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STUDIES ON FLAVIN BINDING IN FLAVODOXINS

STEPHEN G. MAYHEW
Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.)

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SUMMARY

1. Stable apoproteins have been prepared from *Peptostreptococcus elsdenii*, *C. pasteurianum* and Clostridium MP flavodoxins by dialysis of the native proteins against 2 M KBr at pH 3.9 and $3 \cdot 10^{-4}$ M EDTA. The apoproteins each bind 1 molecule of FMN to give complexes identical with the native flavodoxins.

2. Binding causes almost complete quenching of both protein and FMN fluorescence. This property has been used to determine the dissociation constant for the dissociation of *P. elsdenii* flavodoxin into apoprotein and FMN, and to follow the kinetics of the interaction. The dissociation constant determined at pH 6.55 and 20° was $4.26 \cdot 10^{-10}$ M. The maximum rate of binding occurs at pH 4.4 and is described by a second order rate constant of $2.6 \cdot 10^{5}$ $M^{-1} \cdot cm^{-1}$ at 0.5°. The rate is markedly influenced by the salt composition of the solution.

3. Addition of excess apoprotein to FAD, riboflavin or lumiflavin causes only a small decrease in the flavin fluorescence, suggesting that binding of these compounds is weak.

4. Two derivatives of FMN, iso-FMN and 3,4-dihydro FMN form strong, complexes with apoflavodoxin. In the case of iso-FMN and *P. elsdenii* apoflavodoxin the complex is catalytically active, and it is reduced by systems which reduce native flavodoxin. The complex of 3,4-dihydro FMN is catalytically inactive.

5. *C. pasteurianum*, *P. elsdenii* and Clostridium MP flavodoxins contain 1, 2 and 3 sulfhydryl groups, respectively. Experiments with *p*-chloromercuribenzoate and *N*-ethylmaleimide indicate that at least one sulfhydryl group is important for flavin binding.

INTRODUCTION

During the last four years, small flavoproteins termed flavodoxins, have been isolated from several strictly anaerobic bacteria, and shown to function as electron carriers in low potential oxidation–reduction reactions[1–7]. In these reactions, flavodoxins are often interchangeable with the iron–sulfur protein ferredoxin. Flavodoxins

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

have molecular weights of about 15 000, and they contain one molecule of FMN. They give a stabilized neutral flavin semiquinone at half reduction, and studies on the oxidation–reduction properties of three flavodoxins have shown that at pH 7 the two 1-electron oxidation–reduction steps are separated by a potential of at least 0.26 V (refs. 6, 7).

Although apoproteins have been prepared from a number of flavoproteins and in some cases extensively studied (*e.g.* refs. 8–16) the interactions between FMN or FAD and specific apoproteins are not completely understood. Flavodoxins are small proteins and thus they are very suitable for studying such interactions. Flavin binding by these proteins is probably similar to flavin binding in much larger flavoproteins, especially those flavoproteins which like flavodoxin give a stabilized neutral flavin semiquinone[17]. In this respect flavodoxins may serve as useful models for the larger molecules.

This paper describes a procedure for the reversible dissociation of flavodoxins from *Peptostreptococcus elsdenii*[5,6], *Clostridium pasteurianum*[1,2] and Clostridium MP[7] into their respective apoproteins and FMN. It also reports studies on the interaction of flavin with apoflavodoxins. Most of these studies were done with apoprotein prepared from *P. elsdenii* flavodoxin. In some experiments, this apoprotein is compared directly with apoproteins made from *C. pasteurianum* and Clostridium MP flavodoxins. Preliminary accounts of some of these findings have been presented[18,19].

MATERIALS AND METHODS

Preparation of flavodoxins and apoflavodoxins

Flavodoxins from *P. elsdenii*, *C. pasteurianum* and Clostridium MP were prepared from cells grown in iron-poor media ss described previously[5,7].

For the preparation of apoflavodoxin, flavodoxin (approx. 10 mg/ml) in 0.5–3.0 ml was dialyzed for 48 h against four changes of 250 ml of 2 M KBr in 0.1 M sodium acetate buffer (pH 3.9) containing $3 \cdot 10^{-4}$ M EDTA. Apoflavodoxin formed as a white precipitate during this treatment. The dialysis medium was then changed to 0.1 M sodium phosphate buffer (pH 7) containing $3 \cdot 10^{-4}$ M EDTA, to remove KBr and to dissolve the precipitate; this buffer was changed several times during 36 h.

Purification of FMN and other flavin compounds

Commercial preparations of FMN (from Sigma Chemical Co.) contain up to 20% of contaminants fluorescing at about 530 nm. Some of these contaminants can be removed by treatment with DEAE-cellulose, as described by MASSEY AND SWOBODA[20]. However, preparations purified in this way still contain about 15% contamination, as judged by the residual fluorescence after mixing with an excess of apoflavodoxin (flavodoxins have less than 1% of the fluorescence of FMN). Attempts to purify FMN further by treatment on Dowex resin as described by THEORELL AND NYGAARD[9] were not successful. Our best preparations were made by hydrolysis of FAD (Sigma) with snake venom phosphodiesterase (EC 3.1.4.1) (*Naja naja* venom). Approx. 25 mg of FAD in 1 ml 0.1 M sodium phosphate buffer (pH 7.3) was treated in darkness for 12 h at 20° with approx. 0.5 mg of venom. FMN was then separated from other reaction products by chromatography in darkness on a column of DEAE-

cellulose (25 cm × 2 cm) with 0.1 M potassium phosphate buffer (pH 6.8) as eluting agent[20]. Preparations of FMN made by this method showed between 5 and 7% residual fluorescence after treatment with an excess of apoflavodoxin.

