Site-directed Mutagenesis of Glycine 99 to Alanine in L-Lactate Monooxygenase from Mycobacterium smegmatis*

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L-Lactate monooxygenase (LMO) from Mycobacterium smegmatis was mutated at glycine 99 to alanine, and the properties of the resulting mutant (referred to as G99A) were studied. Mutant G99A of LMO was designed to test the postulate that the smaller glycine residue in the vicinity of the α-carbon methyl group of lactate in wild-type LMO has less steric hindrance, leading to the retention and oxidative decarboxylation of pyruvate in the active site, a unique property of LMO in contrast to other members of the FMN-dependent oxidase/dehydrogenase family. G99A has been shown to be readily reduced by L-lactate at a rate similar to that of the wild-type enzyme. The binding of pyruvate to reduced G99A is 4-fold weaker than that to the wild-type enzyme. A dramatic change of this mutation is that G99A has a much lower oxygen reactivity than the wild-type enzyme. Pyruvate-bound reduced G99A reacts with O₂ at a rate ~10⁻⁵-fold slower than the wild-type enzyme, and free reduced G99A reacts with O₂ at a rate ~100-fold slower than the wild-type enzyme. Due to the very low oxygen reactivity of the pyruvate-bound reduced enzyme, G99A has been shown to catalyze the oxidation of L-lactate to pyruvate and hydrogen peroxide instead of acetate, carbon dioxide, and water, the normal decarboxylation products of pyruvate and hydrogen peroxide. Thus, the mutation alters the enzyme from its L-lactate monooxygenase activity to L-lactate oxidase activity. However, compared with L-lactate oxidase, G99A has a much lower reactivity toward oxygen. Our results also reveal that the small steric change around N-5 of the flavin causes a profound change in the electronic distribution in the catalytic cavity of the enzyme and imply that electrostatic interactions in the active site provide an important factor for control of O₂ reactivity.

The physiological role of LMO in the mycobacterial species is unknown. However, the enzyme has been purified in good yields from Mycobacterium smegmatis. The gene of the protein has been subsequently cloned, expressed, and purified from Escherichia coli (3, 4). LMO was found to be an octamer with identical subunits containing one FMN on each subunit (5). The molecular mass of each monomer is ~43 kDa with 393 amino acid residues (3). LMO is a member of a family of FMN-dependent enzymes that catalyze the oxidation of L-α-hydroxy acids, including L-lactate oxidase (6), lactate dehydrogenase (flavocytochrome b₅) (7), glycolate oxidase (8), L-mandelate dehydrogenases (9, 10), and a long chain α-hydroxy-acid oxidase (11). The mechanism of L-lactate monooxygenase has been intensively studied (12).

It has been shown that, within this family, L-lactate monooxygenase is unique in forming a ternary complex (EFlox-pyr-H₂O₂ as shown in Fig. 1) that results in the oxidative decarboxylation of pyruvate. The reaction scheme is depicted as the inner pathway (or coupled pathway) in Fig. 1 with acetate, carbon dioxide, and water as the reaction products. Fig. 1 also shows the reactions catalyzed by the other enzymes as the outer pathway (or uncoupled pathway) with the reaction products of pyruvate and H₂O₂. The unique property of LMO within the family to follow the coupled pathway has been traced to the fact that pyruvate dissociates slowly from reduced LMO, and the complex reacts rapidly with oxygen before pyruvate dissociates from the enzyme.

A difficulty of the study of L-lactate monooxygenase arises from the lack of a crystal structure. However, the crystal structures of flavocytochrome b₅ and glycolate oxidase have been solved (8, 13, 14). The two enzymes show a strong similarity in the folding pattern around the flavin. Based on the fact that there is strong homology in the amino acid sequences within the family and that the six amino acid residues involved in substrate binding and the reaction mechanism are highly conserved (3, 6, 15-17), a basic assumption has been made that the three-dimensional structures of the family of enzymes are similar and that the substrates bind similarly with respect to FMN and the active-site residues. It is believed that all enzymes in this family have a common initialization of reactions, the abstraction of a proton from the α-carbon of the substrate by a histidine residue (for reviews, see Refs. 7 and 12). The protonated histidine is stabilized by a nearby aspartate residue. The resulting carbanion is then proposed to attack FMN at N-5. The anionic form of the flavin could be stabilized by a lysine residue that is close to N-1 of the flavin. There are also two tyrosine residues and one arginine residue that are postulated to be involved in substrate binding as shown in Fig. 2. Based on the crystal structure studies and reactions with 5-deaza-8-hydroxy-FMN-substituted LMO (18, 19), substrates of this family of flavoenzymes have been shown to bind on the si-face of the flavin. The size of the fourth substituent of the substrate α-car-
Mutagenesis of Glycine 99 in Lactate Monooxygenase

Experimental Procedures

Materials—Oligodeoxynucleotide was synthesized with an Applied Biosystems model 380A DNA synthesizer by the Oligonucleotide Synthesis Center at the University of Michigan. The mutagenesis reaction was done by using the Amersham mutagenesis kit (or the Sculptor Center at the University of Michigan). The mutagenesis reaction was performed with a Hi-Tech stopped-flow apparatus. The experimental data were analyzed by a computer program called Program A, which was developed by Dr. D. P. Ballou and G. Ford at the University of Michigan. Rapid reaction studies were performed with a Hi-Tech stopped-flow apparatus. The experimental data were analyzed by a computer program called Program A, which was developed by Dr. D. P. Ballou and G. Ford at the University of Michigan.

