Studies on Lactate Oxidase Substituted with Synthetic Flavins
ISO-FMN LACTATE OXIDASE*

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The natural flavin of lactate oxidase, FMN, was replaced by the artificial flavin, iso-FMN, in which the methyl groups of the benzene ring are located at the 6 and 7 positions, instead of the usual 7 and 8 positions. The iso-FMN enzyme behaves in most ways like the native enzyme. It forms covalent derivatives at the flavin N(5) and C(4a) positions in facile photochemical reactions analogous to those with native enzyme. It is reduced by L-lactate even faster than is native enzyme, to form a rather stable reduced enzyme-pyruvate complex. Like the native enzyme, it also forms a red anionic semiquinone on one-electron reduction, and this radical is remarkably stabilized toward O_2 when it is made in complex with pyruvate. The only striking difference between the iso-FMN enzyme and native enzyme is in the reactivity of the reduced enzyme-pyruvate complex with O_2. With the native enzyme, this complex reacts 200 to 400 times faster with O_2 than does the free reduced enzyme. With the iso-FMN enzyme, the reaction of O_2 is slow, both with the free reduced enzyme and its complex with pyruvate. It is shown that it is this slow reaction with O_2 which is mainly responsible for the different steady state kinetic behavior of the iso-FMN enzyme. It appears that the bulky methyl group at the C(6) position of the flavin in the artificial enzyme does not affect significantly the formation or stability of derivatives covalently linked to the flavin N(5) position.

In recent years, various synthetic riboflavin analogues with modifications in the isoalloxazine ring (1-3) have been made and found to act as substrates for the flavokinase-FAD synthetase system of Brevibacterium ammoniagenes, which brings about their conversion to the FAD and FMN levels (4). These analogues have been substituted for the noncovalently bound natural flavins in simple flavoenzymes, as in the oxidases (p-amino acid oxidase, glucose oxidase, and lactate oxidase (5-11)), monooxygenases (p-hydroxybenzoate hydroxylase (12, 13), and cyclohexanone monoxygenase (14)), dehydrogenases (N-methylglutamate synthetase (15) and lipoamide dehydrogenase (16)), and electron transferases (NADPH-cytochrome P-450 reductase (17) and NADPH-adrenodoxin reductase (18)). In some cases, both 1-deaza- and 5-deazaflavins have provided useful mechanistic information for the roles of the N(1) position (6, 12, 14, 19) and the N(5) position (1, 10, 15, 19) of the flavin. Similar replacement studies in the bacterial luciferase have shown conclusively that the bioluminescence activity is due to the flavin (20).

The earlier attempts by Massey et al. (21, 22) to classify flavoproteins based on their known properties led to the concept of distinctive flavin-protein interactions due to the presence of charged groups near the N(1) and/or N(5) positions of the isoalloxazine ring. This concept received confirmation and amplification from recent work using 8-chloroflavins, 8-mercaptolavins, and 6-hydroxylavins as sensitive active site probes (23) where definite correlations with the classification scheme were found.

In this paper we report our studies on the replacement of FMN in L-lactate oxidase from Mycobacterium smegmati with iso-FMN (6,7-dimethyl FMN). This flavoenzyme catalyses oxidation of L-α-hydroxyacids, such as L-lactate (24) and L-malate (25), while the L-isomers are competitive inhibitors (26). The recent investigations with suicide substrates (27, 28), transition state analogues (29), and photosubstrates (30) strongly suggest the stereospecific orientation of the substrate with the α-carbon position in juxtaposition to the flavin N(5) position. Study of the catalytic reaction with L-lactate did not reveal any intermediate during the rapid conversion of the Michaelis complex (EC_{50}+L-lactate complex) to the charge transfer complex (EC_{50}+pyruvate complex) (24). However, in the reaction of the poor substrate, glycollate, a labile intermediate involving a covalent linkage between the flavin N(5) position and the glycollyl moiety is observed (25).

The objectives of the present study were 2-fold. It was of obvious interest to characterize this reconstituted artificial enzyme in its reaction with the normal substrates, with the hope that mechanistic details inferred from study of the native enzyme might be more clearly visible. The second goal was of more general interest. The primary candidates for covalent intermediates of flavin and substrates are derivatives of the N(5) and C(4a) positions. It is therefore necessary, with any artificial flavin introduced into a flavoprotein, to know the spectral characteristics of such derivatives. Lactate oxidase offers the possibility of a simple way of determining these spectra, owing to the facile photoreactions which it undergoes (30).

