Mandelate Racemase from *Pseudomonas putida*. Subunit Composition and Absolute Divalent Metal Ion Requirement†

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ABSTRACT: Polyacrylamide gel electrophoresis of mandelate racemase (EC 5.1.2.2) in the presence of sodium dodecyl sulfate gives a single protein band corresponding to a molecular weight of *ca.* 69,500. Four protein bands, indicative of four subunits, result from cross-linking of the enzyme with dimethyl suberimidate followed by sodium dodecyl sulfate gel electrophoresis. These and earlier results [Hegeman, G. D., Rosenberg, E. Y., and Kenyon, G. L. (1970), *Biochemistry 9*, 4029] suggest that the enzyme is composed of four identical subunits and has a molecular weight of *ca.* 278,000. The enzyme is completely inactivated when incubated with excess EDTA. Moreover, exhaustive efforts to remove contaminating divalent metal ions from the components of the assay mixture have led to enzyme with complete loss of residual activity. This activity is fully restored upon addition of certain divalent metal ions. Magnesium ion is most effective ($K_m = 2 \times 10^{-5}$ M), followed by Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+} in that order. Other divalent metal ions tested were without effect or prevented activation of metal-free enzyme by Mg^{2+}. These and other results have led to the suggestion that the enzyme has a previously unrecognized absolute divalent metal ion requirement, where the function of the metal ion is to withdraw electron density from the substrate, thereby making it more acidic.

Previous structural studies on mandelate racemase (Hegeman *et al.*, 1970; Hegeman, 1970) had led to the postulate that the enzyme was composed of identical subunits, and a molecular weight of *ca.* 200,000 was estimated for the oligomer from gel filtration experiments. In this paper we report our findings using the more reliable technique of gel electrophoresis in the presence of sodium dodecyl sulfate (Weber and Osborn, 1969), which shows a single staining band for the protein. Knowledge about subunit composition and molecular weight was essential to determining the stoichiometry of affinity labeling of the enzyme by DL-α-phenylglycidate, discussed in an accompanying paper (Fee *et al.*, 1974).

We have also reinvestigated the divalent metal ion requirement for optimal enzyme activity and report explicit instructions for removal of contaminating divalent metal ions from the enzyme and assay mixture. Weil-Malherbe (1966) has previously reported that divalent metal ions, *e.g.*, Mg^{2+}, stimulate binding of substrate. We find here that with great care in divalent metal ion removal completely inactive enzyme can be produced.

Experimental Section

Materials and Methods. General. Both mandelate racemase and L-mandelate dehydrogenase were prepared and assayed as previously described (Hegeman *et al.*, 1970; Hegeman, 1970). Protein was measured by the method of Lowry *et al.* (1951). Samples were compared to standard solutions of bovine serum albumin (Calbiochem). The crystalline material contained 97.5% dry bovine serum albumin by weight.

Polyacrylamide disc gel electrophoresis was performed according to the method of Davis (1964). The pH in the stacking gel was 8.9; the separating gel had a pH of 9.5. The electrophoresis was performed in 6.8-cm tubes in a Canalco Model 6 apparatus. Gels were stained with Aniline Blue Black in 7% acetic acid. Reagents for electrophoresis were obtained as follows: acrylamide (Canalco), methylenebisacrylamide (K & K Laboratories), glycine, Bromophenol Blue, and riboflavine (Matheson, Coleman & Bell), sucrose (Mallinckrodt), and ammonium persulfate (Polyscience, Inc.). Destaining was done by electrophoresis in 7% acetic acid.

When enzyme activity was to be located in the gels, the electrophoresis was done in a cold room (4°) with a current of 3 mA/gel. Then the gels were removed from the tubes, set in the freezer until slightly stiff, and cut into small pieces with a gel slicer. The pieces were dropped unto test tubes containing assay solution. Enzyme was located by the disappearance of color in one tube.

