Total Chemical Synthesis and Catalytic Properties of the Enzyme Enantiomers L- and D-4-Oxalocrotonate Tautomerase

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Abstract: Both the native L-form and the mirror image D-form of the enzyme 4-oxalocrotonate tautomerase (4OT) were prepared by total chemical synthesis. Our results indicate that both enzymes were efficient catalysts and demonstrate, as expected, that the achiral substrate 2-hydroxymuconate (**2**) was processed with equal efficiency by either the D- or the L-enzyme. The stereochemical course of the D-4OT-catalyzed reaction in ^2H_2O was also characterized; and it was found that D-4OT ketonized 2-hydroxymuconate (**2**) to (5*R*)-2-oxo-3(*E*)-[5-^{2}H]hexenedioate (**3**). This finding is consistent with the stereochemical course previously established for the L-4OT-catalyzed reaction and confirms the expectation that the mirror image enzyme molecules D- and L-4OT operate on opposite faces of the dienol intermediate. Furthermore, we have used electrospray ionization time-of-flight (ESI-TOF) mass spectrometry to establish the multimeric state of our synthetic enzymes. Our ESI-TOF results under nondenaturing solution conditions show that each enantiomer formed a noncovalent, homohexameric complex consistent with the previously reported crystallographic analysis of recombinant L-4OT.

Introduction

The ability to prepare enzyme molecules by total chemical synthesis provides a unique and powerful approach to the study of enzymatic catalysis. Recent advances in synthetic peptide chemistry have dramatically increased the number of proteins and enzymes accessible by chemical synthesis.[1] Continuing improvements in solid phase peptide synthesis (SPPS) methods[2] have not only permitted the assembly of larger polypeptides but also enhanced the quality and yield of the end product. These stepwise SPPS methods have allowed the successful synthesis of a number of small biologically active proteins including epidermal growth factor,[3] insulin-like growth factor,[4] rubredoxin,[5] the chemokine IL-8,[6] the cytokine IL-3,[7] and the human immunodeficiency virus (HIV)-1 protease.[8]

Total chemical synthesis enables the construction of protein analogues containing unnatural elements ranging from noncoded amino acids to modified peptide backbones, as well as fixed elements of three-dimensional structure. These synthetic constructs have been used in a variety of studies to probe enzyme structure and function.[9−12] In particular, the assembly of proteins containing all D-amino acids is only possible, at present, through total chemical synthesis. Recently, the synthesis and characterization of the mirror image forms of two different proteins, rubredoxin and HIV-1 protease, have been reported.[5,10] The rubredoxin study illustrated the potential use of racemic protein crystals in improving the quality of X-ray crystallographic data and also established the low immunogenicity of D-proteins *in vivo*.[5] In the case of the homodimeric HIV-1 protease the D- and L-forms of the molecule were equally active, and as expected the two enantiomers showed reciprocal chiral substrate specificity in that the L-enzyme cleaved only an L-peptide substrate and the D-enzyme cleaved only the corresponding D-peptide substrate.[10] Synthetic access to D-enzymes can help further our understanding of biological mechanisms. D-Enzymes are also potentially useful for medicinal purposes because of their nonimmunogenic properties and resistance to catabolic enzymes.

4-Oxalocrotonate tautomerase (EC 5.3.2; 4OT) from *Pseudomonas putida mt*-2 is expressed by certain soil bacteria as part of a set of inducible enzymes that converts aromatic hydrocarbons to intermediates in the Krebs cycle.[13,14] The enzyme catalyzes the 1,3-allylic isomerization of 2-oxo-4(*E*)-

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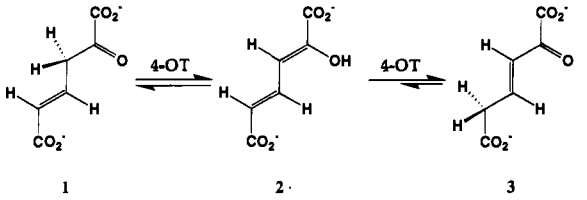
1 10 20 30 40 50 60
PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMAKGHFGIGGELASKVRR

Figure 1. Primary amino acid sequence of the 4OT monomer polypeptide chain from *P. putida mt-2*.[17]

