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The Contribution of the Substrate's Carboxylate Group to the Mechanism of 4-Oxalocrotonate Tautomerase[1]

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4-Oxalocrotonate tautomerase (4-OT) converts 2-oxo-4*E*-hexenedioate (**1**) to 2-oxo-3*E*-hexenedioate (**3**) through the dienol intermediate, 2-hydroxy-2,4-hexadiene-1,6-dioate (**2**). Previous studies established that the isomerization of **1** to **3** is primarily a suprafacial process. It was also suggested that the 6-carboxylate group of the substrate maintains the regio- and stereochemical fidelity of the reaction by anchoring the substrate at the active site. A subsequent study suggested an additional role for the 6-carboxylate group in the mechanism: the enzyme may utilize the binding energy of the carboxylate group to facilitate catalysis. In order to explore the role of the carboxylate group in the mechanism further, the nonenzymatic rate constants for mono- and dicarboxylated substrates were measured and compared to the rates obtained for the corresponding enzymatic reactions. The results show that the missing carboxylate group has a profound effect on enzymatic catalysis as evidenced by the significant decreases (a 10^4- and a 10^5-fold reduction) in the values of k_{cat}/K_m observed for the two monocarboxylated substrates. A comparison of the nonenzymatic rate constants indicates that the reduced k_{cat}/K_m values cannot be explained on the basis of the chemical reactivities. The stereochemical course of the 4-OT-catalyzed reaction was also determined using 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate. The stereochemical analysis reveals that the presence of the carboxylate group improves the stereoselectivity of the enzyme-catalyzed ketonization of 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate to 2-oxo-[3-^{2}H]-4*Z*-heptene-1,7-dioate in ^2H_2O—a result that is consistent with its previously assigned role. These findings provide further evidence that the substrate's carboxylate group contributes to the mechanism of the enzyme in two ways: it anchors the substrate at the active site and it facilitates catalysis by destabilizing the substrate or by stabilizing the transition state.

4-Oxalocrotonate tautomerase (4-OT, EC 5.3.2) catalyzes the isomerization of unconjugated α-keto acids such as 2-oxo-4*E*-hexenedioate (**1**) to its conjugated isomer, 2-oxo-3*E*-hexenedioate (**3**) through the dienol intermediate 2-hydroxy-2,4-hexadiene-1,6-dioate (**2**) (Scheme 1) (*1–3*). The enzyme is elaborated by the soil bacterium *Pseudomonas putida mt-2* as part of a degradative pathway that converts various aromatic hydrocarbons to intermediates in the Krebs cycle (*4*). The entire pathway is encoded by the TOL plasmid and enables bacterial strains harboring this plasmid to use aromatic compounds as their sole sources of carbon and energy (*4*).

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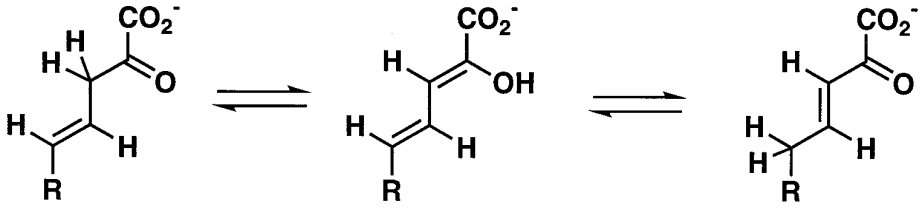
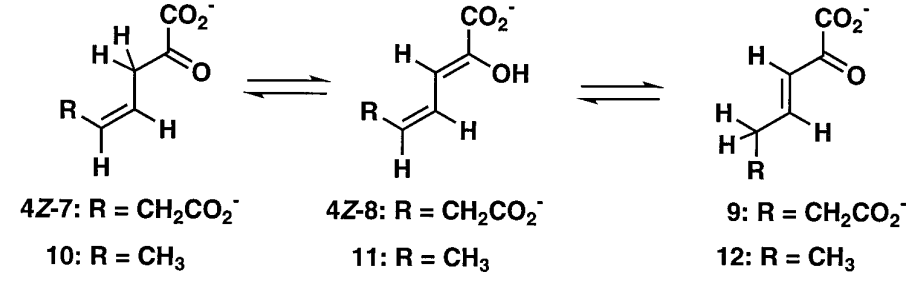


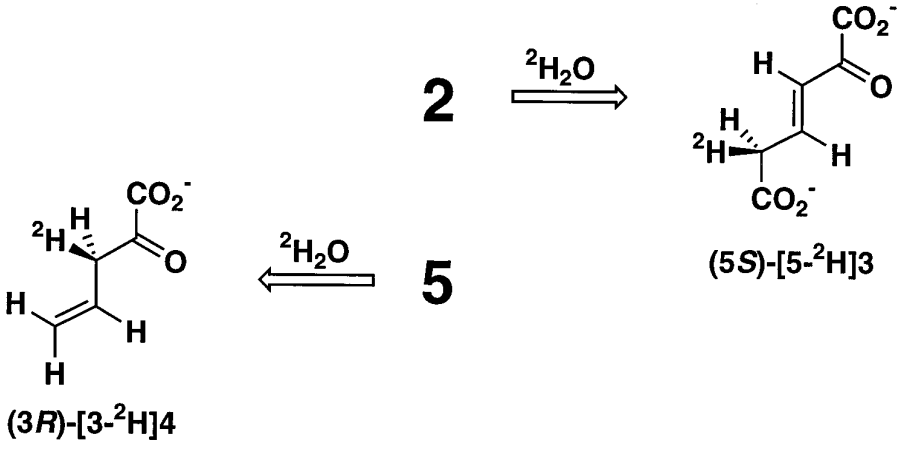
SCHEME 1

The substrate for the enzyme, **1,** cannot be synthesized or isolated—it exists in rapid equilibrium with **2** (*1*). Hence, kinetic, stereochemical, and mechanistic experiments utilize the observation that **2** and the related dienols [2-hydroxy-2,4-pentadienoate, (**5**), 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate (**8**), and 2-hydroxy-2,4-hexadienoate (**11**); Schemes 1 and 2] are partitioned by the enzyme to their respective β, γ- and α,β-unsaturated ketones. Using **2** and **5,** it was demonstrated that 4-OT converts **2** to (5*S*)-[5-^{2}H]**3** and **5** to (3*R*)-2-oxo-[3-^{2}H]-4-pentenoate (**4**), in ^2H_2O (Scheme 3) (*5*, *6*). The stereochemical analysis of 4-OT required the use of two different dienols because only small quantities of [3-^{2}H]**1** were obtained in the enzymatic partitioning of **2** (*5*). Insufficient quantities of [3-^{2}H]**1** presumably result because the equilibrium constant greatly favors the formation of **3** and the facile chemical enolization of [3-^{2}H]**1** results in the formation of [3-^{2}H]**2**. Subsequent ketonization of [3-^{2}H]**2** yields an achiral molecule. On the basis of the stereochemical results, it was concluded that 4-OT catalyzes a suprafacial 1,3-allylic rearrangement consistent with a so-called ''one-base'' mechanism (Scheme 3) (*5*). The single base has been identified as Pro-1 on the basis of affinity labeling and crystallographic studies (*2*, *7*).

