Properties of a High-Potential Flavin Analogue and Its Use as an Active Site Probe with Clostridial Flavodoxin†

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ABSTRACT: The reduction potential of flavin bearing a methylsulfonyl moiety ($MeSO_2$) in place of a methyl group at position 8 is increased by more than 150 mV as compared with normal flavin. This substitution is accompanied by a substantial increase in reactivity with various reductants, including NADH, and greatly (10^3-fold) enhanced susceptibility toward nucleophilic attack by sulfite at N(5). 1,5-Dihydro-8-(methylsulfonyl)riboflavin exhibits two intense, well-resolved absorption bands (λ_{max} = 310, 362 nm) in a region where most other reduced flavins exhibit weak, characterless absorption. This unusual spectrum is attributable to a shift of π-electron density from the N(5) atom into the benzene ring. It is observed only with reduced flavins bearing a strongly electronegative substituent ($MeSO_2$, CN) at the 8-position. The effect is abolished by replacing the hydrogen at N(5) with a bulky group, like sulfite, which interferes with sp^2 hybridization at N(5). Reaction of 8-$MeSO_2$-substituted flavins with thiols results in nucleophilic displacement of $MeSO_2^-$ in a reaction that is about 10^3-fold faster than an analogous nucleophilic displacement reaction observed with 8-halo-substituted flavins. The flavin ring acts as a redox switch in controlling electrophilicity at the 8-position, as judged by the fact that the displacement reactions are observed only with the oxidized flavins. Initial studies to evaluate 8-$MeSO_2$-substituted flavins as active site probes were conducted with flavodoxin from *Clostridium beijerinckii MP*. 8-$MeSO_2$FMN is rapidly bound to apoflavodoxin, accompanied by absorbance and fluorescence changes similar to those observed for FMN binding. 1,5-Dihydro-8-$MeSO_2$FMN flavodoxin exhibits spectral properties (λ_{max} = 323, 382 nm) similar to those of the corresponding free flavin, except for a bathochromic shift due to a change in the polarity of the flavin environment. As judged by peak resolution and intensity, the spectral properties of 1,5-dihydro-FMN flavodoxin (λ_{max} = 311, 362 nm) appear to lie about midway between those observed for the free 1,5-dihydro forms of FMN versus 8-$MeSO_2$FMN. This suggests that the protein environment may favor enhanced resonance delocalization of π-electron density into the benzene ring of bound 1,5-dihydro-FMN, as compared with the free flavin. This hypothesis is consistent with previous NMR studies and with a proposal that electron transfer from reduced flavodoxin to other redox proteins occurs through this region of the ring. 8-$MeSO_2$FMN bound to flavodoxin reacts readily with exogenous thiols but does not react with sulfite. Covalent attachment of 8-$MeSO_2$FMN to the protein, via reaction with a cysteine residue, was not detected during prolonged storage of the reconstituted enzyme. The results are consistent with crystallographic data which show that the 8-position of FMN in the native enzyme is accessible to solvent, that solvent access to N(5) is hindered, and that none of the protein's three cysteine residues is in direct contact with the flavin.

The prosthetic group of many flavoenzymes can be reversibly removed under relatively mild conditions and then replaced, either with the natural flavin or with analogues. Studies with various flavin analogues have yielded a wealth of information regarding the active site environment, especially with respect to the modulation of flavin reactivity and accessibility via interactions with the protein moiety. Modified flavins have also proved useful as probes of the catalytic mechanism (Ghisla & Massey, 1986). Since flavoenzymes catalyze redox reactions, it is not surprising that some of the most useful mechanism probes are derivatives with altered redox potentials. The kind of information that can be obtained by the use of flavin analogues with a range of different potentials is illustrated by elegant studies conducted with xanthine dehydrogenase (Nishino et al., 1989), adrenodoxin reductase (Light & Walsh, 1980), and *p*-hydroxybenzoate hydroxylase (Schopfer et al., 1991). 5-Deaza- and 1-deazaflavins have proved especially powerful and have been widely used as low-potential mechanism probes (Hemmerich et al., 1977; Spencer et al., 1977a,b). However, it is difficult to identify a comparable high-potential flavin probe. The introduction of a small, strongly electronegative group (e.g., CN, RSO_2) at position 8 of the isoalloxazine ring can increase the reduction potential by more than 150 mV (Bruice et al., 1977; Moore et al., 1979), but these analogues have not been available at the FMN[1] or FAD level for use as mechanistic probes in flavoenzyme reactions.

In this article, we report the preparation of FAD and FMN analogues containing a methylsulfonyl moiety in place of a methyl group at position 8. We describe the spectral properties and chemical reactivity of free 8-(methylsulfonyl)-substituted flavins. Initial studies to evaluate 8-(methylsulfonyl)flavin as an active site probe were conducted with flavodoxin from

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; 8-MeS-riboflavin, 8-(methylthio)riboflavin; 8-Cl-riboflavin, 8-chlororiboflavin; 8-$MeSO_2$-riboflavin, 8-(methylsulfonyl)riboflavin; 8-$MeSO_2$FAD, 8-(methylsulfonyl)-FAD; 8-$MeSO_2$FMN, 8-(methylsulfonyl)-FMN; 8-F-riboflavin, 8-fluororiboflavin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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Clostridium beijerinckii MP. Flavodoxins cycle between the fully reduced and semiquinoid states, transferring single electrons to and from other proteins (Mayhew & Tollin, 1992). *C. beijerinckii MP* flavodoxin was selected because the protein is well-characterized and the crystal structure is known for the oxidized, semiquinoid, and fully reduced forms (Burnett et al., 1974; Smith et al., 1977; Ludwig & Luschinsky, 1992).