Scheme 1. 4OT-Catalyzed Reaction



hexenedioate (**1**) to 2-oxo-3(*E*)-hexenedioate (**3**) through the intermediate 2-hydroxymuconate (**2**) (Scheme 1).[15] 4OT is a hexamer of identical 62 amino acid polypeptide chains.[16] The primary amino acid sequence of the 4OT monomer polypeptide chain is given in Figure 1.[17] The relatively small size of the 4OT subunit polypeptide chain makes this multimeric enzyme an ideal target for chemical synthesis. Here we report the total chemical synthesis of the 62 amino acid polypeptide chain of 4OT as well as our characterization of the enzyme as a homohexameric protein. Both mirror image forms of the 4OT molecule were prepared from the D- and L-polypeptide chains. Our structural, kinetic, and stereochemical catalytic activity data for D- and L-4OT reveal that these synthetic polypeptides were readily folded to form active enzyme complexes and that, as expected, both enantiomers are active on the achiral substrate 2-hydroxymuconate, although they react on opposite faces of the molecule.

Results and Discussion

Synthesis and Covalent Characterization of D- and L-4OT. The D- and L-amino acid forms of the polypeptide chains of the 62 amino acid monomer were assembled by highly optimized, stepwise solid phase methods using machine-assisted in situ neutralization protocols for *tert*-butoxycarbonyl (Boc) chemistry.[18] Both syntheses were initiated on 0.2 mmol of Boc-Arg(Tos)-OBzl-4-(carboxamidomethyl)-resin. Protected Boc-amino acids were preactivated [by reaction with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)] to form hydroxybenzotriazole esters, and were then reacted with the trifluoroacetate salt of the resin-bound peptide in the presence of excess diisopropylethylamine (DIEA). The coupling reaction for the addition of each residue was performed for 10 min with the exception of arginine residues which were reacted for 20 min. A qualitative colorimetric assay of residual free amine was performed on peptide–resin samples taken after the addition of each residue in order to evaluate the coupling efficiency of each amino acid; no repeat couplings were required.[19] Removal of side chain protecting groups and cleavage of peptide from the resin were achieved by treatment with anhydrous HF. Subsequent reversed-phase HPLC purification of the crude peptide product gave good yields of high-purity polypeptide chains corresponding to the 62 amino acid sequence of 4OT. Overall yields (based on the loading of the resin) were 4% (D-4OT) and 8% (L-4OT) by weight. Typically, 50–100 mg of high-purity 62-mer could be obtained from a single 0.2 mmol scale synthesis.

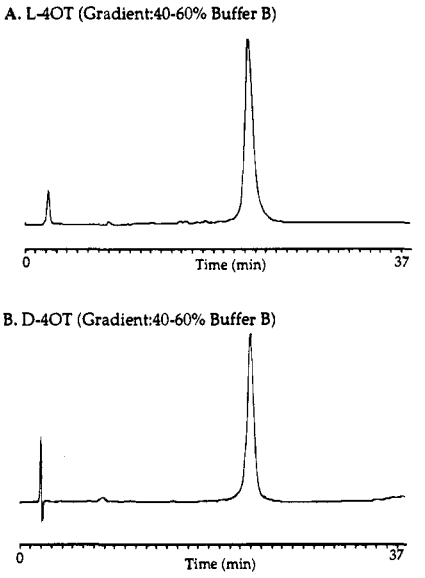


Figure 2. Analytical reversed-phase HPLC analysis of purified D- and L-amino acid forms of the polypeptide chains of the 62 amino acid 4OT monomer: (A) HPLC chromatogram of purified L-4OT recorded with a shallow acetonitrile gradient (40–60% buffer B over 30 min), (B) HPLC chromatogram of purified D-4OT recorded with a shallow acetonitrile gradient (40–60% buffer B over 30 min).

Analytical reversed-phase HPLC analysis was used along with electrospray ionization mass spectrometry (ESI-MS) to confirm the identity and purity of the final polypeptide products. Results for the HPLC analysis of purified D- and L-polypeptides are shown in Figure 2. The purified synthetic peptide products eluted essentially as single peaks under shallow gradient conditions. As expected, the enantiomeric forms of the polypeptide chains had identical retention times. Further characterization of the products by ESI-MS established the high purity of both synthetic products (Figure 3). The monomeric molecular weight of the D- and L-4OT samples were identical within experimental error (6809.7 ± 0.8 and 6809.9 ± 0.7, respectively) and in good agreement with the expected mass, 6810.7 (average isotopic composition).

