Supporting Information for Kinetic and Structural Characterization of a Heterohexamer 4-Oxalocrotonate Tautomerase from *Chloroflexus aurantiacus* J-10-fl: Implications for Functional and Structural Diversity in the Tautomerase Superfamily[†]

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The expression, overproduction, and purification protocols for the heterohexamer 4-oxalocrotonate tautomerase (hh4-OT) are provided in the Supporting Information. The experimental procedures used for the construction, expression, overproduction, purification, and mass spectral analysis of the hh4-OT mutants and the construction and expression of the separate subunits of the hh4-OT are also provided in the Supporting Information. Finally, the molecular modeling studies are described.

Strains. *Escherichia coli* strain DH5 α (Invitrogen Corp. Carlsbad, CA) was used for the propagation of ligated plasmids. *E. coli* strain BL21-Gold(DE3) (Stratagene, La Jolla, CA) was used for the overproduction of the recombinant proteins. Cells for general cloning and overproduction were grown in Luria-Bertani (LB) media supplemented with ampicillin (Ap, 100 μ g/mL) or kanamycin (Kn, 30 μ g/mL), as noted. The composition of LB medium is described elsewhere (1).

General Methods. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations are based on methods described elsewhere (1). The sources

for enzymes, reagents, and kits used in these procedures are reported elsewhere (2,3). DNA sequencing was performed by the DNA Core Facility in the Institute for Cellular and Molecular Biology at the University of Texas at Austin. Mass spectral data were obtained on an LCQ electrospray ion-trap mass spectrometer (Thermo, San Jose, CA) in the Analytical Instrumentation Facility Core in the College of Pharmacy at the University of Texas at Austin. Samples were prepared as described elsewhere (2).

Construction of a pET24a Vector Containing the α -Subunit. The dicistronic clone of the hh4-OT in pET24a was treated simultaneously with the restriction enzymes *EcoRI* and *XhoI* to remove the β -subunit. The larger piece, which contained the α -subunit, the necessary genetic information for kanamycin resistance, plasmid replication and expression of the α -subunit with the T7 system, was isolated and purified. The ragged ends of the isolated DNA piece were filled in and phosphorylated using standard procedures (1), and then treated with T4 DNA ligase for 3 h. The ligation products were recovered by ethanol precipitation and used to transform *E. coli* DH5 α cells by electroporation. Ten colonies were screened by the PCR for an insert of the correct size. All ten colonies were positive for such an insert. Plasmid DNA was isolated from two of the colonies and sequenced. Both were correct. The plasmid was subsequently used to chemically transform *E. coli* BL21-Gold(DE3) cells.

Construction of a pET24a Vector Containing the β -Subunit. The β -subunit was amplified from the genomic PCR product using primers that provided the necessary sequence for cloning into pET21a (not used in this study) or pET24a vectors and for adding the α -subunit to make a dicistronic construct. Accordingly, the sequence of the forward primer is 5'-TAGTAGTAGGAATTCAAGAAGGAGATATAC**ATAT**GCCGATGC-3', which has (in order) nine nonspecific bases, an *EcoRI* restriction site (underlined), a ribosome binding site, an *NdeI* restriction site (in bold, for the initiating methionine codon), and seven additional gene specific bases. The sequence of the reverse primer is 5'-GATGATGATCTCGAG**GGATC**CTCATTATTACTGCTGGTCTGGC-3',

which has (in order) nine nonspecific bases, an *XhoI* restriction site (underlined), a *BamHI* restriction site (in bold), three stop codons (in the 5' to 3' direction TAATAATGA), and ten gene specific bases. The primer design enables the cloning of a single gene into one of the pET vectors using the *NdeI* and *BamHI* restriction sites and then, if desired, the cloning of a second gene between the *EcoRI* and *XhoI* restriction sites. The PCR reaction mixture (100 μ L) reaction contained gel extract from the genomic PCR (1 μ L), primers (0.2 μ M each), dNTPs (0.2 mM each), Vent polymerase (1.0 unit), and the accompanying 10X buffer (10 μ L). Two reactions were run using an amplification protocol that consisted of an initial 2-min denaturation cycle at 94° C, followed by 25 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min, and ended with a 10-min elongation cycle at 72 °C, followed by a hold at 4 °C. The PCR reactions were pooled and the correct size piece was isolated from a gel. The product and the two vectors were treated with *NdeI* and *BamHI* restriction enzymes, gel-purified, and ligated (in separate tubes) using T4 DNA ligase. An aliquot of each ligation mixture was used to transform *E. coli* DH5 α cells by electroporation. Transformants were selected at 37 °C on LB/Kn (30 μ g/mL) plates (pET24a construct). For each clone, five colonies were screened for insert by the PCR. One positive colony from each screen was used to start an overnight culture from which plasmid was isolated and sequenced to confirm the correct coding region.

