Stereospecific Ketonization of 2-Hydroxymuconate by 4-Oxalocrotonate Tautomerase and 5-(Carboxymethyl)-2-hydroxymuconate Isomerase

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Abstract: 4-Oxalocrotonate tautomerase from *Pseudomonas putida mt-2* and 5-(carboxymethyl)-2-hydroxymuconate isomerase from *Escherichia coli C* catalyze the same reaction on substrates that differ only by a carboxymethyl group. While the structural resemblances between the substrates for these two proteins suggest that the enzymes might be evolutionarily related, the existing literature does not indicate an obvious link. It was found that both enzymes ketonize 2-hydroxymuconate (**1**)—the substrate for 4-oxalocrotonate tautomerase. In an effort to uncover similarities in the mechanisms, the stereochemical courses of both enzymatic reactions were examined, utilizing **1** in ^2H_2O. In each case, the product, 2-oxo-3-(*E*)-hexenedioate (**2**), was trapped and processed to [2-$^2H_1$]glutaric acid by chemical degradative procedures. The configuration was established by comparing the molar ellipticity of the isolated glutaric acid to that of a sample generated by a stereoselective synthesis. It is concluded that 4-OT and CHMI ketonize **1** stereospecifically to (*S*)-2-oxo-3-(*E*)-[5-$^2H_1$]hexenedioate. The mechanism and the evolution of 4-OT and CHMI and their respective pathways are discussed in the context of these results.

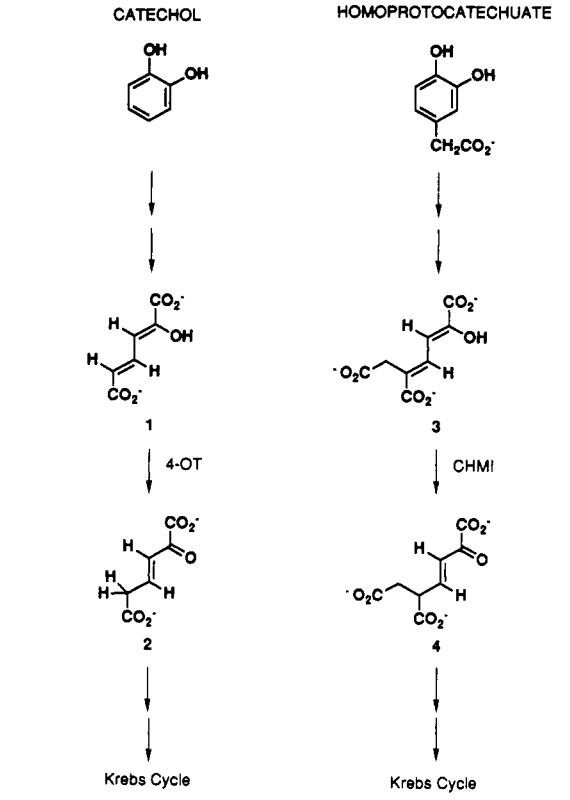
Introduction

One step in the microbial degradation of catechols by a meta-fission pathway is the enzyme-catalyzed ketonization of a dienol. In the catechol meta-fission pathway elaborated by *Pseudomonas putida mt-2* (Scheme I), ketonization of 2-hydroxymuconate[1] (**1**) by 4-oxalocrotonate tautomerase (4-OT) generates the α,β-unsaturated ketone, 2-oxo-3-(*E*)-hexenedioate (**2**), which undergoes decarboxylation and further processing to intermediates in the Krebs cycle.[2,3] In the homoprotocatechuate pathway elaborated by *Escherichia coli C* (Scheme I), ketonization of 5-(carboxymethyl)-2-hydroxymuconate (CHM, **3**) by 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) generates the α,β-unsaturated ketone **4**, which also undergoes decarboxylation and further processing to intermediates in the Krebs cycle.[4,5]

The structural resemblance between **1** and **3** suggest that 4-OT and CHMI might be evolutionarily related and might show similarities in mechanism and structure.[6] The incomplete experimental record indicates that there are mechanistic similarities. Kinetic studies suggest that 4-OT acts as an isomerase transforming a β,γ-unsaturated ketone **5** to its α,β-isomer **2** through a dienol intermediate (**1**, Scheme II).[2] A similar mechanism can be envisioned for CHMI in which the enzyme converts **6** to **4** through **3** (Scheme II), although there are currently no data to support such a mechanism. Nonetheless, both enzymes ketonize a dienol by a net 1,5 proton shift appearing to differ only in specificity for the 5 substituent of 2-hydroxymuconate. Moreover, neither enzyme requires either a metal ion or other cofactor for activity.[2-5]

However, 4-OT and CHMI have very different structures. There is no sequence homology between 4-OT and CHMI.[7] Moreover, 4-OT is a pentamer consisting of monomers having 62 amino acids, while CHMI is a dimer consisting of monomers having 125 amino acids.[4,7] In addition, the purification of 4-OT and CHMI requires different conditions—a reflection of their structural differences.[4,7] Finally, it has been reported that CHMI does not process **1**, indicating that these two enzymes have very narrow substrate specificities.[8] The mechanistic similarities coupled with the structural differences raise several questions regarding each enzyme's mechanism, specificity, and evolutionary lineage. Hence, we initiated a study to explore further the relationship between 4-OT and CHMI.

Scheme I



Scheme II

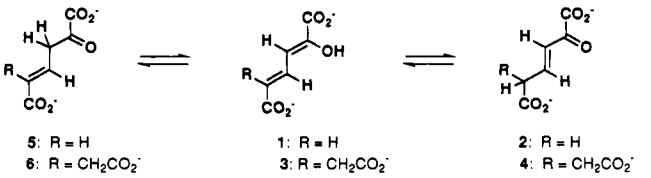


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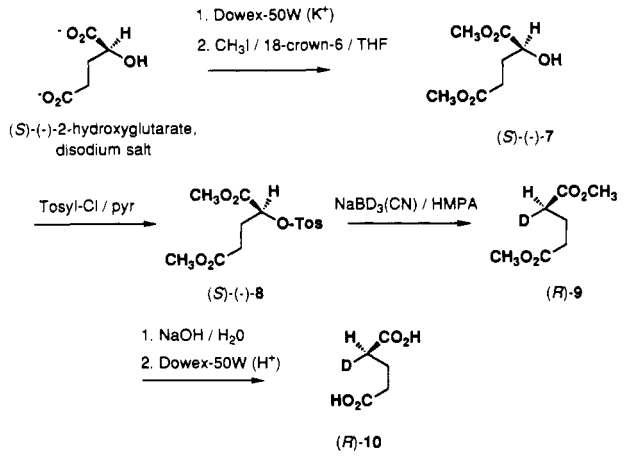
Table I. Kinetic Parameters for 4-Oxalocrotonate Tautomerase and 5-(Carboxymethyl)-2-hydroxymuconate Isomerase Utilizing **1** and **3**

	1			3		
enzyme	K_M,[a] μM	k_{cat}, s^{-1}	k_{cat}/K_M, M^{-1} s^{-1}	K_M,[a] μM	k_{cat}, s^{-1}	k_{cat}/K_M, M^{-1} s^{-1}
4-OT from *P. putida mt-2*	54.1 ± 5.2	3000	5.5×10^7	1010 ● 170	1.2	1.2×10^3
CHMI from *E. coli C*	345 ● 25	68	2.0×10^5	201 ● 8	360	1.8×10^6

[a] Errors are standard deviations.