The reconstructs of the electrospray mass spectra of L- and D-4OT shown in Figure 3 indicate the presence of a minor impurity with a mass 16.9 larger than that of the full length polypeptide chains. This impurity is consistent with oxidation of Met-45 to methionine sulfoxide during sample handling. Unfortunately, the oxidized material coelutes with the pure synthetic peptide product in reversed-phase HPLC. However, the oxidized polypeptide chains were efficiently removed from solution by precipitation during protein folding, as described below.

Protein Folding. The synthetic D- and L-polypeptide chains were separately incubated in assay buffer (20 mM sodium phosphate, pH 7.4, or 5 mM ammonium bicarbonate, pH 7.0) at room temperature for 2 h in order to facilitate formation of the active enzyme complex. Typically, the 62 amino acid polypeptide was dissolved in assay buffer at concentrations of 0.1–0.5 mg/mL. Any precipitate that formed was removed from solution by centrifugation. The precipitated material was characterized by ESI-MS and contained the bulk of the M + 16 impurity. Presumably the precipitated material consisted of misfolded polypeptide chains. Subsequent dilutions of the

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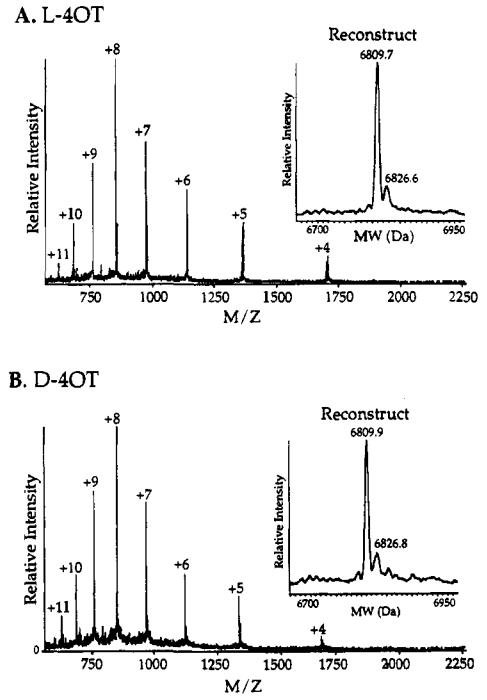

Figure 3. Electrospray mass spectra of HPLC purified L-4OT (A) and D-4OT (B) showing multiple charge states arising from the distribution of protonated residues. The inset in each panel represents the hypermass reconstruction of the spectrum displayed as a single charge state. The L- and D-4OT samples yielded observed masses of 6809.7 ± 0.8 and 6809.9 ± 0.7, respectively. The calculated mass of 4OT is 6810.7 (average isotope composition).

folded enzyme solution were also made at least 2 h prior to the start of kinetic experiments. Kinetic results obtained immediately after diluting the folded enzyme were not reproducible. These observations are similar to those previously reported for the recombinant enzyme.[15] Interestingly, a room temperature incubation step was not necessary for the ultraviolet circular dichroism (UV-CD) and electrospray time-of-flight (ESI-TOF) experiments outlined below. Enzyme solutions analyzed immediately after preparation yielded the same UV-CD and ESI-TOF results as those that were allowed to sit for 2 h at room temperature. These observations suggest that the 4OT enzyme complex is easily assembled from the monomer polypeptide chains. However, approximately 2 h may be required for the enzyme to reach an equilibrium concentration of the active complex. The efficacy of a more rigorous folding protocol involving gradual dialysis from 6 M guanidine to assay buffer was also evaluated. Synthetic L-4OT enzyme folded in this manner was indistinquishable in its physical and kinetic characteristics from enzyme that was folded by direct dilution in assay buffer.