**EXPERIMENTAL PROCEDURES**

**Materials**—L-lactate oxidase from Mycobacterium smegmati was purified by the procedure of Sullivan et al. (31). Apolactate oxidase was prepared by the method of Choong et al. (32). Malonate, tartarate, pyruvate, and then the other compounds noted were purchased from Sigma, and other reagents were of analytical grade and solutions were prepared in glass distilled water.

**Methods**—Absorption spectra and slow kinetics were determined with a Cary 11, 17, or 219 recording spectrophotometer, at 25 °C.
when not otherwise specified. The rapid kinetics measurements were
determined at 25 °C with the modified stopped flow apparatus of
Gibson and Milnes (34). Fluorescence spectra were recorded with a
ratio-recording fluorimeter built by Dr. D. Ballou and G. Ford of the
University of Michigan. Lactate oxidase activity was measured (32)
with an oxygen electrode (Yellow Springs Instrument Co., Model 53)
at 25 °C.

For anaerobic experiments, Thunberg-type cells were used and
anaerobiosis was achieved by many cycles of evacuation and refilling
with nitrogen purified by storage over Pievee's solution. Photochem-
ical reactions and semiquinone formation were carried out at ice
temperature as described previously (35). Pyruvate was assayed using
the NAD-linked L-lactate dehydrogenase (36) and the 2,4-dinitro-
phenylhydrazine reaction (37). The rate of hydrogen peroxide pro-
duction was quantitated by the procedure described by Lockridge
et al. (24).

RESULTS

Binding of Iso-FMN by Apo-lactate Oxidase—When an
excess of apolactate oxidase was mixed with iso-FMN (6,7-
dimethyl-8-nor-FMN) the flavin fluorescence was immedi-
ately quenched. As shown in Fig. 1, the visible absorption spectrum
of the flavin analogue is decreased and red shifted. This
reconstituted enzyme has absorption maxima at 380 nm
(ε = 8900 M cm⁻¹) and 468 nm (εₙ = 7600 M cm⁻¹) and
minima at 315 and 410 nm.

The acid-ammonium sulfate method of preparation of
apoenzyme appears to lead to some limited protein denat-
uration, resulting in a lowered flavin-binding ability for some of
the enzyme (32). As the hydrodynamically stable form of the
enzyme is an octomer (31), the possibility for heterogeneity in
flavin content is obvious. In the case of reconstituted native
enzyme, it was found that fully functional reconstituted en-
zyme could be separated from a lower specific activity fraction
on the basis of solubility of the crystallized reconstituted
enzyme. The same phenomenon exists with the reconstituted
iso-FMN enzyme. The latter was dialyzed versus 1.0 M sodium
acetate buffer, pH 5.4, at 4 °C for more than 24 h. This
resulted in the crystallization of the artificial enzyme, as it
does with native enzyme (31). Centrifugation of the enzyme
resulted in a pellet with two distinctly different colors. The
upper portion, which was intensely yellow, dissolved readily
in 0.01 M imidazole-HCl buffer, pH 7.0. The ratio of the two
absorption minima (315 nm/410 nm) of this fraction was 0.7-
0.85 in various preparations. This material was used in most of
the experiments reported here. It was recrystallized as described above, and stored as the crystalline suspension in
the dark at 4 °C. This lower pellet, paler yellow in color, dissolved much more slowly in 0.01 M imidazole, pH 7.0. It
has higher near-UV absorbance (A₃44/A₄68 > 1.0), analogous
to the minor fraction of low specific activity holoenzyme I
described by Choong et al. (32).

Anaerobic Reduction of the Intermediate by
Lactate oxidase is somewhat unusual among flavoproteins in
its fluorescence properties, the oxidized enzyme being nonflu-
orescent, and the reduced enzyme being strongly fluorescent
(38). Iso-FMN lactate oxidase has similar properties. When
the enzyme and L-lactate are mixed anaerobically, the absorb-
bance of the flavin decreases very rapidly with the concomi-
ant appearance of long wavelength absorbance. The latter
decays slowly to give the fully reduced enzyme. Fig. 3 shows
the typical spectrum of the fully reduced enzyme with the
absorbance maximum at 344 nm and a shoulder at 380 nm.
This is also reflected in its fluorescence excitation spectrum
(λₘₐₓ, 344 nm, plus shoulder at 380 nm; emission maximum,
505 nm). As is the case with native enzyme (24), the long
wavelength absorbance intermediate is shown to be a complex
between reduced enzyme and pyruvate (Fig. 3). The Kₑ value
of the complex, as determined from anaerobic titration of the
reduced enzyme with pyruvate, was 1.25 × 10⁻³ M at 25 °C, a
value very similar to that obtained with native enzyme (24).