Scheme III



In the course of our investigation of 4-OT and CHMI, we found that CHMI does ketonize **1** to **2** although at a significantly slower rate than that observed for 4-OT. This observation alone suggests there are some similarities between the mechanisms of the two enzymes and possibly between their active sites. In order to explore further the extent to which the mechanisms of 4-OT and CHMI may be related, we determined the stereospecificity of 4-OT and CHMI utilizing **1** in ^2H_2O. The product, [5-$^2H_1$]**2**, was trapped and processed to [2-$^2H_1$]glutaric acid by chemical degradative procedures. Comparison of the isolated glutaric acid to a sample generated from a stereoselective synthesis allowed us to assign the configuration and determination that both enzymes ketonize **1** stereospecifically to (*S*)-2-oxo-3-(*E*)-[5-$^2H_1$]hexenedioate (**2**). These findings provide insight into the mechanism of the two enzymes and fuel speculation about their evolutionary origin.

Results

Substrate Specificity of 4-OT and CHMI. The kinetic properties of 4-OT utilizing **3** and CHMI utilizing **1** were determined. The observed values for K_M, k_{cat}, and k_{cat}/K_M are summarized in Table I along with the values for each enzyme utilizing its physiological substrate. A comparison of the parameters measured for 4-OT utilizing **1** to those measured for 4-OT utilizing **3** indicates that the presence of the carboxymethyl group in **3** significantly affects binding and turnover of **3** by 4-OT. Its presence results in a 19-fold increase in K_M and a 2500-fold decrease in the turnover number (k_{cat}). The combination of these factors results in a 46 000-fold drop in the specificity constant. In contrast to these results, a comparison of the parameters determined for CHMI processing **3** to those determined for CHMI processing **1** shows that the absence of the carboxymethyl group in **1** slightly affects binding and turnover of **1** by CHMI. There is a 1.7-fold increase in K_M and a 5-fold decrease in the turnover number. The overall result is a 9-fold drop in the value of k_{cat}/K_M.

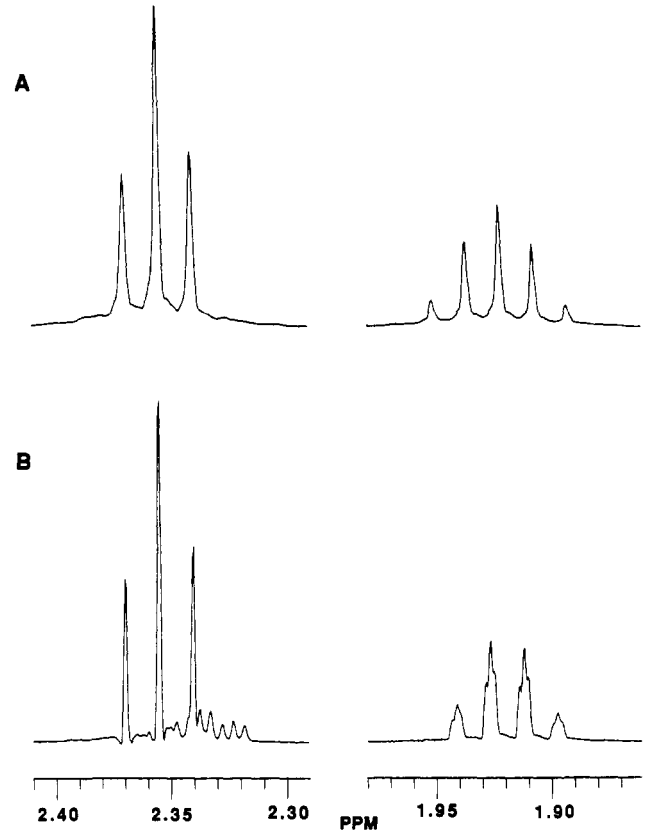


Figure 1. Partial ^1H NMR (500-MHz, $CDCl_3$) spectra indicating the chemical shifts (ppm) and characteristic splitting pattern for the protons on C2, C3, and C4 of (A) dimethyl glutarate and (B) dimethyl [2-$^2H_1$]glutarate.

Synthesis of (*R*)- and (*S*)-[2-$^2H_1$]Glutaric Acids. The sequence or reactions employed for the synthesis of (*R*)-[2-$^2H_1$]glutaric acid from the disodium salt of (*S*)-(−)-2-hydroxyglutarate is shown in Scheme III. The disodium salt was converted into the dipotassium salt and complexed with 18-crown-6 ether. The resulting complex was dissolved in THF; addition of iodomethane yielded an oil which was subjected to flash chromatography to afford the pure dimethyl ester.[9] Subsequently, the alcohol was treated with *p*-toluenesulfonyl chloride to generate the tosylate derivative. Esterification and tosylation do not disturb the configuration of the chiral center. The tosylate group was then displaced by deuteride after heating compound **8** in HMPA with NaB^2H_3CN.[10] It has been well established by Hutchins and

(1) To simplify the discussion of these compounds, we use their trivial names. The systematic names for **1** and **3** are 1-hydroxy-1,3-butanediene-1,6-dicarboxylic acid and 5-hydroxy-2,4-pentadiene-1,2,5-tricarboxylic acid, respectively.

(2) Whitman, C. P.; Aird, B. A.; Gillespie, W. R.; Stolowich, N. J. *J. Am. Chem. Soc.* **1991**, *113*, 3154–3162.

(3) Harayama, S.; Lehrbach, P. R.; Timmis, K. *J. Bacteriol.* **1984**, *160*, 251–255.

(4) (a) Sparnins, V. L.; Chapman, P. J.; Dagley, S. *J. Bacteriol.* **1974**, *120*, 159–167. (b) Roper, D. I.; Copper, R. A. *FEBS Lett.* **1990**, *226*, 63–66.

(5) Hajipour, G.; Johnson, W. H.; Dauben, P. D.; Gillespie, W. R.; Stolowich, N. J.; Whitman, C. P., unpublished results, 1992.

(6) (a) Dagley, S. In *Essays in Biochemistry*; Campbell, P. N., Aldridge, W. N., Eds.; Academic Press: London; 1975; Vol. 11; pp 81–138. (b) Dagley, S. In *The Bacteria: A Treatise on Structure and Function*; Ornston, L. N., Sokatch, J. R., Eds.; Academic Press: New York; 1978; Vol. 6, pp 305–388. (c) Bayly, R. C.; Barbour, M. G. In *Microbiological Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York; 1984; pp 253–294.

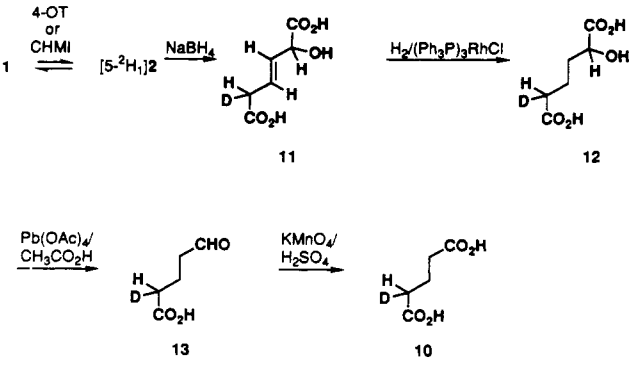
(7) Chen, L. H.; Kenyon, G. L.; Curtin, F.; Harayama, S.; Bembenek, M. E.; Hajipour, G.; Whitman, C. P. *J. Biol. Chem.* **1992**, *267*, 17716–17721.

