Mandelate Racemase from *Pseudomonas putida*. Affinity Labeling of the Enzyme by D,L-α-Phenylglycidate in the Presence of Magnesium Ion†

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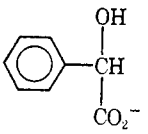
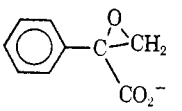
ABSTRACT: D,L-α-Phenylglycidate was synthesized to probe the active site of mandelic acid racemase (EC 5.1.2.2) because of the structural similarity of this compound to the substrate and because of the reactivity of its epoxide moiety toward nucleophiles. In the presence of Mg^{2+} the α-phenylglycidate causes complete and irreversible inhibition of activity. The inhibition follows saturation kinetics. At pH 7.1 K_{inact} is 2 mM compared to K_m = 5 mM for D-mandelate. Both D- and L-mandelate protect the enzyme from inactivation by DL-α-phenylglycidate; the dissociation constant for the substrate in the protection experiments is approximately 5 mM. The results indicate that α-phenylglycidate binds at the active site of the enzyme and that it is therefore an affinity label. When the enzyme was irreversibly inhibited with either β-[^{14}C]- or β-[^{3}H]-α-phenylglycidate, 1 mol of label was incorporated per subunit of enzyme. The label could be removed by incubation at pH 9, and especially rapidly in the presence of hydroxylamine. A portion of the radioactivity from hydrolysis in the absence of hydroxylamine was isolated as α-phenylglyceric acid.

Evidence has been presented favoring a carbanion intermediate in the mechanism of action of mandelate racemase (Kenyon and Hegeman, 1970). This included demonstration of enzyme-catalyzed deuterium exchange in the α position of mandelate which accompanies racemization. Since the enzyme acts without a flavine or pyridine nucleotide cofactor (Hegeman *et al.*, 1970), it was assumed that a basic moiety of the protein itself participated in the generation of this carbanion by abstracting the α proton from mandelate. Mandelate racemase is thus related to the growing group of enzymes (reviewed by Rose, 1966, 1970) which rely upon the basic properties of their active sites to effect proton abstraction and transfer reactions. Of the highly purified enzymes studied in this group proline racemase (Cardinale and Abeles, 1968), hydroxyproline 2-epimerase (Adams and Norton, 1964; Finlay and Adams, 1970), and lactate racemase of *Lactobacillus sake* (Hiyama *et al.*, 1968), each of which catalyzes an epimerization at the carbon α to a carboxylate, are most closely related to mandelate racemase.

In order to probe the active site of the enzyme and possibly to identify this key basic moiety, we selected a close structural analog of mandelic acid (I), namely, α-phenylglycidic acid (II), as a possible affinity label. If the phenyl and carboxylate



I



II

portions of II bind to the enzyme in the same positions as the corresponding portions of I, the epoxide moiety should be situated close to the postulated basic group of the enzyme. The catalytically essential base could then act as a nucleophile toward the epoxide and become covalently attached to it. Epoxide-containing affinity labels have been utilized in the study of several other enzymes, including α-chymotrypsin (Brown and Hartley, 1965), pepsin (Chen and Tang, 1972; Hartsuck and Tang, 1972), lysozyme (Moult *et al.*, 1973), β-glucosidases (Legler, 1968; Legler and Hasnain, 1970), triosephosphate isomerase (Schray *et al.*, 1973), enolase (Schray *et al.*, 1973), phosphoglucose isomerase (O'Connell and Rose, 1973), and phosphoenolpyruvate:uridine 5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose-3-*O*-enolpyruvyltransferase (Cassidy and Kahan, 1973).

In this paper we present evidence that D,L-α-phenylglycidate behaves as an affinity label for mandelate racemase and that this inhibitory action depends upon the presence of a divalent metal ion (*e.g.*, Mg^{2+}). A previously undemonstrated absolute requirement for a divalent metal ion for enzymatic activity, also established in these studies, is described in an accompanying paper (Fee *et al.*, 1974).

Experimental Section

Materials and Methods. General. Many of the methods used and sources of materials used are described in the accompanying paper (Fee *et al.*, 1974). Radioactivity was measured by adding labeled protein to 1.0 ml of 1 M Hyamine hydroxide (Rohm and Haas product obtained from New England Nuclear Corp.) and 15 ml of scintillation fluid in a standard glass scintillation vial. The inside portion of the cap of the vial was either made of poly(vinyl chloride) or lined with Teflon to prevent attack by the Hyamine hydroxide. The total amount

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of enzyme solution plus buffer added to each vial was 0.500 ml. Scintillation fluid contained 1.00 l. of absolute ethanol, 916 ml of toluene, and 84 ml of a solution made from 500 ml of toluene, 50.0 g of 2,5-diphenyloxazole (New England Nuclear), and 0.625 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (New England Nuclear).

