Mechanism of the Reaction Catalyzed by Mandelate Racemase. 3. Asymmetry in Reactions Catalyzed by the H297N Mutant†

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Received April 4, 1991; Revised Manuscript Received June 14, 1991

ABSTRACT: The two preceding papers [Powers, V. M., Koo, C. W., Kenyon, G. L., Gerlt, J. A., & Kozarich, J. W. (1991) *Biochemistry* (first paper of three in this issue); Neidhart, D. J., Howell, P. L., Petsko, G. A., Powers, V. M., Li, R., Kenyon, G. L., & Gerlt, J. A. (1991) *Biochemistry* (second paper of three in this issue)] suggest that the active site of mandelate racemase (MR) contains two distinct general acid/base catalysts: Lys 166, which abstracts the α-proton from (*S*)-mandelate, and His 297, which abstracts the α-proton from (*R*)-mandelate. In this paper we report on the properties of the mutant of MR in which His 297 has been converted to asparagine by site-directed mutagenesis (H297N). The structure of H297N, solved by molecular replacement at 2.2-Å resolution, reveals that no conformational alterations accompany the substitution. As expected, H297N has no detectable MR activity. However, H297N catalyzes the stereospecific elimination of bromide ion from racemic *p*-(bromomethyl)mandelate to give *p*-(methyl)-benzoylformate in 45% yield at a rate equal to that measured for wild-type enzyme; the unreacted *p*-(bromomethyl)mandelate is recovered as (*R*)-*p*-(hydroxymethyl)mandelate. At pD 7.5, H297N catalyzes the stereospecific exchange of the α-proton of (*S*)- but not (*R*)-mandelate with D_2O solvent at a rate 3.3-fold less than that observed for incorporation of solvent deuterium into (*S*)-mandelate catalyzed by wild-type enzyme. The pD dependence of the rate of the exchange reaction catalyzed by H297N reveals a pK_a of 6.4 in D_2O, which is assigned to Lys 166. These observations provide persuasive evidence that the reaction catalyzed by MR does, in fact, proceed via a two-base mechanism in which Lys 166 abstracts the α-proton from (*S*)-mandelate and His 297 abstracts the α-proton from (*R*)-mandelate. Moreover, the facile exchange of solvent deuterium into (*S*)-mandelate catalyzed by H297N demonstrates the formation of a transiently stable *intermediate*. The rate of this exchange reaction and the measured pK_a value of Lys 166 suggests that the pK_a of the α-proton of (*S*)-mandelate in the active site of H297N is ≤ 15 in contrast to the value of 22 recently reported for mandelic acid in solution [Chiang, Y., Kresge, A. J., P. Pruszynski, P., Schepp, N. P., & Wirz, J. (1990) *Angew. Chem. Int., Ed. Engl. 29*, 792]. Since the rates of elimination of bromide ion from *p*-(bromomethyl)mandelate and of exchange of the α-proton of mandelate catalyzed by wild-type enzyme are nearly identical with those observed for the H297N, an *intermediate* is also presumed to lie on the reaction pathway for the racemization reaction catalyzed by wild-type enzyme. These studies demonstrate the power of site-directed mutagenesis in providing otherwise inaccessible detail about the mechanism of an enzyme-catalyzed reaction.

The two preceding papers in this series present chemical and kinetic (Powers et al., 1991) and structural (Neidhart et al., 1991) evidence that the mechanism of the reaction catalyzed by mandelate racemase (MR)[1] utilizes two acid/base catalysts: one to abstract the proton from the α-carbon to generate a putative intermediate having at least partial resonance-stabilized carbanionic character[2] and a second to protonate the opposite face of the intermediate to generate the inverted product. The unequal incorporation of solvent deuterium into the enantiomeric mandelates was persuasive evidence in support of a two-base mechanism (Powers et al., 1991). The conjugate acid of the (*S*)-specific base was, thus, deduced to be polyprotic, e.g., the amino group of a lysine, and the conjugate acid of the (*R*)-specific base was deduced to be monoprotic, e.g., the imidazole group of a histidine. On the basis of the structure of the active site and the position of bound *p*-iodomandelate (Neidhart et al., 1991), we suggested that Lys 166 and His 297 are the acid/base catalysts.

† This research was supported by Grants GM-34572 (J.A.G.), GM-37210 (J.W.K.), and GM-40570 (J.A.G., G.L.K., and J.W.K.) from the National Institutes of Health. This is paper 15 in a series on mandelate racemase; paper 14 is Neidhart et al. (1991).

[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; H297N, the mutant of mandelate racemase in which His 297 has been replaced with asparagine; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MR, mandelate racemase; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

[2] Whether the intermediate is an enolate anion (carbanion) derived by abstraction of a proton from mandelate anion or a charge-neutralized mandelate anion (protonated or metal ion coordinated) or an enol that could be obtained by protonation of an enolate anion is presently unknown. With this distinction is mechanistically important, the studies described in this and the preceding papers (Powers et al., 1991; Neidhart et al., 1991) address only the number and identities of the active site bases that are involved in forming the putative intermediate and not the extent of charge neutralization of the intermediate. Accordingly, the product of proton abstraction from mandelate by an active site base will be referred to as the "intermediate".

0006-2960/91/0430-9274$02.50/0

As the first racemase for which both a high-resolution X-ray structure and substantial mechanistic data are available, MR provides an unusual opportunity to dissect the individual catalytic steps. Thus, the possibility of distinct half-reactions for deprotonation and for reprotonation of a kinetically discrete intermediate may be tested. To this end, we herein describe the properties of the H297N mutant of MR in which the histidine has been "inactivated" by conversion to asparagine by use of site-directed mutagenesis. We demonstrate that while H297N is inactive in the racemization of mandelate, it is remarkably functional in the catalysis of two (*S*)-specific acid/base reactions and nonfunctional in the corresponding (*R*)-specific reactions. These findings strongly point to His 297 as a critical residue for racemization via (*R*)-specific acid/base catalysis and provide support for Lys 166 as the (*S*)-specific acid/base catalyst. Moreover, the mechanistic behavior of H297N provides a striking verification that the overall symmetry of the racemase reaction is the result of two asymmetric half-reactions that share a common intermediate. We further deduce the enzymatically perturbed pK_a values of both Lys 166 and the α-proton of bound mandelate from the catalytic properties of H297N.

