Mathews, M. B. (1980) in *Biology of Collagen* (Viidik, A., & Vuust, J., Eds.) p 193, Academic Press, New York.
Maxam, A., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499.
McKenna, M. C. (1975) in *Phylogeny of the Primates* (Luckett, W. P., & Szalay, F. S., Eds.) p 21, Plenum Press, New York.
Monson, J. M., Friedman, J., & McCarthy, B. J. (1982) *Mol. Cell. Biol. 2*, 1362.
Myers, J. C., Chu, M.-L., Faro, S. H., Clark, W. J., Prockop, D. J., & Ramirez, F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3516.
Olsen, B., & Dickson, L. (1981) in *The Chemistry and Biology of Mineralized Connective Tissues* (Veis, A., Ed.) p 143, Elsevier/North-Holland, New York.
Perler, F., Efstratiadis, A., Lomedico, P., Gilbert, W., Kolodner, R., & Dodgson, J. (1980) *Cell (Cambridge, Mass.) 20*, 555.
Piez, K. A. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) p 1, Plenum Press, New York.
Piez, K. A. (1980) in *Gene Families of Collagen and Other Proteins* (Prockop, D. J., & Champe, P. C., Eds.) p 142, Elsevier/North-Holland, New York.
Prockop, D. J., Kivirikko, K. I., Tuderman, L., & Guzman, N. A. (1979) *N. Engl. J. Med. 301*, 13, 77.
Showalter, A. M., Pesciotta, D. M., Eikenberry, E. F., Yamamoto, T., Pastan, I., de Crombrugghe, B., Fietzek, P. P., & Olsen, B. R. (1980) *FEBS Lett. 111*, 61.
Wilson, A. C., Carlson, S. S., & White, T. J. (1977) *Annu. Rev. Biochem. 46*, 573.
Wozney, J., Hanahan, D., Tate, V., Boedtker, H., & Doty, P. (1981) *Nature (London) 294*, 129.
Yamamoto, T., Sobel, M. E., Adams, S. L., Avvedimento, V. E., DiLauro, R., Pastan, I., de Crombrugghe, B., Showalter, A., Pesciotta, D. M., Fietzek, P. P., & Olsen, B. R. (1980) *J. Biol. Chem. 255*, 2612.

Enzymes of the β-Ketoadipate Pathway in *Pseudomonas putida*: Kinetic and Magnetic Resonance Studies of the *cis,cis*-Muconate Cycloisomerase Catalyzed Reaction†

Ka-Leung Ngai, L. Nicholas Ornston, and Roland G. Kallen*

ABSTRACT: Steady-state kinetic analysis of the divalent metal ion requiring *cis,cis*-muconate cycloisomerase catalyzed interconversion of *cis,cis*-muconate and (+)-muconolactone obeys Michaelis–Menten kinetics and the Haldane relationship from pH 6.2 to 8.3. The pH vs. k_{cat}/K_m profiles suggest free-enzyme apparent pK_a values of 6.2 and 7.4: the reciprocal behavior of the data with respect to the latter pK_a value is consistent with base–acid catalysis by the enzyme involving proton removal from the lactone and protonation of *cis,cis*-muconate, respectively. This catalysis by the enzyme of proton transfer is consistent with the stereospecific incorporation of solvent deuterium into the *pro*-5*R* position of (+)-muconolactone in the enzyme-catalyzed reaction: in reverse, the departure of the carboxylic oxygen atom and proton from the C(4) and C(5) carbon atoms follows a syn (cis) route [Avigad, G., & Englard, S. (1969) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28*, 345, Abstr. 486]. The titration of enzyme freed of divalent metal ion with manganous ion, monitored by electron paramagnetic resonance spectroscopy and steady-state kinetic measurements, indicates a single binding site per subunit characterized by $K_{diss}^{E\cdot Mn} = [E][Mn^{2+}]/[E\cdot Mn^{2+}] = 4.5$ and 3.0 μM, respectively, the latter value analyzed via a rapid equilibrium mechanism. The paramagnetic effects of Mn^{2+} on the $1/T_1$ and $1/T_2$ values for the H-5*S* proton of (+)-muconolactone in the E·ML·Mn ternary complex provide an estimate of the correlation time, τ_c, at 5×10^{-9} s from the T_1/T_2 ratio, indicating that the condition of rapid exchange of (+)-muconolactone in solution with the ternary complex obtains. From the T_2 values, the rate constant for dissociation of (+)-muconolactone from the ternary complex is 3.3×10^4 s^{-1}, which is greater than the catalytic center activity for studies of the reaction in the direction *cis,cis*-muconate to (+)-muconolactone by a factor of about 10^3, consistent with the rapid equilibrium assumption. The distances from the enzyme-bound Mn^{2+} to the H-2, H-3, and H-5*S* protons calculated from the T_1 values for the individual protons are 5.8, 5.3, and 5.2 Å, respectively. The distance from the enzyme-bound Mn^{2+} to the H-5*S* proton of (+)-muconolactone in the ternary complex appears to allow either direct or outer sphere coordination of the metal ion to the C(6)-carboxyl group of (+)-muconolactone. However, EPR spectra of the E·Mn·ML complex at 35 GHz are identical with spectra of the E·Mn complex, making unlikely direct coordination and suggesting that, alternatively, the metal ion may merely serve a structural role.

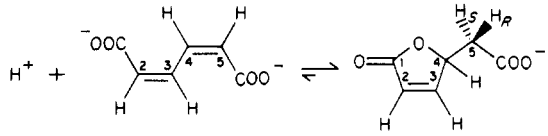
cis,cis-Muconate cycloisomerase[1] (EC 5.5.1.1) is one of the enzymes involved in the catabolism of benzoic acid by means of the pyrocatechol branch of the β-ketoadipate pathway in *Pseudomonas putida*. This enzyme catalyzes the reversible

† From the Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (K.-L.N. and R.G.K.), and the Department of Biology, Yale University, New Haven, Connecticut 06520 (L.N.O.). *Received January 14, 1983; revised manuscript received May 24, 1983.* Supported by Grant GM 13777 from the National Institutes of Health (R.G.K.), Grants GK 27697K (R.G.K.) and PCM 77-24834 (L.N.O.) from the National Science Foundation, Grant RR 00512 from the Middle Atlantic NMR Research Facility, and Research Career Development Award K4 CA 70487 from the National Cancer Institute (R.G.K.). A portion of this work has been published in preliminary form (Ngai & Kallen, 1977, 1979), and further details may be found in a Ph.D. Dissertation (Ngai, 1981).

[1] Abbreviations: CCM, *cis,cis*-muconate (2,4-hexadienedioic acid); E·CCM, ternary complex of enzyme, metal ion, and CCM; E·ML, ternary complex of enzyme, metal ion, and ML; $[E_t]$, total enzyme concentration on a subunit basis; DOD, deuterium oxide; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; EDTA, ethylenediaminetetraacetic acid; ML, (+)-muconolactone [γ-(carboxymethyl)-Δ^{α}-butenolide; 4-(*S*)-hydroxy-2-hexenedioic acid 1,4-lactone]; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance. *cis,cis*-Muconate cycloisomerase has also been referred to as *cis,cis*-muconate lactonizing enzyme (MLE).