In course of these stereochemical experiments, two intriguing observations were made regarding the reaction of **2** and **5** with 4-OT (*5*). First, the enzyme-catalyzed



SCHEME 2



SCHEME 3

partitioning of **5** results in the accumulation of the β,γ-unsaturated isomer, **4,** whereas the enzyme-catalyzed partitioning of **2** results in the accumulation of the α,β-unsaturated isomer, **3.** Although 2-oxo-3-pentenoate (**6**) is the thermodynamically favored product of the reaction of 4-OT and **5,** its rate of formation is substantially slower than the rate of formation of **4** from **5.** Second, the enzyme-catalyzed ketonization of **5** to (3*R*)-[3-^{2}H]**4** is stereoselective, whereas the 4-OT-catalyzed ketonization of **2** to (5*S*)-[5-^{2}H]**3** is stereospecific. These observations suggest that the C-6 carboxylate group of **2** maintains the regio- and stereochemical fidelity of the reaction. In the reaction of **2** and 4-OT, the two carboxylate groups and the hydroxy group presumably anchor the substrate in a fixed position at the active site, thus allowing for facile and stereospecific protonation at C-5 (and presumably C-3). In the reaction of **5** and 4-OT, the substrate may be able to bind in two different modes because of the missing ''anchoring'' carboxylate group at C-6 (the third point of attachment). This allows for the production of both stereoisomers, (3*R*)-[3-^{2}H]**4** and (3*S*)-[3-^{2}H]**4,** in a ratio of 3 : 2. Moreover, it may be easier for the single base to protonate the relatively fixed C-3 position rather than the more mobile C-5 position.

A comparison of the kinetic properties obtained for the reaction of 4-OT with **2** to those obtained for the reaction of 4-OT with **5** provided additional insight into the role of the substrate's carboxylate group. The k_{cat}/K_m value for the conversion of **2** to **3** was determined to be 10^4-fold greater than that observed for the conversion of **5** to **6.** Because the affinity of 4-OT for **2** and **5** differs at most by a factor of 120, it was concluded that the higher value of k_{cat}/K_m was due primarily to a decrease in the overall kinetic barrier for the formation of **3** (*3*). This conclusion suggests that the enzyme utilizes the binding energy of the 6-carboxylate group to facilitate catalysis (*3*).

In order to define further the contribution of this carboxylate group to the mechanism of 4-OT, the nonenzymatic rates for the ketonization of **2, 5, 8,** and **11**

(Schemes 1 and 2) to their respective α,β-unsaturated ketones (**3, 6, 9,** and **12**) were determined and compared to the rates of the corresponding enzymatic reactions. In addition, a stereochemical analysis was performed on the reaction using **8.** The results implicate a role for the carboxylate group in both binding and catalysis. The stereochemical findings provide further evidence that the carboxylate group anchors the substrate while a comparison of the nonenzymatic and enzymatic rates of ketonization suggests that enzymatic catalysis is facilitated by the utilization of the binding energy of the carboxylate group.

EXPERIMENTAL PROCEDURES

Materials. All reagents, enzymes, buffers, and solvents were obtained from either Aldrich Chemical Co. or Sigma Chemical Co. unless noted otherwise. The Dowex anion-exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA). Tryptone and yeast extract were obtained from Difco (Detroit, MI). The YM-3 ultrafiltration membranes and Centricon (10,000 MW cutoff) centrifugal microconcentrators were obtained from Amicon. The syntheses of 2-hydroxy-2,4-hexadiene-1,6-dioate (**2**), 2-hydroxy-2,4-pentadienoate (**5**), and 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate (**8**) have been described elsewhere (*1*, *5*, *8*). 4-OT was purified according to published procedures (*11*).

General methods. Protein concentrations were determined using the commercially available bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) or the method of Waddell (*12*). 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 12.5% gels as described elsewhere (*11*). Kinetic data were obtained on either a Hewlett Packard 8452A Diode Array spectrophotometer or a Perkin–Elmer Model 553 fast scan UV/vis spectrophotometer. Ozone was generated by the passage of oxygen through a Welsbach ozonator. NMR spectra were obtained on a Bruker AM-250 spectrometer or a Varian Unity INOVA-500 spectrometer as indicated. Chemical shifts were referenced as noted below.

Preparation of 2-hydroxy-2,4E-heptadiene-1,7-dioate (*4E-**8***). A mixture containing approximately equivalent amounts of the 4*Z* and 4*E* isomers of **8** was prepared as described previously using 200 mg of 5-(carboxymethyl)-2-hydroxymuconate in dimethyl sulfoxide (1 mL) (*8*). After being heated at 120°C for 5 min, a solution of I_2 (3.6 mg) dissolved in ethyl acetate (1 mL) was added. Subsequently, the reaction mixture was diluted to 5 mL with ethyl acetate and allowed to stir at room temperature overnight under a fluorescent light. The reaction mixture was diluted to 50 mL with ethyl acetate and extracted (5×) with an equivalent amount of a 0.1 M solution of HCl. The organic layer was collected, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was suspended in $CHCl_3$ (25 mL) and stirred for 10 min. The solution was filtered and the filtrate washed with excess

$CHCl_3$ until the purple color was no longer present. A ^1H NMR spectrum of the product showed that it was predominantly 4*E*-**8** (~90% 4*E*-**8** and 10% 4*Z*-**8**) (*8*).

Preparation of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate. Sodium ethoxide was generated by the addition of sodium metal (5.4 g, 0.2 mol) to a stirring mixture of toluene (500 mL) and ethanol (200 mL). The reaction was carried out in a 2-L round-bottom flask under an argon atmosphere following a literature procedure (*13*). Subsequently, the dried sodium ethoxide was chilled in an ice bath and diethyl oxalate (28.5 g, 26.5 mL, 0.19 mol) and ethyl tiglate (25 g, 27 mL, 0.19 mol) were added in succession. The reaction mixture was allowed to warm to room temperature by removal of the ice bath. After being stirred at room temperature for 48 h, the mixture was filtered and the precipitate washed with ether until the filtrate was clear. The precipitate was air-dried to yield 29 g (59%) of the crude diethyl ester of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate: ^1H NMR ($CDCl_3$, 250 MHz) δ 1.30, 1.35 (6H, overlapping triplets, CH_3 of $-OCH_2CH_3$), 1.95 (3H, s, CH_3 at C-5), 4.20 (3H, q, CH_2 of $-OCH_2CH_3$), 4.35 (3H, q, CH_2 of $-OCH_2CH_3$), 6.40 (1H, d, J = 8.3 Hz, H3), 7.60 (1H, d, J = 8.3 Hz, H4); ^{13}C NMR ($CDCl_3$, 250 MHz) δ 13.0 (CH_3), 14.1, 14.3 (CH_3 of $-OCH_2CH_3$), 60.7, 62.8 (CH_2 of $-OCH_2CH_3$), 106.3 (C-3), 129.9 (C-5), 130.2 (C-4), 142.9 (C-2), 164.9 (C-1), 167.9 (C-6).

