Enzymes of the β-Ketoadipate Pathway in *Pseudomonas putida*: Primary and Secondary Kinetic and Equilibrium Deuterium Isotope Effects upon the Interconversion of (+)-Muconolactone to *cis,cis*-Muconate Catalyzed by *cis,cis*-Muconate Cycloisomerase†

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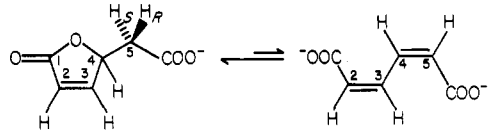
ABSTRACT: Primary and secondary kinetic and secondary equilibrium deuterium isotope effect studies on the *cis,cis*-muconate cycloisomerase catalyzed interconversion of *cis,cis*-muconate (CCM) and (+)-muconolactone (ML) have been performed. The primary and solvent kinetic deuterium isotope effects upon V_{max} for the reactions of (+)-[5*R*-^{2}H]muconolactone in water (HOH) and (+)-muconolactone in deuterium oxide (DOD) to form *cis,cis*-muconate are about 2.5–2.6 with the heavier isotopic reactions being the slower ones. The secondary equilibrium isotope effect for the formation *cis,cis*-[2,3,4,5-^{2}H$_4$]muconate from (+)-[2,3,4,5*S*-^{2}H$_4$]muconolactone is 1.32 for K_H/K_D = ([*cis,cis*-muconate]/[(+)-muconolactone])/([*cis,cis*-[2,3,4,5-^{2}H$_4$]muconate]/[(+)-[2,3,4,5*S*-^{2}H$_4$]muconolactone]) and agrees well with the measured value of 1.45 on the basis of the fumarase reaction [Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4853–4858]. The secondary kinetic deuterium isotope effect determined by the equilibrium perturbation method [Cleland, W. W. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 153–175, University Park Press, Baltimore, MD] for the conversion of *cis,cis*-[2,3,4,5-^{2}H$_4$]muconate to (+)-[2,3,4,5*S*-^{2}H$_4$]muconolactone is 0.66, expressed as $(V_{max}^{CCM(H)}/K_m^{CCM(H)})/(V_{max}^{CCM(D)}/K_m^{CCM(D)})$. From the equilibrium and kinetic secondary deuterium isotope effects, the calculated value for the kinetic secondary deuterium isotope effect for the reverse reaction, $(V_{max}^{ML(H)}/K_m^{ML(H)})/(V_{max}^{ML(D)}/K_m^{ML(D)})$, is about 0.96. The presence of a primary kinetic isotope effect with the absence of a secondary kinetic isotope effect for the reaction of (+)-muconolactone with *cis,cis*-muconate cycloisomerase to form *cis,cis*-muconate is most consistent with a stepwise mechanism involving the rate-determining generation of a carbanion intermediate, EX, from the ternary enzyme–Mn^{2+}–(+)-muconolactone complex, E·ML:

$$E + ML \rightleftharpoons E\cdot ML \underset{\text{step 1}}{\overset{RDS}{\rightleftharpoons}} EX \underset{\text{step 2}}{\rightleftharpoons} E\cdot CCM \rightleftharpoons E + CCM$$

We conclude that concerted or stepwise mechanisms involving carbonium ion or covalent intermediates are therefore not likely to be operative for this enzyme-catalyzed syn (cis) elimination reaction.

cis,cis-Muconate cycloisomerase[1] (EC 5.5.1.1) catalyzes the interconversion of (+)-muconolactone and *cis,cis*-muconate (eq 1). This reaction involves a 4,5-elimination reaction with



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loss of both the C(5) hydrogen and the carboxylate moiety from the C(4) carbon atom as the cyclic lactone opens (Ngai et al., 1983; Rose, 1970). Possible mechanisms for this reaction may be concerted or stepwise, the latter requiring the existence of an intermediate (see below). A choice among the various possible mechanisms including those containing intermediates for this enzyme has become possible on the basis of primary and secondary kinetic isotope effects and related stereochemical studies.

Previous studies of the stereochemical course of the enzymic reaction have shown that the loss of the *pro-R* hydrogen (H_R, eq 1) from the C(5) carbon atom of (+)-muconolactone to solvent water occurs stereospecifically (Avigad & Englard, 1969; Ngai et al., 1983). The occurrence of a proton transfer to and from solvent is consistent with the chemistry of similar elimination reactions (Saunders & Cockerill, 1973) and the failure to identify enzyme-associated prosthetic groups (e.g., redox-active coenzymes) other than the essential divalent metal ion (Ngai et al., 1983).