(8) Sparnins, V. L.; Chapman, P. J.; Dagley, S. *J. Bacteriol.* **1974**, *120*, 159–167.

(9) (a) Jarvest, R. L.; Lowe, G.; Potter, B. V. L. *J. Chem. Soc., Perkin Trans. I* **1981**, 3186–3195. (b) Whitman, C. P.; Craig, J. C.; Kenyon, G. L. *Tetrahedron* **1985**, *41*, 1183–1192. While a synthesis has been reported for enantiomeric methyl esters, the procedure yields only starting material in our hands: Battersby, A. R.; Cardwell, K. S.; Leeper, F. J. *J. Chem. Soc., Perkin Trans I* **1986**, 1565–1580.

(10) Hutchins, R. O.; Kandasamy, D.; Maryanoff, C. A.; Masilamani, D.; Maryanoff, B. E. *J. Org. Chem.* **1977**, *42*, 82–91.

Scheme IV



co-workers that deuteride substitutions of this type are best accommodated by an S_N2 process.[10] Hence, reduction of (*S*)-**8** by NaB^2H_3CN in HMPA occurs with inversion of configuration and results in the formation of (*R*)-**9**. Hydrolysis of (*R*)-**9** with aqueous NaOH generates (*R*)-[2-$^2H_1$]glutarate (**10**). The analogous sequence of reactions was used to generate (*S*)-[2-$^2H_1$]glutaric acid from disodium (*R*)-(+)-2-hydroxyglutarate.

Confirmation of the isotopic substitution was obtained by the distinctive deuterium coupling patterns observed in the ^1H and ^{13}C NMR spectra of **9** (Figure 1).[11] The protons on C-3 of unlabeled dimethyl glutarate present a quintet at 1.92 ppm indicative of coupling to four equivalent protons—those on C-2 and C-4 (Figure 1A). In contrast, the protons on C-3 of the isotopically labeled **9** are observed as a quartet of broadened triplets (1.92 ppm, Figure 1B). Incorporation of a deuterium on C-2 accounts for the broadened triplets via vicinal ^1H–^2H coupling and simplifies the coupling pattern from a quintet to a quartet. Additionally, the protons on C-2 and C-4 of the unlabeled material generate the triplet observed at 2.36 ppm (Figure 1A). In the labeled material, however, the protons on C-4 produce the triplet observed at 2.36 ppm, while the proton on C-2 yields a triplet of triplets (2.34 ppm) which is shifted upfield (Figure 1B). Both effects result from the isotopic substitution at C-2 of **9**. Finally, the most notable feature in the ^{13}C NMR spectrum of **9** (not shown) is appearance of a triplet shifted upfield (32.7 ppm) from the signal corresponding to C-4 (32.9 ppm). The multiplicity of the C-2 signal is due to ^{13}C–^2H coupling, while the upfield shift is consistent with a deuterium isotope shift.[11]

Configurational Assignments of (*R*)- and (*S*)-[2-$^2H_1$]Glutaric Acids. The circular dichroism (CD) spectrum of (*S*)-**10** shows a positive n to π^* Cotton effect at 210 nm.[12] The chiroptical properties of (*S*)-**10** were compared with those of (*S*)-[2-$^2H_1$]-carboxylic acids. Previous work has shown that the 2-deuteriocarboxylic acids exhibit a positive n to π^* Cotton effect.[13] The CD behavior of (*S*)-**10** is entirely consistent with the correlations observed for (*S*)-[2-$^2H_1$]succinic acid, (*S*)-[2-$^2H_1$]propionic acid, (*R*)-[2-$^2H_1$]glycine, and (*R*)-[2-$^2H_1$]glycolic acid. Hence, the configurations of **10** (and therefore **9**) shown in Scheme III are judged to be correct.[14]

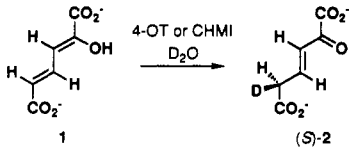
Stereospecificity of 4-Oxalocrotonate Tautomerase. The stereochemical analysis of 4-OT was based on the enzymatic and chemical conversion of **1** in ^2H_2O to a stereospecifically monodeuteriated glutarate (Scheme IV). In ^2H_2O, in the presence of 4-OT, ketonization of **1** afforded [5-$^2H_1$]**2**.[2] A sufficient quantity of enzyme was used in order to minimize the formation of 2-oxo-4-hexenedioate (**5**)—resulting from the nonenzymatic ketonization of **1**.[2] Reduction of 5-[$^2H_1$]**2** by $NaBH_4$ made C-5 of the resulting [5-$^2H_1$]**11** nonracemizable.[2] The reduced product **11** was the major product isolated by anion exchange chromatography as determined by ^1H NMR spectroscopy.[15] Our strategy for the stereochemical analysis of **11** was based on its further chemical conversion to the monodeuteriated glutarate **10**. The fully saturated compound, [5-$^2H_1$]**12**, was obtained by the hydrogenation of **11** in the presence of Wilkinson's catalysts.[16] Homogeneous catalytic hydrogenation prevents the scrambling of the allylic hydrogens which would lead to loss of the chirality at C-5 of **11**.[16] Oxidative decarboxylation of [5-$^2H_1$]**12** by lead tetraacetate resulted in the formation of [5-$^2H_1$]**13**.[17] Glutaric semialdehyde, which was not isolated, was subjected to further oxidation by potassium permanganate to afford the monodeuteriated glutarate **10**.[18]

Table II. Molar Ellipticity of 2-Deuterioglutaric Acids Derived From Stereoselective Synthesis and Enzymatic Reactions

source of (*S*)-[2-$^2H_1$]glutaric acid	$[\theta]_{210}$[a] (deg)
synthetic sample	+108
4-OT from *P. putida mt-2*	+183
CHMI from *E. coli C*	+179

[a] Circular dichroic spectra were recorded in 95% ethanol on a Jasco J-20A automatic recording spectropolarimeter at 25 °C.

Scheme V



The purified [2-$^2H_1$]glutaric acid derived from the above procedure was analyzed by circular dichroism (Table II). Glutaric acid generated in ^2H_2O using 4-OT from *P. putida* exhibits a positive Cotton effect in its circular dichroism spectrum, with a molar ellipticity $[\theta]_{210}$ = +183° at 25 °C. As indicated in Table II, synthetic (*S*)-[2-$^2H_1$]glutaric acid also exhibits a positive Cotton effect, with a molar ellipticity $[\theta]_{210}$ = +108° at 25 °C.[19] We conclude, therefore, that the monodeuteriated glutarate derived from our enzymatic and chemical reaction is also the *S* isomer. Thus, the product of the 4-OT reaction was (*S*)-2-oxo-3-(*E*)-[5-$^2H_1$]hexenedioate (**2**, Scheme V).

