Conversion of L-Lactate Oxidase to a Long Chain α-Hydroxyacid Oxidase by Site-directed Mutagenesis of Alanine 95 to Glycine*

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A mutant form of l-lactate oxidase (LOX) from *Aerococcus viridans* in which alanine 95 was replaced by glycine was constructed as a mimic of l-lactate monoxygenase but proved instead to be a mimic of the long chain α-hydroxyacid oxidase from rat kidney. A95G-LOX keeps oxidase activity with l-lactate at the same level as wild type LOX but has much enhanced oxidase activity, with longer chain l-α-hydroxyacids, α-hydroxy-β-butyric acid, α-hydroxy-β-valeric acid, etc., and also the aromatic α-hydroxyacid, l-mandelic acid. Kinetic analysis of the activity with these substrates indicates that the reduction of the enzyme bound flavin by substrates is the rate-limiting step in A95G-LOX. The affinity of pyruvate for the reduced enzyme is increased, and sulfite binding to the oxidized enzyme is weaker in A95G-LOX than in native enzyme. Wild type LOX stabilizes both the neutral and anionic flavin semiquinones with a pKₐ of 6.1, but A95G LOX stabilizes only the anionic semiquinone form. These results strongly suggest that the environment around the N5-C4a region of the flavin isoalloxazine ring is changed by this mutation.

α-Hydroxyacid oxidizing enzymes, a family of flavoprotein enzymes, share a series of common characteristics. Within the family, the crystal structures of glycolate oxidase and flavocytochrome b₂ have been solved by x-ray diffraction studies (1–4). The reaction mechanisms of L-lactate monoxygenase from *Mycobacterium smegmatis* and flavocytochrome b₂ from *Saccharomyces cerevisiae* have been studied extensively (5, 6). L-Lactate monoxygenase utilizes l-lactate and other l-α-hydroxyacids but is unique in accelerating the oxidative decarboxylation of the products, pyruvate (or corresponding keto acid) and hydrogen peroxide, to acetic acid (or corresponding carboxylic acid), carbon dioxide, and water. New members of this flavoenzyme family have been described including those utilizing aromatic and bulky substrates like mandelate and long chain α-hydroxyacids (7–9).

We are studying one of the new enzymes of this family, L-lactate oxidase from *Aerococcus viridans* (10). This enzyme utilizes l-lactate and oxygen as l-lactate monoxygenase does but forms pyruvate and hydrogen peroxide as the final products instead of catalyzing the oxidative decarboxylation reaction. We believe that lactate oxidase is an ideal enzyme for studying the reaction mechanism of this enzyme family because of its considerable stability, and it is also a useful enzyme for the construction of a lactate sensor for biological applications.

By analogy with the crystal structures of glycolate oxidase and flavocytochrome b₂, it has been pointed out that glycine 99 in l-lactate monoxygenase is unique in the family of enzymes (except now mandelate dehydrogenase (11) also with glycine), because the other family members have an alanine residue at the homologous position (6). This residue, in glycolate oxidase and flavocytochrome b₂, is in close contact with the flavin N-5 position on the re-face of the flavin. It was considered possible that the smaller glycine residue in lactate monoxygenase might allow the keto acid product to remain bound to the reduced enzyme sufficiently long that subsequent reaction with molecular oxygen produced the ternary complex of oxidized flavin, pyruvate, and H₂O₂ from which the oxidative decarboxylation occurs. To test this hypothesis two separate approaches were made. In one, the glycine 99 residue in lactate monoxygenase was changed to alanine (12), and in the present case, the alanine 95 of l-lactate oxidase was changed to glycine. The modified lactate oxidase was not converted to a monoxygenase but significantly was found to have a much broader substrate specificity than the wild type enzyme, becoming a better mimic of long chain α-hydroxyacid oxidase than of lactate monoxygenase.