Samples were counted in a Nuclear-Chicago Mark I scintillation counter. The number of counts per minute was converted to disintegrations per minute either by using an external standard and the efficiency curve calculated for the counter settings used or by using an internal standard of [^{14}C]-toluene (New England Nuclear). Results from the two methods agreed closely.

Melting points were taken on a Büchi melting point apparatus and are uncorrected unless otherwise noted. Infrared spectra were taken of Nujol mulls with a Perkin-Elmer Infracord spectrometer (Model 237). A Varian T-60 nmr spectrometer was used to measure ^{1}H nmr spectra. Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.

Chemical Syntheses. Sodium α-phenylglycidate was best prepared by the saponification of ethyl α-phenylglycidate following the procedure of Singh and Kagan (1970). Ethyl atropate, the precursor of ethyl phenylglycidate, was prepared by adding HCHO to diethyl phenyloxaloacetate in the manner of Ames and Davey (1958).

The sodium salt of diethyl phenyloxaloacetate was made either according to Ames and Davey (1958) or Levene and Meyer (1943). The Levene and Meyer method results in a more rapid reaction. In either case, the starting materials, diethyloxalate (Eastman) and ethyl phenylacetate (Eastman), were distilled before the reaction. The product was stable for several months if it was stored in a freezer over Drierite. The free ester was liberated from the sodium salt by dissolving it in a small amount of H_2O, extracting with ether (to rid it of small amounts of ethyl phenylacetate), and acidifying with concentrated H_2SO_4. The ester was removed from the solution by ether extraction.

Diethyl phenyloxaloacetate was treated according to Ames and Davey (1958) with aqueous formaldehyde to give ethyl atropate. The product was distilled *in vacuo*. Ethyl phenylacetate was a persistent contaminant in the distillation when product was collected over a range of 4–5° (at *ca.* 0.5 mm). The proportion of the ethyl phenylacetate in the first fractions was greater than in the last, as shown by the methylene peak at δ 3.5 in the nuclear magnetic resonance (nmr) spectrum. Because it was possible to remove the contaminant later in the reaction sequence, further attempts were not made at this point. The nmr spectrum of ethyl atropate had peaks at δ 1.12 (3 H, triplet, J = 7 Hz), 4.12 (2 H, quartet, J = 7 Hz), 5.72 (1 H, doublet, J = 1.5 Hz), 6.23 (1 H, doublet, J = 1.5 Hz), and 7.3 (5 H, multiplet).

Ethyl α-phenylglycidate resulted when ethyl atropate was heated at reflux with excess *m*-chloroperoxybenzoic acid (85%, Aldrich) in $CHCl_3$ for 20 hr (Singh and Kagan, 1970). The crude product was purified by silica gel chromatography with petroleum ether (bp 30–60°) as eluent. Ethyl phenylacetate emerged just ahead of ethyl α-phenylglycidate. The nmr spectrum (CCl_4) had peaks at δ 1.12 (3 H, triplet, J = 7 Hz), 2.68 (1 H, doublet, J = 6.5 Hz), 3.24 (1 H, doublet, J = 6.5 Hz), 4.06 (2 H, quartet, J = 7 Hz), and 7.4 (5 H, multiplet).

Sodium α-Phenylglycidate. Ethyl α-phenylglycidate was saponified with sodium ethoxide and H_2O in ethanol (Singh and Kagan, 1970). An nmr spectrum of a D_2O solution of the resulting salt had peaks at δ 3.28 (2 H, br s) and 7.45 (5 H, br). *Anal.* Calcd for $C_9H_7O_3Na$: C, 58.06; H, 3.79. Found: C, 57.86; H, 3.88. This salt was stored in a freezer to minimize decomposition.

Radioactive Sodium α-Phenylglycidate. The syntheses of both sodium [^{3}H]- and [^{14}C]-α-phenylglycidate were based on that described above for the unlabeled compound. Because the label was derived from formaldehyde, the α-phenylglycidate was labeled at the β hydrogens or β carbon (Schinz and Hinder, 1947). The procedure was modified to take into account the low concentration of the formaldehyde solution and the small scale of the reaction. Since only limited amounts of enzyme were available, the starting material was diluted only slightly or not at all.

Formaldehyde was obtained as a 1% aqueous solution from New England Nuclear: [^{3}H]HCHO, 25.0 mCi, 7.5 mg in 0.750 ml of H_2O, lot no. 728-029 (= 100 Ci/mol) and [^{14}C]-HCHO, 1.0 mCi, 3.0 mg in 0.300 ml of H_2O, lot no. 728-016 (= 10 Ci/mol).

