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Role of conserved Met112 residue in the catalytic activity and stability of ketosteroid isomerase

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ABSTRACT

Ketosteroid isomerase (3-oxosteroid Δ^5-Δ^4-isomerase, KSI) from *Pseudomonas putida* catalyzes allylic rearrangement of the 5,6-double bond of Δ^5-3-ketosteroid to 4,5-position by stereospecific intramolecular transfer of a proton. The active site of KSI is formed by several hydrophobic residues and three catalytic residues (Tyr14, Asp38, and Asp99). In this study, we investigated the role of a hydrophobic Met112 residue near the active site in the catalysis, steroid binding, and stability of KSI. Replacing Met112 with alanine (yields M112A) or leucine (M112L) decreased the k_{cat} by 20- and 4-fold, respectively. Compared with the wild type (WT), M112A and M112L KSIs showed increased K_D values for equilenin, an intermediate analogue; these changes suggest that loss of packing at position 112 might lead to unfavorable steroid binding, thereby resulting in decreased catalytic activity. Furthermore, M112A and M112L mutations reduced melting temperature (T_m) by 6.4 °C and 2.5 °C, respectively. These changes suggest that favorable packing in the core is important for the maintenance of stability in KSI. The M112K mutation decreased k_{cat} by 2000-fold, compared with the WT. In M112K KSI structure, a new salt bridge was formed between Asp38 and Lys112. This bridge could change the electrostatic potential of Asp38, and thereby contribute to the decreased catalytic activity. The M112K mutation also decreased the stability by reducing T_m by 4.1 °C. Our data suggest that the Met112 residue may contribute to the catalytic activity and stability of KSI by providing favorable hydrophobic environments and compact packing in the catalytic core.

1. Introduction

Ketosteroid isomerase (3-oxosteroid Δ^5-Δ^4-isomerase, KSI; EC 5.3.3.1) is a homodimer of 14-kDa subunits that performs catalytic reaction by allylic rearrangement of the 5,6 double bond of Δ^5-3-ketosteroid to the 4,5 position by stereospecific intramolecular proton transfer [1,2]. The enzyme is one of the most proficient enzymes, enhancing the rate of the reaction (enolate formation and reketonization) by 11 orders of magnitude [2]. Two bacterial KSIs, one from *Comamonas testosteroni* and one from *Pseudomomas putida*, have been extensively investigated to study the relationships between structure and catalytic activity [3–6]. The active-site structures of these KSIs are almost identical [3,7,8]. Especially, three catalytic residues (Tyr14, Asp38, and Asp99; numbered according to *C. testosteroni* KSI) show sequence and structure conservation. Asp38 abstracts a proton from the steroid; as a result, a dienolate intermediate forms. This intermediate is stabilized by both OH of Tyr14 and COOH of Asp99. Two catalytic residues (Tyr14 and Asp99) form a hydrogen bond network together with Tyr30, Tyr55, and a water molecule in the active site of *P. putida* KSI [9].

The active site of KSI is composed of hydrophobic residues except for three catalytic residues. Some hydrophobic residues, such as Phe54 and Phe101, of the active site in KSI have important roles in catalysis [10,11], but the functions of other hydrophobic residues in the active-site cleft are not known. Met112 is located near the two catalytic residues Asp38 and Asp99 (Fig. 1). Because the Met112 residue is strictly conserved among KSI homologs (Fig. 2) and is buried in the hydrophobic core, it may have important roles in the catalytic activity and stability of KSI.

Methionine is one of the most hydrophobic amino acid residues. Most methionine residues are located in the interior of proteins. The methionine residue is often critical to the function and stability of proteins; e.g., catalysis by phosphite dehydrogenase [12], folding and stability of squash trypsin inhibitor [13], and protein stability and beta-barrel unfolding of outer membrane X [14].

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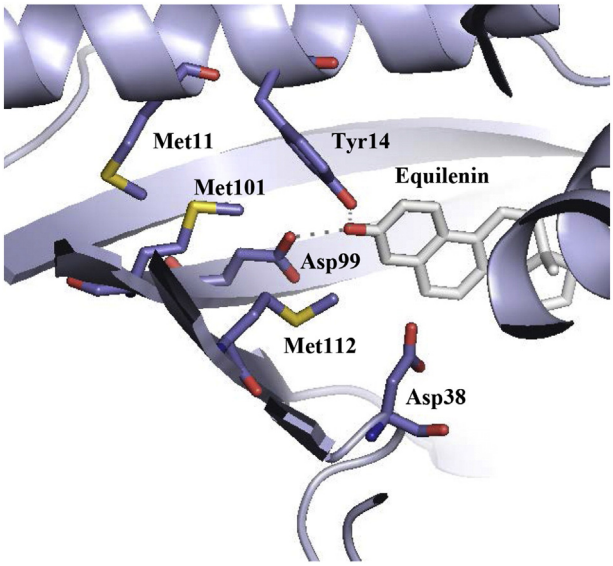

Fig. 1. Close-up view of the active site of KSI. The active site of KSI is composed of three key catalytic residues (Tyr14, Asp38, Asp99) and hydrophobic residues. Three methionine residues (Met11, Met101, Met112) are buried in the catalytic core. Stick models: equilenin and active-site residues; gray dotted lines: hydrogen bonds.

