by Thorneley & Lowe (1983), $MoFe_{red}$–$Fe_{ox}(MgADP)_2$, purportedly resulting from the transfer of an electron from $Fe_{red}(MgATP)_2$ to MoFe. Thorneley & Lowe (1983) suggest that the dissociation of $Fe_{ox}(MgADP)_2$ from the latter complex is rate limiting during nitrogenase turnover with an off rate constant of 6.4 s^{-1}. The weak binding of $Fe_{ox}(MgADP)_2$ to MoFe reported here bears some similarity to that of the Thorneley and Lowe complex even though in our complex the MoFe protein is one electron more oxidized compared to that in their complex.

Registry No. LiCl, 7447-41-8; NaCl, 7647-14-5; KCl, 7447-40-7; NaI, 7681-82-5; NaBr, 7647-15-6; Na_2HPO_4, 7558-79-4; Na_2SO_3, 7757-83-7; nitrogenase, 9013-04-1.

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Symmetry and Asymmetry in Mandelate Racemase Catalysis†

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ABSTRACT: Kinetic properties of mandelate racemase catalysis (V_{max}, K_m, deuterium isotope effects, and pH profiles) were all measured in both directions by the circular dichroic assay of Sharp et al. [Sharp, T. R., Hegeman, G. D., & Kenyon, G. L. (1979) *Anal. Biochem. 94*, 329]. These results, along with those of studying interactions of mandelate racemase with resolved, enantiomeric competitive inhibitors [(*R*)- and (*S*)-α-phenylglycerates], indicate a high degree of symmetry in both binding and catalysis. Racemization of either enantiomer of mandelate in D_2O did not show an overshoot region of molecular ellipticity in circular dichroic measurements upon approach to equilibrium. Both the absence of such an overshoot region and the high degree of kinetic symmetry are consistent with a one-base acceptor mechanism for mandelate racemase. On the other hand, results of irreversible inhibition with partially resolved, enantiomeric affinity labels [(*R*)- and (*S*)-α-phenylglycidates] reveal a "functional asymmetry" at the active site. Mechanistic proposals, consistent with these results, are presented.

Stereospecificity of enzymic catalysis is a key concept in enzymology; that is, except in the case of the interconversion of stereoisomers, enzymes generally process only one enantiomer. Such stereospecificity is not at all surprising since enzymes are proteins composed only (*S*)-amino acids and therefore are inherently asymmetric. Racemases, because of their ability to bind and catalyze either of two enantiomers, present a paradox: How are the groups at the active site so arranged as to catalyze a symmetrical reaction within an inherently asymmetrical environment?

Thus far, there are only limited experimental data for racemases to help answer this question. Although some researchers have reported the K_m and V_{max} values in both directions for racemases, the question of enantiomeric catalysis and binding has been explored in detail only for alanine racemase. Thus, Wang & Walsh (1978, 1981) systematically probed the active site of alanine racemase by using the *R* and *S* enantiomers of both β-chloro- and β-fluoroalanines as so-

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called suicide inhibitors. As a result, the differential tolerance of the active site for these suicide substrates revealed, in their words, the presence of a "functional asymmetry" not apparent in the processing of the natural substrate. In-depth studies of the kinetic properties of other racemases, similar to those of Wang and Walsh, are warranted to provide a clearer understanding of the enzymatic mechanisms involved in the binding and catalysis of enantiomers.

Mandelate racemase (EC 5.1.2.2) catalyzes the racemization of either (*R*)- or (*S*)-mandelate, presumably via a carbanion intermediate (Kenyon & Hegeman, (1979). Because it requires only a divalent cation (e.g., Mg^{2+} or Mn^{2+}) as a cofactor and catalyzes such a simple reaction, it is an attractive enzyme for such studies.

Using the recently developed circular dichroic assay for monitoring mandelate racemase activity (Sharp et al., 1979), we here report initial velocity studies, deuterium isotope effects, and pH profiles all measured in both directions. In addition, we report the results of thehe incubation of the enzyme with the resolved, reversible competitive inhibitors (*R*)- and (*S*)-α-phenylglycerates, as well as the partially resolved, irreversible inhibitors (*R*)- and (*S*)-α-phenylglycidates.

Experimental Procedures

Materials

The following compounds were synthesized and resolved according to the published procedures: (*RS*)-[α-^{2}H]mandelic acid (Sharp et al., 1977), resolution (Manske & Johnson, 1929); (*RS*)-α-phenylglycidate (Fee et al., 1974), resolution and assignment of configuration (Whitman et al., 1985); (*RS*)-α-phenylglycerate (Whitman et al., 1985), resolution and assignment of configuration (Whitman et al., 1985). It was not possible with the methods used to achieve better resolution of the (*R*)- and (*S*)-α-phenylglycidates than 78% pure *R*-(+) isomer and 67% pure *S*-(−) isomer, each enantiomer being contaminated by the other.