The sodium salt of diethyl phenyloxaloacetate (86 mg) was quickly dissolved in 0.6 ml of H_2O and extracted with ether. The ether extract was discarded. The aqueous solution was acidified with 1–2 drops of concentrated H_2SO_4 and extracted with ether. The ether extract was placed into a two-neck 5-ml pear-shaped flask and ether was removed with a stream of dry nitrogen. To the ester in the 5-ml flask was added 0.750 ml of 1% labeled formaldehyde ([^{3}H]HCHO was not diluted; [^{14}C]-HCHO (0.150 ml) was diluted with 0.650 ml of unlabeled 1% HCHO). The flask was fitted with a nitrogen bubbler and a serum cap. Over a period of 0.5 hr, 72 μl of 50% (w/v) K_2CO_3 solution was dropped slowly into the reaction *via* a 100-μl syringe. A water bath around the flask was maintained at 15° during this time. The reaction was allowed to stir for 22–24 hr.

The reaction mixture was extracted with diethyl ether. After the ether layer had dried over Na_2SO_4, it was transferred to a 10-ml pear-shaped flask and ether was removed with a stream of nitrogen. A solution of 60 mg of *m*-chloroperoxybenzoic acid (85%, Aldrich) in 6.5 ml of $CHCl_3$ was added to the flask. The reaction was heated at reflux for at least 24 hr. At the conclusion of the epoxidation reaction, the solution was extracted at least three times with 7.5% $NaHCO_3$ and then dried with Na_2SO_4. Solvent was removed with a nitrogen stream. The yellow liquid was placed on a silica gel column (0.78 g) and eluted with $CHCl_3$. Solvent was again removed with N_2. A solution of 1.00 g of Na in 40.0 ml of absolute ethanol was freshly made. To the epoxide ester was added 0.191 ml of the sodium ethoxide–ethanol solution, 3.8 μl of H_2O, and a small amount of ether. The precipitate (sodium α-phenylglycidate) was collected by centrifugation.

An attempt was made to purify ethyl α-phenylglycidate by preparative thin-layer chromatography on silica gel. This was abandoned because of low recovery. The ethyl α-phenylglycidate resulting from the above sequence was about 60% pure by nmr spectroscopy. The main contaminant is ethyl phenylacetate which is unlabeled and which does not react with the enzyme. Substances which could react with the enzyme (*e.g.*, *m*-chloroperoxybenzoic acid) are removed in this sequence. In conclusion, in the product obtained only α-phenylglycidate is labeled and is capable of reacting with enzyme.

In order to know the final specific activity of [^{14}C]HCHO, it was necessary to compare the concentrations of the labeled solution and the cold solution used for dilution. Schiff's reagent was used for this determination (Walker, 1964). Into six cuvets, each containing 1.25 ml of H_2O, were added the following quantities of a 0.1% HCHO solution: 0, 1, 5, 10, 12,

15 μl. Then 0.25 ml of concentrated H_2SO_4 was added. When the solutions had cooled approximately to room temperature, 1.25 ml of the Schiff's reagent was added to each cuvet. The color was allowed to develop for 45 min before absorbance was read at 570 nm. A calibration curve (concave upward) was drawn from these six values (absorbance *vs.* [HCHO]). [^{14}C]-HCHO was treated in the same way and compared to the calibration curve.

Treatment of Mandelate Racemase with Radioactively Labeled, D,L-α-*Phenylglycidate.* To a solution of mandelate racemase (0.8–2 mg/ml) in 0.05 M Tris-HCl buffer (pH 8.0), containing 0.16 M NaCl and 0.01 M $MgCl_2$, was added an excess of [^{14}C]- or [^{3}H]-α-phenylglycidate dissolved in 0.10 M phosphate buffer (pH 7.0). In a typical experiment 0.525 ml of α-phenylglycidate solution (*ca.* 0.2 mg/ml) was added to 2.0 ml of enzyme solution containing about 0.83 mg of protein/ml. This resulted in an inhibitor:enzyme subunit ratio of approximately 27:1. The reaction was carried out at room temperature until assay revealed that less than 1% enzyme activity remained.

Labeled enzyme was separated from free inhibitor on a Sephadex G-25 medium column which had been swollen in 0.05 M Tris-HCl (pH 8.0), containing 0.10 M NaCl and 0.01 M $MgCl_2$. The bed volume of the column was at least four times the sample volume. The void volume of the column was determined with Blue Dextran (Pharmacia).

