First publ. in: European Journal of Biochemistry 44 (1974), pp. 579-591

Identification and Properties of New Flavins in Electron-Transferring Flavoprotein from *Peptostreptococcus elsdenii* and Pig-Liver Glycolate Oxidase

Stephen G. Mayhew, Carolyn D. Whitfield, and Sandro Ghisla

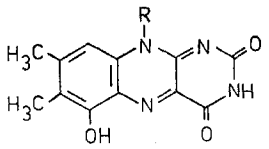
Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan

Marilyn Schuman-Jörns

Fachbereich Biologie der Universität Konstanz

(Received September 25, 1973)

1. New flavins have been isolated from purified preparations of an electron-transferring flavoprotein (ETF) from *Peptostreptococcus elsdenii* and glycolate oxidase from pig liver. The structures of these new species have been established as FAD and FMN derivatives of 6-hydroxy-7,8-dimethyl-isoalloxazine, the chemical synthesis of which is described in the accompanying paper by Schöllnhammer and Hemmerich. The chromophores are yellow at pH 5 and green at pH 9 due to an ionization at pK 7.1.



2. 6-OH-FAD is bound by apo-ETF and its pK is decreased. The complex is reduced by NADH and it couples the oxidation of NADH to the reduction of dichlorophenolindophenol. Unlike the complex of FAD and apo-ETF, 6-OH-FAD · ETF does not couple the oxidation of NADH to the reduction of butyryl-CoA dehydrogenase.

3. 6-OH-FMN is bound by the FMN-specific protein apoflavodoxin from *P. elsdenii* and the pK is increased to $\approx$9. This complex is reduced by sodium dithionite and an intermediate, presumed to be the semi-quinone, is formed at half reduction.

Long-wavelength absorption bands (λ_{max} 500 to 800 nm) have been observed in the spectra of several oxidised flavoproteins (*e.g.* [1—3]), and they have generally been ascribed to charge transfer interaction [4] between the flavin chromophore and a small molecule [1—3]. However, there is evidence that not all long-wavelength bands in oxidised flavoproteins are due to such interactions, and several alternative explanations have been proposed for this phenomenon [5]. In the case of glycolate oxidase from pig liver, Schuman and Massey [6] obtained evidence that the long-wavelength absorption is not due to FMN, and they concluded that there is a second chromophore in the enzyme. They showed that when FMN is selectively removed from glycolate oxidase, the resulting preparation is green, and it retains a broad absorption band centered at 600 nm and also a sharp maximum at 425 nm. The chromophore giving this absorption spectrum was not identified.

More recently we have observed a green chromophore with a similar spectrum in preparations of an electron-transferring flavoprotein (ETF) isolated from the anaerobic bacterium *Peptostreptococcus elsdenii* [7]. This enzyme couples the oxidation of NADH to the reduction of butyryl-coenzyme-A dehydrogenase and resembles the ETF of Beinert [8] which functions in the β-oxidation of fatty acids in aerobic systems. The spectrum of purified ETF from *P. elsdenii*, like the spectrum of glycolate oxidase,

Abbreviations. ETF, electron-transferring flavoprotein; DCPIP, dichlorophenolindophenol.

Enzymes. Butyryl-CoA dehydrogenase (EC 1.3.99.2); NADH dehydrogenase (EC 1.6.99.3); glycolate oxidase or glycolate:O_2 oxidoreductase (EC 1.1.3.1).

shows a weak and broad band of absorption at wavelengths greater than 550 nm in addition to the absorption peaks due to FAD .

In this paper we describe the properties of the chromophores which are responsible for the long-wavelength bands of ETF and glycolate oxidase, and their identification with the FMN and FAD derivatives of 6 hydroxy-7,8-dimethyl isoalloxazine [9], the chemical synthesis of which is described in the accompanying paper by Schöllnhammer and Hemmerich [10].

EXPERIMENTAL PROCEDURE

Enzyme Purification

Glycolate oxidase was purified from pig liver as described by Schuman and Massey [6]. Flavodoxin and apoflavodoxin were prepared from *P. elsdenii* as described elsewhere [11,12]. The purification and properties of ETF will be described in a separate communication (by C. D. Whitfield and S. G. Mayhew). Apo-ETF was prepared by chromatographing 0.5 ml of ETF in 0.05 M sodium acetate, pH 5.5 and 0.3 mM EDTA on a column (1.6×11 cm) of Sephadex G-25 equilibrated with 1.55 M guanidine-HCl (ultrapure from Schwarz/Mann), 0.06 M potassium phosphate pH 6.2 and 0.3 mM EDTA. The apoprotein was then treated on a second column of Sephadex G-25 (1×11 cm) equilibrated with the same buffer but minus guanidine-HCl. Apo-ETF prepared by this method contained traces (about 4%) of residual flavin. The holoprotein was regenerated from apo-ETF and FAD by incubation at 15 °C for 2 h.

Butyryl-coenzyme-A dehydrogenase was prepared from *P. elsdenii* by a procedure similar to that described by Engel and Massey [13].

Enzyme Assays

NADH dehydrogenase was assayed by coupling the oxidation of NADH to the reduction of dichlorophenolindophenol (DCPIP). Reaction mixtures contained in a total volume of 3 ml: 140 μM NADH, 32 μM DCPIP, 0.1 M potassium phosphate buffer pH 7 and NADH dehydrogenase. One unit of activity is defined as an absorbance change of 1 per min at 600 nm and 25 °C.

ETF activity was measured by coupling the oxidation of NADH to the reduction of crotonyl-coenzyme A in the presence of purified butyryl-coenzyme-A dehydrogenase [13]. The incubation mixture contained 0.06 M potassium phosphate buffer pH 6, 100 μM NADH, 30 μM crotonyl-coenzyme A, 1.2 μM butyryl-coenzyme-A dehydrogenase ($A_{710}/A_{430} = 0.44$, $\varepsilon_{430} = 9600\ M^{-1}\ cm^{-1}$ [13]) and ETF in a total volume of 1 ml at 25 °C. The reaction was begun by addition of crotonyl-coenzyme A and followed by measuring the rate of decrease in absorbance at 340 nm. Since NADH is slowly oxidised in the absence of crotonyl-coenzyme A, the rate was first measured in each assay without this substrate. The blank reaction rate was subtracted from the rate in the presence of crotonyl-coenzyme A. Under the conditions described above, the final rate is proportional to the concentration of ETF.

Isolation of the Enzyme Chromophores

The ETF chromophore was extracted from the protein in 0.1 M sodium acetate buffer pH 6 either by heat treatment in a boiling water bath for 3 min, or by precipitation of the apoprotein with 10% trichloroacetic acid at 0 °C, followed by extraction of the trichloroacetic acid with ether. Precipitates of protein were removed by centrifugation (20000×*g* for 10 min). The yellow extract (approx. volume 7 ml; $A_{450\,nm} = 1.3$) was adjusted to pH 8.5 with NaOH and applied to a column of DEAE-cellulose (Whatman DE-32; 16×2 cm) equilibrated with 0.01 M potassium phosphate pH 7. After loading, the column usually showed a narrow green or brown band at the top with a broad yellow and fluorescent band below. It was washed with 0.2 M potassium phosphate pH 5.9 (approx. 100 ml) until the yellow material had been stripped out and the green band had moved into the lower half. The green band was clearly separated from an orange band which remained in the top half of the column, and which contained 8-nor-8-OH FAD [14]. In order to elute the green band into as concentrated a solution as possible, the DEAE-cellulose was carefully extruded from the column, and the green resin cut out and suspended in water. This slurry was poured into a second column (approx. 3×0.9 cm) and the adsorbed material eluted with 0.1 N HCl. Solid K_2CO_3 was added to the yellow acidic eluate until it turned green (pH 7–8). This solution was applied to a further column of DEAE-cellulose (10×2 cm) and the chromatographic and elution procedures described above were repeated. The resulting solution was finally adjusted to pH 9 with solid K_2CO_3 and, when necessary, it was stored at −20 °C.