Electrophoresis of proteins in sodium dodecyl sulfate was done according to the procedure of Weber and Osborn (1969). The normal amount of cross-linker (0.6 g of methylenebisacrylamide/22.2 g of acrylamide) was used. Since mandelate racemase was in solution (Tris–NaCl–$MgCl_2$ buffer, pH 8.0), the procedure was modified so that enzyme solution containing about 0.05 mg of protein was added to an equal volume of dialysis buffer containing twice the usual concentration of components, *i.e.*, 0.02 M sodium phosphate

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(pH 7.0), 0.2% sodium dodecyl sulfate, and 0.2% β-mercaptoethanol. For comparison, crystalline bovine serum albumin and other proteins were first dissolved in Tris–NaCl–$MgCl_2$ buffer.

Sodium dodecyl sulfate and β-mercaptoethanol were from Matheson, Coleman & Bell. Coomassie Brilliant Blue was obtained from Mann. Proteins used as standards were from Miles (bovine α-chymotrypsin, whale skeletal muscle myoglobin, and bovine serum albumin) and Calbiochem (rabbit muscle pyruvate kinase and enolase).

Cross-Linking of Mandelate Racemase Subunits. The cross-linking experiment was based on the procedure of Davies and Stark (1970). Dimethyl suberimidate dihydrochloride was synthesized from suberonitrile (Aldrich) by their procedure. The suberonitrile was first distilled *in vacuo* and collected over molecular sieves. The melting point of the white product was 204–206° (uncor). Davies and Stark's product had a melting point of 214–215°. Nevertheless, no attempt was made to purify the material further. The product was stored over Drierite until it was used.

Triethanolamine buffer was made from redistilled triethanolamine (2,2′,2′′-nitrilotriethanol) obtained from Matheson, Coleman & Bell. The gels (10 × 0.6 cm) contained 6.66% acrylamide, 0.18% methylenebisacrylamide, and running buffer (0.1 M borate, 0.1 M sodium acetate, and 0.1% sodium dodecyl sulfate, pH 8.5) and were polymerized with 0.10% ammonium persulfate and 0.022% *N,N,N′,N′*-tetramethylethylenediamine.

Prior to the cross-linking, mandelate racemase (0.8 mg/ml) in 0.05 M Tris, 0.16 M NaCl, and 0.01 M $MgCl_2$ (pH 8.0) was dialyzed against 0.2 M triethanolamine-HCl (pH 8.5) at 4°. To 0.200 ml of the resulting racemase solution was added 0.040 ml of a freshly made solution of dimethyl suberimidate (12 mg/ml) in the 0.2 M triethanolamine buffer. The reaction was allowed to proceed at room temperature. After 3 hr and after 10 hr 0.120-ml aliquots were withdrawn and denatured by boiling with 100 μl of 5% sodium dodecyl sulfate and 10% β-mercaptoethanol in 0.01 M triethanolamine-HCl buffer (pH 8.5) for 15 min. A sample of mandelate racemase that was not treated with the cross-linking reagent was also denatured in this manner. Another untreated sample of mandelate racemase was denatured with the same reagents without boiling by addition of 4 M urea. The samples were allowed to stand overnight at 37° before electrophoresis was done. Neither bovine serum albumin nor mandelate racemase dissociated completely under the conditions described by Davies and Stark (1970), namely, in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 2 hr at 37°. To dissociate the protein completely, the sodium dodecyl sulfate concentration was therefore increased from 1 to 5%. β-Mercaptoethanol concentration was raised from 1 to 10%, and the ionic strength of the medium was decreased 20-fold. The ionic strength was decreased because increasing ionic strength decreases the interaction between amphipaths (*e.g.*, sodium dodecyl sulfate) and micelles (the enzyme in this case).[1]

Removal of Divalent Metal Ions from Mandelate Racemase and Components of the Assay System. Inorganic chemicals used in these experiments were of reagent grade from Mallinckrodt, unless otherwise noted. Solutions for the enzyme assay were made with distilled, deionized H_2O. After the solutions had been passed through Chelex, they were diluted to the required strength with deionized H_2O and stored in plastic bottles. Glassware (columns, cuvets) was allowed to soak at least 2 hr in 4 N HCl and then rinsed with deionized H_2O. Cuvets were also cleaned with concentrated HNO_3.

