Mechanistic Studies of p-Hydroxybenzoate Hydroxylase Reconstituted with 2-Thio-FAD*

Al Claiborne and Vincent Massey
From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

2-Thio-FAD (oxygen substituent at position 2 is replaced by sulfur) was used to reconstitute the apoenzyme of p-hydroxybenzoate hydroxylase. The 2-thio-FAD enzyme differs from native enzyme in several respects. While the native enzyme catalyzes the fully coupled hydroxylation of p-hydroxybenzoate, the 2-thio-FAD enzyme shows no hydroxylation of this substrate, instead reducing molecular oxygen to hydrogen peroxide. The rate of reduction of 2-thio-FAD p-hydroxybenzoate hydroxylase by NADPH in the presence of substrate was 7-fold faster than with the native enzyme. However, the oxygen reactivity of the reduced 2-thio-FAD enzyme was less than 1% that of native enzyme. This slow oxygen reaction results in the very high K"{o} observed in steady state kinetic studies of the modified enzyme. Stopped flow studies of the oxygen reaction of the reduced 2-thio-FAD enzyme in the presence of substrate confirmed the formation of a transient intermediate. The spectrum of this intermediate is very similar to those of the flavin-C(4a) adducts obtained with 2-thio-FMN lactate oxidase. This evidence suggests that reduced 2-thio-FAD p-hydroxybenzoate hydroxylase forms a flavin-C(4a)-hydroperoxide on reaction with oxygen in a reaction analogous to that with native enzyme, but that the resulting peroxylflavin is incompetent as an oxygenating species, breaking down instead to oxidized 2-thio-FAD enzyme and hydrogen peroxide.

The use of modified flavins as probes of flavoprotein structure and mechanism has been advanced in recent years with considerable success (1, 2). Recent mechanistic studies in this laboratory have employed the 8-mercapto-, 8-chloro-, and hydroxy-FAD analogs with xanthine oxidase placed by page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The 2-thio-FMN analog (oxygen substituent at position 2 is replaced by sulfur; Structure 1) had earlier been used in studies of the charge-transfer complexes of Old Yellow Enzyme (3), as a structural probe of the covalent flavin adducts of lactate oxidase (4), and with the Azoobacter vinelandii flavodoxin (10) and rabbit liver pyridoxamine 5'-phosphate oxidase (11). Previous attempts to apply the 2-thio-FAD coenzyme as a mechanistic probe of adrenodoxin reductase were complicated by the presence of contaminating FAD in the flavin preparations (12). However, recent work in this laboratory has resulted in stable preparations of the 2-thio-FAD coenzyme free of FAD contamination (13). The chemical reactivity of the sulfur substituent at the flavin 2-position toward thiol reagents (methyl methanethiosulfonate; Ref. 13) and peroxides (H2O2, m-chloroperoxybenzoic acid)2 to yield the 2-SSCH3 flavin disulfide and the flavin 2-S-oxide, respectively, makes this flavin a useful probe of active site structure.

The hydroxylation pathway deduced from mechanistic studies with p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens involves three oxygenated flavin intermediates with distinct spectral properties (14). While the structures of intermediates I and III are well established as the flavin-C(4a)-hydroperoxide and the C(4a)-hydroxyflavin, respectively (15), the structure of intermediate II has yet to be established (14, 16). Reconstitution of the enzyme with various FAD analogs might be expected to yield valuable kinetic and spectral information relevant to the structure of this flavin-oxygen derivative.

However, reconstitution of the enzyme with 1-deaza-FAD resulted in a catalytically competent NADPH oxidase which failed to carry out substrate hydroxylation (17). This result was particularly significant in view of the observed formation of a transient enzyme-bound 1-deazaflavin-C(4a)-hydroperoxide. This intermediate, instead of oxygenating bound p-hydroxybenzoate, broke down to yield oxidized enzyme and hydrogen peroxide.