0006-2960/83/0422-5223$01.50/0

interconversion of *cis,cis*-muconate and (+)-muconolactone (eq 1): the reaction constitutes an intramolecular cyclization



$$H^+ + CCM^{2-} \rightleftharpoons ML^- \quad (1)$$

to form a lactone in the forward direction (as written) or, in reverse, a 1,2-elimination reaction.

The enzyme has been isolated from *P. putida* (PRS 2000) in a homogeneous, crystalline form (Sistrom & Stanier, 1954; Ornston, 1966; Meagher & Ornston, 1973) and is composed of six identical subunits each of molecular weight 40 000 containing a single active site (Meagher & Ornston, 1973). The amino acid composition has been reported (Meagher & Ornston, 1973), and amino acid sequence determination is in progress (W.-K. Yeh, personal communication). A divalent metal ion requirement for activity (Sistrom & Stanier, 1954) of the cycloisomerase suggested that this enzyme would be a fruitful subject for detailed kinetic and magnetic resonance studies directed toward the elucidation of its mechanism of action and, especially, the role of the divalent metal ion in catalysis. The first part of this study, the steady-state kinetic and magnetic resonance analysis, is reported herein. The following paper describes primary and secondary kinetic and equilibrium isotope effects and further aspects of the detailed catalytic mechanism (Ngai & Kallen, 1983).

Experimental Procedures

Materials

Chemicals. Hepps, Hepes, Mes, and Pipes (Sigma Chemical Co.), Tris and Tris-HCl (ultrapure, Schwarz/Mann), and other reagents including deuterated acetone, deuterated phenol, and 40% NaOD and 20% DCl in deuterium oxide (where D is deuterium) were of reagent grade or of the highest grade available and used without further purification. Chelex 100 (Na form, 100–200 mesh), AG 1-X8 (chloride form, 200–400 mesh), and columns were from Bio-Rad. DEAE-cellulose (DE-52) was obtained from Whatman. Deionized water of greater that 5×10^5 Ω cm specific resistance was employed throughout.

cis,cis-Muconic acid was prepared by peracetic acid oxidation of phenol (Elvidge et al., 1950; Ngai, 1981). The mp_{uncorr} of the white crystals was 183–184 °C (lit. mp_{uncorr} 184 °C, Elvidge et al., 1950).

(+)-Muconolactone was prepared enzymatically from *cis,cis*-muconic acid with *cis,cis*-muconate cycloisomerase according to the modified method of Ornston (Ornston & Stanier, 1966; Ngai, 1981). The white crystals had a mp_{uncorr} of 76.5 °C (lit. mp_{uncorr} 75 °C, Ornston & Stanier, 1966).

Enzymes. cis,cis-Muconate cycloisomerase was purified to electrophoretic homogeneity (Laemmli, 1970) and crystallized from *P. putida* as described previously (Meagher & Ornston, 1973; Ngai, 1981); the crystalline suspension (sp act. 90–100 units/mg of protein) in 20 mM Tris-HCl buffer, pH 7.4, containing 10 μM manganous chloride and 30% saturated ammonium sulfate, was stored at 4 °C.

Methods

Instruments. Ultraviolet absorbance measurements were made with Beckman DU/Gilford 2000 and Cary 118 spectrophotometers equipped with thermostated cell holders, maintained at 25 ± 0.1 °C. Measurements of pH employed a Radiometer pHM 63 digital instrument with Radiometer GK 2301B or G 222B combined electrodes standardized at pH 4, 7, and 10 with buffers from Beckman. Values of pD in deuterium oxide solutions were obtained by addition of 0.4 to the pH meter reading (Salomaa et al., 1964).

NMR spectra and proton relaxation times were measured at 220 MHz on a Varian HR-220 spectrometer equipped with a Varian computer in the Fourier-transform mode (Middle Atlantic NMR Research Facility) with 0.2–1.0-mL samples in 5-mm sample tubes (Wilmad) at ambient temperature (22–25 °C). Deuterium oxide solutions contained DDS and deuterated aprotic solvents contained Me_4Si as internal references. Chemical shifts are reported as ppm downfield from DSS or Me_4Si, respectively.

EPR spectra were recorded on a Varian E3 spectrometer at 9.1 GHz (X-band) at 25 °C, maintained by nitrogen flow. Samples (50 μL) were contained in quartz capillary (1-mm internal diameter) sample tubes sealed with polyethylene tubing and a Teflon plug.

Substrate Concentrations. cis,cis-Muconate and (+)-muconolactone concentrations were determined by using molar absorptivity values of 1.72×10^4 M^{-1} cm^{-1} at 260 nm and 1.52×10^3 M^{-1} cm^{-1} at 230 nm, respectively, valid for the pH range studied (Sistrom & Stanier, 1954; Ornston & Stanier, 1966).

Enzyme Activity Assays. cis,cis-Muconate cycloisomerase activity was measured spectrophotometrically on the basis of the absorbance at 260 nm of *cis,cis*-muconate (Ornston, 1966). An assay mixture of 100 μM *cis,cis*-muconate and 1 mM manganous chloride in 33 mM Tris-HCl buffer, pH 8.0, was used. A unit of activity is that amount of enzyme that catalyzes the conversion of 1 μmol of *cis,cis*-muconate to (+)-muconolactone per min at 25 °C under the above conditions. Protein concentration was determined colorimetrically (Lowry et al., 1951) with bovine serum albumin as a standard. *cis-cis*-Muconate cycloisomerase subunit (catalytic center) concentration, $[E_t]$, was calculated from the protein concentration and a subunit molecular weight of 40 000 (Meagher & Ornston, 1973). The ionic strength, mainly contributed by the buffers, was in the range 0.01–0.1 M. The enzymatic activity is independent of ionic strength in this range of ionic strength. The enzyme is stable over the pH range 5.2–8.3 under the conditions employed in the present studies.

Removal of Divalent Metal Ion from cis,cis-Muconate Cycloisomerase. Deionized water and 20 mM Hepes buffer were treated with Chelex 100 (about 1 g/100 mL in columns or batchwise) in order to remove divalent metal ions and stored in plastic vessels. Dialysis tubing was boiled in 0.5 M Na_2-EDTA, pH 5–6, for 15 min and washed with the Chelex-treated deionized water. *cis,cis*-Muconate cycloisomerase in 20 mM Hepes buffer, pH 7.4, containing 10 μM EDTA was incubated at 4–5 °C for 1–2 h until no detectable enzyme activity was present. Enyzme activity assays were performed as described above except that Chelex-treated substrate and buffer solutions containing 10 μM EDTA were employed. The EDTA-treated enzyme was placed in the dialysis tubing, dialyzed against 1 L of Chelex-treated buffer with two changes, placed in plastic tubes, and employed as enzyme freed of divalent metal ion.

Equilibrium Measurements. (A) Ionization Constants. Substrate and product ionization constants were determined titrimetrically as described elsewhere (Kallen, 1971).