FMN was also prepared by extraction from pig liver glycolate oxidase (EC 1.1.3.1) (provided by Dr. M. Schuman) and yeast NADPH dehydrogenase (EC 1.6.99.1) (provided by Dr. R. G. Matthews). Cold 5% trichloroacetic acid was used to precipitate protein as described previously for flavodoxin[5]. The trichloroacetic acid extracts were neutralized with K_2HPO_4. Solutions of FMN were stored at −20° until used.

FAD (from Sigma) and iso-FMN (provided by Dr. P. Hemmerich) were purified with DEAE-cellulose before use[20]. Riboflavin (from Sigma) was used without further purification. An extinction coefficient of 12 500 $M^{-1} \cdot cm^{-1}$ at 445 nm was used for FMN and riboflavin[21]; the value used for FAD was 11 300 $M^{-1} \cdot cm^{-1}$ at 450 nm.

3,4-Dihydro FMN was made from L-amino acid oxidase (EC 1.4.3.2) as described by MASSEY *et al.*[22]. Approx. $3 \cdot 10^{-5}$ M *Crotalus adamanteus* L-amino acid oxidase[23] in 1 ml 0.008 M sodium phosphate buffer (pH 7) was treated at 10° with approx. 2 mg $NaBH_4$. After 1 h the solution was heated for 5 min at 90° in a water bath. The solution was centrifuged (23 500 × *g* for 10 min) to remove denatured protein, and then treated at 20° with approx. 0.5 mg *Naja naja* venom to hydrolyze 3,4-dihydro FAD to 3,4-dihydro FMN. The reaction was followed to completion by measuring the increase in fluorescence at 480 nm. The solution was finally heated as above and centrifuged to remove denatured venom protein.

Anaerobic spectrophotometric titrations and fluorescence measurements

Anaerobic titrations with $Na_2S_2O_4$ were performed with the apparatus of FOUST *et al.*[24], using a modified procedure as described previously[7]. Fluorescence measurements were made either with an Aminco Bowman fluorimeter or with a ratio recording fluorimeter[25]. Both instruments were equipped for thermostatic control.

RESULTS

Removal of FMN from flavodoxins; absorption spectrum of apoflavodoxin

Although flavin can be dissociated from flavodoxins with trichloroacetic acid[5,7], some loss of protein occurs by this method. A less drastic procedure for the reversible removal of FMN which gives high yields of apoprotein, has been developed based on a recently described method for the preparation of apoprotein from D-amino acid oxidase (EC 1.4.3.3)[12]. Flavodoxin is dialyzed against a high concentration of KBr at pH 3.9 as described in MATERIALS AND METHODS. The optimum pH for flavin removal has not been determined. However, it was found that at pH 4.5 and above, loss of flavin from *P. elsdenii* flavodoxin is very much slower than at pH 3.9. In order to obtain maximum yields of apoprotein with FMN-binding activity, it is essential to include EDTA in all dialysis media. In the absence of EDTA, a variable amount of inactivation occurs. This inactivation is probably due to the oxidation of protein sulfhydryl groups to disulfides, since FMN-binding activity is restored by treating inactive apoprotein with dithiothreitol, and further, it is known that traces of heavy metals catalyze the oxidation of protein sulfhydryl groups and that this reaction can be prevented with EDTA[25].

With this procedure, yields of active apoprotein from flavodoxin are between 95 and 100%. Preparations are stable at 0°, but some inactivation occurs at −20°. This inactivation is not reversible by dithiothreitol. Freshly made preparations of apoprotein were used for the experiments described in this paper.

The absorption spectra of the apoproteins from *P. elsdenii* and Clostridium MP flavodoxins have a maximum at 278 nm and shoulders at 288 and 275 nm. The extinction coefficients at 278 nm are 26 700 $M^{-1} \cdot cm^{-1}$ and 25 000 $M^{-1} \cdot cm^{-1}$ for *P. elsdenii* and Clostridium MP flavodoxins, respectively. The absorption maximum in the spectrum of apoflavodoxin from *C. pasteurianum* is shifted to a somewhat longer wavelength (282 nm). The shoulders on this peak are 290 nm and 278 nm (Fig. 1). The extinction coefficient for *C. pasteurianum* apoflavodoxin at 282 nm is 25 200 $M^{-1} \cdot cm^{-1}$ (protein concentrations were determined according to LOWRY *et al.*[29] with native flavodoxin as standard). The determined extinction coefficients are very similar to the extinction coefficients calculated by subtraction of the absorption of FMN from the absorption of the native flavodoxins. They suggest that the absorption of FMN in this region of the spectrum is unchanged when FMN is bound to the apoproteins.

Binding of flavin by apoflavodoxins

In common with many other flavoproteins the visible absorption spectra of flavodoxins show marked differences from that of free FMN. Flavodoxins have decreased absorption in the visible region, and in addition they have a shoulder at 465 nm which is not present in FMN. Again like many other flavoproteins, flavodoxins have very low flavin fluorescence (less than 1% of free FMN). Binding of FMN to the apoflavodoxins can therefore be followed by measuring either the decrease in absorption at 445 nm or the decrease in flavin fluorescence. Fig. 1 shows the effect of *C. pasteurianum* apoflavodoxin on the visible absorption spectrum of FMN. The spectra of reconstituted and native flavodoxins are identical (native and reconstituted flavodoxins are also identical in their catalytic and oxidation–reduction properties).