Reaction of Labeled Racemase with NH_2OH. Several attempts were made to remove the radioactive label from the enzyme with NH_2OH. $NH_2OH \cdot HCl$ was obtained as a 10% solution from Sargent-Welch or as reagent grade solid from Baker and Adamson. Solutions were adjusted to the required pH (*ca.* 9) with NaOH, stored at 4°, and used the same day that they were made. Most NH_2OH solutions also contained urea (reagent grade, Mallinckrodt). When the reaction was to be done in urea, the enzyme solution was first passed through a Sephadex G-25 column that had been swelled in a solution containing only urea (also adjusted to a pH of *ca.* 9). To one portion of this enzyme in urea was added NH_2OH–urea solution; to a second portion (the control) was added the same volume of a solution containing only urea. To end the reaction, enzyme was separated from small molecules on a Sephadex G-25 column that had been swelled in 0.05 M Tris-HCl (pH 8.0), containing 0.10 M NaCl and 0.01 M $MgCl_2$.

Kinetic Studies of Irreversible Inhibition. The amount of inhibition of enzyme was measured with the usual quantitative assay. Stock solution containing the correct amounts of phosphate buffer, 2,6-dichlorophenolindophenol, KCN, $MgCl_2$, and H_2O for 25 assays was made daily. Before a run, 2.72 ml of stock solution was pipetted into each of the cuvets to be used in that run (typically six to eight cuvets).

A stock solution of sodium α-phenylglycidate was also prepared daily. Usually this was made with 11.64 mg of sodium α-phenylglycidate (weighed on a microbalance) dissolved in 10.00 ml of 0.10 M phosphate buffer (pH 7.0). Portions of this 6.25 mM solution were then combined with 0.033 ml of 0.30 M $MgCl_2$ and diluted to 10.00 ml with phosphate buffer to give a solution containing 1.0 mM $MgCl_2$ and the required concentration of α-phenylglycidate for a given run. In the protection experiments the necessary amount of 0.10 M mandelate was also added to the second solution. The mandelate solution had been made by adding 10 mmol of mandelic acid to 10 mmol of NaOH and diluting to 100 ml with 0.10 M phosphate buffer.

The enzyme used in all these experiments contained about 0.83 mg of protein/ml in 0.05 M Tris-HCl buffer, 0.16 M NaCl, and 0.010 M $MgCl_2$ (pH 8.0). In order to measure the enzyme activity in convenient quantities, it was necessary to dilute it tenfold. The dilution was done with 0.050 M Tris–0.15 M NaCl, thus giving a final Mg^{2+} concentration of 1.0 mM.

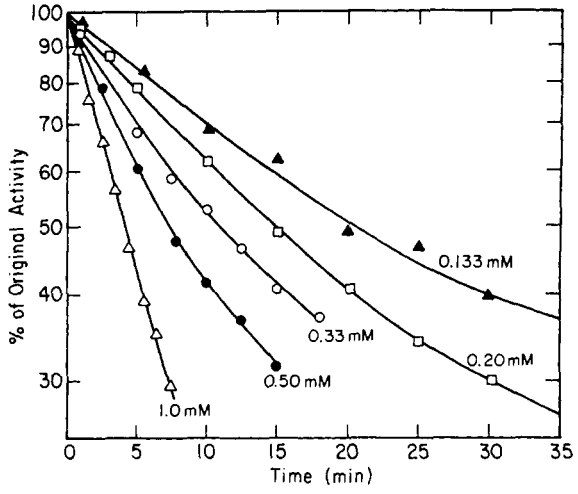

FIGURE 1: Irreversible inhibition of mandelate racemase at various concentrations of D,L-α-phenylglycidate in the presence of 1 mM Mg^{2+}.

The reaction of α-phenylglycidate and enzyme was carried out in a small conical vial (Reacti-vial, Pierce Chemical Co.) at room temperature (23°). At time zero, 25 μl of enzyme in a disposable capillary pipet (Microcaps, Kensington Scientific, Emeryville, Calif.) was added to 100 μl of α-phenylglycidate solution. At known time intervals a 10-μl aliquot was removed (also with a disposable pipet) and added to 2.72 ml of assay solution in a cuvet. Quickly the cuvet was covered with Parafilm and shaken. This solution was allowed to equilibrate for 30 min before 0.250 ml of 0.10 M D-mandelate and 0.025 ml of L-mandelate dehydrogenase were added to begin the assay. This 30-min interval before assay was decided upon because preliminary experiments indicated that the enzyme gains a little activity as it stands.

The results of the assays were converted to per cent of activity remaining and plotted as first-order reactions. The activity of each cuvet corresponded to one point on the semilog plot. The activity corresponding to 100% varied slightly from one run to the next, probably due to pipetting errors. If a plot did not extrapolate to 100%, the value for 100% activity was reestimated and points were correspondingly adjusted. This practice gave reproducible values for $t_{1/2}$ at a given inhibitor concentration. The plots were not perfectly linear. Therefore, the value of $t_{1/2}$ was taken as the time at 50% activity extrapolated from the initial portion of the curve so that $t_{1/2}$ would correspond to the rate constant for the initial reaction. Finally the $t_{1/2}$ values for several different runs were plotted *vs.* the reciprocal of the inhibitor concentration.