Structural Characterization of D- **and** L-**4OT.** Previous attempts to characterize the oligomeric structure of 4OT have yielded variable results. Initial characterization of 4OT isolated from *P. putida mt-2* indicated the enzyme was a 28 000 MW molecule formed by the association of identical subunits with an apparent MW of 3500 as determined by gel electrophoresis.[14] In later studies using recombinantly-derived enzyme, the MW of native 4OT was determined by gel permeation chromatography (37 000) and estimated by ultracentrifugation (32 000).[17] These results suggested that 4OT was a pentamer (calculated M_r = 34 055). More recent X-ray diffraction data have shown that the enzyme is a homohexameric complex in the crystalline state.[16]

Here we have used electrospray ionization time-of-flight (ESI-TOF) mass spectrometry to establish the oligomeric state of

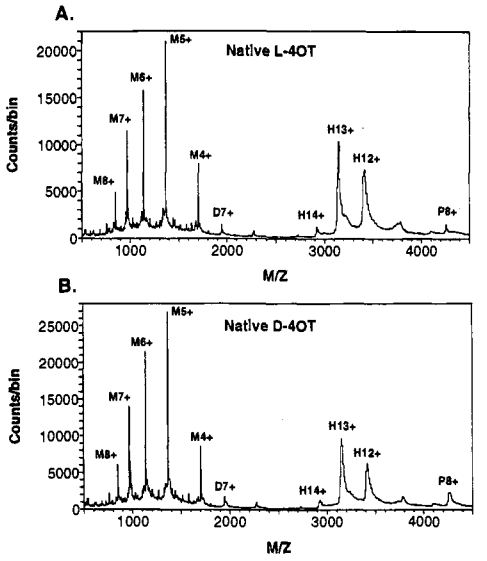

Figure 4. ESI-TOF mass spectra of L-4OT (A) and D-4OT (B) obtained under native electrospray conditions (5 mM ammonium bicarbonate buffer, pH 7.0). In each spectrum peaks corresponding to multiply charged ions of the monomer (MW = 6811), dimer (MW = 13 622), pentamer (MW = 34 055), and hexamer (MW = 40 866) are identified in spectra and labeled M, D, P, and H (respectively).

both the D- and L-forms of 4OT prepared by total chemical synthesis. This recently developed mass spectrometry technique has proven very useful for the analysis of noncovalent complexes of large, multimeric proteins.[20] The ESI-TOF mass spectra obtained for the 4OT protein enantiomers under native electrospray conditions (5 mM ammonium bicarbonate, pH 7.0) are shown in Figure 4. The most abundant signals in each spectrum correspond to those of the multiply charged monomer and hexamer. Only minor signals representative of the dimer and pentamer are observed. In contrast, ESI-TOF analysis of each synthetic version of the enzyme under denaturing conditions (5 mM ammonium bicarbonate, 0.1% acetic acid, pH 4.5) yielded only multiply charged monomer (data not shown). These results suggest that the noncovalent structures of each 4OT enantiomer are similar, and that the predominant form of the enzyme is a hexameric complex of identical subunits. Significantly, our "native" ESI-TOF results provide important evidence for the hexameric structure of 4OT in solution and are consistent with the preliminary X-ray data.[16]

Additional structural studies on D- and L-4OT were performed using UV-CD spectroscopy. UV-CD spectra for both D- and L-4OT were recorded in 50 mM sodium borate buffer, pH 7.0, at 25 °C, and the results are shown in Figure 5. The spectra of the individual D- and L-4OT enantiomers show equal and opposite optical rotations, as is expected for enantiomeric molecules. Furthermore, the molar ellipticity values, $[\theta]$, recorded for each enantiomer at 222 nm are indicative of helical secondary structure. The percent helicities of D- and L-4OT were calculated to be 24% and 26%, respectively. These values are in good agreement with the percent helicity previously reported for recombinant 4OT (21%).[21]

Enzymatic Activity. The catalytic efficiencies of synthetic D- and L-4OT were determined by studying the kinetics of the 4OT-catalyzed conversion of **2** to **3** (Scheme 1). In these experiments the rate of formation of **3** was monitored at 236 nm for six different substrate concentrations ranging from 25

