The Role of Glycine 99 in L-Lactate Monooxygenase from *Mycobacterium smegmatis*\(^*\)

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Glycine 99 in L-lactate monooxygenase (LMO) from *Mycobacterium smegmatis* was mutated to serine and threonine, and the resultant mutants were studied extensively to explore the role of this residue in maintaining monooxygenase activity and in controlling the reactivity with molecular oxygen. Both mutants were observed to lose monooxygenase activity completely and generate H\(_2\)O\(_2\) and pyruvate as reaction products. However, the mutants have much lower activities than a true L-lactate oxidase. The oxygen reactivities of the reduced and semiquinone forms of the mutant enzymes were significantly different from those of wild type enzyme. These results confirm our previous suggestion that the electronic interactions in the active site are a crucial factor that governs the oxygen reactivity of the enzyme (Sun, W., Williams, C. H., Jr., and Massey, V. (1996) *J. Biol. Chem.* 271, 17226–17233). In addition, the mutants cause a dramatic decrease of the rate of flavin reduction by L-lactate compared with the wild type enzyme, mainly due to the much lower stabilization of the transition state.

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\(^1\) The abbreviations used are: LMO, L-lactate monooxygenase; HRP, horseradish peroxidase.

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**EXPERIMENTAL PROCEDURES**

Materials and instrumentation were as described previously (18). All experiments were carried out in 10 mM imidazole-HCl buffer, pH 7.0, and at 25 °C, unless otherwise indicated. Reaction solutions were protected from light as much as possible because LMO is very susceptible to photochemical formation of the semiquinone species.

**Site-directed Mutagenesis**

The mutagenesis of glycine 99 (codon GGG) to serine was performed with oligonucleotide 5’-CGTCCTCCCATACGCTCATATA*GGCGCTGT-GGC-3’ with the codon for serine as underlined. The same oligonucleotide but with ACG at the underlined location was used for the mutagenesis to threonine. A silent mutation at residue isoleucine 101 as marked with an asterisk was also introduced to create an Eco47III site (5’-AGCGCT-3’). Successful mutagenesis was screened for the addition of the Eco47III site. The Neo-l-MunI fragments (321 base pairs) were ligated into the appropriate position of the expression plasmid pUM01. Successful insertions were confirmed by digesting with Eco47III and finally by plasmid DNA sequencing.

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Mutagenesis of Glycine 99 in Lactate Monoxygenase

Cell Growth and Purification of Mutant Enzymes

The mutant enzymes were grown in 6 x 1 liter of Terrific Broth and purified as described previously (8). The purity of the protein was determined by SDS-polyacrylamide gel electrophoresis (20) stained with Coomassie Blue.

Extinction Coefficients

The extinction coefficients for mutant enzymes were determined by denaturing the protein with SDS as described previously (18).

Reductive Half-reaction

The loading and reaction syringes were filled overnight with a mixture of 3,4-dihydroxybenzoate and 3,4-dihydroxybenzoate dioxygenase to scrub oxygen from the stopped-flow apparatus and then rinsed with anaerobic buffer before loading enzyme and substrate solutions. Details of the instrumentation and analysis programs have been described previously (18).

G99S Mutant—The enzyme solution at a concentration of 10 μM was treated with alternate cycles of evacuation and reequilibration with oxygen-free argon in a tonometer. One syringe of the stopped-flow spectrophotometer was filled with the anaerobic enzyme solution, and the other syringe was filled with anaerobic L-lactate with concentrations varied from 5 to 200 mM. The L-lactate was made anaerobic in its syringe 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 reverse reaction, G99S was reduced anaerobically by the addition of a 4-fold excess of L-lactate in a tonometer. One syringe of the stopped-flow apparatus was filled with reduced enzyme solution, and another syringe was filled with anaerobic pyruvate with concentrations varied from 1 to 100 mM. The pyruvate solution was made anaerobic by bubbling with oxygen-free argon for 20 min. The reoxidation of reduced flavin was followed by the absorbance increase at 460 nm. The anaerobiosis of the experiment was checked by mixing the reduced enzyme with anaerobic buffer. The enzyme remained as the reduced form for at least 30 min. This test was carried out at both the beginning and the end of the experiment. Excellent anaerobiosis was maintained during the experiments.

G99T Mutant—The reductive half reaction was performed in an anaerobic cuvette by using 15.7 μM enzyme, and varied concentrations of L-lactate from 1.9 to 101 mM. The reaction was followed spectrally with time with a Hewlett-Packard diode array spectrophotometer (model 8452A). The absorbance decrease at 456 nm versus time was used to obtain the apparent rate constant at each L-lactate concentration.

The reverse reaction was performed in an anaerobic stopped-flow spectrophotometer. G99T (10 μM) was first reduced by a 100-fold excess of L-lactate. One syringe of the stopped-flow apparatus was filled with reduced enzyme, and varied concentrations of pyruvate from 2.9 to 302 mM were loaded onto another syringe. The reaction was followed by the absorbance change at both 456 and 540 nm.

Simulations

The stopped-flow kinetic traces of the reductive half-reaction were simulated by using a computer analysis system called program A, prepared by Dr. D. P. Bailou, Chung-Jen Chu, Rong Chang, and Joel Dinverno (University of Michigan). Initial estimates for the extinction coefficients and rate constants used in the simulation were taken from a quantitative analysis of the experimental data. The initial rate constants were then adjusted by improving the simulated data according to the measured traces until a best fit was obtained.

Oxidative Half-reaction

Reduced G99S (~20 μM) and G99T (~10 μM) were made by the addition of one equivalent and 100-fold excess, respectively, of L-lactate anaerobically in a tonometer. The oxidation of flavin was observed in stopped-flow experiments with one syringe filled with reduced enzyme and the other syringe filled with 10 mM imidazole-HCl buffer, which was bubbled with varied oxygen concentrations for 20 min. The reaction was followed by observing the reoxidation of flavin at 460 nm.

