The Roles of Two Amino Acid Residues in the Active Site of 
L-Lactate Monoxygenase

MUTATION OF ARGININE 187 TO METHIONINE AND HISTIDINE 240 TO GLUTAMINE*

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L-lactate monoxygenase (LMO) catalyzes the conversion of L-lactate to acetate, CO₂, and water with the incorporation of molecular oxygen. Arginine 187 of LMO is highly conserved within the family of l-α-hydroxyacid oxidizing enzymes (Lē, K. H. D., and Lederer, F. (1991) J. Biol. Chem. 266, 20877-20881). By comparison with the equivalent residue in flavocytochrome b₂ from Saccharomyces cerevisiae (Pike, A. D., Chapman, S. K, Manson, F. D. C., Reid, G. A., Gondry, M., and Lederer, F. (1996) in Flavins and Flavoproteins (Stevenson, K. J., Massey, V., and Williams, C. H., Jr., eds) pp. 571-574, University of Calgary Press, Calgary, AB, Canada), arginine 187 might be expected to have an important role in catalytic efficiency and substrate binding in LMO. Histidine 240 is predicted to be close to the substrate binding site of LMO, although it is not conserved within the enzyme family. Arginine 187 has been replaced with methionine (R187M), and histidine 240 has been replaced with glutamine (H240Q).

L-lactate oxidation by R187M is very slow. The binding of L-lactate to the mutant enzyme appears to be very weak, as is the binding of oxalate, a transition state analogue. The binding of pyruvate to the reduced enzyme is also very weak, resulting in complete uncoupling of enzyme turnover, with H₂O₂ and pyruvate as the final products. In addition, anionic forms of the flavin are unstable. The Kₗ for sulfite is increased nearly 400-fold by this mutation. The semiquinone form of R187M is also thermodynamically unstable, although the overall midpoint potential for the two-electron reduction of R187M is only 34 mV lower than for the wild-type enzyme. H240Q more closely resembles the wild-type enzyme. The steady-state activity of H240Q is completely coupled. The kₐₜ is similar to that for the wild-type enzyme.

L-lactate monoxygenase (LMO) from Mycobacterium smegmatis catalyzes the oxidation of L-lactate to pyruvate (1). It is a member of the FMN-dependent family of enzymes that catalyze the oxidation of L-α-hydroxy acids, including L-lactate oxidase (2), flavocytochrome b₂ (3), glycolate oxidase (4), l-mandelate dehydrogenase (5, 6), and long chain a-hydroxy acid oxidase (7). LMO is unique within this family in that dissociation of the initial oxidation product, pyruvate, occurs much more slowly than the reaction of the reduced enzyme-pyruvate complex with oxygen (1, 8, 9). The resultant H₂O₂ decarboxylates pyruvate within the active site of the enzyme and the final products, acetate, CO₂, water, are then released (Fig. 1, inner pathway). The other members release their initial oxidation product rapidly from the reduced enzyme, resulting in the α-keto acid and H₂O₂ as final products in the outer, or uncoupled, pathway.

The gene for LMO in M. smegmatis has been cloned and sequenced (10). The peptide sequence shows considerable homology with other members of this enzyme family (7). On the basis of the known structures of flavocytochrome b₂ (11, 12) and glycolate oxidase (4), which show a strong similarity in the folding pattern around the flavin (13), a model of the active site of LMO has been made (Fig. 2). A number of conserved amino acids have been assigned putative roles in LMO (10), which have been tested by site-directed mutagenesis. Histidine 290 was proposed to be the active site base responsible for the abstraction of the α-proton from L-lactate to form a carbanion intermediate during catalysis (14, 15). Mutation of histidine 290 to glutamate (16) resulted in an enzyme that was able to bind lactate but was unable to catalyze oxidation of L-lactate to pyruvate. Lysine 266 was proposed to be placed near to the N(1)-C(2)O locus of the flavin and responsible for the tight binding of sulfite to LMO (17, 18), as well as stabilization of the flavin anemic semiquinone (19). Mutation of this residue to methionine (20) resulted in a 17,000-fold increase in the Kₗ for sulfite binding to the enzyme and a thermodynamically unstable flavin anemic semiquinone with an unusual absorbance spectrum. The rate of reduction of this mutant form with L-lactate was also significantly decreased as compared with the wild-type enzyme. Three amino acid residues were suggested to be in suitable positions to form hydrogen bonds with L-lactate and thus aid substrate binding. These are tyrosine 44, tyrosine 152, and arginine 293. Both of these tyrosine residues were mutated to phenylalanine, and arginine 293 was replaced with lysine (21). In each case, the binding affinity of L-lactate for the mutant enzyme did not decrease, but the rates of reduction with L-lactate decreased significantly. The stability of the oxalate-enzyme complex, a transition-state analog (14), was also found to be compromised to different extents with the mutant enzymes, and a linear relationship between kₐₜ for L-lactate and Kₗ for oxalate binding was found (21). This provided a clear demonstration that a number of individual residues contribute to the lowering of the energy of the transition state.