The employment of primary and secondary isotope effects in enzymology has provided valuable insight into the mechanism of catalysis and the arrangement of atoms in the transition states of enzymatic reactions [for recent reviews, see Kirsch (1977), Rose (1977), Hogg (1978), Klinman (1978a,b), and Simon & Kraus (1976)].

Primary kinetic isotope effects are attributed to slower rates of cleavage of bonds to an atom substituted by a heavier isotope. Since, as noted above, the conversion of (+)-muconolactone to *cis,cis*-muconate, catalyzed by *cis,cis*-muconate cycloisomerase, requires the cleavage of both a carbon–hydrogen bond and a carbon–oxygen bond, this system presents multiple opportunities for kinetic isotope effect studies.

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[1] Abbreviations: CCM, *cis,cis*-muconate; D, deuterium; E·CCM or E·Mn·CCM, ternary complex of enzyme, divalent metal ion, and *cis,cis*-muconate; E·ML or E·Mn·ML, ternary complex of enzyme, divalent metal ion, and (+)-muconolactone; [E_t], total *cis,cis*-muconate cycloisomerase subunit concentration; EDTA, ethylenediaminetetraacetic acid; ML, (+)-muconolactone; Tris, tris(hydroxymethyl)aminomethane; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMR, nuclear magnetic resonance.

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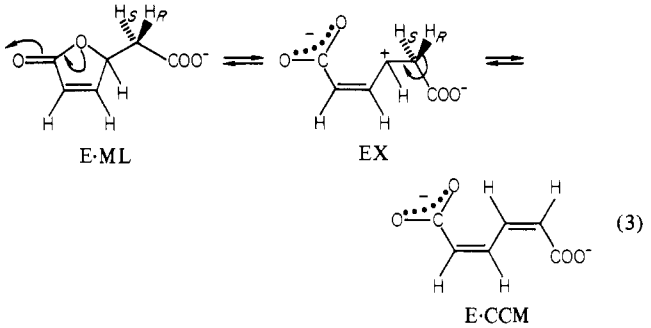
For example, the substitution of deuterium for the *pro-R* hydrogen at the C(5) carbon atom of (+)-muconolactone is expected to lower the rate of the proton-transfer step, but such an effect on the overall rate of reaction will depend on the relative rates of the different steps in the multistep catalytic sequence. Thus, from the presence and the magnitude of the observed primary kinetic isotope effect relative to measured or predicted values for an elementary proton-transfer step, the kinetic importance of the proton-transfer step relative to another (other) step(s) along the enzymatic reaction path may be evaluated (Klinman, 1978a,b; Northrop, 1975).

Secondary isotope effects are attributed to the difference in rate of reaction or position of equilibrium due to isotopic substitution of an atom(s) to which no bonds are broken or formed during the course of reaction or attainment of equilibrium but for which bond hybridization changes occur (Richards, 1970; Kirsch, 1977; Klinman, 1978a; Hogg, 1978). During the conversion of (+)-muconolactone to *cis,cis*-muconate (eq 1), changes in bond hybridization from sp^3 to sp^2 occur at the C(4) and C(5) carbon atoms, enabling study of the secondary kinetic and equilibrium isotope effects upon this reaction. Both the primary and secondary kinetic and equilibrium isotope effects are consistent with the existence of an enzyme intermediate in the catalytic sequence between the Michaelis complexes, E·ML and E·CCM, formed from the enzyme and substrate, (+)-muconolactone (ML), and product, *cis,cis*-muconate (CCM), respectively (eq 2 in which all enzyme species contain divalent metal ion), rather than a concerted mechanism.

$$E + ML \rightleftharpoons E{\cdot}ML \underset{\text{step 1}}{\overset{\text{RDS}}{\rightleftharpoons}} EX \underset{\text{step 2}}{\rightleftharpoons} E{\cdot}CCM \rightleftharpoons E + CCM \quad (2)$$

Three dissimilar mechanisms incorporating such an enzyme intermediate for the pathway of this reaction are presented below and involve carbonium, carbanion, and covalent intermediates, respectively. The first mechanism depicts a carbonium ion intermediate (E1) that is formed by a C(4) carbon–oxygen bond cleavage step prior to the C(5) proton removal step (eq 3). The second mechanism involves the opposite timing of events, namely, proton removal to form a carbanion intermediate (E1cb) prior to the carbon–oxygen bond cleavage step (eq 4). The mechanism involving a covalent intermediate is that of a double displacement by an active site nucleophile, represented by Z in eq 5 (Koshland, 1954).



