(*S*)-Mandelate Dehydrogenase from *Pseudomonas putida*: Mutations of the Catalytic Base Histidine-274 and Chemical Rescue of Activity†

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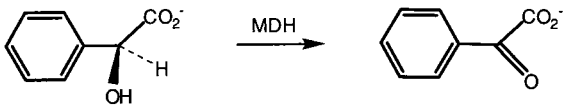
ABSTRACT: (*S*)-Mandelate dehydrogenase from *Pseudomonas putida*, an FMN-dependent α-hydroxy acid dehydrogenase, oxidizes (*S*)-mandelate to benzoylformate. The generally accepted catalytic mechanism for this enzyme involves the formation of a carbanion intermediate. Histidine-274 has been proposed to be the active-site base that abstracts the substrate α-proton to generate the carbanion. Histidine-274 was altered to glycine, alanine, and asparagine. All three mutants were completely inactive. The mutants were able to form adducts with sulfite, though with much weaker affinity than the wild-type enzyme. Binding of the inhibitor, (*R*)-mandelate, was not greatly affected by the mutation, unlike that of the substrate, (*S*)-mandelate, indicating that H274 plays a role in substrate binding. The activity of H274G and, to a lesser extent, H274A could be partially restored by the addition of exogenous imidazoles. The maximum rescued activity for H274G with imidazole was ∼0.1% of the wild-type value. Saturation kinetics obtained for rescued activity suggest that formation of a ternary complex of imidazole, enzyme, and substrate is required for catalysis. pH-dependence studies confirm that the free base form of imidazole is the rescue agent. An earlier study of pH profiles of the wild-type enzyme indicated that deprotonation of a residue with a pK_a of 5.5 in the free enzyme was essential for activity (Lehoux, I. E., and Mitra, B. (1999) *Biochemistry 38*, 5836−5848). Data obtained in this work confirm that the pK_a of 5.5 belongs to histidine-274.

(*S*)-Mandelate dehydrogenase (MDH)[1] from *Pseudomonas putida* catalyzes the oxidation of (*S*)-mandelate to benzoylformate using FMN as cofactor:



MDH is a member of a highly homologous enzyme family that oxidizes α-hydroxy acids to α-keto acids using different sources of the immediate oxidant (*1*, *2*). The oxidases use molecular oxygen, the flavocytochrome b_2s utilize an intramolecular heme, and the bacterial membrane-associated dehydrogenases, including MDH, are oxidized by a component of the electron transport chain in the membrane. The structures of two enzymes in this family, glycolate oxidase from spinach and flavocytochrome b_2 from *Saccharomyces cerevisae*, have been solved (*3*, *4*). A comparison of the active-site residues in the two structures reveals a high degree of similarity (*5*). On the basis of sequence as well as structural homologies, the enzymes in this family appear to be mechanistically related with respect to the substrate oxidation half-reaction.

Two different mechanisms have been proposed for the half-reaction involving the oxidation of the α-hydroxy acids. In the first and more widely accepted mechanism, the reaction proceeds through the formation of a carbanion intermediate generated when an active-site base abstracts the substrate α-proton. In an alternative mechanism, an active-site base removes the hydroxyl proton of the substrate, promoting the loss of a hydride ion from the substrate to FMN. For both mechanisms, the substrate α-proton is ultimately lost to the solvent. A highly conserved histidine residue located in the active site, close to the isoalloxazine part of FMN, has been proposed to be the base that initiates the reaction. The homologous histidine in MDH is H274 (*2*). When the substrate, lactate, was modeled into the active site of flavocytochrome b_2, the N-ϵ of this histidine (H373 in flavocytochrome b_2) was found to be ideally positioned to abstract the α-proton from the substrate, which would generate a carbanion intermediate (Figure 1) (*6*). However, another binding mode for lactate could also be proposed where the histidine abstracts the hydroxyl proton of the substrate, promoting loss of a hydride ion to FMN (*7*). For either mechanism, the conserved histidine appears to be a critical residue.

The importance of the conserved histidine in catalysis has been confirmed by the results of mutagenesis studies of the homologous residues H290 in lactate monooxygenase from *Mycobacterium smegmatis* and H373 in flavocytochrome b_2 (*8*, *9*). In both cases, the replacement of histidine by glutamine resulted in completely inactive mutant proteins, which demonstrated the critical nature of this residue but

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[1] Abbreviations: DCPIP, dichloroindophenol, sodium salt; IPTG, isopropyl β-thiogalactopyranoside; KIE, substrate kinetic isotope effect; LB medium, Luria−Bertani medium; MDH, (*S*)-mandelate dehydrogenase; PCR, polymerase chain reaction; PMS, phenazine methosulfate.

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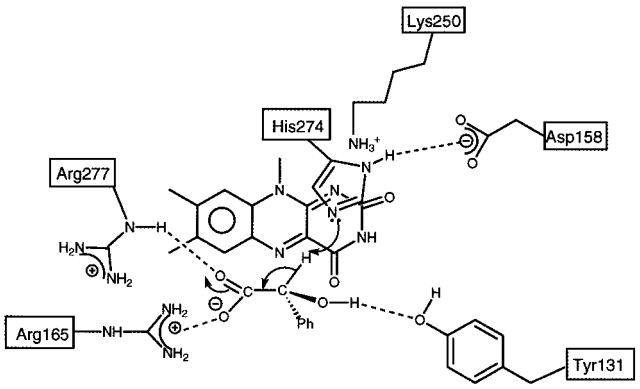

FIGURE 1: Active site geometry of (*S*)-mandelate dehydrogenase with the substrate bound in one possible mode (adapted from ref *6*).

precluded the kinetic characterization of the mutants.