(*R*)- and (*S*)-mandelic acids were purchased from Aldrich Chemical Co. All buffers and biochemical reagents were purchased from Sigma Chemical Co. Solutions were made in distilled and deionized water. All other inorganic salts used in the assay and for the formulation of the Hutner's mineral base were purchased from either Aldrich Chemical Co. or Mallinckrodt.

All inhibitor solutions were prepared fresh daily. The potassium α-phenylglycidate was stored desiccated at −20 °C to minimize decomposition.

Methods

Enzyme Preparations. Mandelate racemase was prepared according to the method of Hegeman (1966, 1970) from *Pseudomonas putida* (A.3.12) (ATCC 12633) except that the heat-treatment step was eliminated. Cultures of this organism were grown on Hutner's mineral medium with ammonium (*RS*)-mandelate as the sole carbon and energy source. In order to achieve affinity labeling results consistent with those of Fee et al. (1974), the enzyme preparation was used after fractionation on a Sephadex G-200 column. It was noticed that later fractions eluting from the DEAE Sephadex A-50 column were not so susceptible to the affinity labeling process as the earlier fractions, and so the earlier fractions were used exclusively in these studies. (*S*)-(+)-Mandelate dehydrogenase was prepared according to the method of Hegeman (1966, 1970) from *Pseudomonas aeruginosa* (ATCC 15692).

Protein determinations were made by the Bio-Rad protein assay with crystalline bovine serum albumin containing 97.5% dry bovine serum albumin by weight. Enzyme solutions were concentrated with Lyphogel. Enzymic purity was determined to be greater than 75% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Measurement of Enzymatic Activity. All assays were performed at 25 °C. (*S*)-(+)-Mandelate dehydrogenase activity was measured as described by Hegeman (1966). Mandelate racemase activity was monitored by two assays, a coupled assay and a CD[1] assay. The coupled assay of Hegeman (1970) was used for measurement of activity of enzyme fractions during the various stages of its purification, reversible inhibition, and irreversible inhibition. Measurements using this assay can only be made in *R* to *S* direction. The following modifications were made in the coupled assay procedure. For the reversible inhibitor experiments, Hepes buffer, potassium mandelate, potassium α-phenylglycerate, DCPIP, KCN, $MgCl_2$, and (*S*)-(+)-mandelate dehydrogenase containing vesicles were added to a sample cuvette. The MDH-containing vesicles, derived by the re-formation of the ruptured cell membranes, catalyze a cytochrome-mediated reoxidation of DCPIP. Addition of the KCN corrects this problem. A reference cuvette was also made up containing all of the above ingredients except the substrate and inhibitor. The two cuvettes were then incubated at room temperature for 70 min in order to eliminate a nonenzymatic rate that is due to several factors as discussed by Sharp et al. (1979). The assay was then initiated by the addition of enzyme.

For the irreversible inhibition experiments, a stock solution of Hepes buffer, KCN, DCPIP, and $MgCl_2$ was made up daily. Sufficient quantities of this stock solution were pipetted into a sample and reference cell to make to total volume of 2.4 mL in each cell after addition of a saturating quantity of potassium mandelate to the sample cell. Then, 0.6 mL of the (*S*-(+)-mandelate dehydrogenase containing vesicles was added to each cell, followed by a 40-min incubation period. The protocol of Fee et al. (1974) prescribed a 30-min incubation period, making a total incubation period of 70 min. The assay was initiated by the addition of a saturating quantity of (*R*)-(−)-mandelate to the sample cuvette.

The CD assay of Sharp et al. (1979) was used to measure the Michaelis–Menten parameters (V_{max} and K_M), the deuterium isotope effects, and the pH profiles. All measurements were made in both directions (*R* to *S* and *S* to *R*) on a Jasco CD spectropolarimeter monitoring changes in ellipticity at 262 nm. Typically, the solutions were made up in disposable test tubes and transferred to the appropriate CD cell. To each test tube was added an appropriate amount of substrate with a sufficient amount of buffer to make a final volume of 3 mL. The solutions were all made 1.0 mM in $MgCl_2$. Enzyme was added to the cells and mixed in by the inversion method.

Deuterium Isotope Effects. Determinations of the V/K_M and V_{max} isotope effects were made from the reciprocal plots generated from initial velocity studies using either (*R*)- or (*S*)-mandelate labeled in the α position with either hydrogen or deuterium. The ratio of the intercepts yielded the V_{max} effect while the ratio of the slopes yielded the V/K_M effect.

pH Studies. Determination of the *V* and *V*/*K* for the (*R*)- and (*S*)-mandelates as a function of pH was made by performing initial velocity studies at the indicated pH. Buffers

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; DCPIP, 2,6-dichlorophenolindophenol; MDH, (*S*)-(+)-mandelate dehydrogenase containing vesicles; CD, circular dichroic.