Materials and Methods

The plasmids that direct the inducible synthesis of MR in *Escherichia coli*, pMRtrc (Ransom et al., 1988), and the constitutive synthesis of MR in *Pseudomonas aeruginosa* ATCC 15692, pMRαpg (Tsou et al., 1989), have been described.

(*R*,*S*)-*p*-(Bromomethyl)mandelate (Lin et al., 1988) and *p*-(chloromethyl)phenylglyoxal (Dirmaier, 1986) were prepared by established procedures.

Restriction endonucleases and polynucleotide kinase from bacteriophage T4 were purchased from New England Biolabs. DNA ligase from bacteriophage T4 and calf intestinal phosphatase were obtained from Boehringer Mannheim. DNA sequence analysis was performed by use of the Sequenase kit and directions supplied by U.S. Biochemicals. Site-directed mutagenesis was performed by the phosphorothioate method with the kit and directions purchased from Amersham. Synthetic oligonucleotides were purchased from the Protein Nucleic Acid Laboratory, University of Maryland, College Park. Standard techniques were employed for isolating plasmid DNA and DNA fragments, cloning and transformation, and resolving protein and DNA samples by electrophoresis.

Site-Directed Mutagenesis. The template for mutagenesis was constructed by ligating the 1.9-kb *Eco*RV–*Sst*I fragment of pMRαpg containing the entire gene for MR (Tsou et al., 1989) with M13mp18 that had been restricted with *Hin*cII and *Sst*I. The synthetic 18-mer d(ATGTCCCAGCAACCTGTTC) was used for constructing the H297N substitution, where the position of the mutagenic mismatch is underlined. The sequence of the entire gene for the H297N mutant was confirmed by use of a series of synthetic primers (Tsou et al., 1989).

Assay of Mandelate Racemase Activity. Mandelate racemase activity was quantitated at 25 °C with use of either (*R*)- or (*S*)-mandelate as substrate with either a polarimetric assay or the circular dichroic assay described by Sharp et al. (1979). The polarimetric assay was performed in a 10-cm cell (8-mL volume) with a JASCO 370-DIP polarimeter; at 25 °C, $[\alpha]_{435}$ = 243° (1.52, H_2O) corresponding to a rotation of 2.43° mM^{-1}.

The dependence of the rate of racemization on pH using either (*R*)- or (*S*)-mandelate as substrate was evaluated in 100 mM buffers, pH 5.5–10.5, containing 10 mM $MgCl_2$. The buffers used were MES, pH 5.5, 6.0, and 6.5; PIPES, pH 6.5, 7.0, and 7.5; HEPES, pH 7.5, 8.0, and 8.5; TAPS, pH 8.5 and 9.0; CHES, pH 9.0, 9.5, 9.75, and 10.0; CAPS, 10.0, 10.25, and 10.5. The use of overlapping pH ranges ensured that neither discontinuities nor artifacts resulted from the use of six different buffers across the pH range examined. Since neither the polarimetric assay nor the circular dichroic assay is sufficiently sensitive to measure velocities accurately at concentrations near and below the estimated K_m value (approximately 0.25 mM at pH 7.5; Whitman et al., 1985), velocities were measured at both 50 and 100 mM concentrations of substrate at pH values below 9.5 and found to be nearly identical (within 10%); therefore, the measured velocities were assumed to be reliable measures for k_{cat}. At higher values of pH, k_{cat} and K_m could be measured, and Lineweaver–Burk plots were used to obtain k_{cat}. Cleland's program HABELL (Cleland, 1979) was used to determine the pK_a values described by the data.

Construction of an Expression System for Wild-Type and H297N Mutant Mandelate Racemases in Pseudomonads Using the trc Promoter. The *Cla*I–*Sac*I fragment from the M13mp18 containing the H297N mutation was isolated and ligated with pMRtrc (Ransom et al., 1988) that had been digested with *Cla*I and *Sac*I and treated with calf intestinal phosphatase. The ligation mixture was transformed into *E. coli* JM105, and several transformants were observed to produce a (catalytically inactive) polypeptide with the same molecular weight as wild-type MR as assessed by SDS–PAGE. The plasmid contained in one of the transformants was designated pMRtrc/H297N.

pMRtrc/H297N was restricted with *Eco*RI and *Hin*dIII, and the fragment containing the intact gene for H297N under the control of the *trc* promoter was ligated with the large *Eco*RI–*Hin*dIII fragment of pKT230. The ligation mixture was transformed into *E. coli* HB101, and transformants were selected by plating on LB plates containing 50 μg/mL kanamycin. The plasmid contained in these cells was designated pKTtrc/H297N. This plasmid was isolated from *E. coli* HB101 and transformed into *P. aeruginosa* ATCC 15692; transformants were selected on LB plates containing 1 mg/mL streptomycin.

This procedure was repeated with the *Cla*I–*Sac*I from M13mp18 containing the wild-type gene for MR to obtain pKTtrc/WT. In addition, both pKTtrc/WT and pKTtrc/H297N (isolated from *E. coli* HB101) were conjugated from *E. coli* HB101 into *Pseudomonas putida* ATCC 17453 in the presence of *E. coli* MM294 transformed with pRK2013. The desired conjugants were selected on minimal medium plates containing benzoate as sole source of carbon (to select for *P. putida* ATCC 17453) and 0.1 mg/mL streptomycin (to select for pKTtrc/WT or pKTtrc/H297N).

Purification of H297N Mandelate Racemase from P. aeruginosa Transformed with pKTtrc/H297N. The purification of H297N from extracts of *P. aeruginosa* ATCC 15692 transformed with pKTtrc/H297N was the same as that described previously for the purification of wild-type MR from the same strain of *P. aeruginosa* transformed with pMRαpg. Since H297N has no measurable MR activity (vide infra), SDS–PAGE was used to verify that column fractions contained the mutant protein.

