Structure based engineering of an NADP⁺-dependent D-amino acid dehydrogenase

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1 Introduction

meso-Diaminopimelate dehydrogenase (DAPDH; EC 1.4.1.16) catalyzes the reversible NADP⁺-dependent oxidative deamination of meso-diaminopimelate (meso-DAP) to produce l-2-amino-6-oxopimelate. The enzyme acts stereoselectively on the D-center of meso-DAP, indicating it recognizes the difference between D- and L-configuration carbons. It was therefore expected that this enzyme would be useful for one-step production of D-amino acids, which are often utilized as source materials for industrial production of medicines, seasonings and agrochemicals. However, its high substrate specificity for meso-DAP and instability have proved to be the major disadvantage for the practical application of DAPDH. We recently identified and characterized a highly stable DAPDH from the thermophilic bacterium Ureibacillus thermosphaericus. By introducing five point mutations, we succeeded in creating a stable NADP⁺-dependent D-amino acid dehydrogenase (DAADH) from U. thermosphaericus DAPDH. This DAADH does not act on meso-DAP but catalyzes the reversible deamination of D-amino acids such as D-cyclohexylalanine (relative activity, 100%), D-isoleucine (73%), D-2-aminoobutyrate (61%) and D-lysine (53%). As the next step, creation of an enzyme exhibiting different substrate specificity and higher catalytic efficiency is a key to the further development of D-amino acid production. We have determined the structures of U. thermosphaericus DAPDH in the apo form and in complex with NADP⁺ and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [1]. However, the information needed for production of novel DAADH mutants with different substrate specificities remains limited.

In the present study, we determined the crystal structure of apo-DAADH. We compared the active-site architecture of the apo-DAADH (open-form) with that of Symbiobacterium thermophilum DAPDH in complex with substrate (closed-form) and sought the amino acid residues responsible for substrate binding. We used site-directed mutagenesis to identify the residues responsible for determining the enzyme’s substrate specificity. As a result, we found that a single mutation (D94A), which has not been tested previously, caused a striking change in the substrate specificity of DAADH. We then succeeded in determining the structures of a D94A/Y224F double mutant in complex with NADP⁺ and in complex with both NADPH and 2-keto-6-aminocaproic acid (KACA), the keto acid analogue of lysine. Based on this structural information, the factors responsible for the difference in substrate specificity between U. thermosphaericus DAADH and its mutant were evaluated [2].

2 Experiment

Diffraction data for crystals of apo-DAADH and the D94A/Y224F mutant enzyme were collected using an ADSC Quantum CCD detector system on the BL-5a and AR-NW12 beamlines at the Photon Factory, Tsukuba, Japan. The structure of the apo-DAADH was solved to a resolution of 1.78 Å by molecular replacement using the MOLREP program in the CCP4 program suite; the structure of chain A from U. thermosphaericus DAPDH (PDB code: 3wyb) served as the search model. The structures of NADP⁺-bound and NADPH/KACA-bound D94A/Y224F mutant enzymes were solved to resolutions of 1.59 Å and 1.74 Å, respectively, by molecular replacement using MOLREP with the apo-enzyme structure (chain A) as a search model.

3 Results and Discussion

The crystal structure of the S. thermophilum DAPDH/NADPH/meso-DAP ternary complex has been reported. Superposition of this structure (PDB code: 3wbf, subunit B) onto that of U. thermosphaericus apo-DAADH (subunit B) enabled estimation of the amino acid residues involved in substrate binding, as well as the induced-fit movement of these residues. In S. thermophilum DAPDH, the side chain of Tyr205 forms a hydrogen bond with the side chain of Asp92 and through this interaction Tyr205 is held at the active-site entrance, where it acts as part of the substrate-binding site. These residues were respectively conserved as Tyr224 and Asp94 in U. thermosphaericus DAADH. Thus, interactions among these residues and the substrate may also occur upon substrate binding to DAADH, though the side chain of Tyr224 is situated about 13 Å farther away from that of Asp94 in the apo-structure. Although both of
the residues are strictly conserved among all known DAPDHs and DAADHs, and may play a key role in substrate binding, there have been no reported mutational analyses of these residues. We therefore constructed D94A and Y224F mutants and examined their substrate specificities.