The glycolate oxidase chromophore was prepared either by enzymic hydrolysis of the ETF chromophore with phosphodiesterase (*Naja naja* venom), or by extraction from glycolate oxidase. Glycolate oxidase was first dialysed *versus* KBr as described by Schuman and Massey [6] to obtain a green FMN-depleted preparation of the enzyme. The protein in 0.01 M potassium phosphate pH 7 was then heat-denatured to give a mixture of FMN and 6-OH-FMN. The extract (4.5 ml, $A_{430\,nm} = 0.15$) was adjusted to pH 9.6 and loaded onto a column (4×0.5 cm) of DEAE-cellulose equilibrated with 0.01 M sodium pyrophosphate pH 9. A band of green material was adsorbed at the top of the column

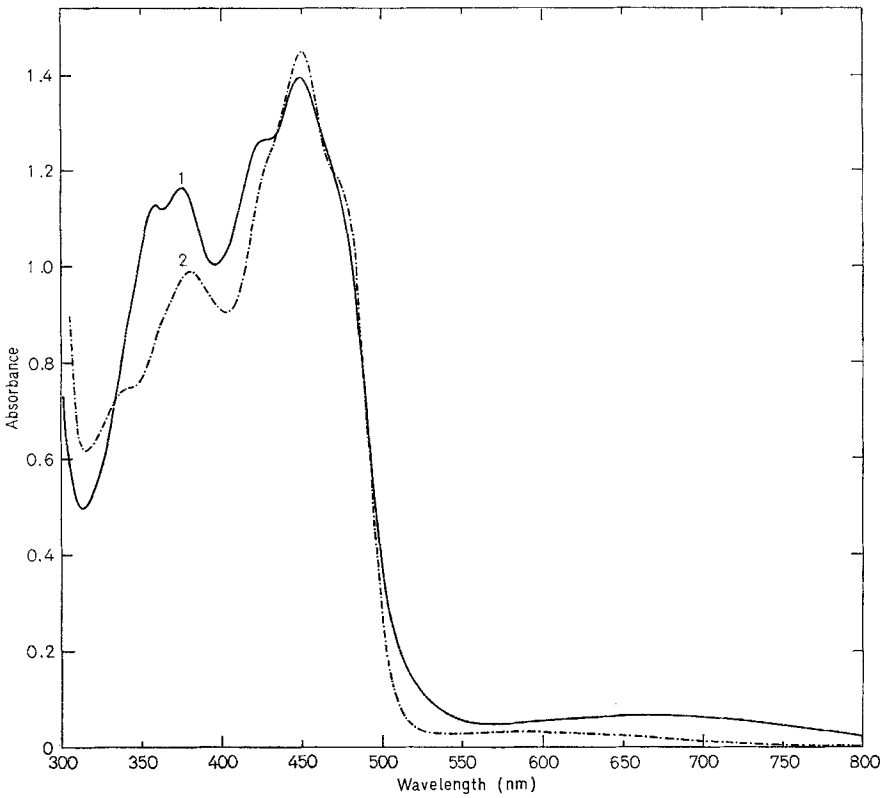


Fig. 1. *Absorption spectra of ETF and glycolate oxidase.* Curve 1, 123 μM ETF dissolved in 0.1 M sodium acetate, pH 5.5. Curve 2, 126 μM glycolate oxidase in 0.04 M potassium phosphate, pH 7

above a broad yellow and fluorescent band (FMN) which was eluted on washing with 0.1 M sodium pyrophosphate pH 9. The column was washed with water to remove excess pyrophosphate, and then with 0.1 N HCl to elute the chromophore as a yellow solution. This was immediately neutralised with K_2CO_3 and when necessary stored at −20 °C.

RESULTS AND DISCUSSION

Absorption Spectra of ETF and Glycolate Oxidase

The spectra of ETF (normal prosthetic group FAD) and glycolate oxidase (normal prosthetic group FMN) have absorption maxima near 450 nm and 375 nm (Fig. 1) similar to the spectra of many flavoproteins. However, they are different from other flavoproteins in certain respects. The most characteristic difference is an additional broad band of absorption which has a maximum between 600 and 660 nm. The intensity of this band, relative to the absorption at 450 nm, varies with the preparation, and preparations which show a more intense long-wavelength band also show two absorption maxima at 330—360 nm and 420—430 nm. In some preparations of ETF, the peak at 430 nm is very pronounced and it is even higher than the absorption at 450 nm.

The spectra of certain other flavoproteins exhibit similar long-wavelength absorption bands due to interaction between the flavin chromophore and another component [1—4]. In such cases, denaturation of the apoprotein causes dissociation of the flavin from the protein and loss of the long-wavelength absorption. In contrast, denaturation of ETF and glycolate oxidase does not remove the longwavelength absorption; when the apoproteins of these enzymes are precipitated by denaturation with heat or trichloroacetic acid, the chromophores in the supernatant still show a band of long-wavelength absorption at high pH. This observation suggested that the long-wavelength bands in ETF and glycolate oxidase are not due to interaction between flavin and either a small molecule or a functional group on the protein, but rather that they arise from discrete chromophores.

Properties and Structure of the Green Chromophores

In order to identify the chromophore with the absorption maximum at 600 nm, extracts from ETF and glycolate oxidase were fractionated as described in the Methods section. It was found that in contrast to FAD and FMN, which are only weakly bound by

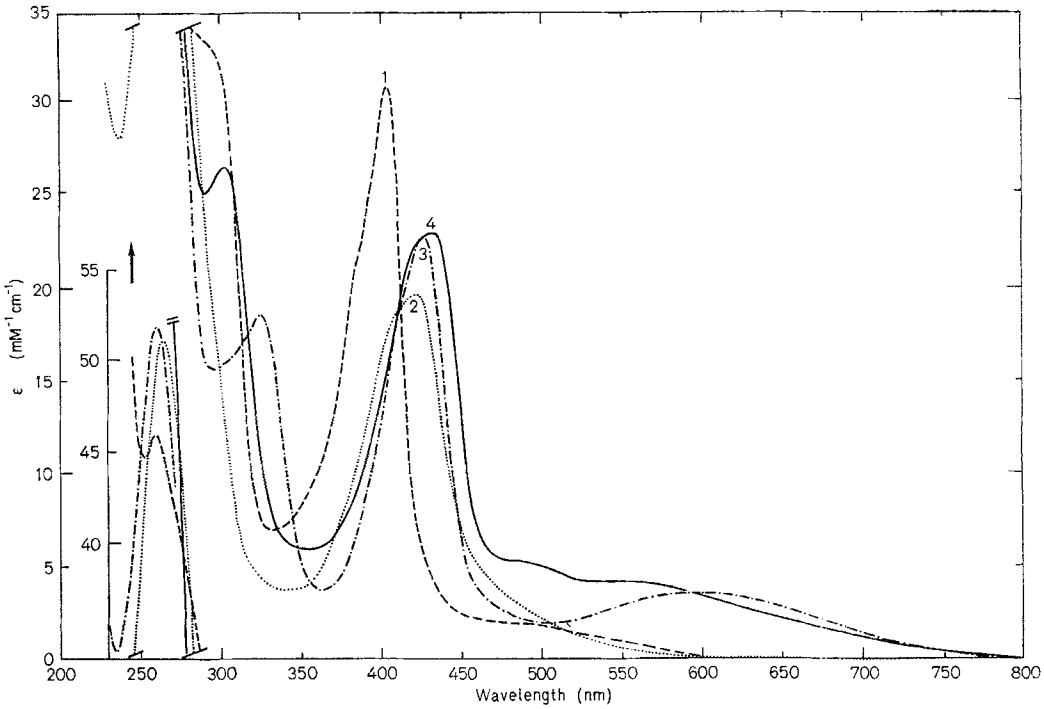


Fig. 2. *Effect of pH on the spectrum of 6-OH-FAD.* 6-OH-FAD was dissolved in 6 N HCl (curve 1), sodium acetate pH 5.4 (curve 2), Tris-HCl pH 9 (curve 3), and 2 N NaOH (curve 4)

DEAE-cellulose, green components in the extracts are strongly retained by the ion-exchange resin at pH values between 7 and 9. This property, which was used extensively in their further purification, suggested firstly that the green compounds are negatively charged, and secondly that they contain more negative groups than FAD and FMN. It was further noted that the color changes from grass-green to yellow between pH 9 and pH 5 respectively. The spectral changes for the pure compound isolated from ETF are shown Fig. 2. The 600-nm band is absent at pH 5 and the remaining transition in the visible region of the spectrum is shifted from 427 nm to 422 nm. These changes are isosbestic (263, 299, 354, 413, 477 and 507 nm) and they correspond to a pK of 7.1. The value for the pK and the behaviour of the chromophore on DEAE-cellulose indicated that the changes are due to the ionisation of a very weak acid.