The anaerobic formation of the intermediate was followed in
the stopped flow spectrophotometer either by the increase
in absorbance at 550 nm or by the fast phase of absorbance
decrease at 460 nm, under the same conditions of 0.01 M
imidazole-HCl, pH 7.0, 25 °C. The apparent first order rate
constants for the formation of the intermediate as a function
of lactate concentrations are shown as a reciprocal plot in Fig.
4. Similar to the data of Lockridge et al. for the native enzyme
(see Fig. 1 of Ref. 24), these results indicate an equilibrium
reaction between iso-FMN enzyme and lactate prior to the
formation of the intermediate.

The values of Kₑ (enzyme-lactate complex) and Kₑ that were
obtained are 8 × 10⁻³ M and 20,000 min⁻¹, values not dissimilar
from those found with native enzyme (24). As will be clear from
following sections, the dramatic difference between Kₑ and
the steady state turnover number can be explained by the
oxygen reactivities of the reduced enzyme-pyruvate complex
and free reduced enzyme. The slow anaerobic decay of the
long wavelength absorbance intermediate (Å₃₄₄nm), which is
To eliminate the problems which would be caused by shift of the photochemical reactivity of the enzyme. The artificial enzyme

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\text{gave predominantly the fully reduced enzyme, as it does with excess substrate in the study of the reaction of reduced iso-FMN lactate oxidase with oxygen, it was decided to utilize the reduced enzyme-pyruvate complex to the free reduced enzyme (24). These slow oxygen reactivities for the iso-FMN lactate oxidase account partly for the low turnover number, and for the uncoupled reaction (cf. "Discussion").}

**Semiquinone Form of Iso-FMN Lactate Oxidase**—Since the native lactate oxidase stabilizes the red anionic semiquinoid form of the flavin (35), it was of interest to see if a similar

**Reaction of Reduced Enzyme Forms with Molecular Oxygen**—To eliminate the problems which would be caused by excess substrate in the study of the reaction of reduced iso-FMN lactate oxidase with oxygen, it was decided to utilize the photochemical reactivity of the enzyme. The artificial enzyme binds d-lactate resulting in spectral perturbation (5-nm red shift of the \( \lambda_{\text{max}} \) 465 and isosbestic points at 500, 475, and 410 nm). Illumination of the complex under anaerobic conditions gave predominantly the fully reduced enzyme, as it does with native enzyme (30). Addition of pyruvate to this species generated the spectrum of the charge transfer complex between reduced enzyme and pyruvate (cf. Fig. 3).

The reaction of \( O_2 \) with free reduced enzyme and with reduced enzyme in complex with pyruvate was monitored in the stopped flow apparatus at 460 nm. The reoxidation was slow, and surprisingly, the second order rate constant for the free reduced enzyme \( (8.4 \times 10^{-3} \text{ M}^{-1} \text{ min}^{-1}) \) was only slightly smaller than that for the intermediate \( (1.42 \times 10^{-2} \text{ M}^{-1} \text{ min}^{-1}) \). In marked contrast, the complex between pyruvate and the reduced flavin of native enzyme (FMN) gives a 200-fold faster rate of reaction with \( O_2 \) than does the free reduced enzyme (24).

**Effect of \( [\text{L-Lactate}] \)** on the observed turnover numbers (moles of substrate oxidized/min/mol of enzyme-bound flavin) and corrected for the uncoupled reaction (cf Fig. 3). The formation of the reduced enzyme-pyruvate complex was followed by the absorbance decrease at 460 nm (●) or the absorbance increase at 550 nm (○).
species was found with the reconstituted artificial enzyme. As illustrated on Fig. 5, the anaerobic photoreduction of the enzyme in the presence of glycine and deazaflavin does indeed generate a red radical with an absorption spectrum very similar (λ_{max} at 378, 402, 480 nm) to that found with native enzyme. On adding pyruvate, the perturbed spectrum of an enzyme radical-pyruvate complex is formed rapidly (λ_{max} at 380, 402, 485 nm) with the long wavelength absorbance extending to 800 nm. This complex can be isolated free from excess pyruvate by Sephadex G-25 chromatography at 4°C and was shown to decay isosbestic to the fully oxidized enzyme, with a half-time of about 38 min (25°C). Thus, as with the native enzyme, the semiquinoid form of the iso-FMN enzyme is stabilized enormously on forming a complex with pyruvate (35). Although we have not tested the binding of this radical to other ketoacids, it is very probable that they behave in the same fashion as with native enzyme (see Ref. 35).