(B) Equilibrium Constant for the Interconversion of cis,-cis-Muconate and (+)-Muconolactone. Apparent equilibrium constant measurements for the interconversion of *cis,cis*-muconate and (+)-muconolactone, K_{app} = [(+)-muconolactone$_t$]/[*cis,cis*-muconate$_t$], where t = sum of all ionic species of the designated compound present at various pH values, were

obtained from either the total *cis,cis*-muconate or (+)-muconolactone concentrations at zero time and the *cis,cis*-muconate concentration at equlibrium, determined spectrophotometrically at 260 nm following the addition of *cis,cis*-muconate cycloisomerase. Below and above pH 7.5, the equilibrium was approached from (+)-muconolactone (initial concentration 1 mM, 1-cm path length) and *cis,cis*-muconate (initial concentration 100 μM, 1-mm path length), respectively. These data have been corrected for minor amounts of *cis,trans*-muconate and *trans,trans*-muconate (<5%) that tend to form spontaneously.

(C) Metal Ion Complexation Equilibrium Constants. Measurements of free manganous ion concentration in aqueous solutions with *cis,cis*-muconate, (+)-muconolactone, or the previously EDTA-treated enzyme (50–100 μM subunit concentration) were made from the amplitudes of the six hyperfine EPR spectral lines that are proportional to the free manganous ion concentrations upon the basis of a constructed standard curve for concentrations >1 μM (Cohn & Townsend, 1954). The complexation equilibrium dissociation constants, $K_{diss}^{Mn\cdot L}$, were calculated from the total amounts of manganous ion and ligand added and the free concentrations of manganous ion at equilibrium, where $K_{diss}^{Mn\cdot L} = [Mn][L]/[Mn\cdot L]$, Mn is manganous ion, L is ligand, and Mn·L is the binary complex.

Kinetic Measurements. The reaction mixture containing substrate, divalent metal ion, and buffer was placed in a 3-mL cuvette of 10-mm path length or a 0.5-mL cuvette of 1-mm path length. Reaction was initiated by addition of a small volume (<25 μL) of a solution containing *cis,cis*-muconate cycloisomerase (about 1 μM subunit concentration), and absorbance readings were recorded at 260 nm for the initial 5 min. The initial velocities at various substrate concentrations were obtained from linear absorbance vs. time traces.

NMR Experiments on Incorporation of Deuterium into cis,cis-Muconate and (+)-Muconolactone in the cis,cis-Muconate Cycloisomerase Catalyzed Reaction. A solution of 1.0 M sodium phosphate buffer in deuterium oxide, pD 8.4, containing 5 μM manganous chloride and 100 mM *cis,cis*-muconate or (+)-muconolactone was lyophilized and redissolved in DOD prior to the addition of *cis,cis*-muconate cycloisomerase. The time dependence of the amplitude of the absorbances assigned to the *cis,cis*-muconate and (+)-muconolactone hydrogens of the ^1H NMR spectra was recorded.

(A) Nuclear Magnetic Resonance Measurements. Cyclic dissolution of chemical compounds and lyophilization in deuterium oxide enabled the replacement of exchangeable protons with deuterons.

(B) Relaxation Measurements. *cis,cis*-Muconate cycloisomerase was dialyzed against three changes of buffer A: Tris-HCl, pD 7.8 (10 mM), and manganous chloride (5 μM) in deuterium oxide. Equal volumes (500 μL) of (+)-[5*R*-^2H]muconolactone and enzyme solutions in buffer A were mixed, the pD was established at 7.4 with NaOD, and the solution was transferred to a 5-mm NMR tube. Measurements for individual resonances of the longitudinal relaxation time, T_1, were obtained by the Fourier-transform saturation recovery method using the "90°–homogeneity spoil–τ–90°" pulse sequence (McDonald & Leigh, 1973) on the Varian HR-220 spectrometer and eq 2:

$$\ln (M_\infty - M_\tau) = -(1/T_1)\tau + \ln M_\infty \quad (2)$$

where M_τ and M_∞ are the magnitudes (peak heights) of the magnetization of the resonance for delay time, τ, and infinite τ, respectively. A least-squares fit for ≥10 data points for τ values ≥7T_1 values provided T_1 values. The transverse relaxation time, T_2, measurements for the individual proton resonances were determined from the line width at half-maximal height of the expansion of the appropriate peaks of a Fourier-transform-enhanced spectrum and eq 3:

$$1/T_2 = \pi W_{1/2} \quad (3)$$

where $W_{1/2}$ is the line width at half-maximal height in hertz.

The paramagnetic contribution to the longitudinal and transverse relaxation rate constants, $1/T_{1P}$ and $1/T_{2P}$, respectively, to a nearby proton resonance was calculated from eq 4:

$$1/T_{1P} = 1/T_1 - 1/T_1^0 \quad (4a)$$

$$1/T_{2P} = 1/T_2 - 1/T_2^0 \quad (4b)$$

where $1/T_1$ and $1/T_2$ represent the respective relaxation rate constants in the presence of the enzyme and $1/T_1^0$ and $1/T_2^0$ represent the respective relaxation rate constants in the absence of the paramagnetic species.

With normalization by the factor f = [(+)-muconolactone$_{bound}$]/[(+)-muconolactone$_{free}$] (Luz & Meiboom, 1964), in the limit of "fast exchange" of ligand, i.e., when T_{1M} and T_{2M} are much greater than the lifetime of the ligand in the E·ML·Mn ternary complex (Swift & Connick, 1962, 1964; Nowak & Mildvan, 1972), the paramagnetic longitudinal and transverse relaxation rate constants for (+)-muconolactone in the first coordination sphere of ternary complex, $1/T_{1M}$ and $1/T_{2M}$, respectively, are given by eq 5.

$$1/(fT_{1P}) = 1/T_{1M} \quad (5a)$$

$$1/(fT_{2P}) = 1/T_{2M} \quad (5b)$$

A derivative of the simplified form of the Solomon–Bloembergen–Morgen equation (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen & Morgen, 1961; Bloembergen, 1957) applicable to manganous ion proton interactions, eq 6,

$$r = \left(CfT_{1P} \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right)^{1/6} \quad (6)$$

was employed for distance measurements, where r is the metal ion–proton distance (cm), C has a value of 2.88×10^{-31} rad^2 cm^6 s^{-2} for the manganous ion–hydrogen nucleus system, τ_c is the dipolar correlation time, and ω_I is the nuclear Lamor precession frequency. Thus, the distance from the Mn^{2+} to the hydrogen of ligand, r, can be calculated from eq 6 with τ_c estimated from the ratio T_{1P}/T_{2P} (James & Cohn, 1974), from eq 7:

$$\tau_c = \frac{(3T_{1P}/2T_{2P} - 7/4)^{1/2}}{\omega_I} \quad (7)$$

A more detailed discussion of the theory and calculations is presented elsewhere (Cohn, 1963; Dwek, 1973; James, 1975; Mildvan & Gupta, 1978; Ngai, 1981).

Data Processing. The kinetic data were in general fitted to the appropriate rate equations by least-squares methods (Cleland, 1967), utilizing a Commodore PET 2001 digital computer with teletype output to provide the kinetic parameters with standard errors.