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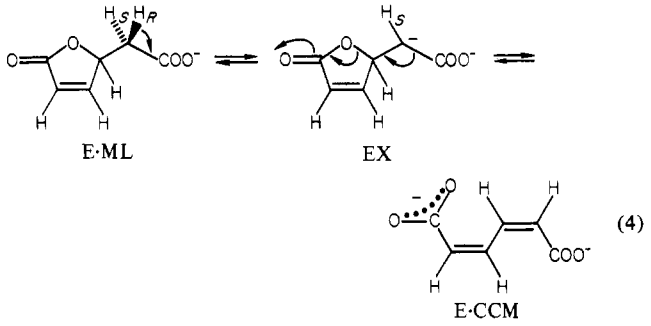

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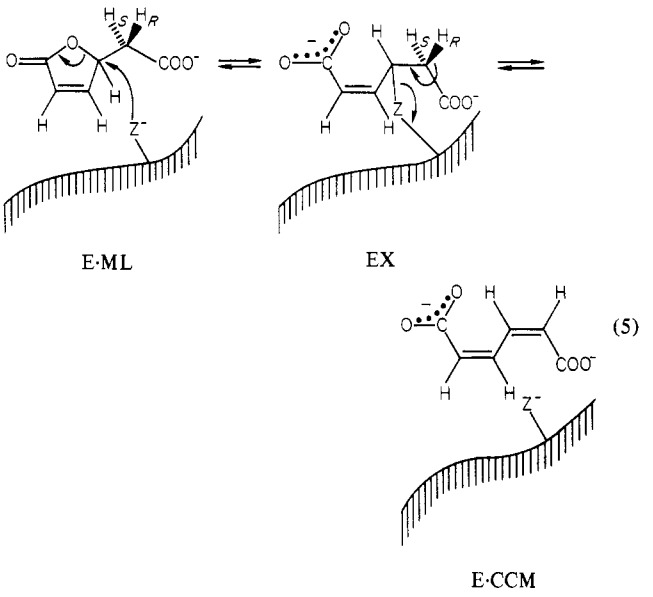

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We report herein the results of kinetic and equilibrium isotope effect studies with *cis,cis*-muconate cycloisomerase that are most consistent with a stepwise mechanism involving a carbanion intermediate (eq 4) in which proton removal (carbon–hydrogen bond cleavage) appears to be the rate-determining step in the formation of *cis,cis*-muconate from (+)-muconolactone.

Experimental Procedures

Materials

Enzymes. *cis,cis*-Muconate cycloisomerase was prepared from *Pseudomonas putida* as described previously (Ngai et al., 1983). EDTA-treated enzyme, prepared as previously described (Ngai et al., 1983), was incubated separately in 100 μM $MnCl_2$ and 1 mM $MgCl_2$ to yield Mn^{2+} and Mg^{2+} forms of the enzyme, respectively.

Chemicals. Hepps and Hepes (Sigma), Tris (Schwarz/Mann), and other chemicals were of reagent grade and used without further purification. DOD (>99.8%), DCl, NaOD (Bio-Rad), and [$^2H_6$]phenol (98%, Merck) were employed as received. Reagents with exchangeable protons were deuterated at the exchangeable sites by cyclical dissolution in DOD and lyophilization prior to the performance of experiments. Deionized water of greater than 5×10^5 Ω cm specific resistance was employed throughout.

cis,cis-Muconic acid and *cis,cis*-[2,3,4,5-$^2H_4$]muconate were prepared by the method of Elvidge et al. (1950) except that in the latter case [$^2H_6$]phenol was employed as the starting reagent. The deuterated product was analyzed by proton NMR spectroscopy.

(+)-[5*R*-^{2}H]Muconolactone was prepared enzymatically from *cis,cis*-muconate in DOD with *cis,cis*-muconate cycloisomerase. *cis,cis*-Muconic acid (2.85 g) was dissolved in 10 mL of DOD, lyophilized, and redissolved in 50 mL of DOD containing 2 mL of 40% NaOD, and the pH was adjusted to 7.0 with 20% DCl. Following the addition of *cis,cis*-muconate cycloisomerase (500 units) and $MnCl_2$ (50 μM), the solution was left in stoppered reaction flask for 10 h at room temperature with the pH maintained at 6.5–7.5 by the intermittant addition of 20% DCl. The pH was lowered to 6.0 with 20% DCl and left for another 2 h to ensure that the reaction had

proceeded to completion. The solution was treated with charcoal, filtered, acidified to pH 2 with 20% DCl, and extracted continuously with ether for about 48 h. The ether extract was evaporated and the crystallization of the yellow oil accomplished by dissolution in ethyl acetate with subsequent addition of benzene: white crystals, about 2 g, mp 75–76 °C (lit. mp 75 °C) (Ornston & Stanier, 1966). Total 5*R*-^{2}H labeling was confirmed by proton NMR spectroscopy. Protio (+)-muconolactone was prepared exactly as described above for (+)-[5*R*-^{2}H]muconolactone except that light water was employed as solvent.

