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2-Thioriboflavin 5′-Phosphate (2-Thio-FMN) Lactate Oxidase

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The natural flavin of lactate oxidase, FMN, was removed and replaced by the synthetic flavin 2-thioriboflavin 5′-phosphate (2-thio FMN). Despite the differences in properties of the flavins, including an oxidation-reduction potential some 80 mV more positive than that of normal flavin the 2-thio-FMN enzyme behaves in practically all respects like the native enzyme. It catalyzes the oxidative decarboxylation of L-lactate with almost the same efficiency as the native enzyme and with similar kinetic constants for individual steps in the catalytic pathway. It forms covalent derivatives at the flavin N(5) and C(4a) positions in facile photochemical reactions analogous to those of the native enzyme. It also forms a flavin anion radical on photoreduction with 5-deazaflavin as catalyst and, as with the native enzyme, this radical is stabilized remarkably on formation of a complex with pyruvate. The spectral properties of the neutral flavin radical form of 2-thioflavin are also reported, as determined by photochemical reduction of 2-thio-FMN flavodoxin. Like native lactate oxidase, the 2-thio-FMN enzyme also forms a flavin N(5)-sulfite adduct in an equilibrium reaction with sulfite. These results demonstrate clearly with this enzyme that the native flavin may be removed and replaced by an artificial flavin, without altering the structural integrity of the protein.

In recent years considerable use has been made of chemically modified flavins as probes of reaction mechanisms of flavin enzymes or of the protein environment around the bound flavin. This has been made possible by the broad specificity of the flavokinase/FAD synthetase system of *Brevibacterium ammoniagenes*, which is able to convert most artificial flavins from the more easily synthesized riboflavin level to the corresponding FMN and FAD level [1]. Fortunately most flavoproteins seem to be very permissive and bind a variety of ring-modified flavins, the main requirement for binding being the nature of the side chain at position N(10): ribityl phosphate if the original flavin were FMN and ribityl-diphospho-adenosine if the original flavin were FAD. Such replacement studies also rely on the ability to remove the native flavin without denaturing the protein, so that on adding flavin to the apoprotein the original holoenzyme is reconstituted. When this can be achieved with the native flavin, it is generally assumed that when an artificial flavin is found to bind to the apoprotein, it does so in a similar way to the native flavin. This is a crucial point in the concept of using such flavins as probes of the reaction mechanism, or as probes of the protein environment surrounding the bound flavin. Hence it is very important to show that the properties of such artificial flavoproteins mimic those of the native enzymes in important details and to show that this can be achieved with a wide variety of flavoproteins. L-Lactate oxidase from *Mycobacterium smegmatis* is a good test enzyme for such studies, since it is unusually sensitive to photochemical formation of N(5) and C(4a) flavin adducts under defined conditions [2, 3] and since its reaction mechanism has been delineated in fair detail [4 – 6]. It is also unusual in that the semiquinoid form, the red anionic semiquinone, is stabilized dramatically against oxidation by O_2 when it is in complex with pyruvate [7, 8]. In a preceding paper [8], we described the results of a study where the native flavin had been replaced by iso-FMN (6,7-dimethyl 8-nor-FMN). That form of the enzyme behaved in most respects like the native enzyme, except for the crucial one of slow reaction of the reduced enzyme-pyruvate complex with O_2. In order to try to evaluate the meaning of these results, we felt it important to examine another flavin derivative and decided to study in detail the enzyme reconstituted with 2-thio-FMN. This decision was also prompted by the finding that the sulfur of 2-thioflavins is quite reactive chemically and promises to be a useful probe of the protein environment around the pyrimidine subnucleus of the flavin [9]. Furthermore, such a study was important in view of the demonstration that 2-thio-FAD *p*-hydroxybenzoate hydroxylase, while catalyzing the substrate-dependent oxidation of NADPH, including the transient formation of the 2-thioflavin C(4a)-hydroperoxide, is not able to catalyze the normal hydroxylation reaction characteristic of the native enzyme [9a]. Lactate oxidase reconstituted with 2-thio-FMN behaves in almost every respect examined remarkably like native enzyme, adding confidence to the use of this flavin as a probe of active-center structure and mechanism.

Dedicated to the memory of Professor Peter Hemmerich, who first awakened our interest in 2-thioflavins as active-site replacements of flavoproteins, and who provided the generous amount of 2-thioriboflavin which made this work possible.

Abbreviation. 2-Thio-FMN, 2-thioriboflavin 5′-phosphate.

Enzymes. Lactate oxidase or lactate 2-monooxygenase (EC 1.13.12.4); flavokinase or riboflavin kinase (EC 2.7.1.26); FAD synthetase or FMN adenylyltransferase (EC 2.7.7.2); *p*-hydroxybenzoate hydroxylase or 4-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2); D-amino acid oxidase (EC 1.4.3.3); glucose oxidase (EC 1.1.3.4); NADPH dehydrogenase or 'old yellow enzyme' (EC 1.6.99.1); NADPH – cytochrome P-450 reductase (EC 1.6.2.4); NADPH – adrenodoxin reductase (EC 1.6.99.–); pyridoxamine-phosphate oxidase (EC 1.4.3.5); xanthine oxidase (EC 1.2.3.2).

MATERIALS AND METHODS

L-Lactate oxidase from *Mycobacterium smegmatis* was isolated by the procedure of Sullivan et al. [10]. The apoenzyme was made by acid/ammonium sulfate treatment as described by

Choong et al. [11]. The apoenzyme of flavodoxin from *Megasphera elsdenii* was prepared as described by Mayhew [12] and standardized by titration versus pure FMN [13]. L-Lactate (lithium salt) and pyruvate were obtained from the Sigma Chemical Co. 2-Thioriboflavin was a generous gift from the late Professor Peter Hemmerich (Konstanz, FRG) [14]. All other reagents were of analytical grade and solutions were prepared in glass-distilled water.

The 2-thioriboflavin was converted to the FAD level by the flavokinase/FAD synthetase system of *Brevibacterium ammoniagenes* [1] and was purified by Bio-Gel P2 and DEAE-cellulose (DE-32) chromatography [9]. Prior to use the 2-thio-FAD was converted to 2-thio-FMN using the phosphodiesterase activity of the snake venom, *Naja naja* (Sigma Chemical Co.). The purification procedure described in [9] separates the non-fluorescent 2-thio-FAD from contaminating FAD. Conversion to 2-thio-FMN does not result in any contamination with FMN.

On a preparative scale, freshly made apolactate oxidase in slight excess over the flavin, was mixed with 2-thio-FMN in 0.1 M Tris/acetate, pH 7.0 at 4 °C. This was stored overnight at 4 °C and then crystallized by dialysis against 1 M sodium acetate, pH 5.4 for >1 day. The problem of heterogeneity of the reconstituted enzyme, first noted with FMN-reconstituted enzyme [11] and then with iso-FMN enzyme, was also observed here and was resolved by differential solubility, in the same way as described for the iso-FMN enzyme [8].