The free acid was prepared by alkaline hydrolysis of the diethyl ester and subsequent acidification as follows. The diethyl ester (29 g, 0.13 mol) was suspended in a solution of 0.5 M NaOH (500 mL) and stirred at ambient temperature for 16 h. The reaction mixture was filtered and the filtrate was adjusted to pH 1 by the addition of concentrated HCl. The precipitate was collected by filtration and suspended in ethyl acetate (~50 mL). After being stirred overnight, the solution was filtered to yield 5.4 g (25%) of the free acid: ^1H NMR (CD_3OD, 250 MHz) δ 1.90 (3H, s, CH_3), 6.40 (1H, d, J = 11.4 Hz, H3), 7.65 (1H, d, J = 11.4 Hz, H4); ^{13}C NMR (CD_3OD, 250 MHz) δ 12.8 (CH_3), 107.1 (C-3), 128.9 (C-5), 132.9 (C-4), 146.9 (C-2), 166.9 (C-1), 171.7 (C-6).

Preparation of 2-hydroxy-2,4-hexadienoate (***11***). 2-Hydroxy-2,4-hexadienoate was generated by the thermal decarboxylation of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate following a similar procedure (*5*). A solution of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate (104 mg, 0.6 mmol) dissolved in anhydrous dimethyl sulfoxide (0.5 mL) was placed in a test tube, sealed with a rubber septum, and purged with argon. The stirring solution was heated at 120°C for 15 min. The reaction mixture was chilled in powdered dry ice, diluted with ethyl acetate (4 mL), and filtered. Subsequently, the filtrate was diluted with ethyl acetate (200 mL) and washed with a solution of 0.1 N HCl (3 × 33 mL). The organic layer was collected, dried over anhydrous Na_2SO_4, and filtered, and the filtrate was evaporated to dryness to give a solid (34 mg, 43%) which decomposes upon prolonged standing (>24 h). The solid was identified as a mixture of 4*Z*-**11** (~55%), 4*E*-**11** (~31%), and 4*E*-**12** (~14%) by ^1H and ^{13}C NMR spectroscopy. 4*Z*-**11**: ^1H NMR (DMSO-d_6, 500 MHz) δ 1.71 (3H, dd, H6), 5.58 (1H, dq, J = 9.3 Hz, H5), 6.07 (1H, d, H3), 6.33 (1H, q, J = 9.5 Hz, H4); 4 *E*-**11**: ^1H NMR (DMSO-d_6, 500 MHz) δ 1.75 (3H, dd, H6), 5.83 (1H, dq, J = 13.5 Hz, H5), 6.26 (1H, d, H3), 6.35 (1H, brd m, H4). 4*Z*-**11** and 4*E*-**11**: ^{13}C NMR (CD_3OD, 250 MHz) δ 13.6, 18.7 (C-6), 108.3, 113.9 (C-3), 123.7, 126.2 (C-4), 129.1, 132.5 (C-5), 140.1, 141.7 (C-2), 167.9 (C-1).

In order to obtain the spectral data for 4*E*-**12,** it was made the predominant product in the mixture described above (4*Z*-**11,** 4*E*-**11,** and 4*E*-**12**) using 4-OT. Accordingly, a solution of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate (68 mg, 0.4 mmol) dissolved in anhydrous dimethyl sulfoxide (0.5 mL) was heated at 120°C for 20 min. A portion of the resulting mixture (30 μL) was added to 100 mM NaH_2PO_4 buffer (0.6 mL, pH 8.8) and transferred to an NMR tube. The addition of the mixture to the buffer adjusted the pH to 7.4. The reaction was initiated by the addition of 4-OT (0.2 mg in 20 μL of 20 mM NaH_2PO_4 buffer, pH 7.1). After 18 h, 4*E*-**12** was the predominant product present in solution as determined by the corresponding NMR signals. Spectra were recorded in 100% H_2O using selective presaturation of the water signal with a 2-s presaturation interval. The lock signal is (methyl sulfoxide)-d_6. Chemical shifts were referenced to (methyl sulfoxide)-d_6. 4*E*-**12**: ^{1}H NMR (100 mM NaH_2PO_4 buffer, 500 MHz) δ 0.88 (3H, t, H6), 2.16 (2H, quintet, H5), 5.99 (1H, d, J = 17.5 Hz, H3), 6.94 (1H, dq, H4); ^{13}C NMR (100 mM NaH_2PO_4 buffer, 500 MHz) δ 12.7 (C-6), 27.5 (C-5), 126.9 (C-3), 161.3 (C-4), 174.0 (C-1), 199.9 (C-2).

Nonenzymatic ketonization of ***2, 5,*** *4E-**8**, 4Z-**8**, and* ***11.*** The nonenzymatic rate for the conversion of each dienol to its β,γ-unsaturated ketone was determined by following the decrease in absorbance at 295 nm (**2**), 264 nm (**5**), 276 nm (4*E*-**8** and 4*Z*-**8**), or 268 nm (**11**). Absorbance readings for the decomposition of each dienol were measured as follows: for **2** every 30 s for 30 min, for **5** every 20 s for 20 min, for 4*Z*-**8** every 10 s for 10 min, for 4*E*-**8** every 20 s for 20 min, and for **11** every 90 s for 60 min. The nonenzymatic rate for the formation of each α,β-unsaturated ketone was determined by following the increase in absorbance at 236 nm (**2** $\rightarrow$ **3,** ε = 6580 M^{-1} cm^{-1}) or at 232 nm (**5** $\rightarrow$ **6,** ε = 5900 M^{-1} cm^{-1}; 4*E*- and 4*Z*-**8** $\rightarrow$ **9,** ε = 8250 M^{-1} cm^{-1}; **11** $\rightarrow$ **12,** ε = 4300 M^{-1} cm^{-1}) (*1*, *5*, *8*). Absorbance readings were collected for each α,β-unsaturated ketone at the indicated wavelength as follows: for **3** every 2.5 min for 3 h, for **6** every 30 min for 16 h, for **9** (from 4*Z*-**8**) every 1 h for 53 h, for **9** (from 4*E*-**8**) every 1 h for 50 h, and for **12** every 1 h for 63 h. All reactions were carried out in 20 mM NaH_2PO_4 buffer (pH 7.3) at 23°C. Kinetic runs were initiated by the addition of 5–17.5 μL of a stock solution (1.8–3.5 mg/mL) made up in ethanol.

