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Unique Cofactor Binding Mode of Homoserine Dehydrogenase from Hyperthermophilic Archaeon *Pyrococcus horikoshii*

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1 Introduction

Homoserine dehydrogenase (HseDH, EC 1.1.1.3) is a key enzyme in the biosynthetic pathway from aspartate to homoserine (Hse), which is a common precursor for the synthesis of three amino acids, methionine, threonine and isoleucine in plants and microorganisms. In this pathway, aspartate is first phosphorylated to β-aspartyl phosphate (β-Ap) by aspartate kinase, after which L-aspartate-β-semialdehyde (Asa) dehydrogenase catalyzes the conversion of β-Ap to Asa. The third enzyme in the pathway, HseDH, catalyzes the NAD(P)H-dependent reduction of Asa to Hse. Subsequent metabolism of Hse yields methionine, threonine or isoleucine, which are all essential in humans. HseDH is thought to be a potential target for the structure-based design of antibiotics or herbicides, as the enzyme is not present in mammals. Moreover, stable HseDH is a potentially useful target for the development of new method for homocysteine biosensing [1].

Up to now, structure of HseDH from Archaea or from hyperthermophiles has not been reported. The structures of putative HseDHs from *Archaeoglobus fulgidus*, *Thermoplasma acidophilum* and *T. volcanium* were deposited in the Protein Data Bank (PDB), but the enzymological properties and structural details of these enzymes have not been reported. Within the genomic sequence of the hyperthermophilic archaeon *Pyrococcus horikoshii*, we found a gene (ORF PH1075) whose predicted amino acid sequence exhibits 31.2% identity to that of the *A. fulgidus* HseDH homolog. In the present study, we expressed that gene, characterized the enzyme produced and revealed the enzyme to be a highly thermostable HseDH. We then determined the crystal structure of this enzyme [2]. Refinement of the structure showed the presence of a bound cofactor, NADP(H), although it was not added during crystallization, which indicates earlier high-affinity binding of the cofactor to the protein. Surprisingly, however, we found that NADP does not act as a cofactor for this enzyme, but as a strong inhibitor of NAD-dependent oxidation of Hse. Structural analysis and site-directed mutagenesis showed that the large number of interactions between the cofactor and the enzyme are responsible for the lack of reactivity of the enzyme towards NADP. This observation suggests this enzyme exhibits a new variation on cofactor binding to a dehydrogenase: very strong NADP binding that acts as an obstacle to NAD(P)-dependent dehydrogenase catalytic activity.

2 Experiment

Diffraction data were collected (λ=1.0 Å) on the beamline AR-NE3A at the Photon Factory. The initial phases for the structure were determined by molecular replacement; the structure of *A. fulgidus* HseDH homologue (PDB entry 3DO5) served as the search model.

3 Results and Discussion

The gene (PH1075) was overexpressed in *Escherichia coli*, and its product was purified and characterized. The expressed enzyme is the most thermostable HseDH yet described, retaining 70% of its activity even after incubation for 10 min at 95ºC. The crystal structure of the enzyme was determined at a resolution of 2.30 Å. Refinement of the structure and mass analysis showed the presence of the bound cofactor NADPH, although it has not been added during crystallization.

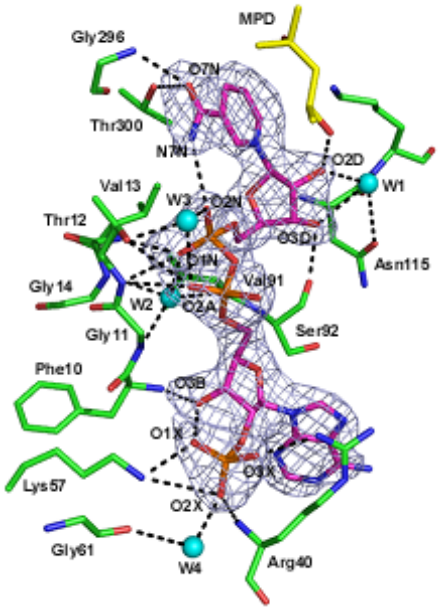

Fig. 1: NADPH-binding site in *P. horikoshii* HseDH.

The C2 phosphate group of the cofactor adenine ribose is tightly held at the nucleotide-binding site through three direct hydrogen bonding interactions with the side chains of Arg40 and Lys57.