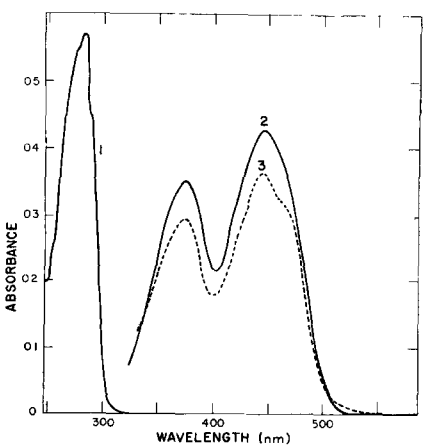


Fig. 1. Absorption spectra of *C. pasteurianum* apoflavodoxin, FMN, and reconstituted flavodoxin. Curve 1, $2.26 \cdot 10^{-5}$ M apoflavodoxin; Curve 2, $3.4 \cdot 10^{-5}$ M FMN; Curve 3, $3.4 \cdot 10^{-5}$ M FMN *plus* $4.1 \cdot 10^{-5}$ M apoflavodoxin. Samples were dissolved in 0.03 M sodium phosphate (pH 7).

When apoprotein was titrated into FMN, the flavin fluorescence decreased linearly and reached a minimum after the addition of 1 mole of apoprotein per mole of FMN. The residual fluorescence in the presence of an excess of apoprotein was between 5 and 7%. Most of this residual fluorescence was probably due to impurity in the FMN, because when FMN prepared by neutralizing a trichloroacetic acid extract from flavodoxin was treated with an excess of apoprotein, the residual fluorescence was only 2.5%. Similarly, when excess apoflavodoxin was added to FMN extracted from the FMN-proteins old yellow enzyme and glycolic acid oxidase, the residual fluorescence was 1.4 and 3.6%, respectively. Furthermore, fluorescent material which is not bound by the apoproteins can be separated either by dialysis or by gel filtration on a column of Sephadex G-25.

Native flavodoxins show very low protein fluorescence. Apoflavodoxins are very much more fluorescent and show a fluorescence excitation maximum at 282 nm and a single fluorescence emission maximum at 345 nm. The fluorescence intensity of *P. elsdenii* apoflavodoxin is equivalent to 90% of the fluorescence of the tryptophan content. This flavodoxin contains four residues of tryptophan and two residues each

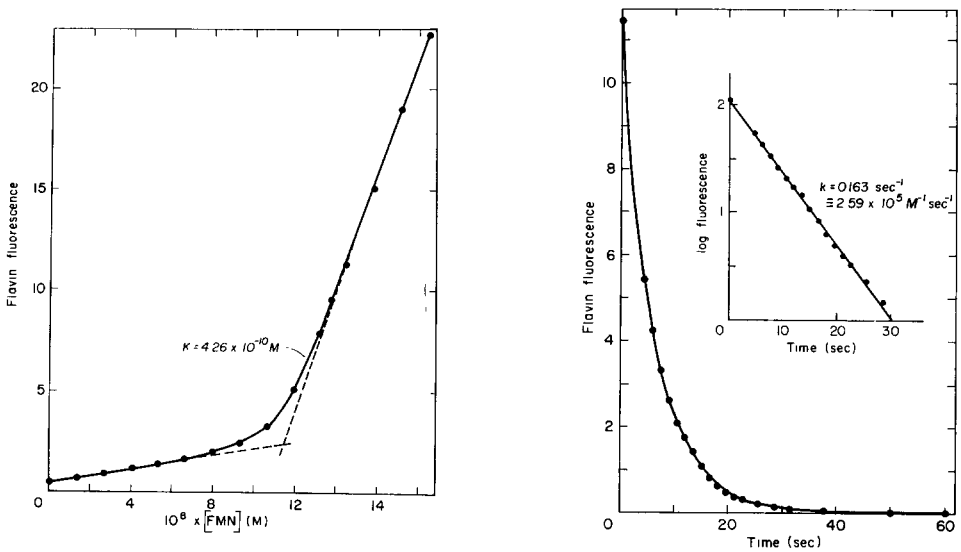


Fig. 2. Determination of the dissociation constant for the dissociation of *P. elsdenii* flavodoxin. Apoprotein ($1.14 \cdot 10^{-7}$ M) in 2 ml 0.025 M sodium acetate (pH 6.55) was titrated with $9.35 \cdot 10^{-6}$ M FMN. Measurements were made with an Aminco Bowman fluorimeter thermostated at 20°. The flavin fluorescence, in arbitrary units, is shown plotted against the FMN concentration. Excitation was at 450 nm, and emission was recorded at 530 nm. After each addition of FMN, the fluorescence was followed with time until a stable value was reached. A value for the dissociation constant of flavodoxin was calculated from the four points in the titration curve which represent the sixth to the ninth additions of FMN. The average value was $4.26 \cdot 10^{-10}$ M. The theoretical curve for this dissociation constant is shown by the solid line drawn through the experimental points.

Fig. 3. Determination of the rate constant for binding of FMN to *P. elsdenii* apoflavodoxin. Apoprotein to give a final concentration of $6.3 \cdot 10^{-7}$ M was added to $9.3 \cdot 10^{-8}$ M FMN in 2 ml 0.01 M sodium acetate (pH 4.4) and the quenching of flavin fluorescence was followed with time. Measurements were made with a ratio recording fluorimeter thermostated at 0.5°. The observed fluorescence, in arbitrary units, has been corrected for the residual fluorescence (7%) remaining at the end of the reaction. The inset shows the first order analysis of the experimental curve. A value for the pseudo first order rate constant (K) was calculated from the slope of the line relating log fluorescence to time.

of tyrosine and phenylalanine[5]. Binding of FMN to apoflavodoxins quenches the protein fluorescence to about 1.5% of the initial value.