Further spectral changes occur at more extreme values of pH. In 6 N HCl, the maximum in the visible region shifts to 403 nm and it increases in intensity. These changes correspond to a protonation with a p$K < 1$. In 2 N NaOH the 427-nm peak is slightly red-shifted and there is an increase of absorption around 500 nm (Fig. 2); this change reflects an ionisation with a p$K > 9$. With the exception of the pK at 7.1, these properties are similar to those of the *iso*-alloxazine system. Thus *iso*alloxazine also has pK values of ≈ 0 and ≈ 10 corresponding to protonation at N(1) and to deprotonation at N(3) respectively [15], and the spectral shifts are in the same direction.

The electronic spectra of the pure chromophores from ETF and glycolate oxidase are closely similar at all pH values, but there are differences in the positions of their absorption maxima, the spectrum of the ETF chromophore being slightly shifted to the red (Table 1).

Both compounds are readily and reversibly reduced by sodium dithionite, indicating that the chromophore is an oxidation-reduction system. The absorption at 422 nm (pH 5.2) decreases linearly during anaerobic titration with sodium dithionite, and there is no evidence for an intermediate. Complete reduction requires two reducing equivalents per molecule of chromophore. The reduced chromophore from ETF at pH 5.2 has only two shoulders of absorption in an otherwise featureless spectrum (Fig. 3). It should be noted that this spectrum is closely similar to the spectrum of reduced FAD, in contrast to the spectra of the two oxidised compounds, which are clearly very different (Fig. 3).

Further information about the chemical nature of the green chromophores was obtained from their reactions with phosphodiesterase, and from binding studies with apoflavodoxin (Fig. 4). When the chromophore from ETF is treated with phosphodiesterase at pH 6.2, the maximum in the visible region increases

Table 1. *Absorption maxima and absorption coefficients of free and protein-bound 6-OH-flavins*

Compound	Conditions or pH	λ_{max} (ε)	pK'
		nm ($mM^{-1} \cdot cm^{-1}$)	
6-OH-FAD from ETF	6 N HCl	260 (46), 403 (31)	<1
	5.6	262 (50.5), 422 (19.6)	
	9.0	260 (51.7), 323 (19.6), 427 (22.6), 600 (3.41)	7.1
	2 N NaOH	302 (26), 434 (22.8), 550 (4.1)	>9
6-OH-FMN from glycolate oxidase	5.5	420 (21.2)	
	9.0	320 (23), 423 (24.9), 600 (3.45)	7.1
6-OH-FAD plus apo-ETF	5.6	350 (17.9), 420 (18.9), 660 (2.26)	
	6.2	350 (18.9), 420 (18.9), 660 (2.36)	<7
	7.9	350 (24.4), 420 (18.9), 660 (3.42)	
6-OH-FMN plus apoflavodoxin	5.4	423 (17.5)	≈9
	10.1	325 (22.2), 429 (19.4), 600 (3.0)	
6-OH-FMN plus apoglycolate oxidase[a]	7	425 600	<7

[a] Data from [6].

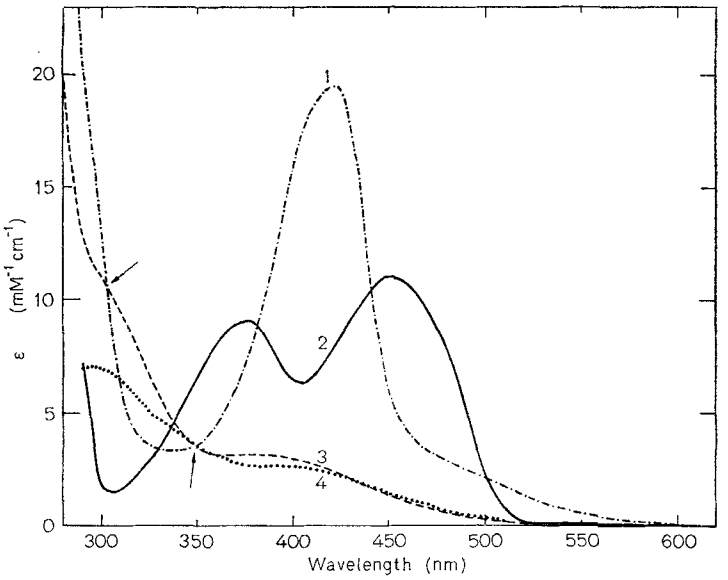


Fig. 3. *Comparison of the absorption spectra of oxidised and reduced 6-OH-FAD and FAD.* Curves 1 and 2, oxidised 6-OH-FAD and FAD respectively. Curve 3, 6-OH-FAD after reduction [16] with equimolar sodium dithionite. Curve 4, FAD after reduction by irradiation with light in the presence of EDTA. All samples were dissolved in 0.1 M sodium acetate pH 5.2. Arrows indicate isosbestic points between oxidised and reduced 6-OH-FAD

in intensity and it shifts from 422 nm to 420 nm (curves 1 and 3, Fig. 4). These changes are analogous to the changes that occur when the pyrophosphate linkage of FAD is hydrolysed to give FMN and AMP [17]; they suggest a similar hydrolysis reaction for the unknown chromophore. The chromophore from glycolate oxidase is not affected by phosphodiesterase and the spectral properties of this compound are identical with those of the hydrolysed chromophore from ETF. Furthermore, the apoprotein from *P. elsdenii* flavodoxin forms a tight complex with these compounds, but it does not bind the untreated chromophore from ETF. Since *P. elsdenii* apoflavodoxin is specific for FMN and FMN derivatives [12], these observations indicate firstly that the green chromophore is a flavin analogue, and secondly that the effect of phosphodiesterase is to convert the green chromophore of ETF from an FAD-like compound to an FMN analogue.

It thus became necessary to consider ways in which FMN and FAD might be modified to produce the spectral properties of the green chromophores.

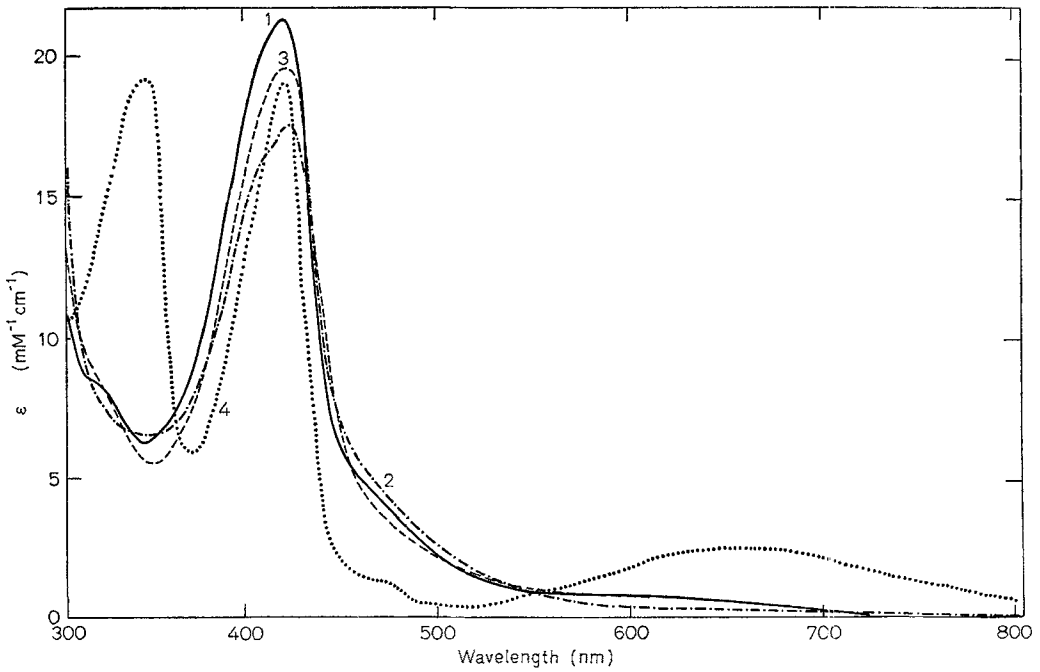


Fig. 4. *Comparison of the absorption spectra of free 6-OH-FAD, 6-OH-FAD · ETF, free 6-OH-FMN and 6-OH-FMN · flavodoxin.* Curve 1, 6-OH-FMN; curve 2, 6-OH-FMN · apoflavodoxin; curve 3, 6-OH-FAD; curve 4, 6-OH-FAD · ETF. Samples were dissolved in 0.06 M potassium phosphate pH 6.2. Curve 4 was obtained from a point in the titration shown in Fig. 6 and it has been corrected for the absorption of apo-ETF