In an earlier work on pH-dependence studies of MDH, we showed that a residue with a pK_a of 5.5 in the free enzyme had to be deprotonated for activity (*10*). We assigned this pK_a to H274, which is consistent with its postulated role as an active-site base. In this work, we report the results of the mutations of H274 to asparagine, alanine, and glycine. In accord with the proposed catalytic role of H274, none of the mutant enzymes had any detectable activity. Chemical rescue of the activity of mutant enzymes by exogenous compounds that replace the side chain of the altered residue was first demonstrated for aspartate aminotransferase and has subsequently been shown for a number of other enzymes (*11*; also see Discussion). In this study, we show the chemical rescue of activity of the H274 mutant proteins, the first demonstration of its kind for flavin-dependent oxidases and dehydrogenases. The unprotonated forms of exogenous imidazoles were able to partially restore the activity of H274G and, to a lesser extent, H274A. Our data indicate that exogenous imidazoles are required to bind in the cavity created at the active sites of H274G and H274A in order to act as surrogates for the missing side chain of H274. This study confirms that H274 is the residue with a pK_a of 5.5 in the free wild-type enzyme.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, T4 DNA ligase, and calf intestine phosphatase were from Boehringer Mannheim Biochemicals. Deoxynucleotides were from United States Biochemicals. Vent DNA polymerase was from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technologies, Coralville, IA. Ni^{2+}−resin was from Invitrogen or Qiagen. All other chemicals were of the highest commercial grade, and were obtained from Aldrich and Sigma. (*R*,*S*)-2-Hydroxy-3-butynoic acid was from TCI.

[α-^{2}H]-(*S*)-Mandelic acid was prepared as described previously (*12*). Imidazole and 1-, 2-, and 4-methylimidazoles (99% pure) were from Aldrich.

Methods. Genetic Engineering Methods. The mutations at residue H274 were engineered in the gene coding for WT MDH by PCR methods. The oligonucleotides used were 5′-TATCAAACGGTGGCGGTCG-3′, 5′-TATCAAACGCTGGCGGTC-3′, and 5′-TATCAAACAATGGCGGTCG-3′ for H274G, H274A, and H274N, respectively. The entire sequences of the three mutant genes were confirmed by DNA sequence analysis. The mutant genes were cloned into the same expression vector as WT MDH (*2*, *10*). The carboxyl-terminus histidyl-tagged WT and mutant genes were expressed by growing transformed cells in LB medium supplemented with 100 μg/mL ampicillin untill the absorbance at 590 nm was 1, followed by overnight induction with 0.5 mM IPTG.

Enzyme Purification and Determination of Protein Concentration. The histidyl-tagged proteins were purified and the protein concentrations estimated as described earlier (*10*). The levels of protein expression and the yields of purified protein for the H274 mutants were similar to WT MDH. Protein concentrations were measured by estimating the free FMN released upon boiling the protein for 5 min at 100 °C. Protein concentrations were also determined with the bicinchoninic acid reagent (Sigma), using bovine serum albumin as standard. The two methods yielded comparable values.

Determination of the pK_a of H274G-Bound FMN. The pK_a was determined as described earlier by titration of H274G with a concentrated potassium hydroxide solution directly in a cuvette (*10*). The data were fitted to a modified version of the Henderson−Hasselbalch equation.

Formation of a Reversible Sulfite Adduct of Enzyme-Bound Flavin. The dissociation constants for the reversible FMN−sulfite adduct for the H274 mutations were determined at 4 °C as described previously for WT MDH (*10*). The mutant proteins (∼15 μM) were incubated in the presence of increasing concentrations of sulfite in 20 mM phosphate, pH 7.5, containing 0.1% Triton X-100 and 10% ethylene glycol. The absorbance change at 460 nm was recorded, and the data were fitted to eq 1, where ΔA and ΔA_{max} are proportional

$$\frac{1}{\Delta A} = \frac{K_d}{\Delta A_{max}[L_{total}]} + \frac{1}{\Delta A_{max}} \quad (1)$$

to the concentrations of the enzyme−ligand adduct and total enzyme, respectively. [L] is the ligand (in this case, sulfite) concentration.

Binding of (R)-Mandelate. The dissociation constants for the binding of (*R*)-mandelate to WT MDH and the H274 mutants were determined at 20 °C by spectral titrations of the enzyme-bound FMN upon addition of the ligand. The buffer used was 20 mM phosphate, pH 7.5, containing 0.1% Triton X-100 and 10% ethylene glycol. The data were fitted to eq 1.

Enzyme Activity Assays. Activity assays for WT MDH were routinely performed at 20 °C, in 100 mM phosphate, pH 7.5, 1 mg/mL BSA, 1 mM PMS, and ∼100 μM DCPIP as the electron acceptor (*10*). An extinction coefficient of 21.6 mM^{-1} cm^{-1} at 600 nm was used for DCPIP at pH 7.5. There was no detectable activity for the H274 mutant proteins under these conditions. Activity could be detected with H274G and H274A when buffered imidazole was added to the assay buffer. In contrast, WT MDH showed no stimulation of activity on addition of low concentrations of imidazole (<100 mM). High concentrations of imidazole (>300 mM) caused inhibition of WT MDH and the H274 mutants, especially below pH 8, possibly due to high ionic strength of the medium. The rescued activity of H274G and H274A did not increase on preincubation with imidazole. Also, the order of addition of imidazole and other assay components did not affect activity for the mutants. Hence, activities for

the H274 mutant proteins were measured in 50 mM phosphate, pH 7.5, in the presence of 0.1% Triton X-100, 1 mg/mL BSA, and 100 μM DCPIP, together with varying concentrations of imidazole. Triton X-100 was included in the assays to avoid aggregation of the relatively large quantities of proteins that had to be used due to the very low activities. The extinction coefficient for DCPIP was remeasured in the presence of 0.1% Triton X-100 to be 20 mM^{-1} cm^{-1} at 600 nm. PMS did not affect the rate for the mutant proteins and was therefore not included in the assays. All activities reported are in the above buffer, unless otherwise stated.