For the formation of the β,γ-unsaturated ketones, the readings were fitted to the equation for a single exponential decay by nonlinear regression analysis. For the formation of the α,β-unsaturated ketones, the readings were fitted to a first-order equation and the rates were determined from the initial portion. Both sets of data were fitted using the grafit program (Erithacus Software Ltd., Staines, UK) obtained from Sigma Chemical Co.

Kinetic parameters of 4-OT using ***2, 5,*** *4E-**8**, 4Z-**8**, and* ***11.*** Enzyme activity was monitored by following the formation of the α,β-unsaturated ketone as indicated by an increase in absorbance at 236 nm (**2** $\rightarrow$ **3,** ε = 6580 M^{-1} cm^{-1}) or by an increase in absorbance at 232 nm (**5** $\rightarrow$ **6,** ε = 5900 M^{-1} cm^{-1}; 4*E*- and 4*Z*-**8** $\rightarrow$ **9,** ε = 8250 M^{-1} cm^{-1}; **11** $\rightarrow$ **12,** ε = 4300 M^{-1} cm^{-1}) (*1*, *5*, *8*). Because the thermal generation of **11** results in a mixture of isomers including the nonreactive **12** (~14%), the substrate concentrations in the kinetic experiments using **11** were corrected accordingly. The cuvettes were mixed by a stir/add

cuvette mixer. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program.

*Stereoselective ketonization of 4Z-**8** to [3-^{2}H]**7** by 4-OT and conversion to [3-^{2}H]**13.*** The ketonization of 4*Z*-**8** to [3-^{2}H]**7** in ^2H_2O by 4-OT was carried out by a modification of a literature procedure (*5*). Accordingly, to a stirring mixture of 4-OT (12 μL, 7.1 mg/mL) and $NaBH_4$ (~5 eq) in buffer (10 mL, 20 mM $Na_2[^2H]PO_4$, p[^{2}H] 8.6) was added a solution of 4*Z*-**8** (8 mg, 0.05 mmol) dissolved in (methyl sulfoxide)-d_6 (0.3 mL). The enzyme solution had been previously exchanged by repeated dilution and concentration in ^2H_2O in a centricon 10 microconcentrator and stored overnight in ^2H_2O. The reaction mixture was stirred for 1 min and a second aliquot of $NaBH_4$ (~5 eq) was added. After being stirred for 30 min, the solution was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 15 cm), eluting with a formic acid gradient (0–4 M formic acid, 60 mL total volume). The product elutes at ~3 M formic acid. Appropriate fractions were pooled and evaporated to dryness. The reaction was repeated three times and the products combined. The ^{1}H NMR spectrum of [3-^{2}H]**13** corresponded to a previously published spectrum (*14*).

*Conversion of [3-^{2}H]**13** to [3-^{2}H]malate (**14**).* The chemical and enzymatic degradation of [3-^{2}H]**13** to [3-^{2}H]malate (**14**) was carried out by a modification of a literature procedure (*5*). Accordingly, a solution of [3-^{2}H]**13** (23.6 mg, 0.13 mmol) in a mixture of 1,4 dioxane (7 mL) and methanol (50 μL) was subjected to a stream of O_3 (1 L/min) for 25 min. After the solvent was evaporated to dryness, H_2O_2 (0.5 mL, 30%) and glacial CH_3CO_2H (2.5 mL) were added to the residual oil, and the reaction mixture was stirred overnight. The solution was evaporated to dryness and the pH was adjusted to ~8 by the addition of a solution of 5% $NaHCO_3$ (2 mL). The diastereomeric [3-^{2}H]malates were purified by anion-exchange chromatography as described above, eluting at ~2.3 M formic acid. The ^{1}H NMR spectrum of [3-^{2}H]malates corresponded to a previously published spectrum (*5*, *8*).

The purified [3-^{2}H]malates were treated with malic enzyme and the resulting (2*R*)-isomers were recovered by anion-exchange chromatography as described elsewhere (*5*, *8*). (2*R*, 3*S*)-[3-^{2}H]**14**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.34 (~0.7 H, dt, $J_{2,3}$ = 11.5 Hz, H3), 4.28 (~0.7 H, dt, $J_{2,3}$ = 11.5 Hz, H2). (2*R*, 3*R*)-[3-^{2}H]**14**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.64 (~0.35 H, dt, $J_{2,3}$ = 3.8 Hz, H3), 4.28 (~0.35 H, brd d, H2).

*Stereoselective ketonization of 4Z-**8** to [5-^{2}H]**9** by 4-OT and conversion to [5-^{2}H]**15.*** A portion (~10 μL) of a solution of 4*Z*-**8** (28.3 mg, 0.16 mmol) dissolved in (methyl sulfoxide)-d_6 (60 μL) was added to seven separate reactions mixtures containing 100 mM $Na_2[^2H]PO_4$ buffer (0.6 mL, p[^{2}H] = 9.2). The addition of 4*Z*-**8** to the buffer adjusted the p[^{2}H] to 7.1. To the mixtures being stirred was added 4-OT (10 μL, 7.1 mg/mL). After 5 min, an aliquot of $NaBH_4$ (~5 eq) was added to each reaction mixture and they were allowed to stand overnight. The final p[^{2}H] of the solution was 9.6. The p[^{2}H] of the solution was adjusted to 8.4 and it was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 14 cm), eluting with a formic acid gradient (0–2 M formic acid, 60 mL total volume). The product, 5-[^{2}H]**15,** elutes as a broad peak from 1.3–2 M formic acid and is recovered in 69%

(20 mg) yield. The ^{1}H NMR spectrum of 5-[^{2}H]**15** corresponded to a previously published spectrum (*15*).

Conversion of [5-^{2}*H*]***15*** *to* [2-^{2}*H*]*succinate* (***16***). A solution of 5-[^{2}H]**15** (20 mg, 0.1 mmol) in a mixture of methanol and 1,4-dioxane was treated with O_3 as described above for 30 min. After the solvent was evaporated to dryness, the residual oil was treated with H_2O_2 and glacial CH_3CO_2H as described above. The [2-^{2}H]succinate (**16**) was purified using anion (formate)-exchange column chromatography. It elutes as a broad peak from 1–1.7 M formic acid and is recovered in 70% yield (9.5 mg): $[\theta]_{210} = 180°$. The ^{1}H NMR spectrum of 2-[^{2}H]**16** corresponded to a previously published spectrum (*16*).

