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4-Thioflavins As Active Site Probes of Flavoproteins

GENERAL PROPERTIES*

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4-Thioflavins (oxygen at position 4 replaced by sulfur) have been studied as potential active site probes of flavoproteins. They react readily with thiol reagents, with large spectral changes, which should be useful for testing the accessibility of the flavin 4-position in flavoproteins. They have an oxidation-reduction potential at pH 7 of -0.055 V, approximately 0.15 V higher than that of native flavins. The spectral characteristics in the fully reduced state show two clear absorption bands, dependent on the ionization state (pK = 4.5). The lowest energy band of the neutral dihydroflavin has a maximum at ~485 nm while that of the anion is ~425 nm. This should be useful in defining the ionization state of the reduced flavin in flavoproteins. The spectral characteristics of the semiquinoid forms of 4-thioflavins have been determined bound to the apoproteins of flavodoxin and D-amino acid oxidase. The neutral radical has an absorption maximum at 730 nm, while the anion radical has an unusually sharp peak at 415 nm. The reduced forms of 4-thioflavins, free and enzyme bound, react with O_2 to regenerate oxidized 4-thioflavin. Reduced 4-thio-FAD *p*-hydroxybenzoate hydroxylase, however, in its reaction with O_2, undergoes a substantial conversion to the native FAD-enzyme. 4-Thioflavins are unusually susceptible to attack by nucleophiles such as hydroxylamine and amines to form the respective 4-hydroxyimino- and 4-aminoflavins, offering the possibility of forming stable covalent flavin-protein linkages with suitably positioned protein residues. Thiols also react with 4-thioflavins, promoting their conversion to the normal (4-oxo) flavin coenzymes. Such reactivity has been found with the apoenzymes of glucose oxidase and lactate oxidase, providing evidence for a thiol residue in the active site of these enzymes.

The ways in which flavin-protein interactions modulate the properties of flavoproteins have received increasing attention in recent years. Considerable progress has been achieved by an experimental approach in which the native coenzyme is replaced by analogs carrying specific modifications at various positions in the flavin ring system. For example, 8-halogen-substituted flavins which are very reactive toward thiolates and sulfide (Moore *et al.*, 1979; Massey *et al.*, 1979) and other nucleophiles (Kasai *et al.*, 1983; Fitzpatrick and Massey, 1983) have been used successfully as probes of solvent accessibility to the flavin position 8 in flavoproteins (Schopfer *et al.*, 1981). With the same coenzyme analog the presence of a reactive cysteine residue near the flavin 8-position was demonstrated in lipoyl dehydrogenase by formation of a covalent linkage of the flavin to the protein (Moore *et al.*, 1978). 8-Mercaptoflavins, 8-hydroxy- and 6-hydroxyflavins are also highly useful probes of the hydrophobic/hydrophilic character of the overall flavin environment (Ghisla *et al.*, 1976; Massey and Hemmerich, 1980) and as indicators of particular protein-flavin charge interactions. In addition, 8-mercaptoflavins react readily with a variety of reagents, offering further possibilities for exploring solvent accessibility to this portion of a protein-bound flavin. They react with alkylating agents such as iodoacetate or iodoacetamide to form 8-S-alkyl flavin derivatives and with methylmethane thiolsulfonate to form 8-S-S-CH_3-flavins (Schopfer *et al.*, 1981), with sulfite to form 8-sulfonyl flavins (Fitzpatrick and Massey, 1983), or with H_2O_2, again to form 8-sulfonyl flavins (Moore *et al.*, 1979). In the latter reaction a blue colored intermediate is observed, whose structure, by analogy to the similar compounds formed with other sulfur-containing flavins (see below) is possibly the flavin-8-S-oxide (Biemann *et al.*, 1983).

With the flavins mentioned above the accessibility and environment of the benzenoid part of the flavin could be investigated. A similar approach using 2-thioflavins was employed to probe the N(1)—C(2)═O—N(3)H positions in various flavoproteins (Claiborne *et al.*, 1982; Choong and Massey, 1983; Biemann *et al.*, 1983). Vargo and coworkers (Vargo *et al.*, 1981) have recently employed 5-deazaflavins and their reactions with peroxides as probes of the flavin 5-position. In this paper we report on the utility of 4-thioflavins[1] as probes of the N(3)H—C(4)═O—C(4a) region. These and 2-thioflavins had first been synthesized by Hemmerich and coworkers (Hemmerich *et al.*, 1959). While it was realized that they were chemically reactive (Müller and Hemmerich, 1966) this reactivity was never utilized as a way of probing the nature of the active site in flavoproteins. Part of the reason for this is probably due to the widespread impression that these flavins are too unstable for practical use, being converted readily to the normal flavin coenzyme by desulfurization. However, we have found that neutral aqueous solutions of these flavins can be kept on ice for weeks or at −20 °C for months without

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[1] For the sake of brevity we use throughout the terms 4-thioflavins and 2-thioflavins. The correct nomenclature is 4-deoxy-4-thio- and 2-deoxy-2-thioflavins. The same convention is used for other functional substituents, *e.g.* 4-deoxy-4-aminoflavins are referred to as 4-aminoflavins.

more than a few per cent conversion to normal flavin, provided that they are protected from light. Hence, with care, they can be incorporated into apoproteins and used to gain information about the active site environment in flavoproteins.

Previous papers in this series have described the properties of 2-thioflavins, both free and protein-bound. 2-Thioflavins react readily with methylmethane thiolsulfonate to form 2-S-S-CH_3 flavins; they also react more slowly with iodoacetate and iodoacetamide to form 2-S-alkyl flavins (Claiborne *et al.*, 1982).They react with H_2O_2 or with organic peracids to form the stable flavin-2-S-oxide, which can be further oxidized to the unstable sulfinate and sulfonate, which in turn undergo hydrolysis to yield normal (2-oxo) flavin (Biemann *et al.*, 1983). In the present paper we show that 4-thioflavins behave in quite analogous ways as reported for 2-thioflavins. They also undergo attack by a variety of nucleophiles, many of which result in stable flavin 4-adducts. These properties appear to offer many interesting new possibilities for determination of the protein environment around the flavin 4-position, as well as offering many new flavin derivatives arising from reaction with 4-thioflavins.

MATERIALS AND METHODS

4-Thiotetraacetylriboflavin—This was synthesized by adaptation of the published synthesis of 4-thiolumiflavin (Müller and Hemmerich, 1966). Better yields were obtained when the P_2O_5 used was treated with charcoal, and recrystallized from pyridine before use. Progress of the reaction was followed with TLC on precoated silica gel plates in *n*-butyl alcohol/glacial acetic acid/water (3:1:1) and the reaction stopped when the desired product had reached optimal concentration. After workup as described by Müller and Hemmerich (1966), the product from 0.9 mol of tetraacetylriboflavin was then applied to a silica gel (Merck) column (60 × 3 cm) and eluted with chloroform/ethyl acetate = 2:1. 4-Thio-, 2-thio-, and 2,4-dithiotetraacetylriboflavin elute in that order. After evaporation of the pooled fractions 4-thiotetraacetylriboflavin was obtained in pure form in 33% yield.

4-Thioriboflavin—This was obtained from the tetraacetyl derivative by hydrolysis for 10 min at 25 °C in 1 N aqueous NaOH and precipitated with glacial acetic acid at 0 °C, filtered, and washed with ether (78% yield).

3-Methyl-4-thiolumiflavin—For the synthesis of this derivative the use of commercially available Lawesson's reagent (EGA Chemie, Steinheim, Germany) proved more convenient: 10 mmol of *N*(3)-methyllumiflavin in 15 ml of dry hexamethylphosphoramide (Janssen, Beerse, Belgium) was heated in the dark at 105 °C for 7.5 h, with 5 mmol of Lawesson's reagent (2,4-bis(methoxyphenyl)1,3-dithia-2,4,diphosphetane-2,4-disulfide), the course of the reaction being followed by thin layer chromatography on silica gel plates in *n*-butyl alcohol/methanol/water (4:3:1). The cooled mixture was poured into 20 ml of water, the precipitate formed was washed several times with water and dissolved in chloroform. The evaporated chloroform phase was chromatographed in small portions on a alumina column (20 × 2 cm) (Merck, Darmstadt) with chloroform as eluent. The 3-methyl-4-thioflavin, starting material, and a violet compound, probably 2,4-dithio-3-methyllumiflavin are eluted, while 2-thio-3-methyllumiflavin, and residual Lawesson's reagent stick to the column. Final purification was achieved on a silica gel (Merck) column using *n*-butyl alcohol as eluent. 3-Methyl-4-thiolumiflavin was obtained in ~20% yield.

Preparation and Purification of 4-Thio-FAD and 4-Thio-FMN—4-Thio-FAD was prepared from 4-thioriboflavin using the FAD synthetase complex, partially purified from *Brevibacterium ammoniagenes* (Spencer *et al.*, 1976). 2.5 mg of 4-thioriboflavin was dissolved in 0.25 ml of dimethylformamide and diluted into 125 ml of 10 mM potassium phosphate, pH 7.5. Solid citric acid was added, after the additions of ATP (2 mM) and $MgCl_2$ (8 mM), to give a final pH of 5.3. Two ml of a fresh FAD synthetase preparation was then added; final pH was 5.5. All operations were carried out in the dark. After adding 75 μl of bovine catalase (Calbiochem-Behring, 20 mg/ml of stock), the mixture was incubated at 37 °C for 16–24 h.

