論文の内容の要旨

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論文題目

Crystal structure of asHPAL as a useful aldolase for the two-step stereoselective synthesis of 4-hydroxyisoleucine: understanding the reaction mechanism and expanding the substrate specificity

(4-Hydroxyisoleucine 合成に関わる酵素 asHPAL の X 線結晶構造解析と基質選択性の拡大)

Chapter 1　Introduction

Aldol reaction is particularly useful because it can generate new chiral centers (one or more) and form a new carbon-carbon bond in a single reaction. Aldolases are a type of enzyme that can catalyze the aldol reaction for specific substrates. The reaction catalyzed by aldolases can proceed under mild conditions and thus has the advantage of low environmental impact. 4-Hydroxy-3-methyl-2-keto-pentanoate aldolase (asHPAL) is an aldolase from *Arthrobacter simplex* strain AKU 626 that belongs to the HpaI/HpcH aldolase subfamily of the class II aldolases. asHPAL uses Mg^{2+} as a prosthetic group to catalyze aldol reactions and has the potential for use in the synthesis of (2*S*,3*R*,4*S*)-4-hydroxyisoleucine (4-HIL) [1], which is a chiral amino acid extracted from the seeds of fenugreek *Trigonella foenum-graecum*. Among the 8 possible enantiomers of 4-HIL, only (2*S*,3*R*,4*S*)-4-HIL potentiates the insulin secretion specifically at supranormal concentrations of glucose in the sense that it becomes ineffective at low or basal glucose concentrations. asHPAL catalyze the first reaction in which 2-ketobutyrate and acetaldehyde are converted to (3*S*,4*S*)-4-hydroxy-3-methyl-2-keto-pentanoate (HMKP). HMKP is then converted to 4-HIL by branched-chain amino-acid aminotransferase (BCAT). Although the first reaction can be catalyzed by some other aldolases like YfaU and YhaF, asHPAL is the only enzyme that can catalyze the synthesis of (3*S*,4*S*)-HMKP preferentially at pH 6.0. The catalytic mechanism of the enzymes of the HpaI/HpcH-aldolase subfamily toward α-keto acids as substrates has been proposed in previous work. However the mechanism of stereo selectivity and substrate specificity remains unclear. asHAPL can utilize only pyruvate and 2-ketobutyrate as substrates, which limits the industrial uses of asHPAL for the synthesis of β-hydroxy carbonyl compounds. Here,

I applied X-ray crystallography to reveal the structural basis of the stereoselectivity and substrate specificity of the aldol reaction catalyzed by asHPAL. Moreover, the obtained structural information was successfully used to improve the stereoselectivity and expand the substrate specificity of asHPAL.

Chapter 2 Crystal structure of asHPAL

asHPAL was produced as a His-tag-fusion protein in the *Escherichia coli* expression system, and was then purified with a Ni-Sepharose 6 Fast Flow column and a Resource Q column [2]. The result of SDS-PAGE showed that the purified asHPAL was sufficiently pure for crystallization experiments and enzymatic analyses. In addition, size-exclusion chromatography affirmed the presence of asHPAL in stable hexamer state solution, and the HPLC analyses of the purified asHPAL demonstrated its lasting aldolase activity.

The purified asHPAL (10 mg ml^{-1}) was used for crystallization experiments. After initial crystallization and 2D grid optimization of crystallization conditions, the optimal crystals of asHPAL were obtained with the reservoir solution containing 100 mM HEPES-Na (pH 7.7), 30% (v/v) PEG 400, and 200 mM $MgCl_2$ at 293 K for 1 day. The crystals diffracted X-rays to 1.60 Å. The initial structure of asHPAL was determined by molecular replacement method using the atomic coordinate of 4-hydroxy-2-oxo-heptane-1,7-dioate aldolase (HpcH) (45% sequence identity to asHPAL; PDB ID, 2V5J; Rea *et al.*, 2007) as a search model. The core of the molecule adopted a triosphosphate isomerase (TIM) barrel fold. An Mg^{2+} was coordinated in combination with oxygen atom of Glu156, Asp182, and four water molecules. Because the divalent metal ion is used by Class II aldolase to stabilize the interaction between substrate and enzymes, the position of Mg^{2+} is suspected to be the active center of asHAPL.

Chapter 3 Structural basis for the stereoselective aldol reaction of asHPAL

In order to determine the mechanisms of asHPAL catalyzing the stereospecific synthesis of (2*S*,3*R*,4S)-4-HIL, the structure of asHPAL was solved at 1.58 Å in complex with 2-ketobutyrate as a substrate. Clear electron density was visible for both the Mg^{2+} and 2-ketobutyrate molecules. In addition, four water molecules appeared nearby Mg^{2+}. The O2 and O3 atoms of 2-ketobutyrate interacted with Mg^{2+} through coordinate bonds. The carbonyl group of 2-ketobutyrate formed a hydrogen bond with the nitrogen atom of Arg77. In addition, Arg77 interacted with the O3 atom of 2-ketobutyrate and a water molecule through an electrostatic interaction and a hydrogen bond, respectively. The position of this water molecule is considered to be the positon of the oxygen atom of acetaldehyde according to docking simulation. The kinetics data suggested the roles of His52, Arg77, and Asp91 in the aldol reaction of the HpaI/HpcH-aldolase subfamily and that the formation of a hydrogen bond between His52 and Asp91 could enhance the catalytic potential of His52 as an intersubunit His52-Asp91 dyad.