Stereospecificity of 5-(Carboxymethyl)-2-Hydroxymuconate Isomerase. The stereochemical analysis of CHMI was also based on the enzymatic and chemical conversion of **1** in ^2H_2O to a stereospecifically monodeuteriated glutarate (Scheme IV). However, CHMI was used in place of 4-OT. Accordingly, the presence of CHMI resulted in the ketonization of **1** to [5-$^2H_1$]**2**. Subsequently, the product was trapped and processed to monodeuteriated glutarate **10** using the reactions described above.

Purified [5-$^2H_1$]glutaric acid derived from the above procedure was analyzed by circular dichroism (Table II). We have determined that the glutaric acid generated using CHMI from *E. coli*

(11) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy: High Resolution Methods and Applications in Organic Chemistry and Biochemistry*; VCH: New York, 1987; p 147.

(12) (*R*)-**10** shows a CD curve which is the mirror image of (*S*)-**10**.

(13) Craig, J. C.; Lee, S.-Y. C.; Fredga, A. *Tetrahedron* **1977**, *33*, 183–190. (b) Ringdahl, B.; Craig, J. C.; Keck, R.; Retey, J. *Tetrahedron Lett.* **1980**, *21*, 3965–3968 and references cited within.

(14) These correlations are valid only if the conformation of (*S*)-**10** is the same as that determined for the other 2-deuteriocarboxylic acids. Several studies indicate that glutaric acid does have the same conformation. Glutaric acid exists in the solid state with the C_2–C_3 bond being syn periplanar with the carbonyl group: (a) Morrison, J. D.; Robertson, J. M. *J. Chem. Soc.* **1949**, 1001–1008. (b) Kanters, J. A.; Kroon, J.; Peerdeman, A. F.; Schoone, J. C. *Tetrahedron* **1967**, *23*, 4027–4033. The results from CD studies on 2-substituted glutaric acids indicate that this conformation prevails for glutaric acid in solution: (c) Listowsky, I.; Avigad, G.; Englard, S. *J. Org. Chem.* **1970**, *35*, 1080–1085. (d) Korver, O.; Sjoberg, S. *Tetrahedron* **1975**, *31*, 2603–2606.

(15) The other product present is 2-hydroxy-4-(*E*)-hexenedioate. This compound results from the $NaBH_4$ reduction of **5**. The unconjugated ketone present results from the nonenzymatic ketonization of **1** as discussed elsewhere.[2]

(16) Osborne, J. A.; Jardine, F. H.; Young, J. F.; Wilkinson, G. *J. Chem. Soc. A* **1966**, 1711–1732.

(17) Pocker, Y.; Davis, B. C. *J. Am. Chem. Soc.* **1973**, *95*, 6216–6223.

(18) Chickos, J. S.; Bausch, M.; Alul, R. *J. Org. Chem.* **1981**, *46*, 3559–3562.

(19) The higher molar ellipticity value measured for the enzyme generated sample relative to that measured for the synthetic sample presumably results from the enzyme's high degree of stereospecificity.[22] Based on the estimated optical purity of the synthetic sample (60–65%), the sample derived from enzymatic reaction is greater than 99% optically pure.

C yields a positive Cotton effect in its circular dichroism spectrum, with a molar ellipticity $[\theta]_{210} = +179°$ at 25 °C. As indicated in Table II, synthetic (*S*)-[2-$^2H_1$]glutaric acid also exhibits a positive Cotton effect with a molar ellipticity $[\theta]_{210} = +108°$ at 25 °C. We conclude, therefore, that the monodeuteriated glutarate derived from our enzymatic and chemical reaction is also the *S* isomer. Thus, the product of the CHMI reaction was (*S*)-2-oxo-3-(*E*)-[5-$^2H_1$]hexenedioate (**2**, Scheme V).

Two experiments rule out the possibility that the preparation of CHMI was contaminated by 4-OT. First, the isolated protein migrates as a single band on SDS-PAGE with an apparent subunit molecular weight of 14000.[4] 4-OT migrates on SDS-PAGE with an apparent subunit molecular weight of 3600.[7] The two bands are readily discernable. Second, two enzymes operating on the same substrate present a characteristic kinetic pattern readily apparent in the Michaelis–Menten and Lineweaver–Burke plots.[20] We were able to produce this pattern using CHMI intentionally contaminated with 4-OT (data not shown). The preparation of CHMI used in these stereochemical experiments did not show this pattern.

Discussion

Our results clearly indicate that 4-OT and CHMI both convert 2-hydroxymuconate into (*S*)-2-oxo-3-(*E*)-[5-$^2H_1$] hexenedioate by the stereospecific incorporation of a solvent deuteron. These findings are fully consistent with the working hypothesis for the mechanisms of 4-OT and CHMI. In addition, the experimental outcome suggests mechanistic similarities between 4-OT and CHMI. Finally, these results may have implications for the evolutionary origin of these two enzymes as well as the hypotheses for the evolution of catabolic pathways.

We have also demonstrated that 4-OT and CHMI process either substrate although at different rates—a reflection of their precisely tailored active sites. It is also apparent from the data that each enzyme has a clear preference for its physiological substrate. Previous reports that **1** was not a substrate for CHMI were puzzling becasue the binding cavity at the active site of CHMI is presumably larger than the binding cavity at the active site of 4-OT. Hence, **1** should "fit" in the active site of CHMI, while **3** may be excluded from the active site of 4-OT by steric hindrance and charge repulsion.[21] Our data are in accord with this analysis. The absence of the carboxymethyl group on the smaller substrate **1** precludes some binding interactions with CHMI which is reflected in the higher value of K_M and the lower value of k_{cat}. However, the consequences of putting a smaller substrate into a larger cavity are not as severe as the consequences of putting a larger substrate into a smaller cavity. The active site of 4-OT clearly discriminates against **3**, as reflected by the 10^5-fold decrease in k_{cat}/K_M. The bulk of the carboxymethyl group, coupled with its additional charge, can easily account for the much higher value of K_M and the much lower value of k_{cat}.

No reason for the discrepancy between our work and the earlier work indicating that **1** is not a substrate for CHMI is readily apparent.[8] It should, however, be noted that in these earlier experiments an ammonium sulfate fraction was used as the source of CHMI.[8] In addition, the assays were carried out in 0.1 M phosphate buffer.[8] We have observed that in the early steps of the CHMI purification process including the ammonium sulfate fractionation step a significant amount of extract is required to generate a perceptible rate of turnover of **1**. Moreover, we have reported that the ketonization of **1** can be catalyzed by phosphate buffer.[2] Perhaps the high background rate of nonenzymatic ketonization of **1** precluded observation of the much slower enzyme-catalyzed reaction.

Our findings also have implications for the mechanisms of catalysis by 4-OT and CHMI. Kinetic studies suggest that 4-OT acts as an isomerase, transforming a β,γ-unsaturated ketone **5** to its α,β-isomer **2** through a dienol intermediate (**1**, Scheme II).[2] A similar mechanism for CHMI can be envisioned in which the enzyme converts **6** to **4** through **3** (Scheme II). The kinetic analysis also indicates that the 4-OT-catalyzed conversion of dienol **1** to product **2** occurs more slowly than does the corresponding conversion of **5** to **2**.[2] The lower rate of conversion observed for the dienol raises the question of whether the dienol is kinetically competent to be an intermediate in the overall reaction.[2] The observation of stereospecific ketonization of **1** to [5-$^2H_1$]$_2$ by 4-OT is compelling evidence that this reaction is enzyme-catalyzed and that **1** is bound and processed by 4-OT.[22] Additionally, these results imply that **3** is processed stereospecifically by CHMI. The steric course of the reaction catalyzed by CHMI is under investigation.

