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Crystal structure of the novel amino-acid racemase isoleucine 2-epimerase from *Lactobacillus buchneri*

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1 Introduction

Free D-branched-chain amino acids (D-BCAAs) such as D-isoleucine (D-Ile), D-leucine (D-Leu) and D-valine (D-Val) are thought to act as specific molecular signals in some bacterial species. For example, D-Ile, D-Leu and D-Val are reportedly involved in regulating cell-wall remodelling in *Vibrio cholera*. It has been suggested that D-BCAAs are produced by a few pyridoxal 5'-phosphate (PLP)-dependent amino-acid racemases with broad substrate specificity, which include arginine racemase from *Pseudomonas graveolens* and amino-acid racemase from *P. putida*. However, these two racemases show much lower activity towards Leu than their main substrate, lysine, and no activity towards Ile or Val. Alanine racemase from *P. putida* is known to catalyze L-Ile racemization, but this enzyme exhibits much lower activity towards L-Ile than L-Ala. Thus, the available biochemical and structural information on the mechanism of bacterial D-BCAA production remains limited. In a recent study, a marked accumulation of D-Leu, D-*allo*-Ile and D-Val was observed in the growth medium of the lactic acid bacterium *Lactobacillus otakiensis* JCM 15040, and the enzyme responsible was identified as a racemase catalyzing the production of D-amino acids from the corresponding L-amino acids [1]. On the basis of the N-terminal amino-acid sequence of the purified enzyme, a gene encoding a homologue of this racemase was identified in the genome of *L. buchneri* JCM 1115 [1], which is closely related to *L. otakiensis* JCM 15040. The product is a PLP-dependent racemase that preferentially catalyzes epimerization between L-Ile and D-*allo*-Ile, but also catalyzes the racemization of nonpolar amino acids, including Leu and Val [1]. This enzyme was therefore named isoleucine 2-epimerase (ILEP). ILEP is a novel enzyme that catalyzes the racemization of BCAAs as its main substrate and is thought to be responsible for the production of D-BCAAs in lactic acid bacteria. *L. buchneri* ILEP has been classified as a fold-type I enzyme on the basis of its amino-acid sequence [1]. Before the detection of *L. buchneri* ILEP, α-amino-ε-caprolactam racemase (ACLR) from *Achromobactor obae* was the only known bacterial racemase that was classified into fold type I; ILEP is the second. Crystal structures of *A. obae* ACLR have previously been reported, but the reaction mechanism of this enzyme remains unclear. A structural analysis of *L. buchneri* ILEP may shed light on the substrate-recognition and reaction mechanisms of fold-type I racemases.

In the present study, we solved structures of *L. buchneri* ILEP bound by the reaction-intermediate analogues *N*-(5'-phosphopyridoxyl)-L-isoleucine (PLP-L-Ile) and *N*-(5'-phosphopyridoxyl)-D-*allo*-isoleucine (PLP-D-*allo*-Ile), in addition to apo and PLP-bound structures. We also evaluate the candidate acid/base catalytic residues for L-Ile epimerization by means of site-directed mutagenesis [2].

2 Experiment

Diffraction data for crystals of PLP-bound, apo form, PLP-L-Ile-bound, and PLP-D-*allo*-Ile-bound ILEP were collected using an ADSC Quantum CCD detector system on the BL-5A and AR-NE3A beamlines at the Photon Factory, Tsukuba, Japan. The structure of the PLP-bound ILEP was solved to a resolution of 1.94 Å by molecular

replacement using the MOLREP program in the CCP4 program suite with the tetramatic structure of GABA-AT from *Sulfolobus tokodaii* (PDB code: 2eo5) serving as the search model. The structures of apo form, PLP-L-Ile-bound, and PLP-D-*allo*-Ile-bound ILEP were solved to resolutions of 2.77, 2.65 and 2.12 Å, respectively, by molecular replacement using MOLREP. As a search model, the structure of chain C from the PLP-bound ILEP was used for the apo and PLP-L-Ile-bound ILEP and the structure of chain A from the PLP-L-Ile-bound enzyme was used for the PLP-D-*allo*-Ile-bound enzyme.

3 Results and Discussion

The *L. buchneri* ILEP assembled as a tetramer, which is consistent with the subunit assembly of the enzyme as estimated by gel filtration and SDS–PAGE (native enzyme 200 kDa, subunit 49 kDa). A very clear electron density corresponding to the PLP molecule was observed within the cofactor-binding site of PLP-bound enzyme, which enabled us to place the ligand with reasonable accuracy (Fig. 1A). The 5'-phosphate group of PLP interacts with the side-chain O and backbone N atoms of Ser116 and Thr309* (the asterisk indicates a residue in the neighbouring subunit), and the backbone N atom of Gly115. The phosphate group also interacts with the backbone N atoms of Lys280 and Thr310* and the side chains of Thr310* and Asn113* via water molecules (W1 and W2). The pyridine ring of PLP is sandwiched between Tyr142 and Val252. Via another water molecule (W3), the 3-hydroxyl group of PLP interacts with the side chains of Glu217 and Asp222 and the backbone N atom of Asp222. The C4 atom of PLP connects covalently to the ε-amino group of Lys280 to form an internal aldimine (Schiff-base) linkage. The pyridine N atom of PLP makes salt bridge with the side chain of Asp250. This interaction is conserved in all known fold-type I enzymes.