Arginine 187 in LMO is also completely conserved within this enzyme family (7). The known structures of recombinant flavocytochrome b₂ (12) and glycolate oxidase in the presence

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‡ The abbreviations used are: LMO, l-lactate monoxygenase; roseo-FAD, 8-dimethylamino-FAD; MMTS, methyl methane-thiosulfonate.
Active Site Mutations of L-Lactate Monoxygenase

EXPERIMENTAL PROCEDURES

Materials and instrumentation were as described previously (24). All experiments were carried out in 10 mM imidazole-HCl buffer, pH 7.0, and at 25 °C, unless otherwise indicated. Enzyme solutions were protected from light as much as possible because LMO is susceptible to photoreduction to the semiquinone species.

Site-directed Mutagenesis—The vectors used for the production of R187M and H240Q and the expression plasmid were as described earlier (20). The mutagenesis of arginine 187 (codon CGG) to methionine was performed with the oligonucleotide 5'-TTCTGGTTGATGCCTAGGGGACCTCTACGA-3' (the codon for methionine is underlined). A silent mutation (marked with an asterisk) was also introduced at residue aspartate 190 to create a DraII site (5'-GGGNCCCT-3'). The mutagenesis of arginine 187 to lysine was performed with the oligonucleotide 5'-TTCTGGTTGAGCCAGGTATCCTACA-3' (the codon for lysine is underlined). A silent mutation (marked with an asterisk) was also introduced for the codon at residue proline 188 to eliminate a StyI site (5'-CCAAGG-3'). The mutagenesis of histidine 240 (codon CAC) to glutamine was performed with the oligonucleotide 5'-CGGATTCTCTTGGGAGGGGCCCTGTCCCT-3' (the codon for glutamine is underlined). This mutation also introduced a DraII site. Successful mutagenesis was screened for by the addition of a DraII site (R187M or H240Q) or elimination of a StyI site (R187K). The SacI-Xcm fragment (486 base pairs), which contains both mutation sites, was ligated into the appropriate position of the expression plasmid pUM01 to give new plasmids pSAS1 (R187M) and pSAS2 (H240Q). Successful insertion was confirmed by digesting with EcoRI and HpaII (R187M or H240Q), which recognizes the DraII site, or with StyI (R187K), and by plasmid sequencing.

Cell Growth and Purification of R187M and H240Q—Escherichia coli strain BL21(DE3), pLysS containing the plasmid pSAS1 or pSAS2 was grown in six 1-liter flasks of Terrific Broth and purified as described previously (20). A typical wet weight yield of cells was 30–55 g. Most of the mutant proteins were obtained in the form of inclusion bodies. Only the soluble fraction was isolated, with a typical yield of 30–40 mg of enzyme. The purity of the protein was determined by SDS-polyacrylamide gel electrophoresis (25) and staining with Coomassie Blue.

Extraction Coefficient—The extinction coefficients for mutant enzymes were determined by denaturing the protein with SDS. 10 µl of 10% SDS was added to 1 ml of enzyme solution. The enzyme solution was then incubated at 45 °C for 10 min. The extinction coefficient for the enzyme-bound flavin was determined from the change in absorbance spectrum due to the release of FMN. The concentration of free FMN was calculated using an extinction coefficient of 12,500 M⁻¹ cm⁻¹ at 445 nm.