Crystallization and Structure Determination of H297N. Crystals of the H297N mutant of MR were grown under conditions identical with those described for the wild-type enzyme (Neidhart et al., 1991) and were isomorphous with

the wild-type crystals. Typical crystal dimensions are 0.5 × 0.5 × 0.3 mm. X-ray diffraction data were collected with the use of Siemen's area detector mounted on a Rigaku rotating anode generator operated at a power of 5 kW with a 0.3 × 3 mm filament and focusing mirrors. The crystal was maintained at a temperature near 5 °C during data collection by use of an FTS crystal cooler. The symmetry *R*-factor for diffraction data to 2.2-Å resolution was 7% on $|F|$. The crystal structure of the H297N mutant of MR was solved by difference Fourier analysis relative to the partially refined 2.0-Å crystal structure of the wild-type enzyme (D. Neidhart, unpublished results). The current *R*-factor for this crystal structure of the wild-type enzyme is 17% for data from 5.0- to 2.0-Å resolution following energy refinement (XPLOR; Brunger et al., 1987) and restrained least-squares refinement (PROLSQ; Hendrickson & Konnert, 1980) with individual isotropic *B*-factors and inclusion of 150 solvent water molecules per subunit. Electron density maps were calculated with coefficients $F_{H297N} - F_c$ and $2F_{H297N} - F_c$, where F_c's and phases were calculated from the refined atomic coordinates of the wild-type enzyme with the side-chain atoms of residue 297 and the sulfate ion removed. These electron density maps clearly revealed the positions of the Asn 297 side-chain atoms and the sulfate ion in the mutant enzyme, so these atoms were added to the atomic model, several active site solvent water molecules were repositioned, and the entire model was subjected to energy refinement (XPLOR). The initial *R*-factor of 0.213 (14 697 reflections from 6.0- to 2.2-Å resolution) was reduced to 0.172 during refinement.

Elimination of Bromide Ion from (R,S)-p-(Bromomethyl)Mandelate. The elimination of bromide ion from (*R,S*)-*p*-(bromomethyl)mandelate catalyzed by H297N was monitored with an Orion Model 811 pH meter equipped with an Orion Model 94-35 bromide ion electrode (Lin et al., 1988). The reaction (1.5 mL) in 100 mM sodium MES, pH 6, containing 1 mM $Mg(NO_3)_2$, and in the absence or presence of H297N (6.4 mg, 0.17 μmol of active sites) was initiated by the addition of 30 μL of an ethanolic solution of (*R,S*)-*p*-(bromomethyl)mandelate to achieve a final concentration of 1.0 mM. Bromide concentration readings were recorded every 30 s for the first 6 min and every 1 min thereafter. When the enzyme-catalyzed reaction was complete, the enzyme was removed by ultrafiltration (Amicon PM-30 membrane), and the deproteinized solution was chromatographed on a Beckman Ultrasphere ODS HPLC column (4.6 × 25 cm) with a mobile phase of 0.5% phosphoric acid/25% methanol at a flow rate of 1 mL/min. The effluent was monitored at 260 nm. The fractions containing *p*-(methyl)benzoylformate (λ_{max} 264 nm, ϵ 14 400 M^{-1} cm^{-1}) and *p*-(hydroxymethyl)mandelate (λ_{max} 222 nm, ϵ 7050 M^{-1} cm^{-1}) were combined separately. The yield of *p*-(methyl)benzoylformate, the enzymatic elimination product, was 0.56 μmol, and that of *p*-(hydroxymethyl)-mandelate, the nonenzymatic solvolysis product, was 0.72 μmol, corresponding to a net recovery of 85% of the starting (*R,S*)-*p*-(bromomethyl)mandelate.

Preparation of (R)- and (R,S)-p-(Hydroxymethyl)-mandelates. The (*R*) enantiomer (Ekwall & Mannervik, 1973; Vander Jagt et al., 1975) of *p*-(hydroxymethyl)mandelate was prepared by the reaction of *p*-(hydroxymethyl)phenylglyoxal with glutathione in the presence of glyoxalases I and II according to the following protocol. A mixture of *p*-(chloromethyl)phenylglyoxal (67 mg, 0.37 mmol) in 1 mL of ethanol and 3 mL of 100 mM sodium phosphate, pH 7, was stirred for 4 h to effect hydrolysis of the benzylic chloride. Aliquots of this solution were then added over a period of 5 h to an unbuffered aqueous solution (20 mL) of glutathione (28 mg, 0.09 mmol), 30 units of glyoxalase I from yeast (Sigma), and 20 units of bovine glyoxalase II (Sigma). The pH was maintained between 6.4 and 7.0 by the addition of NaOH, and the reaction was monitored by the decrease in absorbance at 260 nm. After incubation overnight, the pH was adjusted to 2, and the solution was continuously extracted for 40 h with 75 mL of diethyl ether. The organic extract was dried ($MgSO_4$), and the solvent was removed under reduced pressure. The product was purified by HPLC with the solvent system as described.

(*R,S*)-*p*-(Hydroxymethyl)mandelate was prepared by the hydrolysis of (*R,S*)-*p*-(bromomethyl)mandelate and purified as described above.

Circular Dichroism of p-(Hydroxymethyl)mandelates. The *p*-(hydroxymethyl)mandelates purified by HPLC were extracted into diethyl ether. The solvent was removed under a stream of nitrogen, and the residues were dissolved in 50% hexane in 2-propanol. Molar ellipticities were measured with a JASCO J-500C spectropolarimeter equipped with a JASCO DP-500N data processor.

Exchange of the α-Proton of (R)- and (S)-Mandelates with D_2O. Reactions (0.65 mL) measuring the rate of exchange of the α-proton of an enantiomer of mandelate with D_2O catalyzed by wild-type enzyme or H297N were performed at 25 °C with use of 50 mM (*R*)- or (*S*)-mandelate in 20 mM sodium HEPES, pD 7.5, containing 1 mM $MgCl_2$. The reactions were initiated by addition of sufficient enzyme (wild type or H297N) to give complete exchange in approximately 60 min, and the progress of the exchange reaction was monitored by recording successive ^1H NMR spectra with a Bruker AM-400 NMR spectrometer. The intensity of the resonance for the α-proton (δ = 4.86 ppm) was measured relative to that for the five aromatic protons (δ = 7.2–7.35 ppm), and the slopes of plots of the logarithm of the relative intensity of the resonance of the α-proton as a function of time were used to obtain the rate of exchange.

The pD dependence of the rate of the exchange reaction catalyzed by H297N was evaluated at 28 °C with both 50 and 100 mM (*S*)-mandelate in 20 mM buffers (MES, PIPES, HEPES, and TAPS), pD 5.5–9.0, containing 10 mM $MgCl_2$. H297N was observed to be unstable in D_2O at pH values greater than 9.0, thereby preventing the measurement of the kinetics of the exchange reaction over the same range of acidities used to determining the dependence of k_{cat} on pH for wild-type MR. Two concentrations of (*S*)-mandelate significantly greater than the estimated values for K_m were used to ensure saturation by (*S*)-mandelate. Since the measured values were found to be nearly identical (within 10%), these were assumed to be reliable measures for k_{cat}. Cleland's program HABELL (Cleland, 1979) was used to determine the pK_a value described by the data.