Construction of the hh4-OT Mutants. The β P1A-mutant was constructed by the PCR using the forward primer 5'-TAGTAGTAG**GAATT**CAAGAAGGAGATATACATATGGCGATGC-3' where the *EcoRI* restriction site is shown in bold and the mutated codon is underlined. The forward primer contains nine nonspecific bases, the *EcoRI* restriction site, and 27 additional bases that match the 5'-end of the β -subunit in the pET construct (except for the mutation). The primer was used in combination with the reverse primer (5'-GATGATGATCTGGAGGGAT**CCT**CATTATTACTGCTGGTCTGGC-3'), which has nine nonspecific bases, a *BamHI* restriction site (in bold), and 22 additional bases that match the 3'-end of the β -subunit in the pET construct. The PCR mixture (50 μ L) contained the pET24a vector

with the β -subunit (0.25 μ g), bovine serum albumin (0.5 mM), primers (0.2 μ M each), dNTPs (0.2 mM each), Vent polymerase (0.5 unit), and the accompanying 10X buffer (diluted to 1X). The amplification protocol consisted of an initial 2-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, and ended with a 10-min elongation cycle at 72 °C, followed by a hold at 4 °C. The gel-purified product was used as the template in five subsequent PCR mixtures (100 μ L each) containing the gel extract (1 μ L), primers (0.2 μ M each), dNTPs (0.2 mM each), Taq polymerase (3 units), and the accompanying 10X buffer (diluted to 1X). The products were pooled, purified, and treated with *EcoRI* and *BamHI* restriction enzymes. The pBluescript construct containing the α -subunit was treated similarly and the gel-purified products were ligated using T4 DNA ligase. An aliquot of the ligation mixture was used to transform *E. coli* DH5 α cells by electroporation. Transformants were selected at 37 °C on LB/Ap (100 μ g/mL) plates. Five colonies were screened for insert by the PCR, following the procedure described in the Novagen pET System Manual, using the M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') and the T3 promoter primer (5'-AATTAACCCTCACTAAAGGG-3'). One positive colony from each screen was used to start an overnight culture from which plasmid was isolated and sequenced to confirm the correct coding region.

The remaining four mutants were constructed by overlap extension PCR, as follows and described elsewhere (4). The M13 forward and T3 promoter primers (sequences above) were used as the external primers. For the α R12A mutant, the internal primers were 5'-CTTTTGCTCTGTGCTCGCCCCCTCCAG-3' and 5'-CTGGAGGGGGGCGAGCACAGAGCAAAAG-3' (5'-reverse and 3'-forward, respectively). For the α R40A mutant, the internal primers were 5'-GAATGATCAACGCCACCTCTGCC-3' and 5'-GGCAGAGGTGGGCGTTGATCATTC-3' (5'-reverse and 3'-forward, respectively). For the β R11A mutant, the internal primers were 5'-GTACGGTCTGGCGGGCGATCCCCGC-3' and 5'-GCGGGGATGGCGCCGCCAGACCGTAC-3' (5'-reverse and 3'-forward, respectively). For the β R39A mutant, the internal primers were 5'-

