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8-Mercaptoflavins as Active Site Probes of Flavoenzymes*

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Representative examples of the various classes of flavoproteins have been converted to their apoprotein forms and the native flavin replaced by 8-mercapto-FMN or 8-mercapto-FAD. The spectral and catalytic properties of the modified enzymes are characteristically different from one group to another; the results suggest that flavin interactions at positions N(1) or N(5) of the flavin chromophore have profound influences on the properties of the flavoprotein.

1. The 8-thiolate anion form of 8-mercaptoflavin has an absorption maximum in the region 520 to 550 nm (ϵ ~30 mM^{-1} cm^{-1}). This form is retained on binding to flavoproteins whose physiological reactions involve obligatory one-electron transfers (*e.g.* flavodoxin, NADPH-cytochrome P-450 reductase). In the native form these enzymes stabilize the blue neutral radical of the flavin. A radical form of 8-mercaptoflavin is also stabilized by these proteins.

2. The *p*-quinoid form of 8-mercaptoflavin has an absorption maximum in the range 560 to 600 nm (ϵ ~30 mM^{-1} cm^{-1}). This form is stabilized on binding to flavoproteins of the dehydrogenase-oxidase class (*e.g.* glucose oxidase, D-amino acid oxidase, lactate oxidase, Old Yellow Enzyme). These same enzymes in their native flavin form stabilize the red semiquinone, and have a pronounced reactivity with sulfite to form flavin N(5)-sulfite adducts. These properties of the native enzyme, including the ability to react with nitroalkane carbanions, are not exhibited by the 8-mercaptoflavoproteins.

3. A group of flavoenzymes fails to conform strictly to the above classification, exhibiting some properties of both classes. These include the examples of flavoprotein hydroxylases and transhydrogenases studied.

4. The riboflavin-binding protein of hen egg whites binds 8-mercaptoriboflavin preferentially in the unionized state, resulting in a shift in pK from 3.8 with free 8-mercaptoriboflavin to $\geq$ 9.0 with the protein-bound form.

The previous paper (1) described the ready nucleophilic displacement of the chloro-substituent of 8-chloroflavins by thiolate anions, including sulfide, to produce 8-mercaptoflavins of very different chemical and spectral properties from those of the parent flavin. These results and those obtained by coupling of 8-chloro-FAD with the apoenzyme of lipoyl dehydrogenase (2), suggested that 8-chloro-FMN and 8-chloro-FAD could serve as useful probes of the flavin binding sites of flavoenzymes in several distinctly different ways. First, if the flavin binding site of a particular flavoprotein contained a cysteinyl residue close to the position where the benzene ring of the flavin were normally located, then one might expect that a covalent bond would be formed between the protein and the flavin, by displacement of the 8-chloro group, as was found with lipoyl dehydrogenase (2). If the 8-chloroflavin located itself into the flavin binding pocket without such covalent attachment to the protein, then a second type of probe would be possible. Its subsequent reactivity with free thiols could then be expected to indicate whether the 8-chloro position (and presumably the 8-methyl group of the native flavin) were exposed to solvent or buried within the polypeptide folding of the protein. Studies of this type will be presented in the next paper in this series.[1]

A different type of active site probe was also suggested by the spectral characteristics of 8-mercaptoflavins (1) and by their pH dependence. In analogy to the 8-hydroxyflavin chromophore (3), 8-mercaptoflavins could exist in two basically different tautomeric states (1). Also in their anionic form the negative charge could be located either on the thiolate sulfur, or could be delocalized to a variable extent into the aromatic system, and toward the electron-deficient pyrimidine functions N(1)–C(2)=O. Thus mercaptoflavins could serve as active site probes and, on the one hand, reflect the pK shifts induced by the protein upon the coenzyme. On the other hand they would also reflect the dipole environment and the positions of "action" of hydrogen bridges relative to the flavin at the active center, by showing if a specific tautomeric or mesomeric form is preferentially stabilized.

Such information in turn, could yield clues about the mode of catalysis in different classes of flavoenzymes and should be correlated with those parameters such as the nature of the radical form, or the stability of the sulfite addition products, which have been used previously for classifications of flavoenzymes (4). Some preliminary experiments pointing in this direction, and indicating that the concept might be valid, have been carried out earlier with 8-hydroxyflavins (5). The much better availability of 8-mercaptoflavins at the FAD, FMN, and riboflavin level, and their more pronounced and clear-cut spectral properties prompted us to extend such studies to a representative selection of flavoenzymes from different classes.

MATERIALS AND METHODS

Preparation of 8-Mercaptoflavins—The starting material for these flavins was 8-chlororiboflavin (6), a very generous gift from Dr. John P. Lambooy, University of Maryland. This was converted to 8-chloro-FAD by the partially purified FAD synthetase complex from *Brevibacterium ammoniagenes* following the general procedures of Spencer *et al.* (7), as described previously (2). When the flavin was required

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[1] V. Massey, manuscript in preparation.

at the FMN level, 8-chloro-FAD was hydrolyzed to 8-chloro-FMN by treatment with *Naja naja* venom.

The appropriate 8-mercaptoflavin was prepared just before use by reaction of 8-chloro-FMN or 8-chloro-FAD, buffered at pH 8 to 9, with 5 mM Na_2S. The course of the reaction was monitored at 520 nm and is accompanied by a color change from yellow to bright red (1). The resulting 8-mercaptoflavin is reasonably stable, especially at alkaline pH values. However, it was generally used the same day.

Preparation of 1,10-Ethano-7-methyl-8-chloroalloxazinium Chloride ("Bridged Flavin")—This compound can be prepared conveniently starting from 8-chlororiboflavin (6). The degradation of the ribityl side chain of the latter was carried out by an adaptation of the method of Fall and Petering (8) to synthesis in the 0.1 mmol range. After completion of the oxidation with $HClO_4$ the original procedure (8) was followed up to the step including the filtrations over charcoal; the filtrate was then adjusted rapidly with sodium carbonate to pH ~6, and a small excess of sodium borohydride was added in the dark and at 0°C. After 30 min of stirring at 0°C, a few drops of acetone were added, and the pH was adjusted to 4 to 5 with acetic acid. The brownish precipitate of the 7-methyl-8-chloro-10(2'-hydroxyethyl)isoalloxazine was filtered, washed thoroughly with water, and a little acetone, and then dried under vacuum. The product (yield approximately 50%) contains only minor amounts of impurities as judged from thin layer chromatography on Merck silica (glass) plates in the solvent mixture butanol:acetic acid:water, 4:3:1. The ring closure at the positions N(1) and N(10) was achieved by gentle heating of the 10(2'-hydroxyethyl) derivative in $SOCl_2$ according to the method of Knappe (9). The product, isolated as the chloride, was best used without recrystallization, but after extensive drying under vacuum at 25°C. It was chromatographically pure according to thin layer chromatography in the system described above, and its structure is confirmed by the absorption spectra (λ_{max} 374, 413 nm in acetonitrile) which are characteristic for this type of flavin cation (10).

Preparation of Flavoproteins and Apoproteins—Flavodoxin from *Megasphera elsdenii* (formerly *Peptostreptococcus elsdenii*) was prepared and converted to the apoprotein as described previously (11, 12). NADPH-cytochrome P-450 reductase was prepared from rat liver microsomes as described by Vermilion and Coon (13); it was converted to the FMN-free form (14) by a new procedure which will be published in detail later.[2] NADPH-adrenodoxin reductase from beef adrenal glands (15) was kindly given by Dr. T. Kimura, Detroit. The apoprotein was prepared by dialysis *versus* KBr following the principle of Massey and Curti (16). We are indebted to Mr. J. Parcells and Dr. T. Kimura for practical details (dialysis *versus* 2 M KBr in 0.1 M KP_i, pH 6.2). Lactate oxidase from *Mycobacterium smegmatis* was prepared according to Sullivan *et al.* (17) and the apoprotein according to Choong *et al.* (18). Pig kidney D-amino acid oxidase was prepared by the method of Curti *et al.* (19) and the apoprotein as described in Reference 16. Glucose oxidase was prepared from *Aspergillus niger* by the method of Swoboda and Massey (20, 21). Old Yellow Enzyme was prepared by affinity chromatography (22); details of apoprotein preparation are given in Reference 23. Oxynitrilase from almonds was prepared as described by Becker *et al.* (24); the apoprotein was prepared by an adaptation of the method of Strittmatter (25). (We are indebted to Dr. M. Jorns, Columbus, Ohio for practical details.) Melilotate hydroxylase (26) and *p*-hydroxybenzoate hydroxylase (27) were prepared by Dr. L. M. Schopfer and Dr. M. Husain. The apoprotein of melilotate hydroxylase was prepared as described by Strickland (28), that of *p*-hydroxybenzoate hydroxylase as described by Ghisla *et al.* (29). The electron transfer flavoprotein and D-lactate dehydrogenase, both from *M. elsdenii*, and their apoproteins were prepared as described in References 30 and 31, respectively. The riboflavin binding protein of hen egg whites was prepared in the apoprotein form as described by Becvar (32).