Results and Discussion

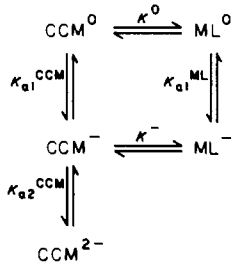
Equilibrium Constant for Interconversion of cis,cis-Muconate and (+)-Muconolactone. The apparent equilibrium constant, K_{app}, for the formation of (+)-muconolactone from *cis,cis*-muconate is pH dependent [see Table I and eq 8 based

Table I: Apparent Equilibrium Constant Values, K_{app}, for Formation of (+)-Muconolactone from *cis,cis*-Muconate at 25 °C

pH	K_{app}[a] I	II
6.0	1060[b]	
6.5	525[b]	
7.0		106.8 ± 4.5
7.4	45 ± 3	
7.5	40.6 ± 4.2	35.5 ± 0.3
	24.7[b]	85.1 ± 25.5
8.0	12.6[b]	9.8 ± 2.0
	11.6 ± 0.4	14.0 ± 0.5
	12.3 ± 0.6	
	11.6 ± 0.2	
8.5	3.9 ± 0.1	4.1 ± 0.1

[a] K_{app} = [(+)-muconolactone$_t$]/[*cis,cis*-muconate$_t$]. pH 6–8, 0.1 M Hepes; pH 8–8.5, 0.1 M Hepps or 0.1 M Tris-HCl. (Column I) Equilibrium approached from *cis,cis*-muconate (1 mM); (column II) equilibrium approached from (+)-muconolactone (100 μM); ± standard errors (four to six determinations at each pH value). [b] Ornston & Stanier (1966).

Scheme I: Equilibria for Isomerization of *cis,cis*-Muconate and (+)-Muconolactone[a]



[a] CCM^0, CCM^-, and CCM^{2-} are the different ionic species of *cis,cis*-muconate; ML^0 and ML^- are the different ionic species of (+)-muconolactone.

upon Scheme I (Ngai, 1981)], as expected for a reaction with net proton uptake over the pH range 6.5–8.5 (see eq 1):

$$K_{app} = \frac{[ML^0] + [ML^-]}{[CCM^0] + [CCM^-] + [CCM^{2-}]} = K^0 \frac{1 + K_{a1}^{ML}/a_H}{1 + \frac{K_{a1}^{CCM}}{a_H} + \frac{K_{a1}^{CCM}K_{a2}^{CCM}}{a_H^2}} \quad (8)$$

where K_{a1}^{ML}, K_{a1}^{CCM}, and K_{a2}^{CCM} are the proton dissociation constants as designated in Scheme I, $K^0 = [ML^0]/[CCM^0]$, and a_H is the hydrogen ion activity. The data in Table I are fit satisfactorily to eq 8 (Scheme I) with K^0 and K^- values of 3.65×10^4 and 1.60×10^4, respectively, with pK_{a1}^{CCM}, pK_{a2}^{CCM}, and pK_{a1}^{ML} values of 3.72, 4.86, and 4.08, respectively.

These data indicate that the neutral (+)-muconolactone is somewhat more stable relative to neutral *cis,cis*-muconate than is the anionic (+)-muconolactone relative to monoanionic *cis,cis*-muconate. A greater inductive effect of the un-ionized carboxyl group compared with the carboxylate anion upon the stabilities of the cyclic lactone and open-chain forms is probable. Nevertheless, at pH values <9, the (+)-muconolactone is the predominant species present in solutions at equilibrium.

Steady-State Kinetic Studies of the cis,cis-Muconate Cycloisomerase Catalyzed Reaction. (A) Relative Effectiveness of Divalent Metal Ions. The dependence of initial velocity upon reactant concentration for the reaction with *cis,cis*-muconate as substrate and the reaction in the reverse direction

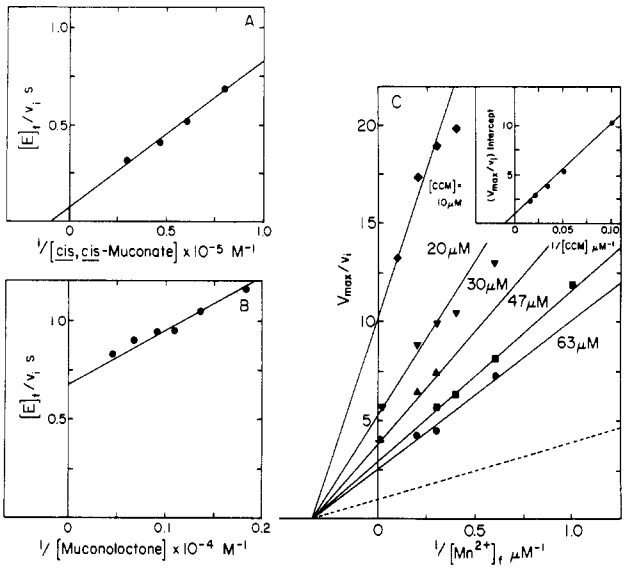


FIGURE 1: Steady-state kinetic analysis of *cis,cis*-muconate cycloisomerase catalyzed interconversion of *cis,cis*-muconate and (+)-muconolactone. (A) *cis,cis*-Muconate as reactant, 20 mM Hepes, pH 7.4, 25 °C; $[Mn^{2+}]$ = 100 μM, $[E_t] = 3 \times 10^{-8}$ M (subunit concentration), k_{cat}^{CCM} = 13.9 s^{-1} and K_m^{CCM} = 105 μM. (B) (+)-Muconolactone as reactant, $[E_t] = 5 \times 10^{-9}$ M (subunit concentration), other conditions as above; k_{cat}^{ML} = 1.4 s^{-1}, K_m^{ML} = 424 μM. (C) Dependence of initial velocity upon divalent metal ion concentration at varying concentrations of *cis,cis*-muconate; K_m^M = 3 μM, $[E_t] = 3 \times 10^{-8}$ M (subunit concentration), and K_m^{CCM} = 105 μM. Solid lines calculated from eq 9 and 10 (with α = 1.0), V_{max}^{CCM} = 0.33 s^{-1} and V_{max}^{ML} = 0.036 s^{-1}.

Table II: Steady-State Kinetic Constants for Interconversion of *cis,cis*-Muconate and (+)-Muconolactone Catalyzed by *cis,cis*-Muconate Cycloisomerase at 25 °C[a]

	constant (units)	value
cis,cis-muconate	K_m^{CCM} (μM)	90 ± 5
		105 ± 10
	k_{cat}^{CCM} (s^{-1})	13.9 ± 1.0[b]
	$pK_{a1}^{E\cdot CCM}$	6.0[d]
Mn^{2+}	$K_m^{E\cdot Mn}$ (μM)	3 ± 1
	$K_m^{E\cdot Mn}$ (μM)	4.5 ± 1[c]
(+)-muconolactone	K_m^{ML} (μM)	424 ± 20
	k_{cat}^{ML} (s^{-1})	1.4 ± 0.2[b]
	$pK_{a1}^{E\cdot ML}$	7.5[d]

[a] Hepes, pH 7.4, 20 mM; for definitions of constants see Scheme II and eq 9; $[Mn^{2+}]$ = 100 μM except for determination of $K_m^{E\cdot Mn}$. [b] k_{cat} expressed per catalytic center. [c] Determined by EPR measurements (see text). [d] pK_{a1}^{E} = 6.2; pK_{a2}^{E} = 7.4.

