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Mandelate Racemase from *Pseudomonas putida.* Magnetic Resonance and Kinetic Studies of the Mechanism of Catalysis†

Edward T. Maggio, George L. Kenyon,* Albert S. Mildvan, and George D. Hegeman

ABSTRACT: The interactions of mandelate racemase with divalent metal ion, substrate, and competitive inhibitors were investigated. The enzyme was found by electron paramagnetic resonance (EPR) to bind 0.9 Mn^{2+} ion per subunit with a dissociation constant of 8 μM, in agreement with its kinetically determined activator constant. Also, six additional Mn^{2+} ions were found to bind to the enzyme, much more weakly, with a dissociation constant of 1.5 mM. Binding to the enzyme at the tight site enhances the effect of Mn^{2+} on the longitudinal relaxation rate ($1/T_{1p}$) of water protons by a factor of 11.9 at 24.3 MHz. From the frequency dependence of $1/T_{1p}$, it was determined that there are ~3 water ligands on enzyme-bound Mn^{2+} which exchange at a rate $\geq 10^7$ sec^{-1}. The correlation time for enzyme-bound Mn^{2+}-water interaction is frequency-dependent, indicating it to be dominated by the electron spin relaxation time of Mn^{2+}. Formation of the ternary enzyme-Mn^{2+}-mandelate complex decreases the number of fast exchanging water ligands by ~1, but does not affect τ_c, suggesting the displacement or occlusion of a water ligand. The competitive inhibitors D,L-α-phenylglycerate and salicylate produce little or no change in the enzyme-Mn^{2+}-H_2O interaction, but ternary complexes are detected indirectly by changes in the dissociation constant of the enzyme-Mn^{2+} complex and by mutual competition experiments. In all cases the dissociation constants of substrates and competitive inhibitors from ternary complexes determined by magnetic resonance titrations agree with K_M and K_i values determined kinetically and therefore reflect kinetically active complexes. From the paramagnetic effects of Mn^{2+} on $1/T_1$ and $1/T_2$ of the ^{13}C-enriched carbons of 1-[^{13}C]-D,L-mandelate and 2-[^{13}C]-D,L-mandelate, Mn^{2+} to carboxylate carbon and Mn^{2+} to carbinol carbon distances of 2.93 ± 0.04 and 2.71 ± 0.04 Å, respectively, were calculated, indicating bidentate chelation in the binary Mn^{2+}-mandelate complex. In the active ternary complex of enzyme, Mn^{2+}, and D,L-mandelate, these distances increase to 5.5 ± 0.2 and 7.2 ± 0.2 Å, respectively, indicating the presence of at least 98.9% of a second sphere complex in which Mn^{2+}, and C_1 and C_2 carbon atoms are in a linear array. The water relaxation data suggest that a water ligand is immobilized between the enzyme-bound Mn^{2+} and the carboxylate of the bound substrate. This intervening water ligand may polarize or protonate the carboxyl group. From $1/T_{2p}$ the rate of dissociation of the substrate from this ternary complex ($\geq 5.6 \times 10^4$ sec^{-1}) is at least 52 times greater than the maximal turnover number of the enzyme (1070 sec^{-1}), indicating that the complex detected by nuclear magnetic resonance (NMR) is kinetically competent to participate in catalysis. Relationships among the microscopic rate constants are considered.

Mandelate racemase (EC 5.1.2.2), a tetramer of molecular weight 278,000, catalyzes the racemization of mandelic acid. This "one substrate" enzyme has recently been shown to exhibit an absolute requirement for divalent metal ions for activity (Fee et al., 1974a); thus it represents one of the simplest examples of metal ion activation of enzymatic catalysis. Activation of the apoenzyme by divalent metal ions may result from stabilization of a catalytically active enzyme conformation, by facilitation and directional coordination of the binding of substrate at the active site of the enzyme, or by direct participation in the catalytic mechanism *via* the ability of electron-withdrawing divalent cations to stabilize carbanions. A possible carbanion intermediate for mandelate racemase has previously been proposed (Kenyon and Hegeman, 1970).

The present study was undertaken to determine the spatial relationship of enzyme, cofactor, and substrate in the fully active ternary enzyme complex and to help elucidate

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the role which divalent metal ions play in the mechanism of catalysis of mandelate racemase. The interactions of Mn^{2+}, substrate, and competitive inhibitors in various binary and ternary complexes with mandelate racemase were investigated by electron and nuclear magnetic resonance (NMR) and kinetic studies. This system has provided one of the very few cases in which the geometry of metal–substrate interactions in a fully active ternary complex could be determined. A preliminary report of these studies has appeared (Maggio et al., 1974).

Experimental Section

Materials

Mandelate racemase and L-mandelate dehydrogenase were prepared as previously described (Hegeman et al., 1970; Hegeman, 1970). Protein concentration was determined by the method of Lowry et al. (1951) by comparison with standard solutions of bovine serum albumin.

1-[^{13}C]-D,L-Mandelic acid was synthesized from benzaldehyde and [^{13}C]NaCN (90 atom % ^{13}C) (Merck Sharp and Dohme, Canada) by a modified procedure based upon that of Fieser (1968a). Thus, benzaldehyde was dissolved in ether and washed three times with Na_2CO_3 solution to remove benzoic acid. The ether was removed. A solution of 5.5 g of $NaHSO_3$ in 15 ml of water was added to 5 ml of benzaldehyde in a separatory funnel. The funnel was flushed with N_2; an excess (1.0 g) of [^{13}C]NaCN was added and the funnel was again flushed with N_2. After shaking the solution for 30 min, 15 ml of ether was added. The solution was then shaken an additional 10 min. The aqueous layer was drawn off, and the ether layer was washed once with saturated NaCl solution. The ethereal solution of mandelonitrile was transferred to a distillation flask, and the ether was evaporated under a stream of N_2. The resulting oil was heated at reflux for 1.7 hr in 10 ml of 6 *N* HCl. The hydrolysate was extracted with ether and the ether extract dried over $MgSO_4$. The ether was removed, and 45 ml of benzene was added. The solution was distilled to remove the benzene–water azeotrope, and, when the volume remaining in the distillation flask was reduced to 20 ml, the solution was decanted and the product allowed to crystallize: mp 118–119°; lit. 118–119° (Fieser, 1968a); ^{1}H nmr (D_2O) δ 5.30 (d, J = 5 Hz, 1 H); 7.41 (s, 5 H); ^{13}C nmr (D_2O) enriched peak at −179.95 ppm from Me_4Si. The ultraviolet spectrum was superimposable on that of authentic mandelic acid. The methyl ester prepared with diazomethane cochromatographed with authentic methyl mandelate upon gas–liquid chromatographic analysis (Kenyon and Hegeman, 1970).