EXPERIMENTAL PROCEDURES

Materials. 8-Chlororiboflavin was a generous gift from Dr. John P. Lambooy. 8-(Methylsulfonyl)riboflavin was kindly provided by Dr. Vincent Massey. 8-Cyano-10-methyl-*N*(3)-(carboxypentyl)isoalloxazine was a generous gift from Dr. John R. Cashman. Flavodoxin from *Clostridium beijerinckii MP* (*Clostridium MP*), expressed as a recombinant protein in *Escherichia coli* (Eren & Swenson, 1989), was a generous gift from Dr. Richard P. Swenson. Sodium thiomethoxide and *m*-chloroperoxybenzoic acid were obtained from Aldrich. Phosphodiesterase (Naja naja snake venom), calf thymus alkaline phosphatase, and FMN were purchased from Sigma.

Chromatography. Reversed-phase HPLC was performed using a Rainin gradient HPLC system and analytical (4.6 × 250 mm) or semipreparative (10.0 × 250 mm) 5-μm Microsorb C_{18} columns from Rainin. To compare 8-(methylthio)riboflavin (8-MeS-riboflavin) with products formed by reacting 8-(methylsulfonyl)riboflavin (8-$MeSO_2$-riboflavin) with DTT or β-mercaptoethanol (see Table III for reaction conditions), samples were injected onto an analytical C_{18} reversed-phase column equilibrated with water. Elution was effected using a linear gradient from 0 to 80% acetonitrile over a period of 27 min. The flow rate was 0.5 mL/min. Peaks were detected by monitoring absorbance at 460 nm. Analytical thin-layer chromatography (TLC) was performed on silica gel plates (Macherey-Nagel) using *n*-butanol/acetic acid/water (2:1:1, v/v) (solvent A).

Spectroscopy. Absorption spectra were recorded using a Perkin-Elmer Lambda 3B spectrophotometer. For most anaerobic experiments, a specially constructed cuvette was made anaerobic, as previously described (Jorns & Hersh, 1975). In anaerobic photoreduction experiments, samples were irradiated at a distance of 2 cm from two black lights (Sylvania F15T8/BLB, 15 W). Dithionite titrations were performed by using an apparatus similar to that described by Burleigh et al. (1969). Dithionite solutions were standardized by titration with lumiflavin-*N*(3)-acetic acid, as described by Foust et al. (1969). The concentrations of 8-MeS-riboflavin ($\epsilon_{474} = 28.5 \times 10^3\ M^{-1}\ cm^{-1}$) and 8-chlororiboflavin (8-Cl-riboflavin) ($\epsilon_{445} = 12.0 \times 10^3\ M^{-1}\ cm^{-1}$) were estimated using extinction coefficients reported by Moore et al. (1979). The extinction coefficient of 8-$MeSO_2$-riboflavin at 450 nm ($\epsilon = 11.2 \times 10^3\ M^{-1}\ cm^{-1}$) was estimated on the basis of a value obtained by Moore et al. at 333 nm. The same extinction coefficient was used for 8-$MeSO_2$FAD and 8-$MeSO_2$FMN.

Kinetic Studies. The second-order rate constant for the reduction of 8-$MeSO_2$-riboflavin with 1,5-dihydro-5-deazariboflavin was determined in 0.1 M sodium pyrophosphate buffer, pH 8.65, at 30 °C, following the method described by Spencer et al. (1976). 5-Deazariboflavin was synthesized and then converted to 1,5-dihydro-5-deazariboflavin by reaction with sodium borohydride as described by Spencer et al. (1976). The kinetics of the reactions of 8-$MeSO_2$-riboflavin or 8-Cl-riboflavin with β-mercaptoethanol at various pH values were measured in aerobic 0.1 M potassium phosphate (pH 6.0–8.0) or 0.1 M sodium borate (pH 8.5–10) buffer at 25 °C by following the increase in absorbance at 490 nm.

Preparation of Apoflavodoxin and Reconstitution with 8-(Methylsulfonyl)-FMN. The concentration of native flavodoxin from *C. beijerinckii* was estimated on the basis of its absorbance at 445 nm ($\epsilon_{445} = 10.4 \times 10^3\ M^{-1}\ cm^{-1}$) (Mayhew, 1971). FMN was removed from the native enzyme by treatment with trichloroacetic acid, and the apoenzyme concentration was determined by titration with FMN, according to procedures described by Wassink and Mayhew (1975). The concentration of FMN was corrected for contaminants, present in commercial FMN preparations, as previously described (Wassink & Mayhew, 1975). For reconstitution studies, apoflavodoxin was titrated with 8-(methylsulfonyl)-FMN (8-$MeSO_2$FMN) in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.3 mM EDTA at 4 °C. Since the fluorescence of free 8-$MeSO_2$FMN is quenched upon binding to apoflavodoxin, the progress of the titration was followed spectrofluorometrically (excitation λ = 450 nm, emission λ = 550 nm), and the end point was detected by a sharp increase in fluorescence due to unbound 8-$MeSO_2$FMN.

To remove any excess flavin, the reconstituted enzyme was quantitatively transferred to a Centricon 3 microconcentrator and then subjected to several cycles of concentration followed by dilution with 0.1 M potassium phosphate buffer, pH 7.0, containing 20 mM EDTA. The extinction coefficient of the reconstituted protein ($\epsilon_{450} = 9.3 \times 10^3\ M^{-1}\ cm^{-1}$) was estimated on the basis of the reconstituted protein concentration. The latter was determined after the initial apoenzyme concentration was corrected for any volume changes during the reconstitution procedure but not for possible protein loss during the microconcentrator step. (Nearly quantitative protein recovery (>90%) was observed in control studies when native flavodoxin was subjected to the same procedure.) 8-$MeSO_2$FMN was prepared by incubating 8-$MeSO_2$FAD (vide infra) for 30 min at room temperature with Naja naja snake venom (100 μg/mL) in 0.1 M potassium phosphate buffer, pH 7.0. The venom was removed using a Centricon 3 microconcentrator and the sample stored at −20 °C.