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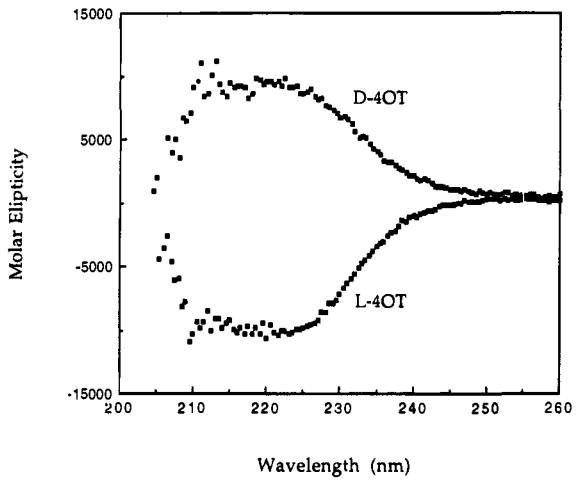


Figure 5. Far UV-CD spectra for L- and D-4OT showing equal and opposite optical rotations, as expected, for the two enzyme enantiomers. The spectra are characteristic of partly helical protein molecules (see text for details).

Table 1. Kinetic Parameters for Synthetic D- and L-4-Oxalocrotonate Tautomerase (4OT) and Recombinant 4OT

enzyme	K_M,[a] μM	k_{cat}, s^{-1}	k_{cat}/K_M, M^{-1} s^{-1}
synthetic L-4OT	90 ± 17	(2.89 ± 0.53) × 10^3	3.2 × 10^7
synthetic D-4OT	103 ± 23	(2.94 ± 0.16) × 10^3	2.9 × 10^7
recombinant L-4OT	89 ± 22	(3.39 ± 0.50) × 10^3	3.8 × 10^7

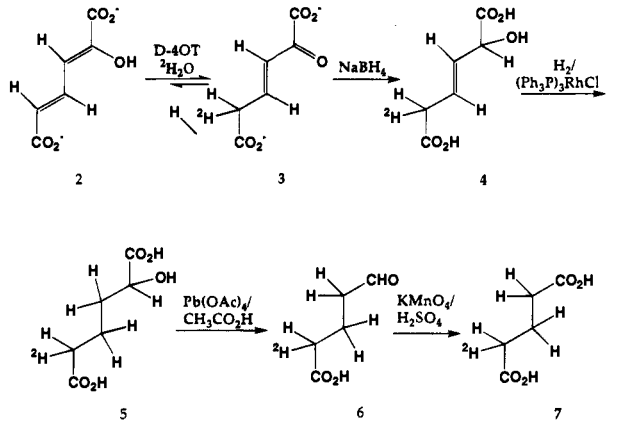
[a] Errors are standard deviations.

to 250 μM. The kinetic parameters, K_M and k_{cat}, were calculated for each synthetic enzyme from double reciprocal plots ($1/v$ vs $1/[S]$). The values obtained for the kinetic parameters K_M, k_{cat}, and k_{cat}/K_M are reported in Table 1, along with results from parallel studies of recombinant 4OT. Essentially identical kinetic parameters were obtained for the synthetic D- and L-enzymes. These results indicate that each enzyme enantiomer catalyzes the ketonization of the achiral substrate **2** to **3** with approximately the same efficiency. The kinetic parameters for synthetic D- and L-4OT are also comparable to those obtained for the recombinant enzyme.

Stereochemical Analysis of the D-4OT-Catalyzed Reaction. As expected, both enantiomers of 4OT acted with equal efficiency on the achiral substrate **2**. As additional proof that the synthesis of the D-enzyme was successful, we characterized the stereospecificity of the synthetic D-enzyme. The L-4OT enzyme molecule has previously been shown to act in a stereospecific manner in the conversion of **2** to **3** in ^2H_2O.[22] Because the newly-created methylene at the C5 position of **3** is prochiral, it would be expected that D-4OT would display the opposite stereospecificity.[10,23] The stereochemistry of the D-4OT-catalyzed reaction was determined by using a previously described strategy, based on stereochemical analysis of [5-^{2}H]-**3** (Scheme 2), which was generated from **2** by D-4OT in ^2H_2O.[22]

The purified [2-^{2}H]glutaric acid product that resulted from the sequence of reactions shown in Scheme 2 was analyzed by circular dichroism. Glutaric acid generated in ^2H_2O using the synthetic D-4OT exhibits a negative Cotton effect in its circular dichroism spectrum, with a molar ellipticity $[\theta]_{210} = -150°$ at 25 °C. It has been previously established that the *S* isomer