2-[^{13}C]-D,L-Mandelic acid was synthesized in the following manner. An ethereal solution of phenylmagnesium bromide (Fieser, 1968b) in a 50-ml flask was fitted with a magnetic stirrer and connected to a vacuum manifold similar to that described by Calvin et al. (1949). Attached to the manifold was a pressure-equalizing addition funnel containing 150 ml of concentrated H_2SO_4, and the funnel was in turn connected to a round-bottom flask containing [^{13}C]$BaCO_3$ (0.103 mol). The manifold was equipped with a manometer capable of measuring pressure up to 1 atm. The ethereal Grignard reagent (0.120 mol) was frozen by immersing the flask in liquid nitrogen, and the system was flushed with prepurified N_2 three times by alternately evacuating and refilling the system. The system was again evacuated and the stopcock connecting the vacuum pump was closed. The Grignard solution was slowly warmed to room temperature. H_2SO_4 from the addition funnel was slowly added to the $BaCO_3$. The rate of addition of acid was so adjusted that the pressure in the system approached, but never exceeded, atmospheric pressure. Both flasks were stirred vigorously by magnetic stirrers while the reaction was proceeding, and when CO_2 uptake ceased, the flask containing the ethereal Grignard solution was again frozen in liquid N_2 to complete the transfer of [^{13}C]CO_2 to the reaction flask. The stopcock connecting the reaction flask was closed, the reaction mixture was warmed to 0°, and the mixture was stirred for 30 min. The reaction mixture was poured over a mixture of 30 ml of ice and 75 ml of 10% H_2SO_4. 1-[^{13}C]Benzoic acid was obtained in 73% yield, mp 120–121°; lit. 122° (Frankel and Patai, 1964). 1-[^{13}C]Benzoic acid was reduced to 1-[^{13}C]benzyl alcohol with $LiAlH_4$ (Nystrom and Brown, 1947) in 97% yield. The 1-[^{13}C]benzyl alcohol was oxidized to 1-[^{13}C]benzaldehyde in 74% yield using CrO_3–pyridine complex (Ratcliffe and Rodehorst, 1970). 1-[^{13}C]Benzaldehyde was converted to 2-[^{13}C]mandelic acid in 66% yield by treatment of the bisulfite addition complex with KCN and subsequent hydrolysis of the nitrile as described above (Fieser, 1968a): mp 118°; ^{1}H nmr (D_2O) δ 5.30 (d, J = 148 Hz, 1 H); 7.41 (s, 5 H); ^{13}C nmr (D_2O) enriched peak at −75.05 ppm from Me_4Si.

D,L-*α-Phenylglyceric acid* was prepared by hydrolysis of the bromohydrin of atropic acid. The precursor, ethyl atropate, was prepared by the method of Ames and Davey (1958). Hydrolysis of ethyl atropate was carried out by heating 98 g of ethyl atropate at reflux in 400 ml of 4 *N* NaOH until an organic layer was no longer apparent (*ca.* 3 hr). The solution was extracted with ether, acidified, and again extracted with ether. After removal of the ether from the acidified extraction, free atropic acid was obtained. The atropic acid was treated with Br_2 in H_2O according to the procedure of Read and Andrews (1921). To 57 g of the product, α-hydroxy-α-phenyl-β-bromopropionic acid, mp 113–114° (lit. 115°, Takahashi, 1956), was added 19.3 g of NaOH in 900 ml of H_2O. The solution was heated for 1.5 hr on a steam bath, and then cooled to 4° in an ice bath and extracted with ether. After acidification to pH 2.0, the solution was extracted several times with ether. The ether fractions from the acidified extraction were evaporated, and the product, D,L-α-phenylglyceric acid, was recrystallized from ethyl acetate–mixed hexanes: mp 149°; lit. 149° (Craig and Henze, 1945); ^{1}H nmr (D_2O) AB quartet δ 3.72, 3.92, 4.12, 4.32 (J_{AB} = 11 Hz, $\Delta\nu$ = 20.8 Hz, 2 H), 7.4 (m, 5 H).

Salicylic acid, 3-hydroxy-2-naphthoic acid, 2-naphthoic acid, and EDTA were purchased from Eastman. 4-Hydroxycoumarin and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)[1] were purchased from Aldrich. 2,6-Dichlorophenolindophenol (DCPIP), sodium salt, was purchased from Calbiochem. All other reagents were either Baker or Mallinckrodt analytical reagents. Organic compounds used in inhibitor studies were recrystallized before use from appropriate solvents (usually H_2O–ethanol) after treatment with activated charcoal.

Removal of divalent metal ion from mandelate racemase and the components of the assay system was carried out as described by Fee et al. (1974a) followed by gel filtration

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCPIP, 2-6-dichlorophenolindophenol, Na^+ salt; PRR, proton relaxation rate of water.

though a Sephadex G-25 column equilibrated with 100 m*M* Na^+-Hepes buffer (pH 7.0) to ensure removal of residual EDTA. Distilled, deionized water was used throughout these studies.

Methods

Enzyme Assays. For assaying the enzymatic activity of mandelate racemase a modification of the procedure of Hegeman et al. (1970) was employed. To 3.00 ml of a buffer solution containing Na^+-Hepes (pH 7.0), DCPIP, $MnCl_2$, and L-mandelate dehydrogenase in a 1.00-cm cuvet was added 100 μl of sodium D-mandelate solution. The assay was initiated by addition of 100 μl of enzyme solution after waiting sufficiently long to ensure temperature equilibration. The concentrations were so adjusted that the final concentrations in the assay solution were the same as those described by Hegeman et al. (1970) (see legend to Figure 2). In experiments measuring inhibition, the inhibitors were included in the buffer solution. Buffer solutions were prepared from stock solutions of the components every few hours; no detectable change in dehydrogenase activity occurred over this period of time.

Magnetic Resonance Measurements. The longitudinal proton relaxation rates ($1/T_1$) of water were measured by the pulsed method described by Mildvan and Engle (1972) at 8, 15, and 24.3 MHz using the NMR Specialities PS 60W spectrometer and at 100 MHz using the Varian XL-100-15-FT spectrometer. The paramagnetic contribution to the longitudinal and transverse relaxation rates, $1/T_{1P}$ and $1/T_{2P}$, respectively, was calculated as described by Mildvan and Cohn (1970) from eq 1 and 2,

$$\frac{1}{T_{1P}} = \frac{1}{T_1} - \frac{1}{T_1^0} \quad (1)$$

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

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

The paramagnetic contributions to the relaxation rates were normalized as described by Luz and Meiboom (1964) by the factor $f = [Mn^{2+}]/[\text{ligand}]$ to yield $1/fT_{1P}$ and $1/fT_{2P}$. The observed enhancement of the paramagnetic effect (ϵ^*) on the relaxation of water is defined as follows (Eisinger et al., 1962)

$$\epsilon^* = \frac{1/T_{1P}^*}{1/T_{1P}} \quad (3)$$

where the superscript (*) indicates the presence of enzyme. The ratio of the concentration of free Mn^{2+} in a mixture of free and bound Mn^{2+} was determined by electron paramagnetic resonance (EPR) (Cohn and Townsend, 1954) using a Varian E-4 EPR spectrometer.

