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Modifying the determinants of α-ketoacid substrate selectivity in *mycobacterium tuberculosis* α-isopropylmalate synthase

Michael F.C. Hunter, Emily J. Parker *

Biomolecular Interaction Centre and Department of Chemistry, University of Canterbury, Christchurch, New Zealand

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ABSTRACT

α-Isopropylmalate synthase (IPMS) catalyses the reaction between α-ketoisovalerate and acetyl coenzyme A (AcCoA) in the first step of leucine biosynthesis. IPMS is closely related to homocitrate synthase, which catalyses the reaction between AcCoA and the unbranched α-ketoacid α-ketoglutarate. Analysis of these enzymes suggests that several differently conserved key residues are responsible for the different substrate selectivity. These residues were systematically substituted in the *Mycobacterium tuberculosis* IPMS, resulting in changes in substrate specificity. A variant of IPMS was constructed with a preference for the unbranched α-ketoacids α-ketobutyrate and pyruvate over the natural branched substrate α-ketoisovalerate.

1. Introduction

The first committed step of both leucine biosynthesis and the α-aminoadipate pathway for lysine biosynthesis is an aldol reaction in which the acetyl group of acetyl coenzyme A (AcCoA) is added to the carbonyl moiety of an α-ketoacid. In leucine biosynthesis this reaction is catalysed by α-isopropylmalate synthase (IPMS) using the α-ketoacid α-ketoisovalerate (KIV) [1], and in lysine biosynthesis the corresponding reaction is catalysed by homocitrate synthase (HCS) using α-ketoglutarate as a substrate [2,3]. Both of these enzyme-catalysed reactions are the first in a sequence of three enzyme-catalysed reactions, the net result of which is the insertion of a single methylene unit between a ketone group and its β-carbon (Fig. 1) [4]. Both enzymes are also feedback inhibited by their pathway's end product amino acid, although by different mechanisms [5–11].

IPMS and HCS both belong to a family of related enzymes that also includes citramalate synthase (CMS) from isoleucine biosynthesis [12], *re*-citrate synthase from the Krebs cycle [13] and methylthioalkylmalate synthase from glucosinolate biosynthesis [14]. Each catalyses a metal-dependent aldol reaction between AcCoA and an α-ketoacid, with their α-ketoacid substrates only differing by the nature of the substituents on the β-carbon. Like HCS and IPMS, each is also the first step in a three enzyme methylene group insertion pathway [7].

As well as the obvious similarities in the reactions that IPMS and HCS catalyse and the pathways in which they operate, the enzymes share strong functional and structural analogies. Both are dimeric, both catalyse attack by AcCoA on the *re* face of their α-ketoacid substrate, and both are dependent upon a divalent metal ion for catalytic activity. The structure of the IPMS from *Mycobacterium tuberculosis* IPMS (*Mtu*IPMS) [11] has been solved, as have those of the HCS enzymes from *Thermus thermophilus* HCS (*Tth*HCS) and *Schizosaccharomyces pombe* [8,10]. Both proteins have a $(\beta/\alpha)_8$ barrel catalytic core with the active site at the C-terminal end, and the residues responsible for binding the divalent metal ion, the acetyl coenzyme A and the α-ketoacid functionality of the variable substrate are all strongly conserved both within and between the two homologues (Fig. 2).

By contrast, the residues that are in close proximity to the different side chains of the substrates of IPMS and HCS have very different sequences, reflecting the differing binding requirements associated with their different α-ketoacid selectivities (Figs. 2 and 3). These residues are believed to be the prime determinants of α-ketoacid specificity, and as such are promising targets for controlling the substrate specificity of these enzymes, either by

Abbreviations: AcCoA, acetyl coenzyme A; HCS, homocitrate synthase; IPMS, α-isopropylmalate synthase; IPTG, isopropyl thio-D-galactoside; KIV, α-ketoisovalerate; PDB, protein data bank; *Mtu*IPMS, *Mycobacterium tuberculosis* IPMS; *Tth*HCS, *Thermus thermophilus* HCS

* Corresponding author. Adrress: Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand. Fax: +64 3 364 2110.
E-mail address: emily.parker@canterbury.ac.nz (E.J. Parker).

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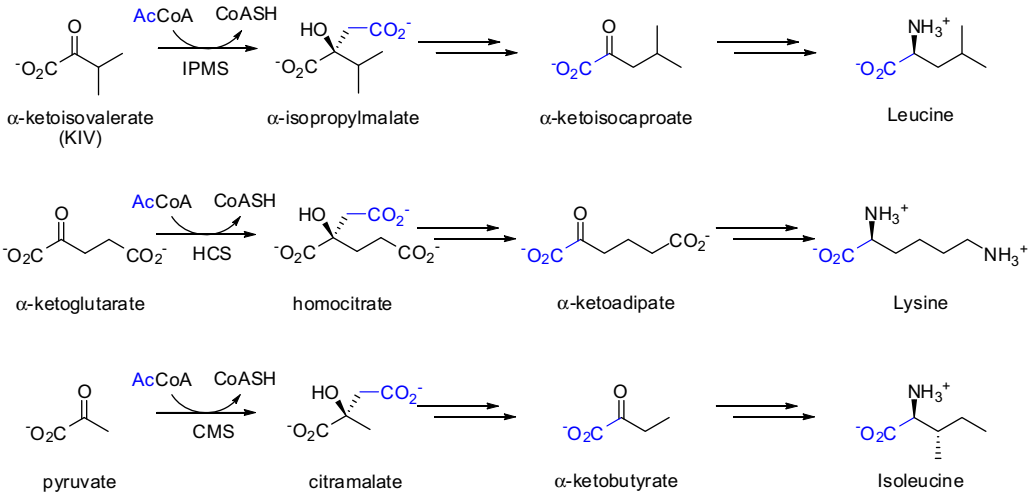

Fig. 1. Reactions catalysed by α-isopropyl malate synthase (IPMS), homocitrate synthase (HCS) and citramalate synthase (CMS).