Methods

Measurements. Measurements of UV absorbance (Gilford Model 2000 and Cary Model 118 spectrophotometers) and pH (Radiometer Model pHM 63 digital instrument equipped with GK 2301B or G 222B electrodes standardized with Beckman pH 4, 7, and 10 reference buffers) were obtained as previously described (Ngai et al., 1983). Values of pD were determined from the relation, pD = pH (meter reading) + 0.4 (Salomaa et al., 1964). IR spectra were recorded with a Perkin-Elmer 421 grating IR spectrophotometer with samples imbedded in KBr pellets. Proton magnetic resonance spectra were recorded with a 220-MHz Varian spectrometer equipped with a Varian computer (Middle Atlantic Research Facility) as previously described (Ngai et al., 1983).

Substrate Concentrations. Both (+)-muconolactone and (+)-[^{2}H]muconolactone concentrations were determined enzymatically with *cis,cis*-muconate cycloisomerase by assuming that the molar absorptivities of *cis,cis*-muconate and (+)-muconolactone at 260 and 230 nm are 1.72×10^4 and 1.52×10^3 M^{-1} cm^{-1} (Ornston & Stanier, 1966), respectively, and that an equilibrium [(+)-muconolactone]/[*cis,cis*-muconate] ratio of 11.5 at pH 8.0 obtained (Ngai et al., 1983).

Enzyme Assays. *cis,cis*-Muconate cycloisomerase assays were performed as described elsewhere (Ornston, 1966; Ngai et al., 1983).

Equilibrium Measurements. The equilibrium constants for the reaction of *cis,cis*-[2,3,4,5-$^2H_4$]muconate to form (+)-[2,3,4,5*S*-$^2H_4$]muconolactone and those for the nondeuterated compounds were determined by starting with *cis,cis*-[2,3,4,5-$^2H_4$]muconate and *cis,cis*-muconate, respectively, as described previously (Ngai et al., 1983).

Equilibrium Perturbation Experiments. Because of the relatively small magnitude of secondary kinetic isotope effects, the equilibrium perturbation method developed recently by Cleland and co-workers (Schimerlik et al., 1975; Cleland, 1977) was employed. To 3 mL of a solution containing *cis,cis*-[2,3,4,5-^{2}H]muconate (~100 μM), 100 mM Tris-HCl, pH 8.0, and $MnCl_2$ (100 μM) in a cuvette (10-mm path length) was added an amount of (+)-muconolactone (about 5 μL of a 1.0 M solution) such that the final solution would be at equilibrium. The exact amount of (+)-muconolactone added was adjusted by trial and error in successive solutions until addition of enzyme produced no absorbance change at 260 nm at chemical and isotopic equilibrium. When that condition had been ascertained, enzyme was added to a duplicate solution, and the transient absorbance change at 260 nm was recorded. Care was taken to maintain the temperature of the solution at 25 °C in order to eliminate artifacts due to temperature changes.

Kinetic Measurements. Initial velocity measurements were made as previously described (Ngai et al., 1983).[2]

Table I: Solvent Isotope Effect upon Apparent Equilibrium Constant Values for *cis,cis*-Muconate Formation from (+)-Muconolactone at 25 °C

pH or pD	K_{app}[a] HOH	K_{app}[a] DOD	$K_{app}^{HOH}/K_{app}^{DOD}$
8.0	(0.081 ± 0.004)	(0.00758 ± 0.00192)	(10.8 ± 2.8)
8.5	0.258 ± 0.005	0.0575 ± 0.0017	4.5 ± 0.1
		0.0535 ± 0.0017	4.8 ± 0.2

[a] 0.1 M Hepps, initial concentration of *cis,cis*-muconate 100 μM, K_{app} = [*cis,cis*-muconate$_t$]/[(+)-muconolactone$_t$], in which t indicates the total concentrations of CCM and ML, i.e., all ionic forms.

Data Processing. The kinetic data were fitted by unweighted linear least-squares method (Cleland, 1967) on a Commodore PET 2001 digital computer with teletype output in order to obtain the kinetic parameters with standard error estimates.

Results

Equilibrium Solvent Isotope Effects upon Interconversion of (+)-Muconolactone and cis,cis-Muconate. The equilibrium solvent isotope effects $K_{app}^{HOH}/K_{app}^{DOD}$ for the formation of *cis,cis*-muconate from (+)-muconolactone were calculated from equilibrium measurements in HOH and DOD, respectively, at 25 °C (Table I). The most reliable values of measured equilibrium solvent isotope effect are about 4.5–4.8-fold and indicate that the reaction is substantially more favorable in HOH than it is in DOD.