A value for the dissociation constant of *P. elsdenii* flavodoxin into apoprotein and free flavin was determined by fluorimetric titration of a known concentration of apoprotein with FMN (Fig. 2). The slope of the line in the first part of the titration was due mainly to contamination in the FMN used (all calculations have been corrected for the contamination which was estimated to be 6% in this experiment). Several points lie off the two linear parts of the curve in Fig. 2. The concentrations of free FMN, bound FMN and apoprotein were determined at each of these points and values for the dissociation constant calculated. The average value was $4.26 \cdot 10^{-10}$ M under the conditions used. The theoretical curve for this dissociation constant is shown by the solid line in Fig. 2. Approximate values for the dissociation constants of flavodoxin semiquinone and fully reduced flavodoxin were calculated from the determined value for oxidized flavodoxin, the previously determined oxidation–reduction potentials for this flavodoxin[6] and published data on the oxidation–reduction potentials of FMN[30]. Values of $3.5 \cdot 10^{-12}$ M and $8.8 \cdot 10^{-11}$ M were calculated for flavodoxin semiquinone and fully reduced flavodoxin, respectively. These values are approximate since the various measurements were made under somewhat different conditions. However, they do indicate that oxidized, semiquinone and fully reduced FMN are all bound very tightly to this apoprotein.

The optimum conditions for binding of FMN to the apoprotein from *P. elsdenii* flavodoxin and the kinetics of the reaction were investigated by fluorimetry. Two kinds of experiment were done to determine the rate constants for flavin binding. First, an excess of apoprotein was added to a known concentration of FMN and the quenching of flavin fluorescence followed with time (Fig. 3). Under these conditions the reaction followed first order kinetics and had a pseudo first order rate constant of 0.163 sec^{-1} at pH 4.4 and 0.5°. Since the apoprotein concentration was $6.3 \cdot 10^{-7}$ M in this experiment, a second order rate constant of $2.59 \cdot 10^{-5}$ M sec^{-1} can be calculated for the reaction. Second, apoprotein was mixed with a small excess of FMN and the progress curve for the quenching of flavin fluorescence analyzed for second order kinetics. Rate constants calculated at $t_{1/4}$, $t_{1/2}$ and $t_{3/4}$ were similar. For example, the values at pH 4.4 and 0.5° were $2.67 \cdot 10^5$, $2.71 \cdot 10^5$ and $2.52 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ at $t_{1/4}$, $t_{1/2}$ and $t_{3/4}$, respectively.

The maximum rate of binding, determined in acetate buffer, was near pH 4.5. However, it was noted that the rate depends on the salt composition of the solution. For example, in sodium phosphate buffer at pH 7, the observed rate was maximal in 0.017 M phosphate; it decreased at lower and higher phosphate concentrations[19]. The rate was increased at all phosphate concentrations by addition of 0.5 M KCl. These salt effects were not extensively studied, but it was observed that the rates measured in 0.01 M sodium acetate (pH 6) and 0.01 M glycine–NaOH (pH 8.4) were also increased by addition of 0.5 M KCl. Similar effects of salt have been reported by THEORELL AND NYGAARD[9] for the binding of FMN to yeast old yellow enzyme. They found that flavin binding was inhibited by polyvalent anions and that the inhibition was reversed by monovalent anions.

The effect of temperature on the rate of binding of FMN to *P. elsdenii* apoflavodoxin was studied between 0.5° and 18° in 0.03 M sodium phosphate buffer

(pH 7). The data gave a linear Arrhenius plot and indicated an activation energy of 8.3 kcal/mole for the reaction.

In addition to binding FMN, apoflavodoxins form strong complexes with the FMN analogues, iso-FMN and 3,4-dihydro FMN (some properties of these derivatives of flavodoxin are described later in this paper). However, from the observed quenching of the flavin fluorescence in the presence of a large excess of the apoproteins it is concluded that FAD, riboflavin and lumiflavin are bound very weakly, with minimum values of 10^{-3} M for their dissociation constants. These flavins are readily dialyzed away from the protein thus excluding the possibility of strong binding without fluorescence quenching.

Effects of sulfhydryl reagents on flavin binding

Amino acid analyses have shown that flavodoxins from *C. pasteurianum*[2], *P. elsdenii*[5] and Clostridium MP[7] contain 1, 2 and 3 half-cystine residues, respectively. Spectrophotometric and amperometric titrations of *P. elsdenii* and Clostridium MP apoflavodoxins with mercurial reagents, showed the presence of 2 and 3 sulfhydryl groups, respectively (Table I), and indicated that there are no disulfide bonds in these proteins. KNIGHT AND HARDY[2] reported that the apoprotein prepared from *C. pasteurianum* flavodoxin by reaction with urea or sodium mersalyl contained 1 sulfhydryl group. This value has been confirmed by analyses of the apoprotein prepared by dialysis *versus* KBr (Table I).