The apparent K_m of the H274G protein for (*S*)-mandelate was determined at 20 °C and pH 7.8. The protein (0.75 μM) protein was assayed in the presence of 60 mM imidazole and varying concentrations of (*S*)-mandelate (0−83 mM). The apparent K_ms of H274G for imidazole and 4-methylimidazole were determined at pH 7.5 with 0.75 and 1.5 μM enzyme, respectively. The (*S*)-mandelate concentration was kept fixed at 40 mM. Imidazole concentrations were varied from 0 to 80 mM and were added to the assay mixture from concentrated stocks adjusted to pH 7.5. The ionic strength was kept constant at 0.13 by the addition of 4 M KCl to the buffer. The data were fitted to eq 2.

$$v = \frac{V_{max}[S]}{K_m(app) + [S]} \quad (2)$$

pH Dependence of the Activities of H274G and WT MDH. The pH dependence of the imidazole-rescued activity of H274G with (*S*)-mandelate as substrate was measured in a buffer containing 25 mM succinic acid, 25 mM potassium phosphate, and 25 mM Tris, 0.2% Triton X-100, 1 mg/mL BSA, and 70−250 μM DCPIP. This range of DCPIP concentrations did not affect the rates of the H274G mutant. The k_{cat} was measured in the presence of a fixed concentration of imidazole or 4-methylimidazole (60 mM) and a fixed saturating concentration of (*S*)-mandelate (typically, >80 mM). The ionic strength was maintained at a constant value of 0.15 at each pH with KCl. The pH dependence of the extinction coefficient for DCPIP was also determined in this buffer and was used to calculate activities from the decrease in absorbance due to the reduction of DCPIP. The reduction of DCPIP was monitored at either 600 or 540 nm, depending on the concentration of DCPIP used in the assay. For comparative reasons, WT MDH was also assayed in this buffer for the pH-dependence studies to ensure that Triton X-100 and imidazole had no effect on activity. For WT MDH, the pK_a values were determined by a nonlinear least-squares fit of the data to eq 3.

$$Y = \frac{Y_{max}}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (3)$$

For the H274G mutant protein, the pK_a values obtained with imidazole and 4-methylimidazole were not well-separated (<2 pH units); in this case, pK_a determinations using eq 3 gave erroneous values. Therefore, a graphical method was used to determine the pK_as of the ascending and descending limbs of the pH profile (*13*, *14*).

Instrumentation and Data Analysis. UV−visible spectra were recorded with a Cary 1 E Varian spectrophotometer.

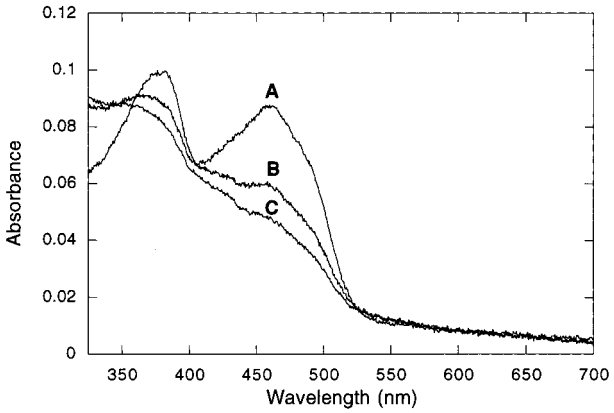

FIGURE 2: Absorbance spectrum of H274G in the oxidized and reduced states: (A) oxidized H274G in 20 mM potassium phosphate, pH 7.5, with 10% ethylene glycol and 0.1% Triton X-100; (B) after 10 mM (*S*)-mandelate and 25 mM imidazole were added; (C) after the sample in (B) was made partially anaerobic.

Data were analyzed with Kaleidagraph for the Macintosh (Synergy Software).

RESULTS

Purification and Spectral Properties of H274G, H274A, and H274N. The three H274 mutant proteins were purified with carboxy-terminal histidyl tags using the same procedure as WT MDH (*10*). All three mutants were similar to WT MDH in terms of stability. Figure 2 shows the absorbance spectrum of the oxidized H274G mutant. This spectrum is very similar to that of WT MDH, with maxima at 382 and 459 nm. On addition of 20 mM (*S*)-mandelate, no reduction of the H274G spectrum was seen under either aerobic or anaerobic conditions. In contrast, oxidized WT MDH is immediately fully reduced under these conditions. However, when 25 mM imidazole was added to H274G under aerobic conditions, the spectrum was immediately partially reduced (Figure 2). This partly reduced spectrum could be maintained under aerobic conditions. Further reduction of the spectrum was achieved when the enzyme was made partially anaerobic. Therefore, the reduction of FMN in the H274G mutant is dependent on the presence of imidazole in the buffer. Moreover, the rate of FMN reduction appears to be similar to the rate of its reoxidation by oxygen, since reduction was only partial in aerobic buffers. In contrast, the rate of FMN reduction in WT MDH is much faster than the rate of its reoxidation by oxygen.

The spectra of oxidized H274A and H274N were also very similar to that of WT MDH (not shown). No reduction of the FMN spectrum was detected with either H274A or H274N following addition of (*S*)-mandelate, in the absence of imidazole. In the presence of imidazole and in partially anaerobic buffers, a small amount of FMN was reduced for H274A. Thus, imidazole-assisted reduction of FMN by substrate was more efficient with H274G than with H274A.

Titration of the enzyme-bound FMN in the H274G mutant showed that the pK_a of the N(3)-position of FMN was unchanged (pK_a > 9.5; data not shown) compared to WT MDH (*10*).

Formation of a Reversible Adduct with Sulfite. WT MDH forms a reversible adduct of FMN with sulfite with a K_d of 30 μM at 4 °C and pH 7.5 (*10*). All three mutant proteins, H274G, H274A, and H274N, also formed FMN−sulfite

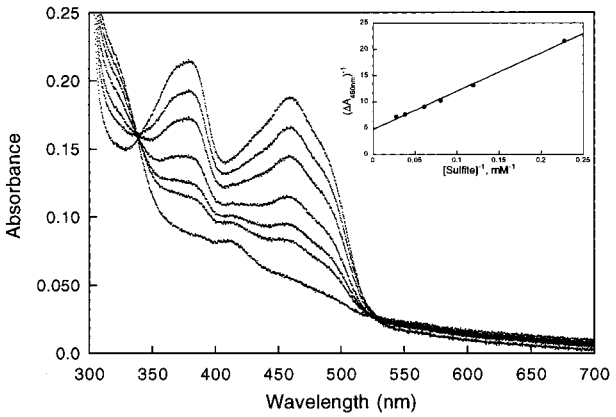

FIGURE 3: Titration of H274G with sodium sulfite. The enzyme (~15 μM) was incubated with 0, 2.4, 4.4, 8.4, 12.4, 16.4, and 36.4 mM sodium sulfite in 20 mM potassium phosphate, pH 7.5, containing 0.1% Triton X-100 and 10% ethylene glycol at 4 °C. The inset shows a double reciprocal plot of the absorbance changes at 460 nm versus the total sulfite added for H274G (●). The solid line is a fit to eq 1.