The calculated contribution due to transfer for the reaction (Schowen, 1972, 1977, 1978; Pentz & Thornton, 1967)

$$ML^- + LOL \rightleftharpoons CCM^{2-} + \text{L}>OL^+$$

where L is protium or deuterium, is given by

$$(K^{HOH}/K^{DOD})_{transfer} = \frac{\phi^{>C-L}\phi^{0-L2}}{\phi^{>O-L^+3}}$$

where ϕ is the fractionation factor for the site indicated (Buddenbaum & Shiner, 1977). Thus

$$(K^{HOH}/K^{DOD})_{transfer} = \frac{0.93 \times 1.00^2}{0.69^3} = 2.83$$

assuming that the net solvation differences between reactant and product are the same. The agreement between calculated and measured values appears satisfactory.

Secondary Equilibrium Deuterium Isotope Effect. At pH 8.0 in 0.1 M Tris-HCl buffer at 25 °C, the equilibrium constant, K_D, for the conversion of (+)-[2,3,4,5*S*-$^2H_4$]muconolactone to *cis,cis*-[2,3,4,5-$^2H_4$]muconate was determined to be 0.066 ± 0.002: the equilibrium constant for the nondeuterated compounds K_H = [*cis,cis*-muconate]/[(+)-muconolactone] = 0.087 ± 0.003. The K_H value is in close agreement with the values of 1/11.6 = 0.086 and 1/12.3 = 0.081 determined at

[2] During the course of reactions with (+)-[5*R*-^{2}H]muconolactone, the deuterium label will be exchanged with solvent (HOH) to form nondeuterated (+)-muconolactone, and the reaction rate is expected to increase as nondeuterated substrate accumulates. The occurrence of this exchange out of deuterium from reactant will be reflected under some circumstances in progressively greater slopes on plots of product concentration vs. time as, for example, labeled dihydroxyacetone phosphate in the presence of triosephosphate isomerase (Leadlay et al., 1976). The initial velocities of reactions involving (+)-[5*R*-^{2}H]muconolactone were measured under conditions in which the extent of reaction was sufficiently small (<0.1%) that no such rate increases were detectable.

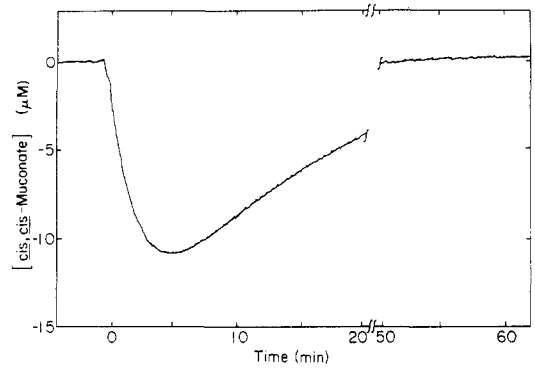

FIGURE 1: Determination of secondary kinetic deuterium isotope effect of reaction catalyzed by *cis,cis*-muconate cycloisomerase by equilibrium perturbation method: $[E_t]$ = 0.05 μM (subunit concentration), [*cis,cis*-[2,3,4,5-$^2H_4$]muconate] = 82 μM, [(+)-muconolactone] = 950 μM, and $[Mn^{2+}]$ = 100 μM in 100 mM Tris-HCl, pH 8.0 and 25 °C. The $(V_{max}^{CCM(H)}/K_m^{CCM(H)})/(V_{max}^{CCM(D)}/K_m^{CCM(D)})$ value was calculated from the maximum perturbation according to Cleland (1980) on the basis of K_{app} = 0.08 (Table I) and β = 1.32 (cf. Cook et al., 1980).

pH 8.0 in solutions containing different buffers (Ngai et al., 1983). Thus, the secondary equilibrium deuterium isotope effect K_H/K_D was calculated at 1.32 ± 0.04 and agrees favorably with the measurement for the comparable change in the fumarase reaction of 1.45 (Cook et al., 1980).

Secondary Kinetic Deuterium Isotope Effect. Representative data for the application of the equilibrium perturbation method to this system with initial concentrations of 82 μM *cis,cis*-[2,3,4,5-$^2H_4$]muconate and about 950 μM (+)-muconolactone at pH 8.0 and 25 °C are shown in Figure 1. The perturbation from chemical equilibrium as isotopic equilibrium is established is due to the more rapid reaction of *cis,cis*-[2,3,4,5-$^2H_4$]muconate to form (+)-[2,3,4,5*S*-$^2H_4$]-muconolactone than the reverse reaction, the formation of *cis,cis*-muconate from (+)-muconolactone. No such perturbations were observed in control reactions employing nondeuterated *cis,cis*-muconate with (+)-muconolactone. The total perturbation corresponds to 10.8 μM *cis,cis*-muconate. This perturbation and the equilibrium isotope effect were used to calculate the secondary kinetic isotope effect of 0.66 for