Reaction of R187M with Sulfite—Additions of a concentrated stock solution of sodium sulfite were made to 1-ml samples of 14 µM R187M to a final sulfite concentration in the range of 5–100 µM. The reactions were followed by bleaching of the enzyme absorbance spectrum, and the decrease in absorbance at 460 nm with time. The reactions were followed by an enzyme-scraping system consisting of photoreduction to the semiquinone species. One syringe of the stopped flow apparatus was filled with an enzymatic oxygen-scraping system consisting of proatechuate dioxygenase (0.2 units ml⁻¹) and protocatechuate (200 µM) to ensure anaerobiosis prior to each experiment. The apparatus was then flushed with anaerobic buffer before loading enzyme and substrate solutions.

Reductive Half-reaction—A solution of R187M at a concentration of 19 µM, containing protocatechuate dioxygenase (0.2 units ml⁻¹), was treated with alternate cycles of evacuation and re-equilibration with oxygen-free argon in a tonometer. Protocatechuate (200 µM) was then added from a side-arm in order to maintain anaerobiosis. One syringe of the stopped flow apparatus was filled with the anaerobic enzyme solution, and the other syringe was filled with an anaerobic solution of l-lactate. The l-lactate was made anaerobic in its syringe by bubbling with oxygen-free argon for 15 min. l-Lactate concentrations in the range of 100–500 mM (before mixing) were used. The total ionic strength of each l-lactate solution was made up to 600 mM by addition of the appropriate concentration of ammonium sulfate. The reduction of enzyme-bound flavin was followed by the decrease in absorbance at 460 nm.

Oxidative Half-reaction—R187M was reduced enzymatically with xanthine oxidase, xanthine, and benzyl viologen. The enzyme solution at a concentration of 19 µM, containing 0.7 µM xanthine oxidase and 1.4

FIG. 1. Kinetic scheme showing the steps involved in the turnover of lactate. The outer loop shows the steps involved in the “coupled” pathway normally carried out by lactate monoxygenase, which results in acetate, carbon dioxide, and water as the final products. The inner loop shows the “uncoupled” pathway, which results in pyruvate and H₂O₂ as the final products.

FIG. 2. Model of the active site of L-lactate monoxygenase based on the known structure of glycolate oxidase (4). The model shows the α-carbon atoms, the side chains of a number of conserved amino acid residues, and the enzyme-bound FMN. Hydrogen atoms have been omitted for clarity.

(22) and absence (4) of bound active site inhibitors show that the equivalent residues in these enzymes (arginine 289 and arginine 164, respectively) are close to the active site and may therefore be involved in catalysis. Mutation of arginine 289 to lysine in flavocytochrome b₂ (23) resulted in a 4-fold increase in kₘ toward l-lactate and a 12-fold decrease in kₐ in steady-state assays.

In the present study, arginine 187 has been replaced with methionine. Attempts were also made to replace arginine 187 with lysine. However, the protein was expressed only in the form of inclusion bodies, and no soluble enzyme was obtained. Methionine has similar spatial requirements to arginine but lacks the positive charge of the guanidinium side chain. The effects of this mutation on the redox properties, and on the binding of substrate and a transition state analog to the enzyme, are discussed.

Histidine 240 has no equivalent histidine residue in lactate oxidase or any other α-hydroxy acid oxidase. However, the present active site model of LMO places this residue in reasonably close proximity to the substrate binding site. Histidine 240 was therefore replaced with glutamine in order to probe potential interactions with the substrate and potential role of this residue in the modulation of oxidase/monoxygenase activity.
μM benzyl viologen, was treated with alternate cycles of evacuation and reequilibration with oxygen-free argon in a tonometer. Xanthine (470 μM) was then added from a side arm, and the anaerobic enzyme solution was left to reach full reduction overnight. One syringe of the stopped flow apparatus was filled with the reduced enzyme solution, and the other was filled with 10 mM imidazole-HCl buffer, pH 7.0, that had been bubbled with varied concentrations of oxygen for 15 min. The oxidation of enzyme-bound flavin was followed by the increase in absorbance at 460 and 375 nm.

Steady-state Production of H₂O₂ by R187M—H₂O₂ formation by R187M was measured colorimetrically by the oxidation of o-dianisidine in the presence of horse radish peroxidase as described previously (9). L-Lactate concentrations in the range of 50–250 mM were used, and the total ionic strength of the assays was made up to 300 mM with ammonium sulfate. All assays were carried out in air-saturated buffer.