Two lines of evidence suggest that the sulfhydryl groups in these flavodoxins

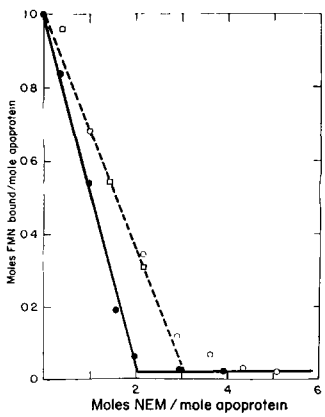
TABLE I

ANALYSES FOR SULFHYDRYL GROUPS IN APOFLAVODOXINS

Amperometric titrations were performed as described by ALLISON AND CECIL[26]. Reaction mixtures contained in 2 ml final volume, 0.1 M sodium phosphate (pH 7.6), $3 \cdot 10^{-4}$ M EDTA, 0.14 M KCl, octanol (10 μl to prevent frothing), and approximately $2 \cdot 10^{-5}$ M apoflavodoxin. Aliquots of $3 \cdot 10^{-3}$ M phenylmercuric acetate (3–4 μl) were added, allowing 5–10 min between each addition and reading. Spectrophotometric titrations were performed according to BOYER[27]. The sample compartment of a Cary spectrophotometer contained in a 1-ml cuvette, 0.1 M sodium phosphate (pH 7) and $1 \cdot 10^{-5}$ M PCMB, in a final volume of 0.75 ml. The reference cuvette contained 0.75 ml of 0.1 M sodium phosphate (pH 7). Aliquots (1 μl) of approx. $5 \cdot 10^{-4}$ M apoprotein were added to sample and reference cuvettes ,and the change in absorption at 250 nm was recorded. For the experiment with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), $2.26 \cdot 10^{-5}$ M apoflavodoxin was treated with $3.3 \cdot 10^{-4}$ M DTNB in 1 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3) at 25°. After 15 min the absorbance at 412 nm was determined *versus* a reagent blank[28].

Source of flavodoxin	*Method used*	*Sulfhydryl groups (moles/mole apoprotein)*
P. elsdenii	Amperometric titration	1.70
	Spectrophotometric titration	
	Expt. 1	1.85
	Expt. 2	2.10
Clostridium MP	Spectrophotometric titration	
	Expt. 1	2.74
	Expt. 2	3.00
C. pasteurianum	DTNB	1.00
	Spectrophotometric titration	1.04

are important in flavin binding. First, although mercurial reagents react very readily with the sulfhydryl groups of apoflavodoxins, they react only slowly with native flavodoxins, and during the reaction, FMN is displaced from the protein[7]. Second, when the apoproteins are treated with an excess of *p*-chloromercuribenzoate (PCMB)[19] or *N*-ethylmaleimide, flavin binding is completely inhibited. The inhibition of flavin binding by PCMB is completely reversible by dithiothreitol. Low concentrations of *N*-ethylmaleimide, which forms a thioether linkage with cysteine sulfhydryl groups, irreversibly inhibits flavin binding by apoflavodoxins. The stoichiometry of this inhibition was determined by treating apoprotein from *P. elsdenii* and Clostridium MP flavodoxins with different amounts of *N*-ethylmaleimide. The flavin-binding sites remaining after reaction were determined by fluorometric titration of FMN with *N*-ethylmaleimide-treated apoprotein. The amount of *N*-ethylmaleimide required to cause complete inhibition of flavin binding was 2 and 3 moles per mole of *P. elsdenii* and Clostridium MP apoflavodoxins respectively (Fig. 4).



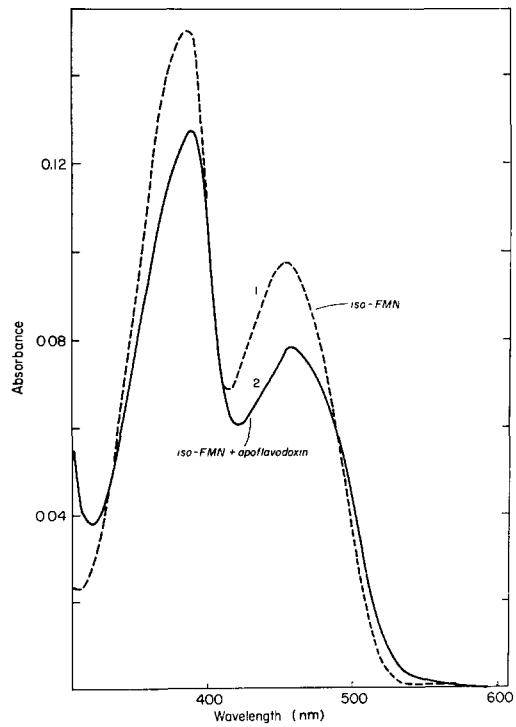


Fig. 4. Effect of *N*-ethylmaleimide on the binding of FMN by apoflavodoxins from Clostridium MP and *P. elsdenii*. Apoflavodoxin ($1 \cdot 10^{-4}$ M) was incubated for 1 h at 20° with *N*-ethylmaleimide in 0.01 M potassium phosphate (pH 6.8) and $5 \cdot 10^{-5}$ M EDTA. The concentration of flavin-binding sites was then determined fluorimetrically by titrating FMN with apoprotein. For the titrations, $1.26 \cdot 10^{-6}$ M FMN was dissolved in 2 ml 0.01 M sodium acetate (pH 6) containing 0.5 M NaCl. The moles of FMN bound per mole of apoprotein is shown plotted *versus* moles of *N*-ethylmaleimide (NEM) added per mole of apoprotein. ●, *P. elsdenii* apoflavodoxin, ○, □, Clostridium MP apoflavodoxin.

Fig. 5. Absorption spectra of iso-FMN and iso-FMN flavodoxin. Curve 1, $1.28 \cdot 10^{-5}$ M iso-FMN; Curve 2, $1.28 \cdot 10^{-5}$ M iso-FMN *plus* $1.45 \cdot 10^{-5}$ M *P. elsdenii* apoflavodoxin. Samples were dissolved in 0.1 M NaCl.