Formation of a 4a,5-Dihydro-iso-FMN Adduct of Iso-FMN Lactate Oxidase by Reaction with L-α-Hydroxybutynoic Acid—2-Hydroxy-3-butyrynic acid is a suicide substrate for both the native enzyme (27, 28) and for enzyme reconstituted with 2-thio-FMN (39) and 1-deaza-FMN (12). With both native and 2-thio FMN enzymes, an intermediate characteristic of the reduced flavin is detected prior to the formation of the stable 4a,5-cyclic flavin adduct (27, 30). With the artificial iso-FMN-containing enzyme, aerobic addition of 1 eq of L-α-hydroxybutynoic acid to the oxidized enzyme caused rapid bleaching of the 468 nm absorption (Fig. 6).

While no intermediate with clearly different spectral properties could be detected, the reaction of the artificial enzyme with α-hydroxybutynoate nevertheless appears to be complex. When enzyme in 0.01 M imidazole HCl, pH 7.0, was mixed at 4°C with 5 × 10^{-4} M DL-α-hydroxybutynoate, the decrease in A_{468} was an exponential process, with a t_{1/2} of approximately 4 s (about 0.07 min). On the other hand, the increase in absorbance at 326 nm was distinctly biphasic, approximately two-thirds of the increase occurring with a t_{1/2} of 0.06 min, and the remaining one-third with a t_{1/2} of 0.23 min. These changes are mirrored by the development of the intense fluorescence characteristic of the reaction product (emission maximum, 485 nm; excitation spectrum closely similar to the absorption spectrum shown in Fig. 6). Approximately 40% of the fluorescence increase (excitation, 320 nm; emission, 485 nm) occurred with a t_{1/2} of about 0.06 min and the remaining 60% with a t_{1/2} of 0.23 min. The rapidity of the reaction has precluded a detailed analysis, which would require stopped flow studies. While this could yield valuable information about the absorption spectra of the enzyme during the two stages, even stopped flow fluorescence studies would not readily yield information about possible differences in fluorescence spectra during the two stages. It should be pointed out that the biphasic time course cannot be attributed to the use of a racemic mixture of the α-hydroxybutynoate, since the disappearance of the absorbance at 468 nm was apparently monophasic.

The rapid spectral changes described above result in the appearance of a product with an absorbance maximum at 325 nm, with a shoulder around 360 nm. Over a period of 1 h there is a slight shift of the wavelength maximum to 328 nm, with the 360 nm shoulder becoming less marked. This phase is accompanied by a small decrease in fluorescence intensity (about 20%) but without any obvious change in the excitation and emission spectra. On more prolonged standing the fluorescence decays in 1 day to less than half the original intensity, but no obvious change in the absorbance or fluorescence spectra can be seen. This long term instability is similar to that observed with the adduct formed with native enzyme (27, 29). The markedly different spectra of the adducts formed with iso-FMN enzyme and α-hydroxybutynoate, and photochemically with reduced enzyme and β-bromopropionate (see later section and Fig. 8), led us to question whether the hydroxybutynoate adduct might have already broken down almost as fast as it was being formed, possibly because of ring strain due to overcrowding in the C(6)-N(5)-C(4a) region of the flavin. While we don’t have a definitive answer, it would appear unlikely that rapid breakdown occurs, since very pronounced spectral changes, presumably due to reduction of the C(10a)-N(1) double bond (39), occur on addition of NaBH₄ (Fig. 6). Thus, in analogy to the reaction with native enzyme (27, 28), the structure of the adduct and the NaBH₄ reduction product are probably as represented in Scheme 1.

It is possible that the differences in spectral properties between the α-hydroxybutynoate adduct (Fig. 6) and the C(4a) carboxyethyl adduct (Fig. 8) are in fact due to ring strain of the former adduct imposed by the 6-methyl group of the iso-FMN enzyme.

Binding of Dicarboxylic Acids to Iso-FMN Lactate Oxidase and Photoreactions of the Complexes—A variety of studies on the binding of substrate and substrate analogues with native enzyme have shown that the carboxylate moiety of the substrate is bound in such a way that the α-carbon atom is in close proximity to the flavin N(5) position, and the β-carbon atom near the flavin C(4a) position (30, 40). In the case of the iso-FMN enzyme, the bulky methyl substituent at the flavin C(6) position could be expected either to interfere with binding of substrate and inhibitors, or to affect the photoreactions of enzyme-inhibitor complexes. Alternately, if the binding and photoreactions proceeded in a way analogous to those with the native enzyme, the stability of N(5) and C(4a) adducts might be different from those formed with native enzyme. In fact, the iso-FMN enzyme appears to behave remarkably like the native enzyme. The properties of products formed in photochemical reactions are summarized in Table I. The dicarboxylic acids, oxalate, malonate, and