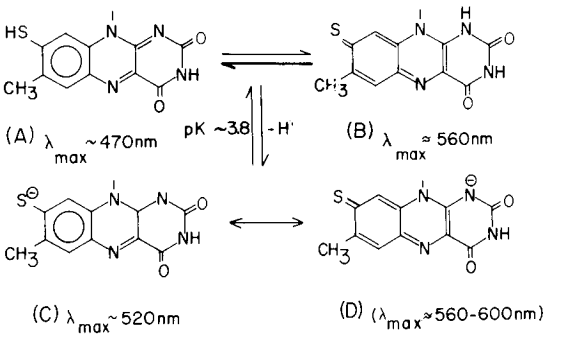
Reconstitution of Apoproteins with 8-Mercaptoflavin—The following general procedure was employed on a preparative scale. A solution of apoprotein, in a buffer appropriate to its stability (generally in the range pH 6 to 8.5) and kept in ice, was mixed with a 2- to 3-fold excess of freshly prepared 8-mercapto-FMN or 8-mercapto-FAD, depending on whether the native enzyme contains FMN or FAD. After keeping for several hours at ice temperature to ensure that the binding process is complete, the mixture was then passed through a Sephadex G-25 column (1.5 × 20 cm) equilibrated with the desired buffer. Where the stability of the apoprotein permitted it, for example with flavodoxin, D-amino acid oxidase, glucose oxidase, Old Yellow Enzyme, riboflavin binding protein, a solution of 8-mercaptoflavin was also titrated with apoprotein. In this manner estimates of K_d and extinction coefficients could be obtained.

[2] J. L. Vermilion, V. Massey, and M. J. Coon, manuscript in preparation.

RESULTS

Attribution of Spectral Properties to the Different Tautomeric and Mesomeric Forms of 8-Mercaptoflavin

In order to interpret the results obtained on binding 8-mercaptoflavins to specific apoproteins it is necessary to differentiate between the spectral properties of the various possible forms of the chromophore. As pointed out in the previous paper (1), 8-mercaptoflavins can exist in two tautomeric forms below the pK, and in various resonance stabilized forms in the anionic state (Scheme 1):



Structure A—This form is the one predominantly present in free solution at pH 3.8. This is demonstrated in a classical way by the close similarity of the mercaptoflavin spectra with those of 8-*S*-alkylflavins (1), and by their marked difference from the spectra of N(1)-, N(10)-substituted, "paraquinoid" 8-thioflavin (*cf.* below). Comparison with the spectra of 8-OH-, 8-O-alkyl, and 8=O,1-alkylflavins (3), leads to the same conclusion.

Structure B—Such a paraquinoid form should not exist as such in the free system, unless positions N(1) or O(2) are blocked by alkylation. Similar criteria have been applied for the parent 8-hydroxyflavin chromophore (3). As is the case with normal flavins, whether the position of alkylation is N(1) or O(2), there should be only minor effects on the chromophore. The spectrum of the 1,10-bridged 8-mercaptoflavin

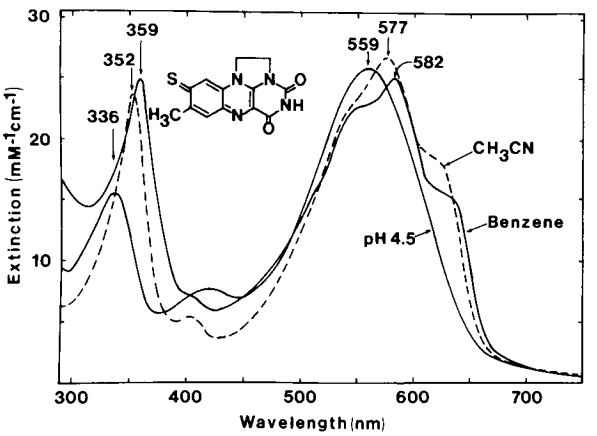
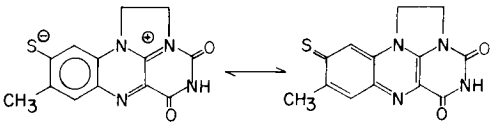


FIG. 1. **Effect of solvent on the spectrum of N(1),N(10)-bridged 8-mercaptoflavin.** The 8-mercaptoflavin was prepared in acetonitrile at 0°C by reaction of the corresponding 8-chloroquinonium salt with a slight excess of aqueous 1 M Na_2S. The reaction mixture was then diluted 1/20 into the solvents shown, and spectrum recorded with the appropriate spectral blank in the reference beam of a Cary 17 recording spectrophotometer. The aqueous sample at pH 4.5 was in 0.25 M sodium acetate buffer.

model (*structure inset*, Fig. 1) is drastically different in shape and wavelength maximum from that of the neutral 8-mercaptoflavin (1). This effect is not sufficient for a structural attribution, however, as the chromophore could exist, in addition to the postulated paraquinoid form (B), in a mesomeric one having zwitterionic character (Scheme 2):



The differentiation between these forms should be easily achieved by an evaluation of the dependence of the spectrum on the polarity of the solvent, as polar structures should be stabilized in polar solvents, and vice versa. Fig. 1, shows that in benezene, as compared to water, the energies of the main transitions are not markedly affected, only a better resolution and a small increase in the extinction of the main band in the near UV is observed. A closely similar spectral effect is observed (results not shown) upon binding of the bridged mercaptoflavin to riboflavin binding protein, a protein which strongly prefers binding of nonpolar species (32, 33).

It can thus be assumed safely that the nonpolar structure of Scheme 2 will best describe the electronic distribution of the bridged flavin chromophore. The pH dependence of its spectra reflects a pK ~7.5; at high pH the bands at 560 and 336 nm show a ~20 nm hypochromic shift (not shown). This ionization is attributed to deprotonation of N(3)-H (pK ~10 with normal flavins (34, 35)), and is within the expected range. In strong acid (5 N HCl), the bridged mercaptoflavin has a λ_{max} at 460 nm (ϵ ~26000 M^{-1} cm^{-1}). 8-Alkylthioflavins, under the same conditions, have an identical spectrum. Thus protonation of the 8-mercapto-1,10-bridged flavin chromophore occurs at position S(8), while 8-alkylmercaptoflavins are protonated at N(1), as with normal (36) and 8-hydroxyflavin (3).