Stereospecific proton transfer has generally been observed in enzymes catalyzing the ketonization of a dienol or an enol intermediate.[23] The example most relevant to this discussion is 3-oxo-Δ^5-steroid isomerase (KSI), which catalyzes the interconversion of the β,γ-unsaturated ketone 5-androstene-3,17-dione to its α,β-isomer, 4-androstene-3,17-dione.[24] There is a considerable body of evidence indicating that the reaction proceeds through a dienol intermediate, including the demonstration that the dienol 3-hydroxy-3,5-androstadiene-17-one is converted to product at a rate comparable to that measured for the overall reaction.[24,25] Moreover, the dienol is stereospecifically protonated, and protonation occurs on the same face as does protonation in the KSI catalyzed conversion of the unconjugated ketone to its conjugated isomer.[25] Although an analogous conclusion cannot yet be drawn for 4-OT and CHMI because the overall stereochemical courses of these reactions are not known, stereospecific protonation of the intermediate dienol **1** by each enzyme is consistent with the proposed isomerase mechanism. The observation that **1** is not kinetically competent in the overall reaction can be explained by two possibilities.[2] The dienol present in solution may either have a different double-bond configuration or a different state of protonation than the dienol generated at the active site of the enzyme. Alternatively, the catalytic groups on the enzyme may not be in the appropriate protonation state for the turnover of **1**.[2]

The central observations of the present work are that 4-OT and CHMI bind and process the same dienol, **1**, and that the ketonization of **1** occurs with the same steric course. The simplest explanation for this result is that the deuteron in the 5 position of **2** arises via transfer from a residue at the active site having exchangeable protons. The fact that the steric course of solvent incorporation is the same for the two enzymes may suggest that this hypothetical residue occupies the same position relative to the bound substrate in both enzymes. The similar net reactions, the common stereochemical courses of protonation, and the observation that 4-OT and CHMI bind and process **1** to generate the same product all hint at mechanistic similarities between the two enzymes. The major differences between the mechanisms are presumably the interactions associated with the differential binding of the two dienols.

These results are not surprising since the two proteins have evolved to bind and ketonize nearly identical dienols. Moreover,

(20) Segel, I. H. *Enzyme Kinetics*; John Wiley & Sons: New York, 1975; pp 64–71. A reviewer has also pointed out that if a 4-OT impurity were the cause of CHMI activity, then the K_M's would be the same for both systems. It should also noted that 4-OT activity has never been reported in *E. coli C.*

(21) Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: San Francisco, CA, 1985; pp 347–350.

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neither enzyme requires a cofactor or metal ion, a fact that is consistent with the suggestion that the two enzymes catalyze proton transfer by a common mechanism. Whether or not there are structural similarities between the two active sites as well as common catalytic groups remains to be demonstrated.

Our results are not unprecedented. There are several examples of families of enzymes that catalyze the same chemical reaction on different substrates. Notable examples include the serine proteases,[26] malate and lactate dehydrogenase,[27] and the penicillin binding proteins and the β-lactamases.[28] The most extensively studied examples are found in the two families of the serine proteases: the trypsin family (trypsin, chymotrypsin, and elastase)[29] and the subtilisin family.[30] The major catalytic features in the active sites of these serine proteases are conserved (the catalytic triad of Asp-His-Ser and the oxyanion binding site) although they are located in different sequential positions.[31] The differences among the active sites of these proteins are found in the specificity pockets, which account for the individual enzymes' substrate preferences.[30-32]

It remains an open question whether the observed similarities between 4-OT and CHMI are the result of divergence from a common ancestor or whether they represent convergence on a structure ideally suited to this reaction type. With regard to the evolution of the catechol and homoprotocatechuate pathways, it has long been speculated that the enzymes within each pathway have a common ancestral gene.[4,6] Convergent evolution implicates two separate progenitors giving rise to the individual enzymes of each pathway by gene duplication and subsequent metabolic diversification.[6,33] Divergent evolution suggests that an enzyme having broad substrate specificity gave rise to 4-OT and CHMI activities,[34] the progenitor presumably having been "recruited" from an existing pathway. Subsequent mutation would lead to progeny with greater specificity. It will be necessary to determine the secondary and tertiary structures of both enzymes and to identify the amino acid residues involved in binding and catalysis before these questions can be satisfactorily answered. The appropriate mechanistic and structural studies are in progress.

Experimental Section

Materials. All chemicals and solvents were purchased from Aldrich Chemical Co. with the following exceptions. (*R*)- And (*S*)-2-hydroxyglutarate (disodium salts) were obtained from Sigma Chemical Co. 2-Hydroxymuconate was prepared according to Lapworth,[35] and 5-(carboxymethyl)-2-hydroxymuconate was isolated according to the procedure described elsewhere.[4] Biochemicals and buffers were obtained from Sigma Chemical Co. Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. 4-Oxalocrotonate tautomerase was purified according to published procedures.[2,7]

Methods. Protein concentrations were determined using the commercially available bicinchoninic (BCA) protein assay kit (Pierce Chemically Co., Rockford, IL). HPLC was performed on a Waters system using a Waters Protein Pak DEAE 5PW anion-exchange column (10-μm particle size), a Bio-Gel Phenyl 5-PW hydrophobic column, or a Pharmacia Superose 12 (HR 10/30) gel filtration column. Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) under denaturing conditions was performed on 15% gels as described elsewhere.[2,7] Kinetic data were obtained on a Perkin-Elmer fast scan UV/vis spectrophotometer Model 553.

NMR spectra were obtained on a Bruker AM-250 spectrometer, a Bruker AM-500 spectrometer, or a General Electric QE-300 spectrometer, as indicated. Chemical shifts were standardized to the $^1H^2HO$ resonance at 4.70 ppm. Optical rotation values (α) were measured at the D line of sodium on a Perkin-Elmer 241 MC polarimeter in a 1-dm cell. The reported concentrations are expressed in g/100 mL. Circular dichroic measurements were carried out in 95% ethanol on a Jasco J-20A automatic recording spectropolarimeter at room temperature. The molar ellipticities are reported in millidegrees.

(*S*)-(−)-2-Hydroxyglutarate, Dimethyl Ester (7). A solution of the disodium salt of (*S*)-(−)-2-hydroxyglutarate (2 g, 10.4 mmol) in water (20 mL) was subjected to chromatography on a Dowex-1 (potassium) column (21 × 2 cm), eluted with water (150 mL). The resulting solution containing the dipotassium salt was evaporated to dryness under mechanical vacuum. The syrupy residue was mixed with 18-crown-6 (5.51 g, 20.8 mmol), and the mixture was dissolved in water (20 mL). After evaporating the solution to dryness, the crystalline residue was suspended in freshly distilled tetrahydrofuran (40 mL), and iodomethane (16.5 mL, 265 mmol) was added. After being stirred at room temperature overnight, the homogeneous yellow solution was concentrated to dryness, and the residue was washed with copious amounts of ether and filtered. The filtrate was dried over anhydrous $MgSO_4$ and filtered, and the filtrate was evaporated to dryness. The residual yellow oil was purified by flash chromatography (50% hexanes, 50% ethyl acetate) to yield **7** in 74% yield (1.36 g): $[\alpha]_D = -1.48°$ (neat) (lit.,[9] −2.48°). Based on the reported optical rotation, the optical purity is estimated as 60%. The ^1H NMR spectrum ($CDCl_3$, 250 MHz) corresponded to that reported for the racemic alcohol.[9]