Photoreduction and Stability of the Semiquinone

L-lactate monoxygenase was photoreduced using glycin as photoreductant and 5-deazaflavin as catalyst, as detailed previously (21, 22). The thermodynamic stability of semiquinone was tested by the addition of 100 μM benzyli viologen. The spectrum of the mixture was followed for days. The concentration of G99S semiquinone remaining in the mixture at equilibrium was calculated by the absorbance change at 380 nm with the extinction coefficients of 16,500 M⁻¹ cm⁻¹ for semiquinone, 8,200 M⁻¹ cm⁻¹ for oxidized enzyme, and 5,470 M⁻¹ cm⁻¹ for reduced enzyme. The G99T semiquinone remaining in the mixture at equilibrium was calculated by the absorbance changes at both 384 and 456 nm. The extinction coefficients for the semiquinone are 18,000 M⁻¹ cm⁻¹ at 384 nm and 3900 M⁻¹ cm⁻¹ at 456 nm; for the oxidized enzyme, coefficients are 8400 M⁻¹ cm⁻¹ at 384 nm and 13,200 M⁻¹ cm⁻¹ at 456 nm; for the reduced enzyme, coefficients are 4500 M⁻¹ cm⁻¹ at 384 nm and 1600 M⁻¹ cm⁻¹ at 456 nm.

Oxidation of Semiquinone by Oxygen

Enzyme semiquinone was made by photoreduction of oxidized enzyme as described above, until maximum semiquinone formation was achieved. One syringe of the stopped-flow apparatus was filled with the semiquinone solution, and the other syringe was filled with buffer, which was bubbled with varied concentrations of oxygen for 20 min. The reaction was followed by both the absorbance decrease at 400 nm and the absorbance increase at 460 nm.

Thermodynamics of the Semiquinone-Pyruvate Complex

Semiquinone was formed by photoreduction and mixed with a large excess of pyruvate from the side arm of an anaerobic cuvette. The mixture was quickly passed through a Sephadex G-25 column at 4 °C to remove excess pyruvate. The remaining semiquinone-pyruvate complex was equilibrated to 25 °C to test its stability to air by following spectral changes with time.

Steady-state Kinetics

Enzyme turnover for the G99S-catalyzed reaction was determined by both the oxygen consumption at air saturation with an oxygen electrode and by the formation of H₂O₂, measuring its incorporation with HRP to form HRP-I at varied oxygen concentrations, as detailed previously (18). The determination of H₂O₂ formation as a function of oxygen concentration at saturated L-lactate concentration yields the K_m for oxygen and K_cat for the L-lactate oxidation reaction catalyzed by G99S.

Enzymatic Assay of H₂O₂

Formation of H₂O₂ as product in the two mutant enzyme-catalyzed reactions was also measured colorimetrically by the HRP-catalyzed oxidation of o-dianisidine, as detailed previously (18). At air saturation, the reaction turnover was determined as a function of the concentration of L-lactate, and the apparent K_m for L-lactate and apparent k_cat of the reaction were therefore obtained.

Reaction with Sulfite

The oxidized G99S and G99T enzymes were titrated with sulfite, and the absorbance changes at 456 nm versus sulfite concentration were used to obtain the dissociation constants. The dissociation constant for the sulfite complex of G99A enzyme was also measured for comparison with those for G99S and G99T, but because of its low value the K_s was determined kinetically. The on-rate was followed by stopped-flow traces at 456 nm at varied sulfite concentrations, and the off-rate was determined by incubation of fully formed complex with excess methyl methanethiosulfonate as described previously (15).
RESULTS

The mutant enzymes were first characterized by their absorption and fluorescence spectra. Details about the environment of the amino acid residues involved in substrate binding with respect to the flavin isoalloxazine ring were then measured by the binding of a transition state analog oxalate and a competitive inhibitor D-lactate. The finding that the mutant enzymes have much reduced reactivities with lactate leads to the test of the binding of sulfite with the mutant enzymes and the measurement of the redox potentials. Furthermore, one-electron reduction of the mutant enzymes was carried out to compare with the properties exhibited by wild type enzyme. The oxygen reactivities of fully reduced enzyme and anionic semiquinone were also measured and compared with wild type enzyme. Finally, reaction turnover measurements show agreement of the direct assay of H2O2 formation with rate constants determined by stopped-flow studies.

Enzyme Purification

In the liquid culture growth, the majority of either mutant enzyme was present as an insoluble form, as has been found for the wild type and other mutant forms of the enzyme (8, 18, 23, 24). Typically, 60–70 mg of G99S and 40–50 mg of G99T were isolated from the soluble portion of the cell culture of 6 liters of culture. Unlike G99A enzyme, which is isolated as a mixture of a flavin N(5)-cysteine adduct and the normal flavin enzyme (18), pure G99S and G99T with normal flavin spectra were obtained by the isolation of enzyme following the usual purification procedure (8).

Spectral Properties

Both G99S and G99T have the normal flavin absorbance characteristics, with the absorbance peak of wild type enzyme at 458 nm blue-shifted to 454 nm and the peak at 373 nm becoming broader and shifted to 371 nm for G99S (Fig. 2A). The absorbance peaks of G99T occur at 375 and 455 nm as shown in Fig. 2B. A shoulder at 472–480 nm for G99S and at 473–480 nm for G99T becomes more pronounced than for the wild type enzyme and for G99A. The ratio of $A_{328}/A_{456}$ is 9.3 for G99S and 9.1 for G99T. The extinction coefficient at 456 nm was determined to be 12,655 $M^{-1} \text{cm}^{-1}$ for G99S and 13,200 $M^{-1} \text{cm}^{-1}$ for G99T. Like wild type enzyme and G99A, the oxidized mutant enzymes are nonfluorescent but become fluorescent upon reduction. The reduced enzymes show a maximum excitation at 365 nm for G99S and 360 nm for G99T and a maximum emission at 515 nm for G99S and 520 nm for G99T, close to the values for the wild type enzyme ($\lambda_{em} = 507$ nm) (25) and G99A ($\lambda_{em} = 510$ nm) (18). The fluorescence of reduced G99S and G99T was about 2- and 4-fold, respectively, weaker than that for G99A.