Following the first incubation, a dark blue precipitate was usually observed on centrifugation and discarded. Progress of the conversion to 4-thio-FAD was monitored on plastic-backed silica gel TLC plates (Brinkmann Instruments,) chromatographed in 5% (w/v) $Na_2HPO_4 \cdot 12\ H_2O$. Extract, catalase, and ATP were added to continue the incubation, until the conversion to 4-thio-FAD was complete.

The 4-thio-FAD preparation was purified over a Bio-Gel P-2 column in 0.01 M NH_4COOH, pH 6.5, and separated from a small amount of contaminating FAD by ion exchange chromatography over DE32. 4-Thio-FAD and FAD were well resolved during elution with 0.2 M NaCl, 0.01 M NH_4COOH, pH 6.5, and the 4-thio-FAD was eluted in a fairly concentrated form. The eluate was further concentrated by evaporation on a rotary evaporator at 40 °C, and was then divided into small aliquots and stored at −20 °C.

4-S-CH_2CONH_2-Riboflavin—Approximately 2 mg of 4-thioriboflavin was dissolved in 1 ml of dimethylformamide, diluted with 1 ml of 0.05 M pyrophosphate, pH 8.5, and 20 mg of iodoacetamide added. (The iodoacetamide was recrystallized from H_2O.) The conversion was complete within 1 h at room temperature. The product was separated from residual iodoacetamide and contaminating riboflavin by passage through a column (45 × 1.5 cm) of Bio-Gel P-2 equilibrated and eluted with H_2O. The iodoacetamide eluted cleanly first, followed by riboflavin, and finally 4-S-CH_2CONH_2-riboflavin. There was some overlap between the two flavins, but by spectral and fluorescence analysis it could be seen that approximately 80% recovery of the derivative was obtained free of contaminating riboflavin.

4-Amino- and 4-Iminoflavins—In general these were prepared only on a small scale, following the reaction spectrophotometrically. In the case of the adducts with glycine and methionine, the reactions were carried out at pH 7 and room temperature over a period of 1–2 days in the presence of 0.1 M amine. Under these conditions there is insignificant breakdown of the starting 4-thioflavin to normal (4-oxo) flavin. In the case of reaction with methylamine and dimethylamine, the high pH (9–10) due to addition of the amine resulted in some breakdown to normal flavin. In these cases the product was purified by high performance liquid chromatography. Typically the flavin was loaded on an Altex Ultrasphere ODS 5-μ column equilibrated with 10% CH_3CN in 0.2 M ammonium acetate, pH 6, and eluted with a linear gradient of 10–25% CH_3CN over a period of 20 min, using a Spectrophysics high performance liquid chromatography instrument. We wish to thank Dr. L. M. Schopfer, University of Michigan, for developing the method and carrying out these separations.

4-Hydroxyiminoflavins—4-Hydroxyimino derivatives of 4-thioriboflavin and *N*(3)-methyl-4-thiolumiflavin were prepared by nucleophilic substitution of 4-thioriboflavin and *N*(3)-methyl-4-thiolumiflavin by hydroxylamine. Stock solutions of the 4-thioflavins in acetonitrile were diluted 1:5 in 0.1 M phosphate buffer, and neutralized $NH_2OH \cdot HCl$ added to a concentration of 0.1 M. The reaction course was followed spectrophotometrically and the end products of the nucleophilic substitution characterized by spectrum and by TLC (on precoated silica gel plates in *n*-butyl alcohol/glacial acetic acid/water (12:3:5)).

Column Separation of Flavin-4-S-oxides—A solution of 4-thiotetraacetylriboflavin in acetonitrite (Baker Analytical grade), concentration 0.1 mM, was titrated with a freshly prepared 5.3 mM solution of *m*-chloroperbenzoic acid in the same solvent until maximal S-oxide was formed. 6 ml of the S-oxide solution was applied to a silica gel column (22 × 1.7 cm) equilibrated with *n*-butyl alcohol/glacial acetic acid/water (10:1:2) and eluted with the same solvent. 4-Thiotetraacetylriboflavin, a violet S-oxide "isomer," tetraacetylriboflavin and a blue isomer eluted from the column in that order with satisfactory separation. The fractions of the two "isomers" were examined by TLC (precoated silica gel plates, *n*-butyl alcohol/glacial acetic acid/water, 10:1:2) and by absorption spectrum.

4-Thioflavoproteins—Riboflavin-binding protein from hen egg whites was prepared as the apoprotein, as described by Becvar and Palmer (1982). It was used to titrate 4-thioriboflavin, or derivatives, generally in 0.1 M phosphate, pH 7.0. D-Amino acid oxidase, from pig kidneys, was prepared as described by Curti *et al.* (1973) and the apoprotein was prepared by dialysis against KBr (Massey and Curti, 1966). The 4-thio-FAD enzyme was prepared by incubation with 4-thio-FAD in 0.1 M phosphate, pH 7, for 10 min at 25 °C. A small amount of denatured protein was removed by centrifuging, and excess 4-thio-FAD was removed by gel filtration with Sephadex G-25, equilibrated with 0.1 M phosphate, pH 7.0. *p*-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* was purified as described by Müller *et al.* (1979) and the apoenzyme prepared as described by the same authors. Reconstitution with 4-thio-FAD was carried out by the

general procedure described by Entsch *et al.*(1980). Glucose oxidase from *Aspergillus niger* was prepared as described previously (Swoboda and Massey, 1965) and the apoprotein was prepared as described by Swoboda (1969a). As described in the text, no satisfactory method was found for preparing a stable 4-thio-FAD enzyme. Old Yellow Enzyme from brewer's yeast, and its apoenzyme, was isolated by the method of Abramovitz and Massey (1976a). The 4-thio-FMN enzyme was reconstituted by incubation with an excess of 4-thio-FMN followed by gel filtration with Sephadex G-25, in 0.1 M phosphate, pH 7.0. Lactate oxidase was isolated from *Mycobacterium smegmatis* by the method of Sullivan *et al.* (1977) and the apoprotein prepared as described by Choong *et al.* (1975). Reconstitution with 4-thio-FMN was tried under a variety of conditions in order to try to overcome the spontaneous desulfurization to normal FMN-enzyme. These will be described in the text; the best method was found to be reconstitution in 0.1 acetate, pH 5.6, for 20 min on ice with a 2-fold excess of 4-thio-FMN, followed by 10 min of incubation at 25 °C. Excess 4-thio-FMN was then removed by gel filtration with Sephadex G-25 equilibrated with the same buffer. Flavodoxin and apoflavodoxin were prepared as described previously (Mayhew and Massey, 1969; Mayhew, 1971). The 4-thio-FMN enzyme was prepared by incubation of apoprotein with a slight excess of 4-thio-FMN in 0.1 M phosphate, pH 7.0, 25 °C, followed by gel filtration on Sephadex G-25, equilibrated with the same buffer.

Photoreductions in the presence of EDTA, with or without 5-deazaflavin as catalyst, were carried out under anaerobic conditions as described by Massey and Hemmerich (1978). Absorption spectra were measured with Cary Model 17, 118, or 219 recording spectrophotometers, or with a Kontron 820 recording spectrophotometer. Fluorescence excitation and emission spectra were measured with a ratio recording instrument designed and built by Dr. David Ballou and Gordon Ford, The University of Michigan.

RESULTS AND DISCUSSION

Spectral Properties of 4-Thioflavins in the Oxidized Form—Like the corresponding 2-thioflavins, 4-thioflavins are nonfluorescent. The neutral and anionic oxidized forms are red in color, with absorption maxima at 495–500 and ~370 nm. By titration with riboflavin-binding protein, or reaction with

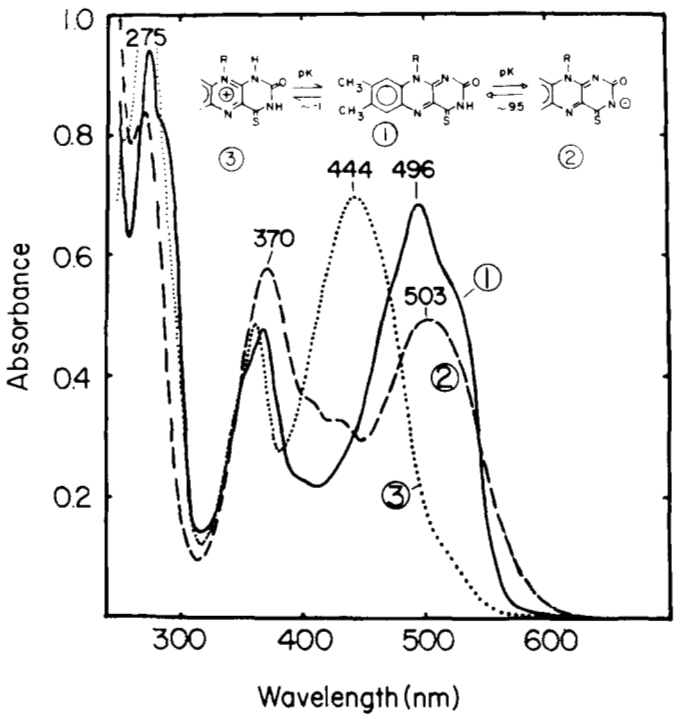


FIG. 1. **Ionization forms of 4-thiotetraacetylriboflavin.** A concentrated stock solution of 4-thiotetraacetylriboflavin in dimethylformamide was diluted equally into the following solutions (final concentration 5% dimethylformamide): *1*, 0.1 M acetate buffer, pH 5.5; 2, 0.1 M carbonate buffer, pH 12; *3*, 54% H_2SO_4 (pH ~ −4).