Syntheses

8-(Methylthio)riboflavin. 8-MeS-riboflavin was synthesized by reacting 8-Cl-riboflavin with sodium thiomethoxide in 0.05 M sodium carbonate buffer, pH 9.85, containing 50% *N*,*N*-dimethylformamide, similar to a procedure described by Moore et al. (1979).

8-(Methylsulfonyl)riboflavin. 8-$MeSO_2$-riboflavin was synthesized by reacting 8-MeS-riboflavin with hydrogen peroxide in acetic acid, as described by Moore et al. (1979). Alternatively, *m*-chloroperoxybenzoic acid (20 mg) was added to a suspension of 8-MeS-riboflavin (3 mg) in 1.5 mL of 75% acetonitrile at room temperature. A clear solution was obtained after the mixture stirred for 5–10 min. After an overnight incubation, the yellow solution was diluted to 10 mL with cold water, stored on ice for 1 h, and then filtered to remove a white precipitate. The pH of the filtrate was adjusted to pH 4.0, and the product was purified by HPLC using a semipreparative C_{18} column. Elution was conducted using a linear gradient of methanol (5–70%) in 5 mM ammonium acetate, pH 5.8 (gradient system I). The flavin product eluted at about 50% methanol and was identified as 8-$MeSO_2$-riboflavin, as judged by its spectral (λ_{max} = 450, 333 nm) and chromatographic [HPLC (gradient system I), TLC (solvent A)] properties. The yield of pure compound was about 90%.

8-(Methylsulfonyl)-FAD. 8-MeS-riboflavin (30 μmol) was converted to 8-MeSFAD by incubation with partially purified

Table I: Enzymic Conversion of 8-(Methylsulfonyl)-FAD to 8-(Methylsulfonyl)riboflavin[a]

flavin	retention time (min)	R_f
8-(methylsulfonyl)-FAD		
untreated	18.07	0.22
phosphodiesterase/phosphatase	21.82	0.68
phosphodiesterase alone	nd[b]	0.41
8-(methylsulfonyl)riboflavin	21.80	0.68

[a] 8-(Methylsulfonyl)-FAD was incubated for 30 min at room temperature in 0.1 M sodium carbonate buffer, pH 9.0, containing 1.0 mM magnesium chloride, 0.1 mM zinc chloride, phosphodiesterase (Naja naja snake venom, 1 mg/mL), and alkaline phosphatase (37 units/mL). A second sample was incubated for 60 min under the same conditions except that alkaline phosphatase was omitted. The samples were analyzed on an analytical C_{18} reversed-phase column equilibrated with 5 mM ammonium acetate, pH 5.8, containing 5% methanol. Elution was conducted using a linear methanol gradient (5–70%) in 5 mM ammonium acetate, pH 5.8. The flow rate was 0.5 mL/min. Thin-layer chromatography was conducted using solvent A. [b] Not determined.

flavokinase/FAD synthetase from *Brevibacterium ammoniagenes*, according to procedures described by Hausinger et al. (1986). Reaction progress was monitored by TLC (solvent A). After 70 h, the conversion to 8-MeSFAD was about 80% complete with the remainder of the flavin present as 8-MeS-FMN. The reaction mixture was centrifuged and then filtered through an Amicon PM-10 membrane. The filtrate (300 mL) was divided into three aliquots, and each aliquot was separately purified by HPLC using a semipreparative reversed-phase C_{18} column. Elution was conducted using a linear gradient of methanol (30–70%) in 5 mM ammonium acetate, pH 5.8. 8-MeSFAD (15 μmol) eluted at about 40% methanol and was well-separated from 8-MeSFMN, which elutes near 50% methanol. The eluate containing 8-MeSFAD was concentrated to 5 mL and then mixed with an equal volume of 0.2 M *m*-chloroperoxybenzoic acid in acetonitrile. After an overnight incubation at room temperature, the reaction mixture was extracted with chloroform to remove *m*-chloroperoxybenzoic and *m*-chlorobenzoic acids and then concentrated to 2 mL. The sample was divided into two aliquots, and each aliquot was separately purified on a semipreparative reversed-phase C_{18} column using a linear gradient of acetonitrile (0–10%) in water as the mobile phase. Fractions containing pure 8-$MeSO_2$FAD (4.8 μmol) (λ_{max} = 450, 333 nm) eluted around 6.5% acetonitrile. These fractions were pooled and stored at −20 °C until use.

RESULTS

Synthesis of 8-(Methylsulfonyl)-FAD. 8-(Methylsulfonyl)riboflavin (8-$MeSO_2$-riboflavin) can be prepared by oxidizing 8-(methylthio)riboflavin (8-MeS-riboflavin) with H_2O_2 in acetic acid (Moore et al., 1979). However, attempts to convert 8-$MeSO_2$-riboflavin to 8-(methylsulfonyl)-FAD (8-$MeSO_2$FAD) using the flavokinase/FAD synthetase from *B. ammoniagenes* were unsuccessful. 8-MeS-riboflavin could be converted to the FAD level using flavokinase/FAD synthetase (50% yield), but multiple products were obtained when 8-MeSFAD was treated with H_2O_2/acetic acid, prompting a search for an alternate oxidant.