The drastic changes in the spectrum indicated that the modifying group (or groups) must be in conjugation with the chromophoric system of the flavin, but a survey of the flavin literature did not reveal any modifications to the flavin chromophore that corresponded with the properties of the green chromophore [18—23]. However, during the course of this work we obtained information about new isoalloxazine derivatives synthesised by Schöllnhammer and Hemmerich [9, 10] in which the 6- or 9-position of the benzene moiety is substituted with an —OH function. The spectra of the naturally occurring chromophores were found to be closely similar to the spectra of the synthetic compounds. The alkyl substituent at position N(10) is different for the models, but the nature of this non-functional substituent is known to have only a minor influence on flavin spectra [18]. The spectral properties of isoalloxazines substituted with an —OH at position 6 are not too different from those of 9-OH isoalloxazines [9, 10]. However, the position of substitution can be determined unambiguously from a comparison of the reactivity of the 6- *versus* the 9-OH isomeric isoalloxazines against metal ions [9, 10, 24]. A phenolate oxygen in position 6, *i.e. ortho* to N(5), should greatly enhance the metal-chelating power of the isoalloxazine system as becomes evident from a general survey of flavin-metal interactions [25]. The metal can be expected to complex with the ligand as shown in IV (*cf.* Scheme 1 below).

The green chromophores from ETF and glycolate oxidase were therefore tested for complex formation with Cu(II) at pH 6 in acetate buffer. Addition of Cu(II) caused a red shift of the main visible absorption band and increased absorption around 320 nm, indicating that in both cases a complex was formed and consequently that the —OH substituent is at position 6 (Fig. 5). The theoretical spectrum of the fully formed complex with the chromophore from ETF (FAD level) was calculated by extrapolation (Fig. 5, curve 4). The dissociation constants (3.3 mM), calculated according to Benesi and Hildebrand [26], were the same for both compounds. Under similar conditions, Cu(II) does not influence the spectra of FMN, FAD and 9-OH-flavins to a detectable extent [9, 10, 24].

Based on the experiments described above and the comparison with the model compounds [9, 10], we therefore assign the 6-hydroxy-7,8-dimethyl-1-ribityl-isoalloxazine structures I $\rightleftarrows$ III to the enzyme chromophores.

From their work on model compounds, Schöllnhammer and Hemmerich [9, 10] attribute structures II and III to the green form of the chromophore and structure I to the yellow form.

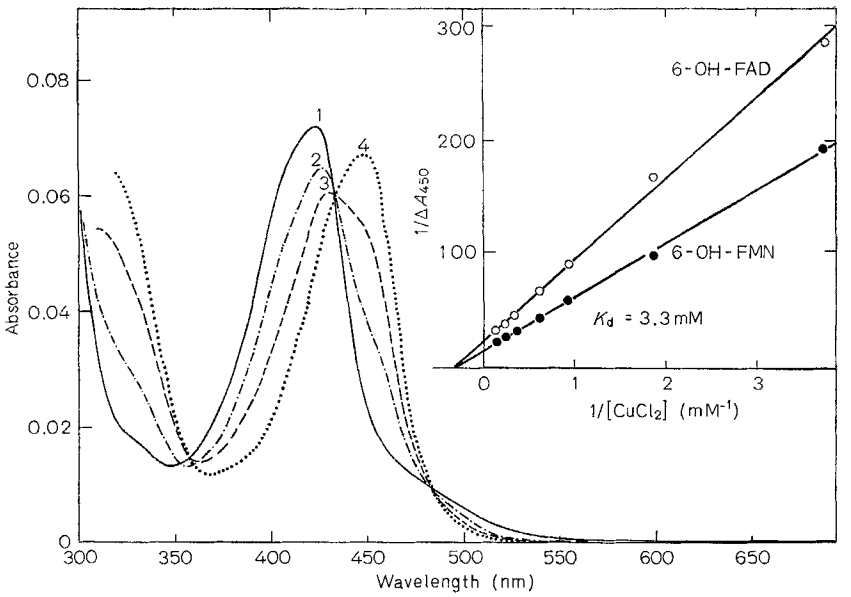
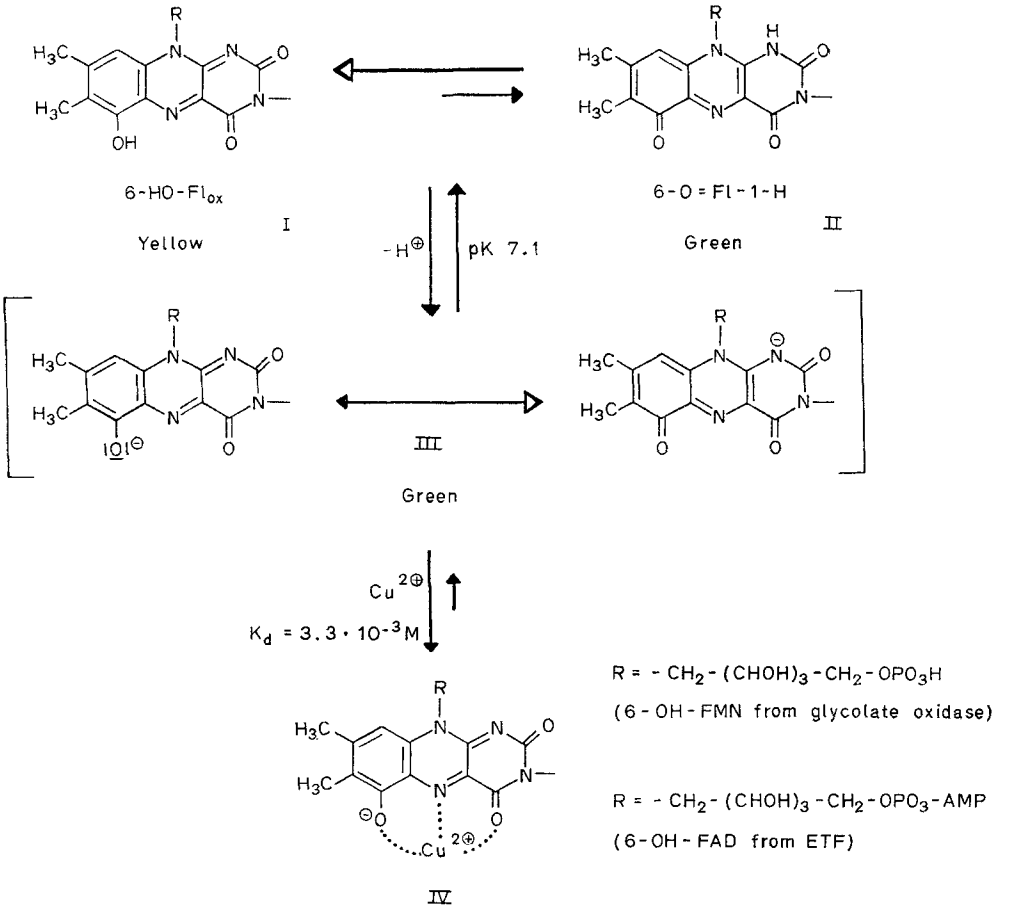


Fig. 5. *Titration of 6-OH-FAD with $CuCl_2$.* The sample cuvette contained 0.75 ml 3.8 μM 6-OH-FAD in 0.5 M sodium acetate buffer pH 6. The reference cuvette contained 0.75 ml acetate buffer. $CuCl_2$ (0.1 M) was added in increments to the sample and reference cuvettes and spectra were recorded after each addition. Curve 1, minus $CuCl_2$; curve 2, plus 1.07 mM $CuCl_2$; curve 3, plus 6.7 mM $CuCl_2$; curve 4, calculated spectrum for the fully formed Cu-complex. The inset shows plots of the reciprocal of the change in absorbance at 450 nm *versus* the reciprocal of the concentration of $CuCl_2$ during titrations of 6-OH-FAD and 6-OH-FMN (3.8 μM)



