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

Cassidy R. Terrell^{a,1}, Elizabeth A. Burks^b, Christian P. Whitman^{b,*}, and David W. Hoffman^{a,*}

Christian P. Whitman: whitman@austin.utexas.edu; David W. Hoffman: dhoffman@c-m.utexas.edu

^aDepartment of Chemistry and Biochemistry, University of Texas, 1 University Station A5300, Austin, TX 78712, USA

^bDivision of Medicinal Chemistry, College of Pharmacy, University of Texas, 1 University Station A1900, Austin, TX 78712, USA

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.

Keywords

Tautomerase; *Methylibium petroleiphilum*; X-ray crystallography; Bioremediation

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

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*Corresponding authors. Fax: +1 512 232 2606 (C.P. Whitman), +1 512 471 8696 (D.W. Hoffman).

¹Present address: Chemistry Department, Regents Hall 362, 1520 St. Olaf Avenue, St. Olaf College, Northfield, MN 55057, USA.

Accession codes

Atomic coordinates and structure factors for tautomerase I and tautomerase II have been deposited with the Brookhaven Protein Data Bank (entries 4FAZ and 4FDX).

²*Abbreviations used:* DEAE, diethylaminoethyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hh4-OT, heterohexameric 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.

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 – – 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 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 DH5⁺ 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).

General methods

Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere [21]. Oligonucleotide primers were synthesized by Sigma. DNA sequencing was performed in the Institute of Cellular and Molecular Biology DNA core facility at the University of Texas at Austin. Mass spectral data was obtained using an LCQ electrospray ion-trap mass spectrometer (Thermo, San Jose, CA) at the Protein and Metabolite Analysis Facility at the University of Texas at Austin. Samples were prepared as described elsewhere [22]. Crystallization and X-ray data collection were carried out in the Macromolecular Crystallography Facility at the University of Texas at Austin. The UV absorbance measurements were carried out using an Agilent 8452A Diode Array spectrophotometer. Non-linear regression analysis was used to fit the data in the GraFit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma–Aldrich. Protein concentrations were determined as indicated in the text.

Cloning of the tautomerase isozymes

Cloning of the tautomerase I—The gene for tautomerase I was amplified by the PCR from the genomic DNA using 5'-TAGTAGTAGCATATGCCGTTTCGCACAG-3' and 5'-GATGATGATCTCGAGTCATTATTAGCGGCCGAG-3' as forward and reverse primers, respectively. The forward primer contains nine nonspecific bases, an *Nde*I restriction site (underlined), and twelve additional bases specific for the gene. The reverse primer contains nine nonspecific bases, an *Xho*I restriction site (underlined), three stop codons, and nine additional bases specific for the gene. Four PCR mixtures were made up with different concentrations of MgSO₄ (2–5 mM). Each mixture (50 µL) contained genomic DNA (0.5 µg), dNTPs (0.2 mM), bovine serum albumin (0.5 mg/mL), primers (0.4 µM each), Vent polymerase (0.5 unit) and 1× buffer made from the 10× buffer supplied with the polymerase. The reaction mixtures were processed in a heated table top PCR thermocycler using a protocol that consisted of an initial 5-min denaturation cycle at 94 °C, followed by 29 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, followed by one cycle at 72 °C for 10 min, ending with a hold at 4 °C. Electrophoresis of the reaction mixtures showed that only the mixture with 2 mM MgSO₄ contained a visible DNA piece of the correct size. The band was excised, processed through a Freeze 'N Squeeze unit, and used as template (2 µL) in four 50 µL PCR reactions, set up as described above with 2 mM MgSO₄. The amplification protocol began with a 2-min denaturation step instead of a 5-min one. The material eluted from the Freeze 'N Squeeze units was pooled, treated with sodium acetate and ethanol and the precipitated DNA recovered by centrifugation. The PCR product was subsequently cloned into a pET24a vector from which the *Bgl*II restriction site had been removed. The gene was inserted between the *Nde*I and *Xho*I restriction sites following procedures described elsewhere [6,7,21]. The ligation product was used to transform *E. coli* DH5⁺ cells by electroporation using a Bio-Rad MicroPulser Electroporation unit. The transformation reaction mixture was plated on LB/Kn (30 µg/mL) plates and grown overnight at 37 °C. Following PCR screening, two positive colonies were used to start individual overnight cultures from which plasmid was isolated and sequenced. One of the two plasmids showed the correct sequence and was subsequently used for protein expression.