Nearly quantitative conversion of 8-MeS-riboflavin (λ_{max} = 474 nm) to 8-$MeSO_2$-riboflavin (λ_{max} = 450, 333 nm) was obtained using *m*-chloroperoxybenzoic acid in aqueous acetonitrile. The reaction was biphasic and proceeded via initial rapid (minutes) formation of a presumed sulfoxide intermediate (λ_{max} = 449, 347 nm), followed by a slower (overnight) conversion to 8-$MeSO_2$-riboflavin, similar to that observed with H_2O_2/acetic acid (Moore et al., 1979). The fluorescent intermediate (R_f = 0.51) was readily distinguishable from

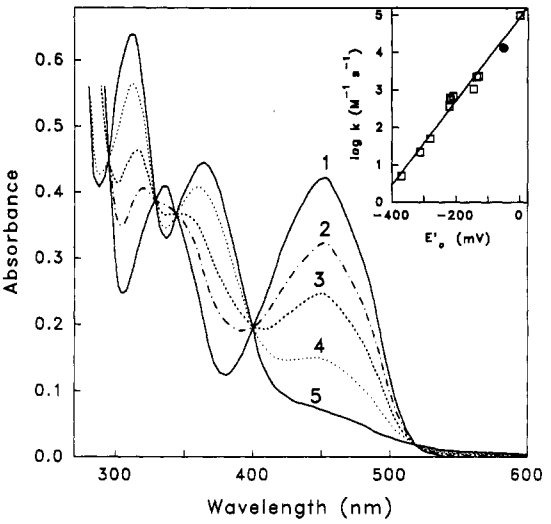


FIGURE 1: Reduction of 8-(methylsulfonyl)riboflavin. Curve 1 is the absorption spectrum of 8-$MeSO_2$-riboflavin in anaerobic 0.1 M potassium phosphate buffer, pH 7.0, containing 20 mM EDTA at 25 °C. Curves 2–5 were recorded after 10, 20, 40, and 110 s of irradiation with black light. The inset shows the linear free energy relationship between the rate of flavin reduction by 1,5-dihydro-5-deazariboflavin and the flavin reduction potential, based on data (□) reported by Walsh et al. (1978). The reduction rate observed with 8-$MeSO_2$-riboflavin (●) is plotted using the reduction potential reported by Moore et al. (1979).

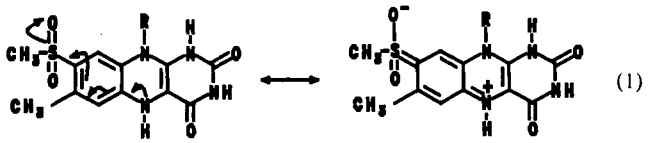
8-MeS-riboflavin (R_f = 0.79, weakly fluorescent) and 8-$MeSO_2$-riboflavin (R_f = 0.68, fluorescent) in thin-layer chromatography studies (solvent A).

Reaction of 8-MeSFAD with *m*-chloroperoxybenzoic acid under similar conditions also proceeded via an intermediate (λ_{max} = 450, 350 nm). The final product was identified as 8-$MeSO_2$FAD, as judged by the following results. Reaction of 8-$MeSO_2$FAD with phosphodiesterase plus alkaline phosphatase at pH 9.0 yielded 8-$MeSO_2$-riboflavin, as evidenced by high-performance and thin-layer chromatography data (Table I). Reaction of 8-$MeSO_2$FAD with phosphodiesterase alone yielded 8-$MeSO_2$FMN, as judged by reconstitution studies with apoflavodoxin (vide infra). Similar spectral properties are observed at pH 7.0 for 8-$MeSO_2$FAD (λ_{max} = 450, 333 nm, ϵ_{450}:ϵ_{333} = 1.11) and 8-$MeSO_2$-riboflavin (λ_{max} = 450, 333 nm, ϵ_{450}:ϵ_{333} = 1.10). No significant spectral change was observed during the enzymic conversion of 8-$MeSO_2$-FAD to 8-$MeSO_2$-riboflavin at pH 9.0, except for small decreases in absorbance probably due to the lability of 8-(methylsulfonyl)flavins under alkaline conditions.

Reduction of 8-(Methylsulfonyl)flavin. 8-$MeSO_2$-riboflavin is readily reducible by dithionite or by photochemical means in the presence of EDTA, similar to that observed for normal flavins. Facile reduction is also observed with sodium borohydride, NADH, or 1,5-dihydro-5-deazariboflavin. Reduction of 8-$MeSO_2$-riboflavin with 1,5-dihydro-5-deazariboflavin (k = 13 300 M^{-1} s^{-1}) is about 20-fold faster than observed for riboflavin reduction under similar conditions (k = 680–770 M^{-1} s^{-1}) (Spencer et al., 1976; Walsh et al., 1978). Walsh et al. (1978) showed that a linear free energy relationship exists between the rate of flavin reduction with 1,5-dihydro-5-deazariboflavin and the reduction potential of the flavin. Using this relationship, a reduction potential of −68 mV was estimated for 8-$MeSO_2$-riboflavin (Figure 1, inset), in fairly good agreement with the value of −50 mV determined by Moore et al. (1979).

The anaerobic photoreduction of 8-$MeSO_2$-riboflavin exhibits isosbestic points at 399, 345, 328, and 296 nm (Figure 1). Similar spectral changes were observed with all other

reductants tested and were fully reversible upon exposure to air. The 1,5-dihydro form (Figure 1, curve 5) exhibits two intense absorption bands at wavelengths greater than 300 nm (λ_{max} = 310, 362 nm), whereas most other 1,5-dihydroflavins in aqueous solution exhibit characterless, weak absorption in this region. Our results agree with previous studies using dithionite as reductant where it was suggested that the unusual absorption spectrum of reduced 8-$MeSO_2$-riboflavin was characteristic of electron-deficient flavins (Moore et al., 1979). While similar spectral properties have been reported for certain high-potential flavins (Table II, compounds 4 and 6), others exhibit featureless spectra in the reduced state (Table II, compounds 3 and 7). The data in Table II suggest that a critical factor might be the presence of an electronegative substituent at the 8-position capable of resonance interaction with the reduced isoalloxazine ring (eq 1).