Scheme 1

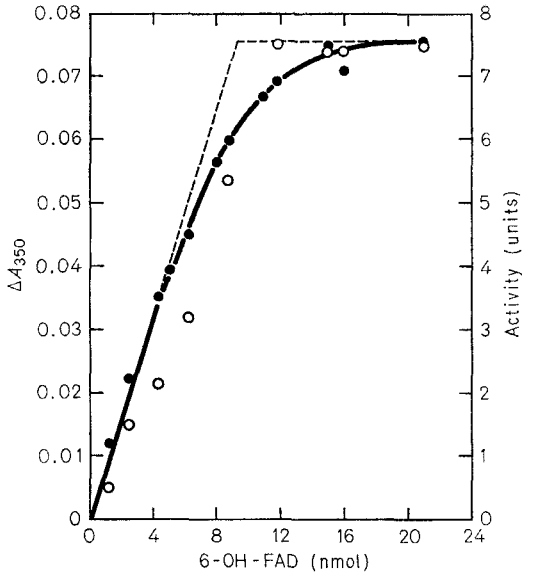


Fig. 6. *Recombination of apo-ETF with 6-OH-FAD.* The sample cuvette contained apo-ETF (0.7—2 mg per ml in different experiments) dissolved in 0.06 M potassium phosphate pH 6.2 at 15 °C. The reference cuvette contained an equal volume of buffer. Increments of 6-OH-FAD or FAD were added to sample and reference cuvettes. Difference spectra were recorded and samples were removed for activity assay after each addition. The concentration of active apoprotein was determined from the titration with FAD. (●) ΔA_{350}; (○) activity

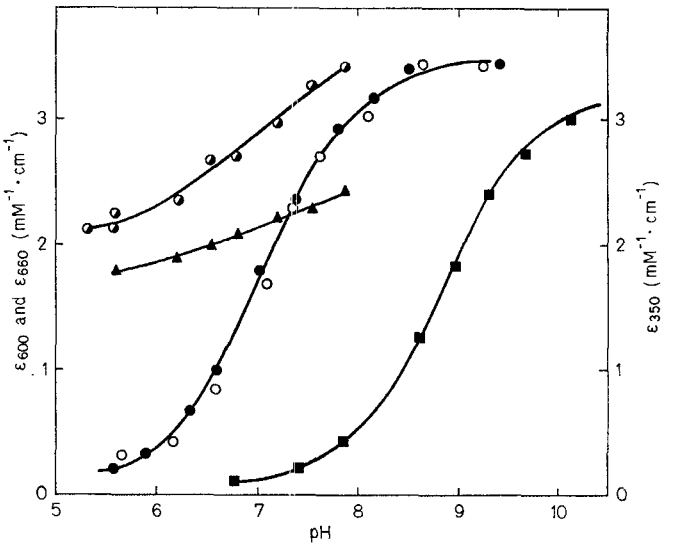


Fig. 7. *pH titrations of free and protein-bound 6-OH-flavins.* The millimolar absorption coefficients at 600 nm (6-OH-FMN, ●; 6-OH-FAD, ○; 6-OH-FMN · flavodoxin, ■), 660 nm (6-OH-FAD · ETF, ◑) and 350 nm (6-OH-FAD · ETF, ▲), are shown plotted *versus* pH. The experiments with the free flavins and 6-OH-FMN · flavodoxin were done at 20 °C in approx. 0.01 M potassium phosphate. These solutions were adjusted to the required pH by addition of solid Tris base or citric acid. The experiment with 6-OH-FAD · ETF was done at 12 °C in 0.01 M sodium acetate, and pH adjustments were made by addition of solid K_2HPO_4 and K_2CO_3, and with 1 N acetic acid. Absorption spectra were recorded at each pH value

Binding of 6-OH FAD to Apo-ETF

In order to show that the long-wavelength absorption of native ETF is due to 6-OH-FAD, we studied the effects of binding this flavin to the apoprotein prepared from ETF. When 6-OH-FAD is added to apo-ETF at pH 6.2, the 423-nm absorption maximum is slightly blue-shifted and decreased in intensity and new absorption maxima are observed at 350 and 660 nm (Fig. 4, Table 1). It should be noted that this spectrum is similar to the spectrum of free 6-OH-FAD at pH 9, although the peaks at 350 and 660 nm are red-shifted compared with the peaks in the free flavin. The three absorption peaks in the complex correspond with the maximum at 660 nm and the shoulders at 360 nm and 425 nm in the spectrum of ETF as it is isolated (Fig. 1). We conclude therefore that 6-OH-FAD contributes to the long-wavelength absorption and also the 360 and 425-nm shoulders in the spectrum of ETF. The content of 6-OH-FAD in this enzyme, estimated from the absorption coefficients determined for free and protein-bound 6-OH-FAD and FAD, is between 5 and 30% of the total flavin in different preparations. It should be noted that the content of 6-OH-FMN in glycolate oxidase also varies with the preparation [6].

The complex of 6-OH-FAD and apo-ETF catalyses the oxidation of NADH and the reduction of DCPIP (Fig. 6), and in fact the activity in this assay is 2.9 times the activity of FAD · ETF (based on activity per mole of flavin bound by the apoprotein). However, in contrast to FAD · ETF, the complex with 6-OH-FAD does not couple the oxidation of NADH to the reduction of crotonyl-coenzyme A in the presence of butyryl-coenzyme-A dehydrogenase. The sequence of reactions in this coupled assay is:

$$\text{NADH} + \text{H}^+ + \text{ETF(ox)} \rightarrow \text{NAD}^+ + \text{ETF(red)}$$

$$\text{ETF(red)} + \text{Butyryl-CoA dehydrogenase(ox)} \rightarrow \text{ETF(ox)} + \text{Butyryl-CoA dehydrogenase(red)}$$

$$\text{Butyryl-CoA dehydrogenase(red)} + \text{crotonyl-CoA} \rightarrow \text{Butyryl-CoA dehydrogenase(ox)} + \text{Butyryl-CoA.}$$

It should be noted that the complex of apoglycolate oxidase and 6-OH-FMN is also inactive in the usual assays for glycolate oxidase [6]. An approximate value for the dissociation constant of 6-OH-FAD · ETF was calculated from points in the spectrophotometric titration of apo-ETF with 6-OH-FAD (Fig. 6) which lie off the two linear regions of the titration curve. The value calculated (0.15 μM) was similar to that of the complex FAD · ETF (Whitfield and Mayhew, unpublished).

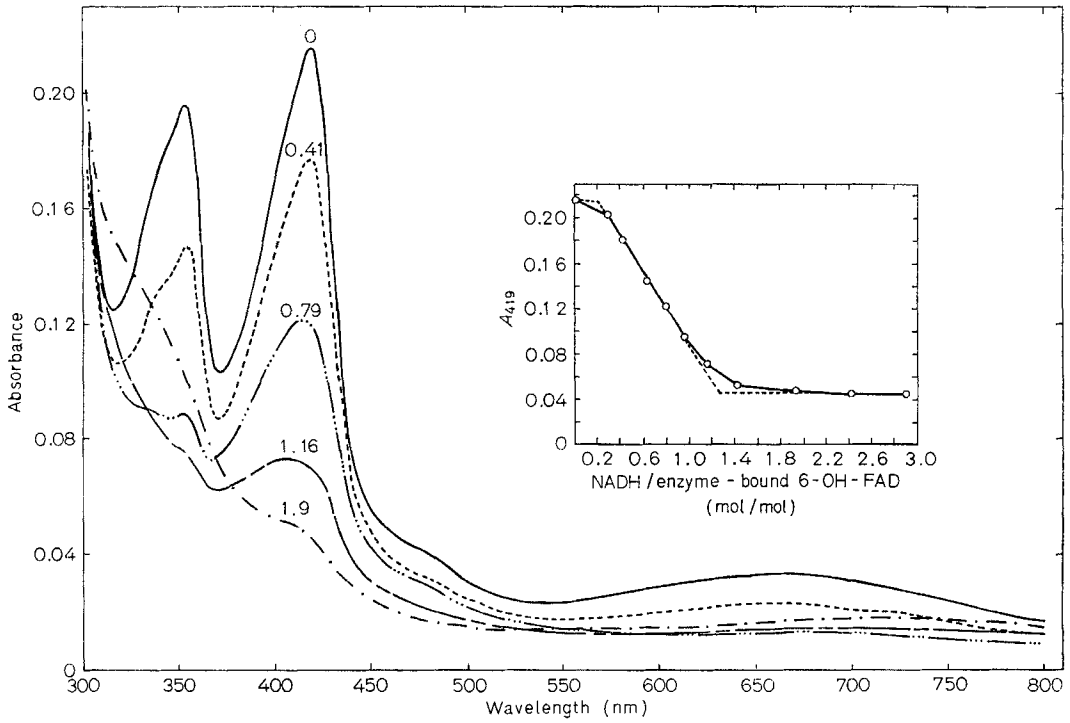


Fig. 8. *Anaerobic titration of 6-OH-FAD · ETF with NADH.* The curves shown are labelled according to the molar ratio of NADH to enzyme-bound flavin. Increments of NADH were added anaerobically [14,29] at 14 °C to 6-OH-FAD · ETF in 0.05 M sodium acetate pH 5.5 and 0.3 mM EDTA. Spectra were recorded after each addition. The preparation of enzyme used for this experiment contained 21.5% of unmodified FAD; curves have not been corrected for this residual flavin. The inset shows a plot of the absorption at 419 nm *versus* the amount of NADH added