Unexpectedly, however, NADP did not act as a cofactor with this enzyme, but as a strong inhibitor of NAD-dependent Hse oxidation. Most strikingly, K57A mutant acquired high reactivity against NADP. The crystal structure of the K57A mutant with bound NADPH was then determined to a resolution of 2.43 Å [2].

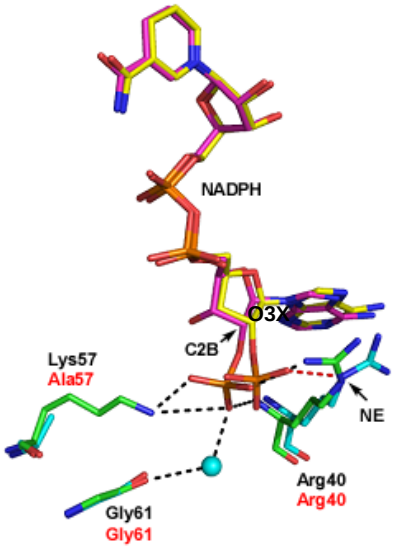

Fig. 2: Comparison of the NADPH-binding site structures in *P. horikoshii* HseDH wild-type (green and black labels) and the K57A mutant (cyan and red labels).

Superposition of the NADPH-bound K57A structure onto the NADPH-bound wild-type structure showed that the NADPH molecule in the mutant structure was positioned/configured nearly identically to the NADPH molecule in the wild-type structure, except for the positioning of the C2 phosphate group of the adenine ribose (Fig. 2). In the wild-type enzyme, the C2 phosphate is tightly held in position through five surrounding hydrogen bonds. In K57A mutant, however, the C2 phosphate group is rotated in a clockwise direction around C2B of NADPH by about 30° relative to the wild-type structure. In addition, the guanidino group of Arg40 in the mutant is also rotated clockwise by about 90° around the NE atom of Arg40 relative to the wild-type structure. As a result, only one interaction between O3X of the C2 phosphate and the NE of Arg40 was observed at the corresponding position in K57A (Fig. 2). Nearly all the interactions between the cofactor NADH moiety and the enzyme are conserved between the two enzymes, except for a few water-mediated interactions. Given that no substantive difference in the domain organization of the two enzymes was found, these observations suggest that the larger number of interactions around the C2 phosphate of the cofactor strengthen the affinity of wild type HseDH for NADP, making the binding too tight to utilize NADP as the cofactor.

Because NAD differs from NADP only in the C2 phosphate group of the adenine ribose, the amino acid residues interacting with this region are thought to be responsible for the cofactor specificity of the enzymes. In the medium-chain dehydrogenase/reductase family enzymes, the coenzyme binding domains possess similar β-α-β motifs centered around a highly conserved GXGXXG/A sequence. Among the amino acid residues of this β-α-β motif, the primary determinant of NAD specificity is the presence of an Asp or Glu residue, which forms hydrogen bonds with both the C2 and C3 hydroxyl groups of the NAD adenine ribose and occupies the space that would be occupied by the C2 phosphate group of the NADP adenine ribose. In NADP dependent enzymes, on the other hand, this residue is usually replaced by a smaller residue such as Gly, Ala or Ser, accompanied by one or more positively charged residues, Arg and/or Lys, which form a binding pocket for the C2 phosphate group. Similar features are also observed in short-chain dehydrogenase/reductase family enzymes. In some enzymes with dual cofactor specificity, replacement of the hydrogen bonds associated with each cofactor binding has been observed. HseDH belongs to an expansive and diverse class of oxidoreductases, and although the overall fold of the catalytic region is unique, the nucleotide-binding domain conforms to the Rossmann fold, like conventional NAD(P)-dependent dehydrogenases. The structure of the nucleotide-binding site in our model indicates that *P. horikoshii* HseDH would have a strong preference for NADP. However, NADP does not act as the cofactor for this enzyme, but as a strong inhibitor for NAD-dependent Hse oxidation. To the best of our knowledge, this is the first reported example of an enzyme in which the very strong binding of NADP is an obstacle to its catalytic activity, in this case NAD(P)-dependent dehydrogenase activity. Although the physiological significance of the inhibitory effect of NADP remains unclear, the present study indicates that the molecular details underlying cofactor preference in NAD(P)-dependent dehydrogenases are more complex than expected, and the cofactor specificity cannot be predicted even from the structural information in the absence of biochemical data.

Acknowledgement

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References

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