^{13}C NMR and relaxation rate measurements were made by the Fourier transform method using a Varian XL-100-15 NMR spectrometer operating at 25.14 MHz. The 1-[^{13}C]- and 2-[^{13}C]mandelate samples were admixed in equimolar amounts for the relaxation rate studies. This procedure, based on that of Fung et al. (1973), permitted the simultaneous measurement of the relaxation rates at both positions and circumvented spectral complications which would have been introduced as a result of ^{13}C–^{13}C coupling if the substrate had been synthesized with two adjacent ^{13}C atoms. The resultant ^{13}C enrichment was 45% at each labeled position in mandelate.

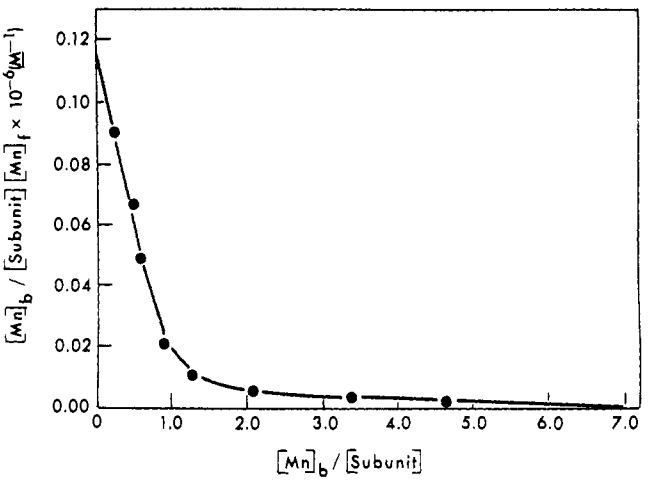

FIGURE 1: Scatchard plot of the titration of metal-free mandelate racemase with $MnCl_2$ in pH 7.0, 75 m*M* Na^+-Hepes buffer. The concentrations of free and bound Mn^{2+} ion, $(Mn)_f$ and $(Mn)_b$, respectively, were determined by EPR spectroscopy. The concentration of mandelate racemase was 69.3 μM in monomers. The parameters used to generate the theoretical curve are given in the text.

The longitudinal relaxation rates ($1/T_1$) were determined from proton-decoupled, partially relaxed Fourier transform NMR spectra using the "homogeneity-spoil" pulse sequence of McDonald and Leigh (1973). For each determination at least ten spectra were obtained with variable intervals (τ) between the 90° pulses. The data were analyzed by the method of Abragam (1962) as described by Fung et al. (1973). The average error in $1/T_1$ determined by this procedure is ±10%.

The transverse relaxation rates of carbon ($1/T_2$) were determined from line widths of the fully relaxed Fourier transform spectra with an average error of 20%. For the ^{13}C NMR studies, the solutions contained 33% D_2O to permit use of the heteronuclear internal field frequency lock of the XL-100-15 NMR spectrometer. No loss of enzyme activity was observed after as much as 12 hr of NMR studies.

The EPR and NMR data were analyzed as previously described by Mildvan and Engle (1972) to determine the number of Mn^{2+} ions bound to mandelate racemase (n), the dissociation constant (K_D), and the enhancement (ϵ_b) of the binary enzyme–Mn^{2+} complex. Titrations of the binary complex with ligands such as D,L-mandelate and D,L-α-phenylglycerate in which $1/T_{1P}$ of water protons was measured at each point were analyzed to yield the dissociation constants (K_S and K_3) and the ehancement values (ϵ_t) of the ternary complexes using the computer program of Reed et al. (1970). The correlation time (τ_c) for the dipolar interaction of enzyme-bound Mn^{2+} with water protons was determined from the frequency dependence of $1/T_{1P}$ (Peacocke et al., 1969).

Results

Manganese Binding Studies. "Metal-free" mandelate racemase (Fee et al., 1974a) was titrated with Mn^{2+}. At each point of the titration, the free Mn^{2+} concentration was measured by EPR spectroscopy and the enhancement (ϵ^*) of $1/T_{1P}$ of water protons was determined. Using the values for the fraction of free and bound Mn^{2+} determined by EPR spectroscopy, the data were plotted according to the method of Scatchard (1949) (Figure 1). The theoretical curve, which fits the data points with an average deviation of ±6.9%, was computed as previously described (Miziorko

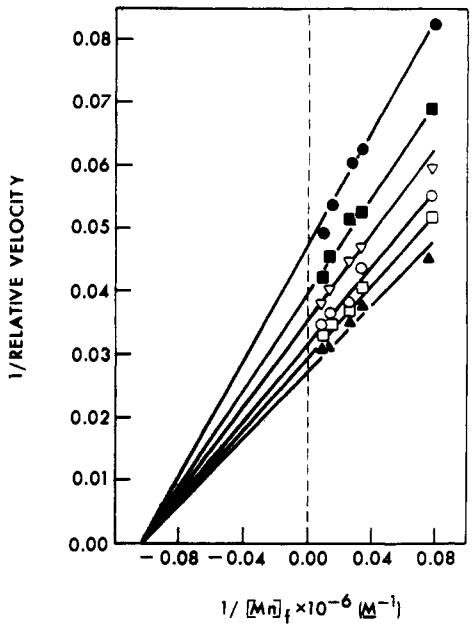

FIGURE 2: Determination of the activator constant K_A of Mn^{2+} at pH 7.0. D,L-Mandelate concentrations were, from top to bottom: 1.03 m*M* (●), 1.54 m*M* (■), 2.07 m*M* (▽), 3.09 m*M* (○), 4.13 m*M* (□), and 8.28 m*M* (▲). The assay solutions also contained 3.3 m*M* KCN, 0.1 *M* Na^+-Hepes buffer (pH 7.0), excess L-manadelate dehydrogenase (0.075 IU/ml), 0.13 m*M* DCPIP, and 3.2×10^{-8} *M* mandelate racemase in a total volume of 3.2 ml. The concentration of free Mn^{2+} in the assay mixture was determined by EPR spectroscopy.