Scheme 2. Stereochemical Analysis of D-4OT-Catalyzed Reaction in ^2H_2O



exhibits a positive Cotton effect in its circular dichroism spectrum, with a molar ellipticity $[\theta]_{210} = +180°$ at 25 °C.[22] We conclude, therefore, that the monodeuteriated glutarate derived from our enzymatic and chemical reaction is the *R* isomer. Thus, the product of the D-4OT-catalyzed reaction was (5*R*)-2-oxo-3(*E*)-[5-^{2}H]hexenedioate (**3**; Scheme 2). The reason for the slightly low value of the molar ellipticity measured for the *R* isomer is not apparent although the overlap with other electronic absorption in this region decreases the sensitivity of the measurement and makes accurate quantitation difficult. The molar ellipticity is, however, unquestionably negative and opposite that observed for the glutaric acid derived from the product of the L-4OT reaction.[22] This reaffirms the stereoselective action of the chiral 4OT molecule on the achiral substrate **2** and completes the characterization of the synthetic enzyme.

Conclusion. We have prepared the enantiomeric enzyme molecules D- and L-4OT by total chemical synthesis. Our kinetic, structural, and stereochemical data for these synthetic enzymes reveal that they fold to form active enzyme complexes and that, as expected, the enzyme enantiomers are equally active on the achiral substrate **2**, although they react on opposite faces of the molecule. Having established synthetic access to 4OT, the stage is set for the construction of a wide variety of 4OT analogues designed to address specific questions about the catalytic mechanism of this highly efficient enzyme. Work is in progress to investigate the structural and mechanistic roles of catalytically significant residues by modulating the chemical properties of their side chain functionalities. The results of this work will help elucidate the catalytic significance of specific residues in the action of 4OT and will increase our understanding of the mechanisms that govern enzyme catalysis.

Experimental Section

Materials and Methods. Machine-assisted solid phase peptide syntheses were carried out on a custom-modified Applied Biosystems 430A peptide synthesizer as previously described.[18] Analytical and semipreparative reversed-phase HPLC was performed on a Rainin dual-pump high-pressure mixing system with 214-nm UV detection using Vydac C-18 analytical (5 μm, 0.46 × 15 cm) and semipreparative (10 μm, 1.0 × 25 cm) columns. Preparative reversed-phase HPLC was performed on a Waters Delta Prep 4000 System using a Vydac C-18 preparative column (15–20 μm, 5.0 × 25 cm). Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% trifluoroacetic acid (TFA) in water, B = 90% acetonitrile in water containing 0.09% TFA) over 30–60 min at 1 mL/min (analytical), 3 mL/min (semipreparative), or 30 mL/min (preparative). Mass spectra were acquired using either a Sciex API-III quadrupole electrospray mass spectrometer or an electrospray ionization time-of-flight instrument. The latter was constructed at the University of Manitoba and has been

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described previously.[24] Circular dichroism spectra were recorded on either a Jasco J-20A or an AVIV 60DS spectropolarimeter. Enzyme kinetic data were collected with a HP 8452A diode-array spectrophotometer fitted with a temperature-controlled cell holder thermostated using recirculating water. NMR spectra were obtained on a Bruker AM-250 spectrometer or a Bruker AM-500 spectrometer as indicated. Chemical shifts are referenced as noted below.

Boc-L-amino acids and HBTU were purchased from Novabiochem (San Diego, CA). Boc-D-amino acid derivatives were obtained from Bachem Bioscience (Philadelphia, PA), Peptides International (Louisville, KY), or Peptide Institute, Inc. (Japan). Preloaded Boc-L-Arg(*p*-tolylsulfonyl)-OBzl-4-(carboxamidomethyl)-resin, (aminomethyl)copoly(styrene−divinylbenzene) resin, and DIEA were purchased from Applied Biosystems (Foster City, CA). Boc-D-Arg(*p*-tolylsulfonyl)-OBzl-4-(carboxamidomethyl)-resin was prepared by loading (aminomethyl)copoly(styrene−divinylbenzene) resin with 4-Boc-D-Arg(*p*-tolylsulfonyl)-OCH_2-phenylacetic acid, prepared as described elsewhere.[25] Synthesis grade DMF was obtained from Mallinckrodt, and AR grade methylene chloride was from Fisher. TFA was purchased from Halocarbon (New Jersey); HF was supplied by Matheson Gas. Ion exchange resins were obtained from Bio-Rad Laboratories. Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. The compound 2-hydroxymuconate (**2**) was prepared according to the method of Lapworth.[26] All other reagents were AR grade or better and were obtained from Aldrich Chemical or Fisher.