Cloning of the tautomerase II—The gene for tautomerase II was amplified by the PCR from the genomic DNA using 5'-TAGTAGTAGCATATGCCCATCATCCAG-3' and 5'-GATGATGATCTCGAGTCATTATTACAGGTCC-3' as forward and reverse primers, respectively. The forward primer contains nine nonspecific bases, an *Nde*I restriction site (underlined), and twelve additional bases specific for the gene. The reverse primer contains nine nonspecific bases, an *Xho*I restriction site (underlined), three stop codons, and seven additional bases specific for the gene. A PCR mixture (50 µL) was set up and processed as described for tautomerase I. The material eluted in the Freeze 'N Squeeze procedure was used as template (1 µL) in four 50 µL PCR reactions, each set up and processed as described above (with an initial 2-min denaturation step). The material eluted from the Freeze 'N Squeeze units was pooled and treated with sodium acetate and ethanol and the precipitated DNA recovered by centrifugation. The PCR product was subsequently cloned into a pET24a vector, as detailed above. Following PCR screening, two positive colonies were used to start individual overnight cultures from which plasmid was isolated and sequenced. One of the two plasmids showed the correct sequence and was subsequently used for protein expression.

Expression and purification of tautomerase I

Single colonies of *E. coli* BL21(DE3) OverExpress cells containing the tautomerase I plasmid were used to inoculate flasks containing 50 mL Luria Broth (LB) and 25 µg/mL kanamycin (Kn). After shaking at 37 °C overnight, the cultures were used to inoculate six 1

1 L flasks of the same LB/Kn medium. The 1 L cultures were grown with shaking at 37 °C. Protein expression was induced using IPTG (100 μ g/mL) when the OD₅₉₀ reached approximately 1.0, and cell growth continued at 37 °C for 6 h. Cells were harvested by centrifugation (10 min at 5000g) at 4 °C, and stored in 0.7 g aliquots at –80 °C. Typically, 12.6 g of cells were harvested from six 1 L cultures [5–7].

In a typical purification procedure, harvested cells (0.7 g) were thawed, suspended in 3 mL of 20 mM HEPES buffer (pH 7.6) containing 50 mM KCl (Buffer A), and frozen at –20 °C. Cell lysis was accomplished by thawing the frozen suspension, and the soluble protein was collected in the supernatant following centrifugation (1 h at 10,000g) [5–7]. The supernatant was applied to a DEAE-Sepharose column (10 mL of resin) equilibrated with Buffer A. After washing with 5 column volumes of Buffer A, the flow-through and wash were combined and applied to an SP-Sepharose column (10 mL of resin) equilibrated with Buffer A. After washing with 5 column volumes of Buffer A, the flow-through and wash were collected and concentrated using Centricon filters. Protein concentration was determined using an extinction coefficient of 12,490 M^{–1} cm^{–1} at 280 nm, obtained from the ExPASy Bioinformatics Resource Portal [23]. This procedure typically yields 30 mg of tautomerase I per 0.7 g of harvested cells, with >95% purity, as assessed by denaturing glycine SDS–PAGE (15% acrylamide) [24]. Mass spectral analysis confirmed that the purified protein had the molecular mass expected for its amino acid sequence, and that the protein had undergone post-translational modification to remove the initiating methionine [25].

Expression and purification of tautomerase II

E. coli BL21(DE3) cells containing the plasmid encoding tautomerase II were grown as described for tautomerase I. After induction with IPTG, each 1 L culture was allowed to grow for an additional 7.5 h at 37 °C. Cells were harvested by centrifugation at 4 °C, and stored at –80 °C in 0.8 g aliquots. Typically, 14.5 g of cells were harvested from six 1 L cultures [5–7].

Tautomerase II was purified by suspending an aliquot of cells (0.8 g cell pellet) in 4 mL Buffer A. Cells were lysed using the freeze–thaw method, and the lysate was cleared by centrifugation (1 h at 10,000g). Nucleic acids were precipitated using 1 mL of a 10% solution of polyethyleneimine, and removed by centrifugation (1 h at 10,000g) [5–7]. Proteins were precipitated by the addition of 2.5 g of (NH₄)₂SO₄ to the supernatant (~5 mL), followed by stirring at 24 °C for 1 h. The precipitated protein was collected by centrifugation (1 h at 10,000g), and subsequently resuspended in 0.8 M (NH₄)₂SO₄ and 20 mM HEPES buffer (pH 7.6) (Buffer B) (20 mL). The homogenous resuspension was then loaded on to a Phenyl-Sepharose column (10 mL of resin) equilibrated with Buffer B. After washing with five column volumes of 20 mM HEPES buffer containing 0.5 M (NH₄)₂SO₄ at pH 7.6 (Buffer C), protein was eluted using a linear gradient [60 mL total, 0.3–0 M (NH₄)₂SO₄ in 20 mM HEPES buffer, pH 7.6]. Fractions containing tautomerase II, as assessed by denaturing glycine SDS–PAGE (15% acrylamide), [24] were collected and concentrated to ~2 mL. This partially purified protein was then exchanged into Buffer A using a PD-10 desalting column. The eluent (~3.5 mL) was applied to a DEAE-Sepharose column (10 mL of resin) equilibrated with Buffer A (20 mL). After washing with 5 column volumes of Buffer A, protein was eluted with a salt gradient (60 mL total, 0.05–1 M KCl, 20 mM HEPES buffer, pH 7.6). Fractions were analyzed by SDS–PAGE and those containing tautomerase II were collected, concentrated and exchanged into Buffer A using a PD-10 desalting column. Since tautomerase II does not absorb strongly at 280 nm, the final protein concentration was determined using the Waddell method [26]. This procedure typically yields 3–8 mg of tautomerase II per 0.8 g of harvested cell pellet with >95% purity, as assessed by denaturing glycine SDS–PAGE (15% acrylamide). Mass spectral analysis