**EXPERIMENTAL PROCEDURES**

Site-directed mutagenesis of lactate oxidase was carried out by the method of Kunkel et al. (13) with a Mut-a-Gen Phagemid in *in Vitro* Mutagenesis version 2 kit (Bio-Rad). From an expression plasmid of the wild type lactate oxidase that was previously constructed (10), a 4.4-kilobase pair EcoRI fragment containing the lactate oxidase gene was excised and subcloned into the EcoRI site in the multiple cloning site of pTV119N. With this recombinant plasmid, *E. coli* CJ236 was transformed to yield the uracil-containing single-stranded DNA by the aid of M13KO7 helper phage. A phosphorylated mutagen oligonucleotide primer to introduce the A95G amino acid substitution (GGCTTAAAATCATGGTGCTGCAATTGGTGCCATG), which was synthesized with an Applied Biosystems 394 DNA synthesizer, was annealed to the single-stranded DNA, and the second DNA was synthesized using T7 DNA polymerase. After the ligation with T4 DNA ligase, *E. coli* DH1 was transformed with the double-stranded heteroduplex DNA. A clone harboring the plasmid containing the mutated lactate oxidase gene was isolated from the transformants, and the introduced base substitution was confirmed by DNA sequencing. The mutated DNA fragment was then excised with EcoRI and subcloned into the EcoRI site of pACYC184 to give an expression plasmid of A95G lactate oxidase. Both the wild type and A95G lactate oxidase were expressed and purified according to the method described before (10).

Initial rate steady state kinetics measurements were made by the addition of enzyme (40–200 pmol) to the assay solution (1.7 ml) in a water-jacketed cell at 25 °C, and the oxygen consumption was monitored by a Gilson medical electronics 56/0xgraph. The initial oxygen regulation by reaction conditions 

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concentration of the assay solution was tuned by the laboratory-made oxygen pump, model SY-1. This instrument is modeled on the oxygen concentration regulator, model SEP-104 from Toray Co. LTD, Shiga in Japan. The assay solution contained varying concentrations of the α-hydroxyc acid substrate, dissolved in 10 mM imidazole buffer, pH 7.0, containing 100 mM KCl.

Rapid reaction studies were carried out with an Otsuka electronics, RA-401 stopped flow instrument or with the laboratory-made stopped flow spectrophotometer described in our previous paper (10). Absorbance and fluorescence spectra for the titrations were measured with temperature-controlled equipment, Hitachi 320 or Hewlett-Packard 8452A and Hitachi 650/60, respectively.

Assay methods for pyruvate and hydrogen peroxide detection, anaerobic, photochemical reduction, the determination of the sulfite binding/dissociation kinetics, and the pyruvate binding measurements were carried out and analyzed as described previously (10). Enzyme concentrations of A95G and wild type lactate oxidase were determined by the molecular extinction coefficient at 457 nm of L-lactate and ultraviolet wavelengths as does wild type enzyme (10). Purified A95G lactate oxidase was purified by the same method as described previously for wild type enzyme (10). All of the reagents were from commercial sources and used without further purification.

RESULTS

Purification of Enzyme and Product Analysis—The mutant enzyme was purified by the same method as described previously for wild type enzyme (10). Purified A95G lactate oxidase shows a similar absorbance spectrum in both the visible and ultraviolet wavelength regions as does wild type enzyme. A95G lactate oxidase is catalytically very active with L-lactate and shows higher activity at higher ionic strength in the same way as wild type enzyme does (10). The addition of catalase to the assay solution after the reaction had progressed for some time resulted in evolution of oxygen, indicating that the primary product of lactate oxidase was pyruvate and hydrogen peroxide. This was confirmed by quantitative analysis of pyruvate and hydrogen peroxide with dinitrophenol-hydrazine and with horseradish peroxidase and dye, respectively, after starting the enzyme assay with 0.05 mM or 0.15 mM L-lactate in air-saturated buffer at 25 °C, pH 7 (10). There was no difference between wild type and A95G lactate oxidase on the production and stability of pyruvate and hydrogen peroxide.

Steady State Kinetics—Enzyme-monitored turnover traces of wild type and A95G lactate oxidase with L-lactate as the substrate at 4 °C and a limiting O2 concentration of 256 μM are shown in Fig. 1. With wild type enzyme the absorbance at 457 nm drops rapidly from 0.052 to the steady state of the turnover between enzyme and substrates where the absorbance of 457 nm remains almost constant around 0.037, until the third phase corresponding to the complete reduction of enzyme, as all the oxygen is consumed. A95G lactate oxidase does not show a clear steady state of turnover as does the wild type enzyme. The extent of the first rapid absorbance decrease at 457 nm from 0.053 depends on the concentration of L-lactate, and the complete reduction of A95G lactate oxidase takes 5–6-fold longer than wild type enzyme. Furthermore, the final absorbance at 457 nm of A95G lactate oxidase is 0.020, three times higher than that of wild type lactate oxidase.