Spectrophotometric experiments were carried out with Cary 17, 118 or 219 recording spectrophotometers, at 25 °C when not otherwise specified. The rapid kinetic measurements were made at 25 °C with the modified stopped-flow apparatus of Gibson and Milnes [15]. Lactate oxidase activity was determined with an oxygen electrode (Yellow Springs Instrument Co., model 53) in 0.01 M imidazole/HCl, pH 7.0 at 25 °C. Enzyme turnover was also determined by stopped-flow experiments where the absorbance of the enzyme was monitored while reacting with different concentrations of L-lactate in the presence of a limiting concentration of O_2. Catalytic centre activity was calculated from the principles enunciated by Chance [16] as described by Gibson et al. [17]. For anaerobic experiments Thunberg-type cells or tonometers were used and anaerobiosis was achieved by repeated evacuation and flushing with N_2 purified over Fieser's solution. Photochemical reactions with 5-deazaflavin as catalyst were done at ice temperature as described previously [18].

RESULTS

Spectral Characteristics of 2-Thio-FMN Lactate Oxidase

As the apoenzyme of lactate oxidase has limited stability and has to be used soon after preparation, it is not possible to titrate a given flavin solution with known concentrations of apoenzyme and calculate binding constant and absorption coefficients. Hence the latter have to be obtained by denaturation of the reconstituted protein and related to the known spectral properties of the released flavin. Fig. 1 shows the spectrum of 2-thio-FMN lactate oxidase and the reneutralized 2-thio-FMN released by addition of 5% (w/v) trichloroacetic acid. The visible absorption maximum of 2-thio-FMN is shifted from 490 nm to 515 nm on binding to lactate oxidase, with the absorption coefficient at the maximum being slightly smaller; $\varepsilon_{490} = 21000\,M^{-1}\,cm^{-1}$ for 2-thio-FMN and $\varepsilon_{515} = 19300\,M^{-1}\,cm^{-1}$ for 2-thio-FMN lactate oxidase. The value for 2-thio-FMN was obtained by titration

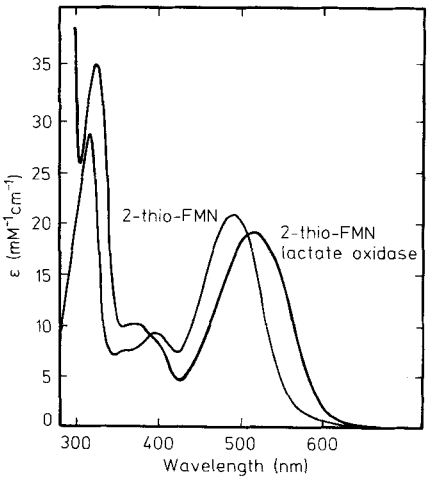


Fig. 1. *Absorption spectra of 2-thio-FMN and of 2-thio-FMN lactate oxidase.* The reconstituted enzyme, prepared as described in Materials and Methods, was dialysed against 0.01 M imidazole/HCl, pH 7.0 and the spectrum recorded. The protein was then denatured by addition of 0.1 vol. 50% (w/v) trichloroacetic acid. After centrifuging, the supernatant solution was reneutralized by addition of solid $NaHCO_3$. The spectrum of the released 2-thio-FMN was recorded and corrected for dilution. The assignment of absorption coefficients was made by an independent titration of 2-thio-FMN with standardized apoprotein of the flavodoxin from *Megasphera elsdenii*

with known concentrations of the stable apoprotein of flavodoxin from *Megasphera elsdenii* (results not shown). This is in good agreement with the value found for 2-thioriboflavin [9]. While the binding constant for 2-thio-FMN to lactate oxidase cannot be measured because of the instability of the apoprotein, it is evident that binding is very tight, with no loss of flavin being observed on extensive dialysis vs 0.01 M imidazole, pH 7.

The 2-thio-FMN enzyme, like native lactate oxidase [4] and free 2-thio-FMN [9, 14] has no visible fluorescence in the oxidized state. It is, however, weakly fluorescent when reduced (see later section).

Catalytic Activity and Steady-State Kinetics

The 2-thio-FMN enzyme is very efficient in carrying out the catalytic reaction brought about by the native enzyme, as will be described below. During the catalytic turnover of L-lactate and O_2 by the artificial enzyme, no pyruvate, as analyzed by rabbit muscle lactate dehydrogenase and NADH [19], or H_2O_2, as analyzed by O_2 evolution on addition of catalase or by the coupled reaction with horseradish peroxidase and *o*-dianisidine [4], was released. Thus, as with native enzyme [4], the reaction is a fully coupled monooxygenase reaction, with acetate, CO_2 and H_2O as products:

$$\text{L-Lactate} + O_2 \rightarrow \text{Acetate} + CO_2 + H_2O\,.$$

This tight coupling is distinguished from the results obtained with the iso-FMN enzyme, where appreciable uncoupling was observed [8].

The steady-state kinetics of the 2-thio-FMN lactate oxidase reaction with L-lactate and O_2, as determined by O_2 uptake using an oxygen electrode, are shown in Fig. 2. A series of parallel Lineweaver-Burk plots was obtained, as with the native enzyme [4]. From the secondary plot (Fig. 2, inset) the maximum catalytic centre activity (V_{max}) is estimated as $4500\,min^{-1}$. The Michaelis constants for O_2 and lactate are estimated

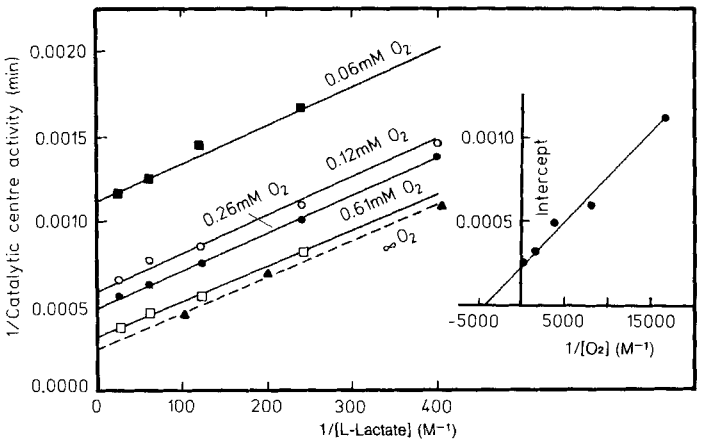


Fig. 2. *Steady-state turnover studies of 2-thio-FMN lactate oxidase with* L-*lactate and* O_2. Oxygen uptake was monitored with a Yellow Springs Instrument Co. oxygen electrode apparatus, with the indicated concentrations of L-lactate and O_2, and a catalytic amount of 2-thio-FMN lactate oxidase, in 0.01 M imidazole, pH 7.0, 25 °C. Also shown are the data points for infinite oxygen concentration (▲) derived from enzyme-monitored turnover studies, analyzed as described by Gibson et al. [17]. The inset shows the intercepts of the primary Lineweaver-Burk plots vs the reciprocal of the O_2 concentration

as 0.22 mM and 8 mM respectively. These values, along with other kinetic constants determined from stopped-flow experiments (see later sections), are listed in Table 1, along with values for native enzyme under the same conditions.