Site-directed Mutagenesis—The vectors used for generating G99A of lactate monooxygenase and the expression plasmid were designed previously (4). The mutagenesis of glycine 99 (codon GGG) was performed with the oligonucleotide 5'-CGCTCCCATCGCGGTCA-3' and finally by plasmid Eco47III site (5'-AGCGCT-3'). Successful insertion was thereby screened for the addition of the Eco47III site.

Oxidative Half-reaction—

Fully reduced G99A (10 mM), which was made anaerobic by bubbling with oxygen-free argon in a tonometer. Another syringe was filled with anaerobic l-lactate at concentrations from 2.5 to 200 mM. The l-lactate was made anaerobic by bubbling with oxygen-free argon for 20 min. The reduction of enzyme-bound flavin was followed by the absorbance decrease at 460 nm.

Reducive Half-reaction—The stopped-flow syringes were filled overnight with a mixture of 3,4-dihydroxybenzoxazole and 3,4-dihydroxybenzoate dioxygenase to scrub oxygen. For the stopped-flow experiment, one syringe was filled with oxidized G99A (10 mM) was made anaerobic by alternate cycles of evacuation and filling with oxygen-free argon in a tonometer. Another syringe was filled with anaerobic l-lactate at concentrations from 2.5 to 200 mM. The l-lactate was made anaerobic by bubbling with oxygen-free argon for 20 min. The reduction of enzyme-bound flavin was followed by the absorbance decrease at 460 nm.

For the oxidation of pyruvate-bound reduced G99A by oxygen, free reduced G99A was grown in 6 l of Terrific Broth and purified as described previously (4). The extinction coefficient of the enzyme-bound flavin of LMO has been reported to be very dependent on the nature of the buffer (2, 5). The magnitude of the majority of the mutant was found in an insoluble form. Only the soluble fraction was isolated and purified. The purity of the protein was determined by SDS-polyacrylamide gel electrophoresis (22) and staining with Coomassie Blue.

Extinction Coefficient—The extinction coefficient for G99A was determined by denaturing the protein with SDS. A 10–20 µl aliquot of 10% sodium dodecyl sulfate was added to 1 ml of enzyme solution in 10 mM imidazole HCl buffer, pH 7.0, at 25 °C. The extinction coefficient for the flavin bound to lactate monooxygenase was determined based on the amount of released FMN. The concentration of free FMN was calculated using an extinction coefficient of 12,500 M⁻¹ cm⁻¹ at 445 nm.

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Cell Growth and Purification of G99A—G99A was grown in 6 l of Terrific Broth and purified as described previously (4). Typically, the wet weight of cells was 30–35 g. The majority of the mutant was found in an insoluble form. Only the soluble fraction was isolated and purified. The purity of the protein was determined by SDS-polyacrylamide gel electrophoresis (22) and staining with Coomassie Blue.

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Oxidative Half-reaction—Fully reduced G99A (~20 µM) was made by the addition of 1 eq of l-lactate from the side arm of an anaerobic cuvette attached to a tonometer. The stopped-flow experiment was performed with one syringe filled with reduced G99A and the other syringe filled with 10 mM imidazole buffer, which was bubbled with varied oxygen concentrations for 20 min. The reaction was followed by the reoxidation of flavin at 460 nm.

For the oxidation of pyruvate-bound reduced G99A by oxygen, free reduced G99A was kept in one syringe, and the other syringe was filled...
with 50 mM pyruvate and varied oxygen concentrations. The reaction was followed by the absorbance increase at 460 nm.

Mechanism-based Inactivation—The reactivity of G99A (15.7 μM) with α,2-hydroxy-3-butyroic acid (23, 24) was tested by tipping in the inhibitor from the side arm of an anaerobic cuvette to a final concentration of 6.6 mM. The reaction was followed spectrophotometrically with time.

Photo-reduction—Lactate monoxygenase was reduced photochemically by the method described previously (20, 25). Glycine was used as photoreductor instead of EDTA since EDTA photochemically breaks down to glyoxalate, which is a substrate for lactate monoxygenase (26). The enzyme in 0.1 M glycine solution was treated with alternate cycles of evacuation and oxygen-free argon in an anaerobic cuvette in the dark. Once the solution was made anaerobic, 5-deazaflavin was tipped in from the side arm to a final concentration of 1 μM. Full semiquinone was obtained by light irradiation with visible light from a sun gun for ~10 min. The thermodynamic stability of the semiquinone was tested by the addition of benzyl viologen (1 μM final concentration), which acts as a mediator between the flavin semiquinone in the octameric protein. The spectra of the mixture were followed for 24 h.