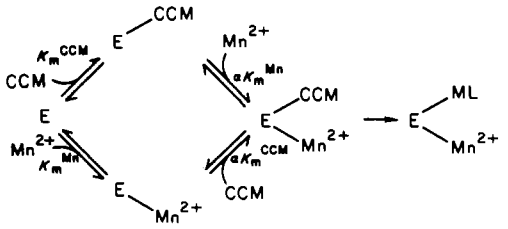
with (+)-muconolactone as substrate at saturating divalent metal ion (manganous ion concentration about 0.1 mM)[2] follow the Michaelis–Menten rate equation (Figure 1A,B double-reciprocal plots):

$$v_i = \frac{V_{max}^S}{\frac{K_m^S}{[S]} + 1} \quad (9)$$

with apparent (pH-dependent) parameters as follows: v_i is the initial velocity; [S] is the reactant concentration; V_{max}^S is the

[2] The equilibrium constants for binary complex formation between *cis,cis*-muconate and (+)-muconolactone and divalent metal ions are sufficiently unfavorable that no correction of the data in this paper for this effect was required.

Scheme II



maximal velocity; and K_m^S is the apparent Michaelis constant for reactant. The kinetic parameters (per catalytic center) of $k_{cat}^S = V_{max}^S/[E_t]$ and K_m^S for *cis,cis*-muconate and (+)-muconolactone, respectively, are contained in Table II. The intervention of apparent inhibition at higher concentrations of *cis,cis*-muconate (>1 mM) limited the range of substrate concentrations studied, but these data are sufficient for the evaluation of valid steady-state constants as indicated by the adherence to the Haldane relationship (see below). That many mono- and dianions are competitive inhibitors of cycloisomerase with adipate the most effective is consistent with the observed substrate inhibition (Ngai, 1981). Similar steady-state kinetic studies conducted at saturating substrate concentrations with other divalent metal ions (not shown) indicate the following order of catalytic effectiveness: Mn^{2+} (100%) ≫ Ni^{2+} (53%) > Co^{2+} (47%) > Mg^{2+} (40%), although the Michaelis constants for *cis,cis*-muconate appear to be independent of the metal ion employed (K_m^{CCM} = 105 μM).

The dependencies of the initial velocity upon the concentration of manganous ion (Figure 1C) for the disappearance of *cis,cis*-muconate, catalyzed by *cis,cis*-muconate cycloisomerase (previously rendered divalent metal ion free by treatment with EDTA), are consistent with a rapid equilibrium mechanism (Scheme II) for the binding of manganous ion and reactant to the enzyme (Segal, 1975). The rate equation (eq 10, where [M] is the metal ion concentration and α is a factor

$$\frac{v_i}{V_{max}^{CCM}} = \frac{1}{\frac{\alpha K_m^M K_m^{CCM}}{[M][CCM]} + \frac{\alpha K_m^{CCM}}{[CCM]} + \frac{\alpha K_m^M}{[M]} + 1} \quad (10)$$

that reflects the extent to which the binding of metal ion affects the binding of *cis,cis*-muconate and vice versa) is derived from Scheme II where $[E_t]$ is the enzyme subunit concentration, k_{cat}^{CCM} is the turnover number per catalytic center, and K_m^M, K_m^{CCM}, and K_m^{ML} are the various dissociation constants for metal, substrate, and product from the several enzyme species. The equation describes the kinetics for the forward reaction and was employed in order to obtain least-squares fits of the experimental data (Table II). The unfavorable equilibrium for the formation of *cis,cis*-muconate from (+)-muconolactone made difficult similar kinetic studies of the reverse reaction. For the data illustrated in Figure 1C, the intersection of the various lines on the abscissa for $V_{max}^{CCM}/v_i = 0$ is consistent with a value of α of 1.0, i.e., no significant influence upon the binding of *cis,cis*-muconate by the presence of metal ion and vice versa (consistent with EPR experiments to be described below).

(B) pH Dependence of Steady-State Kinetic Parameters. The steady-state kinetic parameters are pH dependent (Figure 2) as follows [the pH profile for the formation of *cis,cis*-muconate from (+)-muconolactone is more limited due to the progressively greater difficulty observing *cis,cis*-muconate formation with decreasing pH values]: (i) The k_{cat}^{CCM}/K_m^{CCM} and k_{cat}^{ML}/K_m^{ML} values in the pH region greater than pH 7 appear to be governed in a reciprocal fashion by an apparent

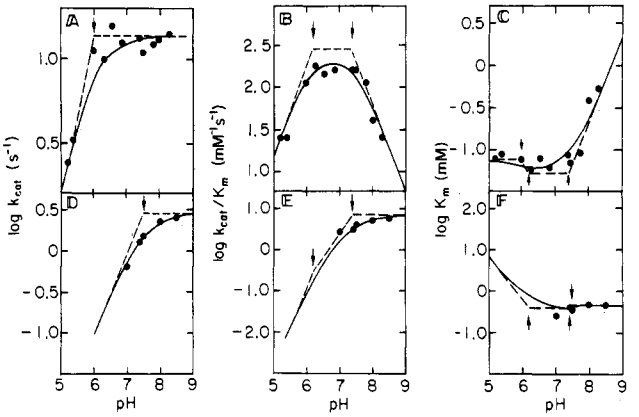
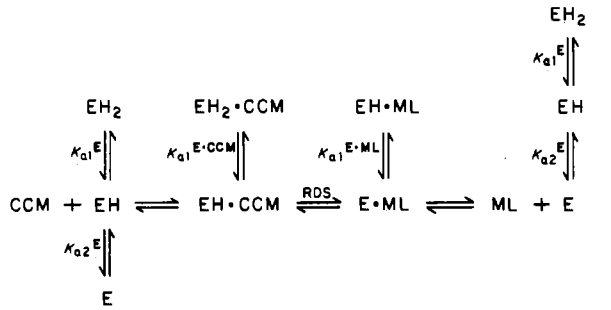


FIGURE 2: pH dependence of steady-state kinetic parameters for *cis,cis*-muconate cycloisomerase catalyzed conversion of *cis,cis*-muconate to (+)-muconolactone [(A–C) $[E_t]$ = 30 nM] at saturating $[Mn^{2+}]$ = 100 μM, 25 °C, and for the conversion of (+)-muconolactone to *cis,cis*-muconate [(D–F) $[E_t]$ = 5 nM]: (A) k_{cat}^{CCM}; (B) k_{cat}^{CCM}/K_m^{CCM}; (C) K_m^{CCM}; (D) k_{cat}^{ML}; (E) k_{cat}^{ML}/K_m^{ML}; (F) K_m^{ML}. Buffers employed were Mes (pH 5.5–7.0) and Hepes (pH 7.0–8.5); ionic strength was 0.025 M. Solid lines are calculated from the equations of Scheme III and the pK values contained in Table II with $k_{cat}^{CCM,0}$ = 14 s^{-1}, $k_{cat}^{ML,0}$ = 2.8 s^{-1}, $K_m^{CCM,0}$ = 48 μM, and $K_m^{ML,0}$ = 435 μM.