CAATTGCAGCGCGCCCCGGTGG-3' and 5'-CCACCGGGCGCGCTGCAATTG-3' (5'-reverse and 3'-forward, respectively). The mutation is underlined in the primer sequences. Typically, the PCR mixtures (total volume 50-100 μ L) consisted of template DNA (300-400 ng plasmid for generating overlap pieces and 2-8 mmol of gel extract containing overlap pieces for overlap PCR), the primers (0.2 μ M), dNTPs (0.2 mM), Vent (0.5 units for generating overlap pieces) or Taq (~4 units for the PCR overlap) polymerase, and the accompanying 10X buffer (diluted to 1X). BSA (0.5 mM) was added if the Vent polymerase was used. For the α R12A and α R40A 5'-pieces, the pBluescript with the α -subunit gene served as the template DNA. For the α R12A and α R40A 3'-pieces, the pBluescript with the dicistronic gene served as the template DNA. For both pieces of the β R11A and β R39A mutants, the pBluescript with the dicistronic gene served as the template DNA. A typical PCR amplification protocol consisted of an initial 2-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, and ended with an elongation cycle at 72 °C for 10 min, followed by a hold at 4 °C. The gel-purified PCR products and the pBluescript vector were treated with *XhoI* and *XbaI* restriction enzymes, purified, and ligated using T4 DNA ligase. An aliquot of each ligation mixture was used to transform *E. coli* DH5 α cells by electroporation. Transformants were selected at 37 °C on LB/Ap (100 μ g/mL) plates. Five colonies were screened for insert by the PCR, following the procedure described in the Novagen pET System Manual using the M13 forward and the T3 promoter primers. One positive colony from each screen was used to start an overnight culture from which plasmid was isolated and sequenced to confirm the correct coding region.

Expression and Purification of hh4-OT and Mutants. One or several colonies (a streak) of the dicistronic pBluescript construct (encoding hh4-OT and the five mutant enzymes) transformed in *E. coli* BL21-Gold(DE3) cells were used to inoculate 10 mL of LB/Ap media (100 μ g/mL) (5). After shaking for ~7 h at 37 °C, an aliquot (4 mL) was used to inoculate a 400-mL LB/Ap culture (100 μ g/mL) in a 2 L flask. The cells were allowed to grow overnight (~16 h) with shaking at 37 °C, collected by

centrifugation (15 min at 11,000g) at 4 °C, and stored at -80 °C. Typically, a 400-mL culture yields 1.6 g of cells.

For crystallization studies, the enzyme was produced using the dicistronic pET24a construct (encoding hh4-OT) transformed in *E. coli* BL21-Gold(DE3) cells (5). Accordingly, a colony or several colonies (a streak) of the construct were used to inoculate 10 mL of LB/Kn media (30 µg/mL). After shaking overnight (~16 h) at 37 °C, an aliquot (4 mL) was used to inoculate a 400-mL LB/Kn culture (30 µg/mL) in a 2 L flask. The cells were allowed to grow at 37 °C with shaking until the OD₆₀₀ reached ~0.8 (~ 2 h), and then induced by the addition of IPTG (final concentration of 0.5 mM). The cells were then allowed grow for an additional 3 h, collected by centrifugation (15 min at 11,000g) at 4 °C, and stored at -80 °C. Typically, a 400-mL culture yields 1.6 g of cells.

The purification protocol was adapted from previously described ones and modified as follows (6,7). In a typical procedure, cells (~3g) were thawed and suspended in ~9 mL of 20 mM HEPES buffer (pH 7.6) in a 15-mL plastic vial, which was placed in ice. Aliquots (1/200th of the suspension mixture volume, ~45 µL each) of two protease inhibitors (from 200 mM phenylmethylsulfonyl fluoride dissolved in ethanol and 200 mM ε-aminocaproic acid in HEPES buffer stock solutions) were added. Cells were disrupted by sonication for 1 min (setting 8) generated by a Heat Systems W-385 sonicator (Heat Systems-Ultrasonics, Inc, Farmingdale, NY) equipped with a 0.5 in tapped horn. After sitting on ice for 10 min, the mixture was sonicated (setting 6) for 30 s. After another 10-min period on ice, the mixture was sonicated four times (30-s each) with an intervening 10-min period on ice between bursts. A second aliquot of the protease inhibitors was added. The mixture was centrifuged (45 min at 30,000g) and the supernatant was removed and centrifuged again (3 h at 264,000g). Subsequently, the supernatant was diluted to 50 mL with the 20 mM HEPES buffer and divided into 5-mL portions in glass tubes (20 × 150 mm). After the addition of an equal volume of a 20 mM HEPES buffer solution made 9% in polyethylene glycol (w/v) and 0.5 M NaCl, the tubes were placed in a water bath set at 80