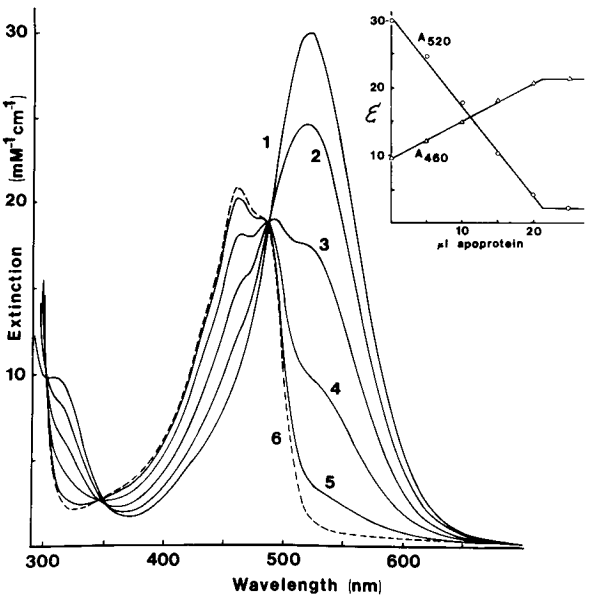


FIG. 2. **Change in absorption spectrum on titrating 8-mercaptoriboflavin with egg white riboflavin-binding protein.** 8-Mercaptoriboflavin was prepared from 8-chlororiboflavin as described under "Materials and Methods," and adjusted to pH 7 with a final concentration of 0.3 M potassium phosphate buffer. The 8-mercaptoriboflavin concentration was 4.77×10^{-5} M. On cooling to 4°C, 1.0 ml of this mixture (*curve 1*) was titrated with 5-μl aliquots of 2.2 mM riboflavin-binding protein (*curves 2* to *6*). The concentration of binding protein was determined in separate experiments by titration of riboflavin. *Inset*, the change in extinction coefficient at 460 nm and 520 nm on addition of binding protein.

Structures C and D—These are valence bond representations of the most likely resonance contributors of the 8-mercaptoflavin anion. In free solution the most likely situation is a charge distribution which will be intermediate between the canonical forms C and D. However, when protein-bound, it would be possible that either of these forms might be favored, depending on the particular microenvironment provided by the protein. The spectral properties of these resonance contributors cannot therefore be deduced in such a clear-cut way as with the tautomeric forms, A and B. The ascriptions we have given are based on the following reasoning. With the analogous 8-hydroxyflavin, a paraquinoid structure such as D is very likely (3). Sulfur, however, is less prone to enter π conjugation, as compared to the elements of the first period, and this would favor, in free solution, a "phenolate" type structure (C). That this assumption might indeed describe the state of electron distribution, at least qualitatively, is borne out by two sets of spectral evidence. First, the spectrum of 8-mercaptoflavin shows on ionization a shift which is usual for the effects obtained upon deprotonation of aromatic thiols. The spectrum of the anion resembles both in its shape and λ_{max} (see in particular the near-UV region) more the phenolic spectrum of Structure A, than that of B, while with 8-hydroxyflavin the reverse is true.

Second, apoflavodoxin from *M. elsdenii* has been shown to stabilize the phenolate mesomer of 8-hydroxy-FMN (3), the pK of the 8-OH function being increased from 4.8 to 6.1 in the complex (5). Binding of 8-mercapto-FMN anion to the same apoflavodoxin causes only a minor shift of the lowest energy transition, also practically no changes in the spectral shape are observed (*cf.* later section). As the apoenzyme of flavodoxin appears to stabilize the phenolate form of 8-hydroxyflavin (of which the paraquinoid form is favored in solution), the same effect appears even more probable in the case of 8-mercaptoflavin, where the sulfur atom should also favor stabilization of the phenolate form.

Hence we ascribe the type of spectrum exhibited on binding to apoflavodoxin to the phenolate form. The ascription of the *p*-quinoid spectrum is based on comparison to those of the neutral bridged flavin shown in Fig. 1 and those obtained with a number of apoflavoproteins (see later sections). These 8-mercaptoflavoproteins not only have absorption maxima in the region of 600 nm, giving them a spectacular blue color, but they also show the distinctively three-banded visible spectrum of the *p*-quinoid bridged flavin.

Binding of 8-Substituted Riboflavins to Egg White Riboflavin-binding Protein

When 8-mercaptoriboflavin at pH 7 is mixed with the

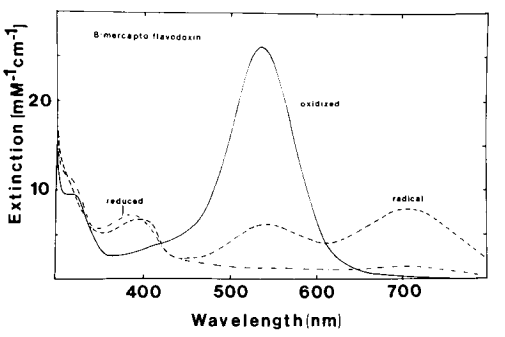


FIG. 3. **Spectra of the oxidized, semiquinoid, and fully reduced forms of 8-mercapto-FMN bound to the apoprotein of *M. elsdenii* flavodoxin.** The radical and fully reduced forms were made anaerobically by the photochemical reducing system employing a catalytic amount of 5-deazaflavin (37).

riboflavin-binding protein of egg whites there is a dramatic change in color from red to yellow. The binding is very tight ($K_d < 10^{-8}$ M), giving a sharp titration of the flavin with the apoprotein (Fig. 2). The change in absorption spectrum appears to be due to the vastly preferential binding by riboflavin-binding protein of 8-mercaptoriboflavin in the neutral, unionized form A (Scheme 1). That this is the case, rather than, for example, oxidation of the flavin to the 8-sulfonate or the disulfide is shown in a number of ways. First, the 8-mercaptoflavin is released in 3 M guanidine, pH 7; it is released quantitatively in the original 8-thiolate anion form (λ_{max} in 3 M guanidine, 530 nm, ϵ_{530} = 28 mM^{-1} cm^{-1}). Secondly, on increasing the pH, spectral changes of the type expected for ionization of the 8-thiol are observed. These effects will be detailed in a later paper, along with similar ones observed for 8-hydroxyriboflavin. In both cases, the pK of the 8-substituted flavin is shifted considerably (pK $\geq$ 9) on binding to the protein.[3]

Binding of 8-Mercapto-FMN to Flavodoxin

On titration of 8-mercapto-FMN with the apoprotein of *M. elsdenii* flavodoxin there is only a minor shift in the absorption spectrum from 520 nm to 535 nm for the protein-bound species (Fig. 3). The titration at pH 8.5, 25°C, gives a sharp endpoint; since the concentration of apoprotein is known, the results yield extinction coefficients for the free and protein-bound 8-mercapto-FMN, as well as an estimate of the K_d value of the association. The extinction coefficients so obtained are 30,000 M^{-1} cm^{-1} for 8-mercapto-FMN, 26,000 M^{-1} cm^{-1} for 8-mercaptoflavodoxin, and a $K_d < 10^{-8}$ M. The tight binding is also evidenced by the fact that the reconstituted protein may be chromatographed on Sephadex G-25 without loss of flavin. The modified flavoprotein, like the native protein, is readily reduced photochemically by the 5-deazaflavin radical-generating system of Massey and Hemmerich (27). Fig. 3 shows the spectrum of the intermediate stable radical form, with a wavelength maximum at 710 nm, which is produced with short irradiation times. The radical also has a maximum around 400 nm; the absorbance in the 500 to 600 nm region shown in Fig. 3 is however complicated by the presence of small equilibrium amounts of the oxidized flavin. On further irradiation the radical disappears; the spectrum of the fully reduced flavin has but a single band in the visible, with a maximum around 380 nm. On aeration of the reduced species the original oxidized spectrum is rapidly regained, with substantial amounts of intermediate radical also being observed. The 8-mercaptoflavin derivative of *Clostridium* sp flavodoxin has also been prepared, and shows very similar properties to those reported above.

Binding of 8-Mercapto-FMN to Cytochrome P-450 Reductase

Cytochrome P-450 reductase is unusual among the simpler flavoproteins in possessing both FMN and FAD as prosthetic groups (38, 13). The FMN may be removed without appreciable loss of FAD to yield the so-called "FMN-depleted" enzyme, and a variety of FMN derivatives may be used to reconstitute catalytically active enzyme (39). This technique has been useful in the identification of FMN as the flavin with high oxidation-reduction potential, and the one responsible for the air-stable semiquinone (14, 39). In experiments in collaboration with Drs. Janice L. Vermilion and Minor J. Coon, which will be reported in full elsewhere, we have also reconstituted FMN-depleted enzyme with 8-mercapto-FMN. The results are shown in Fig. 4 and Table I. As was the case

[3] V. Massey, unpublished data.