We have prepared 2-thio-FAD p-hydroxybenzoate hydroxylase. This modified enzyme, like the 1-deaza-FAD enzyme, is incapable of substrate hydroxylation. The results of stopped flow kinetic studies show the formation of a transient 2-thioflavin-C(4a)-hydroperoxide. Substitution of the N(1) nitrogen or the O(2a) oxygen elements of the flavin pyrimidine nucleus with carbon or sulfur, respectively, thus renders the corresponding flavin-C(4a)-hydroperoxide incapable of substrate oxygenation with this enzyme.

EXPERIMENTAL PROCEDURES

Materials

p-Hydroxybenzoic acid from Sigma was recrystallized before use. NADPH was from P-L Biochemicals; sodium dithionite was from J. T. Baker; and potassium azide was from Eastman Kodak Co. 2-Thioriboflavin was a generous gift of the late Dr. Peter Hemmerich.

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University of Konstanz, West Germany. Bovine catalase was from Calbiochem. Oxygen-nitrogen mixtures were obtained from Matheson, Dayton, OH.

Methods

Preparation of 2-Thio-FAD p-Hydroxybenzoate Hydroxylase—p-Hydroxybenzoate hydroxylase was purified from Pseudomonas fluorescence as previously described (14); the apoenzyme was prepared using the acid ammonium sulfate method of Entsch et al. (17). Highly resolved apoenzyme preparations (containing 0.1-0.3% residual FAD) were required in all catalytic turnover experiments; for single turnover and spectral studies, residual FAD levels of 1-3% were acceptable. 2-Thio-FAD was prepared as previously described from 2-thioriboflavin (15), and was used to reconstitute the apoenzyme following the general procedure of Entsch et al. (17).

Oxygen Incorporation into Substrate—Two procedures were followed in measuring the stoichiometry of substrate hydroxylation by the 2-thio-FAD enzyme. The first method, described in detail by Entsch et al. (17), involves high pressure liquid chromatography analyses of 3,4-dihydroxybenzoic acid formation in reaction mixtures following reoxidation of stoichiometrically reduced enzyme in the presence of substrate. The second method employed catalytic amounts of enzyme with an oxygen electrode (Yellow Springs Instrument Co., model 56), 0.15 µM 2-Thio-FAD enzyme (0.3% residual FAD) and substrate (0.15 mM p-hydroxybenzoate and 0.26 mM O2) at 25 °C. After exhaustion of the limiting substrate (NADPH), catalase was added. The stoichiometry of H2O2 production during NADPH oxidation was measured from the decomposition of peroxide by catalase (18). In the case of complete uncoupling of NADPH oxidation and substrate hydroxylation, 50% of the oxygen initially consumed was returned with the addition of catalase.

Steady State Kinetics—The general procedures of Husain and Massey (19) were followed in initial velocity measurements of 2-thio-FAD enzyme activity with varying oxygen concentrations. The stopped flow spectrophotometer previously described (20) was also convenient for steady state kinetics. The instrument was operated manually; photometer output was channeled to an x-y recorder, giving a direct plot of A(430) versus time, to follow NADPH oxidation, and with easily attainable variation of the initial O2 concentration.

In the enzyme monitored turnover experiments, as in all other rapid kinetic studies, the Nova minicomputer system (21) interfaced with the stopped flow instrument was used to record optical density with time. 2-Thio-FAD enzyme (final concentration = 4.5 µM) was reacted with NADPH (final concentration = 100 µM) in the presence of varying concentrations of p-hydroxybenzoate (final concentrations = 10-6-1 M). Prior equilibration of all solutions with 100% O2 at 25 °C allowed a final O2 concentration of 1.3 mM. Turnover data were analyzed following the general method of Gibson et al. (22), with NADPH taken as the limiting substrate.