$$\frac{V_{max}^{CCM(H)}/K_m^{CCM(H)}}{V_{max}^{CCM(D)}/K_m^{CCM(D)}}$$

Primary Kinetic Deuterium Isotope Effect. The double-reciprocal plots of initial velocities vs. concentrations of (+)-muconolactone and (+)-(5*R*-^{2}H]muconolactone[2] for the reaction catalyzed by *cis,cis*-muconate cycloisomerase (Figure 2) indicate that there is little difference in the abscissa intercept in contrast to the more than 2-fold increase in ordinate intercept for deuterated (+)-muconolactone compared with the nondeuterated substrate at saturating Mn^{2+} concentration. The values for the ratios of $K_m^{ML(H)}/K_m^{ML(D)}$ and $V_{max}^{ML(H)}/V_{max}^{ML(D)}$ from these data are 0.90 ± 0.02 and 2.65 ± 0.06, respectively. Thus, the change in K_m value is minimal while, in contrast, there is about a 2.6-fold decrease in V_{max} value upon deuterium substitution, i.e., a primary kinetic deuterium isotope effect for the reaction catalyzed by *cis,cis*-muconate cycloisomerase with deuterated (+)-muconolactone as reactant. The primary kinetic deuterium isotope effect values were determined over pH range of 7.0–9.0 with Mn^{2+} and Mg^{2+} and *cis,cis*-muconate cycloisomerase, respectively (Table II, the values at the lowest pH value with the smallest changes in absorbance are less accurate and for this reason enclosed in parentheses). The data suggest that

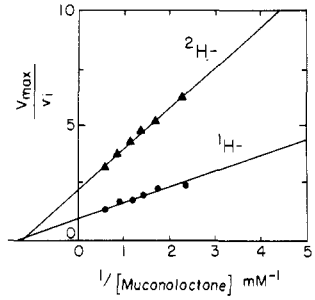

FIGURE 2: Double-reciprocal plots of initial velocities for reactions of (+)-muconolactone and (+)-[5*R*-^{2}H]muconolactone with *cis,cis*-muconate cycloisomerase, both normalized with $k_{cat}^{ML(H)}$: $[E_t]$ = 5 nM (subunit concentration) and $[Mn^{2+}]$ = 100 μM in 20 mM Hepps, pH 8.0 and 25 °C. Solid lines calculated from $v_i = k_{cat}^S[E_t][S]/(K_m^S + [S])$. $k_{cat}^{ML(H)}$ = 2.39 s^{-1}; $k_{cat}^{ML(D)}$ = 0.90 s^{-1}; $K_m^{ML(H)}$ = 0.73 mM; $K_m^{ML(D)}$ = 0.82 mM.

Table II: Primary Deuterium Kinetic Isotope Effects upon Reaction of *cis,cis*-Muconate Cycloisomerase with (+)-Muconolactone and (+)-[5*R*-^{2}H]Muconolactone at 25 °C

pH	$V_{max}^{ML(H)}/V_{max}^{ML(D)}$	$V_{max}^{ML(H)}/K_m^{ML(H)}/V_{max}^{ML(D)}/K_m^{ML(D)}$
	Mn^{2+}-*cis,cis*-Muconate Cycloisomerase[b]	
7.00	2.59 ± 0.38	(5.47 ± 0.62)
7.50	2.39 ± 0.27	3.52 ± 0.17
8.00	2.49 ± 0.11	3.83 ± 0.15
8.00	2.65 ± 0.06	2.97 ± 0.05
8.50	2.63 ± 0.06	2.35 ± 0.05
	Mg^{2+}-*cis,cis*-Muconate Cycloisomerase[c]	
7.00	2.17 ± 0.05	(5.05 ± 0.26)
7.50	2.24 ± 0.10	2.97 ± 0.15
8.00	2.21 ± 0.07	2.67 ± 0.09
8.00	2.35 ± 0.18	2.48 ± 0.18
8.50	2.55 ± 0.19	2.31 ± 0.14
9.00	2.14 ± 0.11	2.38 ± 0.13

[a] pH 7–8, Hepes, 20 mM; pH 8–9, Hepps, 20 mM. [b] 100 μM $MnCl_2$. [c] 1 mM $MgCl_2$.

the primary kinetic deuterium isotope effect for the reaction catalyzed by *cis,cis*-muconate cycloisomerase is independent of pH and the values for Mn^{2+}–enzyme fall within 15% of the values for Mg^{2+}–enzyme.