In the apo ILEP, amino-acid residues –11 to 28, 52 to 54 and 302* to 309* were disordered and were not visible in the electron-density map. When we superimposed the structure of apo ILEP on that of the PLP-bound enzyme, the configuration of the loops around the active site differed greatly between the two enzymes (Fig 1B). Loop 302*–309*, including Thr309*, which forms hydrogen bonds to the 5'-phosphate group of PLP in the PLP-bound structure, is disordered in the apo structure. In addition, loop 81*–87* in the apo structure moves towards the opposite side of the bound PLP molecule. These two loops are derived from the neighbouring subunit, and the elements surrounding the two loops (amino-acid residues –11 to 28 and 52 to 54) are also disordered in the apo structure. It is noteworthy that all of the residues thought to form hydrophobic interactions with the substrate L-Ile (Ile24, Ala54, Ala83*, Tyr84* and Leu307*; see below) are included in the abovementioned loops and elements. As a result, the active-site cavity in the apo structure is much more solvent-accessible than that in the PLP-bound structure. These observations suggest that the binding of PLP to the apo enzyme gives rise to a large structural change around the active site, which in turn provides a solvent-inaccessible environment for the enzyme reaction.

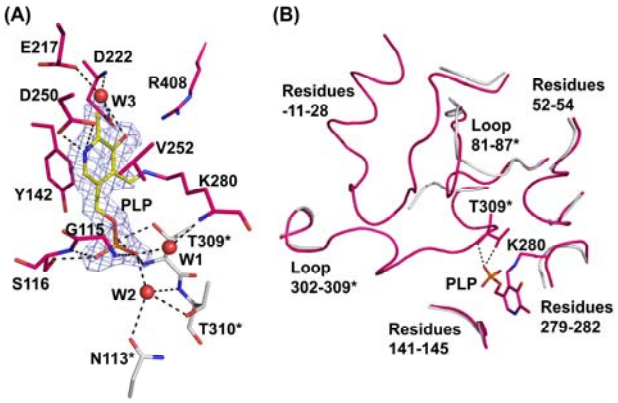

Fig. 1: (A) PLP bound to *L. buchneri* ILEP. The PLP molecule is shown in yellow. The water molecules are shown as red spheres. Residues that interact with PLP are shown in magenta, and those belonging to the neighbouring subunit are in light gray. (B) Close-up views of the loops and elements surrounding the active site. The apo and PLP-bound structure are shown in light gray and magenta, respectively. PLP molecule, Lys280 and Thr309* in the PLP-bound structure is shown as a stick model.

In the initial electron-density map of PLP-L-Ile and PLP-D-*allo*-Ile bound enzyme, extra density was observed within the active-site cavity, and after refinement of the peptide chain, PLP-L-Ile or PLP-D-*allo*-Ile could be modelled into this density (Figs. 2B and C). Comparison of the two structures revealed that the 5'-phosphopyridoxyl moiety of PLP-D-*allo*-Ile occupies almost the same position as that of PLP-L-Ile (Fig. 2A). In addition, the carboxyl group of the substrate isoleucine forms hydrogen bonds to the side chain of Arg408 in both structures. However, the positioning of the covalent bonds connecting the Cα atom of isoleucine and the pyridine ring of PLP is totally different between the two enzymes. The Cβ atom of L-Ile in PLP-L-Ile is rotated in a clockwise direction around the Cα atom by about 49° relative to that of the D-*allo*-Ile in PLP-D-*allo*-Ile. Moreover, the Cδ atom in L-Ile is positioned on the opposite side to that in D-*allo*-Ile. In the PLP-L-Ile-bound enzyme, as a result, the side chain of L-Ile forms a hydrophobic core mainly with Ile24, Tyr142 and Leu307*, while that of D-*allo*-Ile forms hydrophobic interactions mainly with Ala54, Ala83* and Tyr84*.