The absorption spectrum of wild type LMO is perturbed by the presence of most inorganic anions as competitive inhibitors (9, 14, 26). The effect of phosphate ion on the spectrum of the wild type enzyme appears as a large absorbance increase in the range of 350–490 nm with a $K_d$ value of 12.5 mM (14). The presence of phosphate ion was observed to affect the absorption spectra of the oxidized mutant enzymes but in a completely different fashion from that with wild type enzyme. For G99S, the presence of phosphate ion causes an absorbance decrease in the range of 355–375 nm and 402–480 nm and an absorbance increase in the range of 375–402 nm as shown in Fig. 2A. The absorbance maximum at 371 nm was red-shifted to 375 nm upon binding of phosphate. The average $K_d$ value of 90 mM was obtained by measuring the absorbance changes at 320 and 392 nm versus the phosphate concentration, as shown in the inset of Fig. 2A. A similar spectral change as for G99S occurs with G99T, with a $K_d$ value of 110 mM.

Binding of Oxalate

Oxalate has been used as an analog of the presumed carbonation transition state of L-lactate oxidation by LMO (27). The binding of oxalate with LMO induces a pronounced perturbation of the absorption spectrum of the oxidized enzyme. The difference spectrum between the uncomplexed and fully complexed forms of the wild type enzyme shows positive maxima at 391, 425, 450, and 483 nm and negative maxima at 370, 470, and 504 nm, with $\Delta \epsilon = 2000 M^{-1} \text{cm}^{-1}$ at 504 nm. The isosbestic points are at 323, 382, 462, 475, and 485 nm (27). The binding of oxalate with mutant G99A has a difference spectrum similar to that with wild type enzyme. However, the binding of oxalate with either G99S or G99T shows significantly different interactions from those with wild type enzyme.

For G99S, the isosbestic points are at 315, 377, 405, 445, and 510 nm. The positive maxima are at 389, 460, and 490 nm, and the negative maxima are at 350, 370, and 420 nm, with $\Delta \epsilon = 1900 M^{-1} \text{cm}^{-1}$ at 389 nm. The dissociation constant of the G99S-oxalate complex was determined to be $7.4 \times 10^{-4}$ M by monitoring the absorbance change at 389 nm as a function of oxalate concentration (see Table I). Binding of oxalate with G99S is therefore about 50-fold weaker than with the wild type enzyme, where a $K_d$ of $1.6 \times 10^{-5}$ M has been reported (27). A $K_d$ of $3.3 \times 10^{-4}$ M was obtained for G99A (18).

For G99T, the positive maxima are at 390, 425, 451, and 481 nm, with $\Delta \epsilon = 1750 M^{-1} \text{cm}^{-1}$ at 390 nm. The negative maxima are at 355 and 503 nm. The isosbestic points are at 315, 380, and 493 nm. The dissociation constant of the G99T-oxalate complex was determined to be $5.7 \times 10^{-5}$ M by monitoring the absorbance change at 390 nm versus oxalate concentration. Binding of oxalate with G99T is about 3–4 fold weaker than with the wild type enzyme.

The G99S- and G99T-oxalate complexes also form photochemical adducts, as do wild type enzyme and G99A. However, the flavin N(5)-carbonate adducts of G99S- and G99T-oxalate so produced are nonfluorescent and stable for days with oxygen, similar to the adduct with G99A (18), whereas the photoadduct of wild type enzyme is fluorescent and relatively unstable, reforming the oxidized enzyme with a half-life of 3.8 h (28).

Binding of D-Lactate

D-lactate is a competitive inhibitor of LMO and binds with wild type enzyme more strongly than does L-lactate (26). The binding of D-lactate induces a small perturbation of the flavin absorption spectrum of the wild type enzyme. The binding of D-lactate to G99S shows positive maximal changes at 387, 470, and 503 nm. The negative maxima are at 425, 452, and 482 nm, with $\Delta \epsilon = 1400 M^{-1} \text{cm}^{-1}$ at 503 nm. The value of $K_d$ was determined by monitoring the absorbance change at 503 nm as a function of D-lactate concentration and found to be 42 mM. Obviously, the binding of D-lactate to G99S is much weaker than that with wild type enzyme and mutant G99A, both of which have a $K_d$ value of 1.7 mM (Refs. 18 and 26; see Table I).

For G99T, the difference spectra upon D-lactate binding show positive maximal changes at 387, 453, and 480 nm, with $\Delta \epsilon = 2875 M^{-1} \text{cm}^{-1}$ at 453 nm. The dissociation constant of the complex of G99T with D-lactate was determined to be 110 mM by monitoring the absorbance change at 453 nm versus D-lactate concentration.

Reductive Half-reaction

Under anaerobic conditions, the mutant enzymes were reduced slowly by L-lactate. The titration showed that pyruvate was able to reoxidize the reduced enzymes, i.e. that the reductive half-reactions become reversible, namely $k_{-2}$ in Fig. 1
becomes relatively significant. This is in contrast to the findings with wild type enzyme and mutant G99A, where pyruvate was ineffectual in reoxidation of reduced enzyme (14, 18).