Based on the structural and kinetics data, I proposed the catalytic mechanism of asHPAL, which could improve upon the mechanism proposed in previous reports: (1) With the action of the catalytic dyad formed by His52 and Asp91, His52 promotes to remove the H^+ at the O3 atom of 2-ketobutyrate through two warter molecules, forming the nucleophilic enolate; (2) attack of the nucleophilic enolate on the electrophilic C1 carbon of acetaldehyde trapped by Arg77; (3) protonation of the O4 atom of the intermediate accepts a proton from the N1 atom of His52 through the water molecule that forms a hydrogen bond with His52, completing the synthesis of HMKP.

From the structure of asHPAL-2-ketobutyrate complex, it was predicted that the π/C-H-reaction between methyl group of acetaldehyde and benzene ring of Phe26 may ensure the methyl group of acetaldehyde in a particular direction. Thus, acetaldehyde attacks the nucleophilic enolate in the particular direction and syntheses the (3*S*,4*S*)-HMKP preferentially. The loss of stereoselectivity in the F26W and F26L mutants indicated that Phe26 controlled the stereoselectivity to preferably produce (2*S*,3*R*,4*S*)-4-HIL in the coupled reaction with BCAT. Based on the structure, the stereoselectivity of asHPAL was successfully improved by the N219Y/F26N double mutation. asHPAL exhibits a higher stereoselectivity toward (3*S*,4*S*)-HMKP at pH 6.0 than at pH 7.0 or 8.0. The asHPAL crystals at pH 6.0 were obtained with the reservoir solution containing 100 mM Bis-Tris (pH 6.0), 1.5 M $(NH_4)_2SO_4$, and 100 mM NaCl at 293 K. The asHPAL structure at pH 6.0 was determined at 2.15 Å. However, no differences were observed between the structures determined at pH 6.0 and 7.7, which suggested that quantum chemistry approaches may be required to understand the pH dependency of stereoselectivity.

Chapter 4　Mechanism of substrate specificity of asHPAL

Three structures of asHPAL were determined as complexes with pyruvate (1.97 Å), oxamate (1.81 Å), and 4-methyl-2-oxovalerate (1.60 Å) in addition to the complex with 2-ketobutyrate. The structures reveal a detailed structural basis for the binding of α-keto acids as substrates. α-Keto acids possessed the O2 and O3 atoms as common moieties, and the Mg^{2+} coordination of these atoms was a major interaction between asHPAL and α-keto acids. In addition, the binding orientation of substrates at the active site was crucial for the substrate specificity because the aldol reactions can proceed when the C3 atom of an α-keto acid is close to the C2 atom of acetaldehyde.

The structure of asHPAL in complex with 4-methyl-2-oxovalerate and the mutational data revealed that Phe177 restricted the binding of 4-methyl-2-oxovalerate in the active site in an orientation opposite from 2-ketobutyrate. The F177A mutant, which was engineered by the crystal structure of asHPAL and simulation approaches, acquired the catalytic activity for the aldol reaction between 4-methyl-2-oxovalerate and acetaldehyde. Moreover, an additional S181W mutation increased the turnover rate of the F177A mutant toward 4-methyl-2-

oxovalerate as a substrate, which reveals that restricting substrate orientations can enhance the catalytic activity of asHPAL.

Chapter 5 General discussion

We determined the structure of asHPAL in pH 7.7 (low stereo selectivity) and pH 6.0 (high stereo selectivity), asHPAL-2-ketobutyrate (the 1st substrate) complex, asHPAL-4-methyl-2-oxovalerate (Non-substrate of 2-keto acid) complex in this study. Although the structure of asHPAL-acetaldehyde complex was not obtained, the docking simulation of acetaldehyde to asHPAL and the kinetic experiments supported a reaction mechanism promoted by the catalytic dyad formed by His52 and Asp91. Because of the specific direction of acetaldehyde caused by Phe26, (3*S*,4*S*)-HMKP is preferential produced; this finding was supported by the decreased stereoselectivity in the F26W and F26L mutants. Based on this structure, the stereoselectivity of asHPAL was improved by the N219Y/F26N double mutation, which produced (2*S*,3*R*,4*S*)-4-HIL selectively in 91% enantiomeric excess (*ee*) at pH 6.0. The steric hindrance caused by Phe177 makes 4-methyl-2-oxovalerate bind in the active site of asHPAL in an opposite direction, leading to the substrate specificity of asHPAL. Based on the structural basis, the substrate specificity of asHPAL was changed toward 4-methyl-2-oxovalerate by the F177A mutation. Furthermore, the additional S181W mutation could restrict 4-methyl-2-oxovalerate in the same orientation as a preferable substrate, 2-ketobutyrate, and the resulting activity toward 4-methyl-2-oxovalerate was increased by the mutation. Further work is needed to confirm the stereoselectivity of the F177A/S181W double mutant.

References

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