Chemical Synthesis of the L-4OT 62 Amino Acid Polypeptide Chain. The L-enantiomer of the 4OT 62 amino acid polypeptide chain was synthesized from protected L-amino acids in stepwise fashion by highly optimized machine-assisted solid phase methods using *in situ* neutralization/HBTU activation protocols for Boc chemistry.[18] Side chain protection was as follows: Boc-Arg(*p*-tolylsulfonyl)-OH, Boc-Asp(*O*-cyclohexyl)-OH, Boc-Glu(*O*-cyclohexyl)-OH, Boc-His(DNP)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Ser(benzyl)-OH, and Boc-Thr(benzyl)-OH. The synthesis was initiated on a Boc-L-Arg(*p*-tolylsulfonyl)-OBzl-4-(carboxamidomethyl)-resin. After chain assembly was complete His(DNP) groups were removed by treatment of the Boc-peptide-resin with a solution of 20% 2-mercaptoethanol and 10% DIEA in DMF prior to removal of the N-terminal Boc protecting group. Side chain protecting groups were removed and the peptide chain was simultaneously cleaved from the resin by treatment with HF containing 5% (v/v) *p*-cresol and 5% (v/v) *p*-thiocresol for 1 h at 0 °C. After removal of HF under reduced pressure, the crude peptide product was precipitated and washed with anhydrous ether, dissolved in 50% aqueous acetic acid, diluted with water, and lyophilized. The crude product was analyzed by reversed-phase HPLC and electrospray MS and purified by preparative reversed-phase HPLC. Fractions were combined on the basis of electrospray mass spectral analysis. The purified product was characterized by shallow-gradient analytical HPLC (Figure 2) and electrospray ionization mass spectrometry (Figure 3).

Chemical Synthesis of the D-4OT 62 Amino Acid Polypeptide Chain. The synthesis of the D-polypeptide chain was carried out in the same manner as that described above for L-4OT except that protected D-amino acids were used for chain assembly. Side chain protection of D-amino acids was similar to that of L-amino acids with the exception that Boc-D-His(Bom) was used. It should also be noted that in accord with standard nomenclature the side chain chiral centers in Boc-D-Thr(Bzl) and Boc-D-Ile had the configuration opposite that of their L-amino acid counterparts. Chain assembly was initiated on a Boc-D-Arg(*p*-tolylsulfonyl)-OBz-4-(carboxyamidomethyl)-resin. The L-enantiomer content of the Boc-D-amino acid preparation was between 0.01% and 0.08% (manufacturer's specifications).

Mass Spectrometry. Samples for electrospray mass spectrometry were analyzed in HPLC buffer (40% buffer B) at peptide concentrations of approximately 5 μM. ESI-TOF analysis was performed under both denaturing (5 mM ammonium bicarbonate, 0.1% acetic acid, pH 4.0) and native (5 mM ammonium bicarbonate, pH 7.0) conditions. ESI-TOF samples were prepared at concentrations of approximately 60 μM (based on monomer).

Protein Folding. The synthetic D- and L-enzymes were each folded in assay buffer (20 mM sodium phosphate, pH 7.4, or 5 mM ammonium bicarbonate) at room temperature over the course of 2 h. Typically, the 62 amino acid polypeptide was dissolved in assay buffer at concentrations of 0.1−0.5 mg/mL. Any precipitate that formed was removed by ultracentrifugation.

