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Structural and kinetic characterization of two 4-oxalocrotonate tautomerases in *Methylibium petroleiphilum* strain PM1

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ABSTRACT

Methylibium petroleiphilum strain PM1 uses various petroleum products including the fuel additive methyl tert-butyl ether and straight chain and aromatic hydrocarbons as sole carbon and energy sources. It has two operons, dmpI and dmpII, that code for the enzymes in a pair of parallel meta-fission pathways. In order to understand the roles of the pathways, the 4-oxalocrotonate tautomerase (4-OT) isozyme from each pathway was characterized. Tautomerase I and tautomerase II have the lowest pairwise sequence identity (35%) among the isozyme pairs in the parallel pathways, and could offer insight into substrate preferences and pathway functions. The kinetic parameters of tautomerase I and tautomerase II were determined using 2-hydroxymuconate and 5-(methyl)-2-hydroxymuconate. Both tautomerase I and tautomerase II process the substrates, but with different efficiencies. Crystal structures were determined for both tautomerase I and tautomerase II, at 1.57 and 1.64 Å resolution, respectively. The backbones of tautomerase I and tautomerase II are highly similar, but have distinct active site environments. The results, in combination with those for other structurally and kinetically characterized 4-OT isozymes, suggest that tautomerase I catalyzes the tautomerization of both 2-hydroxymuconate and alkyl derivatives, whereas tautomerase II might specialize in other aromatic hydrocarbon metabolites.

Introduction

4-Oxalocrotonate tautomerase (4-OT)[2] isozymes catalyze proton transfer reactions in bacterial catabolic pathways for aromatic hydrocarbons [1–4]. The most extensively studied 4-OT is found in Pseudomonas putida mt-2, where it is one of a group of enzymes known collectively as the meta-fission pathway [1–4]. The genes for the enzymes of the meta-fission pathway are encoded by the TOL plasmid. The presence of this plasmid enables the organism to process simple aromatic hydrocarbons (e.g., benzene, toluene, m- and p-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene) as its sole sources of carbon and energy [1–3].

4-OT transfers a proton from the 2-hydroxy group of 2-hydroxymuconate (1, Scheme 1) and places it at C-5 of 2-oxo-3-hexenedioate (2) [5]. It also processes 2-hydroxymuconate derivatives such as 5-(methyl)-2-hydroxymuconate (3), converting it to 5-(methyl)-2-oxo-3-hexenedioate (4) [6,7].

The enzyme is a hexamer, where each subunit is made up of 62 amino acids [8]. It is a founding member of the tautomerase superfamily, which is a group of structurally homologous proteins characterized by a β–α–β building block and a catalytic amino-terminal proline (Pro1) [9–11]. Kinetic, mechanistic, and structural studies have identified Pro1, the base responsible for proton transfer, as well as Arg11, Arg39, and Phe50 (from different monomers) as key players in the 4-OT-catalyzed conversion of 1–2 (Scheme 2) [12–17]. The results of mutagenesis studies suggest that Arg39 is primarily involved in catalysis and Arg11 is involved in both binding and catalysis [15,16]. Accordingly, Arg39 provides a positive charge to facilitate deprotonation of the 2-hydroxy group and Arg11 binds C-6 and draws electron density toward C-6 to facilitate protonation at C-5. Phe50 is near Pro1 and is partially responsible for the unusually low pK_a of Pro1 (i.e., ~6.4) due to its hydrophobicity [17].

In the course of a database search for new superfamily members, two tautomerase-like amino acid sequences were identified in the thermophile *Chloroflexus aurantiacus* J-10-fl, in a putative meta-fission pathway [6]. One sequence was annotated as a

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[2] *Abbreviations used:* DEAE, diethylaminoethyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hh4-OT, heterohexamer 4-oxalocrotonate tautomerase; IPTG, isopropyl-β-D-thiogalactoside; Kn, kanamycin; LB, Luria-Bertani; NCBI, national center for biotechnology information; 4-OT, 4-oxalocrotonate tautomerase; PEG, polyethylene glycol; rmsd, root-mean-square deviation; SSM, secondary structure matching; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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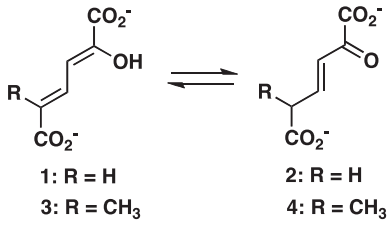


Scheme 1. The substrates for tautomerase I and II.

tautomerase-like protein, but lacked Pro1. The other sequence was not annotated as a tautomerase-like protein but it had Pro1. At first, it was puzzling to find two potential 4-OTs in the same meta-fission pathway because there is no apparent reason for them. The puzzle was solved when co-expression of the two genes produced a stable heterohexamer (verified by the crystal structure) where each dimer consists of an α and β-subunit [6]. Although a different quaternary structure, the so-called heterohexamer 4-OT (hh4-OT) catalyzes the canonical 4-OT reaction using **1** with the same mechanism and efficiency.