When we compared the substrate specificities for oxidative deamination catalyzed by the parent DAADH and the D94A and Y224F mutants (substrate concentration: 30 mM), we found that the substrate specificity of D94A markedly differed from that of the parent enzyme. The D94A mutant utilized d-phenylalanine as the most preferable substrate and had a specific activity of 5.33 μmol/min/mg at 50°C, which is 54 times higher than the parent enzyme. Although the relative activities with D-leucine, D-norleucine, D-methionine, D-isoleucine, D-tryptophan and D-histidine were lower than the activity with the d-phenylalanine, the specific activities with all these substrates were 6-25 times higher than was observed with the parent enzyme. For the reductive amination, D94A also showed significantly higher specific activity toward phenylpyruvate (16.1 μmol/min/mg, 8.3 times), 2-oxo-octanoate (65.5 μmol/min/mg, 5.0 times), 2-oxo-hexanoate (3.8 times), 2-oxo-4-methylthiobutanoate (3.9 times), 2-oxo-4-methylpentanoate (3.3 times), 2-oxo-pentanoate (1.6 times) and 2-oxo-3-methylpentanoate (1.7 times) than the parent enzyme [5 mM of 2-oxo-acid analogues were used except for phenylpyruvate (2 mM)]. On the other hand, Y224F exhibited less activity than the parent enzyme with all substrates, especially for oxidative deamination, though the substrate spectrum was comparable. This suggests Tyr224 is essential for the proper catalytic activity, but is not very important for the substrate recognition.

Reportedly, the H227V mutant created from S. thermophilum DAPDH has the specific activity of 2.4 μmol/min/mg at 30°C with phenylpyruvate in the reductive amination. The Clostridium tetani DAPDH mutant also shows catalytic activity toward the synthesis of d-phenylalanine, but the specific activity with phenylpyruvate is only 0.11 μmol/min/mg at 30°C. The extremely high activity for reductive amination of phenylpyruvate (V_{max} of 92.5 μmol/min/mg at 50°C) is likely a major advantage of using the D94A mutant for direct synthesis of D-phenylalanine.

To examine the factors responsible for the difference in substrate recognition, we modeled the phenylpyruvate molecule into the active site of NADPH/KACA-bound D94A/Y224F based on the orientation of KACA and then minimized the energy of the complex using CNS (Fig. 1). Within this model, an interaction was formed between the carboxyl group of the substrate and the nicotinamide ribose phosphate, as well as between the C2 carbonyl group of the substrate and the side chain of Lys150. On the other hand, the phenyl group of the substrate was observed to rotate 53° around C3 in a clockwise direction relative to the C3–C4 bond of KACA. The phenyl group was held at that position via stacking interactions with Trp148 and the nicotinamide ring, in addition to hydrophobic interactions with the side chains of Lys150 (Cβ and Cε; 8 interactions), Trp123 (CD2, CE3, and CZ3; 5 interactions) and Ala94 (Cβ, 2 interactions). This binding mode of the phenylpyruvate would not be allowed in the parent DAADH, because the phenyl group is supposed to make unusual short contacts with the side chain of Asp94 (the Asp94 side chain is shown in yellow in Fig. 1). The large substrate-binding pocket formed by Trp148, Lys150, Trp123, and Ala94 may also be related to a high activity of the D94A mutant towards 2-oxooctanoate with bulky side chain. Taken together, our observations suggest that the D94A substitution enlarged the substrate-binding pocket and enhanced the hydrophobicity of the pocket around the side chain of the substrate. Consequently, the D94A mutant gained strong reactivity toward hydrophobic D-amino acids such as D-phenylalanine, D-leucine, D-norleucine, D-methionine and D-isoleucine.

Fig. 1: Proposed model for phenylpyruvate binding. NADPH/KACA-bound D94A/Y224F and phenylpyruvate-bound model are shown in white and cyan, respectively. Phenylpyruvate and NADPH molecules in the phenylpyruvate-bound model are in green and magenta, respectively. The hydrogen bonds around phenylpyruvate are shown as dotted lines.

Through the use of U. thermosphaericus DAADH, we have already succeeded in developing novel methods for producing D-branched-chain amino acids, for subsequent generation of compounds labeled with stable isotopes, and for assaying D-isoleucine without interference from the three other isomers. As the next step, creation of an enzyme exhibiting different specificity and higher catalytic efficiency became a key for the further development of D-amino acid production by the method at the industrial level. In this study, we succeeded in the creation of a novel mutant with an extremely high catalytic activity for phenylpyruvate amination. Moreover, the much higher catalytic activity of the mutant observed
with several 2-oxo acid analogues of D-amino acids would be useful for further development of practical applications.

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References

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