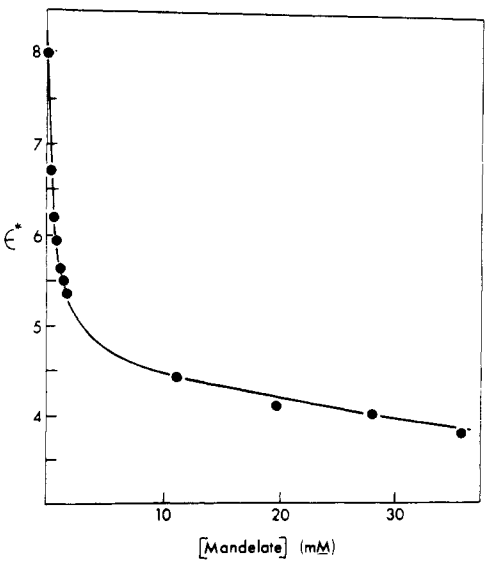

FIGURE 3: PRR titration of the enzyme-Mn^{2+} complex with D,L-mandelate in 75 m*M* Na^+-Hepes buffer (pH 7.0) at 25°. The graph shows the data points and a computer-fitted curve the parameters of which are given in Table I. The concentration of Mn^{2+} and mandelate racemase were 20 and 69.3 μM monomer, respectively.

and Mildvan, 1974) by assuming 0.90 tight binding site per subunit with a dissociation constant of 8.0 μM and 6.0 weak binding sites per subunit with dissociation constants of 1.5 m*M* (Figure 1).

The enhancement of $1/T_{1p}$ of water protons (ϵ_b) resulting from the binding of Mn^{2+} at the tight site was found to be 11.9 ± 1.0. When more than one Mn^{2+} binding site was occupied (2.0–4.6), the average ϵ_b value progressively decreased from 10.8 to 8.9. Correction of these values for the known occupancy of the tight and weak binding sites (Figure 1), and for the known ϵ_b at the tight site, as previously described (Mildvan and Cohn, 1963), yielded an ϵ_b value of 8.8 ± 0.6 for the weak sites.

Table I: Dissociation Constants and Enhancements of Ternary Complexes of Mandelate Racemase, Mn^{2+}, and Ligands Used to Fit the PRR Titration Curves.[a]

Ligand	K_1 (m*M*)	K_S (m*M*)	K_3 (m*M*)	ϵ_t/ϵ_b	% Standard Deviation
D,L-Mandelate	78.3[b]	0.20	0.67	0.72	3.6
D,L-α-Phenylglycerate	105[b]	0.41	1.3	0.96	4.7
Salicylate	0.001[c]	0.72[d]	2.4[d]	1.0	2.8

[a] The dissociation constants of the ligands (L) are defined as follows: $K_1 = [Mn^{2+}][L]/[Mn^{2+} \cdot L]$; $K_S = [E][L]/[E \cdot L]$; $K_3 = [E \cdot Mn^{2+}][L]/[E \cdot Mn^{2+} \cdot L]$. [b] Determined by EPR as previously described. Enhancements of binary Mn^{2+}–mandelate or α-phenylglycerate complexes determined by $1/T_1$ and EPR were both 1.0. The value of ϵ_b used for the binary Mn^{2+}–mandelate racemase complex is 11.9 ± 1.0 and its K_D value is 8.0 μM. [c] Dawson et al. (1969). [d] Determined by competition with D,L-α-phenylglycerate.

In the kinetic study shown in Figure 2, K_M of free Mn^{2+} (9.3 μM) was found to be independent of mandelate concentration. Hence the activator constant of Mn^{2+}, the K_M of free Mn^{2+} extrapolated to zero mandelate concentration as determined from the intersection of the lines in Figure 2, is also 9.3 μM, a value in good agreement with the dissociation constant of 8.0 μM for the tight Mn^{2+} binding site on the enzyme. In the kinetic study, the free Mn^{2+} concentrations in the various solutions were determined by EPR spectroscopy.

Ternary Complexes of Enzyme, Mn^{2+}, and Substrate. Titration of the enzyme-Mn^{2+} binary complex with D-mandelate resulted in a decreased enhancement, ϵ^*, of $1/T_{1p}$ of water protons. From the high dissociation constant (K_1) of the binary Mn^{2+}–mandelate complex, as determined by EPR (78.3 m*M*), this decrease in ϵ^* could not have resulted from the removal of Mn^{2+} from the enzyme but must have resulted from ternary complex formation. Figure 3 shows the experimental points and a computed curve using the program of Reed et al. (1970) which assumes ternary complex formation. The parameters used to obtain the best fit of the experimental data are listed in Table I. The value for the ternary dissociation constant, K_3 = 0.67 m*M* of D,L-mandelate, is very nearly equal to the value of the K_M of D-mandelate extrapolated to infinite free Mn^{2+} concentration (K_M = 0.63 m*M*) as determined by replotting the kinetic data of Figure 2, suggesting that the enzyme-Mn^{2+} complex obeys Michaelis–Menten kinetics with equal affinities for D- and L-mandelate.[2]

[2] Extrapolation of the K_m of D-mandelate to zero concentration of free Mn^{2+} yields a variable value of 4 ± 2 m*M*. The fact that this value does not agree with the K_S of D,L-mandelate (Table I) suggests that D- and L-mandelate have different K_S values, that Michaelis–Menten kinetics may not apply until the enzyme is saturated with Mn^{2+}, or that the binary enzyme-substrate complex detected by NMR represents nonproductive binding. In support of the latter proposal, it has been previously observed that the addition of D-mandelate to the assay prior to Mn^{2+} results in a lag in the onset of activity (Fee et al., 1974a).

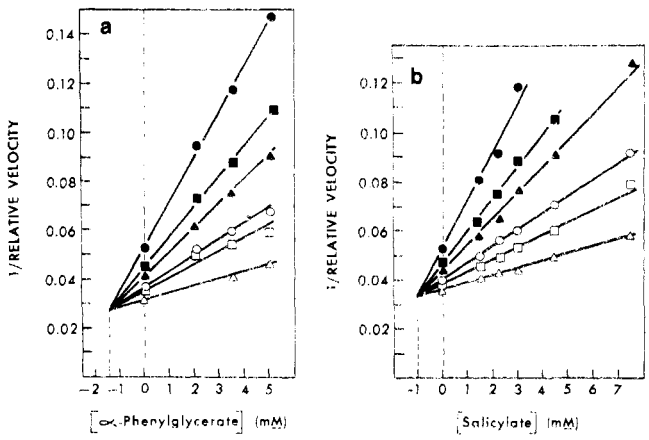

FIGURE 4: (a) Inhibition of mandelate racemase with D,L-α-phenylglycerate in Na^+-Hepes buffer (pH 7.0) at 25°. Enzyme concentration 1.1×10^{-7} *M* (tetramer) (see Figure 2 for details of assay). Data are plotted according to the method of Dixon (1953). Mandelate concentrations from top to bottom are: 1.04 m*M* (●), 1.56 m*M* (■), 2.09 m*M* (▲), 3.12 m*M* (○), 4.17 m*M* (□), and 8.34 (△). (b) Inhibition of mandelate racemase with salicylate in Na^+-Hepes buffer (pH 7.0) at 25°. Enzyme concentration, 9×10^{-8} *M* (tetramer) (see Figure 2 for details of assay). Mandelate concentrations from top to bottom are: 1.04 m*M* (●), 1.56 m*M* (■), 2.09 m*M* (▲), 3.12 m*M* (○), 4.17 m*M* (□), and 8.34 m*M* (△).