°C for 30 min. The tubes were cooled for 5 min at room temperature, placed in ice for additional 10 min, and centrifuged (in polycarbonate tubes for 10 min at 12,000g). The supernatant was collected and divided into two portions (~ 45 mL each). Each portion was made up to 100 mL to make a final concentration of 20 mM HEPES buffer, pH 7.6, and 3M in NaCl. The resulting solution was loaded onto a Phenyl-Sepharose column (1.5 × 10 cm filled with 12 mL of resin) that had been previously equilibrated with 20 mM HEPES buffer made 3 M in NaCl. After the sample loaded, the column was washed with 20 mM HEPES buffer, pH 7.6, made 2M in NaCl (60 mL) followed by 20 mM HEPES buffer, pH 7.6, without NaCl (20 mL). The flow rate was estimated to be ~1.0 mL/min. Fractions (~0.5 mL) were collected and analyzed for activity using 2-hydroxymuconate (**1**). Those showing the highest activity were combined and concentrated about 4-fold using an Amicon Centricon centrifugal filter device equipped with a YM10 ultracell membrane. A portion of the concentrate (~200 µL) was loaded onto a Sephacryl S-100 column (1 × 50 cm filled with 36 mL of resin and topped with 0.5 mL of Sephadex G-25 resin) that had been previously equilibrated with 10 mM potassium phosphate buffer, pH 7.3. The flow rate was estimated to be ~5 mL/h. Fractions (~0.4 mL) were collected and analyzed for activity using **1**. Those fractions with the highest activity and greatest purity (as assessed by SDS-PAGE) were combined and concentrated (for crystallization studies) using a Millipore Ultrafree centrifugal filter device containing a Biomax 5K membrane. Typically, 3 g of cells yields ~3.5 mg of enzyme purified to 95% homogeneity (as assessed by SDS-PAGE).

Mass Spectral Analysis of the hh4-OT Mutants. The purified hh4-OT mutants were analyzed by electrospray ionization mass spectrometry (ESI-MS). Each sample generates two major signals (corresponding to the α - and β -subunits) in the mass spectrometer. The masses (MH^+) for the hh4-OT mutants are as follows: α Arg-12, α 7645 Da (calc. 7647 Da), β 7963 Da (calc. 7965 Da); α Arg-40, α 7645 Da (calc. 7647 Da), β 7963 Da (calc. 7965 Da); β Pro-1, α 7731 Da (calc. 7732 Da), β 7937 Da (calc. 7939 Da); β Arg-11, α 7731 Da (calc. 7732 Da), β 7879 Da (calc. 7880 Da); β Arg-39, α 7731 Da

(calc. 7732 Da), β 7877 Da (calc. 7880 Da). Based on the observed and calculated masses, it can be concluded that the β -subunits have undergone the expected post-translational modification to remove the initiating methionine (enabling Pro-1 to function as a base) whereas the α -subunits have not (8).

Modeling of 2-Hydroxymuconate (1) and Phenylolpyruvate (3) in the hh4-OT Active Site.

Modeling was constructed by manual placement of substrates in the hh4-OT active site and minimization using programs included in Maestro (Schrodinger, Mannheim, Germany). LigPrep was used for the preparation of substrates where the pH was set to 7.0 ± 0.2 . The protein, hh4-OT, was prepared using the protein preparation wizard included in Maestro. All sulfate ions were removed during the protein preparation wizard to prevent them from obstructing the placement of the substrates. The initial orientation of 2-hydroxymuconate in the active site was derived from the 2-oxo-3-pentionate-inactivated 4-OT (1BJP) from *P. putida* mt-2 and the kinetic data in Table 2. Phenylolpyruvate was placed in the active site according to RCSB entry 2OPA and the kinetic data in Table 3. Both hh4-OT-substrate complexes were minimized using Impact Energy Minimization included in Maestro. The dielectric setting for minimization was 1.0. The truncated Newton Algorithm was used for minimization with a convergence criterion of “energy and gradient”.

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