Oxidation of Semiquinone by Oxygen—Enzyme at the flavin semiquinone level was prepared by photoreduction of the oxidized enzyme as described above and put into one syringe of the stopped-flow apparatus. The other syringe was filled with buffer that had been bubbled with varied oxygen concentrations for 20 min. The reaction was followed by both the absorbance decrease at 400 nm and the absorbance increase at 460 nm.

Thermodynamics of Semiquinone-Pyruvate Complex—A large excess of pyruvate (~10 mM) was mixed with enzyme semiquinone (~30 μM) from the side arm of an anaerobic cuvette. On opening to air, the mixture was quickly loaded on a Sephadex G-25 column at 4 °C. Semiquinone-pyruvate complex free of excess pyruvate was thereby obtained in the same way as described previously for the wild-type enzyme (27). The solution was equilibrated to 25 °C, and the stability of the complex to air was followed spectrophotometrically with time.

Reaction Turnover Measured by O₂ Electrode—Enzyme turnover was determined by oxygen consumption measurements with an oxygen electrode. At air saturation, varied concentrations of L-lactate from 5 to 150 mM were used. The reaction was initiated by the addition of G99A to a final concentration as high as 40 μM in order to obtain accurate measurements. When catalase was used in the measurements, it was added to the solution before the addition of G99A. The turnover numbers were calculated from the tangents to the initial portion of the traces.

Enzymatic Assays of H₂O₂ and Pyruvate—H₂O₂ formation was measured colorimetrically by the oxidation of o-dianisidine in the presence of horseradish peroxidase. Typically, 1.0 ml samples containing buffer, 0.4 mM o-dianisidine, 0.1 mM HRP, and various concentrations of L-lactate were used, and the reaction was followed at 460 nm after the addition of enzyme. The extinction coefficient of the oxidized o-dianisidine at 460 nm is 1.16 × 10⁴ M⁻¹ cm⁻¹ (2). Pyruvate formation was followed spectrophotometrically by the formation of the 2,4-dinitrophenylhydrazine derivative (28).

Reaction Turnover Measured by H₂O₂ Formation—The formation of hydrogen peroxide was also determined indirectly by the formation of HRP compound I (HRP-I) at varied oxygen concentrations by stopped-flow experiments. A mixture of 1 μM G99A and 8 μM HRP at air saturation was placed in one syringe of the stopped-flow apparatus. The other syringe was filled with varied oxygen concentrations and L-lactate at concentrations of 9.7, 19.4, 49.7, and 200 mM. The reaction was followed at 398 nm, which is the isosbestic point of HRP-I and HRP-II. The extinction difference of native HRP and HRP-I at 398 nm is 4.35 × 10⁴ M⁻¹ cm⁻¹ (29).

**RESULTS**

Enzyme Purification and Spectral Properties—Pure G99A did not show normal flavin absorbance characteristics in the 370 nm region, having a A₃₈₀/A₄₅₆ ratio of ~16, which is higher than the usual value of ~9.5 for the wild-type enzyme and other mutants (4, 30, 31). Upon addition of an excess of methyl methanethiosulfonate, the flavin absorbance was observed to increase with time as shown in Fig. 3. The reaction mixture shows a final absorption spectrum similar to that of the wild-type enzyme. The plot of A₃₈₀ versus time yields a t₀₂ of 44 min for this reaction. A similar phenomenon has been observed previously with the R293K mutant (32). As with that mutant, it is concluded that the G99A enzyme contains a mixture of a flavin N(5)-cysteine adduct and the normal flavin enzyme free thiol. Methyl methanethiosulfonate reacts with the equilibrium concentration of free thiol, and by doing so, it displaces the equilibrium to abolish completely the N-5 adduct.

The reactivity of G99A (~3.8 mM methyl methanethiosulfonate. Spectra were taken at 0, 10, 33, and 129 min, and the absorbance peaks at 370 and 457 nm were observed to increase with time. The inset shows the absorbance increase at 460 nm versus time. t₀₂ is therefore determined to be 44 min.

The dissociation constant for the reduced G99A-pyruvate complex was determined to be 11.5 mM by following the absorbance increase at 460 nm. The dissociation constant for the reduced G99A-pyruvate complex was determined to be 44 min. A similar phenomenon has been observed with the R293K mutant (32). As with that mutant, it is concluded that the G99A enzyme contains a mixture of a flavin N(5)-cysteine adduct and the normal flavin enzyme free thiol. Methyl methanethiosulfonate reacts with the equilibrium concentration of free thiol, and by doing so, it displaces the equilibrium to abolish completely the N-5 adduct.