(100 mM made 1.0 mM in $MgCl_2$) were used over the following pH ranges: Mes, 6.0–6.5; Pipes, 6.5–7.5; Hepes, 7.5–7.8; Taps, 7.8–9.0; Ches, 9.0–9.5; Caps, 9.5–10.0. All buffers were adjusted to their respective pHs with KOH. In all cases, sufficient overlaps were obtained between buffers either to rule out entirely or to permit minor corrections for buffer effects. In order to ensure metal ion saturation of the enzyme as a function of pH, the concentration was increased 10-fold at the pH extremes. This resulted in no significant changes in the pH profiles.

Competitive Inhibition Studies. An aqueous solution of either (*R*)-(+)- or (*S*)-(−)-α-phenylglycerate was adjusted to pH 7.5 with KOH on the day of the experiment. The solution was kept on ice. Initial velocity studies were performed at four different concentrations of inhibitor (0, 2.0, 3.5, and 5.0 mM).

Kinetics of Irreversible Inhibition. The irreversible kinetic experiments were performed according to the protocol of Fee et al. (1974) with the following modifications. K^+-Hepes buffer at pH 7.5 was used in place of the phosphate buffer. The assay was initiated by the addition of substrate. Irreversible inhibitor concentrations were corrected for optical purity.

Kinetics of (S)-(+)-Mandelate in H_2O and D_2O. To two 2-mL volumetric flasks were added 12 μmol of (*S*)-(+)-mandelate, 177 μmol of Hepes buffer, and 2 μmol of $MgCl_2$. The contents of each flask were dissolved in 2 mL of either H_2O or D_2O. The solutions were than taken to dryness in vacuo. This process was repeated 3 further times in order to ensure complete exchange of all labile protons. The total volumes were made up to 2 mL by addition of either H_2O or D_2O. The final pH was adjusted to pH 7.0 with either NaOH or NaOD. Racemase solution in H_2O [0.1 mL containing 60 μg of protein, 3.5 units mL^{-1} (mg of protein)$^{-1}$] was added, making the final D_2O solution 95% D_2O. The contents of each were immediately transferred to a 1-mm CD capped cell. The change in ellipticity was monitored at 227 nm on a Roussel-Jouan Mark II dichrograph. The chart speed was set at 9 mm/min. The sensitivity was set at 1×10^{-5} optical density unit/mm.

Equilibrium Perturbation Experiment. The experimental procedure employed the "two-pot" method as described by Cleland (1980). To one 10-mL volumetric flask were added 14.5 μmol of (*R*)-(−)-mandelate, 506 μmol of Hepes buffer, and 11 μmol of $MgCl_2$ while to another 10-mL volumetric flask were added 17 μmol of (*S*)-(+)-[α-^{2}H]mandelate, 507 μmol of Hepes buffer, and 9.8 μmol of $MgCl_2$. The contents were dissolved in 10 mL of H_2O, and the pH was adjusted to 7.0 with KOH. A mixture of 3.26 mL of the (*R*)-(−)-mandelate solution and 2.54 mL of the (*S*)-(+)-[α-^{2}H]-mandelate solution resulted in a null at 265 nm in the CD spectrometer indicative of the presence of an equilibrium mixture. Racemase solution [0.2 mL containing 44 μg of protein, 0.7 unit mL^{-1} (mg of protein)$^{-1}$] was added to the mixture to make a final volume of 6 mL. The perturbation was monitored at 265 nm in a 10-cm cell with the chart speed set at 0.1 mm/min and the sensitivity set at 1.0 millideg/cm. A solution of (*R*)-(−)-mandelate used in this experiment produced a molar ellipticity of +513 deg mol^{-1} cm^2 at 265 nm while a solution of (*S*)-(+)-[α-^{2}H]mandelate used in this experiment produced a molar ellipticity of −573 deg mol^{-1} cm^2 at 265 nm.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. The data were fitted to the appropriate equation with the Fortran programs of Cleland (1979). All initial velocity studies, including the individual saturation curves used to obtain pH profiles, were fitted to eq 1, where A is the substrate concentration.

$$v = VA/(K + A) \quad (1)$$

Data for the competitive inhibitor studies were fitted to eq 2, where K_{is} is the inhibition constant and I is the concentration of inhibitor.

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (2)$$

Data for pH profiles that showed a decrease in log (V/K) at both low and high pHs were fitted to eq 3, where H is $[H^+]$

$$\log y = \log [c/(1 + H/K_1 + K_2/H)] \quad (3)$$

and c is the pH-independent value of the parameter y. The parameters K_1 and K_2 represent the dissociation constants for groups on the enzyme.

Because the deuterium isotope effects appeared to be equal on V and V/K, the data were fitted to eq 4, where F_1 is the fraction of deuterium label and VI is the isotope effect minus 1.

$$y = \frac{VA}{(K + A)(1 + F_iVI)} \quad (4)$$

The data obtained from the irreversible inhibition experiments were fitted to the best straight line by linear regression analysis. The isotope effect from the equilibrium perturbation experiment was calculated from eq 26 and Appendix C in Cleland (1977a).