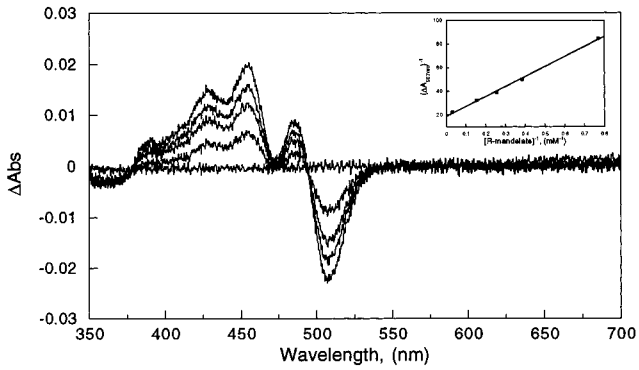

FIGURE 4: Binding of (*R*)-mandelate to WT MDH at 20 °C in 20 mM phosphate, pH 7.5, containing 0.1% Triton X-100 and 10% ethylene glycol. Difference spectra obtained after the addition of 0, 1.3, 2.6. 3.9, and 6.5 mM (*R*)-mandelate to WT MDH are shown. The inset shows a double reciprocal plot of the absorbance change at 507 nm versus the total (*R*)-mandelate concentration. The solid line is a fit to eq 1.

adducts. Figure 3 shows the spectral titration of H274G with increasing concentrations of sulfite. A K_d of 13 ± 1 mM was obtained for the H274G protein (inset, Figure 3). For H274A and H274N, the K_ds for the FMN−sulfite adducts were 0.7 ± 0.1 and 42 ± 3 mM, respectively (data not shown). These K_ds are significantly higher than for WT MDH, indicating a possible role of H274 in stabilizing the FMN−sulfite adduct.

Binding of (R)- and (S)-Mandelates and Inhibitors. (*R*)-Mandelate is a competitive inhibitor of WT MDH. We measured an inhibition constant of 2.5 mM for this inhibitor for WT MDH in steady-state experiments. Binding of (*R*)-mandelate perturbs the oxidized FMN spectrum. Figure 4 shows the difference spectra obtained for WT MDH with increasing concentrations of (*R*)-mandelate. Using this spectral titration, a K_d of 3.8 mM was obtained for WT MDH, in reasonable agreement with the kinetically determined value (inset, Figure 4). (*R*)-Mandelate also produced similar spectral perturbations in the H274 mutant proteins—difference spectra obtained for the mutants were very similar to that of WT MDH (not shown). Dissociation constants determined for the binding of (*R*)-mandelate to H274G and H274N in the absence of any imidazole were 22 and 8 mM, respectively.

(*S*)-Mandelate produced very small changes in the oxidized FMN spectrum of the mutant proteins in the absence of any imidazole; thus, we could not reliably measure K_ds for the substrate.

WT MDH has been shown to be inactivated by the irreversible inactivator (*R,S*)-2-hydroxy-3-butynoate with an inhibition constant of 36 mM (*10*). Addition of this inhibitor (up to 80 mM) did not significantly change the spectrum of the H274G mutant, even in the presence of 60 mM imidazole, probably due to a very high inhibition constant.

Chemical Rescue of Activity by Added Imidazoles. The dehydrogenase activity for the H274 mutant proteins was tested with (*S*)-mandelate as substrate and DCPIP as electron acceptor. Replacement of histidine-274 with asparagine, alanine, or glycine rendered the proteins virtually inactive. These mutant enzymes were at least 10^5-fold lower in activity than WT MDH (k_{cat} = 300 s^{-1}), given the limits of detectability of our dehydrogenase assay. However, the activities of the H274G and H274A mutant proteins with (*S*)-mandelate could be partially restored by including imidazole or 4-methylimidazole in the assay buffer. The H274G protein showed the highest levels of chemically rescued activity of the three H274 mutants; hence, it was chosen for further characterization. Monosubstituted imidazoles tested as rescue agents included 1-methyl-, 2-methyl-, and 4-methylimidazoles; only imidazole and 4-methylimidazole were observed to restore activity. Incubation of H274G with the rescue agent prior to the assay did not affect the amount of rescued activity. The rescued activity of H274G obeyed saturation kinetics with respect to the rescuing agents as well as to the substrate, (*S*)-mandelate (Figure 5A,B). At pH 7.5, the apparent K_ms for imidazole and 4-methylimidazole (both protonated and unprotonated forms) were 72 ± 10 and 100 ± 11 mM, respectively, measured in the presence of 40 mM (*S*)-mandelate (Figure 5A). It is to be noted that the apparent K_ms for the free base forms of both imidazole and 4-methylimidazole at pH 7.5 were 56 and 50 mM, respectively (recalculated from Figure 5A). No spectral change could be detected following addition of imidazole alone; this precluded a determination of the dissociation constants for the binding of imidazole or 4-methylimidazole. The extrapolated activities at saturating imidazole and 4-methylimidazole concentrations in the presence of 40 mM (*S*)-mandelate were 0.2 ± 0.01 and 0.06 ± 0.01 s^{-1}, respectively, at pH 7.5 (Figure 5A). The apparent K_m for (*S*)-mandelate was 13 ± 0.9 mM in the presence of 60 mM imidazole, pH 7.8 (Figure 5B). Assuming independent binding of imidazole and (*S*)-mandelate and given this apparent K_m for (*S*)-mandelate, we can estimate the maximum rescued activity of H274G to be ~0.25 s^{-1} with imidazole at pH 7.5. In contrast, WT MDH has a k_{cat} of ~300 s^{-1}.