Dimethyl (*R*)-(+)-2-hydroxyglutarate was prepared from the disodium salt of (*R*)-(+)-2-hydroxyglutarate (2 g, 2.6 mmol) by the above procedure to yield 1.13 g (62%) of product: $[\alpha]_D = 1.6°$ (neat) (lit.[9] = 2.48°). Based on the reported optical rotation, the optical purity is estimated as 65%. The ^1H NMR spectrum ($CDCl_3$, 250 MHz) corresponded to that reported for the racemic alcohol.[9]

(*S*)-(−)-2-Tosylglutarate, Dimethyl Ester (8). To an ice-cooled solution of **7** (1.36 g, 7.7 mmol) in dry pyridine (7 mL) was added *p*-toluenesulfonyl chloride (1.91 g, 10 mmol) in portions over a 1-h period. After the mixture had been stirred overnight at 4°, it was poured into ice water and extracted with ether. The ether extract was washed with water, aqueous $CuSO_4$, and aqueous $NaHCO_3$ and dried over $MgSO_4$. Following filtration, the filtrate was concentrated to dryness to yield the crude product. The resulting oil was purified by flash chromatography (50% hexanes, 50% ethyl acetate) to yield a solid in 81% yield (2.07 g): ^1H NMR ($CDCl_3$, 250 MHz) δ 2.00–2.30 (2 H, br d m), 2.38 (2 H, t), 2.46 (3 H, s), 3.64 (3 H, s), 3.66 (3 H, s), 4.95 (1 H, dd, J = Hz), 7.35 (2 H, d, J = 7.14 Hz), 7.80 (2 H, d, J = 7.14 Hz); $[\alpha]_D = -40°$ (*c* 0.91, ethanol). Anal. Calcd for $C_{14}H_{18}O_7S$: C, 50.9; H, 5.49; S, 9.70. Found C, 50.65; H, 5.58; S, 9.75.

(*R*)-(+)-2-Tosylglutarate, dimethyl ester was synthesized from (*R*)-(+)-2-hydroxyglutarate, dimethyl ester in an analogous manner to yield 1.17 g (76%) of the pure tosylate, which solidified upon standing. The ^1H NMR spectrum ($CDCl_3$, 250 MHz) corresponded to that reported for its enantiomer.

(*R*)-[2-$^2H_1$]Glutarate, Dimethyl Ester (9). To a 50-mL, three-necked round-bottomed flask equipped with a reflux condenser, drying tube, N_2 inlet, and N_2 outlet was added a mixture of **8** (0.9 g, 2.7 mmol) and NaB^2H_3CN (0.7 g, 10.6 mmol, 98% atom D) in freshly distilled HMPA (4.5 mL). The solution was heated at 100 °C for 23 h under N_2. After the mixture had cooled to room temperature, it was added to H_2O (50 mL) and extracted with ether (2 × 50 mL). The combined ethereal extracts were dried over $MgSO_4$ and filtered, and the filtrate was evaporated to dryness, to yield a mixture of starting material and product (0.44 g) as an oil.

The product mixture was dissolved in 10% (w/w) portions with 60% ethyl acetate 40% hexanes and injected in 1-mL aliquots onto a silica gel Lobar Size B column. Low-pressure liquid chromatography first yielded **9**, with a retention time of 95 min, followed by **8**, with a retention time of 124 min. Fractions containing the same compound were combined and evaporated to dryness, to yield 25% of the desired product (111 mg): ^1H NMR ($CDCl_3$, 500 MHz) δ 1.92 (2 H, q br d t), 2.34 (1 H, tt), 2.38 (2 H, t), 3.65 (6 H, s); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 19.9 ($-OCH_3$), 32.7 (C-2, t), 32.9 (C-4), 51.5 (C-3), 173.2 (C-1, C-5).

(*S*)-[2-$^2H_1$]Glutarate, dimethyl ester was synthesized from (*R*)-(+)-2-tosylglutarate, dimethyl ester by the foregoing procedure to yield

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0.26 g (44%) of the desired product: $[\theta]_{228}$ = 400°. The ^{1}H NMR spectrum corresponded to that reported for its enantiomer. Anal. Calcd for $C_7H_{12}{}^2HO_4$: C, 52.17; H, 6.89.[36] Found C, 51.84; H, 7.29.

(*R*)-[2-$^2H_1$]Glutaric acid (10) was prepared by saponification of **9** (47 mg, 0.3 mmol) by using a solution of NaOH (28 mg, 0.7 mmol) in 5/1 methanol/water (5 mL) stirred at room temperature for 1.5 h. After evaporation of the solvent, the residue was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 7.5 cm) eluted with a formic acid gradient (0–2 M formic acid, 60 mL total volume). Appropriate fractions were pooled and evaporated to dryness to yield 38 mg (97%) of product: ^{1}H NMR (CD_3OD, 300 MHz) δ 1.85 (2 H, q), 2.35 (3 H, t).

(*S*)-[2-$^2H_1$]Glutaric acid was prepared by saponification of dimethyl (*S*)-[2-$^2H_1$]glutarate as described above to yield 39 mg (93%) of product: $[\theta]_{210}$ = 108°. The ^{1}H NMR spectrum (CD_3OD, 300 MHz) corresponded to that reported for its enantiomer.

Purification of 5-(Carboxymethyl)-2-hydroxymuconate Isomerase (CHMI) from *Escherichia coli* Strain C. *E. coli* strain C was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT. Cultures of *E. Coli C* were grown at 30 °C in a medium described elsewhere[37] supplemented with 10 mM 4-hydroxyphenylacetate. After growth to late log phase (24 h), cells were centrifuged at 5000 × g for 12 min, collected, and stored at −78 °C. In a typical procedure, frozen cells (30 g) were suspended in 75 mL of 20 mM Na_2HPO_4 buffer (pH 7.4), containing 1 mM 6-aminocaproic acid, 0.5 mM phenylmethylsulfonylfluoride, 50 μM leupeptin, and 0.067 μM aprotonin in order to limit proteolysis. The cells were disrupted at 4 °C with 10 pulses (1 min each) from a Heat Systems W-385 sonicator equipped with a 0.5" tapped horn delivering approximately 330 W per pulse. The solution was centrifuged at 27 000 × g for 30 min to remove cell debris. Nucleic acids were removed by making the supernatant 1 mg/mL in protamine sulfate (3 mL total volume adjusted to pH 7.4) over 10 min at 4 °C. After centrifugation (27 000 × g for 30 min), the supernatant was adjusted to 1.0 M in ammonium sulfate. After being stirred for 30 min, the mixture was centrifuged, and the precipitate was discarded. The supernatant was adjusted to 2.5 M in ammonium sulfate. After the mixture had been stirred for 30 min, the precipitate was collected by centrifugation. The pellet was dissolved in a small amount of 10 mM ethylenediamine buffer (pH 7.4) and desalted by passage through a column of Sephadex G-75 (3.0 × 50 cm) in the same buffer containing 0.02% sodium azide. Active fractions were pooled and concentrated ~5-fold in a 200-mL Amicon filtration cell using a PM-10 membrane.