Solvent Kinetic Deuterium Isotope Effects. Steady-state kinetic studies of the *cis,cis*-muconate cycloisomerase in DOD with *cis,cis*-muconate provide $V_{max}^{CCM(HOH)}/V_{max}^{CCM(DOD)}$ ratios of about 2.5 at several pH values in the range 7.5–8.5 (data not shown). The Michaelis constants are also altered in DOD although the Haldane relationship (Haldane, 1965) is still obeyed.

Discussion

Secondary Equilibrium Deuterium Isotope Effect. Since the conversion of *cis,cis*-muconate to (+)-muconolactone involves sp^2 to sp^3 bond hybridization changes at the C(4) and C(5) carbon atoms, a secondary equilibrium deuterium isotope effect was expected on the basis of the comparable reaction of fumarase (Cook et al., 1980).

Additionally, both semiempirical vibrational-frequency analysis employing the data of Bellamy (1975) with the Streitweiser approximation (Streitweiser et al., 1958) and computed fractionation factor analyses (Hartshorn & Shiner, 1972; Buddenbaum & Shiner, 1977) yield estimates of the secondary equilibrium deuterium isotope effect of 1.38–1.50 (Ngai, 1981).

Secondary Kinetic Isotope Effects. A ratio of 0.66 was obtained for

$$\frac{V_{max}^{CCM(H)}/K_m^{CCM(H)}}{V_{max}^{CCM(D)}/K_m^{CCM(D)}}$$

by the equilibrium perturbation method for the secondary deuterium isotope effect upon the *cis,cis*-muconate cycloisomerase catalyzed conversion of tetradeuterated *cis,cis*-muconate to tetradeuterated (+)-muconolactone and is almost identical with the value of the equilibrium secondary deuterium isotope effect of 0.69 for the comparable fumarase reaction and is in reasonable agreement with the value of 0.76 for the reaction expressed as the formation of (+)-[2,3,4,5*S*-$^2H_4$]-muconolactone from *cis,cis*-[2,3,4,5-$^2H_4$]muconate, i.e.

$$\frac{1/K_H}{1/K_D} = \frac{1}{1.32} = 0.76$$

From the definition of K_H/K_D, the value of the secondary equilibrium isotope effect of 1.45 (Cook et al., 1980), and the secondary kinetic isotope effect from the *cis,cis*-muconate side of 0.66, the calculated value for the secondary kinetic isotope effect on the conversion of (+)-[2,3,4,5*S*-$^2H_4$]muconolactone to *cis,cis*-[2,3,4,5-^{2}H]muconate is

$$\frac{V_{max}^{ML(H)}/K_m^{ML(H)}}{V_{max}^{ML(D)}/K_m^{ML(D)}} = 1.45 \times 0.66 = 0.96$$

Thus, there is little secondary kinetic isotope effect for the conversion of tetradeuterated (+)-muconolactone to tetradeuterated *cis,cis*-muconate within the limits of our measurements, while an inverse secondary kinetic isotope effect is detectable for the formation of tetradeuterated (+)-muconolactone from tetradeuterated *cis,cis*-muconate. An inverse isotope effect is most straightforwardly interpreted in terms of a major effect of the presence of the isotope upon a preequilibrium step occurring prior to the rate-determining step. Thus, on proceeding from *cis,cis*-muconate to (+)-muconolactone, a secondary isotope effect upon step 2 (eq 2) will be reflected in the rate of formation of (+)-muconolactone if step 1 were the rate-determining step. The further discussion of these results and their interpretation will be deferred until a later section.

Primary Kinetic Isotope Effects. The near equivalence of the observed primary kinetic deuterium isotope effects upon V_{max} and V_{max}/K_m for the formation of *cis,cis*-muconate from monodeuterated (+)-muconolactone over the pH range 7.0–9.0 argues that the substrates are not "sticky" (Cook & Cleland, 1981a,b). A similar value for the solvent isotope effect is obtained for the formation of the latter compound from the former in DOD on the basis of the $V_{max}^{CCM(HOH)}/V_{max}^{CCM(DOD)}$ ratio of 2.5 in the pH range 7.5–8.5. The magnitude of these primary and solvent kinetic deuterium isotope effects is significantly less than the theoretical value of 4–6 expected for a rate-limiting proton-transfer step (Melander, 1960). There are two possible explanations for this result: (1) more than a single step in the catalytic sequence is rate limiting, thereby decreasing the contribution of any single step (for example, a step exhibiting a maximal isotope effect) to the observed rates (Klinman, 1978a,b), or (2) a suboptimal kinetic isotope effect may be observed due to a variety of other transition-state considerations such as "triangular", other nonlinear or asymmetrical transition states, contributions from bending vibrations, and the occurrence of new vibrations in the transition state (Westheimer, 1961). The fact that the primary kinetic isotope effect is approximately the same with Mg^{2+} as it is with Mn^{2+} (Table II), although the absolute rates vary by more than 2.5-fold, makes it less likely that multiple partly rate-determining steps exist since the relative effects of divalent metal ion substitution upon the stability of two (or more) transition states would have to be fortuitously identical. In the case of enolase, metal ion substitution had such different effects on the relative stabilities of transition states that a change in rate-determining step appears to occur in the switch from Mg^{2+} to Co^{2+} (Shen & Westhead, 1973).