To remove the adduct, an ~10-fold excess of L-lactate was used to reduce the enzyme under anaerobic conditions. Following reduction, there was a slow increase in absorbance in the 360 nm region, with a t₀₂ of ~50 min. Thus, as expected, reduction shifts the equilibrium between the normal flavin and the N(5)-cysteine adduct, and eventually, ~100% of the normal flavin enzyme was obtained. The excess L-lactate and keto acid were removed by gel filtration with Sephadex G-25. Such enzyme could be stored at 4 °C for 1 month without re-formation of the adduct. Typically, ~25 mg of G99A was isolated from 6 liters of bacterial growth, compared with ~66 mg of wild-type enzyme.

After treatment and gel filtration, the oxidized enzyme shows absorbance peaks at 457 and 370 nm as shown in Fig. 4. The ratio A₃₈₀/A₄₅₆ has decreased to 9.6, and the spectrum resembles that of the wild-type enzyme. The extinction coefficient at 456 nm was determined to be 11,360 M⁻¹ cm⁻¹. Like the wild-type enzyme, the oxidized enzyme is nonfluorescent, but becomes fluorescent upon reduction. The reduced enzyme shows maximum excitation at 365 nm and maximum emission at 510 nm, similar to the wild-type enzyme (33). The presence of phosphate and chloride ions does not show any effect on the absorption spectrum of oxidized G99A (data not shown), which is in contrast with the wild-type enzyme (2).

Binding of Reduced G99A to Pyruvate—As was found with the wild-type enzyme (2), a charge transfer complex is formed between the reduced enzyme and pyruvate, characterized by an absorbance band extending out to 600 nm. The dissociation constant for the reduced G99A-pyruvate complex was determined to be 11.5 mM by following the absorbance increase at 540 nm as a function of pyruvate concentration. The Kₛ for the wild-type enzyme under the same conditions is 2.5 mM (2). Therefore, pyruvate binds to reduced G99A ~4-fold weaker than to the wild-type enzyme.

Reductive Half-reaction of G99A—Under anaerobic conditions, the enzyme was reduced rapidly by L-lactate. The reaction was monitored by the flavin absorbance decrease at 460 nm with time. Fig. 5 shows a series of time traces at 460 nm at varied L-lactate concentrations. The reaction is biphasic, and it is clear that the first phase is L-lactate concentration-dependent and that the second phase is independent of L-lactate concentration. The kinetic measurements yield the reduction rate.
of G99A by L-lactate to be 13,000 min$^{-1}$, as shown in Fig. 5 (inset). From this kinetic saturation curve, the apparent dissociation constant for binding of L-lactate to G99A was estimated to be 30 mM (34). Compared with the 50 mM dissociation constant for the wild-type enzyme (2), L-lactate binds to G99A tighter than to the wild-type enzyme, even though the binding is still weak (Table I).

The second phase of the stopped-flow traces yields the rate constant of pyruvate release from the reduced enzyme. The average value of 4.7 min$^{-1}$ was obtained, independent of the concentration of L-lactate. Compared with 2.5 min$^{-1}$ for the wild-type enzyme, the mutation thus shows a 2-fold faster release of pyruvate from the reduced enzyme.

Binding of Oxalate to Oxidized G99A—Oxalate has been used as an analog of the presumed carbanion transition state of the reductive half-reaction. The binding of oxalate to the enzyme induces a pronounced perturbation of the absorption spectrum of the oxidized enzyme (35). The difference spectrum between the uncomplexed and fully complexed forms of the wild-type enzyme shows positive maxima at 391, 425, 452, and 482 nm, with $\Delta \varepsilon = 2000$ M$^{-1}$ cm$^{-1}$ at 504 nm. The isosbestic points are at 323, 382, 462, 478, and 485 nm. Fig. 6 depicts the difference spectrum of the titration of oxalate with G99A. It has a shape similar to that of the wild-type enzyme and with the isosbestic points changed to 321, 384, 461, 475, and 489 nm. The positive maxima are at 394, 425, 452, and 482 nm. The negative maxima occur at 371, 468, and 504 nm, with $\Delta \varepsilon = 1800$ M$^{-1}$ cm$^{-1}$ at 504 nm. The dissociation constant of the G99A-oxalate complex was determined to be $3.3 \times 10^{-4}$ M by monitoring the absorbance change at 504 nm. Binding is therefore $20$-fold weaker than with the wild-type enzyme, for which a $K_d$ of $1.6 \times 10^{-5}$ M has been reported (Ref. 35; see Table I). The wild-type enzyme-oxalate complex is converted photochemically to a fluorescent flavin N(5)-carbonate adduct, which decays aerobically back to the oxidized enzyme with a $t_{1/2}$ of 3.8 h at 25 °C (36). In contrast, the photoadduct formed from the G99A-oxalate complex was nonfluorescent and was stable with oxygen for at least 24 h.