The spectrum of 6-OH-FAD · ETF shows changes with pH which are analogous to those of free 6-OH-FAD; the absorption at 350 and 660 nm increases with pH in the range 5.3 to 8 (Fig. 7). We could not extend our measurements to higher or lower values because of dissociation of flavin from the apoprotein. However, at pH 5.6 the 660-nm absorption of bound 6-OH-flavin is about 60% of the maximum observed at pH 8, while free 6-OH-flavin at pH 5.6 shows the absorption of the neutral species (*cf.* I, Scheme 1). In addition, the titration curves for the bound flavin are relatively broad and they suggest that there is an additional ionizable group in the vicinity of the chromophore which has a pK in the same range. Because of these effects we cannot determine the pK for the bound flavin. Nevertheless, it appears that the pK of the 6-OH-flavin is somewhat lowered. This pK shift could be due to a positively charged group (*e.g.* histidine, pK 5—7?) on the protein in the vicinity of the flavin; an acidic group would increase the pK as observed in the case of flavodoxin (see below). It should be noted that native ETF also stabilizes the anionic form of the flavin semiquinone [7] and that positively charged groups have been proposed to explain the stabilization of similar radical anions in other flavoproteins [27].

Since the complex of ETF and 6-OH-FAD shows long-wavelength absorption, the prevalent electronic state of the flavin is most likely III (Scheme 1), and the proposed positively charged group of the protein is probably located near the amide function N(1)-C(2)=O. This interaction could go as far as to stabilize a tautomeric form of the neutral species which is protonated at N(1) (II in Scheme I) and which would be expected to have an absorption around 600 nm [10].

Similar considerations apply in the case of glycolate oxidase. In this enzyme the spectrum of the green chromophore was not influenced by pH in the range pH 6.2—9.6 [6]. Again, either a green neutral form of the modified flavin could be stabilized by the protein, or the pK of the bound flavin is lowered by more than two units. The latter case is supported by strong evidence for a positively charged group(s). Glycolate oxidase binds anions strongly, lowers the pK of the N(3)-H function of FMN by 2.3 units, is highly reactive with sulfite and stabilizes the red anionic flavin semiquinone [6,27,28]. In the case of ETF, there is no evidence for the binding of anions, nor for a pK-shift of the N(3)-H ionization in the bound flavin. However, this enzyme does react weakly with sulfite (C. D. Whitfield and S. G. May-

hew, unpublished results). From these observations we conclude that the different properties of these two enzymes can best be explained by assuming that the basic group(s) has a high pK in glycolate oxidase and a relatively low pK in ETF.

During anaerobic titration of 6-OH-FAD · ETF with NADH, the absorption at 350, 420 and 660 nm decreases linearly following the first additions of NADH (Fig. 8). Towards the end of the titration, a new absorption band appears at long-wavelength with a broad maximum between 700 and 750 nm. The inset in Fig. 8 shows that there is a stoichiometric reduction of 1 mole of enzyme-bound 6-OH-FAD by 1 mole of NADH. Reoxidation by aeration rapidly restores the original oxidized spectrum. The long-wavelength absorption which develops during the titration is similar to long-wavelength bands observed with reduced FAD · ETF and NAD^+ (C. D. Whitfield and S. G. Mayhew, unpublished) and also with certain other flavoproteins [30, 31]. These absorptions have been attributed to charge-transfer transitions between the reduced enzyme and NAD^+ [30, 31].

Photo-irradiation of flavoproteins in the presence of potential electron donors such as EDTA often causes reduction and the accumulation of flavin semiquinone in either its anionic (red) or neutral (blue) forms [32]. A red, anionic semiquinone is observed during photoreduction of FAD · ETF [7]. The complex of 6-OH-FAD and apo-ETF is also reduced by EDTA and light irradiation at pH 8.5, but in this case no intermediate is observed during the reduction.

Properties of 6-OH-FMN Flavodoxin

As mentioned earlier, a tight complex is formed between 6-OH-FMN and apoflavodoxin from *P. elsdenii*. It was of interest to study this complex to obtain more information about the properties of 6-OH-FMN, and also to determine the effects of the —OH group at position 6 on the properties of the holoprotein. As observed with FMN [12], binding of 6-OH-FMN by apoflavodoxin at pH 6.2 decreases the intensity of the visible absorption peak (Fig. 4, Table 1). Absorption coefficients for free and bound 6-OH-FMN were determined by titrating the flavin with apoflavodoxin of known concentration, and by assuming that as in native flavodoxin, only one molecule of flavin is bound per molecule of protein [11, 12]. It was not possible to determine a value for the dissociation constant of the complex because at the concentrations required for spectrophotometric measurements, all of the flavin is bound, and in addition this flavin is not measurably fluorescent. However, we conclude from spectrophotometric titrations at pH 6.2 and pH 9 that the dissociation constant is lower than 50 nM. This strong binding is consistent with the observation that *P. elsdenii* apoflavodoxin will form a strong complex with iso-FMN [12], which has a methyl group at position 6, and also that the flavin in a similar flavodoxin is located in a pocket on the surface of the molecule with one end, which is probably the benzene moiety, relatively exposed to solvent [33].

Like the spectrum of free 6-OH-FMN, the spectrum of the complex with apoflavodoxin depends on pH. Thus, increasing pH causes the color to change from yellow to green, a red shift of the main peak in the visible spectrum and development of a broad absorption band at 600 nm (Table 1). However, the pK for this change is different from that of the free flavin, being shifted from 7.1 to about 9 (Fig. 7). In contrast to 6-OH-FAD · ETF in which the pK appears to be lowered, the increased pK in 6-OH-FMN · flavodoxin may be due to a negatively charged group on the protein that is close to the N(1)-C(2)=O-N(3)H position of the flavin. A negatively charged residue in this region is also suggested by the observed stabilization of the neutral flavin semiquinone [11, 27] and by X-ray crystallographic studies on flavodoxin from *Clostridium* MP (M. Ludwig, private communication).

Again like the free 6-OH flavins, the complex with apoflavodoxin is reduced by sodium dithionite. During an anaerobic titration experiment with dithionite (Fig. 9) the reduction occurred in two steps. In the first part of the titration, corresponding to about 0.5 mol dithionite per mole flavin, the color of the solution changed from yellow to pink, and a new species with an absorption maximum at 544 nm was generated. This absorption disappeared on addition of more dithionite (about 1 mole per mole of flavin) and the solution became pale yellow. Spectra during the first part of the titration were isosbestic at 365 and 495 nm, and during the second part at 340 nm. These isosbestic points suggest that only two species were present during each half of the titration. Aeration of reduced 6-OH-FMN flavodoxin caused a rapid regeneration of the pink-colored intermediate, followed by a slower reaction in which the intermediate decayed to the original oxidised flavin. These changes are analogous to those observed during reoxidation of reduced native flavodoxin, although the pseudo-first-order decay of the intermediate (approximately 0.6 min^{-1}) is considerably faster in the case of the modified protein. The pink intermediate observed at half reduction is probably the neutral semiquinone of 6-OH-FMN, since during the reduction of native flavodoxin the neutral form of FMN semiquinone is generated in a similar way.