RESULTS

Nonenzymatic ketonization of ***2, 5,*** *4E-****8,*** *4Z-****8,*** *and* ***11.*** The ketonization of each dienol (**2, 5,** 4*E*-**8,** 4*Z*-**8,** and **11**) was monitored in aqueous phosphate buffer by observing the decay in UV absorbance at 295, 264, 276, and 268 nm, respectively. In all cases, a rapid drop in absorbance followed by a much slower decrease was observed. For **5, 8** (both isomers), and **11,** the slower decrease was noticeable only after an appreciable time interval. The rapid drop in absorbance corresponds to the ketonization of each dienol to its β,γ-unsaturated ketone (**1, 4, 7, 10**) as has been established in previous studies (*1*, *5*, *8*). The slower decrease in absorbance corresponds to the formation of the α,β-unsaturated ketone (**3, 6, 9, 12**) as previously shown (*1*, *5*, *8*). At equilibrium, **6, 9,** and **12** are the predominant products (>95%) while **3** constitutes ~80% of the equilibrium mixture (*1*, *5*, *8*).

The absorbance data collected during the initial decay were fitted to the equation for a single exponential decay by nonlinear least-squares regression analysis in order to estimate the rate constant (defined as k_1) for the formation of each β,γ-unsaturated ketone from its dienol. The slow rate of formation of **6, 9,** and **12** precluded an accurate measurement of the first-order rate constant. Hence, the initial rate of formation (defined as k_2) for each α,β-unsaturated ketone was measured and compared to that measured for **3.** The rates are summarized in Table 1. Three features are apparent from these rate constants. First, protonation of these dienols occurs more rapidly at the α-carbon (to form the β,γ-unsaturated ketone) rather than the γ-carbon (to form the α,β-unsaturated ketone) under these conditions. Second, the rates for the formation of the β,γ-unsaturated ketones from their respective dienols do not differ significantly. The largest difference is that between the rates of formation for **7** (from *4Z*-**8** and *4E*-**8**) (35-fold). Finally, the initial rates for the formation of the α,β-unsaturated ketones differ somewhat more significantly, with the largest difference in rates being the nearly 320-fold difference observed between **3** and **12** and the 390-fold difference observed between **3** and **9** (generated from 4*E*-**8**).

Kinetic parameters of 4-OT using ***2, 5,*** *4E-****8,*** *4Z-****8,*** *and* ***11.*** The kinetic properties for the 4-OT-catalyzed ketonization of **2, 5,** 4*E*-**8,** 4*Z*-**8,** and **11** to their respective α,β-unsaturated ketones were measured. The observed values for K_m, k_{cat}, and k_{cat}/K_m are summarized in Table 2. A comparison of the parameters measured for

TABLE 1
Rate Constants for the Nonenzymatic Decay of Dienols

Reaction	k_1[a] (s^{-1})	Reaction	k_2[b] (s^{-1})
2 → **1**	3.5×10^{-3}	**2** → **3**	2.8×10^{-4}
5 → **4**	1.2×10^{-3}	**5** → **6**	1.3×10^{-5}
4*Z*-**8** → **7**	6.3×10^{-3}	4*Z*-**8** → **9**	2.7×10^{-6}
4*E*-**8** → **7**	1.8×10^{-4}	4*E*-**8** → **9**	7.1×10^{-7}
11 → **10**	4.4×10^{-4}	**11** → **12**	8.8×10^{-7}

[a] The rate constants are derived by fitting the data to the equation for a single exponential decay.
[b] The rate constants represent the initial rates of the first-order increase in absorbance. For clarity, the standard deviations are not shown. For all rate constants, the standard deviations are ±5%.

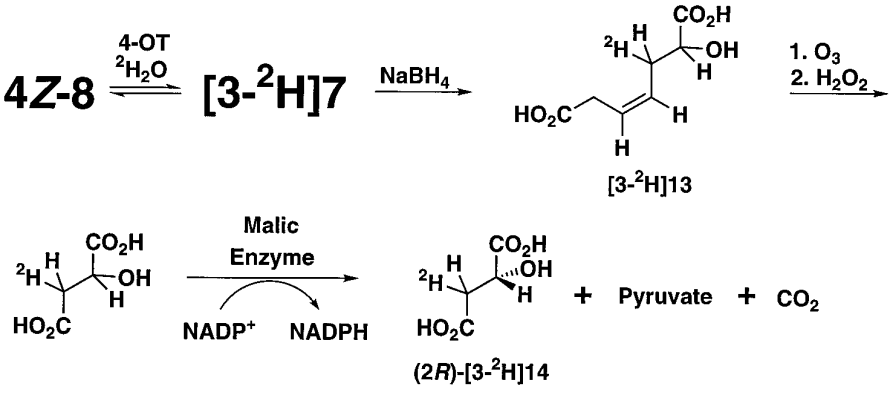
4*Z*-**8** to those measured for **2** shows that K_m increases somewhat (~5-fold) and k_{cat} decreases more significantly (~40-fold). The value of k_{cat}/K_m decreases ~200-fold. The same comparison for **2** to 4*E*-**8** shows that the K_m increases ~3-fold and the k_{cat} decreases 300-fold. The value of k_{cat}/K_m decreases ~800-fold. The kinetic parameters determined for **5** and **11** clearly show that the missing carboxylate group has a profound effect on the enzyme-catalyzed reaction. For **5,** there is a 6-fold increase in K_m and a 9000-fold decrease in k_{cat}, resulting in a 10^4-fold decrease in the value of k_{cat}/K_m. For **11,** there is a 5-fold increase in K_m and a 90,000-fold decrease in k_{cat}, corresponding to a 10^5-fold decrease in the value of k_{cat}/K_m.

Stereochemical analysis of [*3-*^{2}*H*]**7.** The stereochemistry at C-3 of [3-^{2}H]**7** was assigned using a literature procedure which involves its chemical and enzymatic conversion to a monodeuteriated malate as shown in Scheme 4 (5). Ketonization of 4*Z*-**8** in ^2H_2O, in the presence of 4-OT, generated 2-oxo-[3-^{2}H]-4*Z*-heptene-1,7-dioate (**7**). The inclusion of $NaBH_4$ in the reaction mixture trapped [3-^{2}H]**7** by its immediate reduction to [3-^{2}H]**13** and fixed the chirality at C-3 by making the center nonepimerizable. A small-scale reaction was monitored by ^{1}H NMR spectroscopy

TABLE 2
Kinetic Parameters for 4-OT with Various Dienols[a]

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Relative k_{cat}/K_m	k_{cat}/k_2[b]
2	120 ± 10	3600 ± 100	3.0×10^7	1.00	1.3×10^7
4*Z*-**8**	600 ± 70	90 ± 10	1.5×10^5	5.0×10^{-3}	3.3×10^7
4*E*-**8**	320 ± 40	12 ± 1	3.8×10^4	1.3×10^{-3}	1.7×10^7
5	740 ± 130	0.4 ± 0.05	5.4×10^2	1.8×10^{-5}	3.1×10^4
11	600 ± 100	0.04 ± 0.004	7.0×10^1	2.3×10^{-6}	4.5×10^4

[a] The steady state kinetic parameters were determined at 23°C. Errors are standard deviations.
[b] The value of k_{cat}/k_2 is an estimate of the rate enhancement. The rate constant k_2 is defined in Table 1.