Ternary Complexes of Enzyme, Mn^{2+}, and Inhibitors. The interaction of mandelate racemase with inhibitors was investigated kinetically to determine the type and extent of inhibition. As indicated by the Dixon plots (Dixon, 1953) of Figure 4 both D,L-α-phenylglycerate and salicylate are linear competitive inhibitors with K_i values on the order of 1 m*M* (Table II). Table II also summarizes kinetic data for other types of inhibitors of the reaction.

Titration of the enzyme–Mn^{2+} complex with the competitive inhibitor D,L-α-phenylglycerate resulted in a decreased enhancement, ϵ^*, of $1/T_{1p}$ of water protons due to ternary complex formation (Figure 5a, Table I). The dissociation constant for the ternary complex (K_3) formed with α-phenylglycerate was found to be 1.3 m*M* (Table I), which agrees well with the inhibitor constant K_i = 1.5 m*M* (Table II).

Salicylate, unlike mandelate or α-phenylglycerate, produced no change in ϵ^*. A ternary complex could, however, be detected by a competitive titration of the enzyme–Mn^{2+} complex with α-phenylglycerate in the presence of 13.7 ± 1.0 m*M* salicylate (Figure 5b). The parameters for salicylate binding used to generate the theoretical titration curve are summarized in Table I.

The dissociation constant (K_3) for the ternary complex formed with salicylate was found to be 2.4 m*M*. The inhibitor constant (K_i) for salicylate was found to exhibit a slight dependence upon Mn^{2+} concentration. Values of K_i = 1.0 m*M* and 3.5 m*M* at Mn^{2+} concentrations of 1.0 and 0.031 m*M*, respectively, were found. Figure 4b shows the data for salicylate at 1.0 m*M* $MnCl_2$. Hence the K_3 for salicylate, determined at a Mn^{2+} concentration of 0.050 m*M*, falls within this range.

PRR Studies: Determination of q, The Coordination Number for Water on Enzyme-Bound Manganese. The dipolar term of the Solomon–Bloembergen equation may be

$$1/fT_{1p} = q\left(\frac{C}{r}\right)^6\left(\frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right) \qquad (4)$$

used to calculate the coordination number q for water ligands on manganese when the water exchange rate is rapid

Table II: Inhibitors of Mandelate Racemase.[a]

Inhibitor	Inhibition Type	K_i(m*M*)
D,L-α-Phenylglycerate	Competitive	1.5
Salicylate	Competitive	1.0
Benzoate	Mixed	2.7
2-Naphthoate	Mixed	0.30
3-Hydroxy-2-naphthoate	Noncompetitive	0.38
4-Hydroxycoumarin	Noncompetitive	5.50

[a] Conditions are given in Figure 4 and Methods. The concentration of $MnCl_2$ was 1.0 m*M*.

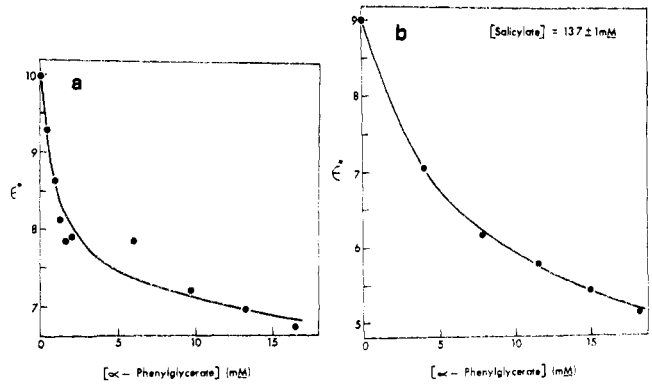

FIGURE 5: (a) PRR titration of the enzyme–Mn^{2+} complex with the competitive inhibitor, D,L-α-phenylglycerate, in 75 m*M* Na^+-Hepes buffer (pH 7.0) at 25°. Data points are shown together with the computer-fitted curve the parameters of which are given in Table I. The concentrations of Mn^{2+} and enzyme are 50 and 69.3 μ*M* monomer, respectively. (b) PRR titration of the enzyme–Mn^{2+} complex with D,L-α-phenylglycerate in the presence of inhibitor, salicylate, in 75 m*M* Na^+-Hepes buffer (pH 7.0) at 25°. Data points are shown together with the computer-fitted curve the parameters of which are given in Table I. The concentrations of Mn^{2+} and enzyme are 40 and 69.3 μ*M* monomer, respectively.

compared to the longitudinal relaxation rate of the coordinated water ligands (Mildvan and Engle, 1972). In eq 4, $f = [Mn^{2+}]/[H_2O]$, C is a product of physical constants numerically equal to 812 for Mn^{2+}-proton interaction and 512 for Mn^{2+}–^{13}C interaction, ω_I is the nuclear precession frequency, τ_c is the dipolar correlation time, and r is the Mn^{2+}-proton distance (Mildvan and Engle, 1972), which is 2.87 ± 0.05 Å from crystallographic data (Reuben and Cohn, 1970). The fast exchange of water protons in the present case is established by the frequency dependence of $1/fT_{1p}$ (Table III) and by the observation that at 24.3 MHz $1/fT_{2p}$ is significantly greater than $1/fT_{1p}$ (Table III). The value of $1/fT_{2p}$ sets a lower limit of $\sim 10^7$ sec^{-1} on the water exchange rate into the coordination sphere of enzyme-bound Mn^{2+}. In the limit of fast exchange, the experimentally determined quantity $1/fT_{1p}$ is a function of three parameters (eq 4), q, r, and τ_c. The correlation time (τ_c) may be calculated from the frequency dependence of $1/fT_{1p}$ (Peacocke et al., 1969; Reuben and Cohn, 1970). From such a study (Table III) $1/fT_{1p}$ was maximal at 24.3 MHz but decreased at higher and lower frequencies indicating that τ_c itself is frequency-dependent and is therefore dominated by τ_s, the electron spin relaxation time of Mn^{2+} (Reuben and Cohn, 1970; Nowak et al., 1973). The frequency dependence of τ_c is given by eq 5 (Bloembergen and