Enzyme Reduction—The rapid anaerobic reduction of the 2-thio-FAD enzyme was accomplished in the stopped flow spectrophotometer. Using general methods previously described (19), enzyme reduction was monitored at wavelengths over the range 450-700 nm in order to determine whether transient species were involved. Kinetic measurements as a function of NADPH concentration were made at 500 nm. The slow anaerobic reduction of the enzyme in the absence of substrate was followed at 500 nm with a Cary 118 double beam spectrophotometer.

Rapid Kinetics of Enzyme Reoxidation—Reoxidation of dithionite-reduced enzyme in the absence or presence of substrate was followed in the stopped flow spectrophotometer. Using general methods previously described (19), enzyme reduction was monitored at wavelengths over the range 450-700 nm in order to determine whether transient species were involved. Kinetic measurements as a function of oxygen concentration at many wavelengths. The use of buffered solutions equilibrated with 100% O2 at 0 °C allowed final O2 concentrations of 1 mM in some of these experiments.

Anaerobiosis—Commercially purified nitrogen was freed of residual oxygen by passage over heated copper turnings. Anaerobic enzyme solutions were prepared by alternate evacuation and equilibration with oxygen-free nitrogen. Anaerobic solutions of 3,4-dihydroxybenzoic acid and protocatechuic acid dioxygenase (23) were used to scrub residual oxygen from the stopped flow system prior to all anaerobic experiments. All other anaerobic techniques are described elsewhere (24).

All experiments with the 2-thio-FAD enzyme, unless otherwise noted, were performed at 4 °C in 50 mM potassium phosphate, pH 7.0, containing 0.3 mM EDTA. Stock solutions of NADPH were kept at slightly alkaline pH to avoid nonenzymatic loss. Absorption spectra were recorded with any Cary 118 or Cary 219 double beam spectrophotometer thermostatted at 4 °C and purged with dry air to avoid fogging.

Calculation of Oxygenated Intermediate Spectrum—For the model two-step mechanism

\[ A + B \xrightarrow{k_1} C \xrightarrow{k_2} D \]

the concentration of intermediate C is given as a function of time by the equation (25)

\[ C(t) = \frac{k_2}{k_1 - k_2} \left(1 - e^{-k_1 t} - e^{-k_2 t}\right) \]

Determination of the rate constants k1 and k2 from stopped flow traces at isosbestic wavelengths during the reaction of reduced 2-thio-FAD p-hydroxybenzoate hydroxylase and oxygen in the presence of substrate and 0.1 mM azide thus allows the calculation of the concentration of the transient intermediate with time. It was determined that 56% of the total enzyme was accumulated as the transient intermediate at 0.4 s of the reaction. The spectrum of the reaction mixture at 0.4 s was then determined from stopped flow traces taken at the wavelengths indicated. Based on the absorbance at 500 nm (isosbestic for formation of intermediate from reduced enzyme), this spectrum was corrected for the absorbance due to oxidized enzyme to give the spectrum of the transient intermediate.

RESULTS

Spectral Properties of 2-Thio-FAD p-Hydroxybenzoate Hydroxylase—The visible absorbance spectrum of the 2-thio-FAD reconstituted enzyme at pH 7.0 is shown in a later section (Fig. 5). Previous studies (15) demonstrated a tight binding affinity of the apoenzyme for 2-thio-FAD (Kd = 10^-7 M or less), and also showed that this association results in a significant decrease in the pK of the flavin N(3) proton. The absorption spectrum is also perturbed on addition of substrate (13). A titration of the enzyme at pH 7.0 with p-hydroxybenzoate gave Kd of 0.13 mM, very similar to the value of 0.16 mM obtained previously at pH 8.8 (13), and which can be compared with the value of 43 µM obtained for native enzyme at pH 6.6 (19). No spectrally observable secondary binding of the substrate was seen up to 0.4 mM p-hydroxybenzoate. No stable semiquinone of the 2-thio-FAD enzyme was observed during photoreduction in the presence or absence of substrate.