Properties of iso-FMN flavodoxin

As reported in an earlier section, apoflavodoxins bind the FMN analogue, iso-FMN, which has methyl groups at positions 6 and 7 in the isoalloxazine ring. This compound shows absorption maxima in the visible spectrum at 452 nm and 384 nm and a fluorescence emission maximum at 550 nm. Binding of iso-FMN to *P. elsdenii* apoflavodoxin causes a decrease in the visible absorption and a shift of the absorption maxima to 457 nm and 388 nm (Fig. 5). During binding, the flavin fluorescence is quenched to 4.7% of the initial value (the residual fluorescence observed may have been at least partly due to contaminants in the flavin sample used). A dissociation constant for the complex was not determined. However, binding is evidently tight since no flavin was lost either during extensive dialysis of iso-FMN flavodoxin against 0.01 M sodium phosphate (pH 7) or during fractionation of the complex with $(NH_4)_2SO_4$.

Extinction coefficients for iso-FMN flavodoxin were determined by fluorimetric titration of iso-FMN with *P. elsdenii* apoflavodoxin of known concentration, and by assuming that one molecule of iso-FMN is bound per molecule of protein. The extinction coefficients calculated were 5800 $M^{-1} \cdot cm^{-1}$ and 9500 $M^{-1} \cdot cm^{-1}$ at 457 nm and 388 nm, respectively.

Iso-FMN flavodoxin replaces native flavodoxin in the phosphoroclastic oxidation of pyruvate by ferredoxin-free extracts of *P. elsdenii*[5], although on a molar basis, iso-FMN flavodoxin was found to be only 31% as effective as flavodoxin at the highest concentration tested (3.3 nmoles)[19].

Iso-FMN flavodoxin is reduced by three systems previously shown to reduce native flavodoxin. These are light irradiation in the presence of EDTA, NADPH in the presence of a catalytic amount of spinach ferredoxin–$NADP^+$ reductase, and $Na_2S_2O_4$. In all cases, an intermediate is generated during reduction. This intermediate shows a free radical which can be detected by EPR (EPR measurements were made by Dr. G. Palmer). The absorption spectrum of the intermediate is similar to the blue semiquinone intermediate previously observed with native flavodoxin[5,6], although the long wavelength absorption maximum is shifted from 580 to 610 nm.

Fig. 6 shows a spectrophotometric titration of iso-FMN flavodoxin with $Na_2S_2O_4$. It can be seen that one mole of protein was reduced by 1 mole of $Na_2S_2O_4$; approx. 0.5 mole of $Na_2S_2O_4$ generated maximum semiquinone. The extinction coefficient of fully formed semiquinone at 610 nm, calculated by extrapolating the linear parts of the titration curve (in the inset to Fig. 6), was 2240 $M^{-1} \cdot cm^{-1}$. The observed intermediate at 50% reduction was 78.7% of the theoretical maximum indicating a semiquinone formation constant[6] of 54.7. This value was used to calculate the difference in oxidation–reduction potential between the couple, oxidized iso-FMN flavodoxin–iso-FMN flavodoxin semiquinone and the couple, iso-FMN flavodoxin semiquinone–fully reduced iso-FMN flavodoxin. The value found was 0.104 V at 25° and pH 6.3. This is lower than the value found previously for native *P. elsdenii* flavodoxin (0.294 V)[6] under similar conditions.

When solutions of fully reduced iso-FMN flavodoxin are aerated, semiquinone is formed very rapidly. The semiquinone, like the semiquinone of native flavodoxin, then decays in a pseudo first order reaction. This reaction is somewhat faster than the reaction of native flavodoxin semiquinone with oxygen, although in both cases the rate increases with increasing pH[6]. Pseudo first order rate constants for the

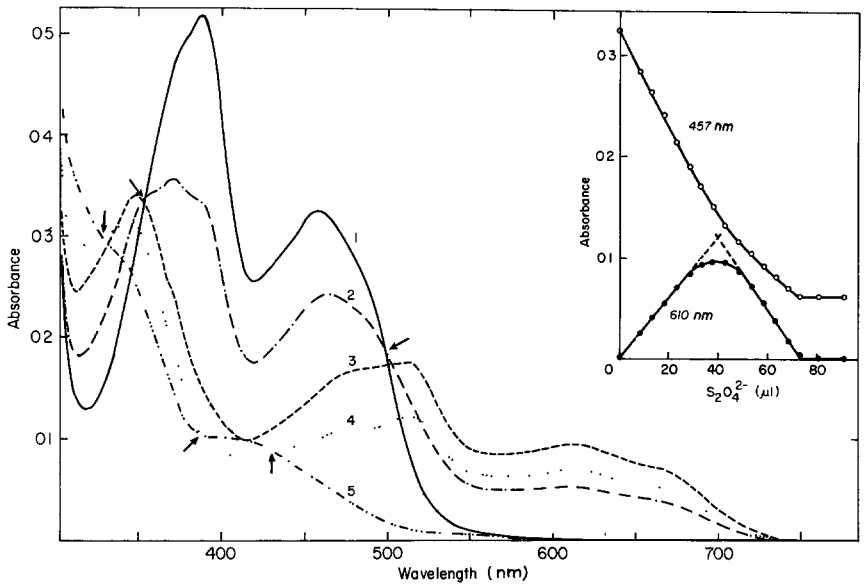


Fig. 6. Spectrophotometric titration of iso-FMN flavodoxin with $Na_2S_2O_4$. Iso-FMN flavodoxin, 0.116 μmole in 2 ml 0.15 M sodium phosphate (pH 6.3) was titrated anaerobically at 20° with $1.82 \cdot 10^{-3}$ M $Na_2S_2O_4$, dissolved in 0.01 M pyrophosphate buffer (pH 8.3). Arrows indicate isosbestic points Spectra are not corrected for dilution. Curve 1, untreated iso-FMN flavodoxin, Curves 2–5, after addition of 0.29, 0.6, 0.84 and 1.16 moles of $S_2O_4^{-2}$ per mole of iso-FMN flavodoxin. The inset shows plots of absorbance at 457 and 610 nm *versus* the $S_2O_4^{2-}$ added. The experimental points in this plot are corrected for dilution.