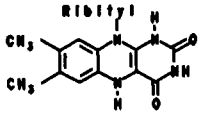
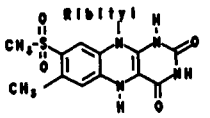
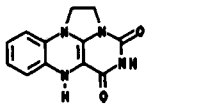
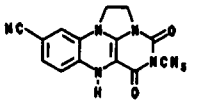
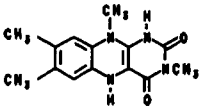
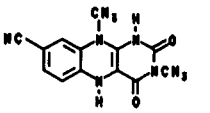
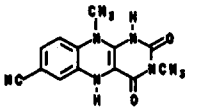
(1)

Flavin-Sulfite Complexes. Delocalization of the lone pair of electrons from the N(5) atom into the benzene subnucleus, as shown in eq 1, requires sp^2 hybridization at N(5). Predominant sp^2 character is observed for the N(5) atom in unsubstituted reduced flavins. However, the introduction of a bulky substituent at N(5) causes peri-overcrowding with the carbonyl at C(4). To escape steric strain, the nitrogen at position 5 will assume more sp^3 character to move the bulky group out of the plane (Hemmerich et al., 1971; Moonen et al., 1984; Dudley et al., 1964).

Sulfite is known to add reversibly to the 5-position of flavins, forming a complex with a structure similar to the corresponding 1,5-dihydroflavin, except that the hydrogen at N(5) is replaced by SO_3^- (Muller & Massey, 1969). 8-$MeSO_2$-riboflavin also reacts with sulfite to form a complex, as judged by the bleaching of the 450-nm absorption band of the oxidized flavin (Figure 2). The reversibility of the reaction was demonstrated in a separate experiment involving partial conversion to the complex, followed by dilution into sulfite-free buffer. Unmodified starting material was recovered as judged by the spectral course of the dissociation reaction which exhibited the same set of isosbestic points as that observed during complex formation (data not shown). The sulfite complex formed with 8-$MeSO_2$-riboflavin ($K_d = 2.0 \times 10^{-3}$ M) is nearly 3 orders of magnitude more stable as compared with the complex formed with riboflavin (K_d = 1.16 M) (Muller & Massey, 1969). A linear free energy relationship has been observed between the stability of flavin-sulfite complexes and the redox potential of the flavin (Muller & Massey, 1969). Using this relationship, a reduction potential of –76 mV is estimated for 8-$MeSO_2$-riboflavin (Figure 2, inset), in fairly good agreement with values estimated on the basis of the rate of reduction by 1,5-dihydro-5-deazariboflavin (–68 mV) or directly measured (–50 mV) in studies by Moore et al. (1979).

The sulfite complex formed with 8-$MeSO_2$-riboflavin exhibits a single absorption band at 306 nm (Figure 2, curve 5), which is dramatically different from the corresponding 1,5-dihydro form, but similar to spectra observed for sulfite complexes formed with other flavins. 8-Cyano-10-methyl-*N*(3)-(carboxypentyl)isoalloxazine also exhibits an unusual absorption spectrum in the reduced state (Figure 3, curve 2) (λ_{max} = 369, 309 nm) similar to other 8-cyano-substituted flavins (Table II), but a "normal" spectrum is observed for the corresponding sulfite complex (Figure 3, curve 3) (λ_{max} = 308 nm). The results obtained with 8-cyano- or 8-(methylsulfonyl)-substituted flavins are quite different from most other flavins. Similar spectral properties are generally observed for sulfite complexes and the corresponding 1,5-dihydro forms, except that the sulfite complexes exhibit little absorption at wavelengths greater than 400 nm. The 1,5-dihydro forms exhibit a weak shoulder in this region which appears to be related to the degree of sp^2 hybridization of the N(5) atom and is largely abolished when the hydrogen at position N(5) is replaced by a bulky substituent (Moonen et al., 1984; Dudley et al., 1964; Hemmerich et al., 1971). Nearly identical spectra are observed for sulfite complexes and the corresponding N(5)-acylated 1,5-dihydroflavins (Muller & Massey, 1969). The results strongly suggest that the unusual absorption spectrum observed for 8-$MeSO_2$-riboflavin and

Table II: Flavin Reduction Potentials and Absorption Properties of the 1,5-Dihydro Forms[a]

	compound	absorption spectrum λ (nm)	absorption spectrum ε (mM^{-1} cm^{-1})	reduction potential (mV)
1		340 (s)	5	–208
2		362 310 265	10.5 15.3 39.8	–50
3		320 (ws)	7.7	–22
4		362 310	13 15.5	> –22
5		350 (s)	4.8	–237
6		370 308 264	13.8 15.0 37	–16
7		310 (s)	8.6	–52

[a] Absorption properties and reduction potentials for compounds 1 (Massey et al., 1978), 2 (Moore et al., 1979), 3 (Muller & Massey, 1969), 4 (Knappe, 1980), and 5–7 (Bruice et al., 1977) are based on literature values. Spectral data were obtained in aqueous solution around neutral pH (pH 7.0–8.15), except for compound 4 where the data were obtained at pH 4. For compounds that do not exhibit absorption maxima at $\lambda > 300$ nm, spectral data at pronounced (s) or weak (ws) shoulders are included for comparison. Unless otherwise noted, 2-electron reduction potentials were determined at pH 7.0 and 25 °C. The data for compounds 5–7 were obtained at pH 9.0 and 30 °C and were reported as $E_{1/2}$ versus the standard calomel electrode. The reported values were recalculated versus the standard hydrogen electrode, and potentials at pH 7 were estimated by adding 59 mV to the pH 9.0 values. The value estimated for *N*(3)-methyllumiflavin (compound 5) is similar to values measured directly at pH 7 with lumiflavin (–216 mV) or lumiflavin-*N*(3)-acetate (–246 mV) (Muller & Massey, 1969). The reduction potential for compound 4 has not been measured but should be significantly greater than that observed with compound 3, as judged by the effect of an 8-cyano substituent in other flavin derivatives.