Results and Discussion

Dependence of k_{cat} on pH for Wild-Type MR. We have determined the dependence of k_{cat} on pH for wild-type MR in both the (*R*) to (*S*) and (*S*) to (*R*) directions using the polarimetric assay described in the Materials and Methods section rather than the circular dichroic assay used previously (Whitman et al., 1985). With either assay, both the low K_m (approximately 0.25 mM at pH 7.5; Whitman et al., 1985) and signal amplitude (rotation or ellipticity) at low concentrations of mandelate prevent a reliable measure of K_m below pH 9.5; however, by measuring velocities at more than one high concentration of mandelate (e.g., 50 and 100 mM) a reliable measure of k_{cat} can be obtained at pH values of 9.5

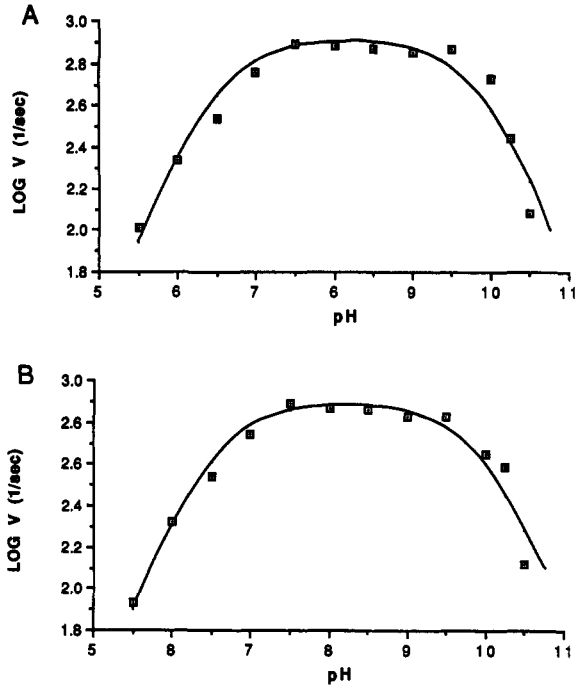


FIGURE 1: (A) The pH dependence of k_{cat} in the (*R*) to (*S*) direction; (B) the pH dependence of k_{cat} in the (*S*) to (*R*) direction.

or below. Above pH 9.5, the increased value of K_m permits both K_m and k_{cat} to be determined reliably by Lineweaver–Burk analysis. The results that were obtained are displayed in Figure 1 [panel A, (*R*) to (*S*); panel B, (*S*) to (*R*)].

In both directions k_{cat} increases with pH to a plateau and then decreases as the pH is further increased; in both directions, the pK_a values described by the data are identical within experimental error and are equal to 6.4 and 10.0. This contrasts with the pH independence of k_{cat} reported previously (Whitman et al., 1985): we have noted that the circular dichroic assay is limited by sample absorbance at modest concentrations of mandelate (<5 mM), and this may explain the new results obtained with the polarimetric assay.

From these data we hypothesize that in each direction both a basic catalyst (pK_a = 6.4) and an acidic catalyst (pK_a = 10.0) are present in the active site. Given the results of the structural studies described in the previous paper (Neidhart et al., 1991), in the (*R*) to (*S*) direction we associate the pK_a of 6.4 with His 297 to produce the basic catalyst required for proton abstraction from (*R*)-mandelate to generate the intermediate and the pK_a of 10.0 with Lys 166, which is required for protonation of the intermediate to generate (*S*)-mandelate. In the (*S*) to (*R*) direction, the pK_a values and roles of His 297 and Lys 166 in catalysis would be reversed. We note that the substrate deuterium isotope effects measured at pH 6.0 (k_H/k_D = 4.1), pH 7.5 (k_H/k_D = 4.3), and pH 10.0 (k_H/k_D = 6.2) are nearly equivalent, thereby demonstrating that the decreases in activity observed at the pH extremes are not due to changes in the rate-determining step. Both the potential effects of active site structure on the pK_a values of Lys 166 and His 297 and the possibility that these kinetically determined pK_a values may not be actual microscopic pK_a values have been discussed in the previous paper (Neidhart et al., 1991).

Construction, Expression, and Purification of the H297N Mutant. The H297N substitution was constructed according to the phosphorothioate methodology. While the mutagenesis was performed in M13mp18 containing the entire gene for MR as well as our previously described promoter that is constitutive in pseudomonads (Tsou et al., 1989), H297N was ultimately expressed by taking advantage of our observation that the *trc* promoter is also constitutive in pseudomonads. In the case of MR, higher levels of protein are produced with the *trc* promoter than with the promoter in pMRαpg that we have utilized previously. Since the mandelate pathway of *P. aeruginosa* ATCC 15692 does not include MR (this host is unable to utilize (*R*)-mandelate as sole carbon and energy source), mutant enzymes isolated from this host cannot be contaminated with any wild-type enzyme.

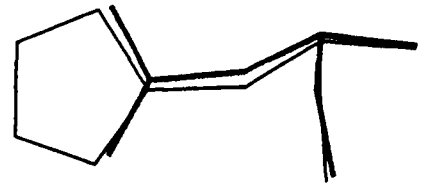
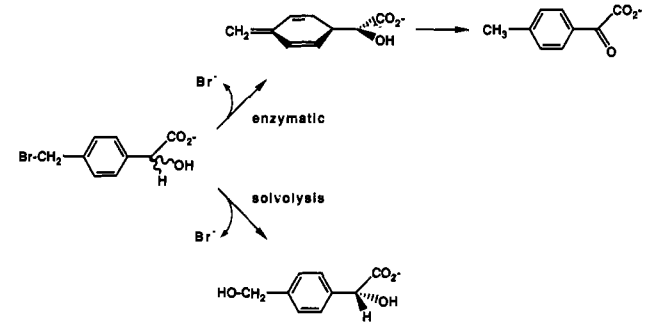


FIGURE 2: Comparison of the positions of His 297 (light lines) and Asn 297 (bold lines) in the active sites of wild-type MR and H297N, respectively.

Scheme I



Although H297N has no MR activity (vide infra), the procedure used to purify wild-type MR could be used to obtain homogeneous samples of the mutant protein: the elution profiles measured by UV for H297N were essentially the same as those for wild type; SDS–PAGE was used to judge the purity of column fractions before these were combined for the next step. In this way, homogeneous enzyme was obtained that was suitable for both mechanistic and crystallographic studies.