Table III: Frequency Dependence of Water Proton Relaxation Rates in Mandelate Racemase Complexes.[a]

Complex	$1/fT_{1p} \times 10^{-6}$ (sec^{-1}) Frequency (MHz) 8	15	24.3	100	$1/fT_{2p} \times 10^{-6}$ (sec^{-1}) 24.3	$B \times 10^{-19}$ [b] (sec^{-2})	$\tau_v \times 10^{12}$ [b] (sec)	$\tau_c \times 10^{9}$ [c] (sec)	q	Average % Error
E–Mn^{2+}	5.15	6.91	7.65	2.39	20.3	6.44	4.65	1.13	3.3	10.3
E–Mn^{2+}–D,L-mandelate	2.39	3.05	3.64	1.17	13.1	6.59	4.55	1.11	2.4	5.9
E–Mn^{2+}–D,L-α-phenylglycerate	3.99	5.14	5.17	1.90	21.8	6.89	3.72	1.13	3.1	11.3

[a] Solutions contained 69.3 μM mandelate racemase subunits, 50 μM $MnCl_2$, 75 mM Na^+-Hepes buffer (pH 7.0), and no ligand, D,L-mandelate (18.8 mM) or D,L-α-phenylglycerate (42.0 mM), T = 25°. Relaxation rates are corrected for bound Mn^{2+} species using the dissociation constants of Table I. [b] Parameters of eq 5 used with eq 4 to fit the relative $1/fT_{1p}$ values with the indicated % error. [c] Correlation time at 24.3 MHz (5.71 kG) computed from eq 5.

Table IV: Paramagnetic Effects of Mn^{2+} and Mandelate Racemase–Mn^{2+} on the Relaxation Rates of the Carboxyl and Carbinol Carbon-13 Atoms of D,L-Mandelate.[a]

Expt	[D,L-Mandelate][1-^{13}C] + [2-^{13}C] (mM)	$[Mn^{2+}]$ (μM)	Enzyme Sites (μM)	Carboxyl-^{13}C $1/T_1$ (sec^{-1})	Carboxyl-^{13}C $1/fT_{1p}$ (sec^{-1})	Carboxyl-^{13}C $1/fT_{2p}$ (sec^{-1})	Carbinol-^{13}C $1/T_1$ (sec^{-1})	Carbinol-^{13}C $1/fT_{1p}$ (sec^{-1})	Carbinol-^{13}C $1/fT_{2p}$ (sec^{-1})
1	100.0			0.030			0.220		
2	99.0	10.60		0.240	1960	7940	0.620	3700	
3	98.0	21.05		0.526	2310	7780	0.885	3100	14,900
4	100.0		55.2	0.039			0.472		
5	98.0	7.82	55.2	0.646	7610	62,500	0.582	1470	
6	96.0	15.40	55.0	0.813	4830	49,810	0.625	950	16,200
7	96.0[b]	15.40	55.0	0.392	2200	31,730	0.500	170	5,300

[a] Solutions also contained 40 mM Na^+-Hepes buffer (pH 7.0) and 33% D_2O for field frequency locking, T = 25°. [b] D,L-Sodium α-phenylglycerate was also present at 212 mM.

$$1/\tau_c = 1/\tau_s = B\left(\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right) \quad (5)$$

Morgan, 1961), in which B is a constant which depends on the symmetry of the ligand field at Mn^{2+}, τ_v is the time constant for distortions of this symmetry, and ω_s is the electron precession frequency. The relative values of $1/fT_{1p}$ (Table III) were fit by computer to eq 4 and 5 to within 11.3%, yielding similar and typical values of B, τ_v, and τ_c for the binary and ternary complexes of mandelate racemase.

Using the values of r and τ_c in eq 4, q was found to be ~3 in the binary enzyme–Mn^{2+} complex (Table III). Hence ~3 water ligands which exchange at a rate $\geq 10^7$ sec^{-1} remain coordinated to the enzyme-bound Mn^{2+} in the binary mandelate racemase–Mn^{2+} complex. The formation of the ternary enzyme–Mn^{2+}–substrate complex with mandelate causes a decrease in q by ~1 indicating the displacement or occlusion of a water ligand such that it exchanges at a rate $\leq 10^5$ sec^{-1} (Table III). In Table III, the relative values of q are more accurate than the absolute values of this parameter. Unlike the substrate, the competitive inhibitor D,L-α-phenylglycerate produced no significant change in q (Table III). Similarly, salicylate, which did not alter ϵ^* yet formed a ternary complex (Figure 7, Table I), apparently did not alter q or τ_c significantly, although no detailed analysis of the frequency dependence was made for the salicylate complex.

Paramagnetic Effects of Mn^{2+} in the Relaxation Rates of 1-[^{13}C]- and 2-[^{13}C]-D,L-Mandelate. As shown in Table IV (experiments 1–3) the addition of micromolar levels of $MnCl_2$ to a solution containing 50 μM 1-[^{13}C]-D,L-mandelate and 50 μM 2-[^{13}C]-D,L-mandelate increased $1/T_1$ of both the carboxyl and carbinol carbon atoms. No effects on the ^{13}C-chemical shifts were detected.

The paramagnetic effects of Mn^{2+} on the relaxation rates, $1/fT_{1p}$ and $1/fT_{2p}$, were significantly greater for the carbinol carbon than for the carboxyl carbon (Table IV, experiments 2 and 3). Correcting the normalized relaxation rates of Table IV for the fraction of Mn^{2+} which was bound to D,L-mandelate (55.7 ± 0.2%) using the dissociation constant of 78.3 μM for the Mn^{2+}–mandelate complex (Table I) yields the longitudinal relaxation rates of 3.8×10^3 and 6.1×10^3 sec^{-1} for the carboxyl and carbinol carbon atoms, respectively, in the binary Mn^{2+}–mandelate complex (Table V). The similarly corrected transverse relaxation rate (2.7×10^4 sec^{-1}) is 4.4–7.0-fold greater than the longitudinal relaxation rates indicating that the latter rates are not limited by chemical exchange, and represent $1/T_{1M}$, the relaxation rates of the coordinated ligand. Hence these rates may be used for distance calculations (Nowak and Mildvan, 1972). The Mn^{2+} to ^{13}C distances (Table V) were