Scheme III: Kinetic Mechanism for *cis,cis*-Muconate Cycloisomerase[a]



$$k_{cat}^{CCM} = k_{cat}^{CCM,0} f(a)$$
$$K_m^{CCM} = K_m^{CCM,0} f(a)/f(e2)$$
$$\frac{k_{cat}^{CCM}}{K_m^{CCM}} = \frac{k_{cat}^{CCM,0} f(a)}{K_m^{CCM,0} f(a)/f(e2)}$$
$$k_{cat}^{ML} = k_{cat}^{ML,0} f(p)$$
$$K_m^{ML} = K_m^{ML,0} f(p)/f(e3)$$
$$\frac{k_{cat}^{ML}}{K_m^{ML}} = \frac{k_{cat}^{ML,0} f(p)}{K_m^{ML,0} f(p)/f(e3)}$$

[a] $f(a) = 1/(1 + a_H/K_{a1}^{E\cdot CCM})$; $f(e2) = 1/(1 + a_H/K_{a1}^{E} + K_{a2}^{E}/a_H)$; $f(p) = 1/(1 + a_H/K_{a1}^{E\cdot ML})$; $f(e3) = 1/[1 + a_H/K_{a2}^{E} + a_H^2/(K_{a1}^{E} K_{a2}^{E})]$.

pK_a value of 7.4, consonant with the assignment of this apparent value to a group that functions as an acid–base catalyst in this reaction (Figure 2B,E). This group presumably removes the *pro-R* proton at the C(5) position of (+)-muconolactone (in the reverse reaction) and in the forward reaction protonates the C(5) carbanionic site to form (+)-muconolactone. (ii) The same conclusion derives from the k_{cat}^{ML} and k_{cat}^{CCM} vs. pH profiles, in the former case formally and mechanistically base catalysis but, in the latter case, mechanistically specific base–general acid catalysis but formally general-base catalysis. (iii) The K_m^{CCM} and K_m^{ML} values for *cis,cis*-muconate and (+)-muconolactone appear to be controlled by apparent pK_{a1}^{E} and pK_{a2}^{E} values of 6.2 and 7.4 and the $pK_{a1}^{E\cdot CCM}$ and $pK_{a1}^{E\cdot ML}$ values of 6.0 and 7.5, respectively (Table II and Figure 2C,F).

These observations are incorporated into kinetic Scheme III, which applies at saturating divalent metal ion concentrations to the forward and reverse reactions, respectively. $k_{cat}^{CCM,0}$ and $K_m^{CCM,0}$ are pH-independent values of k_{cat}^{CCM} and K_m^{CCM}, respectively, and $k_{cat}^{ML,0}$ and $K_m^{ML,0}$ are pH-independent values for k_{cat}^{ML} and K_m^{ML}, respectively. The solid lines have been fitted with acceptable agreement to steady-state kinetic data of Figure 2 (see legend) according to Scheme III.

Haldane Relationships. The Haldane relationship (Haldane, 1965) for the *cis,cis*-muconate cycloisomerase catalyzed reaction at any given pH value at saturating divalent metal ion concentration is given by eq 11:

$$K_{app}^{kinetic} = (k_{cat}^{CCM}K_m^{ML})/(K_{cat}^{ML}K_m^{CCM}) \quad (11)$$

which yields for representative kinetic data at pH 7.4 (Table II) $K_{app}^{kinetic} = (13.9 \times 0.42)/(1.4 \times 0.10) = 42$, comparable to the value of 45 obtained from equilibrium measurements (Table I). The agreement of the experimental data in Figure 2 indicates that the Haldane relationship is obeyed over the entire pH range studied.

Binary Complexes. The interactions of manganous ion with substrates and enzyme to yield Mn^{2+}–substrate and E·Mn complexes, respectively, were investigated by EPR spectroscopy as follows: Titration of manganous ion in solution with increasing concentrations of substrate or EDTA-treated enyzme was performed, and the free manganous concentrations were measured by EPR (Cohn & Townsend, 1954; Mildvan & Cohn, 1963). Values of the binary constant were calculated from the total and free manganous ion concentrations and total ligand concentration (in the case of enzyme, total subunit concentration). The values of the dissociation constants are $K_{diss}^{Mn\cdot ML} = 152 \pm 15$ mM, $K_{diss}^{Mn\cdot CCM} = 31 \pm 3$ mM, and $K_{diss}^{E\cdot Mn} = 4.5 \pm 1.0$ μM. A plot of the latter data according to the method of Scatchard (1949) (not shown) yielded a stoichiometry of 0.95 ± 0.05 Mn^{2+} binding site per subunit (Ngai, 1981). The agreement between this value and the dissociation constant for the Mn^{2+} dissociation from enzyme and binary enzyme–substrate complexes, corresponding to a random equilibrium binding kinetic scheme, supports the correctness of the steady-state analysis.

Ternary Complexes. The data on the magnitudes of the dissociation constants of the binary substrate–manganous ion complexes indicate that the only significant Mn^{2+} complexes are E·Mn, E·CCM·Mn, and E·ML·Mn under the conditions employed in the NMR experiments to be described. That the metal ion dissociation constants for E·Mn and E·ML·Mn are about equal, measured by EPR spectroscopy, is consistent with an α value of about unity in eq 10.

Paramagnetic Effects of Enzyme–Mn^{2+} on Relaxation Rates of Hydrogens of (+)-Muconolactone. From the equilibrium [(+)-muconolactone]/[*cis,cis*-muconate] ratio in DOD of >100 at pD 7.8 [Table I and Ngai & Kallen (1983)], (+)-muconolactone is the predominant species in an equilibrium mixture with the enzyme, enabling the determination of the longitudinal and transverse relaxation rate constants (for the assigned protons, Table III) and, unfortunately, rendering inaccessible comparable studies with *cis,cis*-muconate.