![Fig. 6. Spectral changes accompanying the reaction of iso-FMN lactate oxidase with α-hydroxybutynoate. Curve 1, 4.7 × 10^{-5} M enzyme, in 0.01 M imidazole·HCl, pH 7.0, 4°C. Curve 2, 2 min after aerobic addition of 5 × 10^{-4} M DL-α-hydroxybutynoate. Curve 3, after addition of a few grains of NaBH₄. A spectrum identical with that of Curve 2 was obtained with 5 × 10^{-4} M L-α-hydroxybutynoate.](image)
Iso-FMN Lactate Oxidase

Table I

<table>
<thead>
<tr>
<th>Mode of formation</th>
<th>Adduct structure</th>
<th>Absorption</th>
<th>Fluorescence emission</th>
<th>Rate of formation</th>
<th>Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized enzyme/L-α-hydroxybutyrate (suicide substrate)</td>
<td>–N(5)-C(4a)</td>
<td>325 nm + 370 nm</td>
<td>480 nm</td>
<td>5.8</td>
<td>Very fast</td>
</tr>
<tr>
<td>Oxidized enzyme/L-lactate (substrate)</td>
<td>N(5)-H (reduced)</td>
<td>344 nm + 380 nm</td>
<td>shoulder</td>
<td>505 nm</td>
<td>0.2</td>
</tr>
<tr>
<td>Reduced enzyme/bromoacetate/ν</td>
<td>N(5)-CH_2CO_2^-</td>
<td>355 nm</td>
<td>450 nm</td>
<td>1.2</td>
<td>Slow</td>
</tr>
<tr>
<td>Reduced enzyme/bromopropionate/ν</td>
<td>C(4a)-CH_2CH_2CO_2^-</td>
<td>380 nm + 320 nm</td>
<td>shoulder</td>
<td>525 nm</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Relative to free iso-FMN, pH 7, at its excitation maximum, 375 nm, emission maximum, 550 nm.

*tartronate have been found to bind tightly to native lactate oxidase in a two-step equilibrium reaction, the last step being associated with the uptake of a proton from the medium, and therefore slow at pH values near neutrality (29, 30, 41). The same phenomenon is also evident with the iso-FMN enzyme, but we have not investigated it in such detail. In all the reactions described below, the enzyme has been incubated at a concentration of ligand greater than that of the dissociation constant until no further spectral changes occurred.

*Oxalate*—In 0.01 M 2-(N-morpholino)ethanesulfonic acid, pH 6.0, 25 °C, the $K_D$ value for the binding of oxalate to iso-FMN enzyme was 2.9 × 10^-3 M. This was determined from the spectral shifts on binding of oxalate which were qualitatively similar to those found with native enzyme (29). As with the native enzyme, as adduct is formed very readily on photodissociation of the enzyme-oxalate complex and, by analogy, is presumably an N(5) carbonate adduct, weakly fluorescent, with emission maximum at 450 nm, and with an excitation spectrum identical with the absorption spectrum (no visible absorption peak, but with a marked shoulder at 320 nm).

*Tartronate*—The effect on the absorption spectrum of iso-FMN lactate oxidase by formation of a complex with tartronate ($K_D < 10^{-3}$ M at pH 7.0, 25 °C) is shown in Fig. 7. Binding of this ligand resulted in a 10-nm blue shift of the 465 peak. Photodissociation of this complex under aerobic or anaerobic conditions yielded an adduct with absorbance maximum at 350 nm. This N(5) glycolyl adduct has a strong fluorescence (excitation maximum, 350 nm; emission maximum, 435 nm). The adduct isolated after Sephadex G-25 chromatography at 4 °C decays isothermally to the fully oxidized enzyme, with a half-time of 115 min and 9 min at 0 °C, respectively. The corresponding half-times for decay of the stable glycolyl adduct of native enzyme are 70 min at 0 °C and 20 min at 25 °C (25, 40).