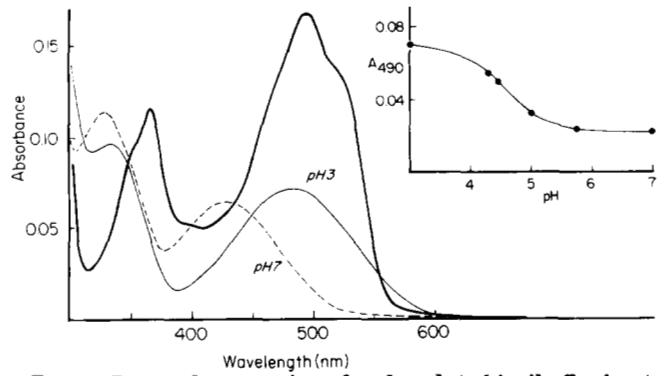


FIG. 2. **Spectral properties of reduced 4-thioriboflavin.** 4-Thioriboflavin (11.1 μM) in acetate and phosphate buffers containing 15 mM EDTA, and under anaerobic conditions, was photoreduced in separate experiments. The *inset* shows a plot of A_{490} of the reduced flavin *versus* pH. The latter was measured at the end of the experiment, after opening to air.

H_2O_2 (see later sections), the extinction coefficient at 495 nm may be estimated as 15,000 M^{-1} cm^{-1}. This value has been used as a reference for calculation of extinction values of the various derivatives to be described. The spectra of the cation, neutral and anion forms of 4-thioriboflavin are shown in Fig. 1. The pK for protonation to the cation was found to be approximately −1; that for deprotonation to the anion is approximately 9.5–10. The latter value is difficult to obtain with accuracy because the hydrolysis rate becomes appreciable as one titrates with alkali through the pH region ≥10.

Spectral Properties of 4-Thioflavins in the Reduced Form—4-Thioflavins, like normal flavins, are reduced readily by light irradiation in the presence of a photochemical donor such as EDTA. Spectra are shown in Fig. 2 for 4-thioriboflavin. The reduced flavin has several quite distinctive features which promise to be of diagnostic value with flavoproteins. First it has two clearly resolved visible absorption bands, with λ_{max} values of 333 and 485 nm for the neutral dihydroflavin and 328 and 425 nm for the anionic form, with a pK ~4.5. These features are much more prominent than those of the corresponding forms of native flavins, which are sufficiently similar that it is often difficult to distinguish whether a particular protein stabilizes either the neutral or anion form of the reduced flavin (Ghisla *et al.*, 1974). The marked difference in spectral behavior from that of normal flavins raises the question of the structure of reduced 4-thioriboflavins. As discussed elsewhere (Hemmerich, 1976), reduction of flavins can lead not only to formation of 1,5-dihydroflavins, but also of other tautomeric forms, resulting from reduction at other positions. In the present case the likelihood of the 1,5-dihydro structure being formed comes from the following observations. (*a*) The reduction product is readily reoxidized by O_2, a behavior typical of 1,5-dihydroflavins. (*b*) Borohydride reduction of 4-thioriboflavins leads to formation of 3,4-dihydro-4-thioflavins,[2] just as is the case with the native flavin coenzymes (Müller *et al.*, 1969). These are readily distinguishable spectrally, and by their intense fluorescence, from 1,5-dihydroflavins, as well as by their insensitivity to O_2. (*c*) The 4a,5-dihydroflavin chromophore of 4-thioflavins is also spectrally and chemicaly different (Table IV).

The observed pK of 4.5 of reduced 4-thioriboflavin is 2 pH units lower than that found with normal flavin (pK ~6.5; Dudley *et al.*, 1965). This effect of sulfur substitution at position 4 on the pK of the reduced flavin is remarkable when compared with the absence of a major effect on the pK of the

[2] V. Massey and S. Ghisla, unpublished data.

TABLE I

Properties of reduced 4-thio-riboflavin and reduced 4-thioflavoproteins

System	Absorbance		Excitation maxima	Fluorescence emission maximum	Intensity % that of oxidized riboflavin
	λ_{max}	ϵ			
	nm	$M^{-1}\ cm^{-1}$	*nm*		
4-Thioriboflavin	333	8,600			~0
pH 3	485	6,400			
pH 7	328	10,200			~0
	425	5,700			
Bound to riboflavin-binding protein	350	8,800			~0
	510	5,400			
4-Thio-FAD-D-amino acid oxidase	335	12,700	345	548	9
	438	6,600	450		
4-Thio-FMN lactate oxidase	330	~11,000	325	525	25
	435	~7,500	430		
Photo product with β-bromopropionate	330 (sh)	~8,000	335	520	11
	410	~8,300	410		
4-Thio-FMN-flavodoxin	350	10,100	355	543	0.5
	466	5,000	463		
4-Thio-FAD *p*-hydroxybenzoate hydroxylase	342	10,900	345	545	1.2
	458	5,200	450		
Plus *p*-hydroxybenzoate	344	11,100	ND	ND	Weak
	466	5,100			
Plus 6-hydroxy nicotinate	460	5,900	ND	ND	Weak
Plus 2,4-dihydroxybenzoate	475	5,500	ND	ND	Weak

[a] ND, not determined.

STRUCTURE 1

neighboring position N(3)H in the oxidized state. It probably reflects a low amide conjugation of the N(3)—C(4)=O subfunctions, as inferred earlier from IR spectra of normal flavins (Hemmerich, 1976). In the reduced 4-thioflavin chromophore, delocalization of the negative charge to the 4-sulfur is probably important (Structure 1). This conclusion is in agreement with the spectral properties of the reduced forms of 4-SCH_2CONH_2-riboflavin, 3-methyl-4-SCH_2CONH_2-lumiflavin and 4-thioriboflavin in its anionic state. These properties will be documented in the next paper in this series.[3]

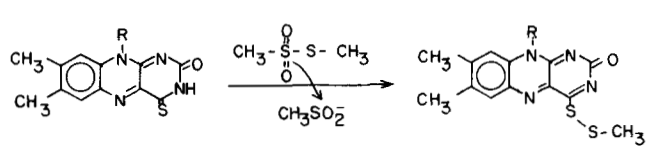
Another distinctive feature of the reduced form of the 4-thioflavins is its intrinsic fluorescence. While normal 1,5-dihydroflavins, free in solution and at room temperature are practically nonfluorescent, the 4-thio analog shows a very weak emission, which often becomes fairly intense on binding to protein. Similar enhancement of fluorescence has been reported previously with normal flavins (Ghisla *et al.*, 1974). Table I shows the results obtained with several flavoproteins substituted with the appropriate 4-thioflavin. The possible significance of the observed spectral shifts will be considered later in the discussion of results with the individual proteins.

In the case of dihydro-4-thioriboflavin and most of the reduced 4-thioflavoproteins, >95% reformation of the oxidized 4-thioflavin is obtained rapidly on admitting air. The reason why complete reversal may not be obtained is due to the further oxidation of 4-thioflavins by H_2O_2, a product of reoxidation of the dihydroflavin by O_2 (see later section). The behavior of individual 4-thioflavoproteins will be considered separately.

[3] M. Biemann, A. Clairborne, S. Ghisla, and V. Massey, manuscript in preparation.

Oxidation Reduction Potential—The oxidation-reduction potential of 4-thio-FAD in 0.1 M potassium phosphate, pH 7.0, 25 °C, was determined by amperometric titration with $Na_2S_2O_4$, using the apparatus described previously (Hille *et al.*, 1983) and recording the spectrum at each stable potential during the titration. The Nernst plot of the results gave a midpoint potential of −0.055 V with an *n* value of 2.3. We are indebted to R. Stewart, University of Michigan, for this determination.

Reaction of 4-Thioflavins at the C(4)-Thio Function with Electrophiles—As is the case with 2-thioflavins, methylmethane thiolsulfonate reacts moderately fast with 4-thioflavins, to yield the corresponding 4-S-S-CH_3-flavin (Scheme 1). As expected for this formulation, the reaction rate increases with increasing pH from 7 to 10, a plot of log k_{obs} *versus* pH yielding a slope of 0.8 and a pK of ~10, in reasonable agreement with the pK value estimated spectroscopically. At pH 7.0, 4 °C, the second order rate constant for the reaction with 4-thioriboflavin was found to be 10 M^{-1} min^{-1}. The spectral changes accompanying the reaction are shown in Fig. 3. The alkylation of 4-thioflavins by methyl iodide was reported by Müller and Hemmerich (1966). We have also found that iodoacetamide reacts slowly with 4-thioriboflavin, with a rate constant of 0.35 M^{-1} min^{-1} at pH 7.6, 25 °C. While this is probably too slow to be of much use as a flavoprotein active site probe, it does permit the easy preparation of a flavin 4-S-R derivative. The spectral properties are given in Table IV.