Steady-state Activity of H240Q—Turnover was measured by following the enzyme absorbance at 468 nm in a stopped flow apparatus. L-Lactate concentrations in the range 2.5–160 mM were used in air-saturated buffer, and so oxygen concentration was limiting. Under these conditions, a steady-state level of reduced enzyme is determined by the relative rates of reduction by l-lactate and oxidation by oxygen, and the absorbance at 458 nm is an indicator of the amount of oxygen consumed at any given time. The data were analyzed according to the method of Gibson et al. (27) to yield values for $k_{cat}$, $K_{m}$, and $K_{cat}/K_{m}$.

Measurements of the $E_{m}/E_{r}$ Redox Potential of R187M by Dye Equilibration—Samples of R187M were slowly reduced with xanthine oxidase, xanthine, and benzyl viologen in the presence of either roseo-FAD (midpoint redox potential $(E_m)_{\text{ox}} = 246$ mV at pH 7.0) or anthraquinone-2,6-disulfonate $(E_m)_{\text{red}} = 184$ mV at pH 7.0). A 1.16-ml sample of 14 mM R187M containing 35 mM xanthine oxidase, 1 mM benzyl viologen, and redox dye (3.3 μM roseo-FAD or 20 μM anthraquinone-2,6-disulfonate) was treated with alternate cycles of evacuation and re-equilibration with oxygen-free argon in an anaerobic cuvette. Reduction of the system was then started by the addition of 36 μl of 10 mM xanthine from a side arm, and the reaction was followed by changes in the absorbance spectrum between 300 and 700 nm. The extent of reduction of roseo-FAD was estimated from the change in absorbance at 509 nm, which is isobestic for the reduction of oxidized enzyme to the semiquinone. The extent of semiquinone formation in the presence of roseo-FAD was estimated from the absorbance changes 375 nm, once the absorbance changes at this wavelength due to dye reduction were subtracted using a ratio of absorbance changes at 509 nm to changes at 375 nm of 8.7.

The extent of reduction of anthraquinone-2,6-disulfonate was estimated from the change in absorbance at 418 nm, which is isobestic for the reduction of the oxidized enzyme to the semiquinone. The extent of enzyme semiquinone formation in the presence of anthraquinone-2,6-disulfonate was estimated from the absorbance changes at 373 nm, once the absorbance changes at this wavelength due to dye reduction were subtracted using a ratio of absorbance changes at 418 nm to changes at 373 nm of 8.7. In order to calculate the difference in redox potential between the dye and the $E_{m}/E_{r}$ couple, the log (ox/red) of the dye was plotted versus the log (ox/sq) of the enzyme-bound flavin according to the method of Minnert (28).

Photooxidation and Stability of the Semiquinone—The mutant enzymes were reduced photochemically using glycine as a photoreductant (30). The mutants were mixed in a spectrophotometer cell (final concentration, 50 μM to 0.5 mM). Ammonium sulfate was added to the oxalate solution to bring the final concentration of oxalate plus ammonium sulfate to 0.5 M in order to maintain a constant ionic strength for all of the data points. Binding of oxalate to the enzyme was followed by recording the absorbance spectrum between 300 and 700 nm until no further change was noted.

RESULTS

Spectral Properties—The absorbance spectra of R187M in its oxidized, semiquinone, and reduced forms are shown in Fig. 3. The spectra of all three species are like those of the wild-type enzyme. The spectrum of oxidized R187M has absorbance peaks at 460 nm ($ε = 10,750$ M⁻¹ cm⁻¹) and at 373 nm and a ratio of $A_{290}/A_{460}$ of 10.6. The spectrum of the semiquinone form of the enzyme is typical of an anionic semiquinone, with absorbance peaks at 372 nm ($ε = 15,530$ M⁻¹ cm⁻¹) and a sharper peak at 404 nm ($ε = 13,150$ M⁻¹ cm⁻¹). A plot of $A_{460}$ versus $A_{290}$ (Fig. 3, inset) indicates that the spectrum shown in Fig. 3 represents at least 90% formation of the anionic semiquinone.