Stopped-flow turnover studies based on the principles detailed by Chance [16] and Gibson et al. [17] were also used to estimate catalytic centre activity. In these experiments, oxidized 2-thio-FMN lactate oxidase, 13.8 μM with respect to 2-thio-FMN, was mixed with different concentrations of L-lactate, both reactants being air-equilibrated. The reaction was monitored at 520 nm and the absorbance vs time curve, obtained as the limiting reactant (O_2) was consumed, was analyzed [17] to generate a Lineweaver-Burk plot of $1/v$ vs $1/[O_2]$ at that concentration of L-lactate. The results were in excellent agreement with those obtained with the O_2 electrode, with parallel Lineweaver-Burk plots being obtained. For the sake of clarity, only the values obtained by extrapolation to infinite $[O_2]$ from such analyses are shown in Fig. 2, along with the O_2 electrode results.

In the enzyme-monitored turnover experiments, in the steady-state region, most of the enzyme is in the oxidized state, indicating, as with native enzyme, that the reaction of O_2 with the reduced enzyme intermediate is not rate-limiting in catalysis. The importance of this observation will be considered in the Discussion.

Anaerobic Reduction Studies with L-*Lactate*

When 2-thio-FMN lactate oxidase and L-lactate are mixed under anaerobic conditions, the red color of the oxidized enzyme is rapidly bleached. When followed in the stopped-flow spectrophotometer the absorbance at 515 nm is seen to decrease rapidly, followed by a secondary slower phase to yield approximately 10% the initial absorbance. Similar biphasic reduction kinetics were observed over the whole visible range, 350 – 600 nm. Fig. 3 shows the time course of decrease in absorbance at 520 nm as monitored in the stopped-flow spectrophotometer when 6.2 μM enzyme and 5 mM L-lactate

Table 1. *Kinetic constants of 2-thio-FMN-substituted and native lactate oxidase*

All data were collected with enzyme in 0.01 M imidazole/HCl, pH 7.0, 25 °C. The values for native enzyme are from [4]. V_{max}, $K_{lactate}$ and K_{O_2} values were obtained from Fig. 2. Individual rate constants are those referred to in Scheme 1. The ratio k_{-1}/k_1 and k_2 were obtained from Fig. 3. The value of k_3 was taken as the slow phase of the reduction experiments of Fig. 3. The value of k_{-3} was derived from the slow phase of reoxidation in the presence of pyruvate (see text for details). The values of k_4 and k_6 were obtained from Fig. 6. The value of k_5 was calculated from the relationship $V_{max} = k_2k_5/k_2 + k_5$ (see [4])

Constant	Value for	
	native enzyme	2-thio-FMN enzyme
V_{max}	6200 min^{-1}	4500 min^{-1}
$K_{lactate}$	22 mM	8 mM
K_{O_2}	0.071 mM	0.22 mM
k_{-1}/k_1	5×10^{-2} M	2.8×10^{-2} M
k_2	1.4×10^4 min^{-1}	1.0×10^4 min^{-1}
k_{-2}	negligible	negligible
k_3	2.5 min^{-1}	105 min^{-1}
k_{-3}	10^3 M^{-1} min^{-1}	8.4×10^3 M^{-1} min^{-1}
k_4	1.1×10^8 M^{-1} min^{-1}	5.4×10^7 M^{-1} min^{-1}
k_5	1.13×10^4 min^{-1} (calc.)	8.2×10^3 min^{-1} (calc.)
k_6	5.4×10^5 M^{-1} min^{-1}	2.9×10^5 M^{-1} min^{-1}
K_{lact} calculated $= \frac{k_3(k_1+k_2)}{k_1(k_2+k_5)}$	22.6 mM	12.6 mM
K_{O_2} calculated $= \frac{k_5(k_2+k_{-2})}{k_4(k_2+k_5)}$	0.055 mM	0.083 mM

(concentrations after mixing) were allowed to react in 0.01 M imidazole/HCl, pH 7.0, 25 °C.

Both phases of reduction were exponential processes. The apparent first-order rate constant of the fast phase is dependent on the concentration of L-lactate. The reciprocal plot in the inset of Fig. 3 shows that this process exhibits saturation kinetics, with a limiting rate at infinite lactate concentration of 167 s^{-1} (10000 min^{-1}). In contrast, the secondary slow phase, with a rate constant of 1.7 s^{-1} (105 min^{-1}) is quite independent of the substrate concentration. These results are similar to those obtained with native lactate oxidase [4] and the iso-FMN enzyme [8], showing that reduction occurs more rapidly than turnover and that an equilibrium complex between oxidized enzyme and lactate precedes reduction. The K_d for this complex, from the data of Fig. 3, is estimated by the method of Strickland et al. [20] to be 28 mM (cf. also Table 1). The nature of the reduced species initially formed, which has no absorbance beyond 600 nm and whose absorbance decreases further in the 500-nm region with a substrate-concentration-independent rate constant of 1.7 s^{-1}, can be identified tentatively as the complex between reduced enzyme and the primary product pyruvate. The analogous complex in the native enzyme is essential to the oxidative decarboxylation mechanism of this enzyme [4]. Further evidence for this ascription is given in the following sections.

Spectral Properties of the Reduced Enzyme-Pyruvate Complex

With both native enzyme [4] and the iso-FMN enzyme [8] the catalytic intermediate of reduced enzyme and pyruvate

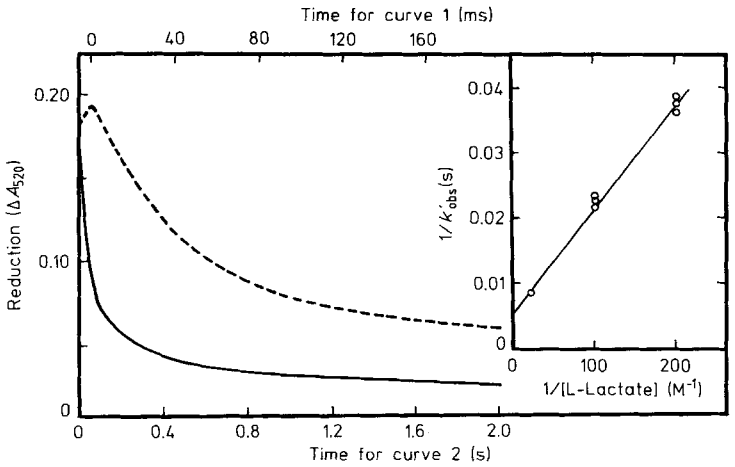


Fig. 3. *Reduction of 2-thio-FMN lactate oxidase by L-lactate under anaerobic conditions.* 2-Thio-FMN lactate oxidase (12.4 μM) was mixed with an equal volume of 10 mM L-lactate, both in 0.01 M imidazole/HCl, pH 7.0, 25 °C, and both anaerobic. The absorbance changes at 520 nm, measured in the 2-cm path length of the stopped-flow spectrophotometer, are biphasic, as shown. Curves 1 and 2 show results with separate reactions, recorded over a 200-ms time scale (top) and over a 2-s scale (bottom) respectively. The inset shows a double-reciprocal plot of the k_{obs} values for the fast phase determined at different concentrations of L-lactate