SCHEME 1

Reaction of 4-Thioflavins with Peroxides—Again in analogy with 2-thioflavins, 4-thioflavins are readily oxidized by H_2O_2 and *m*-chloroperbenzoic acid, first to the S-oxide, then presumably to the unstable sulfinate and sulfonate, which]

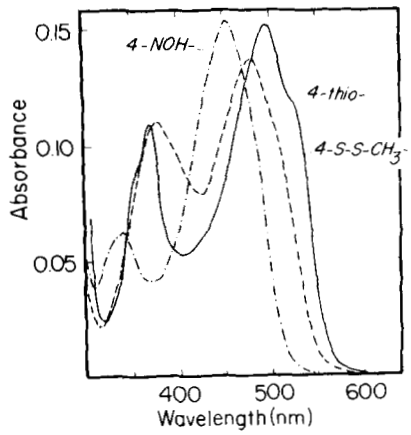


FIG. 3. **Spectra of the derivatives of 4-thioriboflavin obtained on reaction with methylmethane thiolsulfonate and hydroxylamine.** 4-Thioriboflavin (10.0 μM) in 0.1 M phosphate, pH 7.0, was reacted with 2 mM methylmethane thiosulfonate or 0.1 M NH_2OH (neutralized before use).

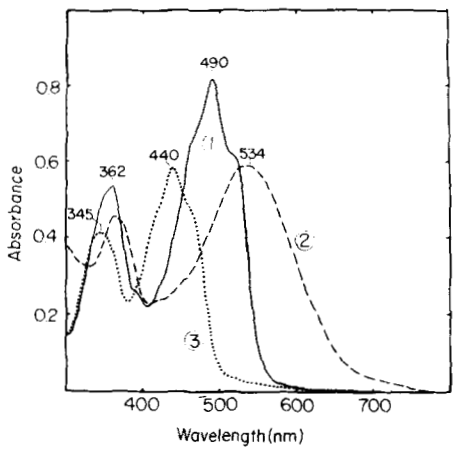


FIG. 4. **Oxidation products of 4-thiotetraacetylriboflavin on titration with *m*-chloroperbenzoic acid.** 4-Thiotetraacetylriboflavin in acetonitrile was titrated with *m*-chloroperbenzoic acid dissolved in the same solvent. *Curve 1*, before additions; *curve 2*, after titration with 1.0 eq of peracid; *curve 3*, final oxidation product after a further 2 eq of perbenzoic acid. The reaction to the S-oxide was fast, requiring only minutes before a stable absorbance change was reached. The further oxidation was very slow, and required 2 days for completion.

lyze with desulfurization to the normal (4-oxo) flavin (Biemann *et al.*, 1983). With H_2O_2 at pH 7.0, very little S-oxide intermediate is observed, and the conversion to normal flavin occurs with pseudo-first order kinetics, with k_{obs} being directly proportional to the H_2O_2 concentration. At pH 7.0, 4 °C, the second order rate constant so obtained is 1.8 M^{-1} min^{-1}. This conversion offers a convenient and accurate way of determining the extinction coefficient of 4-thioflavins. By such means the ϵ_{495} value of 4-thioriboflavin was determined to be 15,000 M^{-1} cm^{-1}, in exact agreement with results obtained by titration with the riboflavin-binding protein (see later section).

With *m*-chloroperbenzoic acid as oxidant it is possible to titrate the 4-thioflavin almost quantitatively to the S-oxide, with the consumption of 1 eq of peracid. The result of such a titration, carried out with 4-thio-tetracetylriboflavin in acetonitrile, is shown in Fig. 4. Further additions of peracid result in the slow disappearance of the long wavelength absorbance, and conversion to tetraacetylriboflavin with consumption of a further 1–2 eq of peracid. Similar results were obtained with 4-thioriboflavin by titrations carried out in aqueous solution

TABLE II

Spectral properties of tetraacetylriboflavin-4-S-oxide

Solvent	λ_{max}	Extinction coefficient
	nm	M^{-1} cm^{-1}
Acetonitrite	363	7,700
	535	12,500
Benzene	356	7,300
	545	12,000
Methanol	366	8,300
	535	11,100
Toluene	367	6,400
	544	10,300
0.1 M Acetate	365	7,000
pH 5.5	535	12,500
0.1 M Na_2CO_3	370	15,000
pH 11	(430)	(9,800)
	605	8,900

TABLE III

R_F values of flavin 4-S-oxides and 2-S-oxides on thin layer chromatography

Plates used were precoated silica gel plates from Merck

Flavin	TLC system[a]		
	A	B	C
TARF[b]-4-S=O major violet isomer	0.91	0.75	0.52
TARF-4-S=O minor blue isomer	0.36	0.16	0.33
Riboflavin-4-S=O violet isomer	0.06		0.41
Riboflavin-4-S=O blue isomer	0.06		0.21
Riboflavin-2-S=O	0.25		0.18

[a] TLC systems: A, methanol/ethyl acetate/chloroform, 4:3:3; B, *n*-butyl alcohol/ethyl acetate/water, 4:3:1; C, *n*-butyl alcohol/glacial acetic acid/water, 10:1:2.

[b] TARF, tetraacetylriboflavin.

at pH 7 and 9.9, where 1 eq of peracid was consumed for full formation of the S-oxide, and a further 1–1.3 eq consumed for conversion to riboflavin. When a concentrated sample of S-oxide, prepared in CH_3CN, is diluted into various solvents, only small perturbations of the spectrum are found, except when aqueous solvent of high pH is used. The latter change is due to a pK of ~8.5 for the S-oxide. At pH values as low as pH 0, no spectral changes are observed, indicating a pK << 0 for protonation. Table II documents some of these effects.

When the 4-thioflavin-S-oxides prepared from both 4-thiotetraacetylriboflavin and 4-thioriboflavin in acetonitrile were analyzed by TLC, a major violet spot and a minor blue spot were obtained (Table III). By analogy with the results previously reported (Biemann *et al.*, 1983) for 2-thioflavins these may represent two (*cis/trans*) isomers.

4-Thioflavin-S-oxides are easily photoreducible. In the presence of 2 mM EDTA and in 0.1 M phosphate, pH 7.0, the reduction proceeds isobestically and quantitatively to yield first oxidized 4-thioflavin. Further illumination yields the dihydro-4-thioflavin as documented in a previous section.

In aqueous solution flavin-4-S-oxides are remarkably stable in the pH range 4–6, providing the sample is protected from light. In this pH range, the $t_{1/2}$ for decay to normal (4-oxo) flavin is of the order of 1 year at room temperature. At pH values below 4, the rate constant for decay is directly proportional to the H^+ concentration, down to pH ~0.5. Above pH 6, the rate again increases directly proportionally to the OH^- concentration until pH 8.5, the pK of the sulfoxide. From pH 8.5 to 11, the rate is virtually unchanged, with a $t_{1/2}$ of

approximately 30 h at 20 °C. In contrast to this remarkable stability, the S-oxide is very photolabile. For example, 5-s illumination with a projector lamp (~2×10^6 ergs cm^{-2} s^{-1}), or 1 hour exposure to room light leads to complete conversion to normal (4-oxo) flavin.

Reaction of 4-Thioflavins with Nucleophiles—One of the main differences between 2-thioflavins and 4-thioflavins appears to be in the much greater susceptibility of the latter to attack by nucleophiles. This is in accordance with the general reactivity of pyrimidine 2 *versus* 4 positions. 4-Thioflavins undergo reaction at neutral pH with a variety of primary and secondary amines, hydroxylamine, thiols, and sulfite. No significant reaction has been observed with imidazole and phenolates except for a small increase in the rate of hydrolysis of the 4-thioflavin to normal flavin. The reaction with sulfite is complex, and will be the subject of a separate paper.[3]

Fig. 5 shows the spectral changes resulting from reaction of 4-thio-tetraacetylriboflavin with methylamine. The resulting product is intensely fluorescent, having a fluorescence intensity approximately 30% that of riboflavin (see Table IV). On acidification to pH 2–3 the fluorescence increases to ~50% that of riboflavin at pH 7. This is in contrast to the behavior of riboflavin, whose fluorescence is quenched markedly in the same pH region. On acidification there is also a large change in the position of the near UV absorption band, which shifts from 358 to 390 nm. By monitoring either the changes in absorption or fluorescence as a function of pH, a pK of 4.6 is calculated for the derivative. No other pH dependence on absorption spectrum was observed up to pH 10.

The properties of the product are similar to those reported earlier by Müller and Hemmerich (1966) for "4-iminoflavins," obtained by reaction of amines with 4-S-CH_3-flavins. The reaction presumably occurs by way of a tetrahedral intermediate resulting from nucleophilic attack of the amine on the flavin 4-position, followed by elimination of sulfur as H_2S, as shown in Scheme 2. The aminoflavin structure shown is supported by several pieces of evidence. First, the same reaction occurs with dimethylamine, and the resulting product has almost identical properties, as detailed in Table IV. While the product from reaction with methylamine could exist in the amino- and imino-tautomeric forms, as shown in Scheme 3, Structures I and II, the preferred tautomer of dimethylamine product should be that shown in Structure III. The second piece of evidence supporting the aminoflavin Structure I comes from results obtained on reaction of amines with *N*(3)-methyl-4-thiolumiflavin. Methylamine does react, but the product is quite unstable at pH values ≥9 and has quite different spectral properties, as shown in Table IV. In this case the only reasonable structure is one similar to that of Scheme 3, Structure II (R=CH_3). In keeping with this, dimethylamine fails to yield a stable product on reaction with 3-methyl-4-thiolumiflavin, the only product observed being normal flavin, and that only at high pH (> pH 10).