Structure of the H297N Mutant. Crystals of H297N were obtained from $(NH_4)_2SO_4$ as described for the wild-type enzyme (Neidhart et al., 1991), and the structure was solved to 2.2-Å resolution by difference Fourier analysis relative to the structure of wild-type enzyme refined to 2.0 Å (D. Neidhart, unpublished data). The position of Asn 297 in the active site of H297N is compared with that of His 297 in the active site of wild type enzyme in Figure 2. The only discernible difference in the active site is the substitution of a carboxamide functional group for the imidazole functional group at residue 297. Since the H297N substitution does not perturb the structure of the enzyme beyond the functional group interchange (data not shown), the mechanistic properties of the mutant enzyme should be useful in characterizing the mechanism of the racemization reaction catalyzed by the wild-type enzyme.

Mandelate Racemase Activity of H297N. The H297N mutant had no detectable MR activity with either (*R*)- or (*S*)-mandelate as substrate. Under the conditions of the polarimetric assay, the k_{cat} of wild-type enzyme was ~750 s^{-1}; an activity 0.01% of this could have been detected for H297N.

Elimination of Bromide Ion from (R,S)-p-(Bromomethyl)Mandelate Catalyzed by H297N. For wild-type MR, the racemization of *p*-(bromomethyl)mandelate is accompanied by the slow elimination of bromide ion yielding *p*-(methyl)benzoylformate as the product after tautomerization

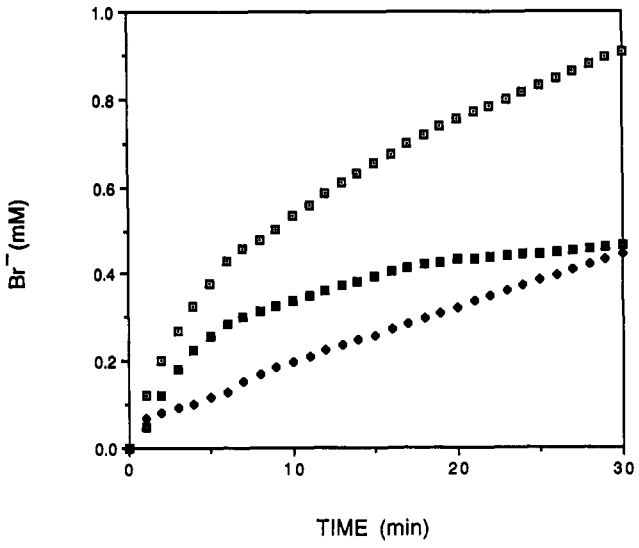


FIGURE 3: Elimination of bromide ion from (*R,S*)-*p*-(bromomethyl)mandelates catalyzed by H297N. Total bromide ion released, open squares; nonenzyme catalyzed bromide ion release, closed diamonds; enzyme-catalyzed bromide ion release, closed squares.

of the transient xylylene intermediate (Scheme I; Lin et al., 1988). In the absence of enzyme, *p*-(bromomethyl)mandelate undergoes slow solvolysis ($t_{1/2} \sim 30$ min at neutral pH) to give *p*-(hydroxymethyl)mandelate (Scheme I). Because elimination of bromide ion from *p*-(bromomethyl)mandelate and racemization of mandelate can both proceed via an intermediate, the processing of *p*-(bromomethyl)mandelate by H297N was studied since removal of one of the catalytic bases would not be expected to alter the elimination of bromide ion even though racemization had been totally inactivated.

The rate of elimination of bromide ion from (*R,S*)-*p*-(bromomethyl)mandelate (1.00 mM) was measured in both the absence and the presence of H297N. The production of bromide ion observed in the absence of enzyme was subtracted from that observed in the presence of enzyme to obtain the enzyme-catalyzed component; the data are shown in Figure 3. Despite the presence of a large amount of the mutant enzyme, the enzyme-catalyzed elimination of bromide approaches 50% completion (0.50 mM) rather than 100% completion (1.00 mM), suggesting that H297N catalyzes the stereospecific elimination of bromide from one enantiomer of the racemic *p*-(bromomethyl)mandelate. The turnover number for elimination of bromide ion catalyzed by H297N was 0.013 s^{-1}; the turnover number for elimination of bromide ion catalyzed by wild-type enzyme was 0.012 s^{-1} (Lin et al., 1988). Thus, the rate of the elimination reaction catalyzed by H297N is equal to that of the reaction catalyzed by wild-type enzyme.

The products of the reaction were separated by HPLC and quantitated. The isolated yield of *p*-(methyl)benzoylformate obtained by enzyme-catalyzed elimination of bromide ion (0.56 μmol) was 78% that of the *p*-(hydroxymethyl)mandelate obtained by competing hydrolysis of the racemic *p*-(bromomethyl)mandelate (0.72 μmol). These relative yields are in good agreement with the observed amount of enzyme-catalyzed elimination of bromide ion (Figure 3).

The absolute configuration of the *p*-(hydroxymethyl)-mandelate isolated from the H297N-catalyzed reaction was determined by circular dichroism. Authentic (*R*)-*p*-(hydroxymethyl)mandelate was obtained by the coupled action of glyoxalase I and II on *p*-(hydroxymethyl)phenylglyoxal, and racemic *p*-(hydroxymethyl)mandelate was obtained by the hydrolysis of (*R,S*)-*p*-(bromomethyl)mandelate. The circular dichroic spectra that were obtained are reproduced in Figure

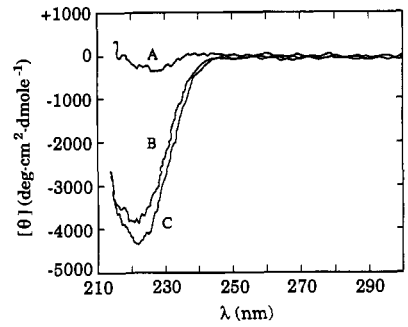


FIGURE 4: Circular dichroic spectra of racemic *p*-(hydroxymethyl)mandelate (A), the *p*-(hydroxymethyl)mandelate produced by the elimination of bromide ion from (*R,S*)-*p*-(hydroxymethyl)-mandelate catalyzed by H297N (B), and authentic (*R*)-*p*-(hydroxymethyl)mandelate produced by glyoxalases I and II (C).