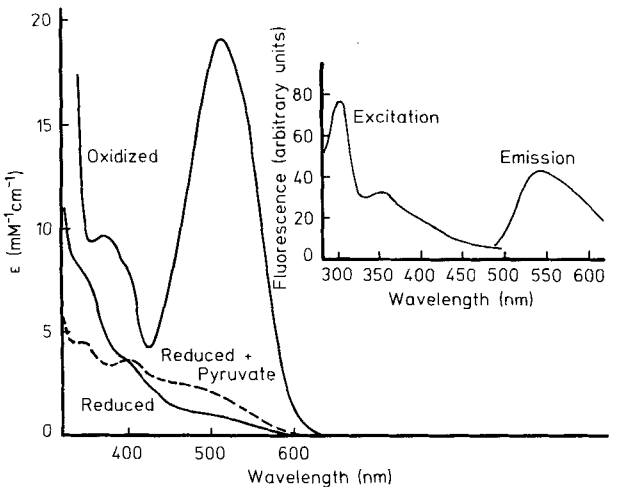


Fig. 4. *Spectra of 2-thio-FMN lactate oxidase and the complex with pyruvate and reduced enzyme.* Oxidized 2-thio-FMN enzyme, in 0.01 M imidazole/HCl, pH 7.0, 25 °C, was mixed with 10 mM L-lactate in an anaerobic cuvette, and the spectrum of the reduced enzyme recorded. Sodium pyruvate was then mixed from a second side arm of the anaerobic cuvette to yield a final concentration of 50 mM. The inset shows fluorescence excitation and emission spectra of the reduced enzyme (excitation spectrum recorded with emission at 530 nm and emission spectrum recorded with excitation at 315 nm). The fluorescence was quenched about 90 % on addition of pyruvate. The oxidized enzyme has no visible fluorescence

exhibits characteristic long-wavelength absorption extending out to 700 nm, and is easily observable, having absorbance at wavelengths where neither oxidized nor free reduced enzyme have any absorbance. In the case of 2-thio-FMN enzyme, owing to the high oxidation-reduction potential of this flavin [21], it would be expected that the charge transfer transition of the complex between reduced enzyme and pyruvate would be of higher energy (shorter wavelength) than with native enzyme and might, therefore, be hidden under the intense absorption band of the oxidized enzyme. Evidence that this is the case is presented in Fig. 4, which shows the spectrum of the enzyme reduced by L-lactate, and the change produced on addition of 50 mM pyruvate. The inset of Fig. 4 shows the fluorescence excitation and emission spectra of the reduced enyzme. The weak fluorescence of the reduced form is quenched almost completely on addition of pyruvate. Similar effects have been reported for native enzyme [5] and iso-FMN enzyme [8]. The absorbance difference spectrum is maximal at 480 nm. Evidence to be presented in the next section indicates that the K_d of the reduced enzyme-pyruvate complex is about 11 mM, so that the changes shown in Fig. 4 should be close to the maximal changes. Hence it can be concluded that the biphasic reduction of the 2-thio-FMN enzyme follows the same pattern as with native [4] and iso-FMN enzyme [8]:

$$\mathrm{EFl_{ox}} + \text{lactate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EFl_{ox}}\,\text{lactate} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EFl_{red}} \cdot \text{pyruvate} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{EFl_{red}} + \text{pyruvate}.$$

Oxidative Half Reaction of Reduced 2-Thio-FMN Lactate Oxidase and of Its Complex with Pyruvate

When 2-thio-FMN enzyme was reduced by a small excess (three equivalents) of L-lactate and reacted in the stopped-flow spectrophotometer with buffer containing various concentrations of O_2, monophasic reoxidation was observed at all wavelengths studied. A typical reaction curve is shown in the upper part of Fig. 5. The observed rate constants as a function of O_2 concentration are shown in Fig. 6, yielding a second-order rate constant of $2.9 \times 10^3\ M^{-1}\ min^{-1}$ for the reaction in 0.01 M imidazole/HCl, pH 7.0, 25 °C. On the other hand, when 10 mM pyruvate was present both with the reduced enzyme and the oxygen-containing buffer, the reaction was markedly biphasic, as illustrated in the lower half of Fig. 5. The extent of the rapid phase was dependent on pyruvate concentration, being greater the higher the pyruvate concentration, and the k_{obs} for this phase was linearly dependent on oxygen concentration as shown in Fig. 6, giving a second-order rate constant of $5.4 \times 10^7\ M^{-1}\ min^{-1}$. The k_{obs} for the fast phase was not dependent on the concentration of pyruvate, only the amplitude changed with pyruvate concentration. These results are very similar to those obtained with native enzyme [4]. A plot

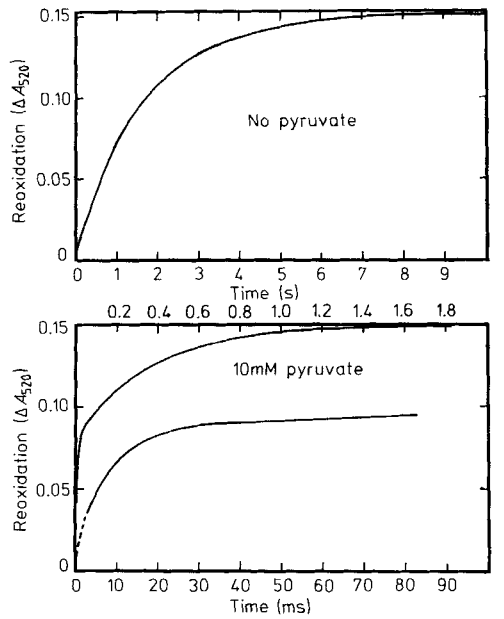


Fig. 5. *Effect of pyruvate on the rate of reoxidation of reduced 2-thio-FMN lactate oxidase by oxygen.* Top: 2-thio-FMN lactate oxidase (12 μM) in 0.01 M imidazole/HCl, pH 7.0, 25 °C was reduced in a tonometer under anaerobic conditions by the addition of 0.04 mM L-lactate and mixed in the stopped-flow spectrophotometer with air-equilibrated 0.01 M imidazole buffer (final O_2 concentration, 0.13 mM). Bottom: same conditions, except that both the reduced enzyme and the buffer contained 10 mM pyruvate. Note the different time scales, that at the top applying to the upper trace and that at the bottom to the lower trace