A similar database search revealed another peculiarity: some organisms have two meta-fission pathways, where each one has a tautomerase homolog, presumably a 4-OT. *Methylibium petroleiphilum* strain PM1 is one such organism [18]. The organism was originally isolated from a biofilter that was used at a sewage treatment plant for treating oil refinery discharges. Subsequent characterization showed that it metabolizes several components of gasoline including aromatic and alkyl hydrocarbons as well as methyl *tert*-butyl ether [19]. These metabolic capabilities make the organism potentially quite useful in the bioremediation of gasoline-contaminated environments.

The presence of two meta-fission pathways in *M. petroleiphilum* raises questions about their specificities and functions. One possibility is that they process similarly substituted aromatic hydrocarbons and that they are redundant, perhaps conferring some selective advantage to the organism. An alternative possibility is that one is a canonical meta-fission pathway and the other one processes differently substituted aromatic hydrocarbons. In this case, the two pathways expand the nutritional versatility of the organism.

As part of an effort to answer these questions, the genes for tautomerase I and II were cloned from *M. petroleiphilum*, the proteins expressed, and the kinetic and structural properties determined. The kinetic analysis shows distinct differences between the enzymes using structurally similar substrates where tautomerase I is a canonical 4-OT and tautomerase II is likely not. The crystal structures show the differences between the active sites although the key players are found in comparable positions. The specificity and function of tautomerase II remains unknown, but these results suggest possibilities, which can be explored in future studies.

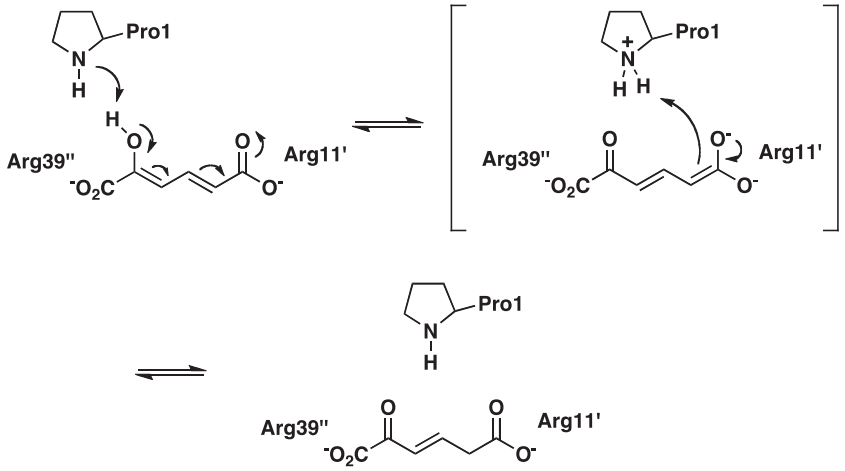
Materials and methods

Materials

Chemicals, biochemicals, buffers and solvents were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Fluka Chemical Corp. (Milwaukee, WI), or EM Science (Cincinnati, OH). Centricon filter devices were purchased from Millipore Co. (Billerica, MA). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Gold BioTechnologies, Inc. (St. Louis, MO). Phenyl Sepharose CL-4B, diethylaminoethyl (DEAE)-Sepharose, and sulfopropyl (SP)-Sepharose Fast Flow resins were purchased from Sigma–Aldrich. Pre-packed PD-10 Sephadex G-25 columns were obtained from GE Healthcare (Buckinghamshire, UK). 2-Hydroxymuconate (**1**) and 5-(methyl)-2-hydroxymuconate (**3**) were prepared as previously described [4,20]. The Freeze 'N Squeeze DNA Gel Extraction Spin Columns were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA).

Bacterial strains and plasmids

One Shot® Mach1™ T1 phage-resistant chemically competent *Escherichia coli* and *E. coli* strain DH5α cells were obtained from Invitrogen (Carlsbad, CA). *E. coli* BL21(DE3) and BL21(DE3) OverExpress cells were obtained from Agilent Technologies (Santa Clara, CA). Plasmids were isolated using the QIAprep Spin Miniprep Kit from Qiagen (Valencia, CA). The genomic DNA from *M. petroleiphilum* strain PM1 was purchased from ATCC #BAA-1232D-5 (American Type Culture Collection, Manassas, VA). The gene sequences for tautomerase I and II were obtained from the NCBI website using the protein accession numbers YP_001021456.1 (GI: 124267452) for tautomerase I and YP_001022511.1 (GI: 124268507) for tautomerase II. The pET vector was obtained from Novagen (Madison, WI). Enzymes and reagents used for molecular biology procedures were obtained from New England Biolabs, Inc. (Ipswich, MA).



Scheme 2. Mechanism for the conversion of 1–2 by 4-OT (where the primes indicate that the residue comes from a different subunit of the hexamer).