The absorbance spectrum of H240Q as isolated from E. coli is shown in Fig. 4. It is similar to the wild-type enzyme, with peaks at 372 and 458 nm. However, there is a slight shoulder at 350 nm, and the extinction coefficient at 458 nm is lower than for the wild-type enzyme, with estimates varying from 8300 to 9400 M⁻¹ cm⁻¹ from different batches of enzyme. Another mutant LMO, R293K (21, 29) was isolated in a form with an extinction coefficient at 456 nm of 6,700 M⁻¹ cm⁻¹. On treatment with methyl methane-thiosulfonate (MMTS), the $A_{460}$ of R293K increased 1.4-fold. This phenomenon was therefore attributed to part of the flavin being in an equilibrium N(5) adduct with a protein cysteine residue (29). On treatment with MMTS, the extinction coefficient of H240Q at 458 nm increased to 11,500 M⁻¹ cm⁻¹ (Fig. 4). The semiquinone spectrum of H240Q closely resembles that of the wild-type enzyme and of R187M, with peaks at 372 and 404 nm (data not shown). The spectrum of H240Q fully reduced with l-lactate is also essentially identical to those of wild-type enzyme and of R187M.

Sulfite Binding to R187M—The absorbance changes observed on the addition of sulfite to R187M (Fig. 5) are typical of the formation of a flavin N(5)-sulfite adduct (16, 17). However, the rate of formation of this adduct and the concentration of sulfite required for its formation indicate that sulfite binding has been adversely affected by this mutation. The association of sulfite with the enzyme is sufficiently slow to allow measurement of its rate in a standard spectrophotometer, and a plot of apparent first order rate constant versus sulfite concentration (Fig. 5, inset) yields a second order rate constant of $k_{on} = 11.6$ M⁻¹ s⁻¹ ("wild-type enzyme, 1.29 × 10⁴ M⁻¹ s⁻¹"). From the y intercept, $k_{off}$ is determined as $2.5$ × $10^{-4}$ s⁻¹ ("wild-type enzyme, 7.2 × $10^{-4}$ s⁻¹). Thus, the $K_d$ estimated from kinetic measurements is 21.5 μM, a value much higher than that determined in the same way for the wild-type enzyme, of 5.6 × $10^{-4}$ μM (1). The observed 380-fold increase in $K_d$ is thus due largely to a decrease in the rate constant of association. The $K_d$ can also be estimated from the total change in $A_{460}$ from the
reaction at the same sulfite concentrations and yields a $K_d$ of 22.2 $\mu M$. This value is in excellent agreement with the kinetically determined value.

**Reductive Half-reaction of R187M**—Under anaerobic conditions, R187M is reduced slowly by $L$-lactate (Fig. 6). The reaction was monitored in a stopped flow apparatus by the decrease in flavin absorbance at 460 nm with time. The observed reaction is monophasic, with little or no saturation behavior observed. A linear fit of $k_{obs}$ versus lactate concentration over the range 50–250 $\mu M$ yields a second order rate constant of 14.7 M$^{-1}$ min$^{-1}$. A hyperbolic fit of the same data yields an apparent $K_d$ for L-lactate of 0.9 $\mu M$ and a limiting rate of 0.27 min$^{-1}$. These data contrast greatly with those obtained for wild-type LMO, in which biphasic reactions were observed (9). The first phase of the reaction showed a clear hyperbolic dependence on lactate concentration, yielding a dissociation constant of 50 $\mu M$ for the binding of L-lactate to the enzyme, and a limiting rate of 230 s$^{-1}$. The rate of the second phase of the reaction was independent of lactate concentration and was shown to be due to the dissociation of the reduced enzyme-pyruvate complex. The present data show no evidence of a reduced enzyme-pyruvate complex.

**Oxidative Half-reaction of R187M**—The reaction of $O_2$ with reduced R187M was monitored by the increase in flavin absorbance at 460 nm and at 375 nm with time. The reaction at 460 nm appeared to be biphasic. The major phase of the reaction resulted in over 90% of the total absorbance change and shows a linear dependence on $O_2$ concentration. The smaller phase of the reaction is very difficult to fit accurately and was attributed to the presence of a small amount of enzyme-bound flavin semiquinone in the sample. This would have arisen from slight reoxidation of the sample by traces of $O_2$ in the stopped flow apparatus, followed by the one-electron reduction of the reoxidized enzyme by the xanthine-xanthine oxidase system used to reduce the enzyme (see under “Experimental Procedures”). The reaction monitored at 375 nm is monophasic and shows the same $O_2$ dependence as the major phase of the reaction observed at 460 nm. Plots of $k_{obs}$ versus $[O_2]$ (data not shown) yield a second order rate constant of $2.6 \times 10^5$ and $2.5 \times 10^5$ M$^{-1}$ min$^{-1}$ at 460 and 375 nm, respectively (wild-type enzyme, $5 \times 10^5$ M$^{-1}$ min$^{-1}$).