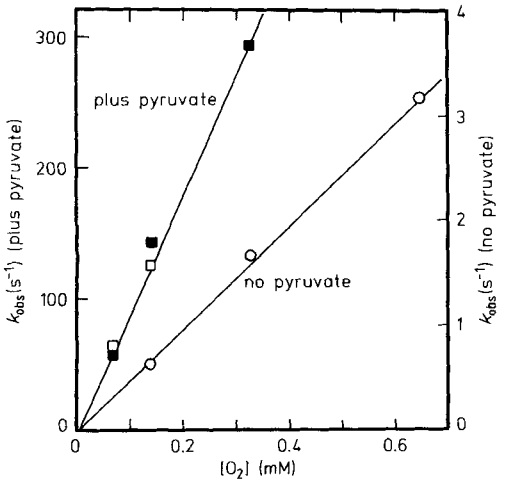
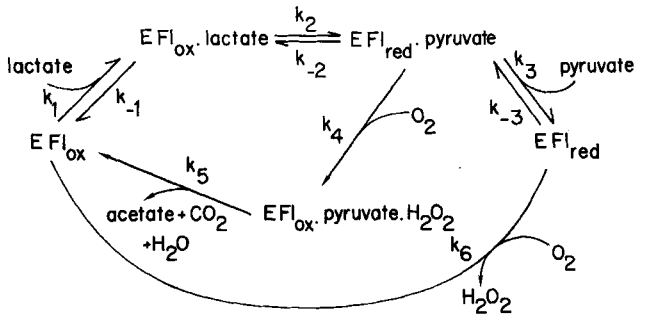


Fig. 6. *Dependence on oxygen concentration of the observed pseudo-first-order rate constants of reduced 2-thio-FMN lactate oxidase, free and in complex with pyruvate.* The scale on the right axis is for values in the absence of pyruvate, and obtained from traces such as shown in the top part of Fig. 5. The scale on the left axis is for the fast phase of the reaction in the presence of 10 mM (■) or 50 mM (□) pyruvate and obtained from traces such as the lower one shown in the bottom part of Fig. 5

(not shown) of the reciprocal of the fraction of the reaction in the fast phase vs the reciprocal of the pyruvate concentration indicates a K_d for the reduced enzyme-pyruvate complex of 11 mM. This compares with the analogous value for the native enzyme of 22 mM [4]. At first sight it would seem reasonable to ascribe the slow phase of reoxidation to reduced 2-thio-FMN



Scheme 1. *Reaction cycle of lactate oxidase*

enzyme not in complex with pyruvate, as was shown to be the case with native enzyme [4]. However, it is evident from Fig. 5 that the rate of the slow phase is significantly faster than that of the free reduced enzyme. The observed first-order rate constants for the slow phase varied not only with the concentration of O_2 but also with the concentration of pyruvate. These results suggested that the slow phase was actually comprised of two parallel reactions:

$$EFl_{red} + O_2 \xrightarrow{k_6} EFl_{ox} + H_2O_2$$

$$EFl_{red} + \text{pyruvate} \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} EFl_{red} \cdot \text{pyruvate} \xrightarrow[k_4]{O_2} EFl_{ox} \cdot \text{pyruvate} \cdot H_2O_2 .$$

In this case, owing to the very rapid reaction of O_2 with the reduced enzyme-pyruvate complex, the observed value for k_{slow} should equal $k_6[O_2] + k_{-3}$[pyruvate]. Indeed, when the contribution of $k_6[O_2]$ (obtained from Fig. 5) is subtracted, the corrected value for k_{slow} is now linearly dependent on the pyruvate concentration, yielding a value for k_{-3} of $1.4 \times 10^2\ M^{-1}\ s^{-1}$. Combined with the value for the K_d of the reduced enzyme-pyruvate complex of 11 mM, k_3 may be estimated as $1.5\ s^{-1}$, in reasonable agreement with the value of the slow phase ($1.7\ s^{-1}$) observed in the reductive half reaction. Taken together, these results provide strong evidence for the sequence of reactions shown in Scheme 1 not only to operate with the native enzyme [4] and iso-FMN enzyme [8], but also with 2-thio-FMN enzyme. As in the case of native enzyme, k_2 and k_5 both contribute significantly to the limiting catalytic centre activity of the 2-thio-FMN enzyme, contrasting sharply with the situation with the iso-FMN enzyme, where k_4 is the rate-determining step in catalysis under most conditions [8]. Individual rate constants for both the native enzyme and the 2-thio-FMN enzyme are collected together in Table 1.

Binding of Pyruvate to Oxidized 2-Thio-FMN Lactate Oxidase

In the experiments described in the preceding section, where reduced enzyme in the presence of pyruvate was reoxidized by O_2, it was noted that the final spectrum was not the same as the initial oxidized enzyme. This was found to be due to a perturbation of the absorption spectrum due to complex formation between the oxidized enzyme and pyruvate. Difference spectra between the complex (sample cuvette) and the free enzyme (reference cuvette) showed maximal negative differences at 570 nm ($\Delta\varepsilon = 3100\ M^{-1}\ cm^{-1}$), 532 nm ($\Delta\varepsilon = 2500\ M^{-1}\ cm^{-1}$), 380 nm ($\Delta\varepsilon = 600\ M^{-1}\ cm^{-1}$) and 337 nm ($\Delta\varepsilon \approx 2300\ M^{-1}\ cm^{-1}$) and maximal positive differ-

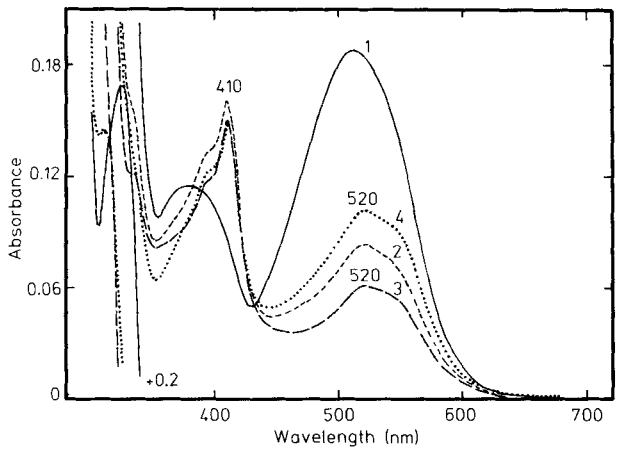


Fig. 7. *Formation of the anionic radical form of 2-thio-FMN lactate oxidase and its complex with pyruvate.* Curve 1: 10 μM enzyme (with respect to 2-thio-FMN) plus 0.1 M glycine and 6 μM 5-deazaflavin, in 0.01 M imidazole, pH 7.0, 4 °C, under anaerobic conditions. Curves 2 and 3: after 40 s and 3 min photoirradiation through a Corning CS-372 filter (cut off below 430 mm). If the cuvette were opened to air at this stage, rapid reoxidation would be obtained (separate control experiments). Curve 4: the same as curve 3, but after mixing with sodium pyruvate from the side arm of the cuvette, to yield a final concentration of 13 mM pyruvate. When air was admitted and mixed the spectrum was practically unchanged; the spectrum of oxidized enzyme was regained after gel filtration on Sephadex G-25 with $t_{1/2}$ of 43 min