The retentate was injected onto a Waters Protein Pak (DEAE-5PW) anion exchange column (15 × 2.15 cm) that had been equilibrated with the ethylenediamine buffer described above at a flow rate of 5 mL/min. The column was washed with buffer for 10 min, and elution was carried out with a NaCl gradient (0–0.25 M over 50 min, followed by 0.25–0.5 M over 10 min) in 10 mM ethylenediamine (pH 7.4). Typically, activity was found to elute at the end of the gradient. The eluent was monitored by UV (280 nm), collected in 10-mL fractions, and assayed for activity. The most active fractions were pooled and concentrated to about 10 mL by ultrafiltration in a 50-mL Amicon filtration cell using a PM-10 membrane.

The concentrate was made 2 M in $(NH_4)_2SO_4$ and injected onto a Bio-Gel Phenyl 5-PW (Biorad) column (75 × 7.5 mm) equilibrated at a flow rate of 5 mL/min with 10 mM ethylenediamine buffer (pH 7.3) made 2 M in $(NH_4)_2SO_4$. The column was washed with the equilibrating buffer for 10 min, and elution was carried out using a $(NH_4)_2SO_4$ gradient (2.0–0.76 M over 10 min, followed by 0.76–0 M over 40 min) in the 10 mM ethylenediamine buffer (pH 7.4). Subsequently, the column was eluted isocratically for 20 min. Typically, activity eluted at 0.1–0 M. Active fractions were pooled and concentrated to about 4 mL by ultrafiltration.

After dialysis of the retentate against 10 mM ethylenediamine buffer (pH 7.3) made 0.15 M NaCl, the solution was injected in 0.5-mL portions onto a Superose 12 (Pharmacia) gel filtration column equilibrated with the same buffer at a flow rate of 0.4 mL/min. The eluent was collected in 0.8-mL fractions. Active fractions were pooled, concentrated in a 10-mL Amicon device, and reinjected onto the Superose column, which was eluted as described above. Again, active fractions were pooled and concentrated. Typically, the activity eluted at 33 min. The final specific activity of CHMI ranged from 1600 to 2500 units/mg. Typically, 200–600 μg of protein was obtained from 60 g of frozen cells. A unit of enzyme produces 1 μmol of **4**/min at pH 7.3 at 30 °C.[5]

Polyacrylamide gel electrophoresis under denaturing conditions was performed on 15% gels as described.[2,7] CHMI migrates as a single band with a M_r of 14 000.

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Stereospecific Ketonization of 1 to 2 by 4-OT and Conversion of 2 to 2-Hydroxy-3-(*E*)-Hexenedioate (11). 2-Hydroxymuconate (**1**, 158 mg, 1.0 mmol) in ethanol-d_5 (15.6 mL) was added in portions (13–1.2 mL portions) to a stirring solution containing 4-OT from *P. Putida* (~1000 units) in 20 mM K[$^2H_2$]PO_4 (130 mL, pD 7.44) in ^2H_2O. Solutions of 4-OT had previously been exchanged by repeated dilution and concentration of the enzyme in ^2H_2O in a Centricon-10 microconcentrator and stored overnight in ^2H_2O. The ketonization process for each 1.2-mL aliquot was monitored spectrophotometrically (λ = 295 nm) and was complete in about 2 min. The mixture was treated with $NaBH_4$ (2 equiv) at two intervals—after half of the solution of **1** (7.8 mL) had been added and after all of the solution has been added. The final pH of the solution was 9.6 (pD 10.0). The solution was evaporated to dryness under mechanical vacuum, and the residue was dissolved in H_2O (100 mL). After the pH had been adjusted to 7.8, the solution was subjected to chromatography on a Dowex-1 (formate) column (20 × 2.0 cm), eluted with a linear gradient of aqueous formic acid (0–2 M, 500-mL total volume). The product eluted at about 1.4–1.6 M formic acid . Appropriate fractions were pooled and evaporated to dryness under mechanical vacuum to yield 131 mg (81%) of [5-$^2H_1$]**11** as the major product.[2,15]

Stereospecific Ketonization of 1 to 2 by CHMI and Conversion of 2 to 2-Hydroxy-3-(*E*)-Hexenedioate (11). 2-Hydroxymuconate (**1**, 139.5 mg, 0.88 mmol) in ethanol-d_5 (10 mL) was added in portions (10–1.0 mL portions) to a stirring solution containing CHMI from *E. coli C* (1500 units) in 50 mM K[$^2H_2$]PO_4 (120 mL, pD 7.62) in ^2H_2O. The solution of CHMI had previously been exchanged by repeated dilution and concentration of the enzyme in ^2H_2O in a Centricon-10 microconcentrator and stored overnight in ^2H_2O. The ketonization process was monitored spectrophotometrically (λ = 295 nm) and was complete in about 30 min. The mixture was treated with $NaBH_4$ as described above. The final pH of the solution was 9.5 (pD 9.9). The solution was evaporated to dryness under mechanical vacuum. The residue was dissolved in H_2O (100 mL), and the pH was adjusted to 8.0. The resulting solution was subjected to Dowex-1 (formate) chromatography as described above to yield 106 mg (75%) of [5-$^2H_1$]**11** as the major product.[2,15]

Catalytic Reduction of 11. To a hydrogenation bottle containing a solution of **11** (131 mg, 0.8 mmol) in 1:1 ethanol/benzene (20 mL) was added $(Ph_3P)_3RhCl$ (11 mg, 0.011 mmol). After the bottle had been flushed with H_2, the mixture was shaken vigorously under H_2 (40 lbs/in^2) in a Parr apparatus at 25 °C for 21 h. The resulting yellow solution was filtered, and the filtrate was evaporated to dryness to yield **12** as the major product (120 mg, 90%): ^{1}H NMR (CD_3OD, 250 MHz) δ 1.60–2.00 (4 H, brd m), 2.34 (2 H, brd m), 4.20 (1 H, brd d); ^{13}C (^2H_2O, 500 MHz) δ 22.5 (C-4), 35.2 (C-3), 35.9 (C-5), 72.5 (C-2), 180.5, 180.9 (C-1, C-6).

Conversion of 12 Derived from the 4-OT Reaction to 2-[$^2H_1$]Glutaric Acid (10). To a stirring solution of **12** (120 mg; 0.7 mmol) in 2 M acetic acid (1.8 mL) was added a solution of lead tetraacetate (0.36 g; 0.8 mmol) in 2 M acetic acid (3.6 mL). After stirring at room temperature for 2 h, the mixture was filtered, and the resulting yellow filtrate was evaporated to dryness. The solid residue was dissolved in 2 M H_2SO_4 (5.4 mL) and oxidized by adding solid potassium permanganate until the dark solution retained by violet color when spotted on filter paper. The reaction mixture was stirred at room temperature for 2 h. Subsequently, 10% $NaHSO_3$ was added until the mixture was clear, and then the solution was extracted repeatedly with ether. The ethereal extracts were combined, dried over $MgSO_4$, filtered, and evaporated to dryness. The crude, solid glutaric acid was purified by chromatography on a Dowex-1 (formate) column (0.8 × 7.5 cm) and eluted with a formic acid gradient (0–2 M formic acid, 60 mL total volume). Glutaric acid eluted at 1.0–1.2 M formic acid. Appropriate fractions were pooled and evaporated to dryness under mechanical vacuum to yield 25 mg (26%) of **10**: $[\theta]_{210}$ = +183°. The ^{1}H NMR spectrum (CD_3OD, 300 MHz) for [2-$^2H_1$]**10** derived from the stereospecific ketonization of **1** by 4-OT corresponded to the spectrum reported above for the sample generated from a stereoselective synthesis.