Hydroxylamine also reacts readily with 4-thioflavins. At pH 7.0, 4 °C, the second order rate constant for the reaction is 4.1 M^{-1} min^{-1}, while at 25 °C it is 26 M^{-1} min^{-1}. This is sufficiently rapid, and the conditions suitably mild, that this reaction holds promise of being a reasonable probe of flavoprotein active sites, although the tetrahedral character of the primary reaction intermediate may be expected to place severe restrictions on the reaction occurring with flavoproteins. The spectrum of the product has somewhat unusual characteristics, with λ_{max} of 452 and 340 nm, as shown in Fig. 3. The structure is probably that shown in Table IV, consistent also with the spectral similarities with the product of reaction of methylamine with 3-methyl-4-thiolumiflavin (*cf.* Scheme 3, Structure II, R=CH_3), and also with the fact that the same spectrum is produced on reaction of hydroxylamine with *N*(3)-methyl-4-thiolumiflavin.

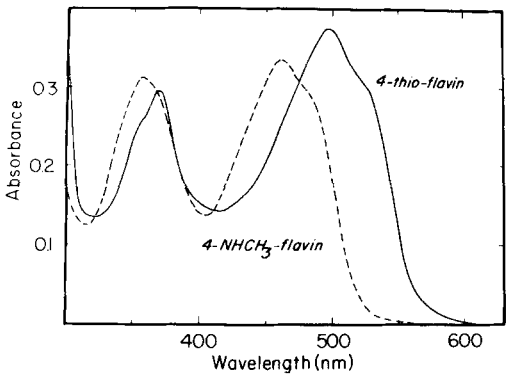
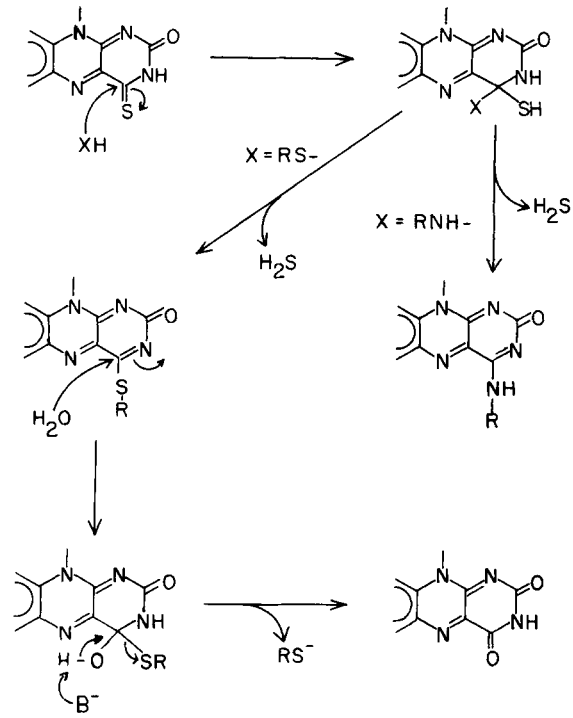
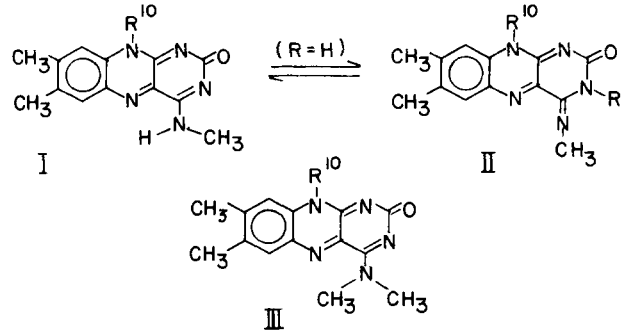


FIG. 5. **Spectrum of 4-methylamino-tetraacetylriboflavin.** 4-Thio-tetraacetylriboflavin (25 μm) in 0.1 M pyrophosphate, pH 8.5, was reacted at 25 °C with 0.1 M methylamine. The reaction was complete in 2 h. Other spectral properties of the product are given in Table IV.



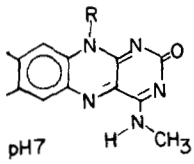
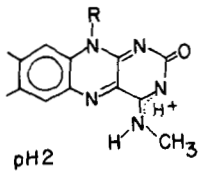
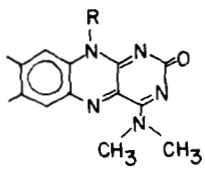
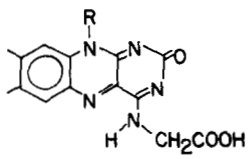
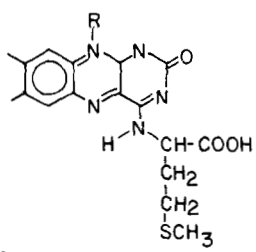
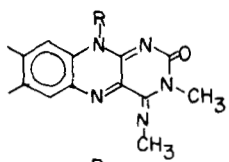
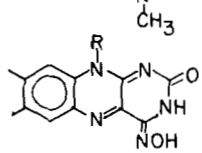
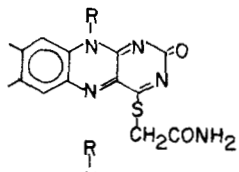
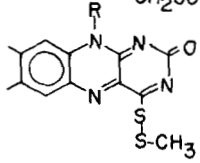
SCHEME 2



SCHEME 3

TABLE IV

Properties of 4-substituted flavins obtained by reaction with 4-thioflavins

Reactant	Product	Absorbance			Fluorescence		
		pK	λ_{max}	ϵ	Excitation max	Emission max	Intensity relative to riboflavin
			nm	$M^{-1}\ cm^{-1}$	*nm*		
Methylamine	 pH7	4.6	357 462	12,900 14,000	365 470	536	28%
	 pH2		388 462	13,800 14,100	395 470	544	48%
Dimethylamine		4.8	pH 7: 358 465 pH 2: 389 462	13,600 13,000 11,100 12,800	365 470 390 475	535 548	31% 70%
Glycine		4.0	pH 7: 360 465 pH 2: 392 470	12,200 13,300 11,700 12,600	365 465 398 470	540 550	4% 11%
Methionine		4.1	pH 7: 360 468 pH 2: 390 465	13,500 13,300 11,900 12,400	365 465 395 470	540 550	3.3% 3%
Methylamine and 3-methyl-4-thiolumiflavin		None	340 439	~10,000 ~15,000	ND[a]	~545	ND
Hydroxylamine		ND	340 452	6,200 15,400			~0
Iodoacetamide		ND	377 478	13,200 15,700	377 476	555	6%
Methylmethane thiolsulfonate		ND	376 478	11,000 13,700			~0

[a] ND, not determined.

Thiols also react readily with 4-thioflavins, promoting their conversion to normal (4-oxo) flavins. For example, on mixing 4-thioriboflavin in 0.1 M phosphate, pH 7.0, 25 °C, with 0.5 mM dithioerythritol, riboflavin is produced in a biphasic reaction. The first stage, which occurs with a $t_{1/2}$ value of approximately 6 min proceeds with isosbestic points at 462 and 371 nm, which appears to be due to formation of an intermediate with an absorption maximum around 460 nm. In the second stage of the reaction, in which riboflavin is the sole product, the initial isosbestic points are lost, being replaced by new ones at 451 and 418 nm. This stage of the reaction has a $t_{1/2}$ of approximately 50 min. The rates of both stages are strongly dependent on the thiol concentration, and a similar series of spectral changes, also resulting in formation of riboflavin, were found using mercaptoethanol and 4-thioriboflavin. That riboflavin was the product was shown by identity of absorbance and fluorescence spectra, and by TLC in *n*-butyl alcohol/acetic/H_2O (10:1:2). While the mechanism of the conversion was not investigated further, a reasonable postulate is shown in Scheme 2, in which thiolate makes a nucleophilic attack at the 4-position to generate a 4-SR flavin, which undergoes further attack by water, eliminating RS^- under influence of general base catalysis with the thiolate as base. Alternatively, water may attack directly at the 4-position of the starting 4-thioflavin under influence of general base catalysis, with the added thiolate anion serving as the base. This route must certainly occur in the promotion of the conversion of 4-thioriboflavin to riboflavin by Na_2S, which also occurs readily at neutral pH (results not shown).

Properties of 4-Thioriboflavin Bound to Riboflavin-binding Protein—When 4-thioriboflavin is titrated with the apoprotein of hen egg white riboflavin-binding protein in 0.1 M phosphate, pH 7.0, large changes in the absorption spectrum occur with a sharp end point, which permit an accurate determination of the extinction coefficient of the free flavin as 15,000 M^{-1} cm^{-1} at 495 nm and 11,000 M^{-1} cm^{-1} at 368 nm. When bound to the egg white-binding protein, the maxima are found at 508 and 368 nm, with extinction coefficients of 12,300 and 7800 M^{-1} cm^{-1}, respectively. The 4-thioflavoprotein shows no reaction with 4 mM NH_2OH at pH 7.0, 4 °C, over a period of 2 days. It does react extremely slowly with H_2O_2 to generate the 4-S-oxide; with 36 mM H_2O_2 the $t_{1/2}$ for formation of the S-oxide was approximately 10 h at pH 7.0, 4 °C. In contrast to this, 2-thioriboflavin bound to the egg white protein reacts readily with H_2O_2 and methylmethane thiolsulfonate (Claiborne *et al.*, 1982). By comparison with the rates of these reagents with free 4-thioriboflavin, it is evident that the 4-position of the flavin must become inaccessible to these compounds on binding to the protein, while the 2-position is free. This is in agreement with results and proposals by other workers (Choi and McCormick, 1980; Becvar and Palmer, 1983).