**Steady-state Production of $H_2O_2$ by R187M**—The rate of formation of $H_2O_2$ was determined by the oxidation of o-diaminobenzidine in the presence of horseradish peroxidase in air-saturated buffer. Measurements were made at L-lactate concentrations in the range 50–250 $\mu M$. The rates of formation of $H_2O_2$ are similar to the pseudo-first order rate constants measured for the reaction of lactate with R187M in Fig. 6. A plot of turnover versus L-lactate concentration has a slope of $14.7$ M$^{-1}$ min$^{-1}$ (data not shown). These data suggest that the reduction of lactate is rate-limiting during enzyme turnover (consistent with the present data on the separate half-reactions) and that all of the enzyme turnover gives rise to $H_2O_2$ as a final product.

**Redox Properties of R187M**—The spectra in Fig. 3 indicate that the one-electron reduction of R187M proceeds via the formation of approximately 90% of an anionic semiquinone. Thermodynamically, this would suggest that the redox potential of the $E_{eq}$, $E_{o-x}$ couple is 150 mV below that of the $E_{eq}$, $E_{o-x}$ couple. However, the apparent stability of the semiquinone form of R187M is due to the slow rate of reaction of this species with reduced methyl viologen rather than its thermodynamic stability.

Flavin anionic semiquinone can also be formed from R187M by photoreduction (see under “Experimental Procedures”). Fig. 7 shows a sample in which 68% of the enzyme was photoreduced to the semiquinone. This species was stable for several hours. On the addition of 100 $\mu M$ benzyl viologen, the sample slowly disproportionated, eventually reaching equilibrium af-
In the present work, two mutant forms of LMO were studied, R187M, and H240Q. Both replacements are semiconservative, involving side chains with spatial requirements similar to those of the wild-type residues but with very different chemical properties. An active site model of LMO (Fig. 2) based on the known structures of flavocytochrome b_{5} (11, 12) and glycolate oxidase (4) features two amino acids previously unstudied in this enzyme that are close to the substrate binding site. Arginine 187 in LMO is conserved throughout the FMN-dependent family of enzymes that catalyze the oxidation of L-\((\alpha, \beta\)-hydroxy acids. The equivalent amino acid residue in glycolate oxidase (arginine 164) and in flavocytochrome b_{5} (arginine 289) is close to the enzyme active site. The present model also places this residue close to the active site of LMO. It is therefore postulated that Arg 187 may have a role in the binding of substrate in the active site of LMO and that the positive charge of the guanidinium side chain may exert direct effects on the electronic properties of the enzyme-bound flavin. Histidine 240 is unique to LMO. In the present model, the imidazole side chain is not immediately adjacent to the FMN prosthetic group, but is potentially in a position to interact with bound substrate. In the present work, two mutant forms of LMO were studied, R187M, and H240Q. Both replacements are semiconservative, involving side chains with spatial requirements similar to those of the wild-type residues but with very different chemical properties. An attempt was also made to replace arginine 187 with lysine, thus replacing a guanidinium side chain with an amine. However, although the R187K protein was expressed in E. coli, it was obtained only in the form of insoluble inclusion bodies. The spectral properties of H240Q as initially isolated and its conversion on treatment with MMTS to a form more closely resembling the wild-type enzyme suggest that this mutant as isolated contains approximately 25% of a flavin thiol adduct. Similar properties were observed for a previously studied mutant of LMO, R293K (21, 29), and were attributed to a
thiol adduct to the N(5) position of the flavin.

