA (green) with the complex structures of type I 3-DHQases of *C. difficile* (blue), *S. typhi* (cyan), *S. aureus* (magenta) and *S. enterica* (dark pink). Superpose of the structures showing that Aq021-TLA is highly similar to that of the other complex structures. Except for Aq021-TLA in all other structures the hH loops adopt closed and ordered conformation. In Aq021-TLA it is disordered (green dotted curve) and also adopts open conformation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed only in the apo and post-dehydration forms of type I 3-DHQases described before [15]. This shows that Aq021-TLA has features of both the pre- [His116(NE2) interaction with TLA(O4)] and post-dehydration [His116(ND1) interaction with Glu65(OE1)] forms and can be considered as a unique intermediate state of the enzyme.

In the complex structures from *S. aureus, S. typhi*, C. *difficile* and *S. enterica*, the carboxylate recognition residue Arg (202/213/214/213 respectively) changes its conformation from apo to ligand-bound structure which favoured interaction of its guanidium group to the 1-carboxyl group of the substrate (Fig. 4A). Light et al. [11] suggested that this conformational change of arginine is loop-dependent so that any steric clash with glutamine (Gln236) of hH loop is avoided during the loop closure event. In the apo structure (*S. typhi*) Gln236 (NE2) of hH loop forms hydrogen bond with Arg213 (NH2) at a distance of 2.9 Å. Once the substrate binds the conformation of the loop changes from open to closed form. Subsequently, the conformation of Gln236 and Arg213 changes thereby breaking the hydrogen bond interaction between them. In the closed loop conformation, Gln236 (NE2) and Arg213 (NH1) form a hydrogen bond with O5 atom of the substrate DHS at a distance of 2.5 and 3.1 Å, respectively. In Aq021, both in apo and complex structures, Gln203 and Arg180 do not form any hydrogen bond. In Aq021-TLA complex, Arg180 forms two hydrogen bonds with the O1 group of TLA via its NH1 and NH2 groups at a distance of 3.2 and 2.7 Å, respectively (Fig. 4B). However, the conformation of Arg180 in Aq021-TLA, was found to be the same as in apo Aq021. Similarly, the conformation of hH loop and Gln203 was also found to be the same in both the apo and complex structures. This structural feature is observed for the first time in type I 3-DHQases.

3.4. Liganded and unliganded structures showed no loop swinging

In type I 3-DHQases the long flexible hH loop acts as a lid that swings out and adopts open conformation facilitating easy access for the substrate and closes when the substrate is in the active site [3]. However, in Aq021-TLA the hH loop showed an unusual feature with the loop possessing a disordered conformation which is also open.

The hH loop has a stretch of six residues (SAPGQI) conserved in almost all the type I 3-DHQases (Supplementary Fig. S2) of which serine and glutamine were seen to directly interact with the substrate as observed in the complex structures from *S. aureus, S. typhi*, C. *difficile* and *S. enterica*. Mutation of Ser232 and Gln236 of *S. enterica* type I 3-DHQase to alanine [11] showed that they are important for the enzymatic reaction. It was also shown that loop closure in the substrate-bound state brings Gln236 in close proximity with Arg213 that induces conformational change in Arg213 and the subsequent formation of a bidentate salt bridge with the 1-carboxyl group of the substrate.

In Aq021-TLA, the loop is in the open form and this has not induced any conformational change in Arg180 (Fig. 4B). Thus, loop swinging was not seen in Aq021-TLA. This is probably because of a bulky phenylalanine in Aq021 (Phe199) in place of the conserved serine in the hH loop of other structures (Supplementary Figs. S2 & S6). If the loop were to close, Phe199 would undergo steric clash with Pro6 which is in the first β-strand of Aq021 (Supplementary