For the 5*S* hydrogen atom of (+)-[5*R*-^{2}H]muconolactone, the transverse relaxation rate constant is an order of magnitude greater than the longitudinal relaxation rate constant, indicating that the latter rate constant is not limited by chemical exchange. The paramagnetic effects of E·Mn on the relaxation of the hydrogen atoms of (+)-[5*R*-^{2}H]muconolactone (Table III) were calculated from eq 4. Since the condition of "fast exchange" of (+)-[5*R*-^{2}H]muconolactone has been established, the longitudinal relaxation rate constants can be used for distance calculations. The correlation time τ_c of the complex was estimated from eq 7 to be about 5.0×10^{-9} to 10^{-8} s. The Mn^{2+} to proton distances of (+)-[5*R*-^{2}H]muconolactone within ternary complex (Table III) were calculated from $1/T_{1P}$ values with eq 6. From those distances, the spatial relationship of enzyme, Mn^{2+}, and (+)-muconolactone in the fully active ternary complex is obtained as follows:

Table III: Peak Assignments, Relaxation Rates, and Paramagnetic Effects on Relaxation Rates of the Proton Resonances of (+)-[5*R*-^{2}H]Muconolactone with *cis,cis*-Muconate Cycloisomerase[a]

	proton (absorption band in ppm)		
constant (units)	H-2 (6.27)	H-3 (7.84)	H-5*S* (2.75)[b]
$1/T_1^0$ (s^{-1})	0.17	0.23	0.28[c]
$1/T_1$ (s^{-1})	1.93	3.38	3.95
$1/T_{1P}$ (s^{-1})	1.76	3.15	3.67
$1/(fT_{1P})$ (s^{-1})	1760	3150	3670[d]
$1/T_2^0$ (s^{-1})			20.4[c]
$1/T_2$ (s^{-1})			53.4
$1/T_{2P}$ (s^{-1})			33.0
$1/(fT_{2P})$ (s^{-1})			33000[d]
r (Å)	5.5–6.1	5.0–5.5	4.9–5.4[e]

[a] Phosphate buffer, pD 6.5, [*cis,cis*-muconate cycloisomerase] = 12.5 μM; [E·Mn·ML] = 4.7 μM. [b] Relative to Me_4Si in [$^2H_6$]acetone. [c] Enzyme and Mn^{2+} absent. [d] Normalization factor; $[ML_{free}] = [ML_t] = 4700$ μM; $[ML_{bound}] = 4.7$ μM; $1/f = [ML_{free}]/[ML_{bound}] = 4700$ μM/4.7 μM = 1000. [e] $\tau_c = 5 \times 10^{-9}$ s or 1×10^{-8} s.

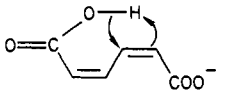
The Mn^{2+} to hydrogen nucleus distances are in the order of H-2 > H-3 > H-5*S* (Table III) and suggest that enzyme-bound Mn^{2+} is closer to the carboxyl rather than the lactone ring of bound (+)-muconolactone. One possible arrangement places the C(6)-carboxylate group closer to the divalent metal ion: the angles between the H-4 and H-5*S* hydrogen atoms and between the H-4 and H-5*R* hydrogen atoms are about 0 and 140°, respectively. These angles correspond almost exactly with those determined for the most stable conformation of (+)-muconolactone in solution, which places the *pro*-5*R* hydrogen syn (cis) to the departing carboxyl group from the C(4) carbon atom in complete correspondence with the known stereochemistry for this enzyme-catalyzed reaction. Distances of this magnitude do not require postulation that a water ligand is positioned between the bound Mn^{2+} and carboxyl of bound substrate (Mildvan, 1977) in contrast to the cases of pyruvate carboxylase (Fung et al., 1973), transcarboxylase (Fung et al., 1974), malic enzyme (Hsu et al., 1976), ribulosediphosphate carboxylase (Miziorko & Mildvan, 1974), alcohol dehydrogenase (Sloan et al., 1975), and mandelate racemase (Maggio et al., 1975). Since the EPR spectrum of the E·Mn complex at 35 GHz is not detectably perturbed upon binding (+)-muconolactone, we have been drawn to the following conclusion: either (+)-muconolactone does not ligand directly to the metal ion or the (+)-muconolactone carboxylate group displaces an active-site carboxyl group and establishes in the ternary complex a comparable ligand geometry about the metal ion. The latter possibility appears less likely but has not been rigorously ruled out.

The largest transverse relaxation rate constant for (+)-[5*R*-^{2}H]muconolactone (Table III) sets a lower limit for the rate constant, k_{off}, for the dissociation of (+)-muconolactone from the ternary complex of 3.3×10^4 s^{-1}. It is of interest that this value is 3 orders of magnitude greater than the k_{cat}^{CCM} for the enzyme reaction with *cis,cis*-muconate (13.9 s^{-1}, Table II). Thus, the release of (+)-muconolactone from the ternary

complex cannot be rate limiting, a result that is consistent with the rapid equilibrium schemes proposed on the basis of steady-state kinetic data. From the value of k_{off} and the dissociation constant, K_m^{ML}, for (+)-muconolactone (about 500 μM), a lower limit for the rate constant for the association of (+)-muconolactone with the E·Mn complex of $\geq 6.6 \times 10^7$ M^{-1} s^{-1} is obtained. It seems, therefore, that the formation of the ternary complex occurs at close to diffusion-controlled rates.

Incorporation of Deuterium into (+)-Muconolactone from Solvent (Deuterium Oxide) in the cis,cis-Muconate Cycloisomerase Catalyzed Reaction. Following the addition of *cis,cis*-muconate cycloisomerase to the solution of *cis,cis*-muconate in DOD, a reciprocal appearance of peaks assigned to the hydrogens of (+)-muconolactone and disappearance of peaks assigned to the hydrogens of *cis,cis*-muconate occurred as equilibrium was attained (not shown). It is noteworthy that the ratio of the integrated areas of the signals for the hydrogen atoms of *cis,cis*-muconate was constant during this experiment. This finding indicates that there is no incorporation of deuterium into *cis,cis*-muconate with *cis,cis*-muconate cycloisomerase and that the hydrogen added to the C(5) carbon atom in the forward direction is the hydrogen atom removed in the reverse direction; i.e., the reaction proceeds stereospecifically. Furthermore, following the addition of *cis,cis*-muconate cycloisomerase to a solution of (+)-muconolactone in DOD, the peak (3.00 ppm) assigned to the 5*R* hydrogen at the C(5) position of (+)-muconolactone disappeared in a time-dependent fashion, providing evidence for the incorporation of deuterium from solvent into this position of (+)-muconolactone catalyzed by *cis,cis*-muconate cycloisomerase.

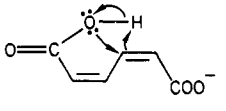
Thus, a functional group on the enzyme stereospecifically removes the 5*R* hydrogen from the C(5) carbon atom of (+)-muconolactone and, as well, exchanges this hydrogen atom with solvent in accord with similar studies in tritiated water (Avigad & Englard, 1969). It has been demonstrated that the carboxylate and proton additions to the C(4) and C(5) result from a syn (cis) addition (Avigad & Englard, 1969). This fact enables one important mechanistic conclusion regarding the enzyme-catalyzed reaction, namely, that the concerted process (i) is ruled out since concerted suprafacial



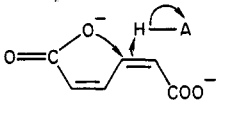
i

syn processes are forbidden from orbital-symmetry considerations (Fukui & Fujimoto, 1966; Miller, 1968).

Although a concerted reaction involving attack on the C(4) carbon atom by a carboxylic acid oxygen atom lone pair (iia)



iia



iib

or one involving proton donation by an amino acid side chain functioning as a general acid catalyst (iib) is not ruled out by orbital-symmetry considerations, there is experimental evidence (Ngai & Kallen, 1983) to indicate that the timing of events at the C(4) and C(5) carbon atoms of *cis,cis*-muconate is such that a stepwise mechanism involving the carbanion intermediate is more likely.