decay of iso-FMN flavodoxin semiquinone in air saturated solution at 20° were 0.29 and 0.53 min^{-1} at pH 5.5 and pH 6.3, respectively. Under similar conditions, the rate constants for the decay of *P. elsdenii* flavodoxin semiquinone were less than 0.01 min^{-1}.

Properties of 3,4-dihydro FMN flavodoxin

It was shown by MASSEY *et al.*[22] that the flavoproteins D- and L-amino acid oxidases react readily with $NaBH_4$ to form a stable product with an absorption maximum at 408 nm. More recent studies with model flavin compounds identified this product as 3,4-dihydro FAD[31]. This FAD derivative is released from the amino acid oxidases by heat denaturation of the apoprotein. Subsequent hydrolysis of the free flavin with snake venom phosphodiesterase gives the FMN analogue. This modified FMN is bound stoichiometrically to apoflavodoxin.

Fig. 7 shows the spectrum of 3,4-dihydro FMN, prepared from $NaBH_4$-treated L-amino acid oxidase, before and after the addition of excess *P. elsdenii* apoflavodoxin. It can be seen that bound 3,4-dihydro FMN shows decreased visible absorption, a shift of the main visible absorption maximum to 410 nm, and development of distinct peaks at 386 nm and 430 nm. Binding of 3,4-dihydro FMN to this apoflavodoxin was accompanied by 98% quenching of the fluorescence at 480 nm. No flavin was lost from the complex during 16 h dialysis against a large volume of 0.1 M sodium phosphate buffer (pH 7), indicating that tight binding had occurred.

This flavodoxin derivative showed between 10 and 20% of the catalytic activity

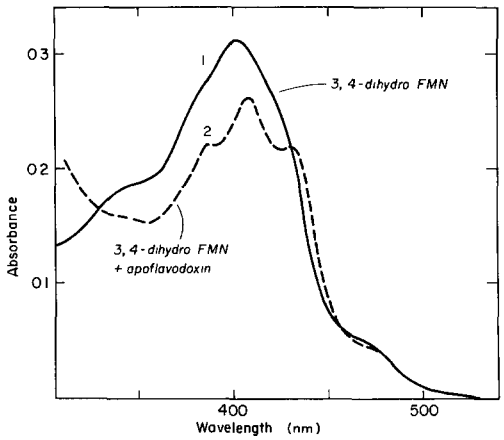


Fig. 7. Absorption spectra of 3,4-dihydro FMN and 3,4-dihydro FMN flavodoxin. Curve 1, $3 \cdot 10^{-5}$ M 3,4-dihydro FMN; Curve 2, $3 \cdot 10^{-5}$ M 3,4-dihydro FMN *plus* $4.7 \cdot 10^{-5}$ M apoflavodoxin. Samples were dissolved in 0.13 M sodium acetate (pH 6).

of native flavodoxin in the phosphoroclastic assay. However, reducing systems known to reduce flavodoxin caused only small changes in the spectrum of the 3,4-dihydro FMN derivative. Anaerobic treatment with EDTA and light irradiation, excess NADPH in the presence of ferrodoxin–$NADP^+$ reductase, or excess sodium dithionite, all caused only a small decrease in absorption at 410 nm and generation of low absorption between 500 and 650 nm characteristic of the semiquinone of native flavodoxin and equivalent to about 10% of the flavin concentration. These changes, and also the low catalytic activity observed, were probably due to unmodified FMN present in the sample of 3,4-dihydro FMN (3,4-dihydro flavin compounds are known to be somewhat unstable to light, which under aerobic conditions slowly converts them to normal flavoquinone)[31]. It is concluded that 3,4-dihydro FMN flavodoxin is catalytically inactive, and cannot be reduced by systems which reduce native flavodoxin. This resistance to reduction distinguishes the 3,4-dihydro FMN derivative of flavodoxin from free 3,4-dihydro flavin compounds, and from $NaBH_4$-modified D- and L-amino acid oxidases. N^3-methyl 3,4-dihydro lumiflavin is readily reduced by EDTA and light irradiation[31], and $NaBH_4$-modified amino acid oxidases are reduced by substrate[22]. With the free flavins and modified amino acid oxidases, several cycles of reduction and reoxidation convert the 3,4-dihydro derivative to normal oxidized flavin.

DISCUSSION

These results show that FMN can be reversibly dissociated from three different flavodoxins. The apoproteins bind FMN to give complexes which have the same light absorption and fluorescence properties, catalytic activity, and oxidation–reduction behavior as the native proteins. The changes in flavin fluorescence which accompany these interactions follow second order kinetics. Additional first order changes similar to those observed during flavin binding by flavoproteins such as D-amino acid oxidase[12] and glucose oxidase[15] have not been detected.