confirmed that the molecular mass is consistent with the amino acid sequence and that the initiating *N*-formyl methionine had been removed [25].

Kinetic studies of tautomerase I and tautomerase II

The kinetic parameters for tautomerase I and tautomerase II using 2-hydroxymuconate (**1**) and 5-(methyl)-2-hydroxymuconate (**3**) were determined as follows and are reported in Tables 1 and 2, respectively. Stock solutions of each substrate (10 mM) were prepared in 100% ethanol, and kept on ice. The linear absorbance range at 236 nm was determined for **1** (10–160 μ M) and **3** (40–360 μ M) [4]. The molar absorptivity of **4** was adjusted to account for the equilibrium composition (43% **3**, 24% **4**, and 33% 5-methyl-2-oxo-4-hexenedioate) as previously described [6,7]. All kinetic assays were carried out in 10 mM potassium phosphate buffer (pH 7.3) at 24 °C.

The initial rate of tautomerization from **1** to **3** was measured by following the increase in absorbance at 236 nm using 1.61 nM tautomerase I. Fresh dilutions of 16.1 nM tautomerase I were made from a stock (1.47 μ M) equilibrated for 1 h in 10 mM potassium phosphate buffer, pH 7.3 (50 mL). The tautomerization of **3** to **4** was measured by following the increase in absorbance at 236 nm using 16.1 nM tautomerase I. For these assays, a 1.61 μ M stock solution of tautomerase I in 10 mM potassium phosphate buffer, pH 7.3, was equilibrated for 30 min prior to making the final dilution to a 16.1 nM solution of tautomerase I (10 mM potassium phosphate, pH 7.3, 30 mL). The final diluted enzyme was allowed to equilibrate for 6 min prior to the assay.

Fresh dilutions of 162 nM tautomerase II (10 mM potassium phosphate buffer, pH 7.3, 50 mL) were made from a 6.0 μ M stock and allowed to equilibrate for less than 5 min prior to each assay with **1**. For the tautomerization of **3** to **4**, a 4.43 μ M stock solution of tautomerase II equilibrated for 30 min prior to the final 111 nM dilution in 10 mM potassium phosphate buffer, pH 7.3 (30 mL). The diluted enzyme solution was allowed to equilibrate for 5 min prior to the assay. For all assays, the substrate was added to aliquots (1 mL) drawn from the solutions of tautomerase I, tautomerase II or buffer without enzyme. The non-enzymatic rates for the conversion of **1** to **2** and **3** to **4** were insignificant. All assays were repeated at least three times.

Crystallization and structure determination of tautomerase I

After optimizing a set of initial crystallization conditions, identified using an Art Robbins Instruments Phoenix liquid-handling robot, the crystallization condition yielding the highest resolution diffraction data was 200 mM tri-ammonium citrate and 10% polyethylene glycol (PEG) 3350. Crystals appeared within 1–3 days. Crystals of tautomerase I used for X-ray diffraction data collection were flash-frozen in liquid nitrogen, and subsequently centered in a stream of dry nitrogen at ~100 K. For crystals yielding the highest resolution images 87 degrees of data were collected, using a 0.5 oscillation angle, resulting in the collection of 174 images each with an exposure time of 120 s. Diffraction data were recorded using an R-Axis IV++ image plate detector (Rigaku), with X-rays generated from a Rigaku MicroMax007 rotating anode generator. Data were processed and reduced using HKL2000 [27]. The phase problem was solved using the PHASER molecular replacement program, [28] with 4-OT from *P. putida* mt-2 (PDB entry 1BJP) as the search model [29]. Model building was performed using Coot, [30] and simulated annealing with REFMAC5 [31] within the CCP4i Program Suite [32]. Model bias was removed by creating a series of omit maps, each with three consecutive residues removed from the structure; corrections to the structure were made using Coot [30]. The final protein structure was evaluated using PROCHECK [33]. Data collection and refinement statistics are summarized in Table 3.