Reaction Catalyzed—Single turnover analysis of 3,4-dihydroxybenzoic acid production by the 2-thio-FAD enzyme demonstrated that this enzyme failed to catalyze the hydroxylation of p-hydroxybenzoate. Thus, no product peak was observed in high pressure liquid chromatograms taken from a sample of the enzyme which had been photoreduced (26), mixed with substrate, and reoxidized in the dark. Based on the recovery of p-hydroxybenzoate in these analyses, as little as 5% conversion of substrate to product would have been detectable. A corollary to the observed failure of the 2-thio-FAD enzyme to carry out coupled substrate hydroxylation is that molecular oxygen is reduced to hydrogen peroxide in turnover, as shown in oxygen electrode experiments, using 2-thio-FAD enzyme containing only 0.3% residual FAD. The addition of catalase to the reaction mixture after exhaustion of the limiting substrate (NADPH) in turnover restored 50% of the oxygen consumed in NADPH oxidation. 2-Thio-FAD p-hydroxybenzoate hydroxylase is thus similar to the 1-deaza-FAD enzyme in its failure to couple NADPH oxidation and substrate hydroxylation.

Steady State Kinetics—Initial velocity measurements of 2-thio-FAD enzyme activity were made first by varying each of the three substrates independently at fixed concentrations of...
the other substrates (19). However, variations in NADPH and p-hydroxybenzoate concentrations over the respective ranges of 14-140 μM and 16-160 μM at 0.26 mM O₂ did not give significant changes in initial velocity. This observation implied that the Kₘ values for NADPH and p-hydroxybenzoate were considerably lower than for the native enzyme (19).

At fixed concentrations of NADPH and p-hydroxybenzoate, an unusual initial velocity dependence was observed with varying O₂ concentration. As the concentration of O₂ was increased from 0.13-0.74 mM, the rate of oxidation of NADPH was found to be directly proportional to the O₂ concentration. This steady state kinetic behavior is consistent with a ternary complex mechanism for the 2-thio-FAD enzyme-substrate complex. In the absence of p-hydroxybenzoate, NADPH reduction of the 2-thio-FAD enzyme occurs at a limiting rate of 0.05 s⁻¹ (results not shown); the binding of substrate thus increases the rate of enzyme reduction by 7000-fold.

In order to investigate the steady state behavior of the 2-thio-FAD enzyme at low concentrations of NADPH and p-hydroxybenzoate, the enzyme monitored turnover method first described by Chance (27) was employed. This technique allows the determination of turnover numbers as the limiting substrate concentration approaches zero. The stopped flow traces shown in Fig. 1 represent the change in enzyme absorbance at 500 nm as oxidized 2-thio-FAD enzyme is mixed with 100 μM NADPH in the presence of excess p-hydroxybenzoate and O₂. The rapid reduction of the enzyme is followed by gradual reoxidation; the latter process is seen to accelerate as NADPH is exhausted. The data were analyzed according to Gibson et al. (22) to generate a series of reciprocal plots (turnover number versus NADPH concentration) at several fixed levels of p-hydroxybenzoate.

An interesting pattern of reciprocal plots over the p-hydroxybenzoate concentration range 10-30 μM was found, consistent with a ternary complex mechanism for the 2-thio-FAD enzyme involving NADPH and p-hydroxybenzoate, as found for the native enzyme (19). However, at higher concentrations of p-hydroxybenzoate, the double reciprocal plots became parallel, and secondary plots of intercept versus reciprocal p-hydroxybenzoate concentration curve downward at higher concentrations (0.1-1 mM), indicating a possible secondary interaction of this substrate with the enzyme. While this activating effect of p-hydroxybenzoate at high concentrations was not further explored, it should be recalled that high substrate concentrations inhibit the native enzyme (28).