To understand the role of the Met112 residue in KSI, we used site-directed mutagenesis to replace the Met112 with alanine, leucine, or lysine, and quantified how these changes affected the catalysis, stability, and structure of KSI. Alanine and leucine were chosen to investigate the effect of decreasing the length of the hydrophobic side chain. Lysine was selected to replace a buried methionine residue with a charged residue because methionine and lysine are similar in overall size and shape, so the substitution minimizes disruption of the structure [15]. The changes caused distinct effects on catalytic activity, stability, and binding affinity toward the steroid. The crystal structure of M112K KSI was determined at 1.6 Å resolution, and showed the structural basis for understanding the effect of the M112K mutation on catalysis. Our studies demonstrated that the conserved Met112 residue might have an important role in the stability and catalytic ability of KSI.

2. Materials and methods

2.1. Materials

Chemicals for buffer solutions were purchased from Sigma (USA). Oligonucleotides were obtained from Genotech Inc. (Korea). 5-Androstene-3,17-dione (5-AND) and equilenin were purchased from Steraloids Inc. (USA). The Superose 12 gel filtration column was obtained from Amersham Bioscience (USA).

2.2. Site-directed mutagenesis, expression and purification of proteins

Site-directed mutagenesis to obtain M112A, M112L and M112K mutant KSIs was performed using a procedure described previously [16]. Expression vector, pKK-KSI (carrying the WT KSI gene) was used as a template with a set of two primers encoding the desired mutation (Supplementary Table 1). All mutations were confirmed by gene sequencing. Each mutant was overexpressed in *Escherichia coli* BL21(DE3) (Novagen). All mutant enzymes were purified using deoxycholate affinity column chromatography and gel filtration chromatography as described previously [16,17]. The purity of protein was confirmed by the identification of a single band in SDS-PAGE.

2.3. Determination of enzyme activity

The catalytic activity of the purified enzyme was determined spectrophotometrically using 5-AND as a substrate as described previously [18]. Various amounts of the substrate were added to the reaction buffer containing 34 mM potassium phosphate, pH 7.0, and 2.5 mM

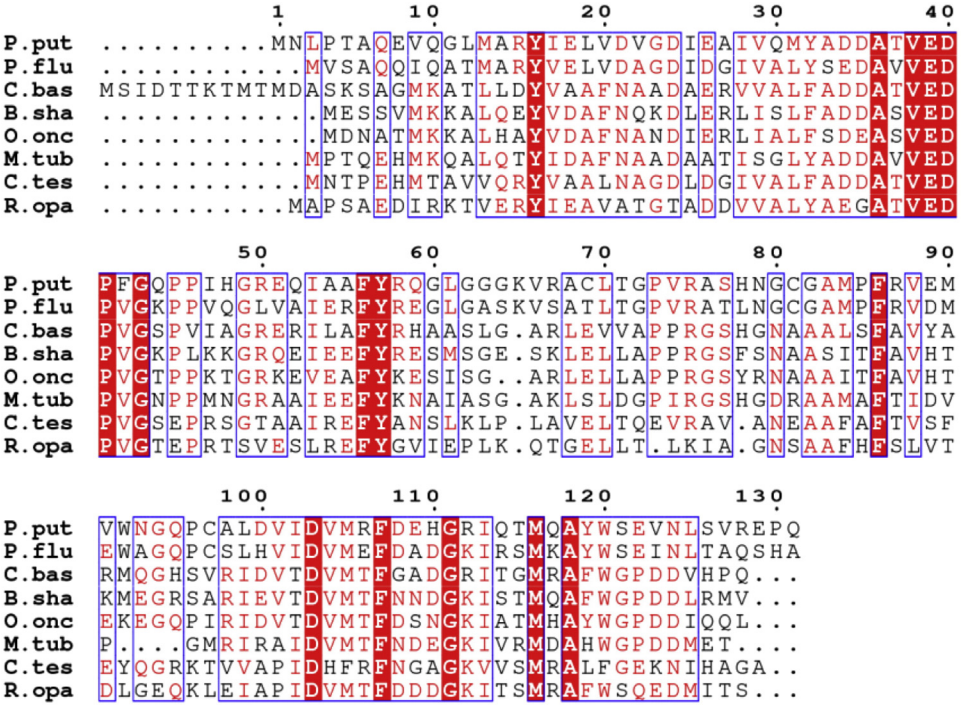

Fig. 2. Multiple sequence alignment of KSI homologs. Abbreviations: P.put: *Pseudomonas putida* (UniProt accession number: P07445); P.flu: *Pseudomonas fluorescens* (A0A0F4V996); C.bas: *Cupriavidus basilensis* (A0A0C4YLD6); B.sha: *Bacillus shackletonii* (A0A0Q3TEP9); O.onc: *Oceanobacillus oncorhynchi* (A0A0A1MPJ2); M.tub: *Mycobacterium tuberculosis* (A0A0T9KXV2); C.tes: *Comamonas testosteroni* (P00947); R.opa: *Rhodococcus opacus* (C1B7K2). White letters on red background: fully conserved residues; red letters on white background: partially conserved residues.