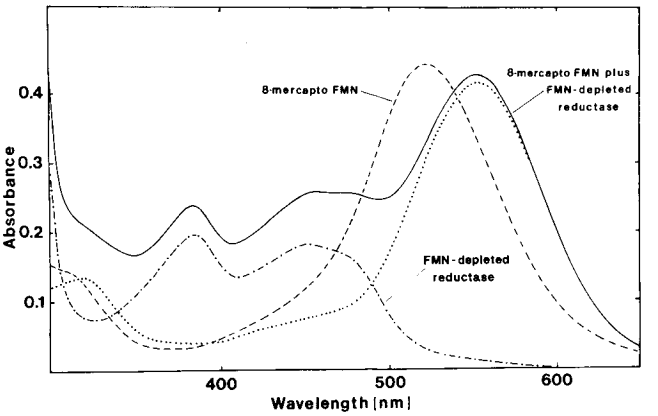


FIG. 4. **Binding of 8-mercapto-FMN to FMN-depleted NADPH-cytochrome P-450 reductase (work in collaboration with Dr. Janice L. Vermilion and Dr. Minor J. Coon).** The FMN-depleted reductase (see "Materials and Methods") in a mixture of 0.2 M phosphate, 10% v/v glycerol, 10^{-4} M EDTA, and 0.1% sodium deoxycholate, pH 7.4 (-·-) was mixed with a slightly substoichiometric amount of 8-mercapto-FMN to yield the *solid curve*. For comparison the spectrum of the same concentration of free 8-mercapto-FMN is shown (- - -). The *dotted line* shows the calculated contribution of the enzyme-bound 8-mercapto-FMN, assuming that the spectrum of the bound FAD is not perturbed from that of the starting enzyme.

with flavodoxin the spectrum of the 8-mercaptoflavin was merely shifted to longer wavelengths on binding to the protein. Like the flavodoxin derivative, the 8-mercaptoenzyme was also converted readily to a radical species, with an absorption maximum at 720 nm. This radical species was produced either photochemically or by reduction with NADPH.[4]

Binding of 8-Mercapto-FAD to Adrenodoxin Reductase

The steroid hydroxylation system of the adrenal gland involves a flavoprotein, NADPH-adrenodoxin reductase, which serves to funnel reducing equivalents from NADPH to adrenal cytochrome P-450 via the one-electron acceptor/donor, adrenodoxin (40). Despite the obligatory 1-electron transfer which must accompany the reaction of the reduced flavoprotein with adrenodoxin, no stabilized radical form of the flavoprotein has ever been observed (41). It was therefore of interest to replace the native flavin of this enzyme by 8-mercapto-FAD. The results are summarized in Table I. The spectral properties of the 8-mercaptoflavin are those of the *p*-quinoid mesomer, and the 8-mercaptoenzyme is rapidly reduced by NAD(P)H or photochemically. In neither case was any long wavelength species observed. The possible significance of these results is considered under "Discussion."

Binding of 8-Mercapto-FAD to D-Amino Acid Oxidase

In contrast to two previous cases, the binding of 8-mercapto-FAD to the apoprotein of D-amino acid oxidase results in dramatic changes in the visible spectrum (Fig. 5). The 535 nm band of 8-mercapto-FAD is shifted to 595 nm, giving a spectacular change in color from red to blue. Particularly notable also are the strong intensification and much better resolution of the two transitions in the 320 to 430 nm region. The similarity of the spectrum of 8-mercapto-D-amino acid oxidase to that of the bridged flavin of Fig. 1 is quite striking, suggesting that on binding to the apoprotein, the 8-mercaptoflavin is stabilized in its *p*-quinoid form. As in the case of flavodoxin, the availability of stable apoprotein of known

[4] J. L. Vermilion, V. Massey, and M. J. Coon, unpublished observations.

TABLE I

Selected properties of native and 8-mercapto-substituted flavoproteins

Enzyme	Classification[a]	Native radical	8-Mercaptoflavoprotein			
			Spectral properties λ_{max} (ϵ, $mM^{-1}cm^{-1}$)	Radical	Reduction by substrate	Photochemical reduction
Flavodoxin	Pure electron transferase	Neutral[b]	~320 (9.6) 400 (Sh) 535 (26)	710 (~10)	N.D.[c]	Facile
NADPH-cytochrome P-450 reductase	Dehydrogenase-electron transferase	Neutral[d]	320 (9.1) 552 (29.1)	720 (7.6)	Fast	Facile
NADPH-adrenodoxin reductase	Dehydrogenase-electron transferase	None stabilized[e]	330 (extinction coef- 445 ficients not de- 580 termined)	None observed	Fast	Facile
D-Amino acid oxidase	Dehydrogenase-oxidase	Anion[f]	342 (12.0) 366 (13.5) 432 (6.9) 595 (29)	None observed	No reduction even in presence of semicarbazide	Very slow
L-Lactate monooxygenase	Dehydrogenase-oxidase	Anion[g]	364 (14) 441 (7.1) 607 (29.1) 655 (22.3) (resolved shoulder)	None observed	Very slow	No reaction
Glucose oxidase	Dehydrogenase-oxidase	Neutral or anion[f] (pK 7.3)	350 Sh (14) 368 (16.6) 438 (8.7) 608 (28.6)	None observed	pH-dependent with 2-deoxyglucose; no reduction with nitromethane anion	No reaction
Old Yellow Enzyme	Function unknown (dehydrogenase-oxidase?)	Anion[h]	335 (11.5) 460 (5.3) 598 (28.5)	None observed	None	Very slow
Oxynitrilase	No known oxidation-reduction function	Anion[f]	350 Sh (extinction 440 coefficients not 605 determined)	None observed		Slow
Melilotate hydroxylase	Dehydrogenase-oxygenase	None stabilized[i]	335 (11.3) 450 Sh (6.3) 565 (25.8) (spectrum perturbed by substrate)	None observed	NADH, medium fast, dependent on melilotate	Facile
p-Hydroxybenzoate hydroxylase	Dehydrogenase-oxygenase	Neutral,[j] but only in presence of certain substrates	320 Sh (extinction 450 Sh coefficients not determined) 547 (perturbed by substrate)	None observed except in presence of tetrafluoro-*p*-hydroxybenzoate (740 nm)	NADPH, medium fast, dependent on *p*-hydroxybenzoate	Facile
D-Lactate dehydrogenase (*M. elsdenii*)	Ambiguous (transhydrogenase?)	Neutral,[k] but only kinetic stabilization	330 (16.0) 450 (10.8) 580 (28.3)	None observed	Rapid	Facile
Electron transfer flavoprotein (*M. elsdenii*)	Ambiguous (transhydrogenase?)	Anion[l]	350 Sh (extinction 460 coefficients not 595 determined)	N.D.	N.D.	N.D.

[a] The classification is based on the suggestions of Hemmerich (60, 61) for a more logical classification of flavoproteins.
[b] Ref. 11.
[c] N.D., not determined.
[d] Ref. 38.
[e] Ref. 41.
[f] Ref. 46.
[g] Ref. 4.
[h] Ref. 62.
[i] Ref. 26.
[j] M. Husain, B. Entsch, D. Ballou, P. Chapman, and V. Massey, unpublished.
[k] Ref. 31.
[l] Ref. 30.

concentration allowed the estimation of extinction coefficients and dissociation constant. The extinction coefficient of 8-mercapto-FAD at its maximum (535 nm) was estimated to be 29,700 M^{-1} cm^{-1} and that of 8-mercapto-D-amino acid oxidase (λ_{max} 595) to be 29,000 M^{-1} cm^{-1}. The binding of 8-mercapto-FAD is tighter than that of the native FAD; while the K_d value for FAD at pH 8.5, 20°C is ~5×10^{-7} M (42), the K_d for 8-mercapto-FAD under the same conditions is 3×10^{-8} M. The modified flavoenzyme is without catalytic activity, due to the fact that the flavin is not reduced by typical substrates such as D-alanine, even on incubation under anaerobic conditions for periods as long as 24 h. The failure of the flavin to be reduced by substrate does not appear to be due to an unfavorable oxidation-reduction equilibrium, since reduction is not observed even in the presence of an imino acid/keto acid trap such as semicarbazide. On the other hand the enzyme is reduced slowly by the 5-deazaflavin photochemical system, but without any observed intermediate which could be ascribed to a radical state.