The pH of the reaction was estimated by mixing Tris buffer and phosphate buffer in the same proportions as the enzyme and inhibitor solutions. A value of pH 7.1 was obtained when 4 ml of 0.10 M phosphate buffer (pH 7.0) was added to 1 ml of 0.05 M Tris-HCl–0.15 M NaCl (pH 8.0).

Results

Irreversible Inactivation of Mandelate Racemase by D,L-α-*Phenylglycidate.* The half-times for inactivation of mandelate racemase were measured for several different concentrations of D,L-α-phenylglycidate at 23° (pH 7.1) in the presence of 1.0

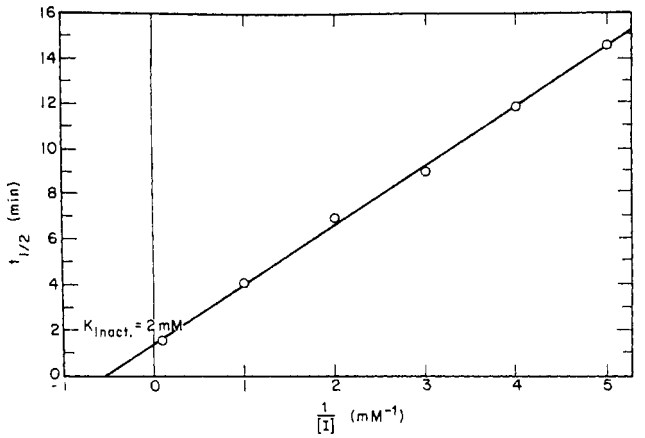

FIGURE 2: Inhibition of mandelate racemase ($t_{1/2}$ values *vs.* $[I]^{-1}$) by D,L-α-phenylglycidate in the presence of 1 mM Mg^{2+}.

mM Mg^{2+}. Figure 1 shows the curves obtained at five different inhibitor concentrations. Because the plots show some curvature at longer reaction times, the initial linear portions were extrapolated to 50% of original activity; those values were taken as $t_{1/2}$ so that $t_{1/2}$ would be proportional to the initial inhibition rate.

Enzyme and affinity label could form a dissociable complex at the active site prior to covalent bond formation and inactivation, in analogy to the formation of an enzyme–substrate complex during the normal enzyme-catalyzed reaction. This process can be represented by the equation (Meloche, 1967)

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_{inact} \xrightarrow{k_2} E_{inact} \qquad (1)$$

where E represents free enzyme, I is the inhibitor, C_{inact} is the enzyme–inhibitor complex, and E_{inact} is the inactivated enzyme. Using the steady-state assumption, Meloche (1967) has derived this rate expression for inactivation:

$$t_{1/2} = (1/[I])(T_{1/2}K_{inact}) + T_{1/2} \qquad (2)$$

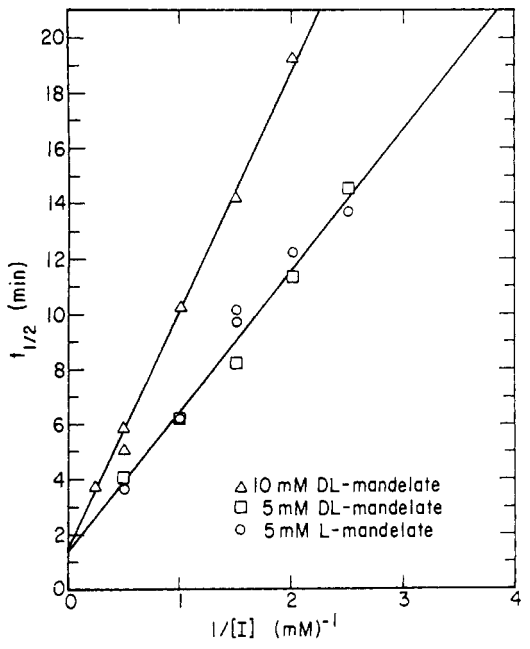

FIGURE 3: Inhibition of mandelate racemase ($t_{1/2}$ values *vs.* $[I]^{-1}$) by D,L-α-phenylglycidate in the presence of 1 mM Mg^{2+} and various concentrations of L- and D,L-mandelate.

TABLE I: Protection of Mandelate Racemase by Mandelate.