Oxidative Half-reaction of G99A—G99A was reduced anaerobically with 1 eq of L-lactate, and the rate of reoxidation of free reduced G99A with $O_2$ was determined by monitoring the absorbance increase at 460 nm with time in the stopped-flow spectrophotometer. Under the experimental conditions of $10 \mu$M G99A, 25 °C, and pH 7.0, the reaction is very slow, requiring $20$ min for completion at the lowest $O_2$ concentration of 61 M. The stopped-flow traces were single exponentials with rates directly proportional to oxygen concentration, yielding a rate constant for reaction of the free reduced enzyme with $O_2$ of $5.1 \times 10^3$ M$^{-1}$ min$^{-1}$, $2$ orders of magnitude lower than that for the wild-type enzyme (2).
When the reduced enzyme was mixed with oxygen and 25 mM pyruvate (~68% of reduced G99A is in the pyruvate-bound form), the stopped-flow traces showed the reoxidation of both free reduced G99A and pyruvate-bound reduced G99A. The rate constant for the pyruvate-bound reduced enzyme reaction with $O_2$ was then determined to be $(1.3 \pm 0.3) \times 10^3 \text{M}^{-1} \text{min}^{-1}$, some 10$^3$-fold slower than that for the wild-type enzyme (2).

**DL-2-Hydroxy-3-Butynoic Acid Reaction with G99A—**2-Hydroxy-3-butynoate is a suicide substrate of lactate monooxygenase, resulting in a ring-closed adduct involving both C-4a and C-6 positions of the substrate (28). The adduct with the wild-type enzyme, which decays to uncharacterized products with a half-life of a few hours, the adduct with the mutant enzyme was stable for days. Similar changes in stability of the adduct have been found for other mutant forms of the enzyme (4, 30, 31); the basis for these differences in stability is unknown.

Binding with d-Lactate—d-Lactate is a competitive inhibitor of LMO, and binding is accompanied by a small perturbation of the flavin absorption spectrum. d-Lactate binds to the wild-type enzyme much more strongly than l-lactate. The binding of d-Lactate to G99A shows a difference spectrum similar to that of the native enzyme, with maximum changes at 372, 390, 427, and 507 nm. The value of $K_d$ was determined by monitoring the absorbance change at 507 nm as a function of d-lactate concentration and was found to be $1.7 \times 10^{-3}$ M, the same as for the wild-type enzyme (Ref. 38; see Table I).

Photoreduction of G99A—Like wild-type enzyme, the G99A is reduced photochemically to a stable semiquinone form. The semiquinone shows the characteristic properties of the anion form, with a maximum at 374 nm, a sharp peak at 406 nm, and a broad band centered around 490 nm, as shown in Fig. 4. The extinction of the semiquinone is high ($\epsilon_{374} \approx 15,000$ M$^{-1}$ cm$^{-1}$) compared with the oxidized species. The isosbestic points of semiquinone and the oxidized enzyme are at 346, 418, and 506 nm. The semiquinone of G99A is thermodynamically stable, as it is with the wild-type enzyme, since it does not disproportionate to the oxidized and reduced enzyme after 24 h in the presence of 1 mM benzyl viologen.

The reactivity of semiquinone with $O_2$ was determined by stopped-flow experiments. Semiquinone was formed by light irradiation in a mixture of 32$\mu$M G99A, 100$\mu$M glycine, and 1.2$\mu$M deazaflavin. The mixture was then reacted with $O_2$ at concentrations varying from 61 to 615$\mu$M. The reaction was monitored by both the absorbance decrease at 400 nm and the absorbance increase at 460 nm. A rate constant of $4.5 - 4.9 \times 10^7$ M$^{-1}$ min$^{-1}$ was obtained from the stopped-flow traces at both wavelengths.

The binding of pyruvate to the G99A semiquinone form was determined. In a mixture of 15.4$\mu$M G99A, 0.1 M glycine, and 1.0$\mu$M deazaflavin, stable semiquinone was formed after photoirradiation for 10 min. The binding of pyruvate to G99A semiquinone results in increases in absorbance in the 400–800 nm range attributable to a charge transfer transition (27). The dissociation constant of the complex was determined to be $2.0 \times 10^{-5}$ M by following the absorbance increase at 540 nm as a function of pyruvate concentration. Pyruvate thus binds to semiquinone with approximately the same strength as to the wild-type enzyme, for which a $K_d$ of $1.4 \times 10^{-5}$ M$^{-1}$ has been reported (Ref. 27; see Table I).