When the reduced form of 4-thioriboflavin at pH 4.3 (near its pK of 4.5; see earlier section) is mixed with an excess of riboflavin-binding protein, the absorption maxima shift to 350 and 510 nm (see Table I) indicative of a shift in pK to higher values. This is consistent with the known preferential binding of neutral flavins over negatively charged ones by this protein (Choi and McCormick, 1980; Blankenhorn, 1978; Massey *et al.*, 1979; Becvar and Palmer, 1983).

Properties of 4-Thio-FMN-Flavodoxin—4-Thio-FMN binds tightly to the apoprotein of flavodoxin from *Megasphera elsdenii*. As shown in Fig. 6, the absorption maxima of the oxidized form are now located at 370 and 503 nm with extinction coefficients of 9700 and 12,600 M^{-1} cm^{-1}, respectively. Like the native protein, the 4-thio-FMN-flavodoxin is reduced readily under anaerobic conditions by the 5-deazaflavin catalyzed photoreduction method (Massey and Hemmerich, 1978). The spectra of the semiquinone and fully reduced forms of the flavoprotein obtained by this method are shown in Fig. 6. As with the native flavin, and all other modified flavins so far studied, the protein stabilizes the blue neutral semiquinone, in this case with an absorption maximum at 725 nm and extinction ~4000 M^{-1} cm^{-1}. In the native protein this stabilization is achieved by hydrogen bonding of the N(5)H of the flavin semiquinone to a backbone carbonyl of the peptide chain (Smith *et al.*, 1977). It would appear that similar stabilization exists with the 4-thio-FMN flavoprotein. In this connection it is interesting to note that although 4-$NHCH_3$-FMN, 4-$N(CH_3)_2$FMN, 4-NOH-FMN, and 4-S-$CONH_2$FMN (see Table IV) all bind tightly to apoflavodoxin, in no case is there any observable stabilization of a semiquinone intermediate on photoreduction (results not shown). This may be due to the bulky residue now located at the 4-position preventing the H bonding contact of the flavin *N*(5)H with the protein.

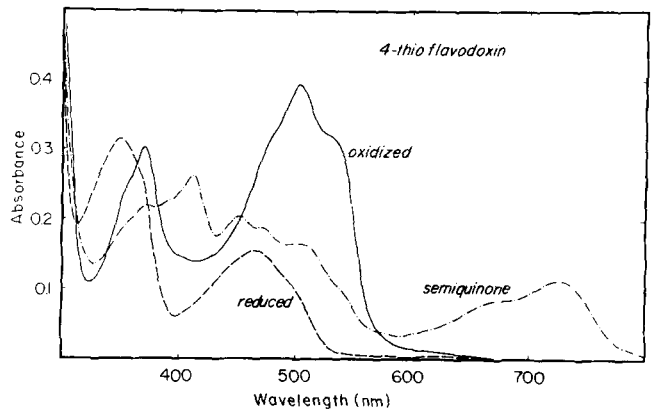


FIG. 6. **Spectra of 4-thio-FMN-flavodoxin.** 4-Thio-FMN-flavodoxin in 0.1 M phosphate, pH 7.0, 4 °C containing 30 mM EDTA and 1 μM deazaflavin, was photoreduced under anaerobic conditions. ——, before irradiation; (— · —), the maximal semiquinone form produced on irradiation for 30 s at an intensity of ~ 2×10^6 ergs cm^{-2} s^{-1}; (– – –), fully reduced form obtained on irradiation for 2 min.

The spectrum of the fully reduced 4-thioflavodoxin is also shown in Fig. 6 and spectral properties are listed in Table I. By comparison with the spectra of free reduced 4-thioriboflavin (Fig. 2), information can be obtained on the ionization state of the reduced enzyme, thus helping to clarify a point of some uncertainty. With native flavodoxin a pK of 5.8 for the reduced enzyme has been found by amperometric titrations; however, the spectrum of reduced flavodoxin is independent of pH (Mayhew *et al.*, 1969). By spectroscopic comparisons with known flavins the flavin of reduced flavodoxin was suggested to be in the anionic form and in a rather planar conformation (Ghisla *et al.*, 1974). However, the pK values of 8-hydroxy-FMN and 6-hydroxy-FMN bound to flavodoxin are increased by 1.3 and 1.9 units comapared with the free coenzymes (Ghisla and Mayhew, 1976; Mayhew *et al.*, 1974). The spectrum of reduced 4-thio-FMN flavodoxin is also consistent with a considerable increase in the pK of the reduced flavin, *i.e.* a stabilization by the protein of the neutral dihydroflavin. Thus the pK of the reduced enzyme observed amperometrically can probably be ascribed to a protein ionization rather than to one of the flavin.

While the reduced forms of 4-thioriboflavin and 4-thio-FMN are nonfluorescent, reduced 4-thioflavodoxin is slightly fluorescent (see Table I). A similar phenomenon is found with the native flavoprotein (Ghisla *et al.*, 1974). On mixing with air, the semiquinone is reformed rapidly, as also occurs with

native protein, and more slowly complete return to the starting oxidized spectrum occurs.

4-Thio-FMN-flavodoxin reacts extremely slowly with NH_2OH, the reaction being about 10% complete in 2 days at pH 7.0, 4 °C, with 4 mM NH_2OH. Under the same conditions 4-thioriboflavin has a half-time of 43 min. Similarly the reaction with H_2O_2 is very slow; approximately 10^{-2} M^{-1} min^{-1} at pH 7.0, 4 °C, compared with the value of 1.8 M^{-1} min^{-1} for free 4-thioflavin. From these results it is apparent that the flavin 4-position must be shielded from attack by these reagents, in agreement with the known structure of the oxidized flavoprotein derived from x-ray diffraction analysis (Burnett *et al.*, 1974).

Properties of 4-Thio-FAD-D-Amino Acid Oxidase—Fig. 7 shows the spectrum of 4-thio-FAD-D-amino acid oxidase, obtained by incubation of 4-thio-FAD with a slight excess of apoprotein for 10 min at 25 °C, removal of denatured apoprotein by centrifuging, and passage through a Sephadex G-25 column equilibrated with 0.1 M phosphate, pH 7.0. No free 4-thio-FAD was detected, indicating tight binding between the flavin and the apoprotein. On photoreduction with EDTA and a catalytic amount of 5-deazaflavin, an intermediate with a very intense and sharp absorption peak at 415 nm was observed, which presumably is the anionic radical species of 4-thio-FAD, since this enzyme has always been found to stabilize anionic flavin radicals (Massey and Palmer, 1966; Massey and Hemmerich, 1980). Further irradiation yields the fully reduced flavoprotein, whose characteristics (see also Table I) resemble those of the anion form of reduced 4-thioriboflavin. The reduced enzyme is quite strongly fluorescent, as detailed in Table I. On admitting air the initial oxidized 4-thioflavin spectrum is regained very rapidly, a property similar to that of native enzyme.

4-Thio-FAD-D-amino acid oxidase is also catalytically active. At pH 8.5, 25 °C, it has a turnover number of 80 min^{-1} in air equilibrated solutions, with D-alanine as substrate, with a K_m apparent of 5 mM. These values should be compared to 500 min^{-1} and 3 mM, respectively, for native enzyme under the same conditions (Massey *et al.*, 1966). When enzyme was mixed with D-alanine under anaerobic conditions, the absorbance at 505 nm was rapidly reduced. The resulting spectrum

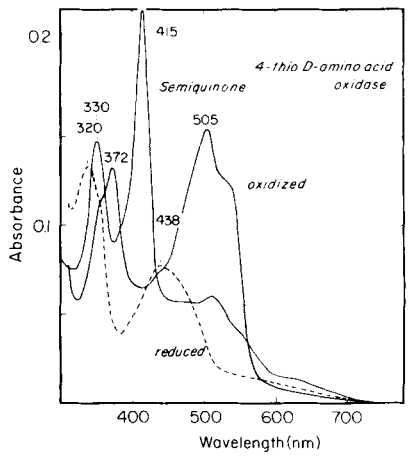


FIG. 7. **Spectra of 4-thio-FAD-D-amino acid oxidase.** The 4-thioenzyme, in 0.1 M phosphate, pH 7, containing 20 mM EDTA and 1 μM 5-deazaflavin was made anaerobic and photoreduced at 4 °C, as described in the legend to Fig. 6. Reduction to maximum semiquinone was rapid, requiring only a 40-s illumination. Further reduction was much more difficult, full reduction being obtained after a 50-min irradiation. Admission of air after this time restored 90% of the initial absorbance at 505 nm.

was similar to that of the photoreduced enzyme shown in Fig. 6, except for a long wavelength tail extending out to 700 nm. At this stage the enzyme was devoid of fluorescence. The fluorescence of the reduced enzyme (Table I) gradually appeared, coincident with the loss of long wavelength absorbance. At pH 7.0, 4 °C, this process had a half-time of approximately 25 min. Similar observations have been found with native enzyme, and are due to a charge-transfer complex between reduced enzyme and imino acid product (Massey and Ghisla, 1974). It is this complex which is the catalytically important form of the reduced enzyme which then reacts with O_2 to complete the catalytic cycle (Massey and Gibson, 1964). In such anaerobic experiments, when catalase is added to destroy H_2O_2, mixing with O_2 gives complete return of the original spectrum, even after the completion of 150 catalytic turnovers.