Preparation of Chelex. Chelex 100, Na form, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.), was washed with at least 10 bed volumes of 0.25 M sodium phosphate buffer (pH 7 or 8) followed by 10 bed volumes of deionized H_2O.

2,6-Dichlorophenolindophenol. DCPIP[2] (15 mg) (Calbiochem) was dissolved in *ca.* 5 ml of H_2O. The solution was applied to a washed Chelex column having a bed volume of 40 ml and eluted with deionized H_2O. The effluent containing the dark blue dye was collected and diluted to a concentration of 1 mM, based on the absorbance at 600 nm [ϵ 20.6 × 10^3 at pH 7.0 (Armstrong, 1964)]. A reddish-purple band remained at the top of the column when the dye had passed through it.

Phosphate buffer, 0.1 M, pH 7.0, made from KH_2PO_4 and $Na_2HPO_4 \cdot 7H_2O$, was also passed through the 40-ml Chelex column.

D-*Mandelate.* D-Mandelic acid (5 mmol; 0.7607 g) (Aldrich, Gold Label) was dissolved in 5 ml of 1 N NaOH and *ca.* 1 ml of deionized H_2O. This solution was passed through a washed Chelex column having a bed volume of *ca.* 100 ml. Effluent fractions of 10 ml each were monitored for mandelate by measurement of the optical density at 263 nm. Highly absorbing fractions were combined and diluted to give a 0.100 M solution based on a molar absorption of ϵ = 156.8 determined from a solution of known concentration.

KCN. Before KCN was applied to a 100-ml Chelex column, washed previously as described above, the column was washed again with 250 ml of 1 N KOH (to change the cation) and then with deionized H_2O. A solution of 0.65 g of KCN in 2 ml of H_2O was applied to the column. The concentration of the effluent was determined by titration with $AgNO_3$ (Blaedel and Meloche, 1963). $AgNO_3$ (*ca.* 0.01 M) was added from a buret to an aliquot of the KCN in 10 ml of a 0.01 M KI–NH_4OH solution (166 mg of KI and 3.4 ml of concentration NH_4OH in 100 ml). The end point was the formation of white precipitate that would not dissolve with stirring or with further addition of NH_4OH. The $AgNO_3$ was standardized against a fresh solution of 0.1 M KCN. Fractions of the KCN solution from the Chelex column were combined and brought to a final concentration of 0.1 M.

L-*Mandelate Dehydrogenase.* The suspension of the particulate fraction normally used in the assay of mandelate racemase was combined with approximately an equal volume of washed Chelex and allowed to stir for 30 min. Then the mixture was centrifuged at low speed to remove the Chelex. This treatment caused no loss of activity of the particles.

Preparation of Chelex–Sephadex Column. Sephadex G-25 medium (Pharmacia), swelled in a buffer of pH 8.0, containing 0.05 M Tris (Schwarz-Mann, Ultra Pure), 0.10 M NaCl, and 0.010 M Na_2EDTA (Mallinckrodt), was packed into a column having a bed volume of 4 ml. The column was then washed with 5 bed volumes of the Tris–NaCl–EDTA (pH 8.0) buffer. Washed Chelex (4–5 ml) was packed on top of the Sephadex. The combination column was washed with more of the Tris–NaCl–EDTA buffer (pH 8.0) and then with a buffer of pH 8.0 containing only 0.05 M Tris and 0.10 M NaCl; the Tris–NaCl elution buffer had been passed through a Chelex column.

Removal of Metal Ions from Mandelate Racemase. Dialysis tubing (diameter = 1/4 in.) was boiled in 30 mM Na_2EDTA

[1] E. Cordes, Indiana University, unpublished results.

[2] Abbreviations used are: DCPIP, 2,6-dichlorophenolindophenol, sodium salt; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonate, sodium salt.