A characteristic feature of native D-amino acid oxidase is its strong binding of aromatic carboxylic acids such as benzoate, which result in spectral changes typical of a hydrophobic environment (43). 8-Mercapto-D-amino acid oxidase also binds benzoate, but with a K_d value several orders of magnitude higher; at pH 8.6, 20°C, this is 4×10^{-3} M compared to the value of 2×10^{-6} M for native enzyme (44). The binding of benzoate to the 8-mercaptoenzyme also results in pronounced spectral changes, particularly the resolution of the shoulder

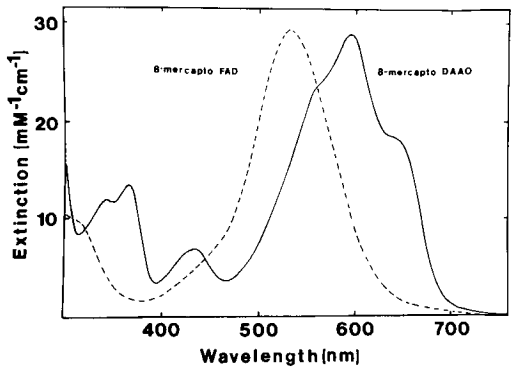


FIG. 5. **Spectral change on binding 8-mercapto-FAD to the apoprotein of D-amino acid oxidase.** The experiment was carried out at 20°C by titration of 8-mercapto-FAD (6.7×10^{-6} M) in 0.02 M pyrophosphate, pH 8.6, with apo-D-amino acid oxidase (*DAAO*). The titration yielded a K_d of 3.1×10^{-8} M and the extinction coefficients shown (see text for more details). Only the initial and final spectra are shown.

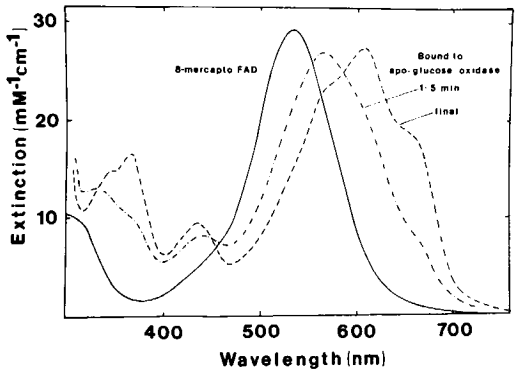


FIG. 6. **Spectral changes accompanying the binding of 8-mercapto-FAD to the apoprotein of glucose oxidase.** The experiment was carried out in 0.02 M pyrophosphate, pH 8.6, 4°C, by mixing 2.7×10^{-5} M 8-mercapto-FAD (*solid curve*) with an excess (7.2×10^{-5} M) of apoglucose oxidase. The spectrum (*curve* -·-) was recorded immediately (1 min at start, 5 min at end) and after the binding reaction was complete, 12 h later (curve - - -).

at ~650 nm. Adding a very high concentration of benzoate (~0.5 M) results in a slow release of the bound flavin. This was shown by return of the spectrum of free 8-mercapto-FAD and loss of the flavin on gel filtration with Sephadex G-25.

8-Mercapto-Glucose Oxidase

When glucose oxidase apoprotein is mixed with 8-mercapto-FAD there is an instantaneous color change from red to magenta, followed by a much slower change to a bright blue. Fig. 6 shows the spectrum of starting 8-mercapto-FAD, that of the intermediate immediately after mixing with apoprotein, and the final stable spectrum which is reached with a $t_{1/2}$ of ~7 min at 25°C and of ~60 min at 4°C. A similar biphasic binding of FAD to apoglucose oxidase has been reported by Swoboda (45). With the native enzyme, however, the spectral changes accompanying the two-stage reaction are subtle, as opposed to the extensive changes obvious with 8-mercapto-FAD. Qualitatively similar phenomena, although less marked, have been found with practically every apoenzyme-8-mercaptoflavin interaction which we have studied. With glucose oxidase the results suggest that 8-mercapto-FAD is bound largely as its thiophenolate mesomer, and that in a slower, probably conformational change, similar to that with native enzyme, the *p*-quinoid mesomer is favored. Glucose oxidase is unusual among the flavoproteins in exhibiting a distinct pK for the semiquinone state, with a value of pH 7.3 (46, 47). This is 1.2 pH units lower than the pK of the flavin semiquinone free in solution (48), implying interaction with a protein base in an ion pair, which would in turn perturb the pK of the protein base by 1.2 pH units higher. Interaction with this base would also be expected to lower the pK of 8-mercapto-FAD by a similar amount. Consistent with this is the fact that the same final spectrum as shown in Fig. 6 is maintained over the whole pH range where the protein is stable, to a pH as low as 3.4, *i.e.* to a pH lower than that of the pK of free mercapto-flavin, pH 3.8 (1).

The modified enzyme is reduced anaerobically by glucose and 2-deoxyglucose. The reduction rate is markedly dependent on pH, in contrast to the reduction of native enzyme, which is practically independent of pH over the range pH 3 to 8 (49). Thus, with 25 mM 2-deoxyglucose, 15°C, the observed reduction rates were 0.18, 1.7, and 10.3 min^{-1} at pH values of 8.4, 7.0, and 5.5, respectively. These results are clearly preliminary, since a study in which the concentration of 2-deoxyglucose is also varied is necessary to define true k_{red} values. However, the trend suggests that the flavin reduction requires the uptake of one proton, *i.e.* that the reaction with substrate requires the unionized form of the flavin.

Stable carbanions such as the nitroalkane carbanions have been found to be good substrates (and therefore good reductants) of native enzyme (50). However, 8-mercaptoglucose oxidase appears to be completely resistant to reduction by such reagents. At pH 7, 15°C, 10 mM nitromethane carbanion had absolutely no effect on the spectrum under anaerobic conditions over a period of 20 h. This is presumably due to charge repulsion by the *p*-quinoid mesomer, actually preventing binding of the carbanion. Similar charge repulsion may account for lack of photoreduction of the 8-mercaptoenzyme by the photochemical 5-deazaflavin radical generating system.

8-Mercapto-Lactate Oxidase

Lactate monooxygenase, although formally catalyzing an oxidative decarboxylation, is really a typical flavoprotein oxidase, being more properly termed an adventitious monooxygenase (51). In keeping with this classification, the native enzyme shows very facile addition of sulfite to the N(5)

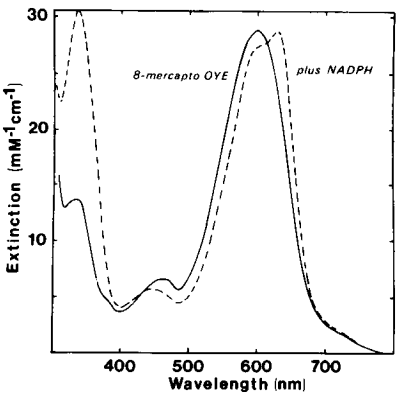


FIG. 7. **Effect of NADPH on Old Yellow Enzyme whose native flavin has been replaced by 8-mercapto-FMN.** The 8-mercaptoenzyme was prepared by addition of a 2-fold excess of 8-mercapto-FMN to the apoprotein followed by gel filtration through Sephadex G-25 equilibrated with 0.02 M pyrophosphate, pH 8.6, to separate the 8-mercaptoenzyme from excess flavin. A solution of this enzyme, 3.5×10^{-6} M with respect to bound flavin (*solid curve*) was mixed anaerobically with 1×10^{-5} M NADPH (*dashed curve*). No further change occurred over a period of 1 h. The *dashed curve* also includes the contribution of excess NADPH. *OYE*, Old Yellow Enzyme.

position (4) and stabilizes, under the influence of artificial reductants, the red anionic semiquinone. In keeping with the results obtained with D-amino acid oxidase and glucose oxidase, it also gives a dramatic shift of absorption spectrum to longer wavelengths when the apoprotein is mixed with 8-mercapto-FMN. The spectral characteristics are listed in Table I. It is reduced very slowly by L-lactate; at pH 7.0, 25°C, with 12.5 mM L-lactate the $t_{1/2}$ for reduction was approximately 70 min. The corresponding $t_{1/2}$ for native enzyme under the same conditions is approximately 100 ms; thus, the 8-mercaptoenzyme is reduced approximately 50,000 times slower than native enzyme. Like the 8-mercaptoflavin-substituted forms of D-amino acid oxidase and glucose oxidase, 8-mercaptolactate oxidase is also quite resistant to photochemical reduction.