Crystallization and structure determination of tautomerase II

A purified aliquot of tautomerase II (12 mg/mL in Buffer A) was screened using the same method and high-throughput screens described for tautomerase I. The crystallization condition yielding the highest resolution diffraction data was 4.3 M sodium formate, where crystals appeared within 10–14 days. Crystals were flash-frozen in liquid nitrogen, and 180 degrees of data were collected and processed at 1.64 Å resolution, as described for tautomerase I. The phase problem was solved using 4-OT from *Pseudomonas* sp. CF600 (PDB entry 1OTF) as the search model [34]. Model building and simulated annealing were carried out as described for tautomerase I. Model bias was removed using a series of omit maps, each made with five consecutive residues removed from the structure. The final protein structure model was assessed using PROCHECK [33]. Data collection and refinement statistics are listed in Table 3.

Results

Sequence alignment

The sequences of the tautomerase I and II are shown in Fig. 1 along with those of the canonical 4-OT from *P. putida* mt-2 [6,7], TomN from *Streptomyces achromogenes*, [7] and the α - and β -subunits of the hh4-OT from *C. aurantiacus* J-10-fl [6]. The sequences, with the exception of the β -subunit from hh4-OT, show identities ranging from 32% to 42% and similarities ranging from 54% to 70%. The BLASTP suite was used to determine these ranges, with both the tautomerase and tautomerase II sequences used as references, separately [35]. Neither tautomerase I nor tautomerase II had significant similarity to the β -subunit sequence of hh4-OT. The alignment shows the conservation of Pro1, Arg11 (Arg12 in the β -subunit of hh4-OT), Arg39, (Arg40 in the β -subunit of hh4-OT) and Trp or Phe50. The alignment shows the sequences giving rise to the secondary elements of the α - β structural unit.

Kinetic characterization of tautomerase I and tautomerase II

The kinetic parameters for tautomerase I and tautomerase II were determined with **1** and **3** (Tables 1 and 2). For comparison, the previously measured kinetic parameters of the canonical 4-OT, the hh4-OT, TomN, and YwhB, a 4-OT homolog from *Bacillus subtilis*, are included [5–7,36]. Both tautomerase I and II catalyze the tautomerization of **1** to **3** (Table 1). The k_{cat} and k_{cat}/K_m values (4600 s^{-1} and $3.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively) for tautomerase I are comparable with those measured for 4-OT and the hh4-OT (but not TomN) [6,7]. The K_m value for tautomerase I is ~ 2 fold higher than the K_m for the two isozymes, but much lower than that of TomN [7]. The k_{cat} and k_{cat}/K_m values for tautomerase II using **1** differ from those of tautomerase I, 4-OT, and the hh4-OT. Although the K_m for tautomerase II is similar to the K_m values for these isozymes, tautomerase II has a k_{cat} that is at least 160-fold lower than that of 4-OT and the hh4-OT (and a 100-fold lower than that of TomN). The reduced k_{cat} value causes a 210-fold decrease in the k_{cat}/K_m value (compared to 4-OT). The activity of tautomerase II with **1** is similar to that of YwhB. A comparison of catalytic efficiencies of these enzymes reveals a 13-fold difference (Table 1). Although tautomerase II has a 16-fold lower K_m than YwhB, the k_{cat} value is comparable to that of YwhB, which does not have Arg39 [36].

Using **3**, tautomerase I and the canonical 4-OT have similar kinetic parameters (Table 2) [6,7]. In contrast, the k_{cat} and k_{cat}/K_m values of tautomerase II using **3** are lower than those of tautomerase I (~ 31 - and 62.5 -fold, respectively) and 4-OT (~ 33 - and 55 -fold, respectively). The kinetic parameters are comparable to those of TomN [6,7]. In summary, the kinetic analysis shows that tautomerase I is comparable to the canonical 4-OT and hh4-OT using **1** and **3**, and is likely a 4-OT. Tautomerase II processes **1** and **3**, but with reduced

efficiencies, primarily due to the lower turnover number. A decrease in k_{cat} can result from an effect on the reaction chemistry, product release or both the reaction chemistry and product release. One possibility is that these substrates (i.e., **1** and **3**) are not optimally aligned in the active site of tautomerase II for efficient proton transfer.

Crystal structure of tautomerase I

Tautomerase I from *M. petroleiphilum* formed high quality crystals that diffracted to high (1.57 Å) resolution (Table 3). The asymmetric unit of the crystal contains three independent protein molecules (chains A–C), with the remaining three chains (D–F) of each hexameric enzyme being generated by the crystallographic 2-fold symmetry. The full orthorhombic unit cell contains two complete hexamers. The electron density map was of sufficient quality to trace the entire peptide backbone of the three crystallographically independent chains. Clear electron density was observed for all side chains, with the exception of Arg62 (Chain A), Lys35 (Chain C) and Glu36 (Chain C) and the backbone carbonyl of Gly61 (Chains A and C). Two residues (Gln25 and Arg39) had relatively large B-factors, and lacked electron density for the functional groups at the ends of their side chains. Although the three monomers within each asymmetric unit are crystallographically unique, they are very similar in structure. The three chains were superimposed with an average rmsd value of only 0.29 Å, using the Secondary Structure Matching (SSM) [37] function in Coot [30].