*Malonate*—The binding of malonate to the artificial enzyme in 0.01 M 2-(N-morpholino)ethanesulfonic acid, pH 6, resulted in a spectral shift similar to that observed with tartronate ($\lambda_{max}$ at 455 nm and 380 nm). Illumination of the complex ($K_D = 7 \times 10^{-4}$ M) gave the strongly fluorescent N(5) carboxymethyl adduct (absorbance and fluorescence excitation maximum at 355 nm, fluorescence emission maximum at 450 nm). This adduct, like that formed with native enzyme (30), is very stable, no decay being observed over a period of several days at 0 °C. Unlike the adduct of the native enzyme, which forms a stable blue radical on further irradiation in the presence of O_2 (30), the adduct with the iso-FMN enzyme does not form a radical. The adduct (like those formed from oxalate and tartronate) is, however, subject to photodecomposition if irradiated with light of wavelengths below 400 nm, to yield oxidized enzyme. Hence, for maximal formation of adduct, the initial photoreaction has to be carried out using a cut-off filter, which does not transmit light of wavelengths below 400 nm, to yield oxidized enzyme. For this purpose we used a Corning CS-372 filter, as described in the text. Curve 3, the spectrum obtained after 28 min of irradiation (Curve 2), calculated spectrum after correction of Curve 3 for the presence of 18% residual oxidized enzyme.

**Photochemical Reaction of Reduced Iso-FMN Lactate Oxidase**—The spectrum of the reduced enzyme is modified significantly on binding to either bromoacetate or β-bromo-
propionate at pH 7.0 (Fig. 8). In each case, the fluorescence emission ($\lambda_{em}$ at 505 nm) for the reduced enzyme is quenched, together with the concomitant loss of the absorption shoulder at 380 nm. Illumination in the presence of these halogenated carboxylic acids resulted in very facile photooxidations.

With bromoacetate, the adduct has a characteristic new absorption maximum at 355 nm and a strong blue fluorescence (emission maximum, 450 nm). This N(5) carboxymethyl dihydro-iso-FMN adduct was stable on admission of air, and even when released from the protein with guanidine-HCl (7.5 M) at 0 °C. No detectable (stable) blue intermediate radical was formed in the presence of guanidine, as was found with the similar adduct of native enzyme (30). It should be noted that the properties of the adduct formed by photoreaction of the reduced iso-FMN enzyme with bromoacetate are the same as those of the adduct produced photochemically from oxidized enzyme and malonate, analogous to the results found with native enzyme (30). Thus, the methyl group at the C(6) position in the iso-FMN enzyme does not appear to affect the binding of ligands to the enzyme or their photooxidations with the N(5) residue of the flavin.

Again analogous to the results with native enzyme, the photochemical reaction of the reduced iso-FMN enzyme with $\beta$-bromopropionate appears to yield the corresponding C(4a)-substituted carboxyethyl adduct (Fig. 8). This has an absorption maximum at 380 and a shoulder at 320 nm. This characteristic absorption spectrum is closely reflected in its fluorescence excitation spectrum (emission maximum, 525 nm) with fluorescence intensity approximately 30% that of the N(5) carboxymethyl adduct. The C(4a) adduct is very stable; even when released from the protein by immersing for 1 min in a boiling water bath it suffers no decay.

**Reaction of Iso-FMN Lactate Oxidase with Glycollate—**

The studies of Massey et al. (25) and Ghisla and Massey (40) on the catalytic reaction of native lactate oxidase with glycollate demonstrated the formation of two diastereomeric flavin N(5) glycollyl adducts. Adduct 1 (R adduct) was shown to be the predominant labile species involved in the catalytic oxidation of glycollate to formate, CO$_2$, and H$_2$O, while Adduct 2 (S adduct) was shown to be the stable adduct which gradually accumulates during turnover, eventually shutting down almost completely the catalytic reaction (25, 40). The latter species was shown to be identical with the photoadduct made with tartronate (30).