Comparison of the traces of wild type and A95G lactate oxidase points out differences in the reductive and oxidative half-reaction rates and affinities of substrates, L-lactate and oxygen, and product, pyruvate in the two enzyme forms. With the wild type enzyme, almost the same steady state level between oxidized and reduced enzyme was obtained, independent of the L-lactate concentration, consistent with a low Km value.

| Substrate       | Turnover number | Km for hydroxyacid | Km for oxygen |
|-----------------|-----------------|--------------------|---------------|
| L-Lactate       | 17000           | 15000              | 0.23          | 1.10          |
| DL-α-OH-β-Butyrate | 660             | 30600              | 18            | 27            |
| DL-α-OH-Valerate | 75              | 3100               | 5.5           | 10            |
| L-Mandelate     | 0.2             | 950                | 0.3           | 20            |
| DL-Glycerate    | 52              | 1880               | 5.0           | 53            |
| L-α-OH-Iso-valerate | ND             | 500                | ND            | 125           |
| L-α-OH-β-Methylvalerate | ND      | 670                | ND            | 140           |

Note: Km values for oxygen could not be determined because of no observed dependence on oxygen concentration in the range used. ND, indicated not tried, because of extremely poor substrates for wild type lactate oxidase. The Km values for the α-hydroxyacids are expressed in terms of the concentrations of the L-isomers. All of the experiments were done in 10 mM imidazole plus 100 mM KCl at pH 7.0 and 25 °C.
for L-lactate and a fast reaction of reduced enzyme with O₂ (10). On the other hand, with the mutant enzyme, the steady state level of oxidized enzyme was very dependent on the L-lactate concentration, indicating a higher $K_m$ value, and probably also a lower reaction rate constant with O₂. Chance (14) and Gibson et al. (15) have devised methods to determine the steady state kinetic parameters from enzyme-monitored turnover traces. Because of the lack of a clear steady state with the mutant enzyme, such analyses could be applied only to the wild type enzyme. Parallel Lineweaver-Burk plots were obtained with wild type and A95G lactate oxidase when the uptake of oxygen was monitored by the oxygen electrode with variation of both L-lactate and oxygen concentrations (Fig. 2a). Secondary plots show no remarkable change in the turnover number but distinct increases in the Michaelis constants both for L-lactate and oxygen between wild type and A95G lactate oxidase (Fig. 2b). This indicates that the kinetic affinities for the substrates L-lactate and oxygen become weaker but the chemistry of the catalytic reaction is not changed by this mutation (Table I).

**Substrate Specificity**—Replacement of alanine 95 by glycine may be expected to make more space around the flavin isoalloxazine ring. The A95G mutant takes up oxygen effectively in the presence of dl-$\alpha$-hydroxy-n-butyric acid or dl-$\alpha$-hydroxy-n-valeric acid (Fig. 3 and Table I). dl-Glyceric acid, l-$\alpha$-hydroxyiso-valeric acid, l-$\alpha$-hydroxy-$\beta$-methylvaleric acid, and l-mandelic acid are also good substrates for A95G lactate oxidase compared with wild type enzyme. Turnover numbers of both wild type and A95G enzyme with substrates that show low activities are insensitive to oxygen concentration, indicating low $K_m$ values, but the insensitivity prevents the determination of $K_m$ values for oxygen. Enzyme-monitored turnover traces of wild type enzyme with poor substrates kept relatively high absorbance at 457 nm, almost the same as the initial absorbance until most of the oxygen was consumed (data not shown). This is because the reduction rate of the enzyme flavin by $\alpha$-hydroxy-acids is relatively slow compared with the reoxidation rate of the reduced enzyme by oxygen.