Table V: Calculation of Distances between Mn^{2+} and ^{13}C of D,L-Mandelate in the Binary Mn^{2+}–Mandelate and Ternary Enzyme–Mn^{2+}–Mandelate Complexes.[a]

Complex	τ_c[b] (sec × 10^9)	$^{13}COO^-$ $1/T_{1M}$ (sec^{-1})	$^{13}COO^-$ r(Å)	$^{13}CHOH$ $1/T_{1M}$ (sec^{-1})	$^{13}CHOH$ r(Å)
Mn^{2+}–D,L-mandelate	0.045	3830 ± 320	2.93 ± 0.04	6130 ± 560	2.71 ± 0.04
Enzyme–Mn^{2+}–D,L-mandelate	6.88[c]	6220 ± 1400	5.5 ± 0.2	1210 ± 260	7.2 ± 0.2

[a] Distances were calculated using eq 4. [b] Correlation time at 23.5 kG, or at 25.14 MHz for ^{13}C. [c] Calculated from eq 5 using the B and τ_v values for the same complex (Table III).

obtained from eq 4, using the correlation time of 4.5 × 10^{-11} sec for the Mn^{2+}–H_2O interaction in the same binary complex as determined from $1/fT_{1p}$ of water. This short correlation time represents the tumbling time of the small binary complex (Mildvan and Cohn, 1970). Because of the sixth root relationship between r and $1/fT_{1p}$ when eq 4 is used to determine distances, the errors in the calculated distances are much lower than those in $1/fT_{1p}$ (Table V). From crystallographic data (Carrel and Glusker, 1973) Mn^{2+} to carbon distances of 2.8–3.2 Å would be expected for carboxylate and carbinol coordination by Mn^{2+}. Hence, the distances of 2.93 and 2.71 Å calculated for the binary complex indicate bidentate coordination or chelation of Mn^{2+} by both the carboxylate and hydroxyl groups of mandelate (Figure 6). A weak chelate complex is also consistent with thermodynamic data since the dissociation constant (K_1) of the Mn^{2+}–mandelate complex (Table I) is 3.4-fold lower than that of the Mn^{2+}–pyruvate complex, which is a monodentate Mn^{2+}–carboxylate complex (Fung et al., 1973).

The corrected transverse relaxation rate of 2.7 × 10^4 sec^{-1} sets a lower limit on the first-order rate constant for dissociation of the Mn^{2+}–mandelate complex. Dividing this value by the dissociation constant, K_1 (Table I), yields a lower limit of ≥3.4 × 10^5 M^{-1} sec^{-1} for the second-order rate constant for formation of this complex.[3]

Paramagnetic Effects of Mandelate Racemase–Mn^{2+} on the Relaxation Rates of 1-[^{13}C]- and 2-[^{13}C]-D,L-Mandelate. Unlike the simple binary system, in the presence of mandelate racemase, Mn^{2+} produced significantly greater paramagnetic effects on the carboxyl than on the carbinol carbon of D,L-mandelate (Table IV, experiments 4–6), indicating a different Mn^{2+}–mandelate conformation on the enzyme. From the disssociation constants of the binary and ternary mandelate complexes (Table I, Figure 3) most of the Mn^{2+} would be expected to be in the ternary complex under these conditions. This point is supported by a displacement experiment with the competitive inhibitor D,L-α-phenylglycerate (Table IV, experiment 7). From the relative K_3 values of the substrate and inhibitor, D,L-α-phenylglycerate at a level which would be expected to displace 53% of the substrate from the ternary complex caused an average decrease of 40 ± 19% in the relaxation rates of the carbon atoms of D,L-mandelate (Table IV, experiment 7). Hence no corrections were made for the distribution of Mn^{2+} in the following calculations.

[3] The rate constants estimated from the NMR studies on a D,L mixture are a composite of the microscopic rate constants for the individual enantiomers.

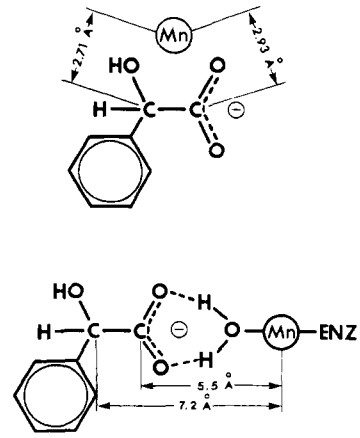

FIGURE 6: Mn^{2+} to ^{13}C distances in the Mn^{2+}–mandelate complex (top) and the enzyme–Mn^{2+}–mandelate complex (bottom).

The fastest transverse relaxation rate, namely that of the carboxyl carbon (5.6 ± 0.6 × 10^4 sec^{-1}, Table IV, experiments 5 and 6) is 9.0–46.4-fold greater than the longitudinal relaxation rates, indicating that the latter rates are not limited by chemical exchange and may be used for distance calculations (Nowak and Mildvan, 1972). The Mn^{2+} to ^{13}C distances in the ternary complex of enzyme, Mn^{2+}, and mandelate (Table V) were calculated with eq 4, using the correlation time of 2.57 × 10^{-9} sec determined for the Mn^{2+}–H_2O interaction in the same complex, from the frequency dependence of $1/fT_{1p}$ of H_2O (Table III). Since the correlation time for the Mn^{2+}–H_2O interaction is dominated by the electron spin relaxation time, a property of the Mn^{2+} itself, the same correlation time would be expected to modulate the Mn^{2+}–^{13}C interaction in this complex (Nowak et al., 1973; Fung et al., 1973). In the ternary complex, the distances from bound Mn^{2+} to the carboxyl and carbinol carbon atoms of bound D,L-mandelate (Table V) are too great for direct substrate coordination by 2.3 ± 0.2 and 4.1 ± 0.2 Å, respectively. These distances are consistent with the observation that $K_s > K_3$ for mandelate; *i.e.*, Mn^{2+} does not tighten but weakens the binding of mandelate to the enzyme (Table I). Enzyme-bound Mn^{2+} is 1.7 ± 0.4 Å farther from the carbinol carbon than from the carboxyl carbon. This difference in distance, which is indistinguishable from either the carbon to carbon single-bond length of 1.54 Å or double-bond length of 1.33 Å (Pauling, 1967), suggests a linear array of Mn^{2+}, C_1, and C_2 of mandelate on the enzyme (Figure 6).