Overall, mutating His-240 to glutamine has had only minor effects on the properties of LMO. The spectra of H240Q in its semiquinone and fully reduced states are very similar to those of wild-type LMO and the semiquinone is thermodynamically stable. From the steady-state kinetic analysis of H240Q it is clear that this mutation has caused only a slight disruption of the enzyme active site. The value of $k_{cat}$ is comparable to that of wild-type LMO, and a 3-fold higher $K_m$ of H240Q for L-lactate suggests only a minor role for His-240 in substrate interaction at the active site. Reactivity of the enzyme with molecular oxygen is also only slightly affected. His-240 also appears to have no role in the stability of the reduced enzyme-pyruvate complex, as indicated by the fact that like wild-type LMO, H240Q turns over completely via the coupled pathway in Fig. 1.

The absorbance spectra of R187M in its oxidized, semiquinone, and fully reduced forms are almost indistinguishable from those of the wild-type enzyme. This is in contrast with K266M (20), in which the removal of a positive charge, the amino acid side chain of lysine 266, from the vicinity of the flavin resulted in an anionic semiquinone species with an absorbance spectrum significantly different from that of the wild-type enzyme. However, in common with K266M, the anionic semiquinone form of R187M is thermodynamically unstable. In comparison with the wild-type enzyme, the $E_{ox-sq}$ couple of R187M has been lowered by 131 mV, and the $E_{sq-red}$ couple has been raised by 63 mV. The wild-type enzyme has redox potentials of $-67$ and $-231$ mV for the $E_{ox-sq}$ and $E_{sq-red}$ couples, respectively (32), and therefore thermodynamically stabilizes the semiquinone. The potential for the 2-electron reduction of the enzyme has decreased by only 34 mV due to this mutation, to $-183$ mV. This leaves the enzyme midpoint potential close to that for the lactate-pyruvate couple of $-189$ mV (30), and so should not constitute a thermodynamic barrier to enzyme turnover.

The binding of sulfite is substantially weakened by this mutation. A 380-fold increase in the dissociation constant of sulfite to R187M in comparison with the wild-type enzyme is caused mainly by a fall in the rate of adduct formation, its rate of dissociation being only 3-fold slower than for the wild-type enzyme. The present data are thus entirely consistent with the close proximity of arginine 187 to the flavin. Indeed, the effects of the present mutation on the redox properties of the flavin are comparable to the effects of a series of mutations made of D. vulgaris from Desulfovibrio vulgaris (33, 34). Tyrosine 98 in flavodoxin from D. vulgaris is known to make extensive van der Waals contacts with the isoalloxazine ring of the FMN cofactor. Mutation of tyrosine 98 to alanine, histidine, or arginine caused the $E_{ox-sq}$ couple to decrease by 25–60 mV. The $E_{sq-red}$ couple was increased by 140 mV in the Y98A mutant and by over 180 mV in the Y98H and Y98R mutants, thus substantially destabilizing the semiquinone form of the enzyme.

The rate of reduction of R187M with L-lactate is greatly decreased compared with that of the wild-type enzyme. Such a large decrease is unlikely to arise from the 34 mV decrease in the potential for two-electron reduction of the enzyme (see above), and is therefore likely to arise from a decreased affinity of the mutant enzyme for substrate, or to a lack of ability of the mutant enzyme to stabilize reaction intermediates. A very low affinity of R187M for substrate is apparent from the failure to observe any clear sign of saturation of the rate of reduction of the enzyme by up to 250 mM L-lactate. A role in the stabilization of the transition state of flavocytochrome b$_2$ for the equivalent residue (arginine 289) has been proposed (23). A study of the reaction of L-lactate oxidase with a series of para-substituted mandelates (35) found a negative Hammet $\rho$ value. This led to the conclusion that the reaction of the enzyme with substrate does not proceed via a mechanism involving the formation of a negative charge during the transition state. The reaction was therefore suggested to proceed via a hydride transfer mechanism or via a carbanion mechanism in which the negative charge of the initial carbanion is neutralized by the two conserved active site arginine residues. In support of the latter mechanism, it was pointed out that the side chain nitrogens of arginine 164 in glycolate oxidase (equivalent to arginine 187 in LMO, 181 in L-lactate oxidase, and 289 in flavocytochrome b$_2$) are 3.0 and 3.2 Å from the carboxylate oxygens of a bound inhibitor (22).