**Kinetics of the Reductive and Oxidative Half-reactions**—The reductive half-reactions of wild type and A95G lactate oxidase by l-lactate were determined by stopped flow absorbance meas-

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**Table II**

| Rate constant | Lactate oxidase at 25 °C | A95G lactate oxidase at 4 °C |
|---------------|--------------------------|-----------------------------|
| $k_{red}$/h<sub>1</sub> (M) | $5 \times 10^{-2}$ | $\sim 6.7 \times 10^{-4}$ |
| $h_1$ (s<sup>-1</sup>) | 230 | 105 |
| $h_2$ (s<sup>-1</sup>) | $\sim 0$ | $\sim 0$ |
| $h_3$ (s<sup>-1</sup>) | 16.7 | 0.35 |
| $h_4$ (M<sup>-1</sup> s<sup>-1</sup>) | $9 \times 10^3$ | $1.5 \times 10^5$ |
| $h_5$ (M<sup>-1</sup> s<sup>-1</sup>) | $1.8 \times 10^6$ | $1.3 \times 10^6$ |
| $h_6$ (s<sup>-1</sup>) | 180 | $1.6 \times 10^5$ |

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**Figure 4.** Kinetics of the oxidative and reductive half-reactions of A95G and wild type lactate oxidase. a. Reductive half-reaction with l-lactate. Equal volumes of 28.3 $\mu$M A95G or wild type lactate oxidase were mixed anaerobically with l-lactate (1, 1.5, 2, 4, or 10 mM, from right to left) in the stopped flow spectrophotometer at 4.1 °C, and the absorbance traces at 456 and 530 nm were monitored (closed circles for A95G and open squares for wild type lactate oxidase). Reduction rate constants at different l-lactate concentrations ($k_{red}$) were analyzed by the faster rate constant from the two-step consecutive curve fitting of the absorbance traces at 456 and 530 nm. Solid lines are the results of the linear regression of data sets of A95G and wild type lactate oxidase, respectively. Only the stopped flow traces with A95G lactate oxidase are shown (See Ref. 10, Fig. 5, for data with wild type enzyme.). b. oxidative half-reaction of A95G and wild type lactate oxidase by molecular oxygen. Enzyme was reduced by two equivalents of l-lactate anaerobically. Buffer equilibrated with 5, 10, 21, 50, and 100% oxygen was prepared at 25 °C. Equal volumes of 28.3 $\mu$M enzyme and oxygen solutions were mixed in the stopped flow spectrophotometer at 4.1 °C, and the reoxidation was followed at 456 nm to yield the pseudo first order rate constants ($k_{o2}$) shown. Solid lines are the results of the linear regression of data sets of A95G and wild type lactate oxidase, respectively.
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FIG. 5. Stability of the reduced enzyme-pyruvate complex of A95G lactate oxidase. a, equal volumes of an anaerobic solution of 12 μM (before mixing) A95G lactate oxidase and different combinations of L-lactate- and oxygen-containing buffer were mixed in the stopped flow spectrophotometer at 4.1 °C. Absorbance spectra at the end of the reactions were measured. The concentrations of L-lactate and oxygen in the solution (before mixing) were: 1, ** 0.5 mM l-lactate and no oxygen; the spectrum shown is that with wild type enzyme. 2, 0.5 mM or 2 mM L-lactate and 10% oxygen. 3, 2 mM L-lactate and 21% oxygen. 4, 4 mM L-lactate and 50% oxygen. 5, 4 mM or 10 mM L-lactate and 100% oxygen. 6, anaerobic buffer with no L-lactate. The inset shows the double reciprocal plot between absorbance difference from the spectrum 1 and pyruvate concentration, which is calculated from the concentration of oxygen present after mixing and consumed in the reaction. b, pH dependence of the dissociation constants of wild type (open symbols) and A95G lactate oxidase (closed symbols) at 25 °C. c, pH dependence of the absorbance spectra of reduced form wild type lactate oxidase-pyruvate complex. A solution of 38 μM wild type lactate oxidase (dotted line) was reduced anaerobically by 5 mM L-lactate (dashed broken line) at 25 °C and then titrated to saturation with pyruvate under anaerobic conditions at different pH values (1, pH 8; 2, pH 7; 3, pH 6; 4, pH 5). d, pH dependence of the absorbance spectrum of reduced form A95G lactate oxidase-pyruvate complex. A solution of 25 μM A95G lactate oxidase (dotted line) was reduced anaerobically by 5 mM L-lactate (dashed broken line) at 25 °C, and then titrated to saturation with pyruvate under anaerobic conditions at different pH values (1, pH 8; 2, pH 7; 3, pH 6). ** A concentration of 6 μM of pyruvate was present in spectrum 1 of panel a based on stoichiometric reduction, and A95G lactate oxidase showed the perturbation of spectra by pyruvate even at this low concentration. Therefore, we used the wild type spectrum 1 for calculation of A absorbance.