The largest transverse relaxation rate of D,L-mandelate (5.6 × 10^4 sec^{-1}, Table IV) sets a lower limit on k_{off}, the

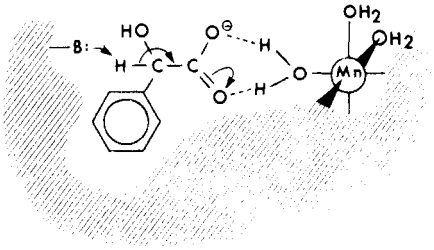

FIGURE 7: Suggested role of metal ion in promoting the enzymatic deprotonation of mandelate.

rate constant for dissociation of the substrate from the ternary enzyme complex,[3] which is 52 times greater than V_{max} (1070 sec^{-1}). From the value of k_{off} and that of the dissociation constant K_3 (Table I), the second-order rate constant for substrate binding to the enzyme-Mn^{2+} complex must be $\geq 8.4 \times 10^7\ M^{-1}\ sec^{-1}$.

Discussion

Examination of the metal ion binding behavior of mandelate racemase by titration of the apoenzyme with Mn^{2+} has revealed that the enzyme has two classes of divalent metal ion binding sites. The enzyme has approximately one tight binding site per subunit with $K_D = 8\ \mu M$ and six weak binding sites with K_D values of 1.5 mM. It has previously been demonstrated that mandelate racemase has an absolute divalent metal ion requirement for activity (Fee et al., 1974a). The close agreement of the activator constant determined from kinetic studies ($K_A = 9.3\ \mu M$) with the dissociation constant for the tight binding site indicates that only the tightly bound Mn^{2+} ion fulfills the enzyme's absolute requirement for divalent metal ion. Furthermore, mandelate racemase exhibits full enzymatic activity at Mn^{2+} concentrations at which weak-binding sites ($K_D = 1.5$ mM) are less than a few percent occupied. This confirms the notion that the weakly bound Mn^{2+} ions do not play an essential role in catalysis.

Figure 6 shows structures consistent with the Mn^{2+}-^{13}C distances obtained from $1/T_{1p}$ of ^{13}C and the frequency dependence of $1/T_{1p}$ of water protons in the ternary complex. It can be seen (top) that the binary mandelate-Mn^{2+} complex is a bidentate chelate with the Mn^{2+} significantly nearer the C-1 than the C-2 position. Thus the Mn^{2+}-mandelate binary complex is clearly an inner sphere complex. In contrast, the ^{13}C-labeled atoms of mandelate in the active ternary enzyme-Mn^{2+}-mandelate complex (Figure 6, bottom) are arranged in an approximately linear array with little or no direct coordination to Mn^{2+}. Alternatively, the lower limit of the Mn^{2+} to carboxyl carbon distance of 5.3 Å could result from the rapid averaging of, at most, 1.1% inner sphere complex ($r = 2.93$ Å) and 98.9% second sphere complex ($r = 5.73$ Å).

From the frequency dependence of $1/fT_{1p}$ of water protons (Table III), it was determined that ~3 fast exchanging water ligands were bound to Mn^{2+} in the binary enzyme-Mn^{2+} complex. In the active ternary complex of enzyme, Mn^{2+}, and D,L-mandelate this value decreased by ~1 despite the formation of negligible amounts of an inner sphere enzyme-Mn^{2+}-mandelate complex. Hence the binding of the substrate D,L-mandelate in the second coordination sphere appears to have occluded an inner sphere water ligand of the enzyme-bound Mn^{2+} such that it exchanges at a rate $\leq 10^5\ sec^{-1}$ (Table III). A water ligand so positioned could, by hydrogen bonding, polarize the carboxylate group of the substrate, and could also protonate this carboxylate group. Either of these effects would facilitate deprotonation at C-2 and carbanion formation (Kenyon and Hegeman, 1970). A suggested mechanism based on this view and consistent with all available data is given in Figure 7.

The possibility remains that the enzyme-bound Mn^{2+} exerts its catalytically important electrophilic effect only on that small fraction of the bound substrate (≤1.1%) which may reside in the inner coordination sphere of Mn^{2+}. However, a role for the divalent cation analogous to that shown for mandelate racemase in Figure 7 is indicated by NMR studies of five other enzymes which, like mandelate racemase, polarize the carbonyl group of their respective substrates. These enzymes are pyruvate carboxylase (Fung et al., 1973), transcarboxylase (Fung et al., 1974), ribulose diphosphate carboxylase (Miziorko and Mildvan, 1974), alcohol dehydrogenase (Sloan et al., 1974), and malic enzyme.[4]

Finally, certain kinetic properties unique to racemase reactions merit comment. The measured $1/fT_{2p}$ values of D,L-mandelate (Table IV) are functions of both k_2 and k_5 in the following minimal kinetic scheme which may be used to describe the mandelate racemase reaction.[3]

$$\text{E} + \text{D-mandelate} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E}\cdot\text{D-mandelate} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E}\cdot\text{L-mandelate} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{E} + \text{L-mandelate} \quad (6)$$

Since the K_M of D-mandelate extrapolated to infinite free Mn^{2+} concentration (0.63 mM) is very nearly equal to $K_3 = 0.67$ mM for the ternary enzyme-Mn^{2+}-D,L-mandelate complex, K_M probably represents a true dissociation constant which may be identical for both enantiomers, namely

$$K_M \sim \frac{k_2}{k_1} \sim \frac{k_5}{k_6} \quad (7)$$

Such a conclusion was suggested by a previous study of the equal competition both of D- and L-mandelate with the irreversible epoxide inhibitor, α-phenylglycidate (Fee et al., 1974b). In the forward direction, the rate-limiting step in eq 6, then, is the conversion of the enzyme-D-mandelate complex to the L-mandelate complex. These views are further supported by the observation of a large primary kinetic deuterium isotope effect of 5–6 on V_{max} but not on K_M in the forward direction (Hegeman et al., 1970). The value of k_3 is therefore the turnover number (k_{obs}) of the enzyme-catalyzed reaction; i.e., $k_3 = 1070\ sec^{-1}$.

The equilibrium constant for the overall reaction, which is equal to unity for racemization, is given by

$$K_{eq} = \frac{k_1 k_3 k_5}{k_2 k_4 k_6} = 1 \quad (8)$$

Rearranging eq 8 one obtains

$$\frac{k_3}{k_4} = \frac{k_2 k_1}{k_5 k_6} \quad (8A)$$

which indicates that if one enantiomer has a larger dissocation constant from its enzyme complex, then this enantiomer will be transformed more rapidly. Of course, if the dissociation constants were equal, k_3 and k_4 would be equal. Such conclusions should be applicable to all racemase reactions which obey Michaelis-Menten kinetics in both directions. In the present case, it follows from eq 7 and 8A that

[4] R. Hsu, C. H. Fung, and A. S. Mildvan, unpublished observations.

$k_4 \sim k_3$; i.e., the enzyme-catalyzed racemization steps for both the D and L enantiomers should be approximately equal.

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