8-Mercapto-Old Yellow Enzyme

The Old Yellow Enzyme of brewer's yeast is a tantalizing enzyme. It is the first discovered and purified flavoprotein (52) but to date its physiological function remains vague. Its only known catalytic activity is the oxidation of NADPH, employing O_2 or artificial electron acceptors as the oxidizing substrate. Stopped-flow kinetic studies have indicated that the low turnover number of the enzyme with O_2 as acceptor ($\sim 40\ min^{-1}$) is because of the rate-limiting reduction of the enzyme flavin by NADPH (53). The reduced enzyme reacts quite rapidly with O_2, without any observable production of flavin semiquinone but with formation of O_2^- (54). On reduction with dithionite, or photochemically (46) the red anion semiquinone is also stabilized similar to many other oxidases (4). However, unlike most other oxidases, it does not form a flavin N(5) adduct with sulfite (4). In view of the results presented above, it was particularly interesting to investigate the properties of the enzyme with 8-mercapto-FMN bound in place of the native FMN. The apoprotein binds 8-mercapto-FMN very tightly, with a shift in the absorption maximum from 520 nm to 595 nm (Fig. 7). The K_d value of the association at pH 8.6, 25°C is $\leq 10^{-8}$ M, and the extinction at 595 nm is determined to be 29,000 $M^{-1}\ cm^{-1}$. The spectrum of the artificial enzyme has many of the characteristics of those described for D-amino acid oxidase, glucose oxidase, and lactate oxidase. Thus its maximum is near 600 nm and the 450 nm band is clearly resolved. However, the near-UV band is nowhere near so intense or resolved as it is with the other oxidases; furthermore, it is not shifted so far to longer wavelengths. The other feature of the spectrum which is distinctively different is that the lowest energy band is quite smooth and unresolved. Nevertheless, 8-mercapto-Old Yellow Enzyme appears to belong to the oxidase classification, since NADPH merely perturbs the spectrum, indicating binding, but does not result in reduction, even over a long period, or when added in considerable excess of that shown in the experiment of Fig. 7. From the spectral changes induced by NADPH, it can be estimated that the K_d for this interaction is less than 10^{-5} M. This is at least an order of magnitude tighter binding than that found with native enzyme where a K_d of 9×10^{-5} M was found under similar conditions (53).

Another characteristic feature of Old Yellow Enzyme is its propensity to form intensely absorbing charge transfer complexes with a wide variety of phenolate anions (55). In these complexes the oxidized flavin was shown to be the charge transfer acceptor and the phenolate anion the charge transfer donor (23). In a study of Old Yellow Enzyme where the native flavin was replaced by 8-hydroxyflavin, it was shown that phenolates, although binding to the enzyme at all pH values tested, would only give charge transfer absorption with the neutral *p*-quinoid form of the bound 8-hydroxyflavin (5). With the *p*-quinoid form of 8-mercapto-FMN, with the negative charge of the anion localized around the N(1) position of the flavin, no charge transfer absorption would be expected on binding phenolate anion. This prediction is borne out by the experimental results shown in Fig. 8. Pentafluorophenolate, which binds with great avidity to native enzyme ($K_d \sim 10^{-7}$ M) producing an intense charge transfer absorption band (23), binds much more weakly to 8-mercapto-Old Yellow Enzyme ($K_d \sim 10^{-3}$ M), the binding merely resulting in a spectral perturbation similar to that found with NADPH. Indeed, similar spectral changes are also observed with *p*-methoxybenzaldehyde, which cannot form a charge-transfer complex with native enzyme because it lacks the phenolate function (23). In this case the binding is tighter to 8-mercaptoenzyme ($\ll$1 mM) than to native enzyme ($\sim$1 mM).

8-Mercapto-FAD Bound to the Apoproteins of Melilotate Hydroxylase and p-Hydroxybenzoate Hydroxylase

The flavoprotein external monooxygenases or hydroxylases, beside differing in catalytic function from the flavoprotein

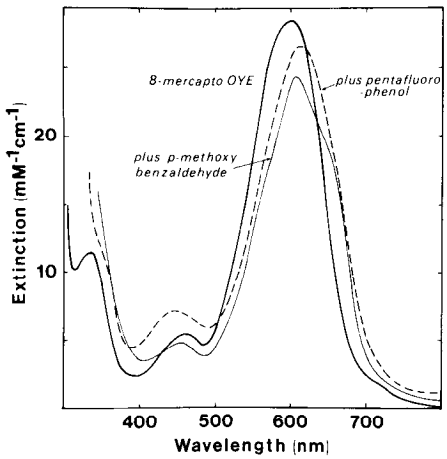


FIG. 8. **The effect of known ligands of Old Yellow Enzyme on the spectrum of the 8-mercaptoenzyme (*heavy solid curve*).** The *dashed curve* shows the spectrum in the presence of 4 mM pentafluorophenol; the *light solid curve* shows the spectrum in the presence of 1 mM or 2 mM *p*-methoxybenzaldehyde. Conditions as in Fig. 7. *OYE*, Old Yellow Enzyme.

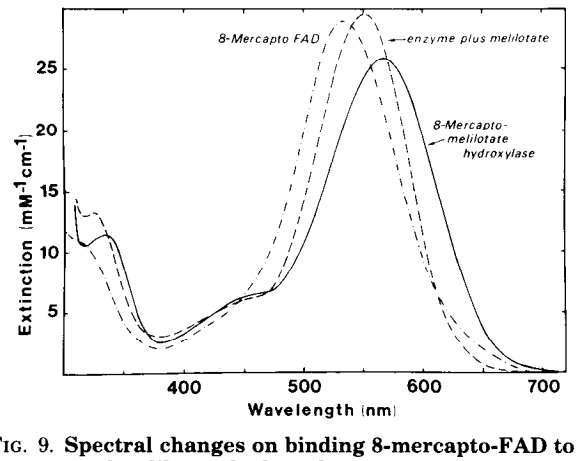


FIG. 9. **Spectral changes on binding 8-mercapto-FAD to the apoenzyme of melilotate hydroxylase.** 8-Mercapto-FAD in 0.1 M phosphate, pH 7.3, 25°C (-·-), was titrated with apoenzyme until no further spectral change occurred (*solid line*). The *dashed line* shows the subsequent change occurring on the addition of 5 mM 2 hydroxyphenylpropionate (melilotate). Almost the same spectrum was found with 0.5 mM melilotate.

oxidases, also differ in several other characteristic ways. The ability of the oxidases to stabilize the red anionic flavin semiquinone and to facilitate the formation of N(5)-sulfite adducts has long been known (4); the hydroxylases in general however have neither of these properties (see Refs. 51 and 56 for recent reviews). Despite considerable variation of conditions, no trace of radical has ever been observed, even transiently, with melilotate hydroxylase (Reference 26).[5] With *p*-hydroxybenzoate hydroxylase, trace amounts of the blue, neutral radical have been observed in the presence of the substrate, 2,4-dihydroxybenzoate (57). More recently, very substantial stabilization of the neutral radical has been observed in the presence of tetrafluoro-*p*-hydroxybenzoate.[6] It was therefore of interest to examine the behavior of these enzymes on binding with 8-mercapto-FAD. Fig. 9 summarizes the changes occurring on binding the flavin to apomelilotate hydroxylase. There is no gross change in the spectrum, the wavelength maximum merely being shifted from 535 nm for 8-mercapto-FAD to 565 nm on binding to the protein. The binding is quite tight ($K_d < 10^{-8}$ M). The modified enzyme, like the native enzyme, also shows marked perturbation of its spectrum on binding with its substrate, melilotate (2-hydroxyphenylpropionate). The 8-mercaptoenzyme is readily reduced by NADH when melilotate is bound, although the reduction rate is much lower than with native enzyme. Thus $t_{1/2}$ values in the range of minutes rather than milliseconds are found. Like with native enzyme, The 8-mercaptoenzyme is very readily reduced photochemically, without any sign of intermediate radicals. We are indebted to Mr. L. M. Schopfer for many of these observations.