Alanine racemase from *Bacillus stearothermophilus*, a fold-type III PLP-dependent enzyme, utilizes a two-base mechanism in which Lys39 and Tyr265* act as general acids/bases. In this mechanism, the Lys residue is responsible for the deprotonation of the substrate D-amino acid, which creates a quinonoid intermediate that is reprotonated by the Tyr residue on the opposite face of the intermediate to produce the L-amino acid. In the reverse reaction (L→D), proton abstraction is performed by Tyr, while reprotonation is accomplished by Lys. Although the overall structure of *A. obae* ACLR is quite different from that of alanine racemase, the position of the

side chain of Tyr137 in *A. obae* ACLR, which is situated on the *re*-face of the PLP ring, symmetrically corresponds to that of Tyr265* in alanine racemase, which is situated on the *si*-face of the PLP ring. Lys39, which forms an internal aldimine with PLP in the alanine racemase, is conserved as Lys267 in *A. obae* ACLR. Therefore, Tyr137 and Lys267 are thought to be candidate acid/base catalytic residues. In addition, another possibility that Asp210, the side chain of which is located near to the side chain of Tyr137, acts as the acid/base catalytic residue instead of Tyr137 has also been described. However, the details of the reaction mechanism of *A. obae* ACLR remain unclear.

The *L. buchneri* ILEP monomer showed high structural similarity to ACLR from *A. obae* (PDB codes: 3dxv and 2zuk). Tyr137, Asp210 and Lys267 in *A. obae* ACLR are strictly conserved in *L. buchneri* ILEP as Tyr142, Asp222 and Lys280, respectively. In the PLP-L-Ile-bound enzyme, the side chain of Lys280 points towards the α-hydrogen of the substrate L-Ile. The interatomic distance from the Cα atom of PLP-L-Ile to the side chain NZ atom of Lys280 is 3.6 Å, which is close enough to cause a reaction, suggesting that Lys280 is a catalytic base that abstracts the α-hydrogen of L-Ile (Fig. 2B). In the D-*allo*-Ile-bound enzyme, on the other hand, the residue whose side chain is most proximate to the Cα atom of D-*allo*-Ile is Asp222: the interatomic distance from the Cα atom of D-*allo*-Ile to the side chain OD2 atom of Asp222 is 3.2 Å (Fig. 2C). The distance between the Cα atom of D-*allo*-Ile and OH of Tyr142 (5.1 Å) is too long to form an interaction. To assess the role of Tyr142 and Asp222, we constructed Y142F, D222A, and D222N mutants and observed that the Y142F mutant retained V_{max} values of 85 and 132 μmol min^{-1} mg^{-1} for the epimerization of L-Ile and D-*allo*-Ile, respectively, which are about 46–56% of the wild-type V_{max} values. In contrast, the activities of the D222A and D222N mutants were almost abolished (about 0.33–0.38% and 0.0047–0.0049%, respectively, of the wild-type V_{max} values). This suggests that, in the case of L-Ile epimerization, Lys280 is responsible for removing the α-hydrogen from L-Ile, while Asp222, but not Tyr142, serves as the catalytic residue adding α-hydrogen to the quinonoid intermediate to form D-*allo*-Ile. In the reverse reaction, proton abstraction and reprotonation would be performed by Asp and Lys, respectively. Although having a low reaction rate, the D222A and D222N mutants still retained catalytic activity. Therefore, the hydroxyl anion in the solvent water may function as the catalytic acid/base, as in the case of alanine racemase from *B. stearothermophilus*. This is the first description of the structure of a BCAA racemase, and our results may provide critical information that will facilitate a better understanding of the reaction mechanism of fold-type I racemases.

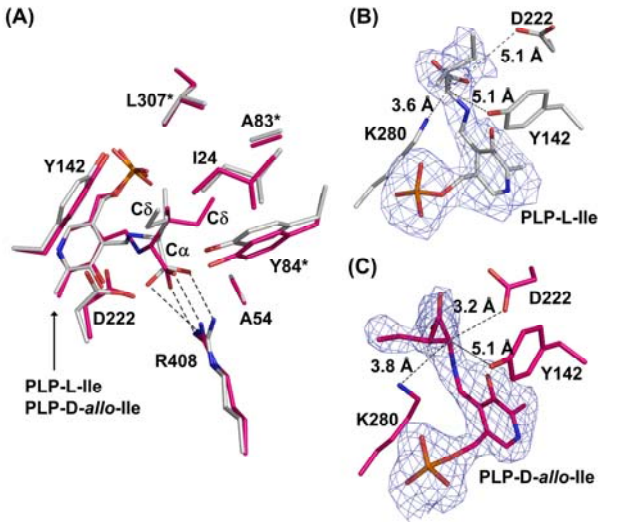

Fig. 2: (A) Comparison of the structures around the PLP-Ile binding site. The structure with bound PLP-L-Ile (gray) is superimposed on that with bound PLP-D-*allo*-Ile (magenta). The hydrogen bonds are shown as dotted lines. (B) PLP-L-Ile binding site. (C) PLP-D-*allo*-Ile binding site. The final σ_A-weighted Fo – Fc OMIT electron-density maps for PLP-L-Ile and PLP-D-*allo*-Ile are shown at the 2.5σ level, respectively. The hydrogen bonds are shown as dotted lines.

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References

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