SCHEME 4

in order to determine the appropriate quantity of enzyme that avoided two potential complications. It was necessary to use a sufficiently large amount of enzyme in order to minimize the facile nonenzymatic ketonization of 4*Z*-**8** to **7** resulting in a stereorandom incorporation of deuterium at C-3, but not so much enzyme such that the conversion of 4*Z*-**8** to **9** becomes the predominant pathway. The reduced product, [3-^{2}H]**13,** was the major product isolated by anion-exchange chromatography as indicated by ^{1}H NMR spectroscopy (*14*). Subsequently, [3-^{2}H]**13** was subjected to ozonolysis and worked up in the presence of H_2O_2 to afford the 2*R* and 2*S* isomers of the monodeuteriated **14.** Treatment of the mixture with malic enzyme and purification by anion-exchange chromatography resulted in the isolation of (2*R*)-[3-^{2}H]malate **14** (*5*).

^{1}*H NMR analysis of* (2*R*)-[3-^{2}*H*]***14.*** Each diastereotopic proton at C-3 of the fully protio malate appears as a doublet of doublets at 2.33 and 2.63 ppm (*17*). Stereospecific incorporation of a deuteron at C-3 results in the loss of one signal and the collapse of the remaining signal into a broadened doublet. The resonances for (2*R*)-[3-^{2}H]malate have been assigned by the reaction of maleic acid with maleate hydratase. When the hydration reaction is performed in ^2H_2O, (2*R*, 3*R*)-[3-^{2}H]malate is obtained (*18*). The resulting ^{1}H NMR spectrum shows the loss of an upfield signal (2.33 ppm) and the presence of a downfield doublet (2.66 ppm).

The ^{1}H NMR spectrum of the purified (2*R*)-[3-^{2}H]malate derived from the 4-OT-catalyzed conversion of 4*Z*-**8** to **7,** in ^2H_2O, is shown in Fig. 1. The two major signals present are centered at 2.33 ppm (a broadened doublet of triplets) and 2.64 ppm (a broadened triplet). The signal at 2.33 ppm corresponds to (2*R*, 3*S*)-[3-^{2}H]malate while the signal at 2.63 ppm corresponds to (2*R*, 3*R*)-[3-^{2}H]malate. The height of the integral for the signal assigned to the (2*R*, 3*S*) isomer is ~2-fold greater than that of the corresponding integral for the (2*R*, 3*R*) isomer. The previously published ^{1}H NMR spectrum of the purified (2*R*)-[3-^{2}H]malate derived from the ketonization of **5** to **4** in ^2H_2O showed the same two signals although the height of the integral for the signal assigned to the (2*R*, 3*S*) isomer is ~1.5-fold greater than that of the

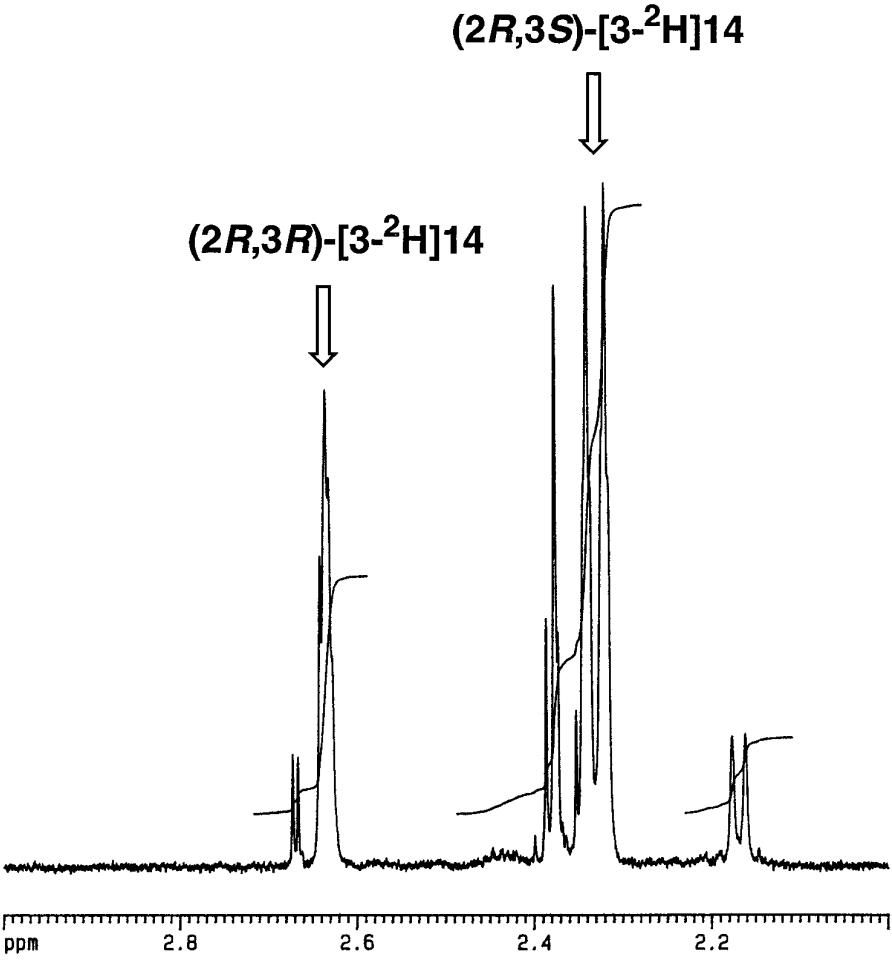
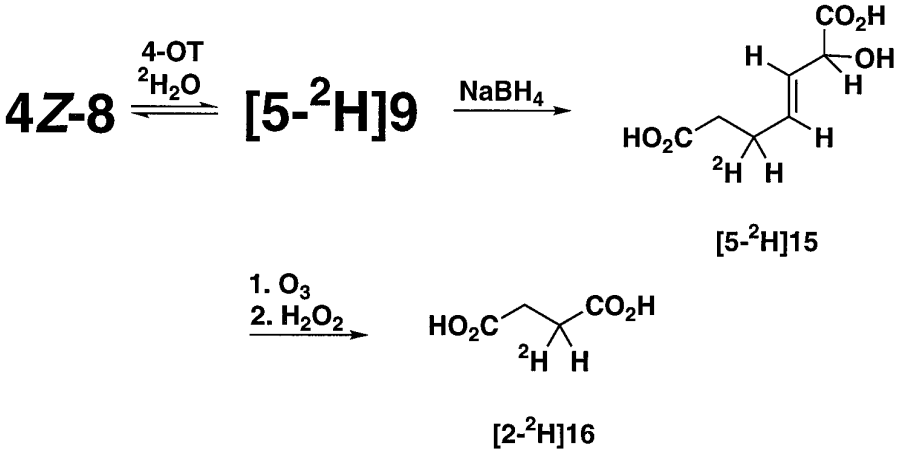