With *p*-hydroxybenzoate hydroxylase, qualitatively similar results were found as with melilotate hydroxylase. They are summarized in Table I and show in this case a shift in the absorption spectrum to longer wavelengths on binding with *p*-hydroxybenzoate. Again the 8-mercaptoenzyme is reduced by its other substrate, NADPH, although more slowly than is native enzyme. Again, like with native enzyme, complex formation with *p*-hydroxybenzoate enhances considerably the rate of reduction. In similarity to melilotate hydroxylase, the 8-mercaptoenzyme is also reduced readily by the photochemical system of Massey and Hemmerich (37), but without any sign of radical intermediate either in the free state or when in complex with *p*-hydroxybenzoate. In marked contrast are the results when tetrafluoro-*p*-hydroxybenzoate is present; an intermediate absorbing maximally at 750 nm is now seen before full reduction is reached. Thus, for whatever reason this compound induces stabilization of the neutral flavin radical in the native enzyme, it does so also with 8-mercapto-FAD bound at the active center instead of FAD.

8 Mercapto-Oxynitrilase

Oxynitrilase is an unusual enzyme among the flavoproteins in having no known direct oxidation-reduction role (it catalyzes the reversible condensation of aldehydes and cyanide to form nitriles (24)). Nevertheless, it does stabilize the red anion flavin radical on artificial reduction (46) and it does facilitate the attack of sulfite at the flavin N(5) position (4). In these respects it behaves like a typical oxidase. It was therefore of considerable interest to determine if it behaved like the other oxidases described in this study when bound with 8-mercapto-FAD. Indeed it does; the color of the resulting protein is of the resolved blue type, with distinct maxima at 440 and 605 nm, and a peak at 350 obscured by light scattering (Table I).

[5] L. M. Schopfer, personal communication.

[6] M. Husain, B. Entsch, D. Ballou, P. Chapman, and V. Massey, unpublished observations.

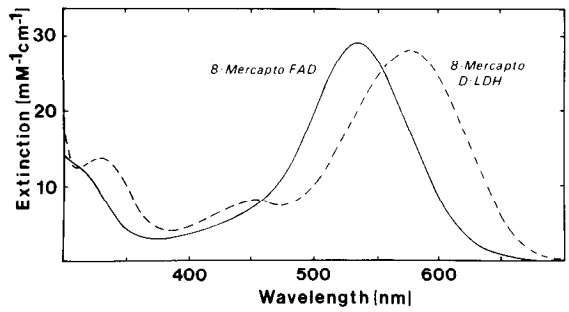


FIG. 10. **Spectral changes accompanying the binding of 8-mercapto-FAD to the apoprotein of the D-lactate dehydrogenase of *M. elsdenii*.** *Solid line*, 8-mercapto-FAD in 0.1 M phosphate, pH 7.0; *dashed line*, after addition of a small excess of apoprotein and $ZnCl_2$, and gel filtration through Sephadex G-25. *D-LDH*, D-lactate dehydrogenase.

The 8-mercaptoenzyme is reduced only with difficulty in the photochemical system employed; no radical was observed (*cf.* Table I).

D-Lactate Dehydrogenase

This enzyme from *M. elsdenii* (58) uses D-lactate as reducing substrate, and oxidized electron transfer flavoprotein as acceptor. The latter enzyme in turn transfers its reducing equivalents to another flavoprotein, butyryl-CoA dehydrogenase (59). By comparison to the other flavoenzymes of Table I the 8-mercapto form of D-lactate dehydrogenase is quite ambiguous in its behavior. The spectral changes accompanying its binding are shown in Fig. 10. While the spectrum has superficially many of the features found with the unequivocal oxidases (long wavelength shift of the 535 nm and 310 nm bands) (Table I), the lack of resolution of the spectrum, and the ratio of the absorption band intensities are comparable to those of the thiolate-type enzymes. Also unlike other members of the dehydrogenase-oxidase class, the 8-mercaptoenzyme is readily reduced by substrate, and photochemically. In neither case is any radical observed.

Electron Transferring Flavoprotein

The 8-mercapto Electron Transferring Flavoprotein behaves according to the pattern found with other flavoproteins where the anion radical is stabilized in the native protein (Table I). Thus it has absorption maxima at 350, 460, and 595 nm, is not readily photo-reduced, and no radical is observed.

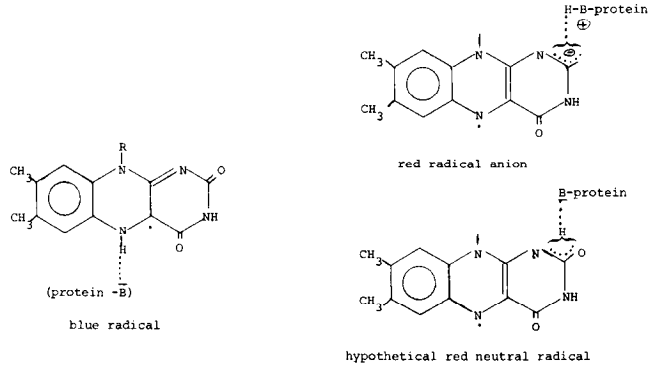
DISCUSSION

From the results presented it is clear that 8-mercaptoflavins offer the possibility of being very sensitive active site probes for flavoproteins. The differences in spectral properties on binding to different flavoproteins are quite remarkable. Quite remarkable also is the correlation of the properties of the bound 8-mercaptoflavin with the function of the flavoprotein studied. This correlation is laid out in Table I. As the number of examples is perforce limited, the conclusions drawn below are clearly only tentative; their validity or general applicability can only be ascertained by testing with many more flavoproteins.

The first conclusion is that in general there is a close correlation between the properties of the 8-mercaptoflavoprotein and the properties of the semiquinoid forms of the native proteins. Thus flavoproteins which in their native state stabilize the red anion radical of FMN or FAD exhibit a blue color when bound with 8-mercapto-FMN or 8-mercapto-FAD; with spectra resembling that of the *p*-quinoid bridged flavin (Fig. 1). These 8-mercaptoflavoproteins are in general difficult

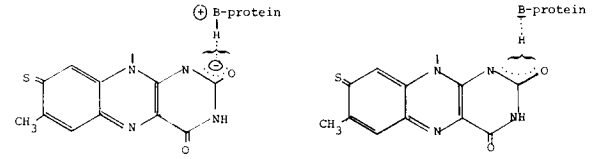
to reduce, and in no case has any stabilization of an 8-mercaptoflavin radical been observed. On the other hand those flavoproteins which stabilize the blue neutral semiquinone of the native flavin exhibit a reddish color when bound with 8-mercaptoflavin. These 8-mercaptoflavoproteins are easily reduced, either by substrate or photochemically. In addition, a long wavelength species, presumably a radical is also stabilized by the protein on partial reduction. For those flavoproteins which in the native state give little or no thermodynamic stabilization of a semiquinoid species, a similar lack of stabilization of a radical is found for the 8-mercaptoflavoprotein. The color of the 8-mercaptoflavoprotein may be reddish or blue, but the main distinguishing feature of this group is that the artificial enzyme is easily reduced by substrate, or photochemically. Particularly interesting in this group is *p*-hydroxybenzoate hydroxylase. In the native state no radical is stabilized on artificial 1-electron reduction. The 8-mercaptoenzyme behaves in a quite similar fashion; no radical is stabilized except in the presence of high concentrations of tetrafluoro-*p*-hydroxybenzoate, when the neutral radical is also stabilized.