urements at 4.1 °C. Stopped flow traces at different wavelengths of A95G lactate oxidase are similar to those of wild type enzyme (Fig. 4a), which indicates the same pathway of the reductive half-reaction as with wild type enzyme, viz. following Michaelis complex formation, conversion to the reduced enzyme-pyruvate complex (with absorbance at 530 nm), and then the dissociation of pyruvate (10). Both enzymes show a linear relationship between the reduction rate and the concentration of l-lactate in double reciprocal plots. (Fig. 4a, inset). The slopes and intercepts of these plots show that the dissociation constant for the binding of l-lactate to A95G lactate oxidase is 10-fold larger than that of wild type enzyme with essentially no change in the reduction rate (Table II). We determined the reduction rate constants for l-lactate and for the alternative substrates DL-α-hydroxy-n-butyric acid and L-glyceric acid as 500, 11.5, and 2.4 s⁻¹, respectively, and their dissociation constants (expressed in terms of the L-isomer) as 1.9, 12, and 7.5 mM, respectively.

Reoxidation of reduced A95G lactate oxidase by oxygen was also measured. The observed rate of reoxidation was directly proportional to the oxygen concentration, indicating that the oxidation step is a simple bimolecular reaction process. The slopes of the plots show that the oxidation rate is decreased 8-fold in A95G lactate oxidase compared with that of wild type enzyme (Fig. 4b and Table II).

Stability of the Reduced Enzyme-Pyruvate Complex—The enzyme-monitored turnover traces of A95G lactate oxidase at 456 nm ended at a higher absorbance, because of the perturbation of the reduced enzyme spectrum by bound pyruvate accumulating during the turnover (Fig. 1, left panel). These results indicated that pyruvate was bound more tightly to the reduced mutant enzyme than to wild type enzyme. In order to determine the dissociation constant, an anaerobic solution of A95G lactate oxidase was mixed with different concentrations of oxygen in the presence of an excess of l-lactate in the stopped flow spectrophotometer (Fig. 5a). Under these conditions, lactate oxidase forms different amounts of the reduced enzyme-pyruvate complex depending on the amount of pyruvate resulting from turnover with the different limiting concentrations of oxygen. The Kd value of the reduced A95G lactate oxidase-pyruvate complex at 4 °C and pH 7.0 is 0.1 mM, approximately 11 times lower than that for wild type enzyme (10).

Unlike the wild type enzyme, which exhibits a modest dependence of the value of Kd for this complex on the pH (Fig. 5b), the Kd for the mutant enzyme is almost the same at all pH values examined. Because of instability, it was not possible to measure the dissociation constant at pH 5. The spectral shape of the complex with wild type lactate oxidase was also influenced by pH (Fig. 5c), but that of the complex with A95G enzyme is similar at all pH values, as shown in Fig. 5d.

Sulfite Binding—Flavoprotein oxidases bind sulfite tightly and form a flavin N-5 adduct with sulfite (16). The Kd for sulfite binding is sufficiently low that more accurate values can be obtained by determination of association and dissociation rate constants than by equilibrium titration. The affinity of sulfite binding for wild type and A95G lactate oxidase was therefore estimated from the association (kcat) and dissociation (kcat) rate
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Comparison of results between wild type and A95G lactate oxidase with L-lactate confirmed that the reaction pathway is not changed by the replacement of alanine 95 with glycine in the presence of L-lactate, with similar $k_{cat}$ values (Tables I and II). Therefore if the oxidation rate, $k_{cat}$, changes to a smaller value in A95G lactate oxidase, the $K_m$ for oxygen becomes larger as in Table I. For substrates with lower $k_{cat}$ values, the $K_m$ for oxygen has a direct relation with $k_{cat}$, which is determined by the values of $k_5$ and $k_7$. We determined the $k_5$ values of wild type lactate oxidase at 25°C with L-lactate and also the poor substrates DL-$\alpha$-hydroxy-n-butryic acid and L-$\alpha$-glycric acid as 500, 11.5, and 2.4 s⁻¹, respectively. They show a direct relationship with the turnover numbers of 283, 11, and 0.87 s⁻¹, respectively. The reaction rate of A95G lactate oxidase with DL-$\alpha$-hydroxy-n-butryic acid still depends on oxygen concentration because of the compensation between the decrease in $k_7$ and the increase in $k_{cat}$. Both A95G and wild type L-lactate oxidase with poor substrates, therefore, have lower $K_m$ values for oxygen, resulting in insensitivity of reaction rate to oxygen concentration (Fig. 3).