Tautomerase I exhibits the canonical elements of a typical tautomerase superfamily member [11,29,38]. The monomeric unit is comprised of the classic $\alpha\beta\alpha$ fold (Fig. 2) [39]. Beginning with Pro1, the first α -strand (1) is linked to helix 1 via a short loop. The proceeding 3_{10} helical turn leads into strand, through another short loop, which is directly connected to the second 3_{10} helical turn. This is followed by a short strand (3), a loop region, and the final secondary structural element, helix 2. The dimeric interface consists of a hydrogen bond network, where the two stranded parallel β -sheets of each monomer align anti-parallel to one another, and create a four-stranded β -sheet. Additionally, the 1 helix from each monomer aligns in an anti-parallel manner through hydrophobic contacts on one face of the four-stranded β -sheet. The quaternary structure, a trimer of dimers (Fig. 2), is driven by the strongly hydrophobic nature of the β -strands, creating a protein core of β -sheet structure. The six 1 helices surround the hydrophobic core and complete the classic $\alpha\beta\alpha$ barrel motif. The shorter secondary structural elements make important stabilizing quaternary contacts between the dimers. The 3_{10} turn forms a hydrogen bond with the amide hydrogen of Gly53 from the adjacent dimer. Also, the backbone amide hydrogen and carbonyl oxygen of Gly54, in the loop region of one dimer, form hydrogen bonds with residues in the 1 helix of the adjacent monomer. Lastly, 3 forms hydrogen bonds anti-parallel to the four-stranded β -sheet formed by the adjacent dimer and creates a five-stranded β -sheet. The hydrophobic core, coupled with the stabilizing hydrogen bonds, drives formation of the quaternary structure, and thus the construction of the six enzyme active sites.

Active site of tautomerase I

Pro1 is situated at the center of each active site, flanked by the two electropositive amino acids (Arg11' and Arg39' where the primes indicate that these residues are contributed by different chains within the hexameric enzyme), and oriented across from a key hydrophobic residue Trp50 (corresponding to Phe50 in 4-OT from *P. putida*) (Fig. 2). Clear electron density was observed for the active sites of tautomerase I (Fig. 3). Based on crystallographic results, the six active sites within each tautomerase I hexamer can be grouped into three types, depending on the orientation of specific chains and the presence of non-protein molecules. In the first type of active site, a sulfate ion is bound less than 4 Å from the Pro1 nitrogen and the side chains of Arg11' and Trp50 (Fig. 3). This active site also contains a

bound water molecule within hydrogen bonding distance of the Pro1 nitrogen. The second type of active site lacks the sulfate ion, but contains water molecules coordinated through hydrogen bonding with side chains of Asn37 and Arg39. In the third type of active site, the protein backbone atoms of Pro1 and Asn37 are within hydrogen bonding distance to a water molecule. Further inspection of the different active sites identified two differences: when the sulfate ion is present, the side chain of Arg62 is solvent exposed, and the C-terminal carboxylate group is oriented toward the enzyme active site. In active sites that lack the sulfate ion, the side chain of Arg62 is directed toward the active site center, stabilized by hydrogen bonding, and the phenyl portion of the indole side chain of Trp50 is rotated in toward Pro1.

Crystal structure of tautomerase II

Crystals of tautomerase II were grown in 4.3 M sodium formate, and diffracted to a resolution of 1.64 Å. The asymmetric unit of the crystal contains two independent monomers (chains A and B), with the complete hexameric enzyme being generated by the crystallographic symmetry (Fig. 4). The hexagonal unit cell contained a total of 24 protein monomers. Clearly interpretable electron density was observed for the enzyme active sites (Fig. 5), as well as all protein backbone and side chain atoms up to residue Gln61 of chain A, where electron density was lacking beyond the C of the Gln61 side chain. Chain B, on the other hand, showed continuous electron density through the amide nitrogen of Ala64. Chains A and B superimpose with an rmsd of 0.29 Å, using SSM within Coot [30].

The secondary, tertiary and quaternary structural elements confirm that tautomerase II is a member of the 4-OT family. Each monomer exhibits the familiar $\alpha\beta$ fold, with Pro1 at the start of α_1 , the first α -strand (Fig. 4). A small loop segment leads into the major helix α_1 , which connects to strand α_2 through a short loop and a 3_{10} helical turn. A short α -strand (α_3), which is followed by another loop, leads into the final helix α_2 . The overall hexameric structure of tautomerase II conforms to the canonical trimer of dimers with a classic $\alpha\beta$ -barrel motif, as described for tautomerase I.