Reductive Half-reaction—The slower rate of reduction of the 1-deaza-FAD enzyme by NADPH was attributed to the lower oxidation-reduction potential of -280 mV measured for 1-deazariboflavin, compared to the potential of -208 mV for riboflavin (17). The higher oxidation-reduction potential of -126 mV measured for 2-thioriboflavin (12), therefore, might predict a faster rate of NADPH reduction with the 2-thio-FAD enzyme. This prediction was confirmed in stopped flow studies of the reductive half-reaction of this enzyme.

The reduction of the 2-thio-FAD enzyme by NADPH in the presence of 1.2 mM p-hydroxybenzoate is shown in Fig. 2A. Traces at all NADPH concentrations used were markedly biphasic; subtraction of the slow [NADPH]-independent phase (kslow = 1.6 s⁻¹) results in an [NADPH]-dependent pseudo-first order trace. The double reciprocal plot of Fig. 2B indicates a rapid association of NADPH with the 2-thio-FAD enzyme-substrate complex (Kₐ = 0.16 mM) followed by flavin reduction. The first order rate constant of 324 s⁻¹ obtained from the intercept can be compared with the value of 46 s⁻¹ reported for the native enzyme-p-hydroxybenzoate complex at pH 6.6 (19).

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The slow [NADPH]-independent phase was observed over the wavelength range 450-650 nm, but not beyond 600 nm. The spectral properties of this species and the concentration-independent nature of its decay are consistent with a charge-transfer intermediate of the reduced 2-thio-FAD enzyme-p-hydroxybenzoate complex and oxidized pyridine nucleotide (NADP⁺). The shorter wavelength maximum for such an intermediate with the 2-thio-FAD enzyme would be predicted from the higher oxidation-reduction potential of the 2-thioflavin (29). The corresponding charge-transfer intermediate with the native FAD enzyme is observed over the range 690-750 nm, and has been documented in both reductive half-reaction (19, 28) and catalytic turnover experiments (28). It should be noted that a spectral intermediate similar to that seen in the reduction of 2-thio-FAD p-hydroxybenzoate hydroxylase has been observed in both rapid kinetic and static experiments with 2-thio-FMN lactate oxidase; the charge-transfer acceptor in this case is the bound pyruvate molecule.

Oxygen Reactivity of the Modified Enzyme—The results of stopped flow kinetic experiments in which dithionite-reduced 2-thio-FAD enzyme was reacted with oxygen in the absence and presence of 1.2 mM p-hydroxybenzoate are shown in Fig. 3.
2-Thio-FAD p-Hydroxybenzoate Hydroxylase

Fig. 3. The relationship between oxygen concentration and the rate of reduced 2-thio-FAD enzyme oxidation in the absence (A) and presence (B) of substrate. Dithionite-reduced 2-thio-FAD enzyme (5.4-9 μM) was reacted with oxygen in the stopped flow spectrophotometer as described under "Experimental Procedures" in 50 mM phosphate buffer, pH 7.0, with 0.15-0.3 mM EDTA at 4 °C, in the absence and presence of 1.2 mM p-hydroxybenzoate. Pseudo first order rates were measured at 500 nm; the slopes of lines A and B give respective second order rate constants of 733 and 2100 M⁻¹ s⁻¹.

3. The respective second order rate constants, taken at 4 °C, of 733 and 2100 M⁻¹ s⁻¹ show that the oxygen reactivity of the 2-thio-FAD enzyme is much slower than with native enzyme. Comparable values for the native FAD enzyme, taken at pH 6.6 and 4 °C (14), are 2.6 × 10⁶ and 2.6 × 10⁵ M⁻¹ s⁻¹ respectively; the reduced 2-thio-FAD enzyme reacts 120-fold more slowly with oxygen than does the native enzyme in the presence of substrate. Despite the poor oxygen reactivity, the presence of substrate does accelerate the oxidation rate to a modest extent (3-fold) with the 2-thio-FAD enzyme; the size of this effect with native enzyme is 10-fold (14).