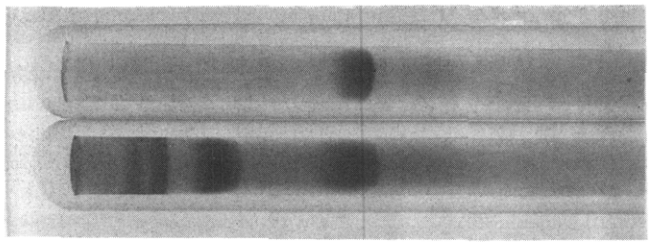

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electropherograms of sodium dodecyl sulfate treated enzyme before and after cross-linking with dimethyl suberimidate. The most forward band migrated 5.2 cm from the origin.

for about 15 min. A solution of mandelate racemase (0.500 ml containing 0.83 mg/ml) in 0.05 M Tris–0.16 M NaCl–0.01 M $MgCl_2$ (pH 8.0) was dialyzed against 250 ml of 0.05 M Tris–0.10 M NaCl–0.005 M EDTA (pH 8.0) in a plastic container. The buffer was changed once after at least 5 hr. Then the dialysis buffer was changed to 0.05 M Tris–0.10 M NaCl–0.001 M EDTA (pH 8.0). The final dialysis buffer was 0.05 M Tris–0.10 M NaCl (pH 8.0) that had been passed over Chelex. About 0.5 g of washed Chelex was added to this buffer. In some trials the dialysis against buffer containing 0.005 M EDTA was omitted. After dialysis for a period of 4 days, solution was placed on the Chelex–Sephadex column (described above), eluted with 0.05 M Tris–0.10 M NaCl (pH 8.0) (previously passed through Chelex), and collected in 1.0-ml fractions in small plastic vials. The dialysis and the column elution were carried out at 4°. The enzyme was located by measuring absorbance of the fractions at 280 nm.

Results

Polyacrylamide Disc Gel Electrophoresis (without Sodium Dodecyl Sulfate). An important criterion of purity for the enzyme preparation was an analysis by disc gel electrophoresis. The main protein band that appeared after staining had a mobility of 0.26, where mobility is the distance protein moves through the separating gel divided by the distance the tracking dye moves through the separating gel. That this band was mandelate racemase was proven by the location of mandelate racemase activity in a duplicate, unstained gel. A faint band having a mobility of about 0.7 was also present.

Polyacrylamide Disc Gel Electrophoresis (with Sodium Dodecyl Sulfate). Mobilities upon sodium dodecyl sulfate gel electrophoresis of mandelate racemase and the proteins used as standards are shown in Table I. A plot of logarithm of molecular weight *vs.* mobility of protein (not shown) was linear. α-Chymotrypsin did not completely dissociate; a band appeared for the undissociated protein as well as two bands for the two large subunits. It can be concluded that the molecular weight of each mandelate racemase subunit is slightly greater than that of bovine serum albumin, *i.e.*, about 69,500.

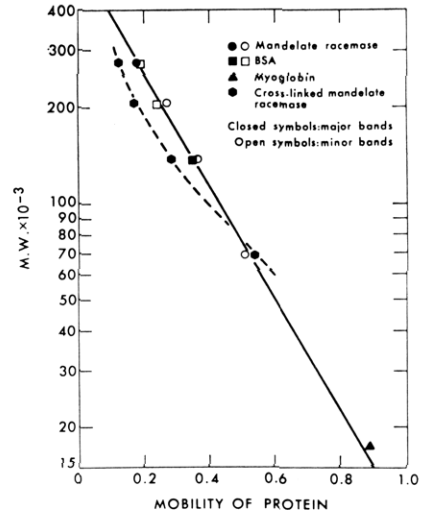

FIGURE 2: Logarithm of molecular weight *vs.* mobility for various proteins upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Cross-Linking of Mandelate Racemase Subunits with Dimethyl Suberimidate. Native mandelate racemase was cross-linked with dimethyl suberimidate in the manner of Davies and Stark (1970) and was then dissociated with sodium dodecyl sulfate. Cross-linking occurs between subunits of the same molecule much more readily than between subunits of different molecules because of the difference in concentration. In a protein having four identical subunits, then, four main bands should appear following electrophoresis, corresponding to the monomer, dimer, trimer, and tetramer. Such a four-band pattern did result with mandelate racemase (Figure 1). Thus, mandelate racemase consists of four subunits, each having a molecular weight of *ca.* 69,500; the total molecular weight of the enzyme is *ca.* 278,000.