Iso-FMN lactate oxidase also recognizes glycollate as a poor substrate, with apparent $K_m$ and $V_{max}$ values of approximately $1.3 \times 10^{-1} \text{ M}$ and $6 \text{ min}^{-1}$ in 0.01 M imidazole-HCl buffer, pH 7.0, 25 °C. The reaction has a very high Arrhenius activation energy of 26 kcal/mol for the turnover of glycollate. This value is twice that for the oxidative decarboxylation of lactate by native enzyme (26, 29), and 5 kcal mol$^{-1}$ higher than that for the decay of the native enzyme N(5) glycollyl adduct (40). The catalytic turnover of glycollate, determined by the effect of catalase on the O$_2$ electrode traces, is substantially uncoupled, over 60% of the reaction proceeding to produce glyoxylate and H$_2$O, rather than formate, CO$_2$, and H$_2$O. Glyoxylate, as the hydrate, is also a substrate for the native enzyme (25, 40) and for the iso-FMN enzyme, with oxalate and H$_2$O as products. These properties serve to give different results for the aerobic turnover of glycollate with native and iso-FMN enzymes. Turnover with native enzyme leads to substantial accumulation of the stable glycollyl Adduct 1, identical with that produced by light irradiation of the oxidized enzyme-tartronate complex (30). With iso-FMN enzyme, the predominant species seen on addition of glycollate is the free reduced enzyme, with absorbance and fluorescence properties identical with those found on reaction with L-lactate. Continued turnover leads not to accumulation of the highly fluorescent N(5) glycollyl adduct, but instead to accumulation of the weakly fluorescent reduced enzyme-oxalate complex. The latter dissociates quite slowly, and may be isolated in approximately 70% yield by passing the reaction mixture through Sephadex G-25 at 4 °C. The $t_{1/2}$ for decay of the complex at 4 °C, measured by the return of the absorption of oxidized enzyme, was 24 min. That the product was oxalate was shown by photoinirradiation of the reoxidized mixture, yielding the same weakly fluorescent N(5) carbonate adduct described in a previous section. The same results were obtained when enzyme was reduced with L-lactate and incubated with oxalate. Again the fluorescence of the reduced enzyme was quenched on complex formation with oxalate ($K_d$ about $10^{-7}$ M in 0.01 M imidazole-HCl, pH 7.0, 4 °C), and the complex could be isolated by passage through Sephadex G-25, decaying with a $t_{1/2}$ of 20 min at 4 °C.

**DISCUSSION**

It is well to reiterate that the holoenzyme of L-lactate oxidase from *M. smegmatis* is composed of eight identical subunits, with 1 mol of FMN/43,500 molecular weight subunit (31). The kinetics of reconstitution (binding of apolactate oxidase and FMN) is biphasic, a very rapid step followed by a slow step which is associated with further spectral perturbation of the complex (32). In this case, the absorption maximum at 455 nm is resolved with the appearance of shoulders at 420 and 480 nm. As illustrated on Fig. 1, the FMN analogue, iso-FMN, binds tightly to the apoprotein, resulting in the red shift and quenching of the flavin fluorescence. However, in this case, no slow secondary spectral changes were observed. In contrast, distinctive secondary spectral changes have been demonstrated on binding apolactate oxidase with 8-mercapto-FMN (42) and with 6-hydroxy-FMN.$^1$

Several important points emerged from this study. First, by the different solubility and centrifugation properties, we have found a way to resolve the problem of heterogeneity in making reconstituted lactate oxidase with flavin analogues. The ability to store the artificial enzyme as a crystalline suspension without loss of activity is very convenient for preparation of

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$^1$ Y. S. Choong and V. Massey, unpublished observations.
large amounts of enzyme, such as were used in this work, since the apoprotein is unstable (32).

This artificial enzyme appears to catalyze the oxidation of L-lactate via the same pathway as that proposed by Lockridge et al. (24) for the native enzyme (Scheme 2). The reductive half-reaction data of Fig. 4 show saturation kinetics very similar to that of native enzyme, with a limiting velocity ($k_b$) at infinite lactate concentration of about 20,000 min$^{-1}$ for the formation of the long wavelength-absorbing intermediate, which in turn is formed from an enzyme-substrate complex with a $K_d (k_1/k_i)$ of $8 \times 10^{-2}$ M.

Long wavelength absorbance in reduced flavoproteins frequently arises from the interactions between the reduced flavin as charge transfer donor and an electron-deficient ligand as the charge transfer acceptor (43). Such charge transfer complexes, with characteristic long wavelength absorption, have been demonstrated with the native enzyme in the case of the reduced enzyme-pyruvate complex (24) and the anionic flavosemiquinone-a-ketoacid complex (35). The iso-FMN lactate oxidase shows the same types of complexes, including a spectroscopically detectable transient long wavelength intermediate on anaerobic reduction of the enzyme with L-lactate. The static titration results of Fig. 3 confirm the intermediate to be the charge transfer complex between the fully reduced iso-FMN enzyme and pyruvate. As measured in the stopped flow spectrophotometer, the rate of decay (decrease at 550 nm) of the intermediate to free reduced enzyme is independent of substrate concentration. The rate of 2.6 min$^{-1}$ is the same as that found for native lactate oxidase (24) and like the latter, is too small for free reduced enzyme to be involved significantly in catalysis.