Substrates other than (*S*)-mandelate were also tested with H274G. Imidazole-assisted activity could be measured with (*R,S*)-*p*-chloromandelate; however, 20 mM (*R,S*)-3-phenyllactate, 60 mM (*R,S*)-2-hydroxy-3-butynoate, or 40 mM (*R,S*)-vinylglycolate were not oxidized at a measurable rate. A k_{cat} of 0.03 s^{-1} was obtained with 50 mM (*R,S*)-*p*-chloromandelate in the presence of 20 mM imidazole, in contrast to a k_{cat} of 0.09 s^{-1} for (*S*)-mandelate under similar conditions. However, the (*R*)-enantiomers appear to be strong

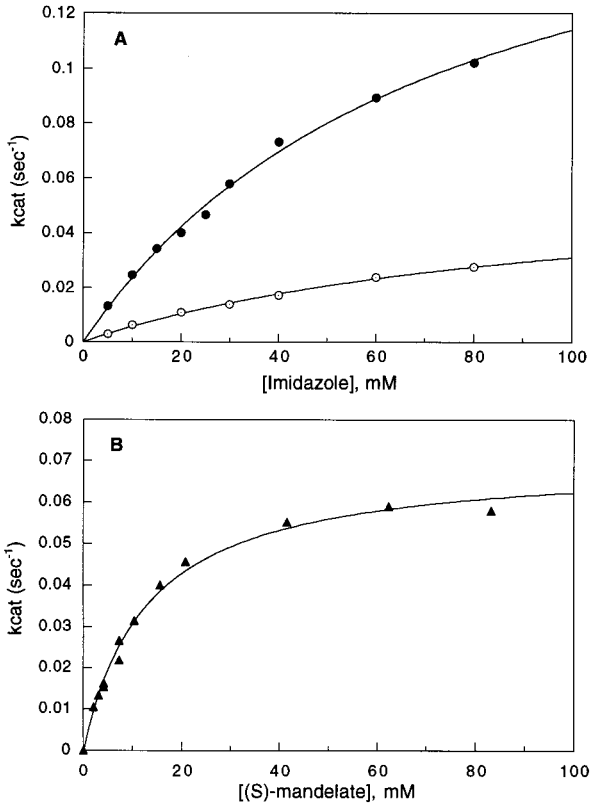

FIGURE 5: Activity of H274G as a function of total imidazole concentration (protonated and unprotonated forms) and (*S*)-mandelate. (A) Determination of the apparent K_ms of imidazole (●) and 4-methylimidazole (○) in the presence of 40 mM (*S*)-mandelate. Activity was measured in 50 mM phosphate, pH 7.5, with 0.1% Triton X-100, 1 mg/mL BSA, and 100 μM DCPIP. (B) Determination of the apparent K_m for (*S*)-mandelate in the presence of 60 mM imidazole, in 50 mM phosphate, pH 7.8, containing 0.1% Triton X-100, 1 mg/mL BSA, and 100 μM DCPIP. The lines are fits to eq 2.

inhibitors of the (*S*)-enantiomeric substrates for the H274 mutants. Hence, the lower activity with (*R,S*)-*p*-chloromandelate relative to (*S*)-mandelate for H274G is possibly due to inhibition by (*R*)-*p*-chloromandelate.

The ability of imidazoles to rescue the activity of both H274A and H274N were also examined. Imidazole was able to rescue the activity of H274A slightly. The activity obtained for H274A in the presence of imidazole and (*S*)-mandelate, both at 80 mM, at pH 7.5, was only 0.015 s^{-1}, much lower than the activity measured for H274G under similar conditions. Due to the very low level, this rescued activity of H274A was not characterized further. There was no measurable activity for H274N with imidazole or 4-methylimidazole.

pH Dependence of the Imidazole-Rescued Activity. The pH dependence of the dehydrogenase activity of H274G in the presence of 60 mM of imidazole or 4-methylimidazole was studied. This imidazole concentration is below the K_ms determined at pH 7.5 (Figure 5A) due to the fact that high concentrations of imidazoles were found to be deleterious for the activity of WT MDH due to the high ionic strength of the buffers. At 60 mM imidazole, the rates were 10−100-fold higher than the background rate and could be measured accurately. Figure 6 shows the pH profiles for k_{cat} for H274G in the presence of imidazole and 4-methylimidazole. For comparison, the pH profile for WT MDH

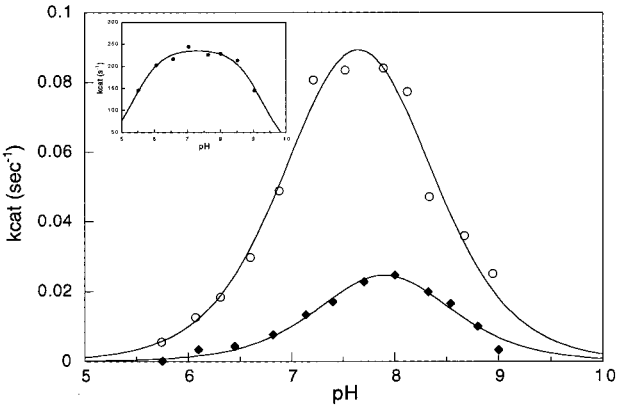

FIGURE 6: pH dependence of the rescued activity for H274G in the presence of 60 mM imidazole (○) or 60 mM 4-methylimidazole (◆). The buffer composition and assay conditions are described in Materials and Methods. The solid lines are smooth lines joining the data points and do not represent fits. Inset: pH profile of the activity of WT MDH in the same buffer containing 60 mM imidazole and 1 mM PMS. The solid line is a fit to eq 3.

measured under these conditions is also shown in the inset in Figure 6. For WT MDH, a residue with a pK_a of 5.3 has to be deprotonated for activity and a second residue with a pK_a of 9.3 has to be protonated for activity. These pK_a values are very close to those obtained under different buffer conditions in a previous study (5.1 and 9.6, respectively) (*10*). The pH profiles obtained for H274G differ significantly from that of WT MDH, especially in the low pH range. For H274G, the lower pK_as obtained for imidazole and 4-methylimidazole are 6.9 and 7.6, respectively. These pK_a values agree well with the solution pK_a values of imidazole (pK_a ∼ 7.0) and 4-methylimidazole (pK_a ∼ 7.5) (ACD Labs). Thus, it is clear that the unprotonated form of the imidazoles are important for restoring the activity of H274G.