The constancy of the kinetic primary (Table II) and solvent isotope effects (not shown) over a 10-fold range of hydronium ion activity is further evidence that a single step may be rate determining in the catalytic sequence since, were two steps to be partly rate determining, the pH dependencies of the two activation energy barriers would be required to be identical in order to maintain the kinetic isotope effect constant. Furthermore, the possibility that substrate dissociation from the enzyme is partly rate limiting can be ruled out on the basis of an estimate of the lower limit for the rate constant for lactone dissociation that greatly exceeds the catalytic center activity (Ngai et al., 1983) and from the near equivalence of V_{max} and V_{max}/K_m values at all pH values studied (Cook & Cleland, 1981a,b). In view of the constancy of the kinetic isotope effects with changing pH and metal ion, it may be that a single step is rate limiting and that the low kinetic isotope effect of about 2.5 derives from alternative explanations (Westheimer, 1961). Direct measurements of the intrinsic isotope effect would resolve this issue (Hermes et al., 1982).

The Mechanism. In summary, (a) the primary and solvent isotope effects are consistent with the assignment of rate-determining proton transfer to and from the C(5) carbon atom, and (b) the secondary kinetic isotope effect is fully expressed in the conversion of tetradeuterated *cis,cis*-muconate to tetradeuterated (+)-muconolactone while there is little detectable secondary kinetic isotope effect upon the reverse reaction.

In terms of the four mechanisms proposed, the concerted and carbonium ion mechanisms appear to ruled out by the absence of a secondary kinetic deuterium isotope effect on the forward reaction. Thus, the concerted mechanism is expected to exhibit a secondary kinetic isotope effect in both directions since there are hybridization changes occuring at both the C(4) and C(5) carbon atoms in the elimination reaction. Similarly, the carbonium ion mechanism is expected to show detectable secondary kinetic deuterium isotope effects (about 1.4, Dahlquist et al., 1968) in both directions regardless of which step is rate determining since there are bond-hybridization changes in each of the two steps of the stepwise sequence (eq 3).

The second steps in the carbanion or covalent intermediate mechanisms cannot be rate determining due to the absence of secondary isotope effects on *cis,cis*-muconate formation since bond-hybridization changes occur at both C(4) and C(5) carbon atoms (eq 4 and 5). With the first steps in the carbanion and double-displacement mechanisms assigned as the rate-determining step, no kinetic secondary deuterium isotope effect is expected since there are no net hybridization changes in the formation of EX (eq 4 and 5). The observed primary kinetic deuterium isotope effect would then establish the carbanion intermediate mechanism since in the first step of the double-displacement mechanism there is no involvement of the C(5) hydrogen in the formation of EX from E·ML (eq 5) and thus no possible primary kinetic isotope effect (contrary to observation). In the reverse reaction, the formation of the carbanion intermediate in a rapid prior equilibrium step from E·CCM exhibits the full secondary isotope effect due to the hybridization changes at the C(4) and C(5) carbon atoms (sp^2 to sp^3), prior to the rate-determining proton-transfer step. This

secondary isotope effect on the prior equilibrium step to form the carbanion intermediate is probably approximately equal to the overall equilibrium secondary isotope effect since the bond hybridizations in the formation of the carbanion intermediate and (+)-muconolactone, both from *cis,cis*-muconate, are quite alike. This mechanism is also consistent with the failure to detect release of the deuteron from (+)-[5*R*-^{2}H]-muconolactone at rates greater than the rate of generation of *cis,cis*-muconate.

The placement of Mn^{2+} in juxtaposition to the C(6)-carboxyl group, perhaps with an interposed water molecule, would enable the metal ion to interfere with the carboxylate resonance stabilization and make available the C(6)-carbonyl group in order to stabilize both the carbanion intermediate and the transition state leading to that intermediate as has been shown for the exchange of methylene hydrogen atoms in EDTA–metal ion complexes (Terrill & Reilley, 1966). However, evidence presented elsewhere suggests that the metal ion in this enzyme may in fact not be in a position to play such a direct catalytic role (Ngai et al., 1983).

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