**G99S Mutant**—Under anaerobic conditions, the reduction of oxidized G99S by L-lactate was carried out by mixing of 10 mM of G99S with varied concentrations of L-lactate from 5 to 200 mM. The reaction was monitored by the flavin absorbance decrease at 460 nm (shown in Fig. 3). The reaction is biphasic, and the first phase is dependent on the L-lactate concentration. The second phase, which originates from the release of pyruvate from the reduced enzyme, is independent of substrate concentration. However, a reliable value for the rate constant of this phase ($k_2$ in Fig. 1) cannot be determined because the absorbance change is very small, less than 5% of the total. Simulations of these kinetic traces revealed the apparent dissociation constant for L-lactate binding to oxidized G99S to be 4.3 mM and the rate constant $k_2$ to be 51 min$^{-1}$ and $k_3$ to be 1.5 min$^{-1}$. Clearly, L-lactate binds to G99S much more tightly than to the wild type enzyme, which has a $K_d$ value of 50 mM (14).

For the reverse reaction, fully reduced G99S was made by the addition of a 4-fold excess of L-lactate to oxidized G99S under anaerobic conditions. The reaction of 5 mM reduced G99S with varied concentrations of pyruvate from 0.5 to 50 mM was followed by monitoring the absorbance increase at 460 nm. The kinetic traces were simulated with the $k_2$ and $k_3$ values as well as the $K_d$ for L-lactate binding to the oxidized enzyme determined from the forward reaction. Simulations of the kinetic traces yielded the rate constants $k_2$ of 3 min$^{-1}$ and $k_3$ of $3 \times 10^2$ M$^{-1}$ min$^{-1}$. Therefore, the apparent dissociation constant of pyruvate to the reduced G99S is 10 mM (Table I).
the apparent dissociation constant of L-lactate to be 4.3 mM, and simulations of these curves revealed concentrations after mixing). The apparent rate constant of the fast phase was dependent on L-lactate concentration and yielded the sum of $k_2$ to be 4.3 min$^{-1}$ and $k_{-2}$ to be 1.5 min$^{-1}$.

**Figs. 3.** The time traces of the absorbance at 460 nm of 5 µM G99S in the reductive half-reaction with L-lactate concentrations of 2.5, 5, 10, 25, and 100 mM (curves from right to left, concentrations after mixing). Simulations of these curves revealed the apparent dissociation constant of L-lactate to be 4.3 mM, $k_2$ to be 51 min$^{-1}$, and $k_{-2}$ to be 1.5 min$^{-1}$.

**G99T Mutant**—The reduction of oxidized G99T by L-lactate is very slow and was carried out in anaerobic cuvettes by mixing 15.7 µM G99T with 1.9, 3.8, 19, 51, and 101 mM L-lactate. The reaction was followed spectrally with time. Fig. 4 shows the reduction process of 15.7 µM G99T by 1.9 mM L-lactate occurring over a period of 55 min. No absorbance in the long wavelength region due to charge transfer interaction between the reduced enzyme and pyruvate was observed in the reduction. The absorbance at 456 nm versus time was plotted to obtain the apparent rate constant at each L-lactate concentration. At all concentrations used, a linear plot of log $A$ at 456 nm versus time can only be obtained after subtraction of a slow phase from the main phase. The apparent rate constant of the fast phase was dependent on L-lactate concentration and reached a plateau at high L-lactate concentration. A tangent to the initial part of the plot of apparent rate constant versus L-lactate concentration gave an estimation of $k_1$ of 131 m$^{-1}$ min$^{-1}$. The maximal rate constant of 1 min$^{-1}$ from the plot yielded the sum of $k_2$ and $k_{-2}$. The amplitude of the slow phase is less than 5% of the total absorbance change and did not yield any reliable data.

The reoxidation of reduced G99T by pyruvate was performed in anaerobic stopped-flow experiments. Reduced G99T was made by mixing 10 µM oxidized enzyme with 1 mM L-lactate anaerobically. The reduced enzyme (5 µM after mixing) was reacted with 2.9, 11.6, 30.2, 151.1, and 302.3 µM pyruvate. The reaction was monitored at both 456 and 540 nm. Fig. 5 displays the stopped-flow traces at the two wavelengths with 30.2 µM pyruvate. The first phase of the stopped-flow trace is dependent on the pyruvate concentration. The amplitudes of this phase at both wavelengths were plotted versus pyruvate concentration to obtain a dissociation constant of the reduced G99T-pyruvate complex of 5.4 mM as shown in the inset of Fig. 5. The extinction coefficients of the reduced G99T-pyruvate complex were estimated to be 3800 M$^{-1}$ cm$^{-1}$ at 456 nm and 1770 M$^{-1}$ cm$^{-1}$ at 540 nm. A plot of the apparent first order rate constant of the first phase versus the pyruvate concentration reaches a plateau of 540 min$^{-1}$, and a tangent to the initial part of the plot gives an estimation of $k_3$ of 3.3 × 10$^3$ M$^{-1}$ min$^{-1}$. The second phase of the stopped-flow trace is not dependent on pyruvate concentration and yielded the rate constant of $-0.12$ min$^{-1}$.

The kinetic traces of the stopped-flow experiment of the reduced enzyme reaction with pyruvate at both 456 and 540 nm were simulated with the following model in which the reduced enzyme exists in two forms, but only one of them binds pyruvate to cause the reoxidation.

$$
E_{red} + pyruvate \rightleftharpoons E_{red-pyruvate}
$$

$$
\frac{k_3}{k_2} E_{red-pyruvate} \rightleftharpoons E_{red-lactate} \rightleftharpoons E_{red} + L-lactate
$$

**Reaction 1**

The same phenomenon has been reported for L-lactate oxidase from *A. viridans* (1). The finding that the apparent first
order rate constant of the first phase depends on pyruvate concentration and reaches a plateau of 540 min\(^{-1}\) requires that the rate constant \(k_6\) be equal to the maximal value of 540 min\(^{-1}\). Simulations yielded the values of the rate constants for each step as well as the apparent dissociation constants of \(l\)-lactate binding to oxidized enzyme and pyruvate binding to the reduced enzyme shown in Table I. The value of \(k_7\) equal to 180 min\(^{-1}\) was also obtained.