Although the 1-deaza-FAD enzyme does not hydroxylate substrate, the appearance of a 1-deaza-FAD-C(4a)-hydroperoxide intermediate has been documented (17) in stopped flow studies of the oxidation of reduced 1-deaza-FAD enzyme in complex with p-hydroxybenzoate, p-aminobenzoate, and the effector 6-hydroxynicotinate. This peroxylavin, however, is unable to carry out oxygenation of substrates, instead breaking down to yield the oxidized 1-deaza-FAD enzyme and hydrogen peroxide.

Stopped flow studies of the reduced 2-thio-FAD enzyme in the presence of p-hydroxybenzoate, on reoxidation with 0.65-1 mM O₂ at 4 °C, were made in order to establish whether this modified enzyme also formed a C(4a)-peroxylavin intermediate. At most wavelengths, the return to the absorbance of the oxidized 2-thio-FAD enzyme-substrate complex was monophasic (kₐ = 1.4 to 1.6 s⁻¹ at 0.65 mM O₂). At other wavelengths, notably in the region below 390 nm, the reoxidation was not monophasic, indicating the formation of intermediate species. The failure to observe a clear 2-thio-FAD-C(4a)-peroxylavin intermediate under these conditions may well be a consequence of the poor oxygen reactivity of the reduced 2-thio-FAD enzyme, so that the formation of the hydroperoxide is slower than its decay. This is not an unreasonable possibility, since decay rates with native and 1-deaza-FAD enzymes occur in the range 4.6-10.7 s⁻¹ at 2-5 °C (14, 17).

Previous studies of the oxygen reactivity of the native enzyme (14) had shown that the kinetic resolution of intermediates could be greatly improved by the addition of 0.1 M azide to reaction mixtures. For example, the oxidation of reduced native enzyme at pH 6.6 and 4 °C in the absence of substrate was found to proceed (14) via a kinetically invisible intermediate in the absence of azide, due to the rate of decay of the flavin hydroperoxide being faster than the rate of its formation:

$$\text{EF}_{\text{red}} + \text{O}_2 \rightarrow \text{EFHOOH} \rightarrow \text{EF}_{\text{ox}} + \text{H}_2\text{O}_2$$

However, in the presence of 0.1 M azide, the course of reoxidation with the native enzyme at appropriate wavelengths was markedly biphasic (14), demonstrating the transient formation of an intermediate between reduced and oxidized enzyme. Determination of the spectrum of this intermediate allowed its identification as the C(4a)-hydroperoxide. Azide...
was found to have no effect on the [oxygen]-dependent rate \( k_1 \), but dramatically decreased \( k_1 \) to the level where the intermediate could be observed. Therefore, we undertook a stopped flow study of the oxidation of the 2-thio-FAD enzyme-substrate complex in the presence of 9.1 M potassium azide and 1 mM \( O_2 \).

A clearly biphasic course of oxidation with the reduced 2-thio-FAD enzyme-substrate complex was observed under these conditions. At wavelengths in the range 340–370 nm, there was a rapid increase in absorbance followed by a slower return to that of the oxidized enzyme-substrate complex. The absorbance change was greatest at 355–360 nm. By following the reaction at appropriate isobestic wavelengths (see below), it was established that the rapid increase in absorbance was [oxygen]-dependent (\( k_{abs} = 4.1 \text{s}^{-1} \) at 1 mM \( O_2 \)), while the slower decay rate was [oxygen]-independent (\( k_{abs} = 1.5 \text{s}^{-1} \)).

At wavelengths beyond 480 and below 340 nm, the kinetics of reoxidation consisted of a single monophasic reaction trace at the [oxygen]-independent rate of 1.3–1.6 s\(^{-1}\). These wavelengths were thus isobestic for the formation of the intermediate. The reaction at 375 nm consisted of a monophasic increase in absorbance at the [oxygen]-dependent rate of 4.1 s\(^{-1}\), and thus was isobestic for the decay of the intermediate to oxidized enzyme. Representative reaction traces at 360 and 500 nm are given in Fig. 4.