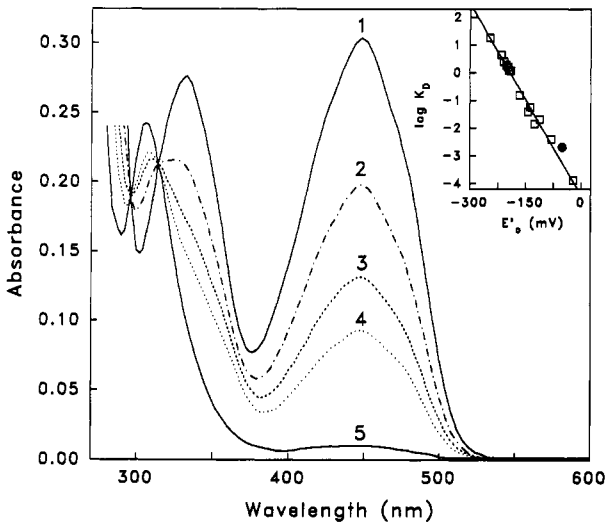


FIGURE 2: Reaction of 8-(methylsulfonyl)riboflavin with sulfite. Curve 1 is the absorption spectrum of 8-$MeSO_2$-riboflavin in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C. Curves 2–5 were recorded 15 min after adding 1.0, 2.0, 6.0, and 154 mM sodium sulfite, respectively. The inset shows the linear free energy relationship between sulfite complex stability and flavin reduction potential, based on data (□) reported by Muller and Massey (1969). The dissociation constant observed with 8-$MeSO_2$-riboflavin (●) is plotted using the reduction potential reported by Moore et al. (1979).

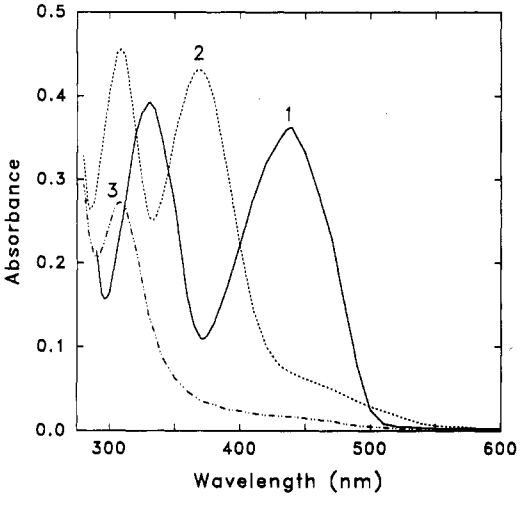


FIGURE 3: Spectral properties of the 1,5-dihydro form and the sulfite complex formed with an 8-cyanoflavin. Curve 1 is the absorption spectrum of 8-cyano-10-methyl-*N*(3)-(carboxypentyl)isoalloxazine in anaerobic 0.1 M potassium phosphate buffer, pH 7.0, containing 20 mM EDTA at room temperature. Curve 2 is the spectrum of the 1,5-dihydro form obtained after photoreduction for 180 s with black light, as detailed in the Experimental Procedures. Curve 3 is the absorption spectrum of the sulfite complex formed in a separate experiment by mixing the same concentration of the 8-cyanoflavin with 200 mM sodium sulfite in aerobic 0.1 M sodium phosphate buffer, pH 7.0.

related flavins in the 1,5-dihydro form is attributable a shift of π-electron density from the N(5) atom into the benzene ring, a process that is blocked when the hydrogen at N(5) is replaced by SO_3^-.

Nucleophilic Displacement Reactions with Thiols. As shown in Figure 4, the reaction of 8-$MeSO_2$-riboflavin with β-mercaptoethanol under aerobic conditions results in the isosbestic conversion to a product (λ_{max} = 474 nm) with spectral properties similar to those observed for flavins bearing an alkylthio substituent at position 8 (Moore et al., 1979). This suggested that the reaction might involve either a 4-electron reduction of the 8-$MeSO_2$ substituent to yield 8-MeS-

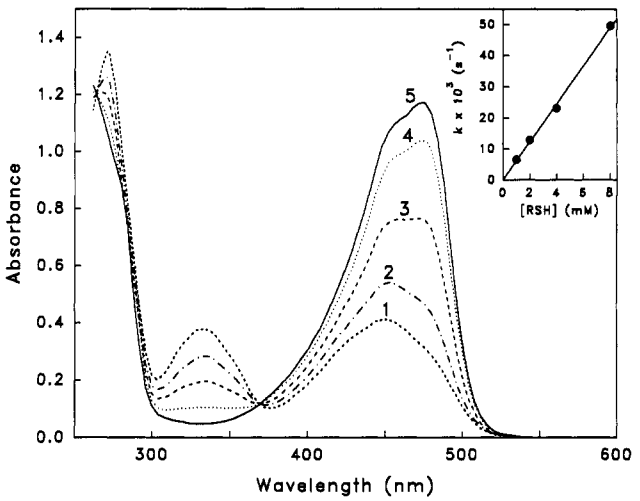


FIGURE 4: Reaction of 8-(methylsulfonyl)riboflavin with β-mercaptoethanol. Curve 1 is the absorption spectrum of 8-$MeSO_2$-riboflavin in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C. Curves 2–5 were recorded immediately, 3.5, 11, and 41 min, respectively, after mixing with 0.5 mM β-mercaptoethanol. The inset shows a plot of pseudo-first-order rate constants observed at various concentrations of β-mercaptoethanol in a separate series of experiments under otherwise similar conditions.