4. The racemic material, as expected, does not rotate circularly polarized light at 222 nm. The molar ellipticity of authentic (*R*) enantiomer was −4300 deg cm^2 $dmol^{-1}$. The molar ellipticity of the *p*-(hydroxymethyl)mandelate obtained from the H297N-catalyzed reaction was −3910 deg cm^2 $dmol^{-1}$, demonstrating that it has the (*R*) configuration with an enantiomeric excess of 82% (assuming that material produced by the coupled action of glyoxalases I and II has an enantiomeric excess of 100%).

Thus, H297N catalyzes the stereospecific elimination of bromide ion from (*S*)-*p*-(bromomethyl)mandelate. On the basis of the structure of the active site of wild-type racemase (Neidhart et al., 1991), which suggests that Lys 166 catalyzes abstraction of the α-proton of (*S*)-mandelate and His 297 catalyzes abstraction of the α-proton of (*R*)-mandelate, the elimination of bromide ion from (*S*)- but not (*R*)-mandelate is the expected result and confirms the positioning of mandelate in the active site. That the elimination reaction catalyzed by H297N occurs at effectively the same rate as that catalyzed by wild-type enzyme indicates that the rate of abstraction of the α-proton from the (*S*) enantiomer is unaffected by the H297N substitution; this is in accord with the observation that the structure of the active site is unaffected by the substitution with the exception of the substitution of His for Asn. That elimination of bromide ion can occur in the complete absence of racemization suggests that with *p*-(bromomethyl)mandelate elimination and racemization may involve partitioning of a common intermediate produced by α-proton abstraction (vide infra); in H297N protonation of this intermediate by the conjugate acid of Asn 297 cannot occur but elimination of bromide ion is unaffected.

Exchange of the α-Proton of (S)- but Not (R)-Mandelate with D_2O Catalyzed by H297N. Since a concerted mechanism for elimination of bromide ion catalyzed by either wild-type enzyme or H297N cannot be unequivocally excluded, the exchange of the α-proton from (*S*)- and (*R*)-mandelates catalyzed by H297N was studied under conditions in which proton abstraction is the rate-determining step so that unambiguous evidence in support of formation of an intermediate could be obtained.

In wild-type enzyme three modes are available for incorporation of solvent deuterium: (1) into product via racemization; (2) in substrate via product (either with or without release from the active site); and (3) into substrate via reversion of an intermediate subsequent to deuterium exchange of the active site base. Only the last option is available for a mutant of MR in which one of the two acid/base catalysts has been removed. On the basis of previous work (Powers et al., 1991; Neidhart et al., 1991) the possibility of deuterium exchange

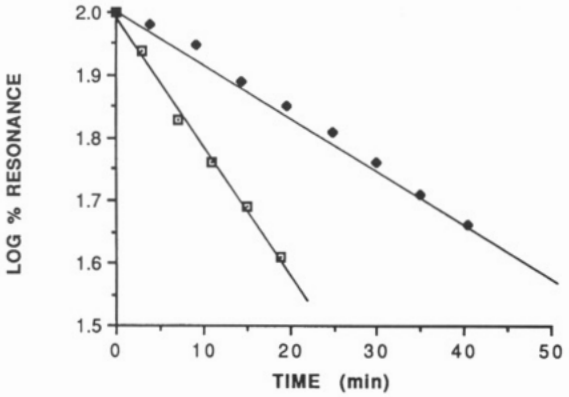


FIGURE 5: Comparison of the rates of exchange of (*S*)-[α-^{1}H]-mandelate with D_2O catalyzed by wild-type MR (open squares) and H297N (closed diamonds).

involving the conjugate acid of an active site base and an intermediate is viable only for the polyprotic Lys 166. Therefore, the possibility of a suprafacial hydrogen-exchange reaction catalyzed by H297N was explored.

The exchange reactions were monitored by ^{1}H NMR spectroscopy, and the data for the reactions using 50 mM (*S*)-mandelate catalyzed by wild-type racemase and H297N are compared in Figure 5. The rate for the reaction catalyzed by H297N, 53 s^{-1} at pD 7.5, is only 3.3-fold less than that observed for the reaction catalyzed by wild-type enzyme, 175 s^{-1}. At 10 mM (*S*)-mandelate, the rate of the exchange reaction catalyzed by H297N is 37 s^{-1}, demonstrating that H297N is effectively saturated with (*S*)-mandelate at the 50 mM concentrations used in these experiments. [Due to technical limitations, the K_m for the exchange reaction cannot be readily measured in these NMR experiments. However, the K_m of wild-type enzyme for either (*S*)- or (*R*)-mandelate is approximately 0.25 mM (Whitman et al., 1985).]

Under the same conditions, no detectable exchange catalyzed by H297N was observed for 50 mM (*R*)-mandelate, even after several weeks of incubation. When the exchange reaction catalyzed by H297N was conducted with 100 mM (*R*,*S*)-mandelate, the observed rate of exchange, 14 s^{-1} (data not shown), was 3.9-fold less than that observed for 50 mM (*S*)-mandelate. Therefore, (*R*)-mandelate can bind to the active site of H297N, but the mutant enzyme cannot catalyze the exchange of its α-proton with solvent.

Given the inability of H297N to catalyze racemization but its ability to catalyze a suprafacial hydrogen exchange with (*S*)- but not (*R*)-mandelate, it is reasonable to assign specific roles for His 297 in the racemization reaction: abstraction of the α-proton from (*R*)-mandelate to generate an intermediate in the (*R*) to (*S*) direction and protonation of the intermediate in the (*S*) to (*R*) direction. In the active site of H297N, the (*S*)-specific base, Lys 166, remains fully functional in abstracting the α-proton from (*S*)-mandelate in the suprafacial hydrogen-exchange reaction. These conclusions are in agreement with the previously described chemical and kinetic (Powers et al., 1991) and crystallographic (Neidhart et al., 1991) evidence and provide strong support for the formation of an intermediate of sufficient lifetime to allow rotation about the C–N bond in the conjugate acid of Lys 166 so that suprafacial hydrogen exchange can occur (Scheme II; for illustrative purposes the reaction is shown to proceed via a carbanionic intermediate).[2]

Estimate of the pK_a Values of Lys 166 and (S)-Mandelate Bound in the Active Site of H297N. The simplicity inherent in the exchange reaction catalyzed by H297N permits estimation of ionization constants for *both* Lys 166 and (*S*)-