Results

Comparison of the Kinetic Constants. The initial velocity plots for mandelate racemase were determined by the circular dichroic assay in both the R to S and S to R directions. The K_M for (*R*)-mandelate was 0.23 ± 0.02 mM, and the K_M for (*S*)-mandelate was 0.26 ± 0.03 mM. The $V_{max\,R\to S}$ was 0.75 ± 0.02 μmol min^{-1} mL^{-1}, and $V_{max\,S\to R}$ was 0.83 ± 0.03 μmol min^{-1} mL^{-1} under identical conditions at pH 7.5. For the chemically symmetrical reaction (*R*)-mandelate ⇌ (*S*)-mandelate, the equilibrium constant must be unity, and this has been validated with other racemases (Adams, 1976). The Haldane relationship allows one to compute a K_{eq} value from the experimentally derived V/K values (Briggs & Haldane, 1925):

$$K_{eq} = \frac{(V_{max}/K_M)_{(R)\text{-mandelate}}}{(V_{max}/K_M)_{(S)\text{-mandelate}}} = 0.99 \pm 0.09 \quad (5)$$

Deuterium Isotope Effects. The deuterium isotope effects were measured by reciprocal plots as determined by the CD assay in the R to S and S to R directions. The measured isotope effect in the R to S direction was 3.20 ± 0.11 while it was 3.56 ± 0.12 in the S to R direction. The V_{max} and V/K_M isotope effects were found to be equal.

pH Profiles. By use of the CD assay, pH profiles were generated in both directions for mandelate racemase. As shown in Figure 1A, the $V/K_{(R)\text{-mandelate}}$ profile decreases at high pH with a pK_a of 7.8 ± 0.2. The $V_{R\to S}$ profile does not vary over the pH range 5.5–10.0. Likewise, as shown in Figure 1B, $V/K_{(S)\text{-mandelate}}$ decreases at high pH with a pK_a of 7.6 ± 0.2. Again, there was no variation in the $V_{S\to R}$ profile. In both directions the V/K profiles begin to break at low pH, suggesting a second pK_a value. Without additional data points at the lower pH values, it is not possible to assign a pK_a value. The enzyme is unstable below pH 5.5.

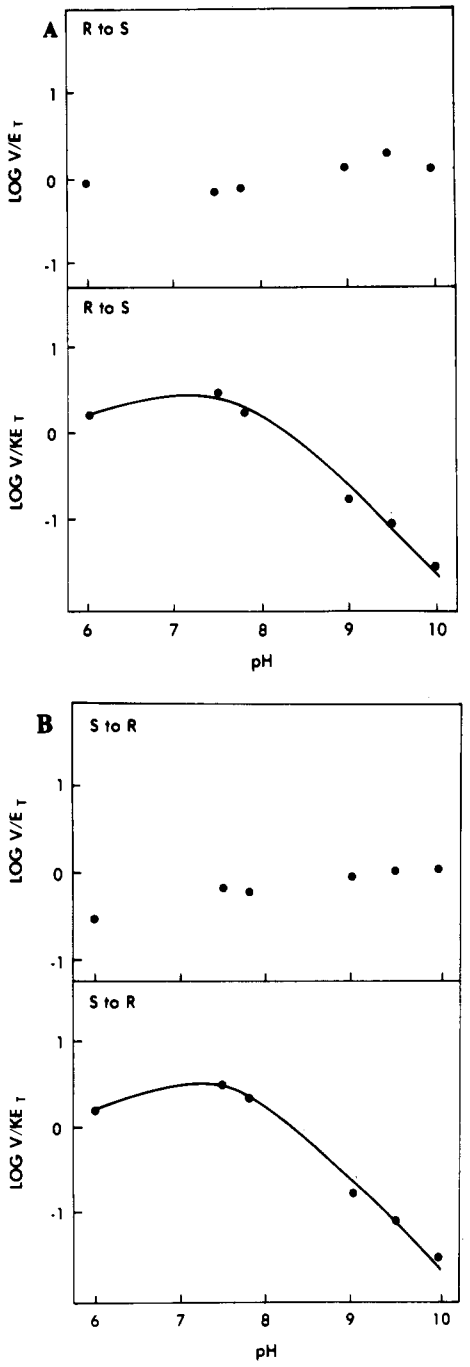

FIGURE 1: (A) pH dependence of $V/K_{(R)\text{-mandelate}}$ and $V_{(R)\text{-mandelate}}$ for mandelate racemase in the $R \rightarrow S$ direction. (B) The same pH dependence for the kinetic parameters of (*S*)-mandelate in the $S \rightarrow R$ direction.

Competitive Inhibition Studies. The racemic mixture of (*RS*)-α-phenylglycerate was resolved, and the separate enantiomers were tested for competitive inhibition of the enzyme. The compounds were judged to be of equal optical purity as determined by their equal and opposite $[\alpha]_D$ values. With the spectrophotometric assay, strictly competitive inhibitions were shown for both enantiomers. The K_I for (*R*)-(+)-α-phenylglycerate was determined to be 1.25 ± 0.12 mM while the K_I value for (*S*)-(−)-α-phenylglycerate was 1.29 ± 0.11 mM.