Circular Dichroism Spectroscopy. CD spectra of folded D- and L-4OT were measured in 50 mM sodium borate buffer at pH 7.0 at a concentration of approximately 10 μM using the AVIV 60 DS instrument. CD spectra for these protein samples were recorded from 190 to 260 nm at 20 °C using a 3.0-mL quartz cuvette with a 1-cm path length. The results are presented as a plot of mean molar ellipticity per residue ($[\theta]$, deg·cm^2·dmol^{-1}) versus wavelength in 0.5-nm increments. The percent helicity in each sample was estimated from $[\theta]_{222}$ using eqs 1 and 2.[27]

$$\% \text{ helix} = [\theta]_{222}/[\theta]_{max} \times 100 \quad (1)$$

$$[\theta]_{max} = \pm 39500[1 - (2.57/n)] \text{ where } n = \text{number of amino acids per chain} \quad (2)$$

CD measurements on [2-^{2}H]glutaric acid were carried out in 95% ethanol on the Jasco J-20A instrument at room temperature. The molar ellipticities for these samples are reported in millidegrees. Protein concentrations were established by amino acid analysis after acid hydrolysis.

Enzymatic Activity of D- and L-4OT. The kinetic parameters reported here for D- and L-4OT were obtained with the substrate **2** as previously described.[15] Briefly, each enzyme enantiomer was assayed spectrophotometrically at 30 °C by following the rate of appearance of product **3** (ϵ = 6.58 × 10^3 M^{-1} cm^{-1} at 236 nm) immediately following the addition of **2** to an assay mixture containing enzyme appropriately diluted in 20 mM phosphate buffer (pH 7.4). Final enzyme concentrations were typically between 1 and 2 nM. Stock solutions of the enzyme were greater than 1 μM as losses in activity were observed in enzyme stock solutions of less than 1 μM. Initial velocities for the enzyme-catalyzed isomerization of **2** to **3** were measured at six substrate concentrations ranging from 25 to 250 μM, and the data were used to generate double reciprocal plots ($1/v$ vs $1/[S]$ from which V_{max}, K_M, and k_{cat} values were extracted. Enzyme concentrations were established by amino acid analysis after acid hydrolysis.

Ketonization of 2 to [5-^{2}H]3 by D-4OT in ^2H_2O and Conversion of [5-^{2}H]3 to 2-Hydroxy-3(*E*)-[5-^{2}H]-hexenedioate (4). The stereochemical analysis of [5-^{2}H]**3** was based on its chemical conversion to a stereoselectively monodeuteriated glutarate (**7**) according to the procedure of Whitman *et al* (Scheme 2).[22] A solution of **2** (162 mg, 1.02 mmol) in dimethyl-d_6 sulfoxide (0.36 mL) was added in aliquots (0.01 mL) to 36 individual reaction vials containing 100 mM Na_2[^{2}H]-PO_4 (0.6 mL, p^{2}H 9.66) buffer made up in ^2H_2O. The addition of **2** adjusted the p^{2}H of the buffer to about **7**. Subsequently, a portion of D-4OT (0.01 mL, 1.7 mg/mL) was added to each stirring solution and allowed to react for 2.5 min. The reaction mixture was worked up as previously described.[22] Ultimately, 110 mg (66%) of [5-^{2}H]**4** was recovered as the major product, the identity of which was confirmed by ^{1}H NMR spectroscopy.[22]

Catalytic Reduction of [5-^{2}H]4. The fully saturated compound [5-^{2}H]**5** was obtained by the hydrogenation of [5-^{2}H]**4** in the presence of Wilkinson's catalyst as described previously.[22] Briefly, $(Ph_3P)_3RhCl$ (12 mg, 0.011 mmol) was added to a hydrogenation bottle containing a solution of [5-^{2}H]**4** (110 mg, 0.7 mmol) in 1:1 ethanol/benzene (24 mL). This procedure resulted in the recovery of 58 mg (52%) of [5-^{2}H]-**5** as the major product.

Conversion of [5-^{2}H]5 to [2-^{2}H]Glutaric Acid (7). To a stirring solution of [5-^{2}H]**5** (58 mg, 0.35 mmol) in 2 M acetic acid (1 mL) was added a solution of lead tetraacetate (0.17 g, 0.38 mmol) suspended in 2 M acetic acid (2 mL). After stirring at room temperature for 2 h, the reaction was worked up as previously described to yield 17 mg

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(36%) of [2-^{2}H]**9**: $[\theta]_{210} = -150°$.[22] The ^{1}H NMR (250 MHz, $C^2H_3O^2H$) spectrum corresponded to the previously reported spectrum.[22]

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