Table III: Identification of Products Formed in the Reactions of 8-$MeSO_2$-riboflavin with Thiols

expt	reaction components[a]	retention time (min)
A	8-$MeSO_2$-riboflavin + β-ME	13.22
B	8-Cl-riboflavin + β-ME	13.26
C	8-$MeSO_2$-riboflavin + DTT	13.55
D	8-Cl-riboflavin + DTT	13.55
E	mixture of A and C	13.22, 13.57
F	mixture of A and B	13.26
G	mixture of C and D	13.53
H	8-$MeSO_2$-riboflavin alone	11.92
I	8-Cl-riboflavin alone	13.47

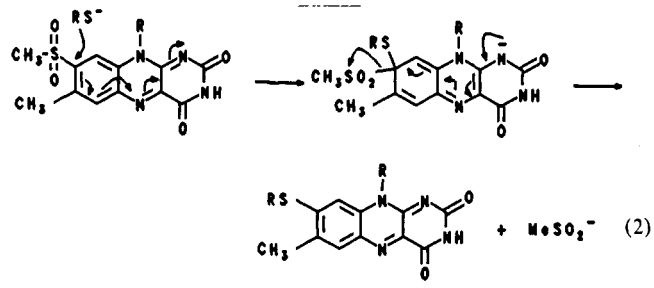
[a] The samples were incubated for 60 min at room temperature with 0 or 10 mM β-mercaptoethanol (β-ME) or DTT in 0.1 M potassium phosphate, pH 7.0 (8-$MeSO_2$-riboflavin), or 0.1 M sodium pyrophosphate, pH 8.5 (8-Cl-riboflavin), buffers containing 10% acetonitrile. Reaction aliquots were injected alone or in pairs onto an analytical C_{18} reversed-phase column equilibrated with 5 mM ammonium acetate, pH 5.8, containing 5% methanol. Isocratic elution was performed for 30 min using 80% methanol in 5 mM ammonium acetate, pH 5.8, as the mobile phase. The flow rate was 0.5 mL/min.

riboflavin or nucleophilic displacement of $MeSO_2^-$ by β-mercaptoethanol to yield 8-[(2-hydroxyethyl)thio]riboflavin.

The reaction of 8-$MeSO_2$-riboflavin with excess β-mercaptoethanol exhibits pseudo-first-order kinetics, and the observed rate is directly proportional to the thiol concentration (Figure 4, inset). The observed first-order kinetics with respect to β-mercaptoethanol argues against a 4-electron reductive reaction since thiols typically function as 1-electron reductants under the mild conditions used in these studies (pH 7.0, 25 °C). Similar spectral changes were observed when the reaction with 8-$MeSO_2$-riboflavin was conducted using DTT in place of β-mercaptoethanol (data not shown). HPLC analysis (detailed in Experimental Procedures) showed that a single product was formed in each reaction. However, the products formed with DTT or β-mercaptoethanol were not identical, and neither product cochromatographed with authentic 8-MeS-riboflavin (data not shown). The results show that the reaction of 8-$MeSO_2$-riboflavin with thiols cannot involve reduction of the 8-$MeSO_2$ moiety, consistent with the fact that sulfones are generally stable toward all but the strongest reducing agents (March, 1977).

Nucleophilic displacement of halide has been observed upon reaction of 8-chlororiboflavin (8-Cl-riboflavin) or 8-fluororiboflavin (8-F-riboflavin) with thiols (Moore et al., 1978; Kasai et al., 1983). To determine whether an analogous reaction occurred with 8-$MeSO_2$-riboflavin, the products formed with DTT or β-mercaptoethanol were compared with standards prepared by reacting the sime thiols with 8-Cl-riboflavin. Each product was subjected to HPLC analysis, and product identity was verified by the observation of a single symmetric peak when products from different reactions were mixed prior to chromatography. The data in Table III show that the same product is formed when a given thiol is reacted with either 8-Cl- or 8-$MeSO_2$-riboflavin.

The results indicate that reaction of thiols with 8-$MeSO_2$-riboflavin involves substitution of $MeSO_2$ by RS, presumably via an aromatic nucleophilic substitution mechanism (eq 2).



(2)

Second-order rate constants observed for the reaction of 8-$MeSO_2$-riboflavin with β-mercaptoethanol at various pH values (k_{obsd}) were used to estimate the pH-independent rate constant (k) and the acid dissociation constant of β-mercaptoethanol (K_a), assuming that thiolate anion is the reactive species ($k_{obsd} = kK_a(K_a + [H^+])$). The estimated values for these parameters (k = 272 M^{-1} s^{-1}, $K_a = 1.78 \times 10^{-9}$) were then used to simulate the expected variation of k_{obsd} as a function of pH. The data observed with 8-$MeSO_2$-riboflavin show a good fit to the simulated curve (Figure 5, curve 1). A similar fit was obtained in analogous studies with 8-Cl-riboflavin (Figure 5, curve 2). The pK_a values estimated for β-mercaptoethanol, on the basis of the kinetic data obtained with 8-$MeSO_2$-riboflavin or 8-Cl-riboflavin (pK_a = 8.75 or 8.84, respectively), are somewhat low as compared with a value directly measured (pK_a = 9.5) (Jencks & Regenstein, 1976) or one estimated in previous kinetic studies with 8-Cl-riboflavin (pK_a = 9.37) (Moore et al., 1978). The rate constant estimated for the reaction of 8-$MeSO_2$-riboflavin with β-mercaptoethanol (k = 272 M^{-1} s^{-1}) is more than 3 orders of magnitude larger than the value obtained with 8-Cl-riboflavin [k = 0.198 M^{-1} s^{-1} (this work), k = 0.2 M^{-1} s^{-1} (Moore et al., 1978)].

The reaction of 8-F-riboflavin with *N*-acetylcysteine is 23-fold faster than that with 8-Cl-riboflavin. 8-F-riboflavin also reacts with other nucleophiles, but its reactivity is diminished as compared with thiols (*N*-acetylcysteine > *N*-acetyltyrosine > glycine) (Kasai et al., 1983). When 8-$MeSO_2$-riboflavin was reacted with glycine (13.3 mM) under conditions similar to those used for the reaction with 8-F-riboflavin (0.1 M sodium carbonate, pH 8.8, 40 °C), a slow loss of the compound's visible absorption bands was observed ($t_{1/2}$ = 3 h), accompanied by the formation of a new band at 305 nm (data not shown). Similar spectral changes have been reported for the reaction of 8-Cl-riboflavin with ethylamine (Moore et al., 1978). The results provide no evidence for the formation of 8-amino-substituted flavins since these derivatives absorb strongly near 480 nm (Kasai et al., 1983). The spectral changes observed with 8-$MeSO_2$-riboflavin were independent of the presence of glycine and probably reflect hydrolysis of the isoalloxazine