Mandelate racemase that had not been treated with dimethyl suberimidate dissociated very little under the conditions used by Davies and Stark prior to electrophoresis. One dark band appeared at a mobility equal to 0.166. Faint bands having higher mobilities also were present. The faint bands in the racemase gels were assumed to result from partial dissociation of the enzyme; the mobilities were plotted *vs.* the logarithm of the molecular weight (of monomer, dimer, trimer); a straight line resulted (see Figure 2). The

TABLE I: Sodium Dodecyl Sulfate Polyacrylamide Disc Gel Electrophoretic Mobilities of Mandelate Racemase and Reference Proteins.

Protein	Mobility	Mol Wt	Reference
Bovine serum albumin	0.196, 0.192, 0.186, 0.205	69,000	Tanford *et al.* (1967)
Rabbit muscle pyruvate kinase	0.222, 0.265	57,200	Darnall and Klotz (1972)
Rabbit muscle enolase	0.287	41,000	Weber and Osborn (1969)
Bovine α-chymotrypsin	0.558	25,000	Weber and Osborn (1969)
	0.794	13,000	
	0.829	11,000	
Sperm whale myoglobin	0.763, 0.754, 0.743, 0.769	17,200	
Mandelate racemase	0.191, 0.182, 0.197		

dark protein band corresponded to tetramer on this plot. Bovine serum albumin and myoglobin were treated under the same conditions. Myoglobin exists as a monomer (Weber and Osborn, 1969); the mobility found for it fits on the same plot. Bovine serum albumin has appeared in oligomeric forms in other studies using sodium dodecyl sulfate electrophoresis (Shapiro *et al.*, 1967; Dunker and Reuckert, 1969). If the main band in the bovine serum albumin gel was assumed to be dimer and if the two minor bands were trimer and tetramer, the mobilities also fit on the plot (Figure 2). The mobilities of three of the four main bands (corresponding to dimer, trimer, and tetramer) obtained after treatment of mandelate racemase are also shown in Figure 2.

Lack of Enzyme Activity in the Presence of Excess EDTA. When the usual assay, except for $MgCl_2$ addition, was performed after incubation of the buffered enzyme for 30 min in the presence of 3.33 mM NaEDTA and no added $MgCl_2$, no detectable activity was observed. A control vessel containing no EDTA which had instead been incubated with 1.0 mM $MgCl_2$ retained full activity. In a separate experiment enzyme and assay materials which had not been treated with Chelex to remove contaminating metal ions (see Experimental Section) generally showed *ca.* 30% of the control activity when the usual assay was performed in the absence of added $MgCl_2$. The presence of EDTA was shown in a separate experiment not to affect the activity of the L-mandelate dehydrogenase; Weil-Malherbe (1966) had previously shown that EDTA did not affect L-mandelate dehydrogenase using a more complex coupled assay system.

Reduction of Enzymatic Activity by Removal of Contaminating Divalent Metal Ions. Extensive efforts to remove contaminating divalent metal ions from the enzyme and components of the assay mixture (see Experimental Section) have led to enzyme with no detectable residual activity. Addition of 1.0 mM Mg^{2+} to the assay mixture restored full activity. The time of reactivation of the enzyme was noticeably dependent on the order of addition of Mg^{2+} and Na^+ D-mandelate. When Mg^{2+} was added to the enzyme in a cuvet containing all components of the assay system except D-mandelate, followed within *ca.* 1 min by the addition of D-mandelate and assay, the reactivation was apparently complete before the measurement could be made, *i.e.*, reactivation occurred in less than 5 min. On the other hand, when the order of addition of Mg^{2+} and D-mandelate was reversed, the enzyme first showed a slight increase in activity only after 5 min; the activity gain was very gradual in the following minutes.