FIG. 1. ^{1}H NMR (500 MHz, ^2H_2O) spectra of (2*R*)-[3-^{2}H]malate obtained from the chemical and enzymatic conversion of 2-oxo-[3-^{2}H]-4*Z*-heptene-1,7-dioate (**7**) generated by the 4-oxalocrotonate tautomerase-catalyzed ketonization of 2-hydroxy-2,4-heptadiene-1,7-dioate (**8**) in ^2H_2O. The other signals in the spectra correspond to fully protio malate and other impurities. The fully protio malate results from the presence of H_2O in the reaction mixture.

corresponding integral for the (2*R*, 3*R*) isomer. Both analyses show that the enzymatic reaction is stereoselective and that the (2*R*, 3*S*) isomer predominates. However, the enzyme-catalyzed conversion of 4*Z*-**8** to **7** is clearly more highly stereoselective than the enzyme-catalyzed conversion of **5** to **4.**

Stereochemical analysis of [5-^{2}*H*]**9.** The stereochemistry at C-5 of [5-^{2}H]**9** was determined by its chemical and enzymatic conversion to a monodeuteriated succinate as shown in Scheme 5. In ^2H_2O, the ketonization of 4*Z*-**8** produced 2-oxo-3-[5-^{2}H]heptene-1,7-dioate (**9**) in the presence of a large excess of 4-OT. The chirality



SCHEME 5

at C-5 was fixed by the reduction of [5-^{2}H]**9** to [5-^{2}H]**15** using $NaBH_4$. The reduced product, [5-^{2}H]**15,** was the major product isolated by anion-exchange chromatography as indicated by ^{1}H NMR spectroscopy (*15*). Subsequently, treatment of [5-^{2}H]**15** with ozone generated an ozonide which was processed to the monodeuteriated succinate **16** by an oxidative work-up using H_2O_2 (*5*).

The purified [2-^{2}H]**16** derived from the above procedure was analyzed by circular dichroism (Table 3). The circular dichroism (CD) spectrum of (2*S*)-[2-^{2}H]succinic acid shows a positive n to π^* Cotton effect at 210 nm. As indicated in Table 3, the molar ellipticity of the authentic sample is $[\theta]_{210} = +228°$ at 25°C (19). The monodeuteriated succinic acid obtained from the chemical degradation of [5-^{2}H]**9** exhibits a positive Cotton effect with a molar ellipticity of $[\theta]_{210} = +180°$ at 25°C. It is therefore concluded that the *S* isomer of [2-^{2}H]**16** has been obtained.

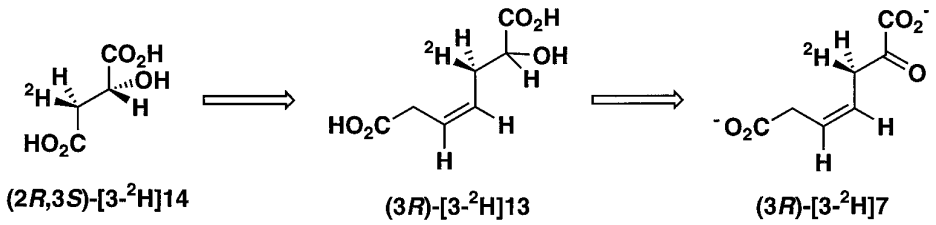
Assignment of the stereochemical course of the 4-OT-catalyzed reaction. The stereochemistry at C-3 of [3-^{2}H]malate indicates that the stereochemistry at C-3 of

TABLE 3
Molar Ellipticity of 2-Deuteriosuccinic Acid

Source of (*S*)-2-[^{2}H]succinic acid	$[\theta]_{210}$[a]
Authentic sample[b]	228°
4-OT-catalyzed conversion of **8** to **9**	180°

[a] Circular dichroic spectra were recorded in 95% ethanol on a Jasco J-20A automatic recording spectropolarimeter at 25°C. The units of $[\theta]$ are in degrees M^{-1} cm^{-1} and the errors are $\pm 10°$ M^{-1} cm^{-1}.

[b] Ref. (*19*).

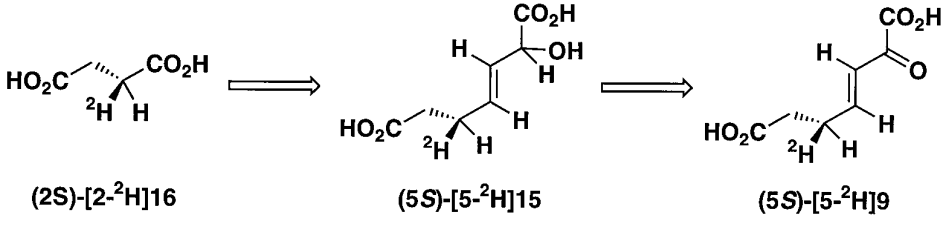


SCHEME 6

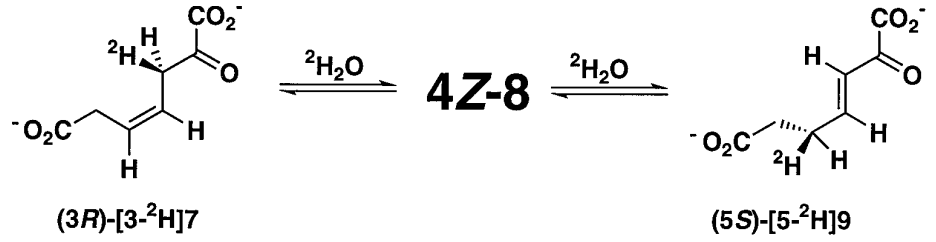
[3-^{2}H]**13** is *R*. The priority numbering changes because there is a double bond instead of a carboxylate group at C-4. This, in turn, indicates that 4-OT ketonizes 4*Z*-**8** to (3*R*)-[3-^{2}H]**7** (Scheme 6). The stereochemistry at C-2 of [2-^{2}H]succinate indicates that the stereochemistry at C-5 of [5-^{2}H]**15** is also *S* (Scheme 7). This, in turn, indicates that 4-OT ketonizes 4*Z*-**8** to (5*S*)-[5-^{2}H]**9.** It can be reasonably concluded from these stereochemical findings that the isomerization of **7** to **9** is predominantly a suprafacial process in accord with the previous studies (Scheme 8).