The pK_as obtained in the alkaline range of the k_{cat} pH profile for H274G with imidazole and 4-methylimidazole are 8.6 and 8.7, respectively. These values are lower than the pK_a of 9.3 obtained for WT MDH and are similar to the alkaline pK_a obtained in the k_{cat}/K_m pH profile for WT MDH (*10*). Due to the large errors associated with measurement of the extrapolated K_ms for (*S*)-mandelate at saturating imidazole concentrations, the k_{cat}/K_m pH profile for H274G was not measured.

Substrate Kinetic Isotope Effect. WT MDH displays a substrate kinetic isotope effect (KIE) of 2.5 when the activities of α-protio and α-deuterio (*S*)-mandelates are compared (*10*). This KIE is constant at acidic and neutral pHs, increasing at pH > 8.5. The imidazole and 4-methylimidazole-rescued activity of H274G showed KIEs of ∼6.0 in the pH range 6−9.

DISCUSSION

As depicted in Figure 1, structural studies of glycolate oxidase from spinach and flavocytochrome b_2 from *S. cerevisiae* have shown that the FMN-dependent α-hydroxy acid oxidases/dehydrogenases have a conserved histidine at the active site. This histidine is positioned to act as the base that initiates the reaction by abstracting the α-proton to form a carbanion. In the alternative "hydride transfer" mechanism, the substrate would bind with its hydroxyl group pointing toward the histidine, which would then abstract the hydroxyl

proton. In either case, the conserved histidine is critical for catalysis to occur. Consistent with this postulate, the replacement of this histidine, H274 in MDH, by asparagine, alanine, or glycine, resulted in proteins that were unable to catalyze the oxidation of (*S*)-mandelate to benzoylformate within the limits of detection of our assays. Similar results have been reported with two other enzymes in this protein family, lactate monooxygenase and flavocytochrome b_2. The H290Q mutant in lactate monooxygenase had 10^5-fold lower activity compared to the wild-type enzyme. In flavocytochrome b_2, the mutation H373Q resulted in an inactive protein (*8*, *9*).

Enzyme-Bound FMN and the Adduct with Sulfite. To ascertain that the loss in activity for H274G, H274A, and H274N was not due to dramatic structural changes, we characterized these mutants further. The FMN spectra of the three mutants were identical to that of WT MDH. WT MDH has a high pK_a for the N(3) position of the FMN; this pK_a in H274G was unchanged compared to that of WT MDH (*10*). The binding of sulfite to FMN for the three mutants was tested. The FMN−sulfite adduct is stabilized by interactions with many residues at the active site as indicated by structural studies of flavocytochrome b_2 complexed with sulfite, including the active-site histidine (*15*). It was expected that the H274 mutants in MDH would bind sulfite if the overall structure of the active site was intact; however, the dissociation constants for the adducts would increase relative to WT MDH, since the interaction with the histidine side chain would be missing. Consistent with this expectation, all three mutants, H274G, H274A, and H274N, were able to bind sulfite, with K_ds of 13, 0.7, and 42 mM, respectively, compared to a K_d of 30 μM for WT MDH (*10*). Similarly, the H373Q mutant of flavocytochrome b_2 had 10-fold lower affinity for sulfite than the wild-type enzyme whereas, for lactate monooxygenase, the K_d increased 1000-fold in the H290Q mutant (*8*, *9*). It is evident that the histidine contributes significantly to the strength of the FMN−sulfite adduct, but the magnitude of its contribution is different in the various enzymes, perhaps reflecting slightly different modes of interaction among the histidine side chain, the sulfite, and the flavin. The K_d for the FMN−sulfite complex for H274A is ∼25-fold higher than for WT MDH, whereas the K_d for H274G increased 450-fold relative to WT MDH. These differences are probably due to both enthalpic as well as entropic effects, that is, the absence of favorable interactions with the histidine side chain as well as steric factors. The mutations H274G and H274A are likely to create large cavities at the active site that may not allow strong interactions of the adduct with other active-site residues. Surprisingly, the affinity for sulfite decreased most for the semiconservative mutation, H274N. It is possible that the electron-rich side chain of the asparagine residue disfavors binding of the sulfite or decreases the electrophilicity of the flavin. The imidazole side chain of histidine becomes protonated when the adduct is formed; however, the amide side chain of asparagine cannot be protonated (*15*).

Binding of (R)- and (S)-Mandelates. (*R*)-Mandelate is a competitive inhibitor of WT MDH with an inhibition constant of 2.5 mM determined in steady-state kinetic experiments and 3.8 mM as measured from perturbations of the FMN spectrum upon binding. WT MDH has a K_d of 0.2 mM for (*S*)-mandelate, which is equal to the K_m (B.M. unpublished observation); hence the binding of (*R*)-mandelate is ∼15-fold weaker than that of (*S*)-mandelate for WT MDH. The binding of (*R*)-mandelate to the H274 mutants produced difference spectra that were very similar to WT MDH. The dissociation constants for H274G and H274N were 22 and 8 mM, respectively, ∼7- and ∼3-fold higher than for WT MDH. Thus, the active sites of WT MDH and the mutant enzymes bind (*R*)-mandelate in similar ways, and H274 appears to contribute only weakly to the binding of (*R*)-mandelate. Due to the very small changes produced in the FMN spectra on binding of (*S*)-mandelate, we could not reliably measure dissociation constants for the mutants. However, the apparent K_m for (*S*)-mandelate obtained with H274G in our rescue experiments is ∼13 mM in contrast to a K_m of 0.2 mM for WT MDH. Given the dependence of the rescued rate on the chemical bond-breaking step as indicated by the high KIE, it is probable that the apparent K_m represents the apparent K_d for (*S*)-mandelate for H274G. Therefore, in contrast to the binding of (*R*)-mandelate, H274 does appear to contribute significantly to the binding of (*S*)-mandelate. In fact, our results indicate that the 15-fold weaker binding of (*R*)-mandelate relative to (*S*)-mandelate in WT MDH may partly result from an interaction of H274 with (*S*)-mandelate that is absent when the (*R*)-enantiomer binds. It is to be noted that the H373Q mutation in flavocytochrome b_2 resulted in a 10-fold weaker binding of D-lactate and a 2-fold weaker binding of the product, pyruvate (*9*). The homologous mutation, H290Q in lactate monooxygenase, produced a 2-fold increase in the K_d for D-lactate binding and a 2.5-fold *increase* in the binding affinity for L-lactate (*8*). This last observation is in contrast to our findings in this study.