The complex of 6-OH-FMN and apoflavodoxin substitutes for native flavodoxin as an electron carrier in the phosphoroclastic oxidation of pyruvate which is catalysed by crude extracts of *P. elsdenii* [11, 29]. However, at saturating levels of the electron carriers, 6-OH-FMN · flavodoxin is only 8.5% as active as FMN · flavodoxin. This

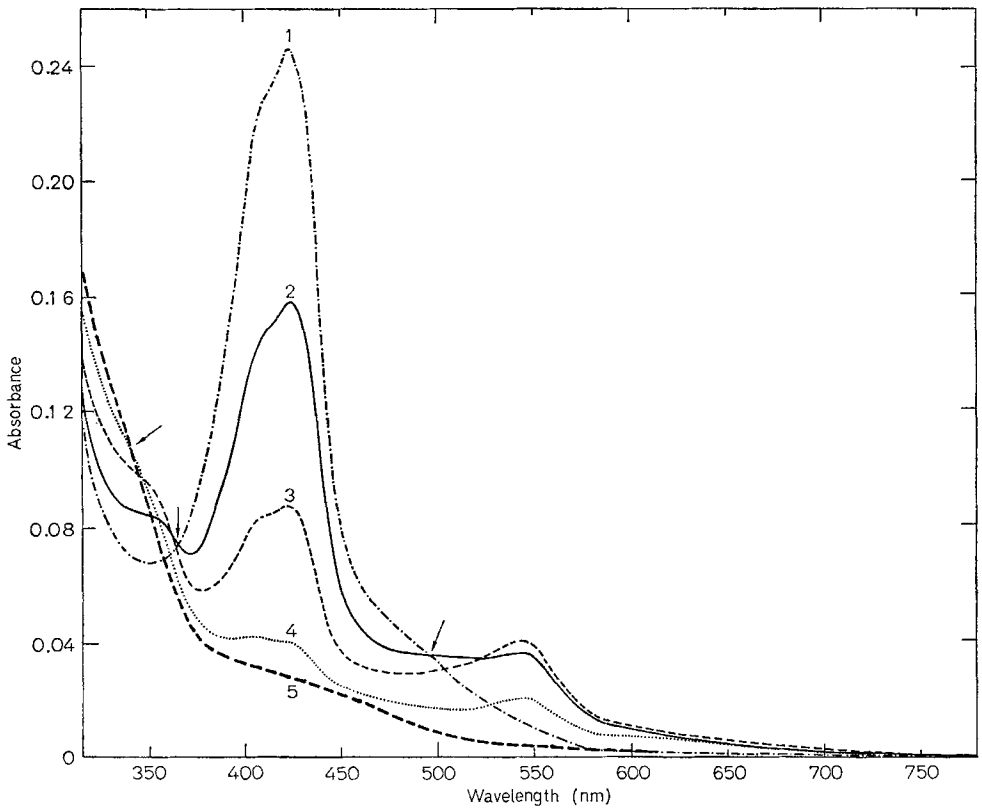


Fig. 9. *Spectrophotometric titration of 6-OH-FMN · flavodoxin with sodium dithionite.* Increments of sodium dithionite (1.5 mM) in 0.01 M sodium pyrophosphate pH 8.5 were added under anaerobic conditions [14,29] to 6-OH-FMN · flavodoxin in 0.05 M potassium phosphate pH 6.5 at 20 °C. Curves 1—5 were obtained after the addition of 0, 0.34, 0.56, 0.81 and 1.1 mol dithionite respectively per mol 6-OH-FMN

difference in activity might be due to differences in the oxidation-reduction potentials of bound 6-OH-FMN and FMN, as discussed elsewhere for the complex with iso-FMN [12,34].

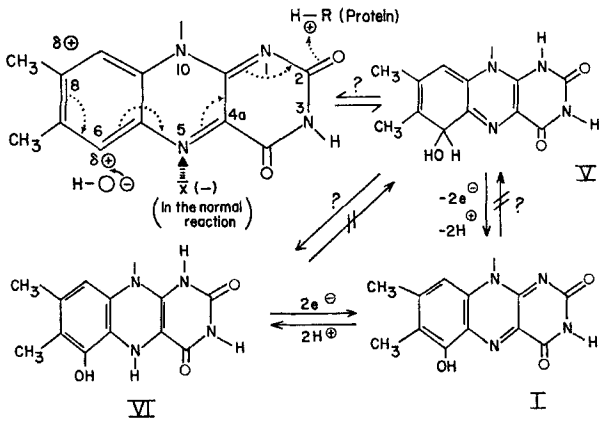
Possible Origins and Functions of 6-OH-Flavin

Following the identification of flavin (7,8-dimethyl-isoalloxazine) as the yellow chromophoric group of old yellow enzyme [35—37], it took 30 years before modifications to this chromophore were established in certain flavoproteins. These modifications occur at the methyl group in position 8 of the isoalloxazine nucleus [20,38—40], and in all cases the spectral properties of these compounds are not very different from those of FMN. Recently we reported the first positive identification of a modified flavin prosthetic group (8-nor-8-OH-FAD) in which the modifying group is in full resonance with the nucleus [14]. This paper describes two further modified flavins which have a similar substituent to 8-nor-8-OH-FAD, but markedly different spectral properties. The two enzymes from which these flavins have been isolated are from rather diverse sources and they catalyse quite different oxidation-reduction reactions. Thus glycolate oxidase is from pig liver and it catalyses the oxidation of short chain aliphatic hydroxy acids by oxygen according to the general reaction:

$$RCHOHCOOH + O_2 \rightarrow RCOCOOH + H_2O_2.$$

ETF on the other hand is from a strictly anaerobic bacterium and it couples the oxidation of NADH to the reduction of the flavoprotein butyryl-CoA dehydrogenase.

All of our preparations of glycolate oxidase and ETF contain unmodified flavin as a main component in addition to 6-OH flavin. We do not yet know why the proportions of 6-OH flavin and unmodified flavin vary in these enzymes, and a number of possible explanations have to be considered. Since 6-OH-FAD · ETF and 6-OH-FMN · glycolate-oxidase lack catalytic activities of the native proteins, it is possible that the nature of the prosthetic group is varied to control the reaction catalysed by the holoenzyme. However, it is also possible that 6-OH-flavins are formed during purification of the enzymes by



Scheme 2

exposure to conditions not encountered *in vivo*. ETF is from an anaerobic organism and it is not exposed to oxygen until after extraction from the cell; exposure of the reduced enzyme to oxidative conditions in the extract may generate 6-OH-flavin. If this is the case with the crude enzyme, we have not been able to reproduce the effect by reaction of the purified enzyme with oxidising agents. It is also conceivable that 6-OH-flavin is produced in the enzymes in a photochemical reaction similar to that used in their chemical synthesis [10]. However, all attempts to generate 6-OH-flavin by light irradiation of pure preparations of ETF and glycolate oxidase have failed, and conversely, although our enzymes are routinely purified with minimal exposure to room light, they still contain the modified flavin. A final possibility which is difficult to assess is that 6-OH-flavin is produced slowly in a side reaction of the normal reaction mechanism.

Although we do not yet know why glycolate oxidase and ETF contain 6-OH-flavin, a mechanism for the formation of this flavin can be envisaged based on the known chemical reactivity of isoalloxazine. It has been shown that positions 6 and 8 in isoalloxazine are activated and susceptible to chemical reaction, with sulfite for example, which can lead to nucleophilic substitution at these positions [41]. Further, although theoretical calculations predict that the most electrophilic position of the oxidized flavin molecule is position N(5), they also show that C(6) is a possible candidate for a nucleophilic attack (P. S. Song, private communication). It is possible therefore that a 2-electron reduction of the flavin may occur not only by attack of a carbanion species [42,43] or hydride [44] at position N(5) [C(4a)] as has been proposed, but also by a similar reaction of OH^- for example, at the vinylogue position C(6). It has been suggested that protonation or a hydrogen bond to the N(1)-C(2)=O group of the flavin is required for activation in its catalytic redox function [45]; the spectral properties of 6-OH-FAD · ETF indicate the presence of such a positively charged group in ETF. An addition reaction at C(6) would lead to the 1,6-dihydroflavin V, which could then either tautomerize to the 1,5-dihydro species VI or lead directly to the oxidized form of the green flavin I in an oxidative process.

A similar addition of R-OH to the benzene moiety of the flavin, but catalysed by light, has been reported for model compounds [46]. The above mechanism would explain how green flavin could be produced in the enzymes as a result of an irreversible side pathway to the normal enzyme mechanism.

The proposed function of a positive charge near N(1)-C(2)=O agrees well with the observed high reactivity of sulfite with flavoprotein oxidases [27] and model compounds [47], since as outlined above, the interaction of a positive charge at this position would enhance the nucleophilic attack of sulphite at N(5). Furthermore, model compounds in which protonation at N(1) is simulated by alkylation, exhibit greatly enhanced association constants with sulfite [47].