These results have important implications concerning the structures of the neutral and anion radicals of normal flavins and the protein forces leading to their stabilization. The structure of the neutral blue radical has been shown to be due to blocking of the N(5) position by either aklylation or protonation (63) (Scheme 3):



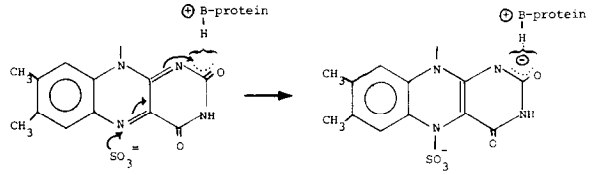
In flavoenzymes the blue neutral radical might reflect a strong hydrogen bridge between the protein backbone and the flavin position N(5). The recent x-ray crystallographic structure elucidation of flavodoxin has provided direct support for this hypothesis; with the blue semiquinoid form of the flavodoxin from *M. elsdenii*, the N(5) position of the flavin is within hydrogen bonding distance of a carbonyl residue of the protein polypeptide chain (64). It should be pointed out however, that in absence of a specific hydrogen bridge between protein and the flavin position N(5), a blue radical can be stabilized by a simple preferential binding of the neutral form (to a hydrophobic binding site) leading to a corresponding pK increase. Such a thermodynamic stabilization by at least 3 pK units has been demonstrated by Blankenhorn with riboflavin-binding protein (65). Various proposals have been put forward for the electronic structure of the red flavin anion radical. A particularly attractive one, which fits nicely with the results presented here, has been proposed by Hemmerich (66). It suggests that there may be two types of red flavin radical. The true anion is envisaged as being stabilized by hydrogen bonding from a H-bond donor of the protein to the radical anion, with its negative charge localized in the N(1)–C(2)=0 position (Scheme 3). An alternative red neutral radical can be envisaged by H-bonding from the same locus to a H-bond acceptor of the protein. The evidence for this interesting suggestion arises from the spectra of O(2) alkylated model compounds in their radical state (67).

The existence of a suitable base (B) in the proximity of the flavin N(1)–O(2) position, would also favor the *p*-quinoid forms of 8-mercaptoflavin (Scheme 4):



Clearly then, the ionization of the flavin chromophore will depend directly on the ratio of its microscopic pK and that of the protein base. For these reasons, a clear correlation would be expected between stabilization of the *p*-quinoid (blue) forms of 8-mercaptoflavin and the formation of a red radical in the case of the native flavoprotein. The results summarized in Table I are extremely suggestive. Without exception, the expected correlation discussed above is found.

The correlation, previously noted (4) between the ability of a particular flavoprotein to form a stable red radical and the ease of formation of a sulfite adduct to position N(5) of the flavin, also deserves comment in view of the results presented here. With the conclusion that flavoproteins which form stable red anion radicals do so because of the proximity of a protein protonated base to the flavin N(1) position, the correlation is readily explained. This protein group would be expected to exert an inductive effect, facilitating the nucleophilic addition of sulfite to the flavin N(5) position (Scheme 5):



The relevance of this observation lies in the fact that it would parallel the catalytic event involving uptake of reducing equivalents (carbanions) through the flavin position N(5). On the other hand, a protein H-bond acceptor would not promote such an inductive effect, offering an explanation of why some flavoproteins which stabilize red radicals do not form sulfite adducts. In light of these considerations, it would be particularly interesting to examine the binding of 8-mercapto-FAD to putrescine oxidase, which does stabilize a red radical, but does not form a sulfite adduct (68). However, it should be pointed out that other considerations than those enumerated may influence the formation of sulfite adducts. Trivial reasons, such as a negatively charged protein residue repelling approach of the sulfite ion, could of course be responsible for such failure.

Perhaps the most striking feature of the results is the clear differentiation of properties of 8-mercaptoflavins bound to different classes of flavoproteins. The four oxidases listed in Table I when bound to their specific 8-mercaptoflavin produce dramatic spectral shifts, indicative of stabilization of the *p*-quinoid mesomer. In keeping with this formulation is the difficulty of reduction of the chromophore, which in the *p*-quinoid form, is quite different from that of normal flavin. The only case where reduction occurred readily was 8-mercaptoglucose oxidase; there the pH dependence of reduction suggests that the species which actually reacts may be unionized 8-mercaptoflavin. The difficulty of reduction among this group is relevant in view of the evidence supporting the transient existence of substrate carbanions in the normal catalytic pathways of glucose oxidase (50), D-amino acid oxidase (69), and L-lactate oxidase (70). A negatively charged carbanion intermediate, while able to react readily with the

neutral normal oxidized flavin chromophore, might not be formed or not react at all with the negatively charged *p*-quinoid 8-mercaptoflavin. Indeed, just as in the case of sulfite-adduct formation, the existence of an appropriate protein base in the vicinity of the N(1) locus would be required to catalyze the attack of the carbanion at the N(5) position of normal flavin to form a flavin N(5) covalent linkage to the substrate (71, 72). Such an intermediate has been documented in the case of lactate oxidase and the substrate, glycollate (71, 72).

Table I also lists three enzymes, flavodoxin, NADPH-cytochrome P-450 reductase, and NADPH-adrenodoxin reductase, where the physiological role of the enzyme requires the flavin to participate in catalysis as the semiquinone. The first two of these enzymes show a marked thermodynamic stabilization of the flavin radical (11, 38) while surprisingly, adrenodoxin reductase does not (41). This behavior is mirrored in the properties of the 8-mercaptoflavin forms of the enzymes (Table I), where the first two appear to stabilize the 8-thiolate form, and where a radical species with λ_{max} ~700 to 720 nm is also stabilized. The 8-mercapto form of adrenodoxin reductase appears spectrally to have the *p*-quinoid structure. However, it is rapidly reduced by NAD(P)H, suggesting either that the flavin does not carry a negative charge (*cf.* the neutral protonated form B of Scheme 1) or that the reactive species is a small equilibrium concentration of the 8-thiolate anion form. This situation may exist with all the 8-mercaptoflavoproteins listed in Table I which are reduced more or less readily by their substrate, or photochemically.

Finally, the hypothesis that stabilization of the *p*-quinoid mesomer of 8-mercaptoflavin and the stabilization of the red anionic semiquinone of native flavin are manifestations of the same protein interaction leads to the prediction that this protein residue should also lead to stabilization of the anion form of the native dihydroflavin. In free solution the pK of this ionization is approximately 6.5 (34). Unfortunately the spectral characteristics of dihydroflavins distinguishing the neutral and anion species are often confused by end absorption from the aromatic residues of the apoprotein, and are complicated by the presence of even small amounts of oxidized flavin (73). However, the fully reduced forms of lactate oxidase (73), D-amino acid oxidase,[3] glucose oxidase (73), and Old Yellow Enzyme (37) have spectra clearly indicative of the dihydroflavin anion. On the other hand the reduced forms of flavodoxin (73) and NADPH-cytochrome P-450 reductase (14) have the spectral characteristics of neutral dihydroflavins. Thus, the predicted correlation appears to be fulfilled.

Acknowledgments—We are indebted to Dr. John P. Lambooy for a very generous gift of 8-chlororiboflavin, and to various colleagues for providing us with flavoproteins or the corresponding apoproteins; Dr. Mazhar Husain for *p*-hydroxybenzoate hydroxylase, Dr. Larry Schopfer for melilotate hydroxylase, Dr. Steven Olson for D-lactate dehydrogenase, and Drs. Jeff Parcells and Tokuji Kimura for adrenodoxin reductase. We also wish to thank Drs. Janice L. Vermilion and Minor J. Coon for permission to use some of our unpublished results here. Finally we wish to acknowledge the many valuable discussions, extending over many years, which we have had with Dr. Peter Hemmerich. These have been very helpful in formulation of many of the ideas expressed here.

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