The similarities between the FMN spectra of all three mutants and WT MDH, as well as the formation of the sulfite adduct, confirm that the overall structure of the active site surrounding the FMN cofactor has been relatively unaltered by the mutations. The mutants were able to bind (*R*)-mandelate with affinities similar to that of WT MDH, though the binding of (*S*)-mandelate appears to be considerably weaker.

Complete Inactivation Due to Removal of Histidine and Rescue by Exogenous Imidazoles. The replacement of H274 by alanine, asparagine, and glycine completely abolished activity. Given the limits of our assay system, the mutant proteins are at least 10^5-fold less active than WT MDH. This dramatic loss in activity appears to be due to the removal of the imidazole side chain of H274 and not because of any gross structural changes in the active site. The H274N mutation is expected to be a semiconservative mutation. Structurally, asparagine can substitute well for a histidine because the side chain can form hydrogen bonds with neighboring residues or ligands. However, asparagine cannot be protonated and thus cannot act as a base. In the H274A and H274G mutations, large cavities are expected to be formed in the place occupied by the imidazole side chain.

We were able to partially rescue the activity of the H274A and H274G mutants by exogenous imidazoles but not of H274N. The extrapolated maximum activity obtained at saturating imidazole and (*S*)-mandelate concentrations for H274G is 0.25 s^{-1} or ∼0.1% of the wild type activity. This is low but significant because it represents at least a 100-fold increase over the rate in the absence of imidazole; this imidazole-dependent increase is probably much higher since

the H274G mutant is virtually dead. The rate of oxidase activity of WT MDH is ~0.1 s^{-1}, compared to a rate of FMN reduction of ~300 s^{-1} (Y. Xu and B.M. unpublished observation). In the case of H274G, the rescued activity of ~0.25 s^{-1} is of similar magnitude to the oxidase activity of ~0.1 s^{-1}, as evidenced from Figure 2, where the FMN spectrum does not get fully reduced in aerobic buffers; in other words, the rate of imidazole-dependent reduction of FMN by (*S*)-mandelate is similar to the rate of its reoxidation by oxygen. Thus the $FMNH_2$ reoxidation reaction appears not to be affected in this mutant protein. Similarly, the H290Q mutant in lactate monooxygenase was as competent as the wild-type protein in the reoxidation of the reduced flavin (*8*). H274G exhibited saturation behavior with respect to both imidazole and 4-methylimidazole; this suggests the formation of a ternary complex between the enzyme, the imidazole, and the substrate. Such saturation behavior is not always observed in rescue experiments but usually indicates the requirement for the formation of a discrete complex prior to catalysis. An example of similar saturation behavior has been observed in the chemical rescue of the K329A mutant of ribulose 1,5-bisphosphate carboxylase (*16*). In the case of MDH, following abstraction of a proton from the substrate by H274, electrons are transferred to FMN either from the carbanion intermediate or directly as a hydride ion. Therefore, we expect that for catalysis to occur, the imidazoles are required to bind to the active site, presumably in the cavity created by the mutations, in close juxtaposition to FMN and other critical catalytic residues. For example, if a carbanion intermediate is indeed formed, it has to be generated in such a way that it is stabilized through interactions at the active site and is also able to transfer its electrons to FMN. Therefore, for the reaction to proceed, the reactive components—imidazole, FMN, and substrate—have to be held in a proper orientation relative to each other at the active site, sequestered from the solvent. Hence, formation of a ternary complex is essential.

Though a number of imidazoles and amines were tested, only imidazole and 4-methylimidazole were able to restore activity; 1-and 2-methylimidazoles could not rescue activity at a detectable level. An explanation for this observation may lie in the presence of an aspartate residue in the active site, D158, that forms a catalytic dyad with H274 (Figure 1). This aspartate has been proposed to correctly orient the catalytic histidine as well as neutralize the charge on the imidazolium cation following proton abstraction (*17*). The interaction between the histidine and the aspartate may explain why 1- and 2-methylimidazoles were unable to rescue activity. 1-Methylimidazole may bind to the active site such that the incorrect tautomer is presented to the substrate. 2-Methylimidazole may also not be properly oriented for catalysis, even though it may bind just as well as imidazole. Only 4-methylimidazole, which most closely mimics the side chain of histidine, and imidazole were found to rescue activity. The apparent K_ms for the free base forms of both imidazole and 4-methylimidazole were ~50 mM, indicating that both the rescue agents bind with similar affinity. However, imidazole proved to be more efficient at rescue than 4-methylimidazole. This may again be due to a more favorable geometry achieved by imidazole, (*S*)-mandelate, and the enzyme relative to 4-methylimidazole. Therefore, the complete lack of activity observed with 1- and 2-methylimidazoles may indicate that these ligands were unable to form a catalytically competent ternary complex with H274G. Unfortunately, we were unable to measure binding of the imidazoles directly due to the lack of any spectroscopic change in the mutant proteins upon binding and, therefore, cannot be certain whether 1- and 2-methylimidazoles bind and do not rescue activity or whether they do not bind at all. Rescue of activity by imidazole was not very efficient for the H274A mutant and undetectable for the H274N mutant. This is probably due to the fact that, only in the case of H274G, the cavity created at the active site is large enough to accommodate imidazole.