Active site of tautomerase II

The six active sites of tautomerase II have the catalytic base (Pro1) and substrate binding residues Arg11 and Arg39 found in 4-OT family members. The main catalytic residue, Pro1, resides in the core of the active site, and the two key arginine residues, Arg11 and Arg39, flank Pro1 (Figs. 5 and 6). Three separate monomers are responsible for bringing these three active site residues together: Pro1 (chain A), Arg11 (chain B) and Arg39 (chain D) constitute one of the six active sites. The Pro1 nitrogen is approximately 6.7 Å from the carbon in the side chain of Phe50, one residue responsible for the low pK_a (~6.4) of the prolyl nitrogen of 4-OT from *P. putida* [13]. The most significant difference between the six active sites can be found in the electron density near Pro1. The active site containing Pro1 from Chain A coordinates only one water molecule, while Pro1 in chain B has four water molecules within the core of the active site, causing the side chain guanidinium groups of Arg11 and Arg60 to angle toward Pro1.

Discussion

Structural comparison of tautomerase I and tautomerase II

The high quality crystal structures of tautomerase I and II, at 1.57 Å and 1.64 Å resolution, respectively, offer a detailed comparison of these isozymes. Although these enzymes are present in the same organism, they share only 34.5% sequence identity, but their structures can be superimposed with a strikingly small rmsd of 0.78 Å for their C α atoms. As such, it isn't surprising that the secondary, tertiary and quaternary structural elements are quite

similar. The most significant difference is the absence of a second 3_{10} helical turn in tautomerase II, which instead has a region that lacks regular secondary structure. An examination of the active sites reveals other potentially significant differences between the two tautomerase. First, inspection of the known catalytic and substrate binding residues shows that the locations of Pro1, Arg11, Arg39, and Phe/Trp50 are similar in each isozyme, but the orientations of some side chains differ (Fig. 6). For example, the guanidinium groups of the arginines are tilted slightly in different directions, likely due to coordination with different active site water molecules. Trp50 and Phe50, which are primarily responsible for placing the hydrophobic environment near Pro1 in tautomerase I and II, respectively, are in the same general positions in each tautomerase (Fig. 6), and when a sulfate ion occupies the active site of tautomerase I, the side chain of Trp50 aligns well with that of Phe50 (in tautomerase II). However, in the tautomerase I active sites where a water molecule takes the place of the sulfate ion, the ring of the tryptophan is rotated toward the core of the active site and toward the N-terminal proline.

Tautomerase I and II differ significantly in the shape of the hydrophobic surface that surrounds their respective active sites (Fig. 7). Tautomerase I contains an extensive network of aromatic amino acids (Phe2, Tyr6, Trp41 and Trp50) that crowds the core of the active site. Of these residues, only one (Phe50) is aromatic in tautomerase II; the other three corresponding residues (Ile2, Asn6 and Leu41) are smaller in tautomerase II, with Asn6 providing a significantly less hydrophobic active site environment (Fig. 7).

Tautomerase I and II also exhibit significant differences in the shape and sign of the electrostatic surfaces near their respective active sites (Fig. 8). For example, in tautomerase II, Arg34 and Gln37 provide hydrogen bonding opportunities that differ significantly from those present in tautomerase I, where the corresponding residues are Pro33 and Asn36. In addition, residue 60 is a non-polar leucine in tautomerase I, where tautomerase II has a positively charged residue (Arg60) at this position. Arg60 of tautomerase II is a feature reminiscent of the *P. putida* 4-OT, which also has an arginine at the corresponding position near the active site. Residue 62 of tautomerase I contains a positive charge (Arg62) adjacent to its active site, whereas tautomerase II has an alanine at this position. Another electrostatic difference between the tautomerase occurs at residue 32, which is an aspartate in tautomerase II, and a glycine in tautomerase I, and located in a loop connecting α_1 with the 3_{10} helix. Finally, Lys47 in tautomerase I corresponds to a non-polar valine in tautomerase II. Lys47 is ~ 13 Å from Pro1 and is located on a loop connecting α_2 to α_3 . In summary, the structural results show that although the backbones of tautomerase I and tautomerase II are highly similar, the differences present in their respective amino acid side chains might provide each tautomerase with a distinct active site environment that could confer distinct substrate preferences.