Pig liver was the only source of glycolate oxidase used in the present work, but it is possible that preparations from hog renal cortex [48] and etiolated plants [49] also contain 6-OH-flavin. These preparations and also ETF from monkey liver [50] have absorption maxima between 410 and 420 nm. It is possible that other flavoproteins also contain this flavin. Proteins which stabilize the green form of the 6-OH-flavin would show a characteristic band of absorption at long-wavelength. However the intensity of this band is low and small amounts of the flavin would not be detected easily. Similarly, in a protein such as flavodoxin, which stabilizes the yellow form of 6-OH-flavin, long-wavelength absorption would not be observed at neutral pH, and the peak at 420 nm would be masked by the absorption of unmodified flavin. In all such cases, 6-OH-flavin would only be detected if the spectrum of the protein-free extract was carefully examined at different pH values. Changing the pH from 6 to 9 would cause the appearance of a low band of long-wavelength absorption.

We are very grateful to Dr V. Massey for providing advice and facilities and to Drs G. Schöllnhammer and P. Hemmerich for discussing their unpublished work with us. This work was supported by USPHS Grant GM 11106 to Dr V. Massey, an American Cancer Society Fellowship (PF 670) to C.D.W., a grant from *Schweizerische Naturforschende Gesellschaft* to S. G. and American Cancer Society Fellowship (PF 640) to M.S.J.

REFERENCES

1. Matthews, R. G. & Massey, V. (1969) *J. Biol. Chem. 244*, 1779–1786.
2. Engel, P. & Massey, V. (1971) *Biochem. J. 125*, 889–902.
3. Massey, V. & Ganther, H. (1965) *Biochemistry, 4*, 1161–1173.

4. Massey, V. & Ghisla, S. (1973) *Proc. N. Y. Acad. Sci.*, in press.
5. Hemmerich, P. & Schuman-Jorns, M. (1973) in *Enzymes: Structure and Function* (Veeger, C., Drenth, J. & Osterbaan, R. A. eds) pp. 95–117, North-Holland, Amsterdam.
6. Schuman, M. & Massey, V. (1971) *Biochim. Biophys. Acta, 277*, 500–520.
7. Whitfield, C., Mayhew, S. & Massey, V. (1972) *Fed. Proc. 31*, 447.
8. Beinert, H. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H. & Myrbäck, K., eds) vol. 7, p. 467, Academic Press, New York.
9. Schöllnhammer, G. & Hemmerich, P. (1972) *Z. Naturforsch. 27b*, 1030–1031.
10. Schöllnhammer, G. & Hemmerich, P. (1974) *Eur. J. Biochem. 44*, 561–577.
11. Mayhew, S. G. & Massey, V. (1969) *J. Biol. Chem. 244*, 794–802.
12. Mayhew, S. G. (1971) *Biochim. Biophys. Acta, 235*, 289–302.
13. Engel, P. & Massey, V. (1971) *Biochem. J. 125*, 879–887.
14. Ghisla, S. & Mayhew, S. G. (1973) *J. Biol. Chem. 248*, 6568–6570.
15. Hemmerich, P., Veeger, C. & Wood, H. C. S. (1965) *Angew. Chem. Int. Ed. 4*, 671–687.
16. Foust, G. P., Burleigh, D. B., Jr., Mayhew, S. G., Williams, C. H., Jr & Massey, V. (1969) *Anal. Biochem. 27*, 530–535.
17. Bessey, O. A., Lowry, O. H. & Love, R. H. (1949) *J. Biol. Chem. 180*, 755–769.
18. Dudley, K. H., Ehrenberg, A., Hemmerich, P. & Müller, F. (1964) *Helv. Chim. Acta, 47*, 1354–1383.
19. Müller, F., Massey, V., Heizmann, C., Hemmerich, P., Lhoste, J. M. & Gould, D. C. (1969) *Eur. J. Biochem. 9*, 392–401.
20. Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S. & Hartmann, U. (1972) *Eur. J. Biochem. 26*, 267–278.
21. Ghisla, S. & Hemmerich, P. (1971) *FEBS Lett. 16*, 229–232.
22. Ghisla, S., Hemmerich, P., Hartmann, U. & Müller, F. (1972) *Helv. Chim. Acta*, in press.
23. Hemmerich, P., Ehrenberg, A., Walker, W. H., Eriksson, L. E. G., Salach, J., Bader, P. & Singer, T. P. (1969) *FEBS* Lett. *3*, 37–40.
24. Ghisla, S., Hemmerich, P. & Jefcoate, C. (1972) *J. Chem. Soc. Perkin Trans. I*, 1564.
25. Lauterwein, J. & Hemmerich, P. (1973) in *Inorganic Biochemistry* (Eichhorn, L. ed.) vol. 2, pp. 1168–1189, Elsevier Publishing Co., Amsterdam.
26. Benesi, H. A. & Hildebrand, T. A. (1949) *J. Am. Chem. Soc. 71*, 2703–2707.
27. 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.
28. Schuman, M. & Massey, V. (1971) *Biochim. Biophys. Acta, 227*, 521–537.
29. Mayhew, S. G. (1971) *Biochim. Biophys. Acta, 235*, 276–288.
30. Massey, V. & Palmer, G. (1962) *J. Biol. Chem. 237*, 2347–2358.
31. Massey, V., Matthews, R. G., Foust, C. P., Howell, L. C., Williams, C. H., Jr, Zanetti, G. & Ronchi, S. (1969) in *Pyridine-Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.) pp. 393–411, Springer Verlag, Heidelberg.
32. Massey, V. & Palmer, G. (1966) *Biochemistry, 5*, 3181–3189.
33. Andersen, R. D., Apgar, P. A., Burnett, R. M., Darling, G. C., Le Quesne, M. E., Mayhew, S. G. & Ludwig, M. L. (1972) *Proc. Natl. Acad. Sci. U. S. A. 69*, 3189–3191.
34. Mayhew, S. G. (1971) in *3rd Int. Symp. Flavins and Flavoproteins* (Kamin, H., ed.) pp. 187–205, University Park Press, Baltimore.
35. Karrer, P., Schöpp, K. & Benz, F. (1935) *Helv. Chim. Acta, 18*, 426–429.
36. Kuhn, R., Reinemunde, K., Weygand, F. & Ströbele, R. (1935) *Chem. Ber. 68*, 1765–1774.
37. Theorell, H. (1935) *Biochem. Z. 278*, 263.
38. Hendriks, R., Cronin, J. R., Walker, W. K. & Singer, T. P. (1972) *Biochem. Biophys. Res. Commun. 46*, 1262–1269.
39. Walker, W. H., Kearney, E. B., Seng, R. L. & Singer, T. P. (1971) *Eur. J. Biochem. 24*, 328–331.
40. Brühmüller, M., Möhler, H. & Decker, K. (1972) *Eur. J. Biochem. 29*, 152–155.
41. Hevesi, L. & Bruice, T. C. (1972) *J. Am. Chem. Soc. 94*, 8277–8278.
42. Walsh, C. T., Schonbrunn, A. & Abeles, R. H. (1971) *J. Biol. Chem. 246*, 6855–6866.
43. Porter, D. J. T., Voet, J. G. & Bright, H. H. (1973) *J. Biol. Chem. 248*, 4400–4416.
44. Brustlein, M. & Bruice, T. C. (1972) *J. Am. Chem. Soc. 94*, 6548–6549.
45. Hamilton, G. A. (1971) *Progr. Bioorg. Chem. 1*, 83–157.
46. Te Nijenhuis, B. & Berends, W. (1967) *Photochem. Photobiol. 6*, 491–494.
47. Müller, F. & Massey, V. (1969) *J. Biol. Chem. 244*, 4007–4016.
48. Robinson, J. C., Keay, L., Molinari, R. & Sizer, I. W. (1962) *J. Biol. Chem. 237*, 2001–2010.
49. Baker, A. L. & Tolbert, M. E. (1967) *Biochem. Biophys. Acta, 131*, 179–187.
50. Hoskins, D. D. (1966) *J. Biol. Chem. 241*, 4472–4479.

S. G. Mayhew's present address: Laboratorium voor Biochemie, Landbouwhogeschool, De Dreijen 11, Wageningen, The Netherlands

C. D. Whitfield and S. Ghisla, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A. 48104

M. Schuman-Jörns' present address: Department of Biochemistry, University of Texas Southwestern Medical School, Harry Hines Boulevard, Dallas, Texas, U.S.A. 75235