The maximum rate of binding to *P. elsdenii* apoflavodoxin occurs at a pH value where only one of the two hydroxyl groups in the phosphate of FMN is ionized. In this respect this flavodoxin resembles the Shethna flavoprotein from Azotobacter[14] rather than NADPH dehydrogenase[9] (FMN is the natural prosthetic group in both of these proteins). In the case of NADPH dehydrogenase[9], the maximum rate of binding is near pH 9, a pH region where both hydroxyl groups are ionized. The importance of the phosphate group for flavin binding by the apoflavodoxins is emphasized by the observed lack of significant interaction with riboflavin. In their specificity for FMN and FMN analogues, these apoflavodoxins differ markedly from flavodoxin isolated from *Desulfovibrio gigas*. DUBOURDIEU AND LEGALL[4] have recently reported that an apoprotein prepared from *D. gigas* flavodoxin forms a catalytically active complex with FAD. During photo-reduction of this complex with EDTA, an intermediate similar to the semiquinone intermediate of native flavodoxin is generated. Although the fluorimetric experiments with *P. elsdenii*, *C. pasteurianum* and Clostridium MP apoflavodoxins suggest that these proteins do not bind FAD or riboflavin, some weak interaction cannot be ruled out; equilibrium dialysis experiments to rigorously test this possibility have not yet been done.

The strong interaction observed between apoflavodoxins and either iso-FMN or 3,4-dihydro FMN, implies that the 4, 6 and 8 positions in the isoalloxazine ring are not critical for complex formation. However, X-ray crystallographic studies on crystals of the iso-FMN derivative of Clostridium MP flavodoxin[32] have suggested that the crystal structure of this complex may be somehat different from the structure of native flavodoxin. The low catalytic activity of iso-FMN flavodoxin compared with native *P. elsdenii* flavodoxin in the phosphoroclastic oxidation of pyruvate is probably due to an unfavorable oxidation–reduction potential in this complex. The difference in oxidation–reduction potentials calculated for the two 1-electron reduction steps in iso-FMN flavodoxin is 0.104 V at pH 6.3. Assuming that the oxidation–reduction potentials of free iso-FMN are similar to those of FMN[30], an approximate value of −0.26 V can be calculated for the oxidation–reduction potential of the second step (*i.e.* the reduction of the semiquinone to fully reduced iso-FMN flavodoxin). This value may be too positive for efficient coupling of iso-FMN flavodoxin in the phosphoroclastic assay. It is considerably higher than the oxidation–reduction potential (−0.370 V) determined for the second step in the reduction of native flavodoxin at the same pH.

Rather different redox effects may explain why 3,4-dihydro FMN flavodoxin does not substitute for flavodoxin in the phosphoroclastic assay, and why reducing systems which reduce native flavodoxin do not cause significant reduction of 3,4-dihydro FMN flavodoxin. Treatment of native flavodoxin with EDTA and light irradiation or with NADPH and ferredoxin $NADP^+$ reductase gives high yields of the blue neutral semiquinone[5,6]. Further reduction to fully reduced flavodoxin is difficult with these systems. There is no evidence for the formation of the neutral form of the semiquinone of 3,4-dihydroflavins; only the cationic radical has been observed[31]. It is thus possible that at neutral pH, 3,4-dihydro FMN flavodoxin can be reduced only by a 2-electron reduction step to give 1,3,4,5-tetrahydro FMN flavodoxin. The oxidation–reduction potential for this system might be much lower than for free 3,4-dihydroflavins, and also lower than the second step in the reduction of native flavodoxin (*i.e.* the reduction of semiquinone to fully reduced flavodoxin).

It is not too surprising that sodium dithionite does not reduce 3,4-dihydro FMN flavodoxin; free 3,4-dihydroflavins[31], and also the $NaBH_4$-modified amino acid oxidases (V. MASSEY and coworkers, unpublished observations) are only slowly reduced by $Na_2S_2O_4$.

The effects of mercurial reagents and *N*-ethylmaleimide on the interaction of apoflavodoxin and FMN indicate an important role of sulfhydryl groups in the complex. Since *C. pasteurianum* apoflavodoxin contains only one sulfhydryl group, it is possible that in *P. elsdenii* and Clostridium MP flavodoxins only one of the thiols is critical for flavin binding; the experiments described in this paper do not distinguish between them. In addition, the results of these experiments do not necessarily imply a direct interaction between the flavin prosthetic group and a cysteine residue of the protein. In protein molecules as small as the flavodoxins, modification of a sulfhydryl group remote from the flavin-binding site may sufficiently alter the structure of the protein to prevent flavin binding.

It has been reported recently that when native flavodoxin from *C. pasteurianum* is reacted with *N*-bromosuccinamide at pH 4, two of the four tryptophan residues are oxidized, with loss of flavin from the protein and loss of catalytic activity[33]. A similar treatment but in the presence of 8 M urea caused the oxidation of all four tryptophan residues. It was suggested that these results indicate that at least one tryptophan residue could be tightly complexed with FMN. However, it is very likely that at pH 4 FMN is lost from this flavodoxin even in the absence of *N*-bromosuccinamide; this control experiment was not reported.

There is evidence that one or more tyrosyl residues are important for flavin binding by a number of flavoproteins[34,11,14]. Preliminary data suggest that this is also the case in flavodoxin. Nitration of one of the two tyrosines in *P. elsdenii* apoflavodoxin with tetranitromethane under conditions which leave the sulfhydryl groups intact, completely inhibits flavin binding. However, the nitrated apoprotein has not yet been completely characterized, and the possibility remains that some other group or groups in the protein are also affected by this treatment.

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