Ability of Metal Ions to Restore Activity. Enzyme from which divalent metal ions had been removed was assayed with divalent metal ions and Li^+. At 1 mM concentrations in several buffer systems (Tris-Cl, imidazole-Cl, sodium phosphate, and NaHepes) at several pH values, Ca^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, Sr^{2+}, Cu^{2+}, and Li^+ were unable to restore activity. Mg^{2+}, Co^{2+}, Ni^{2+}, and in some buffers Mn^{2+} and Fe^{2+}, were effective in restoring activity, in that order of effectiveness. Some K_m's and relative V_{max} values for effective metals are given in Table II. Because of complex formation and salt precipitation all divalent metals were not tested under all conditions. Mn^{2+} and Fe^{2+} did not show restorative effects in phosphate or in Tris at high pH values; their effectiveness could be demonstrated in imidazole chloride or buffers other than phosphate at pH 7.0. Although they do not restore activity to demetalized enzyme, certain divalent metals (*e.g.*, Cd^{2+}, Ba^{2+}, Mn^{2+} in phosphate) prevent restoration and activity by Mg^{2+} added at a later time. If Mg^{2+} is added to demetalized enzyme prior to the other metal, no inhibition is observed.

TABLE II: Activation of Mandelate Racemase by Divalent Metal Ions in Several Buffer Systems:[a] K_m and V_{max}.

Metal Ion[b]	K_m (mol $l.^{-1}$)	Rel V_{max}
Mg^{2+}	5×10^{-5} [c]	1.0[c,d]
	2×10^{-5} [d]	
	1.5×10^{-5} [e]	
Co^{2+}	2×10^{-4} [c]	0.38[c]
		0.60[d]
Ni^{2+}	3×10^{-4} [c]	0.30[c]
		0.59[d]
Mn^{2+}	3×10^{-5} [c]	0.25[c]
	2×10^{-5} [d]	0.48[d]

[a] Either imidazole chloride or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate sodium salt (Hepes) replaced phosphate in the standard assay system (Hegeman *et al.*, 1970) in an equimolar amount at pH 7.0. Metal salts were preincubated in the reaction mixture complete except for substrate for 10 min; the reaction was started by adding D-(−)-mandelate. [b] Added as the chloride salts. [c] Imidazole chloride. [d] Hepes (Na salt). [e] Phosphate (pH 7.0).

Discussion

The results of the experiments with electrophoresis of mandelate racemase in the presence of sodium dodecyl sulfate are consistent with a molecular weight for each subunit of *ca.* 69,500. The error of this method is generally less than or equal to 10% (Weber and Osborn, 1969). That only one band appeared is evidence that mandelate racemase is composed of identical subunits, a conclusion which had been reached earlier (Hegeman *et al.*, 1970).

When the enzyme was cross-linked using dimethyl suberimidate (Davies and Stark, 1970) and then dissociated with sodium dodecyl sulfate, four bands appeared upon sodium dodecyl sulfate gel electrophoresis, corresponding to monomer, dimer, trimer, and tetramer. Thus, we conclude that mandelate racemase is composed of four identical subunits, and therefore that the molecular weight of the oligomeric native enzyme is *ca.* 278,000. Even without cross-linkage, mandelate racemase showed faint bands corresponding to monomer, dimer and trimer upon sodium dodecyl sulfate gel electrophoresis, and a plot of logarithm of the molecular weight *vs.* mobility of protein was linear (Figure 2). The mobilities of three of the four main bands (corresponding to dimer, trimer, and tetramer) obtained following treatment of mandelate racemase with dimethyl suberimidate, however, do not fit on the straight line in Figure 2. Davies and Stark (1970) mention that the cross-linking increases apparent molecular weight by reducing the amount of sodium dodecyl sulfate bound to the protein. In addition, extensive cross-linking can add significantly to the molecular weight. The curved plot obtained for mandelate racemase is similar to the curved plots obtained for other proteins after cross-linking with dimethyl suberimidate (Davies and Stark, 1970) and with glutaraldehyde (Griffith, 1972). The band corresponding to the dimer appears to be a doublet (Figure 1); this effect is probably due to differing amounts of cross-linkage.