Irreversible Inhibition Kinetics. The half-times for inactivation of mandelate racemase were measured for several different concentrations of (*R*)-(+)- and (*S*)-(−)-α-phenylglycidates at 25 °C (pH 7.5) in the presence of 1.0 mM $MgCl_2$. The formation of a dissociable complex between enzyme and affinity label at the active site prior to covalent

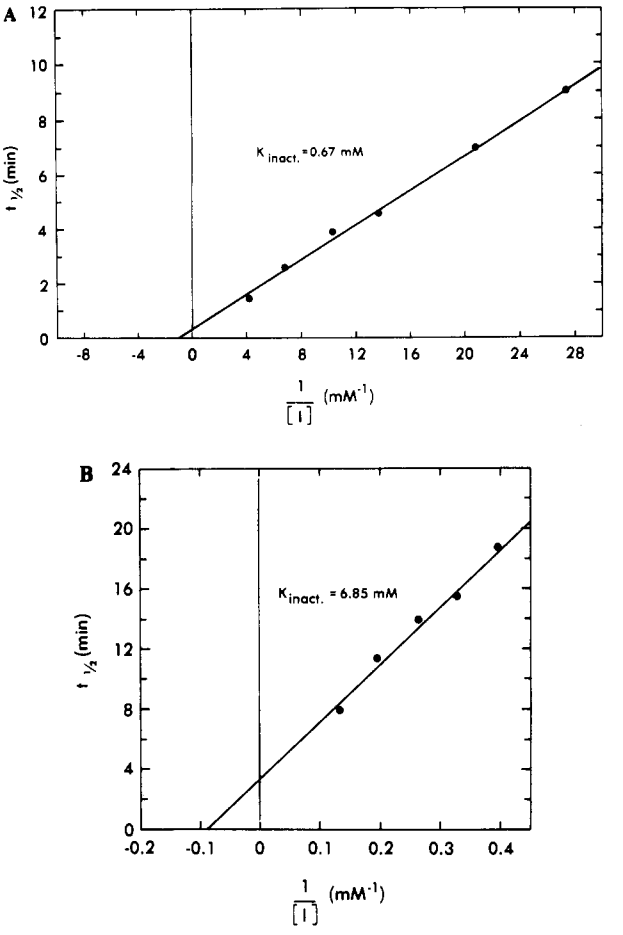

FIGURE 2: (A) Inhibition of mandelate racemase ($t_{1/2}$ values vs. $[I]^{-1}$) by partially resolved (*R*)-(+)-α-phenylglycidate. (B) Inhibition of mandelate racemase ($t_{1/2}$ values vs. $[I]^{-1}$) by partially resolved (*S*)-(−)-α-phenylglycidate.

bond formation—analogous to the formation of the enzyme–substrate complex in Michaelis–Menten kinetics—has testable kinetic consequences. This process and its consequences were discussed in some detail by Fee et al. (1974).

The $t_{1/2}$ values for (*R*)-(+)- and (*S*)-(−)-α-phenylglycidates were plotted against a series of inhibitor concentrations, according to the equations derived by Meloche (1967), in parts A and B of Figure 2, respectively. Both (*R*)-(+)- and (*S*)-(−)-α-phenylglycidates form complexes with the enzyme as indicated by the finite vertical intercepts (i.e., $T_{1/2} > 0$). For (*R*)-(+)-α-phenylglycidate, $T_{1/2}$ = 0.5 min whereas it is 4.8 min for (*S*)-(−)-α-phenylglycidate— a 9.6-fold difference. These values correspond to a first-order rate constant *k* of 2.31 × 10^{-2} s^{-1} for (*R*)-(+)-α-phenylglycidate and *k* = 2.41 × 10^{-3} s^{-1} for (*S*)-(−)-α-phenylglycidate. The value for K_{inact} for (*R*)-(+)-α-phenylglycidate was 0.67 mM whereas it was 6.85 mM for (*S*)-(−)-α-phenylglycidate— a 10.2-fold difference.

Kinetics of the Racemization Reaction in D_2O and H_2O. In Figure 3 the course of the reaction as monitored by the change in ellipticity at 227 nm is shown for (*S*)-(+)-mandelate both in H_2O and in D_2O. In contrast to the results of Cardinale & Abeles (1968) for proline racemase and Finlay & Adams (1970) for hydroxyproline epimerase, it is seen that the approach to equilibrium in D_2O is asymptotic for mandelate racemase. That is, it is not characterized by an overshoot region followed by a return to equilibrium as was the case for these other two enzymes.