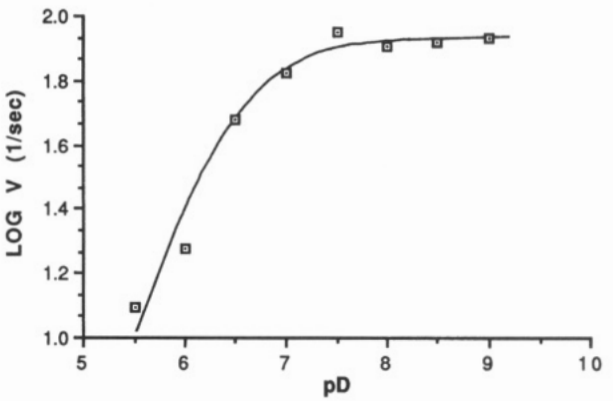
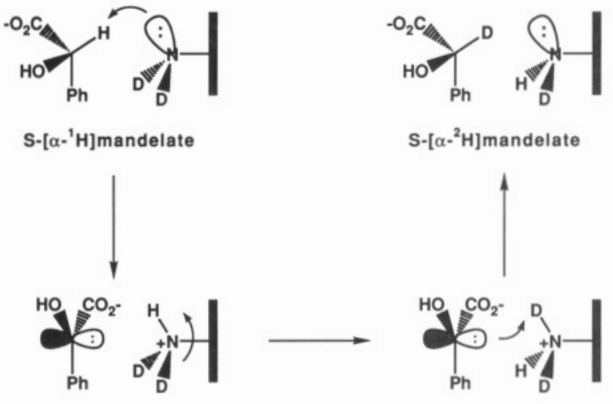


FIGURE 6: The pD dependence of the rate of exchange of the α-proton of (*S*)-mandelate catalyzed by H297N.

Scheme II



mandelate as described in this section.

The pD dependence of the rate of the exchange reaction catalyzed by H297N was studied by ^{1}H NMR spectroscopy so that the microscopic pK_a value of Lys 166 could be estimated. At the high concentration of (*S*)-mandelate used in these experiments (50 mM), changes in the observed rates of exchange should measure changes in k_{cat} for the exchange process; this assumption was verified by also measuring the rates of the exchange reactions using 100 mM (*S*)-mandelate at pDs 6, 7.5, and 9, and the values that were obtained were within 10% of those reported in Figure 6. In addition, the observation of substantial substrate deuterium isotope effects at pH 6, 7.5, and 10 (vide infra) suggests that substrate/product association/dissociation will not contribute significantly to the k_{cat} measured for the exchange reaction catalyzed by H297N. The data obtained are reproduced in Figure 6. The dependence of the rate of the exchange reaction on pD is described by both a maximum value for the exchange reaction, 85 s^{-1}, and a pK_a of 6.38 ± 0.08 that we attribute to the ε-ammonium group of Lys 166. For reactions in H_2O, the pK_a of Lys 166 could be approximately 5.8 since N–D bonds are approximately 5-fold stronger than N–H bonds (Laughton & Robertson, 1969); however, that the pK_a for the general basic catalyst observed in the dependence of k_{cat} on pH for wild-type MR measured in H_2O is also 6.4 does suggest that the pK_a is not significantly altered by the identity of the solvent hydrogen isotope. That the pK_a value of Lys 166 is significantly depressed by the active site structure is in accord with the proposal (Neidhart et al., 1991) that Lys 164 and 166 are sufficiently close in space for interresidue electrostatic interactions to reduce the first pK_a value of this pair of ε-ammonium groups.

With an estimate of the pK_a value of Lys 166, the measured rate of exchange of the α-proton of (*S*)-mandelate with solvent

can be used to estimate an upper limit for the pK_a value of the α-proton in the active site of H297N and, presumably, wild-type racemase. We assume the reaction

$$\text{E-ND}_2 + \text{H-Mdl} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{E-ND}_2\text{H}^+ + \text{Int} \qquad (1)$$

where k_f is the rate constant for abstraction of the α-proton of mandelate (H-Mdl) by the base in the active site (E-ND_2) and k_r is the rate constant for protonation of the intermediate derived from mandelate (Int) by the conjugate acid of the active site base (E-ND_2H^+). We assume that k_f is the maximal rate of exchange of the α-proton of mandelate catalyzed by H297N, 85 s^{-1}. The occurrence of the suprafacial hydrogen-exchange reaction necessitates that the intermediate have a lifetime comparable to or greater than the rate of rotation about the C–N bond of Lys 166 (Scheme II). This reasoning can be used to select an *upper limit* for the value of k_r that assumes an activation energy barrier of ~3 kcal/mol for the rotation about the C–N bond. Thus, k_r can be estimated to be no larger than 6.2×10^{10} s^{-1} ($kT/100h$, where k is Planck's constant and h is Boltzmann's constant).

Equation 1 describes the proton transfer reaction in the active site whose equilibrium constant is given by

$$K_{eq} = \frac{k_f}{k_r} \qquad (2)$$

Using the estimated values of k_f and k_r, K_{eq} is 1.5×10^{-9}.

The equilibrium constant for equation (1) can also be written as

$$K_{eq} = \frac{[\text{E-ND}_2\text{H}^+][\text{Int}]}{[\text{E-ND}_2][\text{H-Mdl}]} \qquad (3)$$

Since the ionization constant of Lys 166 (pK_a ~ 6) is represented as

$$K_a = \frac{[\text{E-ND}_2][\text{H}^+]}{[\text{E-ND}_2\text{H}^+]} \qquad (4)$$

and the ionization constant of the α-proton of (*S*)-mandelate bound in the active site is represented as

$$K_a = \frac{[\text{Int}][\text{H}^+]}{[\text{H-Mdl}]} \qquad (5)$$

the pK_a of the α-proton of (*S*)-mandelate bound in the active site is $\leq$15.