[Mandelate]	Slope (min mM)[a]	K_S (mM)[a]
5.0 mM DL 5.0 mM L	5.11	5.07
5.0 mM D	6.07	3.67
10.0 mM DL	17.64	4.12

[a] Determined from plots of inhibition of enzyme by DL-α-phenylglycidate ($t_{1/2}$ values *vs.* $[I]^{-1}$) using various concentrations of D-, DL-, or L-mandelate (see Figure 3).

where $t_{1/2}$ is the inactivation half-time at a given inhibitor concentration [I], $T_{1/2}$ is the minimum inactivation half-time at infinite inhibitor concentration, and K_{inact} is $(k_{-1} + k_2)/k_1$. K_{inact} is also the concentration of the inhibitor which gives the half-maximum saturation rate and therefore is similar to the Michaelis constant. If the inhibitor forms a complex with the enzyme prior to inactivation, a plot of $t_{1/2}$ *vs.* $[I]^{-1}$ should be linear and the value of $T_{1/2}$ should be greater than 0.

Values of $t_{1/2}$ for a series of inhibitor concentrations were averaged and plotted according to eq 2, and the plot is shown in Figure 2. The D,L-α-phenylglycidate apparently does form a complex with the enzyme since the plot has an intercept at a minimum inactivation half-time of 1.34 min. This value corresponds to a first-order rate constant k of 8.61×10^{-3} sec^{-1}. The value of K_{inact} determined from this plot is 1.92 mM.

Since it was of interest to compare K_{inact} to the Michaelis constant K_m, the apparent K_m for D-mandelate was determined at pH 7.0, 23°, in the presence of 1.0 mM Mg^{2+} with the same assay used for enzyme partially inhibited by α-phenylglycidate. A value of K_m = 5 mM was obtained. This K_m is different than that determined by Weil-Malherbe (1966), 0.15 mM. The assay systems used, however, were quite different for the two determinations. Weil-Malherbe used imidazole-HCl buffer, whereas the data here were determined in phosphate buffer. Since phosphate buffer inhibits the enzyme (Weil-Malherbe, 1966), it is not surprising that the K_m is different in phosphate buffer. In addition, Weil-Malherbe used a more complex coupled assay which depended on the presence of benzoylformate decarboxylase.

Substrate Protection against Inhibition by D,L-α-*Phenylglycidate.* The eq 1 and 2 shown above for irreversible inhibitor kinetics were expanded by Meloche (1967) to include the case where substrate S (or a competitive inhibitor of the usual enzymatic reaction) is present along with the irreversible inhibitor. The half-time of inactivation will then be a linear function of the reciprocal of the inhibitor concentration

$$t_{1/2} = (1/[I])\ T_{1/2}[K_{inact} + (K_{inact}[S]/K_S)] + T_{1/2} \qquad (3)$$

where K_S is the equilibrium constant for the dissociation of enzyme and substrate, S.

Mandelate racemase was treated with various concentrations of α-phenylglycidate in the presence of 1.0 mM Mg^{2+} and 2.0 and 10.0 mM DL-mandelate, 5.0 mM L-mandelate, and 5.0 mM D-mandelate. The plot shown in Figure 3 shows results of experiments with L- and DL-mandelate. The plot for results of experiments with D-mandelate is not shown.

The slopes of the plots and the values of K_S determined from the slopes are shown in Table I. It is interesting that the K_S values are close to the K_m value of 5 mM determined for D-mandelate. Thus, K_m apparently is a dissociation constant for the substrate and a subsequent step to binding is rate

limiting. That the slope of the plot for 5.0 mM D-mandelate is slightly greater than that of 5.0 mM L-mandelate and 5.0 mM DL-mandelate is probably not of significance. The K_S value of 3.7 mM is close to that determined for 10.0 mM DL-mandelate. That the substrate competed with α-phenylglycidate is shown by the fact that the plot (Figure 3) had the same y intercept as did the plot without mandelate (Figure 2).

Behavior of Mandelate Racemase with Glycidate. For 30 min at room temperature a solution containing 1.0 mM sodium glycidate in 0.10 M phosphate buffer (pH 7) was incubated with 1.7×10^{-5} mM mandelate racemase. At the end of this interval, the enzyme showed no measurable inhibition.