Equilibrium Perturbation. In Figure 4 it is seen that upon addition of enzyme to an equimolar mixture of (*S*)-(+)-[α-^{2}H]mandelate and (*R*)-(−)-mandelate there is an equilibrium

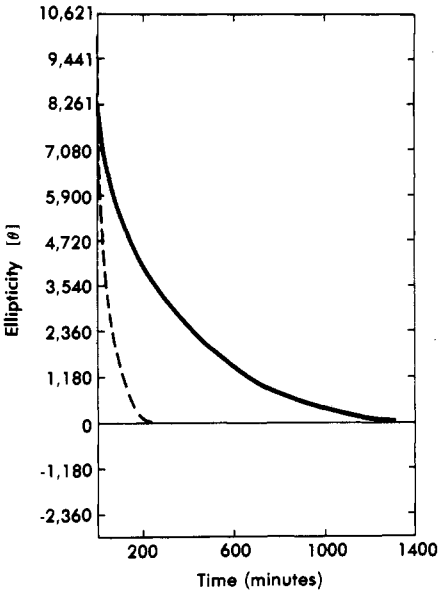

FIGURE 3: Racemization (ellipticity vs. time) of (*S*)-(+)-mandelate in H_2O (--) and D_2O (—). Measurements were made at 227 nm and 25 °C by circular dichroism as described under Methods.

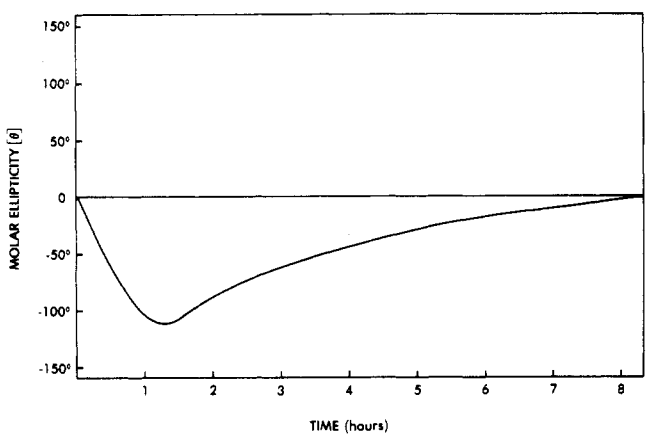

FIGURE 4: Equilibrium perturbation (ellipticity vs. time) with mandelate racemase added to an equimolar mixture of (*S*)-(+)-[α-^{2}H]mandelate and (*R*)-(−)-mandelate. Measurements were made at 265 nm and 25 °C by circular dichroism as described under Methods.

perturbation followed by an asymptotic return to equilibrium. The perturbation was measured by monitoring the change in ellipticity at 265 nm in the CD. The size of the perturbation, corresponding to the production of 0.33 mM of (*S*)-(+)-[α-^{2}H]mandelate, is halved due to the concomitant depletion of the (*R*)-(−)-mandelate. Using the resulting value of 0.17 mM in eq 26 and Appendix C of Cleland (1977a), one is able to calculate an isotope effect of 3.6, a result in good agreement with the value calculated from the comparison method (see above). This experiment lends added significance to the results shown in Figure 3 (see Discussion).

Two additional controls were performed in order to ensure that the equilibrium perturbation experiment itself was not an artifact. First, the same experiment was carried out without enzyme in order to observe the stability of the base line. There was no drift. Second, the same experiment was run with nonisotopically labeled mandelate. No perturbation was observed in this experiment.

Discussion

Kinetic Parameters and Symmetry in Mandelate Racemase Catalysis. The results of the initial velocity studies, the competitive inhibition studies, the deuterium isotope effect experiments, and the pH studies all suggest that the active site of mandelate racemase, although inherently asymmetric, appears both to bind and to process the enantiomeric substrates and reversible inhibitors with a remarkable symmetry. The enzyme binds either (*R*)- or (*S*)-mandelate equally; it catalyzes the rates of reaction equally; it binds either (*R*)- or (*S*)-α-phenylglycerate with equal affinity; and finally, groups with either the same or very similar pK_a values groups appear to be involved in the binding of the substrate. These results are consistent with previous data suggesting a one-base rather than a two-base acceptor mechanism for mandelate racemase catalysis as discussed by Kenyon & Hegeman (1979).

This kinetic symmetry displayed by mandelate racemase goes beyond the mandates of the Haldane relationship, which states only that V/K values of the racemase reaction must equal one another. That is, it is possible for V_{max} to be higher in one direction, but then, K_m must also be increased proportionally. For mandelate racemase, V_{max} and K_m values are virtually the same in both directions, a result that had been inferred from earlier kinetic measurements of Maggio et al. (1975).

The large deuterium isotope effects of ca. 3.5 in both directions indicate that carbon–hydrogen bond breaking is at least partially rate limiting in the reaction. Intrinsic kinetic deuterium isotope effects are often in the 6–8 range, so there may well be partially rate-limiting internal commitments to catalysis preceding the bond-breaking steps in both directions.

The absence of measureable changes in the V_{max} profile and the presence of pK_a values in the V/K profiles indicate the involvement of a protonated group on the enzyme for maximal binding and catalysis. The pK_a values of amino acid residues can be greatly perturbed from those observed in solution, and so no firm assignments of these groups can be made at this time.