Binding of Sulfite

The reduction of flavin by \(l\)-lactate is postulated to be initiated by proton abstraction from the \(\alpha\)-carbon atom of \(l\)-lactate by histidine 290 in LMO, and the resultant carbocation intermediate forms an adduct at the flavin N(5), which is then stabilized by the interaction of negative charge at the flavin N(1) position with lysine 266 (27, 29, 30). The strength of the interaction between the carbocation and lysine residue could be reflected by the tendency of sulfite binding to the enzyme. Wild type LMO binds with sulfite strongly to form the flavin N(5)-sulfite adduct (31, 32) with the dissociation constant \(K_d\) of 5.8 \(\times\) 10\(^{-8}\) M (15). Binding of sulfite to G99S and G99T results in the abolition of the flavin visible absorption spectrum, as with the wild type enzyme. Plots of absorbance change at 456 nm versus the sulfite concentration yielded the dissociation constant of 6.1 \(\times\) 10\(^{-3}\) M for G99S and 2.5 \(\times\) 10\(^{-5}\) M for G99T. Therefore, sulfite binding to G99S and G99T is considerably weaker than to the wild type enzyme.

For comparison with G99S and G99T, the \(K_f\) value of sulfite binding to G99A was also determined. As with wild type enzyme, the \(K_f\) for equilibrium formation of the flavin N(5)-sulfite adduct of G99A is so low that it can only be determined by measuring the second order rate constant for formation of the adduct \((k_{\text{on}}\) by stopped-flow measurement at 456 nm and the dissociation rate constant \((k_{\text{off}}\) by reacting the equilibrium concentration of released sulfite with methylmethane thiosulfonate and following the return of the flavin absorbance spectrum (15). The determined \(k_{\text{on}}\) was 2.1 \(\times\) 10\(^4\) M\(^{-1}\) s\(^{-1}\), and \(k_{\text{off}}\) was 4.2 \(\times\) 10\(^{-4}\) s\(^{-1}\); thereby, \(K_f\) was 2.0 \(\times\) 10\(^{-5}\) M for G99A. Therefore, a small increase of \(k_{\text{on}}\) and a small decrease of \(k_{\text{off}}\) occur for G99A compared with the wild type enzyme, where \(k_{\text{on}}\) was 1.3 \(\times\) 10\(^4\) M\(^{-1}\) s\(^{-1}\) and \(k_{\text{off}}\) was 7.2 \(\times\) 10\(^{-4}\) s\(^{-1}\) (15).

Redox Potential

The equilibrium titration of fully reduced enzyme with pyruvate was carried out to determine the redox potential for two-electron reduction, \(E_{\text{red/ox}}\) for both mutants. Fig. 6 shows the titration of fully reduced G99S with pyruvate. The reduced G99S was made by mixing with 10-fold excess of \(l\)-lactate, and the titration was followed spectrally, which clearly demonstrated the flavin reoxidation. The Nernst plot of log [oxidized/reduced] LMO versus log [pyruvate]/[lactate] yielded a unit slope with an intercept equivalent to 54 mV at log [pyruvate]/[lactate] = 0 as shown in the inset of Fig. 6. Therefore, the mid-point potential of oxidized/reduced G99S can be calculated to be –131 mV by using the pyruvate/lactate couple as a reference (–185 mV; Ref. 33). A similar titration with G99T revealed the midpoint potential of oxidized/reduced G99T to be –167 mV. The mutations of G99S and G99T therefore result in little change in the redox potential for the two-electron reduction of the flavin from that of wild type enzyme (–149 mV (34); see Table I).

Photoreduction of Mutant Enzymes

Like wild type enzyme and mutant G99A, G99S and G99T are readily reduced photochemically to the anionic form of the flavin semiquinone. The semiquinone of G99S shows a maximum absorbance at 380 nm, a small peak at 404 nm, and a broad band centered around 490 nm. The extinction of the semiquinone is 16,500 M\(^{-1}\) cm\(^{-1}\) at 380 nm, higher than the oxidized species, as shown in Fig. 7A. The peak at 404 nm becomes less obvious than those of wild type enzyme and G99A. The isosbestic points of semiquinone and oxidized enzymes are at 352, 414, and 498 nm. Unlike wild type enzyme and G99A, the semiquinone of G99S is thermodynamically unstable, since it reacts bimolecularly to form an equilibrium mixture consisting of semiquinone and fully oxidized and fully reduced enzyme, although the disproportionation is a very slow process. About 57% of the semiquinone was disproportionated in the presence of 100 \(\mu\)M benzyl viologen when the equilibrium was reached in 3 days, as shown in Fig. 7A. The redox potentials of the oxidized/semiquinone and semiquinone/reduced couples can therefore be calculated to be –121 and –141 mV, respectively, from the concentrations of each enzyme species at the equilibrium and the known redox potential of –131 mV for the two-electron reduction for G99S. Compared with the wild type enzyme, where the redox potential for the oxidized/semiquinone couple was reported to be –67 mV and that for the semiquinone/reduced couple to be –231 mV (34), the mutation of glycine to serine has caused a large change in the redox potentials of the two one-electron transfer processes as summarized in Table I.

The binding of pyruvate to G99S semiquinone affects the absorption spectrum of the semiquinone. The absorption peak at 404 nm shifts to 406 nm and becomes sharp upon binding, as shown in Fig. 7B. The binding of pyruvate with G99S semiquinone results in increases in absorbance in the 400–700 nm range attributable to a charge transfer transition (35). The dissociation constant of the complex was determined to be (1.5 \(\pm\) 0.3) \(\times\) 10\(^{-5}\) M by following the absorbance increase at 540 nm as a function of pyruvate concentration as shown in the inset of Fig. 7B. Pyruvate thus binds to G99S semiquinone with approximately the same strength as to the wild type enzyme, where a \(K_f\) of 1.4 \(\times\) 10\(^{-5}\) M has been reported (35), and to mutant G99A, which has a \(K_f\) of 2.0 \(\times\) 10\(^{-5}\) M (18).