Effect of Added Mg^{2+} on the Rate of Irreversible Inhibition by D,L-α-Phenylglycidate. In an accompanying paper, we have shown (Fee *et al.*, 1974) that enzyme with divalent metal ions removed is completely inactive and that full activity can be restored by addition of divalent metal ion (*e.g.*, Mg^{2+}) to this inactive enzyme. In an attempt to deactivate enzyme with D,L-α-phenylglycidate after removal of metals, 25 μl of divalent metal-free enzyme solution (in 0.05 M Tris and 0.10 M NaCl) was incubated with 25 μl of 0.03 M NaCl and 100 μl of 0.10 M phosphate buffer (pH 7.0) for 10 min. Then 100 μl of 0.883 mM α-phenylglycidate in 0.10 M phosphate buffer (the α-phenylglycidate had not been treated with Chelex) was added and 10-μl aliquots were removed at 2-min intervals from 1 to 11 min after the addition. The aliquots were diluted into 2.72 ml of assay solution containing 1.0 mM Mg^{2+}. After 30 min the assay was begun with the addition of 0.250 ml of 0.10 mM D-mandelate and 25 μl of L-mandelate dehydrogenase suspension. In a control experiment the enzyme was first incubated with 25 μl of 0.010 *m* $MgCl_2$ and 100 μl of 0.10 M phosphate buffer. In the presence of Mg^{2+} the α-phenylglycidate caused a rapid loss of activity of the enzyme; $t_{1/2}$ for inactivation was 4.25 min (see Figure 4). The results show that after 2.58 half-lives (corresponding to *ca.* 80% activity loss in the presence of Mg^{2+}) no inactivation occurred in the absence of Mg^{2+}. In another similar experiment enzyme was treated with an excess of α-phenylglycidate in the presence of Mg^{2+} for several hours. Complete inhibition of catalytic activity was observed.

Binding of Radioactively Labeled α-Phenylglycidate to Mandelate Racemase. When D,L-α-phenylglycidate radioactively labeled with either ^3H or ^{14}C in the β-carbon methylene position was incubated with the enzyme in the presence of Mg^{2+}, radioactivity was incorporated into the protein. With β-[^{3}H]-D,L-α-phenylglycidate 0.98 mmol of inhibitor was incorporated per mmol of subunit and with β-[^{14}C]-D,L-α-phenylglycidate the value was 1.09 mmol per mmol of subunit.

After 12 days of storage at 4° in 0.05 M Tris-Cl, 0.10 M NaCl, and 0.010 M $MgCl_2$ (pH 8.0), the enzyme treated with β-[^{3}H]-α-phenylglycidate was passed through Sephadex G-25 again and the ^3H specific activity was redetermined. About 60% of the counts per minute was lost from the protein. Enzymatic activity was not restored, however. Moreover, when enzyme treated with β-[^{14}C]-α-phenylglycidate was carried through the same sequence, no radioactivity was lost.

Partial Removal of Affinity Label from Enzyme. Basic hydroxylamine solution was incubated with mandelate racemase which had been labeled with β-[^{14}C]-α-phenylglycidate. After 38 hr at 33° in 7 M urea–2 M NH_2OH (pH 9) about 20% of the label was lost. In a control experiment with NH_2OH deleted about 10% of the label was lost. In the latter case the low molecular weight fraction was collected from a Sephadex column, acidified, and extracted with ether. Unlabeled α-phenylglyceric acid (Craig and Henze, 1945) was added to the residue left from evaporation of the ether. The material was recrystallized twice from ethyl acetate–hexane and a third time from toluene to constant specific activity (9.22 cpm/mg).

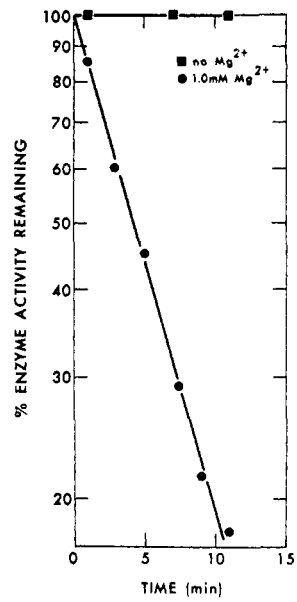

FIGURE 4: Time course (percent remaining enzyme activity *vs.* time) for inactivation of divalent metal-free mandelate racemase with D,L-α-phenylglycidate in the presence and absence of 1.0 mM Mg^{2+}.

Discussion

In order for a compound to be an active-site-directed irreversible inhibitor (affinity label) of an enzyme, it must satisfy several requirements (Meloche, 1967). It should inhibit the enzyme completely and irreversibly. In addition the inactivation should obey saturation kinetics. The normal substrate or a competitive inhibitor should protect the enzyme from the inactivation in a competitive manner. Finally, the binding of the inhibitor should be stoichiometric; *i.e.*, one inhibitor molecule should bind per active site. D,L-α-Phenylglycidate satisfies all of these requirements in its action upon mandelate racemase. Moreover, a comparison of the K_m for D-mandelate (5 mM) and K_{inact} for D,L-α-phenylglycidate (2 mM) indicates that mandelate racemase has approximately the same affinity for the substrate and the inhibitor. That the structural similarity of α-phenylglycidate to mandelate is important in this affinity of enzyme for inhibitor is shown by the failure of glycidate to inhibit the enzyme under conditions in which α-phenylglycidate rapidly inactivates it. Finally, there is apparently a parallel absolute metal ion requirement for both enzymatic catalysis of mandelate racemization and enzyme inactivation by the epoxide analog.