Affinity Labeling by (R)- and (S)-α-Phenylglycidate and "Functional Asymmetry" of Mandelate Racemase. The differential tolerance of mandelate racemase for the affinity labels potassium (*R*)- and potassium (*S*)-α-phenylglycidates implies as asymmetric active site for binding and processing of the enantiomers. Thus, these affinity labeling results parallel those found by Wang & Walsh (1978) for alanine racemase.

Although it is apparent that the (*R*)-(+)-α-phenylglycidate is the more potent affinity label of mandelate racemase, it is possible that this isomer, once optically pure, could be even more potent as an irreversible inhibitor, while the *S*-(−) isomer, once optically pure, may be essentially inactive. That is, the inhibitory activity observed in the studies using the *S*-(−) isomer may be due entirely to the presence of contaminating *R*-(+) isomer, with the *S*-(−) isomer acting to protect mandelate racemase from irreversible inhibition by the *R*-(+) isomer. At the concentrations and optical purities of the *S*-(−) isomer used in these experiments, there was a 10-fold greater concentration of *R*-(+) isomer present than is normally necessary to inhibit irreversibly the enzyme. Fee et al. (1974) used a racemic mixture of α-phenylglycidate to inhibit mandelate racemase. They proposed that the less reactive enantiomer of α-phenylglycidate could be protecting against inhibition by the other enantiomer, resulting in pronounced curvature in their percent original activity vs. time plots. Similar plots with the partially resolved inhibitors (not shown) were linear, presumably because the enantiomeric purities of the inhibitors were substantially greater. Finally, the results of the competitive inhibition studies, in conjunction with the earlier NMR studies of Maggio et al. (1975), strongly rein-

force the idea that one enantiomer of α-phenylglycidate can protect against irreversible inhibition by the other. The NMR studies of Maggio et al. (1975) indicated that (*RS*)-α-phenylglycerate was able to form a ternary complex with the enzyme and Mn^{2+}. The kinetic studies presented here (see above) demonstrate that the resolved enantiomers of α-phenylglycerate are competitive inhibitors with essentially identical K_I values. The similarities in structure of the α-phenylglycerates and α-phenylglycidates enable one to infer that the *S*-(−) isomer of α-phenylglycidate may also be able to form a Michaelis complex with the enzyme and, in a competitive manner, thereby protect against irreversible inhibition by the *R*-(+) isomer. To test this hypothesis, (*R*)- and (*S*)-α-phenylglycidates of even greater optical purity must be obtained.

Racemization in D_2O. The results of the racemization experiment (Figure 3) provide strong confirmatory evidence in favor of a one-base rather than a two-base acceptor mechanism. By circular dichroic measurements it was shown that there is no overshoot in the racemization of either (*R*)- or (*S*)-mandelate in D_2O. Such overshoots were observed with both proline racemase (Cardinale & Abeles, 1968) and hydroxyproline epimerase (Finlay & Adams, 1973), both enzymes thought to function by a two-base acceptor mechanism.

The lack of overshoot in this experiment could also be consistent with a two-base mechanism accompanied by very rapid isotopic exchange of the proton α to the carboxylate. However, earlier experiments with mandelate racemase (Kenyon & Hegeman, 1970; Sharp et al., 1977) indicate that such isotopic exchange is not particularly rapid, and only partial washout of label accompanies racemization. In one of these experiments (Kenyon & Hegeman, 1970) unlabeled (*R*)-mandelate was subjected to partial enzymatic racemization in the presence of tritiated water. Both (*R*)- and (*S*)-mandelates were then separately analyzed for specific radioactivity. Approximately the same amount of tritium was incorporated into each enantiomer. This experiment provides supporting evidence for the one-base mechanism.

The equilibrium perturbation experiment with mandelate racemase (Figure 4) demonstrates that observation of an overshoot region is indeed possible under appropriate experimental conditions. Cleland (1977b) has shown that the overshoot region seen in the course of racemization of proline by proline racemase and in the course of an equilibrium perturbation experiment results from the same phenomenon, i.e., the accumulation of one enantiomer that is isotopically labeled, due to an isotope effect. However, the origin of the isotopically labeled material is different for the two differnet types of experiments. In the racemization experiment, the enzyme synthesizes the isotopically labeled enantiomer as an inherent feature of its mechanism whereas in the equilibrium perturbation experiment the isotopically labeled enantiomer is added to the reaction mixture by the experimenter. Because of this difference, one can use the equilibrium perturbation experiment as a control for the racemization experiment. If one does not observe an overshoot region upon enzymic racemization in D_2O but does observe the equilibrium perturbation, then one is assured that the racemization experiment is valid, even if no overshoot region is observed, as is the case here with mandelate racemase. That is, if such an overshoot were possible as a result of an inherent feature of the mechanism, one should have been able to observe an overshoot region in the racemization experiment.