The semiquinone of G99T shows an absorbance peak at 384 nm with a shoulder at 400 nm and a broad band centered at 454 nm. The extinction of the semiquinone is 18,000 M\(^{-1}\) cm\(^{-1}\) at 384 nm. The isosbestic points of semiquinone and oxidized enzymes are at 336, 411, and 500 nm. A change of the flavin environment for the G99T semiquinone is reflected by the diminished absorbance peak at 406 nm as compared with the wild type enzyme. Similar to the G99S semiquinone, the semiquinone of G99T is also thermodynamically unstable. About 82% disproportionation in the presence of 100 \(\mu\)M benzyl...
viologen occurred when the equilibrium was reached in 9.5 h (results not shown). The redox potentials of oxidized/semiquinone and semiquinone/reduced couples can be calculated to be 2170 mV and 2164 mV, respectively, based on the known redox potential of 2167 mV for the two-electron reduction of G99T.

The binding of pyruvate to G99T semiquinone has similar effects on the flavin absorption spectrum to binding with G99S semiquinone; i.e. the shoulder at 400 nm becomes a peak, and absorbance at 400–700 nm increases. The dissociation constant of pyruvate binding to the reduced enzyme to be 5.4 mM.

Reactions with Oxygen

The Oxidative Half-reaction—The reduced G99S and G99T were made anaerobically by the addition of one equivalent and a 100-fold excess of L-lactate, respectively, and the rate of reoxidation of the reduced enzymes with oxygen was determined by monitoring the absorbance increase at 460 nm with time in the stopped-flow spectrophotometer. Under the experimental conditions of 10 μM G99S, 25 °C, and pH 7.0, the reaction is very slow, requiring ~50 min for completion at the lowest O2 concentration of 30 μM. The stopped-flow traces were single exponentials with rates directly proportional to oxygen concentration, yielding a rate constant of the free reduced G99S with O2 of 2.2 × 103 M⁻¹ min⁻¹, about 2 orders of magnitude lower than that of 5.4 × 106 M⁻¹ min⁻¹ for the wild type enzyme (14) and more than 2 times lower than that of...
5.1 \times 10^3 \text{ m}^{-1} \text{ min}^{-1} for mutant G99A (18). The same experiment with G99T yielded a rate constant of the free reduced G99T with O_2 of 1.1 \times 10^4 \text{ m}^{-1} \text{ min}^{-1}, about 50 times slower than for the wild type enzyme. It should be noted that because of the slow reduction of enzyme by L-lactate, the presence of excess lactate in these experiments is without effect on the reaction with O_2.

Reaction of Semiquinone with Oxygen—The reactivity of semiquinone with O_2 was determined by stopped-flow experiments. Semiquinone of G99S was formed in a tonometer under anaerobic conditions by photoreduction of a mixture of 38 \mu M G99S, 0.1 M glycine, and 1.25 \mu M 5-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 absorbance increase at 460 nm. The stopped-flow traces were single exponentials with rates directly proportional to oxygen concentration. A rate constant of 1.64–1.74 \times 10^6 \text{ m}^{-1} \text{ min}^{-1} was obtained from the stopped-flow traces at both wavelengths. Therefore, the semiquinone of G99S reacts with O_2 at a rate constant more than 3-fold larger than the wild type enzyme (14) and mutant G99A (18). Similar stopped-flow experiments were performed for G99T, yielding the rate constant of 4.6 \times 10^7 \text{ m}^{-1} \text{ min}^{-1}, which is 2 orders of magnitude larger than that of 4.5 \times 10^5 \text{ m}^{-1} \text{ min}^{-1} for the wild type enzyme (14).

No obvious disproportionation reactions of the flavin semiquinones occurred during the course of the experiments.

The Reactivity of Semiquinone-Pyruvate Complex with Oxygen—The reactivity of the semiquinone-pyruvate complex with O_2 was determined. In a mixture of 19.4 \mu M G99S, 1 \mu M 5-deazaflavin, and 0.1 M glycine, semiquinone was formed by light irradiation for 15 min in an anaerobic cuvette. Mixing of pyruvate from the side arm to a final concentration of 9.64 mM yielded the semiquinone-pyruvate complex. Excess pyruvate was removed by gel filtration with a G-25 Sephadex column, and the reoxidation of the remaining complex was measured spectrophotometrically as shown in Fig. 8. A half-time of 33 min for the first order reoxidation of the complex was obtained by plotting the absorbance change at 457 nm versus time (Fig. 8, inset). Therefore, like wild type enzyme and mutant G99A, the semiquinone-pyruvate complex of G99S is probably 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. This mutation, therefore, has not caused any change in its slow release of pyruvate, as with the wild type enzyme (35).

In a similar experiment with 23.6 \mu M G99T semiquinone mixed with 5.9 mM pyruvate, the spectrum of fully oxidized G99T was observed after the mixture was passed through a G-25 Sephadex column. This indicates that the release of pyru-
The rate of O$_2$ consumption was determined colorimetrically by monitoring the rate of H$_2$O$_2$ formation employing H$_2$O$_2$ and HRP to form HRP-I. One syringe was filled with 1.4 $\mu$M G99S and 8 $\mu$M HRP at air saturation, and the other syringe was filled with 10 mM L-lactate and varied O$_2$ concentrations. The rate of H$_2$O$_2$ formation was determined by monitoring the formation of HRP-I at 398 nm, which is the isosbestic point between HRP-I and HRP-II. This is necessary to avoid complications resulting from the further conversion of HRP-I to HRP-II. The reaction turnover numbers were obtained at varied O$_2$ concentrations at a saturated concentration of L-lactate. The plot of turnover number versus O$_2$ concentration yielded a hyperbolic curve, from which a $k_{cat}$ of 3.4 $\pm$ 0.5 min$^{-1}$ and $K_m$ of 1.3 $\pm$ 0.3 mM for oxygen can be calculated. These determined values are comparable with the theoretical values of $k_{cat}$ of 2.8 min$^{-1}$ and $K_m$ of 1.25 mM for oxygen as displayed in Table II.