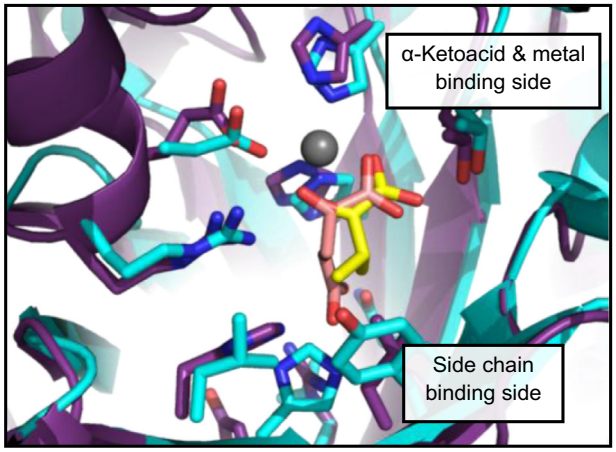

Fig. 2. The α-ketoacid binding site of *Mtu*IPMS (protein data bank (PDB) code 1SR9) in cyan and *Tth*HCS (PDB code 2ZYF) in purple. The KIV substrate of IPMS is shown in yellow and the α-ketoglutarate substrate of HCS is shown in pink. Zn^{2+} is shown in grey.

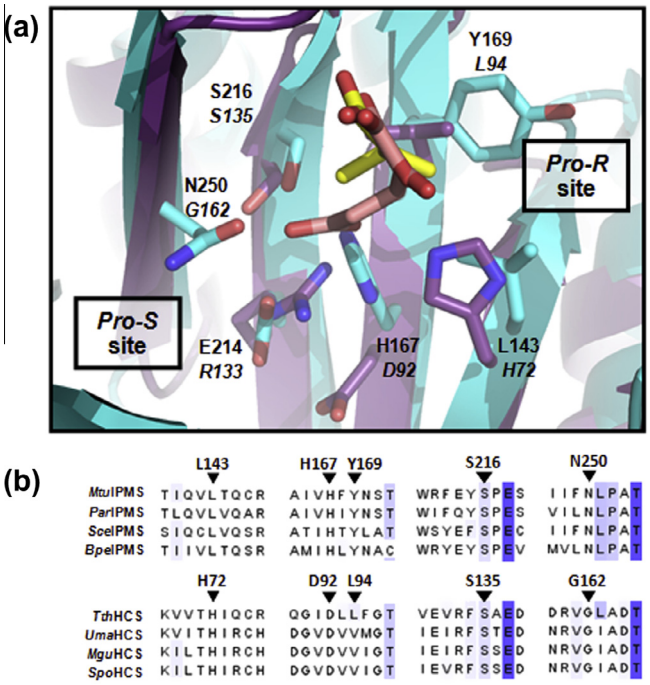

Fig. 3. (a) Overlay of the binding sites of *Mtu*IPMS and *Tth*HCS. Crystal structure of *Mtu*IPMS (PDB code 1SR9) is in cyan and of *Tth*HCS (PDB code 2ZYF) in purple. Residue labels in italics are for *Tth*HCS. Metal ions are omitted for clarity. The substrate of *Mtu*IPMS (KIV) is shown in yellow and the substrate of *Tth*HCS (KG) is shown in pink. (b) Partial alignment between IPMS and HCS sequences. Dark blue indicates positions conserved in IPMS, HCS and CMS. The residue numbers are for *Mtu*IPMS and *Tth*HCS. Full alignment is provided in the supplementary material (Fig. S1).

introducing unnatural residues or by replacing residues from one homologue with the corresponding residues from the other.

Several studies have been conducted that probe the natural selectivity of enzymes in this family in order to exploit the C–C bond forming capabilities of this enzyme for wider application [1,15–20]. Most notably, *Escherichia coli* strains producing IPMSs that have been altered to have a larger substrate-binding pocket have shown increased activity towards longer α-ketoacids, demonstrating that IPMS can be adapted to catalyse different reactions [4,20–22].

Here we examine in detail the determinants and malleability of substrate selection in IPMS by modifying the residues that line binding site of the KIV substrate's isopropyl group in *Mtu*IPMS. We find that specific substitutions were able to enhance activity with unbranched α-ketoacid substrates while significantly attenuating the ability of the enzyme to utilise efficiently the branched chain KIV.

2. Materials and methods

2.1. Mutagenesis

PproExHta-LeuA plasmid bearing the gene encoding *Mtu*IPMS was generously provided by Dr. Chris Squire (University of Auckland, New Zealand). Quikchange site directed mutagenesis (Stratagene) was used to introduce mutations into the plasmid (primer sequences are provided in Supplementary Material, Table S2). Following sequence verification plasmids were transformed into chemically competent BL21 (DE3) Star *E. coli* cells for protein expression.

2.2. Protein expression and purification

LB media was inoculated with 50 mL of overnight culture per litre. This culture was then grown at 37 °C until it reached an