From the coupling constants determined for (+)-muconolactone (Figure 3), an attempt was made to specify

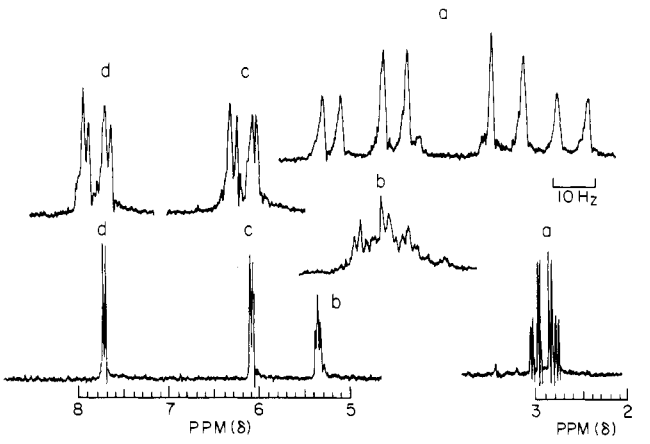


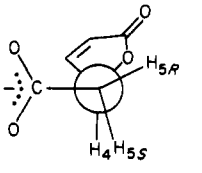
FIGURE 3: Proton magnetic resonance spectra of (+)-muconolactone at 220 MHz in aqueous solution, pH 7.4, 20 °C. This spectrum was simulated with the following parameters:

chemical shift (ppm)	H site
2.75 (dd, J = 8 and 17 Hz)	5*S*
3.00 (dd, J = 5 and 17 Hz)	5*R*
5.61 (dd, J = 2 Hz, J_R = 5 Hz, J_S = 8 Hz)	4
6.27 (dd, J = 6 Hz)	2
7.84 (dd, J = 6 and 2 Hz)	3

the most stable conformer of (+)-muconolactone in aqueous solution. This was accomplished by applying the Karplus equation:

$$J = 4.22 - 0.5 \cos\theta + 4.5 \cos 2\theta \qquad (12)$$

where J is the vicinal coupling constant for the H–C–C–H system and θ is the dihedral angle formed by the H–C bonds of the adjacent carbon atoms (Karplus, 1963). With $J_{4,5S}$ and $J_{4,5R}$ values of 5 and 8 Hz, respectively, $\theta_{4,5S}$ and $\theta_{4,5R}$ values are 38 or 138° and 10 or 159°, respectively. Since only the pair $\theta_{4,5S}$ = 10° and $\theta_{4,5R}$ = 138° are allowed, an eclipsed conformer (iii) of (+)-muconolactone appears to be the dominant conformer in solution. While this conformation is consistent with the designation of the stereochemical course for the reaction (Avigad & Englard, 1969) with the leaving groups (the proton and carboxylate) in a syn (cis) position (iii), it may or may not be the actual conformation of substrate in an enzyme–substrate complex.



iii

Acknowledgments

We thank Dr. Terry Newirth and Sonal Mazumdar for aid in the performance of the initial experiments with this system. We are grateful to Drs. G. G. McDonald and G. H. Reed and A. Wand for help with the NMR and EPR experiments and for helpful discussions.

Registry No. CCM, 1119-72-8; ML, 1124-48-7; Mn, 7439-96-5; *cis,cis*-muconate cycloisomerase, 9023-72-7.

References

Avigad, G., & Englard, S. (1969) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28*, 345, Abstr. 486.

Bloembergen, N. (1957) *J. Chem. Phys. 27*, 572–573.

Bloembergen, N., & Morgan, L. O. (1961) *J. Chem. Phys. 34*, 842–850.
Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.
Cohn, M. (1963) *Biochemistry 2*, 623–629.
Cohn, M., & Townsend, J. (1954) *Nature (London) 173*, 1090–1091.
Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry: Applications to Enzyme Systems*, pp 174–246, Oxford University Press, London.
Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H., & Pattison, D. B. (1950) *J. Chem. Soc.*, 2228–2235.
Fukui, K., & Fujimoto, H. (1966) *Bull. Chem. Soc. Jpn. 39*, 2116–2126.
Fung, C. H., Mildvan, A. S., Allerhand, A., Komoroski, R., & Scrutton, M. C. (1973) *Biochemistry 12*, 620–629.
Fung, C. H., Mildvan, A. S., & Leigh, J. S. (1974) *Biochemistry 13*, 1160–1169.
Haldane, J. B. S. (1965) *Enzymes*, MIT Press, Cambridge, MA.
Hsu, K. Y., Mildvan, A. S., Chang, G. G., & Fung, C. H. (1976) *J. Biol. Chem. 251*, 6574–6583.
James, T. L. (1975) *Nuclear Magnetic Resonance in Biochemistry*, pp 173–234, Academic Press, New York.
James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 3519–3526.
Kallen, R. G. (1971) *J. Am. Chem. Soc. 93*, 6227–6248.
Karplus, M. (1963) *J. Am. Chem. Soc. 85*, 2870–2871.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosebrough, N. H., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Luz, Z., & Meiboom, S. (1964) *J. Chem. Phys. 40*, 2686–2692.
Maggio, E. T., Kenyon, G. L., Mildvan, A. S., & Hegeman, G. D. (1975) *Biochemistry 14*, 1131–1139.
McDonald, G. G., & Leigh, J. S. (1973) *J. Magn. Reson. 9*, 358–363.
Meagher, R. B., & Ornston, L. N. (1973) *Biochemistry 12*, 3523–3530.
Mildvan, A. S. (1977) *Acc. Chem. Res. 10*, 246–252.
Mildvan, A. S., & Cohn, M. (1963) *Biochemistry 2*, 910–919.
Mildvan, A. S., & Gupta, R. K. (1978) *Methods Enzymol. 49*, 322–359.
Miller, S. I. (1968) *Adv. Phys. Org. Chem. 6*, 185–332.
Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 2743–2750.
Ngai, K.-L. (1981) Ph.D. Dissertation, University of Pennsylvania.
Ngai, K.-L., & Kallen, R. G. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 666, Abstr. 2081.
Ngai, K.-L., & Kallen, R. G. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 721, Abstr. 2598.
Ngai, K.-L., & Kallen, R. G. (1983) *Biochemistry* (following paper in this issue).
Nowak, T., & Mildvan, A. S. (1972) *Biochemistry 11*, 2819–2828.
Ornston, L. N. (1966) *J. Biol. Chem. 241*, 3795–3810.
Ornston, L. N., & Stanier, R. Y. (1966) *J. Biol. Chem. 241*, 3776–3786.
Salomaa, P., Schaleger, L. L., & Long, F. A. (1964) *J. Am. Chem. Soc. 86*, 1–7.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Segal, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Sistrom, W. R., & Stanier, R. Y. (1954) *J. Biol. Chem. 210*, 821–836.
Sloan, D. L., Young, J. M., & Mildvan, A. S. (1975) *Biochemistry 14*, 1998–2008.
Solomon, I. (1955) *Phys. Rev. 99*, 559–565.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261–266.
Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys. 37*, 307–320.
Swift, T. J., & Connick, R. E. (1964) *J. Chem. Phys. 41*, 2553–2554.