Comparison of tautomerase I and II with other 4-OTs

With the inclusion of the results reported here, kinetic and structural studies have been carried out on five 4-OTs from a diverse set of organisms with different biological niches. (A 6th 4-OT from *Pseudomonas* sp. CF600 shares 77% similarity with the *P. putida* mt-2 4-OT [34].) Previous studies describe the 4-OT from *P. putida* mt-2 (PDB ID: 1BJP) [8,29], the hh-4OT (PDB ID: 3MB2) [6], and the homolog TomN (PDB ID: 3RY0) [7]. Taken together, these studies provide insight into the range of characteristics found in the 4-OT family. Although these five enzymes vary significantly in primary sequence (Fig. 1), an alignment of their secondary, tertiary and quaternary structures shows a remarkable degree of conservation. An alignment of the backbone atoms of chain A from all five enzymes shows an average of under 1 Å rmsd in position. Consequently, the α -barrel motif is present in all structures, formed by a hexamer comprised of a trimer of dimers, with six

active sites. The positions of the key catalytic and substrate binding residues Pro1, Arg11, Arg39 and Phe/Trp50 align well through all five structures.

While the alignment of the key active site residues highlights their importance, the five structurally characterized tautomerase contain a number of subtle differences in their active site environments. In all of the structures, a leucine, isoleucine, or phenylalanine directly follows Pro1 in the primary sequence. The side chains of these residues form a backdrop for the active site that is located distal to the prolyl-1 nitrogen (where each hexameric enzyme contains six active sites). Additionally, residues 31, 33 and 38 are all conserved hydrophobic residues within 4 Å of Pro1. Residue 45 is also a contributor to the active site environment and is particularly interesting when comparing enzymes with a tryptophan as opposed to a phenylalanine at position 50. In the enzymes with Trp50 (TomN, tautomerase I and hh4-OT), the bulk of the tryptophan side chain is accommodated by smaller side chain from Val45 (tautomerase I and hh4-OT) or Thr45 (TomN). In tautomerase I and 4-OT, which both have Phe50, the larger side chains of Leu45 or Met45, respectively, fill the additional space. Additionally, residue 37, which is situated across part of the active site opening and within 4 Å of Pro1, is only conserved with respect to polarity. In 4-OT, this residue is serine, in tautomerase I it is asparagine, while tautomerase II and the hh4-OT have a glutamine at position 37. In the tautomerase II structure, Gln37 protrudes into the active site significantly more so than the corresponding residue in the 4-OTs of the other species. 4-OT, tautomerase I, tautomerase II, and TomN each have uniquely positioned arginines on their surface. In tautomerase II, Arg34, which corresponds to a proline in all other structures, protrudes across the opening of the active site. While the other structurally characterized 4-OT homologs have an arginine as the C-terminal residue, tautomerase II has an alanine at this position. Residues Arg61 (in 4-OT) and Arg62 (tautomerase I), whose side chains are similarly positioned, appear to hinder entrance to the active site. In TomN, Arg61 is in a unique orientation, and also occludes part of active site. hh4-OT differs in that no unique arginine or similarly positioned amino acid appears to partially obstruct the active site.

M. petroleiphilum is unusual in that it has two 4-oxalocrotonate tautomerase, encoded in two distinct operons (*dmpI* and *dmpII*, where dmp stands for dimethylphenol) that provide the enzymes for a pair of *meta*-fission pathways [18,19]. Presumably, the presence of these parallel catabolic pathways imparts an ability to use a wider variety of aromatic molecules as carbon and energy sources. Multiple pathways in the same organism for the degradation of aromatic compounds have previously been reported [41]. These reports examined the upper pathway enzymes (conversion of the aromatic compounds to catechols), but not the lower pathway enzymes (conversion of catechols to Krebs cycle intermediates). Multiple pathways in these organisms augment their nutritional capabilities and enable them to use a wider variety of aromatic compounds including benzene, toluene, ethylbenzene, halogen-substituted species, [42] or naphthalene as growth substrates. Tautomerase I could process the canonical 4-OT substrates, whereas Tautomerase II could process 3- or 4-substituted 2-hydroxymuconates (derived from the growth substrates), but not the 5-substituted ones. The current results provide a foundation for an in-depth exploration of this possibility.

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Fig. 1.

Amino acid sequence alignment of known 4-OTs. Sequences are for tautomerase I and II from *M. petroleiphilum*, 4-OT from *P. putida*, TomN from *S. achromogenes*, and *C. aurantiacus* beta subunit* and alpha subunit**. Secondary structure elements present in most 4-OT family members are indicated. Amino acids located in the enzyme active site are in bold. Sequences were aligned using BLAST [40].

Fig. 2. Structural overview of tautomerase I from *M. petroleiphilum*. Shown are a ribbon diagram of the monomer unit, dimer, and two views of the hexameric structure of the enzyme. The active site Pro 1 is shown in blue. Ribbon diagrams were prepared using PyMoL [39].

Fig. 3.
 (A) Electron density map (2Fo-Fc) the active site region of tautomerase I from *M. petroleiphilum*, contoured at a sigma level of 1.0. (B) A second view of the same map, centered on the water molecule and sulfate ion in the enzyme active site. The figure was prepared using PyMoL [39].