The enzyme appears to require a divalent metal ion (*e.g.*, Mg^{2+}) for activity. This conclusion is based on the following evidence. First, in the presence of excess EDTA, no activity is observed. Second, after exhaustive removal of contaminating divalent metal ions from the enzyme and other components of

the assay mixture, residual activity was reduced to zero; this demetalized enzyme could be restored to full activity by addition of Mg^{2+}. Third, a parallel divalent metal ion requirement for inactivation of the enzyme by the affinity label D,L-α-phenylglycidate has been found (Fee *et al.*, 1974).

The fact that the order of addition of mandelate and Mg^{2+} makes a difference in the time needed for reactivation of the enzyme is consistent with the postulate that free Mg^{2+}, rather than a magnesium–mandelate complex, reactivates the enzyme. D-Mandelate may slow the reactivation either by binding at the active site, thereby sterically inhibiting binding of Mg^{2+}, and/or by binding to Mg^{2+} in the solution, reducing the concentration of free Mg^{2+}. In either case, this result seems to rule out a coordination scheme in which the substrate, D-mandelate, acts as a bridge between the enzyme and the metal ion (Mildvan, 1970). The failure of Ca^{2+} to restore activity to mandelate racemase further substantiates this; Ca^{2+} often activates enzymes which form substrate bridge complexes but inhibits enzymes in which metal ion is the bridge (Mildvan, 1970).

Li^+ sometimes behaves like Mg^{2+} chemically (Phillips and Williams, 1966). The behavior of the two ions with this enzyme is not similar, however, since Li^+ does not produce catalytic activity.

Besides Mg^{2+}, Co^{2+}, and Ni^{2+}, Mn^{2+} and Fe^{2+} (in some buffers) are capable of reactivating demetalized enzyme. This finding is in agreement with earlier work of Weil-Malherbe (1966). Certain divalent metals unable to restore activity (*e.g.*, Cd^{2+}, Ba^{2+}, and Mn^{2+} in phosphate buffer) may prevent reactivation by Mg^{2+} added later; yet these metals do not inactivate enzyme to which Mg^{2+} has been restored. This finding suggests that under appropriate conditions an inactive complex may be formed by the enzyme with certain divalent metals.

It is not possible to rule out an enzyme–bridge complex with the data at hand. Nevertheless, it seems that a metal bridge is the likely coordination scheme. Metal ions which bind a carboxylate oxygen and the α-amino nitrogen of an amino acid are able to catalyze racemization by making the α-hydrogen more labile. For example, the α-hydrogen of glycine and of alanine in $[Co(ethylenediamine)_2(glycine)]^{2+}$ and $[Co(ethylenediamine)_2(alanine)]^{2+}$ are rapidly replaced with deuterium in slightly basic D_2O, as shown by nuclear magnetic resonance spectroscopy (Williams and Busch, 1965). Mg^{2+} may likewise catalyze racemization of mandelate by withdrawal of electron density from the carbanionic intermediate species.

Chelates of mandelate with Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, isolated from methanol, have been reported (Kovalenko *et al.*, 1971). The postulate that divalent metal ion binds near to the substrate at the enzyme active site is also reasonable from the standpoint of known enzyme chemistry. In several enzymatic reactions, metal ions have been found to assist in the formation of carbanionic species. Among these enzymes are yeast aldolase, pyruvate carboxylase, histidine deaminase, aconitase, and enolase (Mildvan, 1970).

The geometry of the enzyme–metal–mandelate chelate is probably important in determining the rate of the reaction. Weil-Malherbe (1966) noted that HPO_4^{2-} inhibited the enzymatic racemization and that this inhibition was not competitive with Mg^{2+}. It is known that HPO_4^{2-} and other oxyanions can alter the conformation of chelates [*e.g.*, tris(ethylenediamine)cobalt(III) (Duesler and Raymond, 1971)] by forming hydrogen bonds with the ligands attached to the metal. It is possible, then, that HPO_4^{2-} inhibits racemization by changing the conformation of the metal–mandelate chelate.

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