By using the methods previously described (14) and modified as under “Experimental Procedures,” knowledge of the rate constants for the reaction scheme

\[
A + B \rightarrow k_1 \rightarrow C \rightarrow k_2 \rightarrow D
\]

allowed the calculation of the absorbance spectrum of the transient intermediate, as shown in Fig. 5. This spectrum is similar to that of the covalent C(4a) adducts of 2-thio-FMN lactate oxidase with \( \beta \)-bromopropionate and \( \alpha \)-hydroxybutyrate (9).

**DISCUSSION**

The 2-thio-FAD reconstituted \( p \)-hydroxybenzoate hydroxylase catalyzes the \( p \)-hydroxybenzoate-stimulated oxidation of NADPH by a mechanism similar to that observed for both the 1-deaza-FAD enzyme (17) and for the native enzyme in the presence of nonhydroxylatable effectors (14, 28). This mechanism is summarized in Scheme 1.

\[
\begin{align*}
EFl_{ox} + PHB & \xrightarrow{k_1} EFl_{ox}-PHB \\
EFl_{ox}-PHB + NADPH & \xrightarrow{k_2} EFl_{ox}-PHB + NADP^+ \\
EFl_{ox}-PHB + O_2 & \xrightarrow{k_3} EFl_{ox}-PHB + H_2O_2 \\
\end{align*}
\]

**Scheme 1**

The kinetic constants determined by steady state and stopped flow methods for the 2-thio-FAD enzyme and for the native enzyme (Table I) suggest that the low oxygen reactivity observed with the modified enzyme is responsible for the unusual \( O_2 \) dependence seen in steady state studies. This kinetic behavior precludes the determination of accurate values for \( V_{max} \), \( K_{mPHB} \), \( K_{mNADPH} \), as well as \( K_{mO_2} \). However, we can estimate (30) that the \( K_{mO_2} \) value for the 2-thio-FAD enzyme must be at least 10 mm or higher. Similar steady state oxygen dependencies were noted previously in studies of the 7,8-dichloro-FAD D-amino acid oxidase (4) and the iso-FMN lactate oxidase (5). In both cases, the reactions of \( O_2 \) with the respective reduced enzyme-imino acid or enzyme-pyruvate complexes were much slower than with the native enzymes. The low oxygen reactivity of 2-thio-FAD \( p \)-hydroxybenzoate hydroxylase is not due to an inherent poor oxygen reaction of the 2-thioflavin, since the reaction of \( O_2 \) with reduced 2-thio-FMN lactate oxidase is rapid.

There does not appear to be any correlation between the oxidation-reduction potentials of the free flavins and the rates of oxidation of \( p \)-hydroxybenzoate hydroxylase (as the enzyme-substrate complex) containing 1-deaza-, native, or 2-thio-FAD. The data of Table I show that the 1-deaza-FAD enzyme reacts with \( O_2 \) with a second order rate constant less than 30% that of the native enzyme under identical conditions. The 2-thio-FAD enzyme at pH 7.0 and 4 °C reacts more slowly than either the 1-deaza-FAD enzyme or the native enzyme.

On the other hand, a reasonable correlation appears to exist between the limiting rate of two-electron NADPH reduction of the coenzymes bound to \( p \)-hydroxybenzoate hydroxylase and the oxidation-reduction potentials of the free flavins (Table I). A plot of log \( k_0 \) versus \( E^0 \) (for the free riboflavin derivative) for these three enzyme forms is linear with a slope of \( 1.13 \times 10^{-2} \text{ mV}^{-1} \), i.e. a 10-fold increase in rate constant results from an 88 mV increase in flavin potential. This observation also suggests that these flavin potentials are not differentially affected on binding to the apoenzyme.