The steady state data of Fig. 2 are consistent with a ternary complex mechanism as shown in Scheme 2. With native enzyme, all the substrates tested so far, except glycollate, yield a series of parallel Lineweaver-Burk plots (24, 25). As pointed out previously (44, 45), the condition for a ternary complex mechanism to yield a series of parallel Lineweaver-Burk plots, rather than the expected converging plots, is that the last term in the denominator of the initial rate equation become a series of parallel Lineweaver-Burk plots (24). The kinetic expression for $K_{0i}$ is $k_5 (k_5 + k_2 + k_3)$. The most striking difference between native and iso-FMN enzymes is in the value of $k_b$, the rate constant for formation of the reduced enzyme-pyruvate complex with $O_2$. 1.1 $\times 10^8$ M$^{-1}$ min$^{-1}$ for native enzyme, and $1.4 \times 10^8$ M$^{-1}$ min$^{-1}$ for iso-FMN enzyme. Given the measured rate constants listed in Table II, it is evident that the value of $k_b$, the rate constant for the ternary complex oxidative decarboxylation reaction, could be unchanged from that of native enzyme. If this were the case, the predicted $K_{0i}$ would be $5.1 \times 10^7$ M, well within the limits imposed by the data of Fig. 2. Whatever the actual value of $k_b$, it is clear that the main factor limiting the rate of catalysis with the artificial enzyme is the poor reactivity of the reduced enzyme-pyruvate complex with $O_2$. These findings are qualitatively similar to those obtained with D-amino acid oxidase where the native flavin FAD had been replaced by 7,8-dichloro-FAD (8). In that case, the reaction of the reduced enzyme-imino acid complex with $O_2$ was also greatly lowered compared with the native enzyme, and became rate limiting under most experimental conditions.

The observation of partial uncoupling of the normal oxidative decarboxylation can be explained partially by the sequence of steps $k_3$ and $k_6$ of Scheme 2. However, the rate of $H_2O_2$ production is generally greater than that of $k_6$, the dissociation of the reduced enzyme-pyruvate complex. Therefore, some uncoupling must also occur at the level of the ternary complex of oxidized enzyme, pyruvate and $H_2O_2$. With native enzyme the oxidative decarboxylation of pyruvate by $H_2O_2$ is accomplished in this complex (24). However, if either $K_{0i}$ or $K_6$ were obtained from the same experiment by following the decay of $A_{450}$, the values of $k_6$ and $k_i$ were obtained from stopped flow experiments by measuring the dependency on oxygen concentration of the reoxidation of the reduced enzyme-pyruvate complex and free reduced enzyme, respectively. The value of $k_6$ was estimated from the kinetic expressions for $V_{max}$ and $K$ (see text for more details). All data were collected with enzyme in 0.01 M imidazole/HCl pH 7.0, 25 °C. The values for native enzyme are from Ref. 24. The values for the iso-FMN enzyme were calculated with correction for the uncoupled production of $H_2O_2$ and pyruvate.

### Table II

<table>
<thead>
<tr>
<th>Iso-FMN enzyme</th>
<th>Native enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>Large but indeterminate</td>
</tr>
<tr>
<td>$K_{lactate}$</td>
<td>Large but indeterminate</td>
</tr>
<tr>
<td>$K_{i}$</td>
<td>Large but indeterminate</td>
</tr>
<tr>
<td>$K_3$</td>
<td>Negligible</td>
</tr>
<tr>
<td>$k_1/k_i$</td>
<td>$8 \times 10^2$ M</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$2 \times 10^4$ min$^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$2.6 \times 10^2$ min$^{-1}$</td>
</tr>
<tr>
<td>$k_6$</td>
<td>$1.4 \times 10^4$ min$^{-1}$</td>
</tr>
<tr>
<td>$k_8$</td>
<td>Large but indeterminate</td>
</tr>
<tr>
<td>$k_9$</td>
<td>$8.4 \times 10^5$ min$^{-1}$</td>
</tr>
</tbody>
</table>
H₂O₂ or pyruvate dissociated from this complex at a rate competitive with the decarboxylation reaction, some uncoupling would result.

The similarity of most of the rate constants for native and iso-FMN enzymes, listed in Table II, indicates that the bulky methyl group at the flavin position C(6) in the iso-FMN enzyme interferes very little or not at all in the reductive half-reaction. This is also consistent with the finding that the same photochemical reactions occur with the native enzyme also occur with the iso-FMN enzyme. The adducts which are formed appear to be, in most cases, even more stable than the corresponding adducts with the FMN of native enzyme. With the latter, the N(5) carboxymethyl and C(4a) carboxyethyl derivatives, while very stable while enzyme bound, decay fairly rapidly on release from the protein. In the case of the dihydro enzyme, pyruvate must serve to flatten the normal enzyme-bound explanation is correct in the present context, then binding of the latter, the

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