4-Thio-FAD-D-amino acid oxidase also forms a complex with benzoate, with pronounced perturbation of the absorption spectrum, with a large increase in extinction ($\Delta\epsilon$ ~4600 M^{-1} cm^{-1}) at 550 nm, isosbestic points at 503 and ~415 nm, and a decreased absorbance between these wavelengths ($-\Delta\epsilon_{460}$ approximately 1300 M^{-1} cm^{-1}). Using the absorbance increase at 550 nm as a function of benzoate concentration, a K_d of 2.7×10^{-5} M at pH 7.0, 4 °C, was calculated. This is approximately 10 times greater than that observed at the same pH, but at 19 °C, for native enzyme (Quay and Massey, 1977).

The 4-position of the flavin in 4-thio-FAD-D-amino acid oxidase appears to be quite accessible to solvent-borne reagents, a result not surprising in view of the long known reactivity of the native enzyme with sulfite at the neighboring position *N*(5) (Massey *et al.*, 1969). When 4-thio-FAD enzyme was reacted with H_2O_2 at pH 7.0, 4 °C, the S-oxide was formed with a rate constant of 2.6 M^{-1} min^{-1}, and was subsequently converted to native FAD enzyme at a rate of 0.88 M^{-1} min^{-1}.

When the reaction was carried out with 4-thio-FAD enzyme containing 3 mM benzoate the rate was slowed dramatically. No S-oxide was observed and the half-time for conversion to native enzyme by 18 mM H_2O_2 was approximately 3 days at pH 7.0, 4 °C.

Similar effects were seen in reaction of 4-thio-FAD enzyme with NH_2OH. At pH 7.0, 4 °C, the rate constant was 0.56 M^{-1} min^{-1}, which compares not unfavorably with the value of 4.1 M^{-1} min^{-1} with free 4-thioriboflavin, and indicates reasonable accessibility of the flavin 4-position to NH_2OH. However, in the presence of 2 mM benzoate, at most a 10% reaction was observed in 1 day with 2 mM NH_2OH. These and the results with H_2O_2 indicate that in the benzoate complex, either the flavin 4-position is shielded by benzoate, or a conformational change occurs which brings part of the protein peptide chain over this portion of the flavin. It is not possible to say what the effect of benzoate is due to in the present case. However, in view of the finding that benzoate has a similar protective effect on reactions of 8 substituted flavins bound to the apoprotein (Schopfer *et al.*, 1981), it is clear that a significant conformational change in the enzyme has to occur on binding of benzoate.

Properties of 4-Thio-FMN Lactate Oxidase—While the 4-thioflavoproteins described above are stable for at least 1 week on ice and at least 3 months at −20 °C, 4-thio-FMN reconstituted into the apoprotein of lactate oxidase at pH 7 undergoes appreciable changes even during gel filtration with Sephadex G-25. The spectrum changes gradually toward that of native flavoenzyme, but in addition has a broad long wavelength band centered around 700 nm (Fig. 8). Incubation with a low concentration of KCN (10 mM) resulted in the

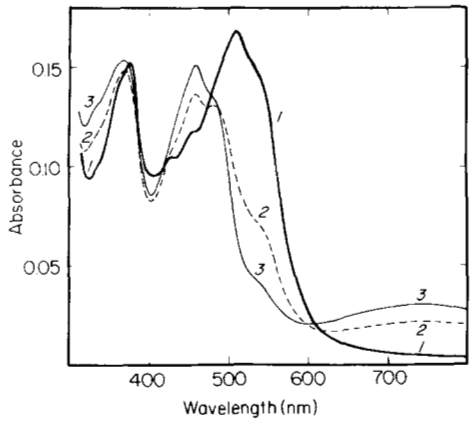


FIG. 8. **Slow spectral changes accompanying conversion of 4-thio-FMN lactate oxidase to FMN enzyme.** Freshly prepared apo-lactate oxidase was mixed with approximately an equimolar concentration of 4-thio-FMN in 0.1 M imidazole, pH 7, 4 °C. After 20 min, the mixture was subjected to gel filtration on Sephadex G-25 equilibrated with 0.01 M imidazole, pH 7. *Curve 1*, immediately after Sephadex; *curve 2*, afer 2 days at 0 °C; *curve 3*, after 6 days.

disappearance of the long wavelength band, and the resulting enzyme had spectral characteristics very similar to that of native enzyme (λ_{max} 372 and 456 nm). Denaturation with 5% tricholoroacetic acid resulted in all the flavin appearing in the supernatant; on neutralization the absorbance and fluorescence properties were indistinguishable from those of FMN. Analysis of the supernatant with acid ferric nitrate by the method of Sörbo (1957) revealed the presence of thiocyanate. With two separate batches of aged 4-thio-FMN enzyme, the amounts of thiocyanate detected were 1.20 and 0.48 mol/FMN. The larger content was estimated from the relatively dilute sample shown in Fig. 8 and is only approximate. The finding of thiocyanate is indicative of a protein persulfide residue being generated in the course of conversion of the 4-thioflavin to FMN, and forming a charge-transfer complex with the protein-bound FMN. A charge-transfer complex with similar spectral properties has recently been identified in the butyryl-CoA dehydrogenase of *M. elsdenii*, and shown to be due to interaction of coenzyme A persulfide with enzyme-bound FAD (Williamson *et al.*, 1982). In the case of lactate oxidase, the conversion of bound 4-thio-FMN to FMN is presumably promoted by a protein thiolate, in the same way as occurs with free 4-thioflavins and thiols (see previous section). In this case, however, the sulfide released from the 4-thioflavin is presumably captured by the protein thiol to form a persulfide. Such a process would involve a net 2-electron oxidation of a sulfur atom, which could be accomplished at the expense of reduction of the enzyme flavin.

The 4-thio-FMN enzyme is reduced rapidly by L-lactate under anaerobic conditions, and the reduced enzyme is strongly fluorescent. The spectral and fluorescence characteristics are given in Table I. As with the native enzyme (Ghisla *et al.*, 1974), the reduced flavin is clearly stabilized in the anionic form. Like with the native enzyme, the reduced 4-thio-FMN enzyme also undergoes a photochemical reaction with β-bromopropionate. With native enzyme a flavin C(4a)-propionate adduct is formed, which is not reactive with O_2 (Ghisla *et al.*, 1979). The adduct formed with 4-thio-FMN enzyme is similarly O_2-stable, and is presumably also a C(4a)-adduct. Its spectral and fluorescence properties are also given in Table I.

The 4-thio-FMN enzyme reacts rapidly with H_2O_2 to form catalytically active normal FMN enzyme, without any detectable S-oxide intermediate. This reaction is approximately 10 times faster than with free 4-thioriboflavin, indicating ready accessibility of H_2O_2 to the flavin 4-position. However, surprisingly methylmethane thiolsulfonate reacts extremely slowly, its main effect being to slow down dramatically the conversion to normal FMN enzyme, and to prevent completely the development of the long wavelength band shown in Fig. 8. The most logical explanation of these results is that methylmethane thiolsulfonate reacts rapidly with the protein thiolate which promotes the conversion to FMN, and in doing so blocks access of further reagent molecules to the flavin 4-position.

Properties of 4-Thio-FMN-Old Yellow Enzyme—Again the reconstituted 4-thio-FMN enzyme is not stable, undergoing very slow conversion to normal FMN enzyme over a period of several weeks at ice temperature. The initially formed holoenzyme has absorption maxima at 378 and 508 nm, and a very pronounced shoulder at 540 nm. While its catalytic turnover has not been determined, it is reduced rapidly by both NADH and NADPH, to give a weakly fluorescent reduced species. Like 4-thio-FMN lactate oxidase, it also reacts more rapidly with H_2O_2 than does free 4-thioriboflavin, and as with the lactate oxidase, reacts extremely slowly with methylmethane thiolsulfonate.

The 4-thio-FMN enzyme, like the native enzyme, also forms highly colored complexes with a variety of phenols. The absorption spectra of these complexes have been ascribed to charge transfer transitions between the bound phenolate anions and the oxidized flavin (Abramovitz and Massey, 1976b); this ascription has recently been questioned by Eweg *et al.* (1982). The results obtained with enzyme reconstituted with 4-thio-FMN and derivatives which can be easily prepared from it (see Table IV) fully support our original conclusions. This work will be presented in a separate paper.[4]

Reaction of 4-Thio-FAD and the Apoprotein of Glucose Oxidase—All attempts to prepare a stable 4-thio-FAD glucose oxidase have failed. When apoprotein is mixed with 4-thio-FAD at pH 7.0, 15 °C, the 4-thio-FAD is converted to FAD with a $t_{1/2}$ of approximately 40 min. Times of this magnitude are required for reformation of holoenzyme even with the native flavin, FAD (Swoboda, 1969b). If the incubation is carried out at pH 5.6 and 4 °C for 70 min, and the mixture is then passed through Sephadex G-25, approximately 35% binding of 4-thio-FAD to the apoprotein is achieved with only partial conversion to FAD. However, even at this pH the isolated 4-thio-FAD enzyme was unstable, and on warming to 25 °C was converted quantitatively to FAD-enzyme with a $t_{1/2}$ of approximately 15 min. The resulting enzyme behaves in all ways tested like normal enzyme. In distinction to the results with lactate oxidase, no long wavelength absorption is associated with the conversion of the 4-thio-FAD enzyme.