Conversion of 12 Derived from the CHMI Reaction to [2-$^2H_1$]Glutaric Acid (10). Using the procedure described above, **12** (108 mg, 0.67 mmol) was converted to 13.9 mg (16%) of **10**: $[\theta]_{210}$ = +179°. The ^{1}H NMR spectrum (CD_3OD, 250 MHz) for 2-[$^2H_1$]**10** derived from the stereospecific ketonization of **1** by CHMI corresponded to the spectrum reported above for the sample generated from a stereoselective synthesis.

Kinetic Parameters and Substrate Specificities of 4-OT and CHMI. In the course of purification, 4-OT and CHMI were assayed spectrophotometry at 30 °C by following the rate of disappearance of substrate **1** ($\epsilon = 24.1 \times 10^3$ M^{-1} cm^{-1} at 295 nm) or the rate of appearance of product **2** ($\epsilon = 6.58 \times 10^3$ M^{-1} cm^{-1} at 236 nm).[2] The kinetic parameters reported for 4-OT and CHMI utilizing **1** were obtained by following the rate of appearance of product (**2**) using the nonequilibrium technique described elsewhere.[2] The kinetic parameters reported for CHMI utilizing **3** were

obtained by following the rate of appearance of product **4** (ϵ = 7.07 × 10^3 M^{-1} cm^{-1} at 236 nm) using the nonequilibrium technique discussed elsewhere.[2] Stock solutions of **1** and **3** in ethanol were made up just prior to the start of the experiment. Detectable decompsition of these solutions was not observed during the lifetime of an experiment. Dilutions of 4-OT were made 2 h prior to the start of an experiment; otherwise inconsistent results were obtained.[2] No significant inhibition of the enzymes by ethanol was observed at ethanol concentrations below 2.5% (v/v). All results were reproducible in multiple runs. The kinetic data were fitted by nonlinear regression data analysis with Enzfitter (Elsevier Science Publishers, Amsterdam).

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Rate of Decarboxylation, Monitored via the Key Enzyme-Bound Enamine, of Conjugated α-Keto Acids by Pyruvamide Activated Pyruvate Decarboxylase Is Kinetically Competent with Turnover

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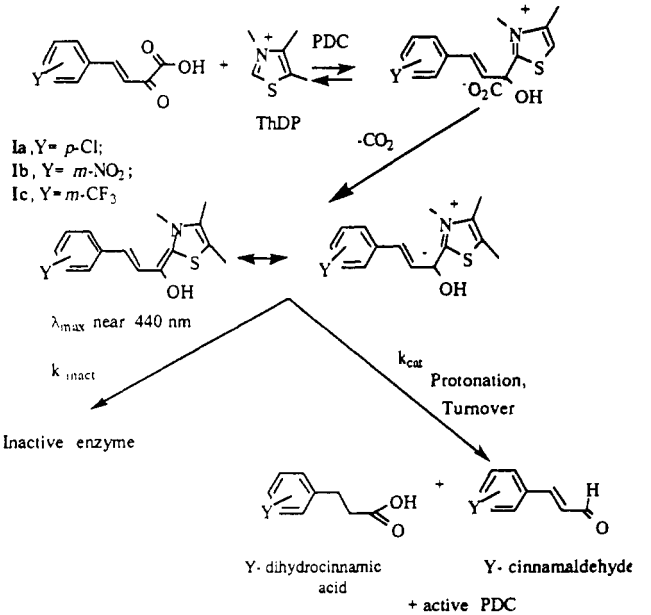
Abstract: The rate of formation of the covalent thiamin diphosphate-bound enamine/C2-α-carbanion intermediate monitored at 440 nm from conjugated mechanism-based inhibitors of the structure (*E*)-$YC_6H_4CH{=}CHCOCOOH$, where Y = *p*-Cl, *m*-NO_2, *m*-CF_3, was determined in the absence and presence of the allosteric activator pyruvamide on brewers' yeast pyruvate decarboxylase (PDC, E.C. 4.1.1.1). For all three compounds the first-order rate constant for enamine formation was accelerated from 15–150-fold by conversion of the enzyme to its activated form. The rate constant for enamine formation is 10^2–10^3 times faster than those estimated for inactivation. Comparing the k_{cat} (0.44 s^{-1}) to the rate constant for decarboxylation (0.653 s^{-1}) for Y = *p*-Cl leads one to conclude that enamine formation is kinetically competent to participate in the turnover pathway. Based on the maximum absorbance developed at 440 nm, and the ϵ = 10^4 at this wavelength for a model compound (Y = H), there appear to be four active sites per tetrameric holoenzyme. The k_{cat}/active site for pyruvate is estimated at ca. 40 s^{-1} at 20 °C, and the decarboxylation rate constant for pyruvate can be estimated to be 80 s^{-1}/active site at 20 °C, assuming decarboxylation and product release are equal in kinetic significance. The rate constants for decarboxylation by activated PDC for Y = *m*-NO_2, *m*-CF_3 (53 and 69 s^{-1}) are comparable to this estimated decarboxylation rate constant for pyruvate (80 s^{-1}). The *k*'s for Y = *m*-NO_2, *m*-CF_3 are also similar in magnitude to the decarboxylation rate constant (62–80 s^{-1} at 22 °C) reported for pyruvate oxidase, an enzyme with considerable sequence homology to PDC, and one that follows the same mechanism through decarboxylation.

Introduction

Quantitative aspects of substrate activation of brewers' yeast pyruvate decarboxylase (PDC, EC 4.1.1.1) were recently reported based on a variety of kinetic measurements.[1] It had also been demonstrated that pyruvamide, a nondecarboxylatable pyruvate surrogate is capable of fully activating PDC.[2] Evidence from this laboratory has been presented concerning the putative thiamin diphosphate (ThDP)-bound enamine/C2-α-carbanion intermediate that accumulates and is detectable on PDC (with a λ_{max} near 440 nm, Scheme I) when derived from conjugated α-keto acids with the basic structure of (*E*)-$YC_6H_4CH{=}CHCOCOOH$ (**I**), where Y is a *p*- or *m*-substituent.[3] Synthesis of an appropriate model compound for such enamines with identical absorption maximum gave support for the structure of the PDC-bound enamine intermediate.[4] Kinetic studies of the rate of formation of the enamine in the absence and presence of pyruvamide would provide direct evidence of enzyme activation and the PDC catalyzed decarboxylation process.

We report pre-steady-state kinetic results on the formation of the enamine intermediate on PDC from three conjugated substrate analogs (**Ia**, Y = *p*-Cl; **Ib**, Y = *m*-NO_2; **Ic**, Y = *m*-CF_3) in the absence and presence of saturating pyruvamide. These analogs are known to be mechanism-based inactivators (they are decarboxylated and also inhibit the enzyme),[5,6] and we hypothesized

Scheme I. Reactions of (*E*)-2-Oxo-4-phenyl-3-butenoic Acids with Pyruvate Decarboxylase



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