The most remarkable change caused by this point mutation is on the affinity and reactivity of small molecules. The dissociation constant for L-lactate was increased 8-fold. The second order rate constants of reoxidation of reduced enzyme by oxygen and of binding rate of sulfite decreased to one-eighth and one-eleventh, respectively, the values for wild type enzyme (Table II). The affinity of pyruvate for the reduced enzyme, on the other hand, is increased. Even the proton concentration of the solution, the pH, becomes insensitive for influence of the stability of semiquinone forms and of the reduced enzyme-pyruvate complex in A95G lactate oxidase.

The replacement of a methyl group with a hydrogen in A95G lactate oxidase has permitted the reaction with longer chain or bulky hydroxyacids. The replacement did not induce the monoxygenase activity that characterizes L-lactate monoxygenase from M. smegmatis. A95G lactate oxidase, however, shows a 2-fold higher turnover number with DL-$\alpha$-hydroxy-n-butryic acid than with L-lactate. Turnover numbers with DL-$\alpha$-hydroxy-n-meric acid, DL-$\gamma$-glycric acid, and L-mandelic acid are also relatively high compared with those of wild type lactate oxidase (Table I). L-Lactate monoxygenase from M. smegmatis also shows relatively high activity with aromatic and also branched longer chain hydroxyacids (17). Although the $K_m$ values for these long chain $\alpha$-hydroxyacids with A95G lactate oxidase (Table I) are somewhat higher than those reported by Urban et al. (18) for the rat kidney L-$\alpha$-hydroxyacid oxidase, the ratio $k_{cat}/K_m$ is quite comparable for most substrates except for L-mandelate, which has a 20-fold higher value for the rat kidney enzyme than for A95G lactate oxidase. The values of $k_{cat}/K_m$ given by Cromartie and Walsh (19) for the rat kidney enzyme are some 2 orders of magnitude lower than those for the mutant enzyme described here, due mainly to higher $K_m$ values than those reported by Urban et al. (18).

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Fig. 6. Dissociation constant of sulfite adduct of A95G and wild type lactate oxidase, a, formation of the sulfite adduct. A solution of 9 µM wild type (open) or 8.4 µM A95G (filled) lactate oxidase in 0.1 M potassium phosphate buffer, pH 7.0, and 5–50 fold sulfite in the same buffer were mixed in the stopped flow spectrophotometer at 25°C, and the absorbance decrease at 457 nm was monitored. The traces fit to pseudo first order rate constant ($k_{cat}$). The $k_{on}$ values of 1.8 × 10⁻⁵ M⁻¹ sec⁻¹ and 1.6 × 10⁻⁴ M⁻¹ sec⁻¹ for wild type and A95G lactate oxidase, respectively, were obtained from the dependence of $k_{on}$ on sulfite concentration, b, decay of sulfite adduct by methyleneaminone sulfonate. Sulfite adducts were formed by the addition of 5-fold sulfite to 18 µM wild type or 16.8 µM A95G lactate oxidase in 0.1 M potassium phosphate buffer, pH 7.0. Methyleneaminone sulfonate was added in 30- and 40-fold excess, and the regain of absorbance at 457 nm was followed for the determination of $k_{cat}$. Regression analyses were done and shown as solid lines.

Scheme I. Reaction pathways for l-lactate monooxygenase (inner pathway) and L-lactate oxidase (outer pathway). This scheme was adapted from Ref. 14.
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