DISCUSSION

The results of the stereochemical studies of 4-OT using 4*Z*-**8** provide two pieces of mechanistic information. First, they show that the overall stereochemical course of the reaction is suprafacial when the same dicarboxylated dienol intermediate is partitioned to a β,γ-unconjugated ketone (**7**) and its α,β-isomer (**9**). Because the previous stereochemical study examined the enzymatic partitioning of the dicarboxylated **2** (to **3**) and the monocarboxylated **5** (to **4**), the conclusion relied on the assumption that **2** and **5** bind comparably at the active site (*5*). These results validate this assumption. Second, the results of this study provide additional experimental evidence supporting the anchoring hypothesis. The underlying premise of this hypothesis is that the C-6 carboxylate group interacts with an active site residue to fix the substrate at the enzyme's active site allowing for regio- and stereospecificity (*5*). Thus, if it were possible to determine the stereochemical course in both direc-



SCHEME 7



SCHEME 8

tions using **2,** the anchoring hypothesis predicts that the reaction would be highly stereoselective in both directions as enzymes are generally believed to be stereospecific when utilizing their physiological substrates (*20*). Indeed, the formation of **3** from **2** is highly stereoselective, whereas the formation of **4** from **5** is not. Using 4*Z*-**8,** an increase in the degree of stereoselectivity is observed for the formation of **7** and a decrease in the degree of stereoselectivity is observed for the formation of **9.** The improvement in stereoselectivity presumably results because the carboxylate group at C-7 of **8** provides a third point of attachment to the enzyme which favors one mode of binding while the decrease in stereoselectivity presumably results because of a less favorable interaction between 4-OT and **8** (compared to 4-OT and **2**).

The amino acid residue responsible for binding the C-6 carboxylate group of **2** (and presumably the C-7 carboxylate group of **8**) was initially identified by crystallographic studies (*7*). The crystal structures of an isozyme of 4-OT (73% homologous) and the structurally homologous enzyme 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) show that two arginine residues and the amino-terminal proline are among the few conserved residues in both enzymes' active site region. In the active site of CHMI, these two arginine residues are involved in the binding of two sulfate ions. Moreover, the distance between the sulfate ions (6.7 Å) compares favorably to the distance between the two carboxylate groups of substrate in an extended conformation (6.0 Å). For these reasons, it was proposed that Arg-11 of 4-OT interacts with the C-6 carboxylate group (*7*).

Because these two crystal structures were solved in the absence of a bound ligand (other than the fortuitously placed sulfate ions), the proposed interaction was examined further by NMR studies of 4-OT complexed with a mono- and a dicarboxylated compound (*21*). The key finding of this study is that binding of the dicarboxylated compound, *cis*, *cis*-muconate, a competitive inhibitor of 4-OT, results in changes to the backbone NH chemical shift of both Arg-11 and Arg-39, whereas the covalent modification of 4-OT by the monocarboxylated compound 3-bromopyruvate results only in changes to the chemical shifts of Arg-39 (*21*). These observations support the suggestion that Arg-11 interacts with the C-6 carboxylate group of the substrate.

Thus far, the discussion has focused on the role of the carboxylate group at C-6 (of **2**) or C-7 (of **8**) in binding the substrate to the active site of 4-OT. However,

it appears that this noncovalent, electrostatic interaction must also contribute to catalysis as there is a significant difference between the enzyme's activity utilizing a dicarboxylated dienol (**2** and the two isomers of **8**) compared to its activity using a monocarboxylated dienol (**5** and **11**). While it is well known that noncovalent interactions contribute to the overall rate acceleration of an enzyme-catalyzed reaction (22), it must first be established that the observed differences in rates are not simply the result of a difference in the chemical reactivities.

The nonenzymatic properties of three dienols (**2, 5,** and 4*Z*-**8**) discussed in these studies have been characterized in previous studies (*1*, *5*, *8*). In aqueous buffer, there is a rapid interconversion between these dienols and their respective β,γ-unsaturated ketones (**1, 4,** and **7**) before a much slower conversion of the mixture to the α,β-unsaturated isomers (**3, 6,** and **9**). At equilibrium, the α,β-unsaturated ketone is the predominant (~80% for **3**) or exclusive product (**6** and **9**). As shown in this study, the nonenzymatic properties of 4*E*-**8** and **11** indicate that they are also in rapid interconversion with their β,γ-unsaturated ketones before a much slower conversion to their α,β-unsaturated isomers which are the exclusive products at equilibrium.

A quantitative analysis of these observations is provided by the rate constants measured for the formation of the β,γ-unsaturated ketones and the α,β-unsaturated ketones. The rate constants for the formation of the β,γ-unsaturated ketones differ only by a factor of 10 while the rate constants for the formation of the α,β-unsaturated ketones differ only by a factor of 100. These observations indicate that the intrinsic chemical reactivities do not account for the rate differences seen in the enzymatic reactions. For example, there is a 21-fold difference in the nonenzymatic rate constant determined for the formation of **3** compared to that determined for **6.** This difference cannot account for the 10^4-fold decrease observed in the corresponding k_{cat}/K_m values. Moreover, the rate constant for the nonenzymatic formation of **9** from 4*E*-**8** is comparable to that measured for the formation of **12** from **11.** Yet, the presence of the carboxylate group on 4*E*-8 results in a 500-fold increase in its k_{cat}/K_m value.

How can a noncovalent interaction such as that between the carboxylate group of substrate and Arg-11 of enzyme facilitate catalysis? Jencks, Ray, and others have identified some of the factors that might be involved (*9*, *10*, *24*, *25*). Among these factors are the strength of substrate binding, the increased reactivity of the substrate due to its destabilization, the exact positioning of the substrate at the active site, and a conformational change introduced by binding.

How these factors contribute to catalysis is not known but the nonenzymatic behavior of these dienols provides some clues. The partitioning factors for these dienols indicate that protonation is favored at C-3, which has been attributed to the greater charge density at this position. In general, the planarity of the π system will affect the delocalization of the negative charge of a dienolate intermediate (*23*). If the two double bonds are planar to each other, the negative change is very effectively delocalized throughout the π system so that protonation at C-3 is only slightly favored. If the two double bonds are not planar to each other, then the negative charge is localized at C-3 and protonation at C-3 is favored. These observations suggest that the positioning of the substrate at the active site coupled with

other factors which increase the charge density at C-5 contributes to its reactivity. Hence, the interaction between the C-6 carboxylate group of the substrate and Arg-11 of 4-OT may bear two responsibilities: it maintains the planarity of the dienol and it acts as an electron sink. Both effects may increase charge density at C-5 and favor protonation at this position. Hence, this interaction destabilizes the substrate leading to the transition state or it stabilizes the transition state. In addition, the neutralization of Arg-11 by the C-6 carboxylate group of substrate may induce a conformational change which facilitates this process.

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