At high inhibitor to enzyme ratios the inactivation reaction should be first order (Meloche, 1967). It is evident in Figure 1 that there is some deviation from linearity in the inhibition of mandelate racemase by α-phenylglycidate. It is possible that the enzyme has different reactivities with and affinities for the D and L forms of the α-phenylglycidate, and that the less reactive enantiomer can protect against inhibition of the enzyme by the more reactive enantiomer. Attempts are currently underway to separate the D and L forms of α-phenylglycidate to investigate this hypothesis. Another possible explanation for this behavior is protection from inactivation by the diol α-phenylglycerate formed by nonenzymatic hydrolysis

of α-phenylglycidate. This latter seems unlikely since we have determined by nuclear magnetic resonance spectroscopy (J. A. Fee and G. L. Kenyon, unpublished results) that hydrolysis of this epoxide is far too slow in the phosphate buffer used in these experiments.

Another possible explanation for the deviation from pseudo-first-order kinetics is that initial reaction with the inhibitor may cause a conformational change in the enzyme which would slow reaction of the other subunits with the compound. In other words, the deviation may be due to negative cooperativity (Koshland, 1970). Negative cooperativity has been reported to occur in some reactions of enzymes with affinity labels. The most striking effect of this type has been the reported complete loss of enzymatic activity when only half of the active sites have been labeled by the affinity label; further incorporation of label does not occur (Levitski *et al.*, 1971). When the kinetics of inactivation have been measured for half-of-the-sites labeling, deviation from pseudo-first-order kinetics, similar to that found with mandelate racemase, has occurred in the latter part of the reaction. Examples are the labeling of phosphoribosyl pyrophosphate amidotransferase with 6-diazo-5-oxo-L-norleucine (Hartman, 1963), of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with β-(2-furyl)acryloyl phosphate at high NAD^+ concentrations (Malhotra and Bernhard, 1968) and of cytidine triphosphate synthetase with 6-diazo-5-oxonorleucine (Levitski *et al.*, 1971). Although measurement of the stoichiometry of binding shows that α-phenylglycidate binds to all, rather than half, the subunits of mandelate racemase, it seems plausible that negative cooperativity could occur in this reaction, just as negative cooperativity apparently explains the binding of NAD^+ to each of the subunits of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland, 1968). The existence of more than one reactive conformation may in fact explain the deviation from pseudo-first-order kinetics observed in the labeling of 2-keto-3-deoxy-6-phosphogluconic aldolase by bromopyruvate, which reacts at both active sites (Meloche, 1967). The existence of two different active-site conformers has been postulated to explain the attachment of the label either to a carboxylate or to a sulfhydryl group (Meloche, 1970).

The affinity-labeling process is greatly aided by the addition of Mg^{2+}. This result is consistent with the discovery of an absolute divalent metal ion requirement for normal enzymatic activity (Fee *et al.*, 1974). A metal bridge coordination scheme is attractive in explaining this Mg^{2+} effect. By binding near to α-phenylglycidate, metal ion could aid in polarizing the epoxide ring, thereby assisting attack of an amino acid side chain moiety of the enzyme.

Lewis acids, including Mg^{2+}, are known to catalyze reactions of epoxides (Hartman and Rickborn, 1972; House and Reif, 1957). The inactivation of enzyme by α-phenylglycidate probably does not occur in the complete absence of divalent metal.

There is uncertainty as to the identity of the amino acid residue or residues of the enzyme which bond covalently to the α-phenylglycidate. In previous cases where epoxide-containing affinity labels have been employed the carboxylate group of either an aspartic acid or glutamic acid residue has been most commonly implicated (Hartsuck and Tang, 1972; Moult *et al.*, 1973; Legler, 1968; Legler and Hasnain, 1970; Schray *et al.*, 1973; O'Connell and Rose, 1973). If an ester linkage from a carboxylate on mandelate racemase were formed upon affinity labeling, then hydrolysis should have released the diol α-phenylglyceric acid from the enzyme. In fact a portion of the radioactive product was isolated as [^{14}C]-α-phenylglyceric acid. The yield was low, however, since after hydrolysis at pH 9 in 7 M urea for 38 hr at 33° only about 10% of the ^{14}C label was lost from the enzyme. Even after 24 hr in 7 M urea in 0.1 M NaOH at 25°, only about 30% of the ^{14}C label was lost. The fact that added hydroxylamine accelerated the rate of loss of the ^{14}C label is also consistent with the idea that an ester linkage is involved in the covalent attachment. Efforts are being made to determine more conclusively the group or groups on mandelate racemase modified by α-phenylglycidate.

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