We were unable to detect any rescued activity with substrates other than (*S*)-mandelate and ring-substituted mandelates. This is not surprising considering that β-saturated as well as small substrates for WT MDH have k_{cat}s ranging from 0.06 to 1.2 s^{-1}, at least 500-fold lower than for (*S*)-mandelate (*10*). Therefore, if the stringent substrate specificity displayed by WT MDH is preserved in the H274G mutant protein, the rescued activity with substrates such as 3-phenyllactate and vinylglycolate would be too low to be detectable. (*R,S*)-2-Hydroxy-3-butynoate is a substrate as well as an inhibitor of WT MDH with a 150-fold lower k_{cat} and a 65-fold higher K_m than (*R,S*)-mandelate. In the presence of 60 mM imidazole, H274G did not utilize (*R,S*)-2-hydroxy-3-butynoate as a substrate even at the highest concentration tested, 80 mM. Not surprisingly, we did not detect any time-dependent inactivation of H274G by this inactivator in the presence of the rescue agent. The lack of activity with (*R,S*)-2-hydroxy-3-butynoate may be due to a very low k_{cat} or a very high K_m or both.

pH Dependence of Rescued Activity. In a previous study with alternate substrates and substrate kinetic isotope effects, we have shown that MDH appears to follow a mechanism involving formation of a carbanion intermediate which results from abstraction of the α-proton by an active-site base (*10*). Using pH-dependence studies of k_{cat}, k_{cat}/K_m, and substrate isotope effects, we concluded that, in WT MDH, a residue with a pK_a of ~5.5 has to be deprotonated for activity, while a residue with a pK_a ~8.9 has to be protonated for activity in the free enzyme. These pK_as were shifted to 5.1 and 9.6 in the enzyme−substrate complex. Using competitive inhibitors, we demonstrated that the higher pK_a belongs to R277 or R165. The lower pK_a appeared to be that of H274, though we had no direct proof for this assignment. Therefore, in this work, we studied the pH dependence of the activity rescued by imidazoles for the H274G mutant, to confirm that the assignment of the pK_a of 5.0−5.5 to H274 was correct.

From the postulated role of H274, the activity is expected to be dependent on the free base form of the imidazoles. Therefore, if the pK_a of H274 is indeed ~5.1 in the enzyme−substrate complex, we should see shifts of the lower pK_a from 5.1 to that of the rescue agent in the pH profiles of the imidazole-rescued activities of the H274G mutant. Consistent with this expectation, the rescued activity of H274G decreases at low pH with pK_as of 6.9 and 7.6, for imidazole and 4-methyl imidazole, respectively. The solution pK_as of imidazole and 4-methyl imidazole are 7.0 and 7.5, respectively, showing that the rescued activity is dependent on the unprotonated form of the exogenous imidazoles. Thus the pH dependence of the rescued activities of H274G clearly

establishes that the pK_a of 5.0−5.5 seen in the pH profile of WT MDH is due to H274. We cannot eliminate the possibility that the protonated forms of the imidazoles also bind to the active site, since we were unable to measure binding of imidazole directly; however, protonated imidazoles would not be able to rescue activity.

The pH profile of H274G in the alkaline range deserves mention. The alkaline pK_a obtained for H274G with the two imidazoles, 8.6−8.7, is somewhat lower than that obtained for WT MDH under the same condition, 9.3. In our previous pH studies of k_{cat}/K_m and k_{cat} for WT MDH, we have shown that the free enzyme has a pK_a of 8.9, assigned to R277 or R165, that is elevated to 9.6 when the substrate, (*S*)-mandelate, binds. The pK_a of 8.6−8.7 obtained for the rescued activity of H274G is from the k_{cat} pH profile and, therefore, represents a pK_a in the enzyme−substrate complex. This pK_a is similar to the pK_a of the free enzyme for WT MDH. Therefore, unlike WT MDH, substrate-binding does not appear to raise the pK_a of the active-site arginine. This is not surprising considering that (*S*)-mandelate binding appears to have been affected by the H274G mutation, and therefore, the substrate may not interact with the active-site arginine in the same way as in WT MDH. We did not obtain the k_{cat}/K_m pH profiles for the H274G mutation because of the large errors associated with measuring the extrapolated K_ms for (*S*)-mandelate at saturating imidazole concentrations.

WT MDH has a substrate KIE of 2.5 over the optimal pH range, indicating that the carbon−hydrogen bond-breaking step is only partly rate-limiting (*10*). A higher KIE of 5−6 is obtained with slow substrates, for which this step does become fully rate-limiting (*10*). The KIE for the rescued activity of H274G was ∼6 in the pH range 6−9, for both imidazole and 4-methylimidazole. This shows that the carbon−hydrogen bond-breaking step is fully rate limiting when imidazole acts as a surrogate base for H274. This result and the low levels of rescued activity obtained suggest that though exogenous imidazoles are able to bind at the active site and catalyze the reaction, the complex of imidazole and substrate is not in the exact position occupied by the substrate and the side chain of H274 in the wild-type enzyme. This conclusion is supported by the inability of 1-methyl and 2-methylimidazoles to rescue activity; these may bind to the active site as well as imidazole but not in the correct geometry.

There have been many examples of chemical rescue experiments in the recent literature; the extent of restored activity varies greatly among enzymes and rescue agents. Other instances where mutants of a histidine residue have been rescued by added imidazoles or amines include but are not limited to the following: the H42A and H170A mutations in horseradish peroxidase (*18*, *19*), the H44A mutant of bacterial luciferase (*20*), and the H64A mutant of carbonic anhydrase (*21*). For bacterial luciferase, as well as for the H42A mutant of peroxidase, activity could be restored by a number of imidazoles as well as amines in the former case. In these cases, a Bronsted analysis could be performed to calculate the charge developed on the imidazole in the transition state. Unfortunately in our case, only two imidazoles were able to restore activity; hence a Bronsted analysis was not possible.

Conclusion. The postulated active-site base in MDH, H274, was mutagenized to glycine, alanine, and asparagine. All three mutant proteins were totally inactive. The FMN cofactor in the mutant proteins appeared to be unaffected relative to WT MDH. They were able to form adducts with sulfite, though with lower affinity. Binding studies indicate that H274 does not contribute significantly to the binding of (*R*)-mandelate; however, it does play an important role in the binding of the substrate, (*S*)-mandelate. The activity of H274G and, to a lesser extent, H274A could be rescued by exogenously added imidazoles—the first demonstration of chemical rescue of a catalytic residue in the flavin-dependent oxidase/dehydrogenase enzymes. Rescue of activity was dependent upon formation of a ternary complex of imidazole, substrate, and the enzyme. pH-dependence studies show that the free base forms of the imidazoles are the rescue agents. Additionally, pH-dependence studies of the rescued activities of H274G presented in this work, together with pH profiles of WT MDH measured in a previous study, confirm that H274 has a pK_a of ∼5 in the enzyme−substrate complex and it is the active-site base that initiates the reaction.

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