In order to test the possibility that reaction intermediates may also be destabilized by this mutation, the binding of oxalate to R187M was investigated. Oxalate has long been considered to be a transition state analog for LMO (14), and a series of investigations has shown a linear relationship between the affinity of a number of mutant forms of LMO for oxalate and $k_{red}$, the limiting rate of reduction of the enzyme by L-lactate (20, 21). The binding of oxalate to the enzyme is clearly disrupted by this mutation, with formation of the oxalate-enzyme complex occurring much more slowly than for the wild-type enzyme and requiring substantially higher concentrations of oxalate. The $K_d$ for the binding of oxalate to R187M of 106 mM would suggest a value of 0.42 min$^{-1}$ for $k_{red}$ should the linear relationship between $k_{red}$ and oxalate binding hold true for R187M. A hyperbolic fit to the present data (Fig. 6, inset) yields a value of 0.27 min$^{-1}$ for $k_{red}$, which, although slower than the predicted rate, is of the same order of magnitude. In addition, from the wild-type values of 230 s$^{-1}$ for $k_{red}$ and a $K_d$ of 50 mM for the binding of L-lactate to the oxidized enzyme, an initial slope of approximately 2.75 x $10^5$ M$^{-1}$ min$^{-1}$ can be estimated at low substrate concentrations compared with the value of 13.5 M$^{-1}$ min$^{-1}$ for R187M. Thus, this mutation has given rise to a 2 x 10$^4$-fold decrease in the second order rate constant for the reaction of L-lactate with LMO and a 7 x 10$^5$-fold increase in the $K_d$ for oxalate to the enzyme. That these values are only a factor of three apart supports the notion that oxalate is a transition state analog for LMO, and therefore favors a carbanion mechanism for the reduction of the enzyme by L-lactate.

The anaerobic reduction of LMO by L-lactate is known to give rise to a complex of reduced enzyme and pyruvate (1, 9). The formation of this complex is very fast and characterized by a long wavelength absorbance. It is relatively stable with dissociation rate constant (k$_d$ in Fig. 1) of 2.5 min$^{-1}$. The reaction of this complex with molecular oxygen (k$_o$) is rapid, with a second order rate constant of 1.1 x $10^6$ M$^{-1}$ min$^{-1}$ (9). Thus under aerobic conditions, the enzyme turns over with lactate via a ternary complex mechanism (the coupled pathway in Fig. 1), to give acetate, CO$_2$, and water as final products. The present data show no indication of a pyruvate-reduced enzyme complex for R187M. The reduction of R187M by L-lactate proceeds via a slow, monophasic reaction with no sign of slow wavelength absorbance. This would imply that the dissociation of pyruvate from the reduced enzyme (k$_d$) is faster than the rate of reduction of the enzyme by L-lactate (k$_o$). Attempts to measure a $K_d$ for the pyruvate-reduced R187M complex were also carried out by the anaerobic addition of pyruvate to fully reduced enzyme (data not shown). This resulted only in the slow reoxidation of the enzyme, and no complex was observed.

Destabilization of the reduced enzyme-pyruvate complex might be expected to uncouple turnover and give rise to steady-state production of pyruvate and H$_2$O$_2$ as final products. Measurement of H$_2$O$_2$ during the steady-state turnover of R187M at varying concentrations of L-lactate and air-saturated O$_2$ gives rates that closely match the pseudo-first order rate constants
for the anaerobic reduction of the enzyme by L-lactate. This observation suggests that enzyme turnover proceeds completely via the uncoupled, or outer, pathway in Fig. 1. Thus $k_3$ is considerably faster than $k_2$. For this hypothesis to be valid, the reductive half-reaction must be rate-limiting during turnover. This is strongly borne out by the present data.

In summary, the data presented for R187M and H240Q are completely consistent with their positions in the present model of the active site of LMO. Histidine 240 is probably located on the opposite side of the flavin from the substrate binding site. The only major effect of the H240Q mutation on the flavin is the equilibrium formation of a flavin-thiol adduct. Arginine 187 appears to interact strongly with the substrate, transition state, and pyruvate when bound to LMO. Thus mutation of arginine 187 appears to be much closer to the enzyme-bound flavin and consequently has a much more profound effect on its properties. Anionic forms of the flavin are highly destabilized in comparison with wild-type LMO due to removal of the positively charged guanidinium side chain. Arginine 187 also appears to interact strongly with the substrate, transition state, and pyruvate when bound to LMO. Thus, mutation of arginine 187 to methionine has converted LMO to an extremely ineffective L-lactate oxidase.

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