Fig. 4. Structural overview of tautomerase II from *M. petroleiphilum*. The monomer unit, dimer, and two views of the hexameric structure are shown. The active site Pro 1 is shown in pink. Ribbon diagrams were prepared using PyMol [39] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 5. (A) Electron density map (2Fo-Fc) showing polar groups in the vicinity of the active site of tautomerase II from *M. petroleiphilum*, contoured at a sigma level of 1.0. (B) A second view of the same map, showing water molecules near the enzyme active site's canonical catalytic and substrate binding residues. The figure was prepared using PyMoL [39].

Fig. 6. Canonical active site residue alignments of tautomerase I (pink) and tautomerase II (blue) from *M. petroleiphilum*. The figure was prepared using PyMoL [39] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 7. Superposition of residues near an active site of tautomerase I (pink) and tautomerase II (blue) from *M. petroleiphilum*, showing the significant differences in hydrophobicity in each active site environment. The figure was prepared using PyMoL [39] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 8. (A) Stereoview of the electrostatic surface at the active site tautomerase I and (B) tautomerase II from *M. petroleiphilum*. The figure was prepared using PyMoL [39].

Scheme 1.
The substrates for tautomerase I and II.

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).

Table 1Kinetic parameters of 4-OTs using 1.^a

Enzyme	k_{cat} (s ⁻¹)	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
Tautomerase I	4600 ± 200	140 ± 10	3.3×10^7
Tautomerase II	18.5 ± 0.7	60 ± 6	3.1×10^5
4-OT ^b	4000 ± 182	62 ± 8	6.5×10^7
hh4-OT ^c	3000 ± 100	70 ± 8	4.3×10^7
TomN ^b	1850 ± 630	512 ± 225	3.6×10^6
YwhB ^d	26.0 ± 1.4	940 ± 70	2.8×10^4

^a Steady-state kinetic parameters were obtained in 10 mM potassium phosphate buffer, pH 7.3, at 24 °C, as described in the text. Errors are standard deviations from a minimum of three assays.

^b Kinetic parameters for 4-OT from *P. putida* and TomN are from Ref. [7].

^c Kinetic parameters for the hh4-OT are from Ref. [6].

^d Kinetic parameters for YwhB are from Ref. [36].

Table 2Kinetic parameters of 4-OTs using 3.^a

Enzyme	k_{cat} (s ⁻¹)	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
Tautomerase I	107 ± 3	105 ± 8	1.0 × 10 ⁶
Tautomerase II	3.4 ± 0.1	211 ± 15	1.6 × 10 ⁴
4-OT ^b	111 ± 6	125 ± 17	8.9 × 10 ⁵
TomN ^b	8.8 ± 0.7	306 ± 45	2.9 × 10 ⁴

^aSteady-state kinetic parameters were obtained in 10 mM potassium phosphate buffer, pH 7.3, at 24 °C, as discussed in the text. Errors are standard deviations from a minimum of three assays.

^bKinetic parameters for 4-OT from *P. putida* and TomN are from Ref. [7].

Table 3Summary of crystallographic and structural statistics for tautomerase I and tautomerase II.^a

Statistic	Tautomerase I	Tautomerase II
PDB ID	4FAZ	4FDX
Space group	C222 ₁	P6 ₃ 22
Unit cell dimensions (Å)	<i>a</i> = 60.17 <i>b</i> = 76.17 <i>c</i> = 69.88	<i>a</i> = 50.24 <i>b</i> = 50.24 <i>c</i> = 158.1
Protein molecules per asymmetric unit	3	2
Matthews coefficient (Å ³ /Dalton)	1.96	1.83
Maximum resolution	1.57 Å	1.64 Å
Data collection wavelength	1.54 Å	1.54 Å
Total reflections	163,467	286,243
Unique reflections	24,295	15,338
<i>R</i> _{sym} (%)	4.5 (45.9)	7.5 (44.5)
Completeness (%)	98.5	99.8
<i>I</i> / σ	15.9 (4.4)	36.7 (11.4)
Resolution range for refinement (Å)	47.2–1.57	158.1–1.64
<i>R</i> _{work} (%)	22.8	22.6
<i>R</i> _{free} (%)	27.4	26.3
Protein atoms	1523	998
Water molecules	82	37
Sulfate ions	1	0
Average B-factors (Å ²)		
Protein	26.9	23.1
Solvent	27.1	26.6
Rmsd from ideal geometry, covalent bonds	0.021 Å	0.025 Å
Rmsd from ideal geometry, covalent angles	2.16°	2.32°
Ramachandran plot		
Residues in favored regions	99.4%	100.0%
Residues in allowed regions	0.6%	0.0%
Residues in generously allowed regions	0.0%	0.0%
Residues in disallowed regions	0.0%	0.0%

^aValues in parentheses refer to the highest (10%) resolution shell of the X-ray data.