The reactivity of the semiquinone-pyruvate complex with $O_2$ was also determined. In a mixture of 28.6$\mu$M G99A, 0.1 M glycine, and 0.2$\mu$M deazaflavin, semiquinone was formed by light irradiation for 10 min in an anaerobic cuvette. The mixing of pyruvate from the side arm for a final concentration of 10$\mu$M yields the formation of the semiquinone-pyruvate complex. Excess pyruvate was removed by gel filtration with Sephadex G-25, and the reoxidation of the remaining semiquinone-pyruvate complex by $O_2$ was measured spectrophotometrically with time. As shown in Fig. 7, a half-time of 71 min for the first-order reoxidation of the complex was obtained by plotting the absorbance change at 460 nm versus time. Therefore, like the wild-type enzyme, the semiquinone-pyruvate complex of G99A is in fact unreactive with $O_2$, and the slow oxidation observed is due to the reaction of $O_2$ with free semiquinone formed as pyruvate dissociates from the complex (27). The mutation thus alters the release of pyruvate from its complex with the semiquinone, with G99A being 2.8-fold slower than the wild-type enzyme.

**Turnover Measurement by $O_2$ Consumption—**Under steady-state conditions, the $O_2$ consumption was monitored with time at varied concentrations of l-lactate from 5 to 150$\mu$M with 38$\mu$M G99A at air saturation. The initial rate of $O_2$ disappearance was observed to be independent of l-lactate concentration. The average turnover number was $0.89 \text{m}^{-1}$.$\text{min}^{-1}$. In the presence of 9.1$\mu$g/ml catalase (760 units), the initial rate of $O_2$ disappearance was also independent of l-lactate concentration. The average value was $0.42 \text{m}^{-1}$. Within experimental error, this clearly indicates that the oxidation of l-lactate catalyzed by G99A results in the production of pyruvate and $H_2O_2$, rather than the oxidative decarboxylation catalyzed by the native enzyme. At air saturation and 5$\mu$M l-lactate, the initial rates of $O_2$ consumption were also measured in the presence of increasing concentrations of chloride and phosphate ions. It was observed that these anions did not have any effect on the turnover number of G99A (data not shown), which is in contrast to the inhibition found with wild-type enzyme (2).

**Assays for Formation of $H_2O_2$ and Pyruvate—**From the effect of catalase in the $O_2$ consumption studies, it is expected that the products of l-lactate oxidation will be $H_2O_2$ and pyruvate. The rate of free $H_2O_2$ formation from the reaction of l-lactate and $O_2$ catalyzed by G99A was determined colorimetrically by the oxidation of o-dianisidine in the presence of horseradish peroxidase. At air saturation, the rate of $H_2O_2$ formation was observed to be independent of l-lactate concentration from 300$\mu$M to 100$\mu$M, with a turnover number of $0.85 \text{m}^{-1}$.$\text{min}^{-1}$, in good agreement with the value obtained from measurement of $O_2$ consumption. At lower concentrations of l-lactate (from 5 to
TABLE II

| G99A          | Observed | Wild-type LMO<sup>b</sup> |
|---------------|----------|---------------------------|
| Calculated<sup>a</sup> |          |                           |
| \( k_{\text{cat}} = \frac{k_3 k_1}{k_2 + k_{-2} + k_3} \) | \( k_3 = 4.7 \text{ min}^{-1} \) | \( 4.3 \text{ min}^{-1} \) |
| \( k_{\text{cat}}^{\text{obs}} = \frac{k_2 + k_{-2} + k_3 k_1}{k_{-3} + k_3 k_1} \) |          |                           |
| \( K_m^{\text{O}_2} = \frac{k_3 k_1}{k_0 (k_2 + k_3)} \) | \( K_m^{\text{O}_2} = 0.92 \text{ mM} \) | \( 0.83 \text{ mM} \) |
| \( K_m^{\text{O}_2} = \frac{k_0 (k_2 + k_3)}{k_3 k_1} \) |          |                           |
| \( K_m^{\text{O}_2} = 20 \mu\text{M} \) |          |                           |
| \( K_m^{\text{O}_2} = 14 \mu\text{M} \) |          | \( 22 \mu\text{M} \)     |
| \( K_m^{\text{O}_2} = 55 \mu\text{M} \) (calculated) |          | \( 71 \mu\text{M} \) (observed) |

(100 \mu\text{M}), a variation of rate was observed. The plot of turnover number versus \( L\)-lactate concentration yielded an apparent \( K_m \) for \( L\)-lactate of \(-14 \mu\text{M}\) and an apparent \( k_{\text{cat}} \) of \(-0.85 \text{ min}^{-1}\) (data not shown). The determined \( K_m \) for \( L\)-lactate is close to the calculated value of \(-20 \mu\text{M}\) derived from the rapid reaction studies, as shown in Table II.

Pyruvate formation from the reaction of \( L\)-lactate and \( O_2 \) catalyzed by G99A was determined colorimetrically by the formation of the 2,4-dinitrophenylhydrazine derivative. The absorbance at 530 nm was used to calculate the lactate concentration from the reaction mixture according to a standard curve. At air saturation and a \( L\)-lactate concentration varied from 5 to 100 m\text{M} with 1.2 \mu\text{M} G99A, the rate of pyruvate formation was observed to be independent of \( L\)-lactate concentration. The average turnover number was measured to be 1.1 \text{ min}^{-1} (data not shown).