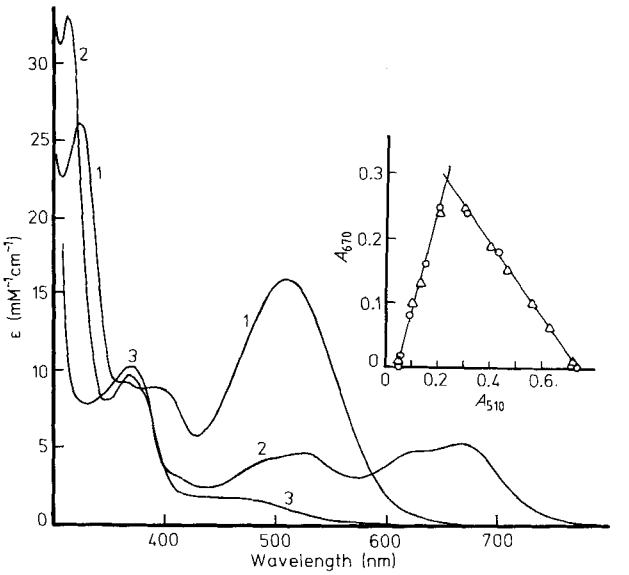


Fig. 8. *Spectra of 2-thio-FMN flavodoxin in its different oxidation states.* 2-Thio-FMN flavodoxin in 0.1 M potassium phosphate, pH 7.0, 25 °C, in the presence of 30 mM EDTA and 1 μM 5-deazaflavin, was made anaerobic in the dark and then reduced by irradiation with visible light. Curve 1: oxidized enzyme, before irradiation. Curve 2: neutral semiquinoid form, produced by irradiation for 75 s at a light intensity about 0.2 J cm^{-2} s^{-1}. Curve 3: fully reduced form, produced by irradiation for 3 min. The inset shows the absorbance at 670 nm versus that at 510 nm during photoreduction (○) and on reoxidation with small amounts of O_2 admitted to the cuvette (△)

ence at 415 nm ($\Delta\varepsilon = 1\,100\ M^{-1}\,cm^{-1}$). Spectral titration of the enzyme with pyruvate gave a value for the K_d of 0.1 mM at pH 7.0, 25 °C (results not shown). This is the same as that obtained for the analogous complex with native enzyme [4].

Semiquinone Form of 2-Thio-FMN Lactate Oxidase and Its Stabilization by Pyruvate

On photoreduction with 5-deazaflavin as catalyst [18] both native lactate oxidase and the iso-FMN enzyme exhibit spectra typical of the flavin anionic semiquinone [7, 8]. In both cases the semiquinone is rapidly reoxidized on mixing with O_2 and with both forms this oxygen reactivity is dramatically slowed by formation of a complex with pyruvate [7, 8]. This effect is so great that it is possible to isolate the 1 : 1 complex of pyruvate and semiquinoid enzyme by gel filtration with Sephadex G-25. It was therefore of particular interest to see whether the 2-thio-FMN enzyme showed similar properties.

Indeed the 2-thio-FMN enzyme undergoes facile photochemical reduction to the radical state, as illustrated in Fig. 7. In this experiment curve 2 was reached on 40 s irradiation and curve 3 on 90 s irradiation (same at 3 min). Continued irradiation resulted in very slow further reduction. If air is admitted at this stage, rapid reoxidation to the starting spectrum is obtained. When 12.5 mM pyruvate was added under anaerobic conditions, a marked increase in absorbance in the 500-nm range was observed with very little change in the absorbance at 410 nm (Fig. 7, curve 4). Similar spectral changes have been observed previously on the addition of pyruvate to the semiquinoid forms of native [7] and iso-FMN lactate oxidase [8]. As in those cases, the complex with pyruvate can also be isolated by gel filtration with Sephadex G-25. The isolated complex reoxidized with a $t_{1/2}$ of 43 min at 4 °C. Thus, although it is also dramatically stabilized compared with the free enzyme, where the half-time of reoxidation is of the order of seconds, it is not as stable as the corresponding complexes with native enzyme and iso-FMN enzyme, where $t_{1/2}$ values under the same conditions were ≈ 2000 min at 0 °C and ≈ 35 min at 25 °C for native enzyme [7] and ≈ 38 min at 25 °C for iso-FMN enzyme [8].

Spectral Properties of the Neutral Semiquinoid Form of 2-Thioflavin

While lactate oxidase is known to stabilize the anionic flavin radical form, a stablization postulated to be due to hydrogen bonding interactions from a positively charged protein base and the negatively charged N(1) – C(2α) locus of the flavin [22], flavodoxins are known to stabilize the neutral flavin radical, because of hydrogen bonding from the flavin N(5) – H to a peptide backbone carbonyl of the protein [23]. Since the spectral properties of thioflavins are quite different from those of normal flavins, it was of obvious interest to determine the spectrum of the stabilized neutral radical, in order to compare it with the presumed anion form of lactate oxidase. Fig. 8 shows the spectrum of 2-thio-FMN flavodoxin in its three oxidation states. Again, 5-deazaflavin-catalyzed photoreduction was used to generate the semiquinoid and fully reduced forms, as can be done with the native protein [18]. As with the native protein [24], mixing the fully reduced form with O_2 results in the very rapid reformation of semiquinone (and O_2^-) and the very much slower reoxidation of the semiquinone to the oxidized form. The inset of Fig. 8 shows a plot of the absorbance at 670 nm versus that at 510 nm during photoreduction (circles) and reoxidation (triangles). It can be seen that almost quantitative amounts of radical can be obtained, as with native flavodoxin [18].