Symmetry and Asymmetry in a One-Base Acceptor Mechanism. From the above results, a mechanistic scheme for mandelate racemase must acccount for a high degree of symmetry, some asymmetry, and a one-base acceptor mechanism. Previous experments with (*RS*)-α-phenylglycidate (Fee et al., 1974) had suggested that a carboxylate group of either a glutamate or an aspartate residue was the group attacking the epoxide ring of the affinity label. Namely, the diol α-phenylglycerate was released upon basic treatment of affinity-labeled enzyme. This putative carboxylate group may also be involved in the abstraction of the α-proton of mandelate in the normal enzymic mechanism. In addition, the required protonated group (general acid catalyst) reflected in the pH study may be involved in binding of either the α-hydroxyl group or carboxyl group of mandelate through hydrogen bonding. Kenyon & Hegeman (1979) had postulated the placement of the phenyl group of mandelate in a hydrophobic pocket and the carboxyl group of mandelate tethered, either through a water molecule or directly, as a ligand to the required divalent metal ion. Such an involvement of the required divalent metal ion emerged from magnetic resonance and kinetic studies of Maggio et al. (1975) on the Mn^{2+}-mandelate–enzyme ternary complex.

When the racemase binds (*R*)- and (*S*)-mandelates and (*R*)- and (*S*)-α-phenylglycerates, a pseudosymmetry must prevail since the enzyme's active site is inherently asymmetric. The enzyme may attenuate any differential binding of these enantiomeric pairs by utilizing flexibility of the side-chain residues involved.

A degree of active site asymmetry is revealed in the experiments using the enantiomeric affinity labels of mandelate racemase. The structural similarities between the α-phenylglycidate molecule and the mandelate molecule suggest that the two molecules may employ similar modes of binding at the active site. This could place the oxygen of the epoxide moiety in the vicinity of the required protonated group on the enzyme. It has been postulated for several enzymes labeled by epoxides that an acid catalyst is required in the vicinity of the epoxide oxygen to assist the nucleophilic ring opening by a base on the enzyme (Cassidy & Kahan, 1974; Rose & O'Connell, 1969; Schray et al., 1973). The constraint of the epoxide oxygen confines the oxygen of the *R* and *S* isomers to different regions of the active site. The (*R*)-(+)-epoxide is placed, evidently, within the vicinity of a general acid catalyst to enhance ring opening and labeling of the enzyme, while the (*S*)-(−)-epoxide apparently is not.

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Bacterial Luciferase: Demonstration of a Catalytically Competent Altered Conformational State following a Single Turnover†

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ABSTRACT: Ziegler-Nicoli et al. [Ziegler-Nicoli, M., Meighen, E. A., & Hastings, J. W. (1974) *J. Biol. Chem. 249*, 2385-2392] reported that a highly reactive cysteinyl residue on the α subunit of bacterial luciferase resides in or near the flavin binding site such that the enzyme–flavin complex is protected from inactivation by alkylating reagents. These authors also observed that injection of reduced flavin mononucleotide ($FMNH_2$) into an air-equilibrated solution of enzyme protected the enzyme from alkylation for much longer than the lifetime of the 4a-peroxydihydroflavin intermediate resulting from reaction of enzyme-bound $FMNH_2$ with O_2. Two related explanations were offered: either (1) the product flavin mononucleotide dissociated from the enzyme much more slowly following a catalytic cycle than would be predicted from the K_d measured by equilibrium binding or (2) the enzyme itself, without bound flavin, was in an altered conformational state in which the thiol was less reactive following a catalytic cycle. Either explanation involves a slow return of the enzyme to its initial state following a catalytic cycle. We have investigated this phenomenon in more detail and found that rapid removal of the flavin from the enzyme by chromatography following catalytic turnover did not return the enzyme to its original state of susceptibility to either alkylating reagents or proteolytic enzymes. The flavin-free enzyme returned to the susceptible conformation with a half-time of ca. 25 min at 0 °C. Inactivation of the enzyme at intermediate times of relaxation by either a proteolytic enzyme or an alkylating reagent showed biphasic kinetics, indicative of a mixture of the protected and susceptible forms. Our results demonstrate that the enzyme is in an altered conformational state following a single catalytic cycle and that this altered conformational state slowly relaxes to a conformer resembling the original in susceptibility to alkylating reagents and proteolytic enzymes.

Bacterial luciferase is a flavin monooxygenase which catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and long-chain aldehydes in the presence of oxygen, producing blue-green light (λ_{max} 490 nm) [see Ziegler & Baldwin (1981) for a review]. The structure of the enzyme has been studied in some detail, primarily through the use of chemical modification and mutant enzyme analyses. Ziegler-Nicoli et al. (1974) have shown that the enzyme is rapidly inactivated by alkylating reagents due to the high reactivity of a specific thiol in or near the active center. Modification of this thiol, which resides on the α subunit, results in loss of measurable reduced flavin binding, while binding of oxidized flavin protects the thiol from the alkylating reagents. Another protection experiment reported by these authors led to an unexpected result. Injection of $FMNH_2$ into a solution of luciferase in air-equilibrated buffer resulted in the formation of a species of enzyme that was inactivated with *N*-ethylmaleimide in a pseudo-first-order reaction with a half-time of 40 min under conditions in which the native enzyme alone

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