**DISCUSSION**

The introduction of serine or threonine in place of glycine 99 of LMO was to explore further the essential role of glycine 99 in maintaining monooxygenase activity and the factors that control enzyme reactivity with molecular oxygen.

Substitution of Gly$_{99}$ by Ala, Ser, Thr, and Val results in increasingly severe changes in the properties of the enzyme. The substitution of Ala did not change significantly the reduced enzyme-pyruvate complex by a factor of 10$^2$ (18). This results in a change of mechanism so that the enzyme now follows the outer pathway of Fig. 1 rather than the inner pathway employed by wild type enzyme (14). The replacement of Gly$_{99}$ by either Ser or Thr, in addition to the changes to the oxidative half-reaction, causes also large decreases in the rate of flavin reduction by L-lactate (Table I). An even more drastic effect is caused by substitution of the glycine residue by valine. With this mutant form, the enzyme is expressed as an apoprotein, unable to bind FMN.

A significant change in the flavin environment of G99S and G99T is first reflected by the flavin absorption spectra of the
two mutant enzymes, which contain a more notable shoulder at 472 or 473–480 nm than in wild type enzyme or G99A. The intensity of the fluorescence of the reduced enzyme becomes weaker as the size of the introduced residue is increased. A change of flavin environment upon the mutation is also implied by the observation that the effect of phosphate ion on the spectrum of the oxidized G99S or G99T is completely different from that with the wild type enzyme (14) in that phosphate ion causes less perturbation on the absorption spectra of the two mutants. The binding affinities of phosphate ion to G99S and G99T are about 7- and 9-fold weaker, respectively, than to the wild type enzyme, and the presence of phosphate ion does not have any effect on the turnover number of G99S, which is also in contrast to the inhibition found with wild type enzyme (14).

A further evidence of the change in the flavin environment in G99S and G99T is that the spectral features of flavin semiquinone are only slightly separated in G99S and are even inverted in G99T. More importantly, unlike the wild type enzyme and G99A, the semiquinones of G99S and G99T are not thermodynamically stabilized but are only kinetically favored upon photoreduction. The thermodynamic stability of the semiquinone depends on the relative reduction potential for each one-electron step. These are widely separated in the case of wild type enzyme but are only slightly separated in G99S and are even inverted in the case of G99T (see Table I). The change of thermodynamic stability of flavin semiquinone implies that the interactions of flavin with the proteins are significantly altered by the mutations and therefore that the equilibrium as well as the rate at which it is attained is affected. The fact that G99S and G99T do not form a flavin N(5)-cysteine adduct, whereas G99A does, indicates that the orientations of the flavin isoalloxazine ring with respect to the polypeptide backbone are different in G99S and G99T from that in G99A. All of the above evidence illustrates that the flavin environment or specifically the electronic interactions in the active site of the enzyme are varied among the glycine 99 mutants and wild type enzyme. Therefore, G99S and G99T are good models for the purpose of this study.

The mechanistic studies of G99S and G99T show that, overall, both mutations cause a change in the reaction pathway from the coupled reaction (in which O$_2$ consumption is coupled to production of acetate, CO$_2$, and H$_2$O) characterized by the wild type enzyme to the completely uncoupled reaction (in which O$_2$ consumption leads to pyruvate and H$_2$O$_2$ production), which is characteristic of L-lactate oxidase. Measurements of the H$_2$O$_2$ formation rates at various L-lactate and oxygen concentrations yield the values of turnover number and $K_m$ for lactate and oxygen, which are very close to the theoretical values calculated from individually measured reaction rate constants (Table II).

Unlike mutant G99A, mutations of glycine 99 to serine and threonine show substantial decreases in the rate of flavin reduction by L-lactate. The rate constant of flavin reduction by L-lactate was changed from 14,000 min$^{-1}$ for wild type enzyme (14) to 51 min$^{-1}$ for G99S and 0.84 min$^{-1}$ for G99T, but with only little decrease for G99A (18). Pyruvate is able to reoxidize reduced G99S and G99T, which was not observed with the wild type enzyme (14) and G99A (18). However, the binding affinity of substrate with oxidized G99S and G99T increases about 12- and 2-fold, respectively, compared with the wild type enzyme (see Table I). With G99S, the mutation does not have much effect on the rate of the release of pyruvate from reduced enzyme, but pyruvate binding to the reduced G99S is about 4-fold weaker than to the wild type enzyme. The replacement of glycine with threonine increases the rate of the release of pyruvate from reduced enzyme 7-fold compared with the wild type enzyme, but pyruvate binding to the reduced G99S is about 2-fold weaker than to the wild type enzyme.

Results indicate that steric hindrance around flavin N(5) has some influence on the binding of pyruvate to the enzyme but is not a major factor.

Sulfite binds to wild type LMO strongly to form the flavin N(5)-sulfite adduct (31, 32), which has been proposed to be related to the inductive effect of a positively charged residue of the protein located near the flavin N(1) position (31, 36), namely lysine 266 in LMO (7). The binding affinity of sulfite to