Turnover Measurements by \( H_2O_2 \) Formation at Varied \( O_2 \) Concentrations—Under steady-state conditions, the reaction turnovers were determined by measuring the rate of \( H_2O_2 \) formation from the reaction of \( H_2O_2 \) and HRP to form HRP-I. One syringe was filled with 1 \mu\text{M} G99A and 8 \mu\text{M} HRP at air saturation; the other syringe was filled with 4.85 m\text{M} \( L\)-lactate and varied \( O_2 \) concentrations. The rate of \( H_2O_2 \) formation was determined by monitoring the formation of HRP-I at 389 nm, which is the isosbestic point between HRP-I and HRP-II (Fig. 8). The same experiment was carried out at three other \( L\)-lactate concentrations (9.7, 24.85, and 100 m\text{M}). Therefore, the reaction turnover numbers were obtained at varied \( O_2 \) and lactate concentrations. The plot of turnover number at each \( L\)-lactate concentration versus \( O_2 \) concentration yields a hyperbolic curve, from which the \( K_m \) and \( k_{\text{cat}} \) for \( O_2 \) can be calculated. The average \( k_{\text{cat}} \) is 4.3 \text{ min}^{-1}, which can be compared with the determined \( k_3 \) of 4.7 min\textsuperscript{-1}. The average \( K_m \) for \( O_2 \) is 8.3 \times 10\textsuperscript{-4} M, which is close to the calculated value of 9.2 \times 10\textsuperscript{-4} M, as shown in Table II.

FIG. 8. Spectral changes of HRP in the oxidation of \( L\)-lactate catalyzed by G99A. The absorbance decrease at 403 nm with time clearly indicates the formation of HRP-I. The inset shows an example of the time trace of the absorbance at 398 nm. The initial rate of \( H_2O_2 \) formation was determined from the slope of the time trace.

DISCUSSION

Mutant G99A of LMO was designed to test the postulate that the smaller glycine residue in the vicinity of the \( \alpha \)-carbon methyl group of lactate in wild-type LMO has less steric hindrance, leading to the retention and oxidative decarboxylation of pyruvate in the active site, a unique property of LMO in contrast to other members of the FMN-dependent oxidase/ dehydrogenase family.

G99A and the wild-type enzyme show similar spectral and fluorescence characteristics. However, the absorption spectrum of the oxidized wild-type enzyme had been shown to be substantially affected by inorganic anions, which are competitive inhibitors of the enzyme (2), whereas the presence of phosphate and chloride ions does not show any effect on the absorption spectrum of G99A or on its turnover number. This clearly indicates that G99A has different electrostatic interactions in the catalytic cavity of the enzyme compared with the wild-type enzyme.

This family of flavoproteins, including \( L\)-lactate oxidase, glycolate oxidase, flavocytochrome \( b_2 \), and \( L\)-mandelate dehydrogenase, shares a common primary and tertiary structure particularly in the active site, and all members catalyze the oxidation of \( L\)-\( \alpha \)-hydroxy acids. However, they have substantially different reactivities with \( O_2 \); compare glycolate oxidase, for which oxygen is a substrate, with flavocytochrome \( b_2 \), which catalyzes a reaction that does not involve oxygen at all (12). It is unlikely that \( O_2 \) reactivity in this class of flavoproteins is governed by control of the access of \( O_2 \) since \( O_2 \) can probably diffuse easily to almost any location within the active site of the enzymes. The real factor that controls the \( O_2 \) reactivity in this family of flavoproteins remains unknown. In this study, based on the stopped-flow results of the oxidative half-reactions, the \( O_2 \) reactivities of the reduced G99A-pyruvate complex and of free reduced G99A were decreased 10\textsuperscript{5}- and 100-fold, respectively, compared with those of the wild-type enzyme. Therefore, the replacement of a hydrogen by a methyl group in LMO brings about a dramatic change in oxygen reactivity rather than an increase in the rate of pyruvate dissociation, as in lactate oxidase (6). The reduced enzyme-pyruvate complex in the wild-type en-
zyme reacts with O$_2$ at a rate $\sim$200-fold faster than the free reduced enzyme. It was speculated that the rate enhancement might come from the favorable electronic effect, from the charge transfer complex between the reduced enzyme and pyruvate, on the spin inversion steps required for reaction of triplet oxygen (12). The observations that G99A has different electrostatic interactions in the active site compared with the wild-type enzyme and that reduced G99A has a much lower oxygen reactivity than the wild-type enzyme provide evidence that the electronic effect is a crucial factor for the oxygen reactivity. Obviously, the electronic distribution in G99A is unfavorable for the oxygen activation. Thus, our observations point to a key factor that controls the oxygen reactivity in this family of flavoproteins.