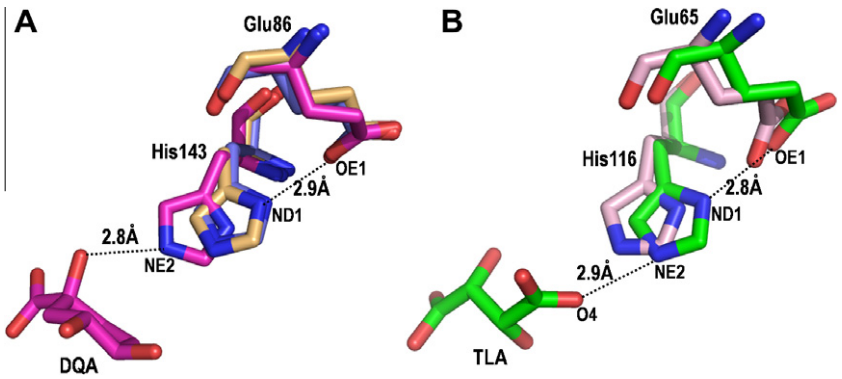

Fig. 3. Conformational change of histidine in the active site region with respect to the apo and complex structures of type I 3-DHQases. (A) His143 of *S. enterica* (dark pink) in the pre-dehydration intermediate state conformation makes hydrogen bond with the hydroxyl group of the substrate, DQA. However, as seen in the apo (orange) structure from *S.enterica* and post-dehydration intermediate state structure from *C. difficile* (blue), His143 is displaced away from the catalytic site and makes hydrogen bond with Glu86. (B) His116 (ND1) of apo Aq021 (light pink) and Aq021-TLA (green) interact with the respective Glu65 (OE1) via a hydrogen bond. In Aq021-TLA, His116 (NE2) form a hydrogen bond TLA (O4). His143 and Glu86 of *S. enterica* type I 3-DHQase are homologous to His116 and Glu65 of Aq021 respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

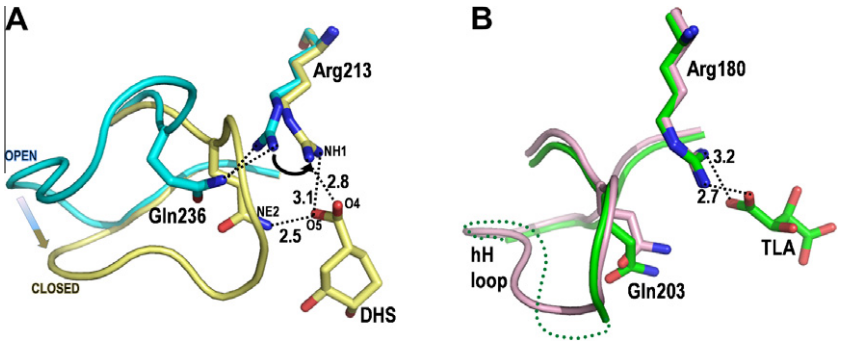

Fig. 4. Conformational change of arginine in the active site of apo and complex structures of type I 3-DHQases. (A) Open (cyan) and closed (light yellow) conformation of the hH loop in *S. typhi* type I 3-DHQase. During loop closure, in the presence of the substrate DHS, Gln236 comes in close proximity with Arg213 which induces the conformational change in Arg213 (shown by the curved arrow). (B) In apo Aq021 (light pink) and Aq021-TLA (green), hH loop showed no swinging irrespective of the presence or absence of the substrate. Arg180 therefore retains the same conformation. In Aq021-TLA, the hH loop is disordered (shown in dotted curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. S6). Such a notable feature of the loop was also seen in the case of *Plasmodium falciparum* triosephosphate isomerase [PfTIM, 16] where the catalytic loop of the enzyme did not close when the substrate bound to the active site in order to avoid steric clash between Ile172 and Phe96. However, this did not affect the activity of the enzyme [16]. The present Aq021-TLA complex structure therefore suggests that hH loop closure is probably not required for the enzyme action as there was no conformational change with respect to the loop in both the liganded and the unliganded forms.

3.5. Thermostability of Aq021

A major hurdle in the industrial application of proteins lies in formulating them into therapeutics that can overcome certain intrinsic and extrinsic factors surrounding the protein environment [17]. Amongst these factors the thermodynamic stability of a protein is of utmost importance as unfolded or aggregated forms of a protein, besides being non-functional, are toxic or immunogenic [18]. Aq021 being an enzyme from a hyperthermophilic bacterium was compared to its mesophilic homologues and analyzed for differences that may contribute to the thermostability of the enzyme. This is discussed in detail in supplementary data.

Briefly, the percentage composition of the residues Glu, Arg, Tyr, Ser and Gly were found to be high, while that of residues Met, His, Asn and Gln were found to be low in the thermophiles when compared to the mesophiles (Supplementary Fig. S7; Supplementary Table 2). Other notable features that might impart thermostability to Aq021 include high number of intramolecular and intermolecular interactions, ion pairs and disulphide bond (Supplementary Table 2). Knowledge on the factors that impart thermostability to this enzyme will be useful in enhancing structural stability to it during industrial applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.099.

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