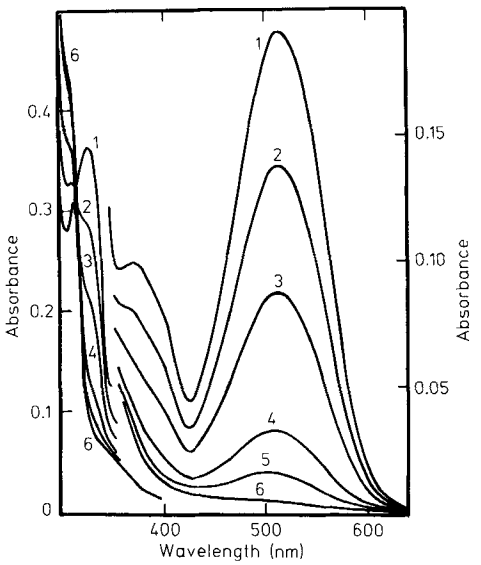


Fig. 9. *Formation of sulfite adduct of 2-thio-FMN lactate oxidase.* Curve 1: 9.94 μM 2-thio-FMN lactate oxidase in 0.01 M imidazole/HCl, pH 7.0, 25 °C. Curves 2–6: after addition of $NaHSO_3$ to concentrations of 2.99 μM, 5.96 μM, 8.92 μM, 11.86 μM and 3 mM respectively. Note the different absorbance scales for the visible (right-hand axis) and near-ultraviolet (left-hand axis)

Formation of an N(5)-Sulfite Complex with 2-Thio-FMN Lactate Oxidase

Flavins in the oxidized state react with sulfite to form an equilibrium complex in which the sulfite is covalently linked to the flavin N(5) position [25]. This reaction is enhanced and stabilized in the case of the flavoprotein oxidases in general [22, 26]. As a representative of this class of enzymes, lactate oxidase forms a very strong complex with sulfite, with the K_d in the neighborhood of 0.1 μM (S. Ghisla and V. Massey, unpublished). (The value of 4 μM given in [26] is high because it was determined in the presence of 0.1 M phosphate, which was shown later [4] to be a competitive inhibitor.) Sulfite also interacts strongly with 2-thio-FMN lactate oxidase as shown in Fig. 9. The K_d calculated from these results was 0.1 μM. The spectrum of the complex was similar to that shown in Fig. 4 for reduced enzyme, and to that determined earlier for other N(5)-substituted derivatives of the 2-thio-FMN enzyme [3]. It should be noted that the spectra of C(4a) derivatives of 2-thio-FMN lactate oxidase are distinctly different from those of N(5) derivatives [3]. Hence it can be concluded that as with the native enzyme, sulfite forms an adduct at the 2-thioflavin N(5) position.

Photochemical Reactions with 2-Thio-FMN Lactate Oxidase

The ability of lactate oxidase to undergo facile photochemical reactions is very useful in determining the spectral characteristics of covalent adducts of artificial flavins. In the case of 2-thio-FMN, there are pronounced differences in the spectra of C(4a) and N(5) adducts. The latter resemble the spectrum of the reduced form (cf. Fig. 4), while the former have absorbance maxima with relatively high absorption coefficients at about 360 nm and 455 nm [3]. With native enzyme [3] and with the iso-FMN-substituted form [8] photochemical reaction with D-lactate, instead of giving a stable adduct, results in the facile reduction of the enzyme flavin. By contrast, when 2-thio-FMN lactate oxidase is illuminated through a Corning CS-370 filter, which transmits light only at wavelengths greater than 490 nm, the enzyme is bleached rapidly in the presence of D-lactate, but instead of being reoxidized rapidly on mixing with air, the oxidized enzyme is returned very slowly. This is not due to the formation of a complex between reduced enzyme and D-lactate; when enzyme was reduced by L-lactate and then mixed with the same concentration of D-lactate as used in the photochemical reaction (20 mM), subsequent mixing with air resulted in the rapid reoxidation of the enzyme flavin. The adduct which is formed is sufficiently stable to withstand aerobic gel filtration through Sephadex G-25 in the cold. It breaks down to reform oxidized 2-thio-FMN enzyme with a $t_{1/2}$ of 7 min at 25 °C and 220 min at 0 °C. The adduct is presumably one involving the flavin N(5) position, since the spectrum resembles closely that of other N(5) adducts [3]. It has weak fluorescence, similar to that of reduced enzyme (cf. Fig. 4) and other N(5) adducts [3]. By analogy with the photochemical reactions of native lactate oxidase, which involve decarboxylation of the photo-substrate, the adduct from D-lactate is presumably the N(5)-$CHOHCH_3$ adduct.

DISCUSSION

From the results presented it is clear that the native flavin (FMN) of lactate oxidase may be removed and replaced by 2-thio-FMN and that the resulting holoenzyme has properties remarkably similar to those of the native enzyme. Thus the 2-thio-FMN enzyme catalyzes the oxidative decarboxylation of lactate, as shown in Scheme 1, with a catalytic centre activity only 30% smaller than that of native enzyme, and with individual kinetic constants not appreciably different from those of native enzyme (cf. Table 1). Of particular note is the effect of complex formation with pyruvate on the rate of reoxidation of the reduced enzyme with O_2. This complex is a crucial intermediate in the catalytic mechanism, as it leads to the ternary complex of oxidized enzyme, pyruvate and H_2O_2 from which the final products, acetate, CO_2 and H_2O arise [4]. With native enzyme, the reduced enzyme-pyruvate complex reacts some 200-fold faster with O_2 than does the free reduced enzyme. Almost the same rate enhancement is found with the 2-thio-FMN enzyme. In the catalytic reaction, the rate-limiting steps, for both native and 2-thio-FMN enzymes, are reduction (k_2) and product release (k_5), consistent with the observation that in enzyme-monitored turnover experiments, the enzyme exists largely in the oxidized form during the steady state.

Other features of the native enzyme are also displayed by the 2-thio-FMN enzyme. These include formation of an anionic semiquinone on photochemical reduction with 5-deazaflavin as catalyst [18] and the remarkable stablization of this radical species against O_2 by formation of a complex with pyruvate [7]. Also the 2-thio-FMN enzyme forms a flavin N(5) adduct with sulfite with comparable avidity to that of the native enzyme [26]. As reported previously, both it and the native protein also undergo facile photochemical reactions to form flavin N(5) and C(4a) adducts [3]. The only apparently different photochemical reaction is that involving D-lactate. With native enzyme [3] and with the iso-FMN enzyme [8], irradiation in the presence of D-lactate produces the fully reduced flavin-enzyme, while with the 2-thio-FMN enzyme, a flavin N(5) adduct is formed. This apparent difference, however, is probably one of stability of the adduct rather than a difference in mechanism of the photochemical reaction. Even with free flavins, such photochemical reductions involving decarboxylation of the photosubstrate appear to involve intermediate flavin adducts [28].