Pyruvate binds to the anionic semiquinone of G99A slightly less tightly than to the wild-type enzyme. However, the semiquinone is shown to have a similar O$_2$ reactivity as the wild-type enzyme. The apparent difference between the fully reduced enzyme and anionic semiquinone is at N-5, which should have tetrahedral character in the fully reduced enzyme and planar (sp$^2$) character in the anionic radical (12). It is possible that the different orientations of the lone pair of electrons in semiquinone could have some compensation on the altered electronic distribution in G99A. Obviously, further evidence is needed to elucidate this phenomenon.

The oxygen consumption results during turnover in the presence of catalase show that the net oxygen consumption is half that observed in the absence of catalase and that hydrogen peroxide is the primary product. Thus, G99A works as an L-lactate oxidase by following the uncoupled pathway, although G99A has a much lower reactivity with O$_2$ compared with L-lactate oxidase from Aerococcus viridans (6). This observation is consistent with the stopped-flow kinetic data. From the stopped-flow data, it can be calculated that at air saturation, the pyruvate-bound reduced enzyme reacts with oxygen at a rate of 0.33 min$^{-1}$, which is much smaller than the rate of pyruvate dissociation from the reduced enzyme ($k_3 = 4.7$ min$^{-1}$). Therefore, pyruvate is released from the reduced enzyme before the complex of the pyruvate-bound reduced enzyme can be oxidized by O$_2$. As expected, the formation of H$_2$O$_2$ and pyruvate was observed. The measurements of the H$_2$O$_2$ formation rate at varied L-lactate and oxygen concentrations yield the values of K$_m$ for lactate and oxygen, which are very close to the theoretical values as summarized in Table II. The mutation has also caused a dramatic decrease in the K$_m$ for L-lactate and an increased K$_m$ for oxygen.

The binding of oxalate, which mimics the structure of the presumed carbanion transition state of the lactate oxidation by LMO (35, 39), shows that oxalate binding to G99A is 20-fold weaker than to the wild-type enzyme. A linear relationship between the K$_d$ for binding of oxalate and the rate of flavin reduction by L-lactate was observed previously (31). Namely, it is that the tighter the oxalate binds the enzyme, the faster the reduction of the flavin by L-lactate. The values for G99A do not lie on the line defined by data from the wild-type enzyme and four-active site mutants.

The G99A-oxalate complex undergoes photochemical adduct formation similar to that found with the wild-type enzyme. However, the resulting photoadduct does not show reoxidation by oxygen, as does the photoadduct of the wild-type enzyme (24). Taken together, the results of the oxalate binding studies and the effects of anions support the conclusion that the small steric change around N-5 of the flavin causes a profound change in the electronic distribution in the catalytic cavity of the enzyme.

L-2-Hydroxy-3-butynoic acid reacts with LMO to form a cyclic adduct at flavin C-4a and N-5 (23, 37). Thus, this reagent should further document any change in the electronic state of the C(4a)-N(5) area of the flavin. This adduct is formed rapidly in the mutant, as in the wild-type enzyme. However, the striking difference is that the complex of the wild-type enzyme slowly decays, whereas the mutant complex does not. Clear, the state of the flavin in the area nearest the mutation, C(4a)-N(5), is markedly different in the mutant.

The mutation shows little change in the reduction of the oxidized flavin by L-lactate. The dissociation constant for L-lactate decreases to 30 mM from 50 mM for the wild-type enzyme. Therefore, L-lactate binding to G99A is slightly stronger than to the wild-type enzyme. Pyruvate is released from the reduced enzyme at a rate approximately two times faster and has a dissociation constant $\sim$4-fold larger than for the wild-type enzyme. These results are consistent with the original hypothesis that the steric hindrance around flavin N-5 has an influence on the binding of pyruvate to the enzyme.

Overall, the mutation of glycine 99 to alanine in LMO alters the activity of the enzyme from that of an L-lactate monooxygenase to that of an L-lactate oxidase. However, the change in the enzymatic reaction is not due to a rapid release of pyruvate from the reduced enzyme, as in the L-lactate oxidase of A. viridans. Unexpectedly, this mutation causes an extraordinary decrease in the oxygen reactivity of the pyruvate-bound reduced enzyme, which is the origin of the change in the enzymatic reaction. Free reduced G99A also reacts with oxygen at a rate $\sim$100-fold slower than wild-type LMO. Furthermore, although G99A acts as a lactate oxidase, it has a much lower oxygen reactivity than a true lactate oxidase (6). In addition, the finding that the electronic distribution in the active site of the enzyme is altered upon mutation suggests that electrostatic interactions are an important factor in controlling oxygen reactivity. This mutant demonstrates that enzymes with strong homology in primary and tertiary structure may have very striking differences in their functions.

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