The pK_a of the α-proton of mandelic acid was recently determined to be 22 (Chiang et al., 1990), but that of the α-proton of mandelate anion has not been measured. Since mandelic acid presumably binds to the active site of the mandelate racemase as the carboxylate anion, the latter is the more relevant value for describing the increase in α-proton acidity induced by the environment of the active site. Values for the pK_a and enolization constants of cyclopentadienyl-1-carboxylic acid in H_2O were recently reported (Urwyler & Wirz, 1990). The pK_a of the cyclopentadienyl proton is 8.0 for the acid and 13.7 for the carboxylate anion. This difference, 5.7 pK_a units, suggests that the pK_a of the α-proton in mandelate anion may be approximately 28. Confirmation of this estimate is provided by the recent measurement of the pK_a of the α-proton in the lithium salt of phenylacetic acid, 30.2, although this value was measured in a mixture of hexamethylphosphoramide and tetrahydrofuran (Renaud & Fox, 1988). However, as described in the previous paper (Neidhart et al., 1991), an additional consideration is that the carboxylate group of the bound mandelate is likely coordinated to the essential Mg^{2+}, thereby lowering the charge density on the carboxylate group and increasing the acidity of the α-proton. Irrespective of its mode of binding to the enzyme, the pK_a value of the α-proton of mandelate is evidently lowered significantly by binding to the active site of MR.

The upper limit on the pK_a for mandelate bound in the active site, 15, is sufficiently low that the intermediate deduced from the observation of the suprafacial exchange reaction would, in fact, have a lifetime sufficiently long to undergo the exchange reaction catalyzed by H297N. In the absence of other information, the assumed value for k_r may well represent an upper limit, and the actual value for the pK_a of mandelate may well be significantly less than 15.

Conclusions. The observation that H297N is inactive as a racemase but nearly as active as wild-type racemase in catalyzing both elimination of bromide from (*S*)-*p*-(bromomethyl)mandelate and exchange of the α-proton of (*S*)-mandelate with solvent strongly suggests that the mechanism of the reaction catalyzed by wild-type racemase requires two acid/base catalysts, Lys 166 and His 297, identified crystallographically. The observed reactivities of the (*S*) but not (*R*) enantiomers of *p*-(bromomethyl)mandelate and mandelate also support the conclusion regarding the enantioselectivities of Lys 166 and His 297 in abstracting the α-protons from the enantiomers of mandelate bound in the active site, in accord with the chemical, kinetic, and structural experiments presented earlier (Powers et al., 1991; Neidhart et al., 1991). While elimination of bromide ion from *p*-(bromomethyl)-mandelate could occur in a concerted process without the formation of an intermediate having some carbanionic character, the observed suprafacial exchange of the α-proton of (*S*)-mandelate with solvent catalyzed by H297N cannot occur without the formation of a transiently stable intermediate. The observed exchange rate implies that the pK_a of the α-proton of mandelate is no greater than 15 in the active site of H297N, demonstrating that the active site is capable of significantly enhancing the acidity of the α-proton. A quantitative structural explanation for the enhanced acidity is not clear, although it is undoubtedly the result of electrostatic interactions in the active site. Since the rates of elimination of bromide ion from *p*-(bromomethyl)mandelate and of exchange of the α-proton of mandelate catalyzed by H297N do not differ significantly from those measured for the wild-type enzyme, an intermediate is also likely to occur in the reaction catalyzed by the wild-type enzyme.

Relationship to Other Applications of Site-Directed Mutagenesis for Establishing Mechanism. The mechanistic utility of the H297N mutant is unusual for a site-directed mutation in the active site of an enzyme. In many other enzymes, mutation of a functional group in the active site significantly decreases the rate of the enzymatic reaction. In addition, only rarely are structures available for both wild-type enzyme and active site mutants. Thus, quantitative interpretations of the kinetic properties of mutant enzymes are often prone to considerable uncertainty. If structural information is available and conformational changes within the active site accompany the substitution, the kinetic properties of the mutant enzyme cannot be used to comment even qualitatively on the mechanism of the reaction catalyzed by the wild-type active site (Hibler et al., 1987; Loll & Lattman, 1990). For this reason, many practitioners of site-directed mutagenesis regard substitutions that have no effect on the kinetic properties as the "best" kind of mutation since such studies allow residues that are unimportant in catalysis to be identified, e.g., Tyr 248 in

the active site of carboxypeptidase A (Hilvert et al., 1986). In the present situation, the H297N substitution has been demonstrated to be without effect on the conformation of the active site, although the racemase activity has been totally inactivated by the substitution. However, with the use of two mechanistic probes for abstraction of the α-proton from (*S*)-mandelates, the H297N mutant enzyme is catalytically indistinguishable from the wild-type enzyme. This retention of full activity in a partial reaction allows the impairment observed in the overall racemization reaction to be assigned to the absence of His 297 and its role as a general acid/base catalyst in generating or protonating a kinetically viable intermediate.

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Conformational Stability of Pig Citrate Synthase and Some Active-Site Mutants†

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Received January 11, 1991; Revised Manuscript Received July 3, 1991

ABSTRACT: The conformational stabilities of native pig citrate synthase (PCS), a recombinant wild-type PCS, and six active-site mutant pig citrate synthases were studied in thermal denaturation experiments by circular dichroism and in urea denaturation experiments by using DTNB to measure the appearance of latent SH groups. His^{274} and Asp^{375} are conserved active-site residues in pig citrate synthase that bind to substrates and are implicated in the catalytic mechanism of the enzyme. By site-directed mutagenesis, His^{274} was replaced with Gly and Arg, while Asp^{375} was replaced with Gly, Asn, Glu, or Gln. These modifications were previously shown to result in 10^3–10^4-fold reductions in enzyme specific activities. The thermal unfolding of pig citrate synthase and the six mutants in the presence and absence of substrates showed large differences in the thermal stabilities of mutant proteins compared to the wild-type pig citrate synthase. The functions of His^{274} and Asp^{375} in ligand binding were measured by oxalacetate protection against urea denaturation. These data indicate that active-site mutations that decrease the specific activity of pig citrate synthase also cause an increase in the conformational stability of the protein. These results suggest that specific electrostatic interactions in the active site of citrate synthase are important in the catalytic mechanism in the chemical transformations as well as the conformational flexibility of the protein, both of which are important for the overall catalytic efficiency of the enyzme.

Citrate synthase catalyzes the stereospecific condensation of acetyl coenzyme A and oxalacetate to form citrate. It is an excellent enzyme for studying the relationships between the chemical structure of the protein and its physical, chemical, and functional properties. The sequence of the enzyme from pig heart was derived from amino acid sequence analysis (Bloxham et al., 1981, 1982); the mammalian enzyme has a high degree of homology with the citrate synthases isolated from yeast, plant, and bacterial sources (Alter et al., 1990); the three-dimensional structures of the pig heart citrate syn-

† This research was supported by grants from the Department of Veterans Affairs Medical Center, the U.S. Public Health Service, and the National Science Foundation.

0006-2960/91/0430-9281$02.50/0