The rapid conversion of 4-thio-FAD to FAD appears to be due to a single thiol residue which is exposed in apoprotein but is buried in holoenzyme. Analysis with 5,5′-dithiobis(nitrobenzoic acid) showed 1.28 thiols/mol of funtionally active aproprotein and 0–0.18 thiols/mol of native glucose oxidase. Reaction with 5,5′-dithio(nitrobenzoic acid) abolished the ability of apoprotein to bind flavin.

Properties of 4-Thio-FAD-p-Hydroxybenzoate Hydroxylase—The holoenzyme formed by incubation of 4-thio-FAD with apo-*p*-hydroxybenzoate hydroxylase is stable for prolonged periods, in the absence of added reactants, when stored frozen at −20 °C, and has an extinction coefficient at 497 nm of 14,900 M^{-1} cm^{-1}. The 4-thio-FAD enzyme reacts with H_2O_2 at approximately the same rate as does free 4-thioflavin; the

[4] V. Massey, L. Schopfer, and W. R. Dunham, manuscript in preparation.

rate of further oxidation of the S-oxide is slowed, however. At pH 7.0, 4 °C, the rate of conversion to the S-oxide is 1.8 M^{-1} min^{-1}; the further conversion to FAD enzyme occurs at a rate of 0.16 M^{-1} min^{-1}. Under the same conditions with free 4-thioriboflavin only a trace of S-oxide intermediate is observed and the overall conversion to riboflavin occurs at a rate of 1.8 M^{-1} min^{-1}. This must represent the rate of S-oxide formation, with the subsequent reactions occurring more rapidly. The 4-thio-FAD enzyme also reacts rapidly with methylmethane thiolsulfonate. At pH 7.0, 4 °C, the reaction is strictly second order, with a rate of 340 M^{-1} min^{-1}. This is 34 × faster than the rate with free 4-thioriboflavin, and may be explained at least partially by a lowering of the pK of the enzyme bound 4-thio-FAD, as was observed in the case of the 2-thio-FAD enzyme (Claiborne *et al.*, 1982). The 4-thio-FAD enzyme binds the substrate *p*-hydroxybenzoate with a perturbation of the absorption spectrum characterized by a large negative difference at 548 nm and positive difference maxima at 492 and 508 nm. Using these differences a K_d for binding of *p*-hydroxybenzoate of 20 μM at pH 7.0, 4 °C, was obtained. In the presence of 1 mM *p*-hydroxybenzoate the rate of reaction with methylmethane thiolsulfonate was slowed approximately 20-fold to a value of 15 M^{-1} min^{-1}. The presence of 1 mM *p*-hydroxybenzoate also slows the reaction with H_2O_2, the conversion to S-oxide occurring at a rate of 0.14 M^{-1} min^{-1} and the subsequent conversion to FAD-enzyme at a rate of 0.04 M^{-1} min^{-1}. The S-oxide can be formed practically quantitatively by titration with *m*-chloroperbenzoate (λ_{max} = 383, 541 nm; ϵ_{541} = 12,300 M^{-1} min^{-1}). The ready conversion of the 4-thio-FAD enzyme to normal FAD-enzyme by H_2O_2 should be contrasted to the results obtained in the reaction of 2-thio-FAD enzyme, where covalent attachment of the flavin to the protein was found (Claiborne *et al.* (1983).

Like native enzyme, the 4-thio-FAD enzyme is reduced only slowly by NADPH in the absence of substrate; however, in the presence of *p*-hydroxybenzoate, reduction is very rapid. Unfortunately, it has not been possible to determine if the 4-thio-FAD enzyme is catalytically viable, since on reduction and reoxidation it is converted to normal FAD enzyme. The mechanism of this intriguing and unusual reaction is under investigation and will be documented separately.[5] The basic phenomenon is, however, illustrated in Fig. 9. Here the enzyme was photoreduced under anaerobic conditions in the absence of substrate and the presence of 2.5 μg/ml of catalase. The resulting reduced enzyme is slightly fluorescent, with characteristics listed in Table I. On admitting air, the spectrum changes rapidly to that shown in curve 3, which can be fitted well by a mixture of 53% native FAD enzyme and 47% 4-thio-FAD enzyme. Curve 4 shows the results of three cycles of photoreduction and reoxidation, where >90% conversion to native enzyme is found. The degree of conversion to native enzyme on reaction of the reduced enzyme with O_2 is markedly dependent on the type of substrate or effector present.[5] This sensitivity to conversion of the reduced 4-thioflavin to normal oxidized flavin as a result of reaction with O_2 is in marked contrast to the stability already noted in previous sections of 4-thio-FMN flavodoxin and 4-thio-FAD-D-amino acid oxidase, as well as free 4-thioriboflavin, to cycles of reduction and reoxidation.

During the photoreduction of 4-thio-FAD-*p*-hydroxybenzoate hydroxylase in the absence of substrate, a spectral intermediate is observed in small amounts similar to the anion semiquinone of 4-thio-FAD-D-amino acid oxidase. In the presence of 2,4-dihydroxybenzoate or tetrafluoro-*p*-hydroxybenzoate extensive formation of the blue semiquinone, similar to that of 4-thio-FMN flavodoxin, is observed. The spectrum of the reduced 4-thio-FAD enzyme is also quite dependent on bound subtrate (see Table I). This is particularly notable in the case of 2,4-dihydroxybenzoate where the absorption maximum is shifted from 458 nm in the uncomplexed enzyme to 475 nm. Thus it would appear that there may be a correlation between ligand binding and the stabilization of both the neutral semiquinone and the neutral reduced flavin. Such a correlation can also be made in the case of native enzyme, although the effects are not so clear (Entsch *et al.*, 1976; Husain *et al.*, 1980). This would imply a conformational change in substrate binding which brings the flavin N(5)H of these reduced forms in closer contact to a suitable protein residue which would permit stabilization through hydrogen bonding. Such a stabilization might also exist for the flavin 4a-hydroperoxide formed on reaction of the reduced flavin with O_2, and perhaps play an important role in catalysis.

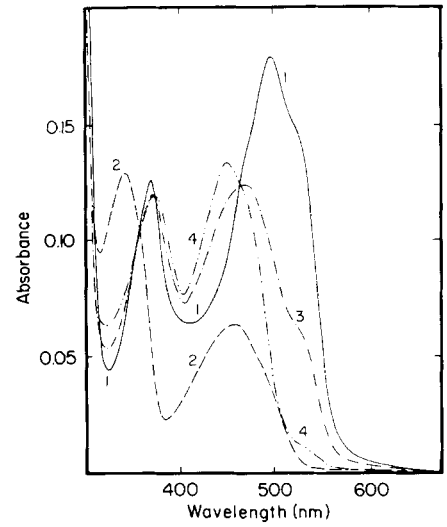


FIG. 9. **Conversion of 4-thio-FAD-*p*-hydroxybenzoate hydroxylase to native enzyme by repetitive cycles of photoreduction and air oxidation.** 4-Thio-FAD enzyme (11.8 μM; *curve 1*) in 0.05 M phosphate, pH 7, containing 35 mM EDTA, 2.5 μg/ml of catalase, and 1 μM 5-deazaflavin, was irradiated under anaerobic conditions to yield the reduced enzyme (*curve 2*). *Curve 3* was obtained on air reoxidation. *Curve 4* was obtained after three cycles of anaerobic photoreduction and air oxidation. The resulting enzyme had the same specific activity in the hydroxylation of *p*-hydroxybenzoate as native enzyme. That the 4-thio-FAD was converted to FAD was shown by identical absorbance and fluorescence properties in 4 M guanidine HCl, pH 7, and by TLC (on silica gel plates developed with 5% $Na_2HPO_4/12H_2O$ (w/v)) of the flavin released by precipitation of the protein with 5% trichloroacetic acid.

CONCLUSIONS

The ability of 4-thioflavins to react readily with comparatively innocuous reagents such as H_2O_2 and NH_2OH makes them attractive candidates for testing solvent accessibility to this region of the flavin in flavoproteins. In this respect it joins 8-mercapto- and 2-thioflavins as very useful flavin analogs for exploring the active site environment of flavoproteins. In addition, 4-thioflavins have the possibility of forming stable covalent linkages with lysine residues or N-terminal amino acid residues, if they were properly located in the active site so as to permit formation of the presumed tetrahedral intermediate on the way to the final aminoflavin product (*cf.* Scheme 2). So far no such covalent link has been found in the proteins that we have tested. However, very suggestive evidence has been obtained for a thiol residue in the flavin

[5] A. Clairborne and V. Massey, unpublished data.

binding site both of glucose oxidase and lactate oxidase, where the 4-thioflavin coenzyme is converted to the native (4-oxo) flavin. In the case of glucose oxidase, this conversion is very rapid, suggesting that the active site thiol is optimally positioned for attack at the flavin 4-position. In the native holoenzyme this thiol is not exposed to reaction with thiol reagents, and so must be covered by the flavin, or buried as a result of the conformational changes observed to accompany formation of catalytically active holoenzyme (Swoboda, 1969b). In the case of lactate oxidase, the conversion of 4-thio-FMN to FMN is accompanied by the development of a long wavelength band which appears to be due to conversion of an active site thiol to a persulfide. It is interesting to note that the spectral characteristics of the resulting enzyme are very similar to those of the green form of a bacterial butyryl-CoA dehydrogenase, where the long wavelength band has been shown to be associated with a charge-transfer interaction between a tightly bound coenzyme A-persulfide and the oxidized FAD coenzyme (Williamson *et al.*, 1982).

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