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### Table II

| Enzyme | G99S | G99T |
|--------|------|------|
| $k_{cat}$ | 2.8 min$^{-1}$ | 0.62 min$^{-1}$ |
| $K_m^{lac}$ | 1.25 mM | 1.3 mM |
| $k_{cat,app}$ | $\frac{1}{k_2 + k_{-2} + k_3 + \frac{1}{k_{d(O_2)}}}$ | $\frac{1}{k_2 + k_{-2} + k_3 + \frac{1}{k_{d(O_2)}}}$ |
| $K_m^{lac,app}$ | $\frac{1}{k_{cat} + \frac{1}{k_{d(O_2)}}}$ | $\frac{1}{k_{cat} + \frac{1}{k_{d(O_2)}}}$ |
|  | G99S | G99T |
| --- | --- | --- |
| Calculated | 0.47 min$^{-1}$ | 0.51 min$^{-1}$ |
| Observed | 0.55 min$^{-1}$ | 25 mM | 63 µM | 27.6 mM |
| Wild type | (3.4 ± 0.5) min$^{-1}$ | (1.3 ± 0.3) mM | 55 µM (calculated) | 71 µM (observed) |
oxidized G99S and G99T is found to be about 3 orders of magnitude and 430-fold, respectively, weaker than to the wild type enzyme. However, sulfite binds to G99A at a similar strength as to the wild type enzyme. Thus, the change of sulfite binding affinity for G99S and G99T is correlated with the observed decreases in the rate of flavin reduction for the two mutants as compared with wild type enzyme and G99A. The reduction of flavin is postulated to be initiated by proton abstraction from the α-carbon atom of the substrate by a histidine residue, and the resultant carbanion intermediate forms an adduct at the flavin N(5), which is then stabilized by the interaction of negative charge at the flavin N(1) position with lysine 266 (27, 29, 30). Weakening of this salt bridge due to the mutations could result in a substantial decrease in the rate constant for flavin reduction. A similar phenomenon of a substantial decrease in the rate of flavin reduction by substrate was observed with mutant K266M of LMO where the salt bridge was removed (8). However, the lower stabilization of the transition state for reduction of G99S and G99T by L-lactate is obviously not the only factor that affects the reduction rate, because G99T binds to sulfite stronger than does G99S but with even slower reduction by L-lactate (see Table 1).

As stated earlier, the electronic interactions between the flavin and protein polypeptide backbone are different among the constructed glycine 99 mutants and the wild type enzyme. Consistently, changes of the oxygen reactivity were observed among these mutants and the wild type enzyme. The reactivity of reduced G99A, G99S, and G99T with molecular oxygen is about 100-, 245-, and 50-fold smaller than that of wild type enzyme. This indicates that the electronic distributions in the mutant enzymes are more unfavorable for the spin inversion steps required for the reaction of triplet oxygen than in wild type enzyme.

The observation that the semiquinones of G99S and G99T react with molecular oxygen at a rate 3- and 100-fold faster, respectively, than wild type enzyme and mutant G99A provides a further evidence for the suggestion that the electrostatic interactions in the active site of the enzyme control the oxygen activation. Obviously, the electronic interactions in the catalytic cavity of the G99S and G99T semiquinones are more favorable for the oxygen activation than in wild type enzyme and mutant G99A.

Oxalate, which is considered to be a transition state analog of substrate oxidation by LMO, binds to oxidized G99A, G99S, and G99T 21-, 50-, and 3.5-fold more weakly, respectively, than to the wild type enzyme. The difference spectra show that oxalate binds to G99S and G99T in different ways with respect to the flavin isoalloxazine ring than with the wild type enzyme and G99A. This presumably reflects changes in the orientation of amino acid residues involved in the substrate binding with respect to the flavin isoalloxazine ring. A linear relationship between the $K_a$ for binding of oxalate and the rate of flavin reduction by l-lactate was observed previously for a series of active site mutant enzymes (24). The values for mutants at glycine 99 do not lie on the line defined by the previous data. The relationship of the binding affinity of oxalate to glycine 99 mutants and the wild type enzyme with the reduction rate is not readily interpreted.

The G99S- and G99T-oxalate complexes also undergo photochemical adduct formation similar to that found with wild type enzyme and G99A. However, like mutant G99A, the resulting photoproducts do not show decay in the presence of oxygen as does the photoadduct of the wild type enzyme.

The binding of l-lactate to G99S and G99T cause different perturbations on the flavin spectra from those to the wild type enzyme and G99A, and the binding capacity to G99S and G99T is about 24- and 65-fold weaker, respectively, than to the wild type enzyme and G99A. These results clearly indicate again that the orientations of the amino acid residues involved in substrate binding with respect to the flavin isoalloxazine ring are altered upon mutation.

Pyruvate binds to the anionic semiquinone of LMO, and the complex is inactive with molecular oxygen (35). The slow reoxidation of the complex is due to the reaction of oxygen with free semiquinone formed as pyruvate dissociates from the complex, with a half-life of 33 min for the wild type enzyme (35). Like the wild type enzyme, the semiquinone-pyruvate complex of G99S is also unreactive with oxygen, and the mutation has not caused any change in the rate of release of pyruvate from its complex with semiquinone. However, in contrast to the wild type enzyme and mutants G99A and G99S, a much weaker binding of pyruvate to semiquinone and a fast release of pyruvate from its complex with semiquinone was observed with G99T.

In summary, the mutations of glycine 99 to serine and threonine cause a change in the kinetic pathway to a completely uncoupled reaction as in L-lactate oxidase, and the same as mutant G99A but with still much lower activities. Therefore, the three mutant enzymes lose the monooxygenase activity but with only small changes in the rate of pyruvate release from reduced enzyme ($k_4$ in Table 1). In this respect, the mutant enzymes are quite different from L-lactate oxidase of A. viridans, where the oxidase activity has been shown to be associated with the very fast release of pyruvate from the reduced enzyme-pyruvate complex (1). Unlike mutant G99A, the mutations to serine and threonine cause substantial decreases in the rate of flavin reduction by l-lactate, which can be correlated with the weaker binding of sulfite. A large number of observations illustrate changes of the electronic interactions of the flavin in the active site of the enzyme. The greatly decreased oxygen reactivities with reduced enzymes and increased oxygen reactivities with semiquinone forms of the mutant enzymes thus appear to be associated with the altered electronic interactions in the active site. Glycine 99 in LMO is therefore a key residue in the active site to maintain the monooxygenase activity of the enzyme.

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