The difficulty encountered in detecting a clear oxygenated flavin intermediate with the 2-thio-FAD enzyme in the absence of azide can be attributed to its poor oxygen reactivity; the 1-deaza-FAD enzyme reacted 30-fold faster with oxygen to yield a well resolved intermediate in the absence of azide. The similarity between the absorption spectrum calculated for the transient intermediate in oxidation of the 2-thio-FAD enzyme (Fig. 5) and the C(4a) adduct of 2-thio-FMN lactate

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Table I

| Kinetic constants for 2-thio-FAD and native \( p \)-hydroxybenzoate hydroxylase |
|------------------------------------------|
| 2-thio-FAD enzyme | Native enzyme |
| \( V_{max} \) | Indeterminate | 550 m\( \text{s}^{-1} \) |
| \( K_{mPHB} \) | Indeterminate | 5.5 \times 10^{-3} \text{ M} |
| \( K_{mNADPH} \) | Indeterminate | 2.1 \times 10^{-5} \text{ M} |
| \( K_{mO_2} \) | Large but indeterminate | 3.1 \times 10^{-3} \text{ M} |

\( k_0/k_1 \) | 1.3 \times 10^{-4} \text{ M} | 4.2 \times 10^{-3} \text{ M} |

\( k_0/k_2 \) | 1.6 \times 10^{-4} \text{ M} | 1.8 \times 10^{-3} \text{ M} |

\( k_3 \) | 324 s\(^{-1}\) | 46–50 s\(^{-1}\) |

\( k_5 \) | 1.6 s\(^{-1}\) | 46 s\(^{-1}\) |

\( k_7 \) | 2.1 \times 10^{-4} \text{ M} \(-1\text{s}^{-1}\) | 2.6 \times 10^{-4} \text{ M} \(-1\text{s}^{-1}\) |

\( k_9 \) | 0.07 s\(^{-1}\) | 4 s\(^{-1}\) |

\( k_6 \) | 7.4 s\(^{-1}\) | 10.7 s\(^{-1}\) |

\( k_{7'} \) | 1.3 \times 10^{-4} \text{ M} | 2.55 \times 10^{-4} \text{ M} \(-1\text{s}^{-1}\) |

\( k_{7''} \) | 0.05 s\(^{-1}\) | 3.3 \times 10^{-4} \text{ s}^{-1} |

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*All data for the 2-thio-FAD enzyme were obtained in 50 mM potassium phosphate, pH 7.0, plus 0.3 mM EDTA at 4 °C.*

*All values for the native enzyme are from Refs. 14 and 19, and were obtained at pH 6.5–6.6, 3.5–5 °C.*

*The value for \( k_0 \) is taken from Ref. 14, and was determined in the presence of the nonhydroxylatable effector 6-hydroxynicotinate.*

*Rate constants \( k_7 \) and \( k_7' \) refer to the rates of oxidation and reduction, respectively, of the enzyme in the absence of \( p \)-hydroxybenzoate.*

*The abbreviation used is: PHB, \( p \)-hydroxybenzoate.*


The formation of C(4a)-peroxyflavin intermediates in the oxygen reactions of native, 1-deaza-FAD, and 2-thio-FAD p-hydroxybenzoate hydroxylase represents a common first step in their respective oxidative mechanisms. However, neither the 2-thio-FAD nor 1-deaza-FAD enzymes carry out substrate hydroxylation. While our results with the 2-thio-FAD p-hydroxybenzoate hydroxylase do not serve to distinguish between the carbonyl oxide mechanism of Hamilton (32), or the alternative ring opening suggested by Entsch et al. (14), and the model suggested by Bruice (31) involving inductive polarization of the peroxy moiety during oxygen transfer, we can suggest that slower rates of substrate oxidation should be observed for the synthetic N(5)-alkyl-2-thioflavin-C(4a)-hydroperoxide as compared to the normal peroxyflavin. Further studies of p-hydroxybenzoate hydroxylase reconstituted with suitably modified flavin should allow closer examination of the influence of electronegative character on the course of peroxyflavin decay.

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