The present results add confidence to the conclusion that, with care, the native flavin of a flavoprotein can be removed and replaced by artificial flavins with various modifications of the isoalloxazine ring system, without altering the structural integrity of the protein and its active-site geometry. It is particularly important that this principle be documented, so that when examples are found where a particular flavin derivative introduced into a protein shows modified behavior, the differences from the native enzyme can be considered in proper perspective. In the case of *p*-hydroxybenzoate hydroxylase, for example, we have found that the 2-thio-FAD-substituted enzyme, although it readily forms a flavin C(4a)-hydroperoxide, cannot support the full sequence of reactions catalyzed by the native enzyme [9a]. Similar results have been found for this enzyme substituted with 1-deaza-FAD [27]. The possibility of interpreting the significance of such findings relies in no small way on examples like the present one, where the same flavin derivative is shown to be fully functional. A number of examples exists where modified flavoenzymes have been prepared which possess many of the properties characteristic of the native protein. These include D-amino acid oxidase [9, 29–31], glucose oxidase [29–31], lactate oxidase [3, 8, 30, 31], 'old yellow Enzyme' [30–32], flavodoxin [30, 31], *p*-hydroxybenzoate hydroxylase [30, 31]. NADPH–cytochrome P-450 reductase [33], NADPH–adrenodoxin reductase [21, 30], pyridoxamine 5′-phosphate oxidase [34] and xanthine oxidase [35]. In many of these cases it has been taken for granted that the modified flavin is bound to the apoprotein in the same way as the natural flavin. In the present case, it is clear that despite the differences in electronegativity at the flavin 2α position, and the significant difference in oxidation-reduction potential [21], 2-thio-FMN lactate oxidase and native enzyme are virtually identical in their catalytic properties and have remarkably similar rate constants for individual steps in the catalytic cycle. At first sight the similarity of the 2-thio-FMN enzyme and the native enzyme might seem surprising, since the higher redox potential of 2-thioflavin could be expected to result in a greater stability of adducts arising from attack of nucleophiles at the flavin N(5) position [25]. Indeed, the enantiomeric flavin N(5)-glycollyl adducts formed with the 2-thio-FMN enzyme [3, 6] are several times more stable than those found with the native enzyme [5, 6]. As detailed in this paper, the N(5) adduct formed photochemically with 2-thio-FMN enzyme and D-lactate has finite stability, whereas with native enzyme the corresponding derivative, if formed, must be much less stable. The finding that the rate of reduction of the 2-thio-FMN enzyme by lactate is only marginally slower than with native enzyme may be a consequence of two compensating effects, a faster formation of the postulated N(5)-lactyl intermediate, because of the higher redox potential of 2-thio-FMN, and a slower breakdown of the adduct to the reduced enzyme-pyruvate complex, because of the enhanced stability of the adduct.

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REFERENCES

1. Spencer, R., Fisher, J. & Walsh, C. (1976) *Biochemistry*, *15*, 1043–1053.
2. Ghisla, S. & Massey, V. (1975) *J. Biol. Chem. 250*, 577–584.
3. Ghisla, S., Massey, V. & Choong, Y. S. (1979) *J. Biol. Chem. 254*, 10662–10669.
4. Lockridge, O., Massey, V. & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097–8106.
5. Massey, V., Ghisla, S. & Kieschke, K. (1980) *J. Biol. Chem. 255*, 2796–2806.
6. Ghisla, S. & Massey, V. (1980) *J. Biol. Chem. 255*, 5688–5696.
7. Choong, Y. S. & Massey, V. (1980) *J. Biol. Chem. 255*, 8672–8677.
8. Choong, Y. S. & Massey, V. (1981) *J. Biol. Chem. 256*, 8671–8678.
9. Claiborne, A., Massey, V., Fitzpatrick, P. F. & Schopfer, L. M. (1982) *J. Biol. Chem. 257*, 174–182.

9a. Claiborne, A. & Massey, V. (1983) *J. Biol. Chem.* in the press.

10. Sullivan, P. A., Choong, Y. S., Schreurs, W. J., Cutfield, J. F. & Shepherd, M. G. (1977) *Biochem. J. 165*, 375–383.
11. Choong, Y. S., Shepherd, M. G. & Sullivan, P. A. (1975) *Biochem. J. 145*, 37–45.
12. Mayhew, S. G. (1971) *Biochim. Biophys. Acta*, *235*, 289–302.
13. Mayhew, S. G. & Strating, M. J. J. (1975) *Eur. J. Biochem. 59*, 539–544.
14. Föry, W. & Hemmerich, P. (1967) *Helv. Chim. Acta*, *50*, 1766–1774.
15. Gibson, Q. H. & Milnes, L. (1964) *Biochem. J. 91*, 161–171.
16. Chance, B. (1943) *J. Biol. Chem. 151*, 553–577.
17. Gibson, Q. H., Swoboda, B. E. P. & Massey, V. (1964) *J. Biol. Chem. 239*, 3927–3934.
18. Massey, V. & Hemmerich, P. (1978) *Biochemistry*, *17*, 9–16.
19. Hohorst, H. J. (1963) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.) p. 266, Academic Press, New York.
20. Strickland, S., Palmer, G. & Massey, V. (1975) *J. Biol. Chem. 250*, 4048–4052.
21. Light, D. R. & Walsh, C. (1980) *J. Biol. Chem. 255*, 4264–4277.
22. Massey, V. & Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246–257.
23. Ludwig, M. L., Burnett, R. M., Darling, G. D., Jordan, S. R., Kendall, D. S. & Smith, W. W. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed.) pp. 393–404, Elsevier, Amsterdam.
24. Mayhew, S. G. & Massey, V. (1969) *J. Biol. Chem. 244*, 794–802.
25. Müller, F. & Massey, V. (1969) *J. Biol. Chem. 244*, 4007–4016.
26. Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G. & Foust, G. P. (1969) *J. Biol. Chem. 244*, 3999–4006.
27. Entsch, B., Husain, M., Ballou, D. P., Massey, V. & Walsh, C. (1980) *J. Biol. Chem. 255*, 1420–1429.
28. Hemmerich, P., Knappe, W.-R., Kramer, H. E. A. & Traber, R. (1980) *Eur. J. Biochem. 104*, 511–520.
29. Spencer, R., Fisher, J. & Walsh, C. (1977) *Biochemistry*, *16*, 3594–3602.
30. Massey, V., Ghisla, S. & Moore, E. G. (1979) *J. Biol. Chem. 254*, 9640–9650.
31. Schopfer, L. M., Massey, V. & Claiborne, A. (1981) *J. Biol. Chem. 256*, 7329–7337.
32. Abramovitz, A. S. & Massey, V. (1976) *J. Biol. Chem. 251*, 5327–5336.
33. Vermilion, J. L., Ballou, D. P., Massey, V. & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266–277.
34. Merrill, A. H., Kasai, S., Matsui, K., Tsuge, H. & McCormick, D. B. (1979) *Biochemistry*, *18*, 3635–3641.
35. Hille, R., Fee, J. A. & Massey, V. (1981) *J. Biol. Chem. 256*, 8933–8940.

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