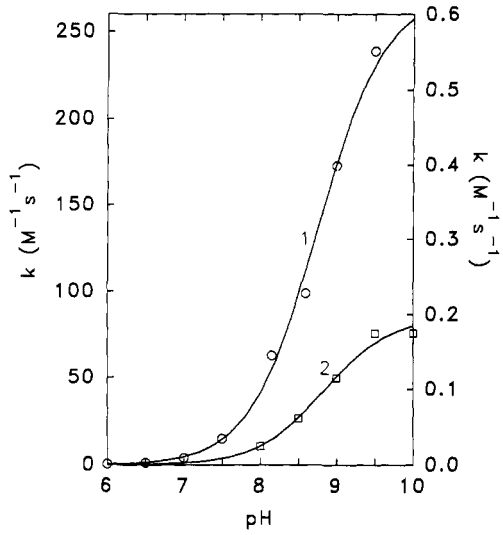


FIGURE 5: Effect of pH on nucleophilic displacement reactions with 8-substituted flavins and β-mercaptoethanol. Rate constants for the reaction of 8-$MeSO_2$-riboflavin (○) or 8-Cl-riboflavin (□) with β-mercaptoethanol were determined as described in the Experimental Procedures and used to estimate values for the pH-independent rate constant (k) and the dissociation constant of β-mercaptoethanol (K_a). Curves 1 and 2 were calculated, as described in the text, using values for k and K_a estimated from kinetic data with 8-$MeSO_2$-riboflavin and 8-Cl-riboflavin, respectively. Data points and curves for the reactions with 8-$MeSO_2$-riboflavin and 8-Cl-riboflavin are plotted according to the left- and right-hand axes, respectively.

ring, analogous to that observed under similar conditions with other flavins bearing a strongly electronegative substituent at the 8-position (Bruice et al., 1977). The results suggest that the high reactivity of 8-$MeSO_2$-riboflavin with thiols does not extend to amines as nucleophiles.

Flavin as a Redox Switch. The initial addition step in nucleophilic aromatic substitution reactions disrupts the aromatic π-system, is generally rate-determining, and is accelerated by electron-withdrawing groups in the ortho and para positions. In the absence of electron-withdrawing groups, nucleophilic aromatic substitution reactions occur only under extreme conditions (Bunnett et al., 1957; March, 1977). The isosbestic spectral course observed for the reaction of 8-$MeSO_2$-riboflavin with β-mercaptoethanol (Figure 4) is consistent with a rate-determining addition of thiolate anion since a nonisosbestic spectral course would be expected if the Meisenheimer intermediate accumulated in appreciable amounts. The reactivity of the 8-position toward nucleophilic aromatic substitution, observed with 8-(methylsulfonyl)- and 8-halo-substituted flavins in the oxidized state, is likely to reflect stabilization of the Meisenheimer intermediate via tricyclic resonance delocalization (eq 2). Analogous resonance stabilization is not possible with the corresponding 1,5-dihydroflavins, suggesting that the isoalloxazine ring might act as a redox switch in controlling electrophilicity at position 8. To test this hypothesis, we sought to determine whether thiol displacement of 8-$MeSO_2$ or 8-Cl substituents could occur with flavin in the reduced state.

8-$MeSO_2$FAD reacts rapidly with 10 mM DTT under anaerobic conditions (0.1 M potassium phosphate, pH 6.8, 10 °C) to yield the corresponding 1,5-dihydro form, as judged by its characteristic absorption spectrum. This spectrum, which is readily distinguishable from the featureless spectra observed with 8-(alkylthio)flavins in the reduced state (Moore et al., 1979), remained unchanged during a 1-h incubation with 10 mM DTT (data not shown). In a control reaction conducted with oxidized 8-$MeSO_2$FAD under aerobic but otherwise similar conditions, displacement of $MeSO_2$ by DTT

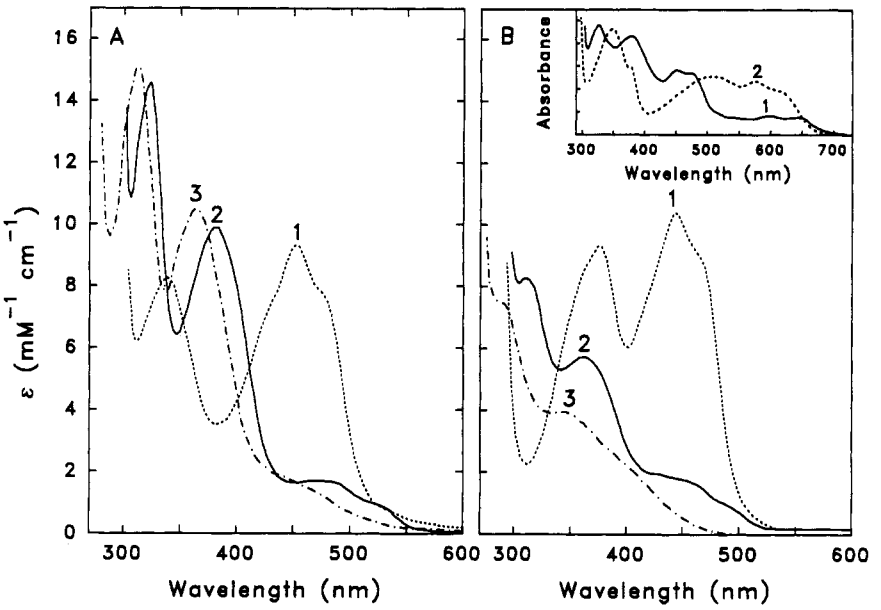


FIGURE 6: Comparison of the effect of flavodoxin binding on the spectral properties of 8-(methylsulfonyl)-FMN versus FMN in various redox states. (A) Curve 1 is the absorption spectrum of oxidized 8-$MeSO_2$FMN bound to flavodoxin in anaerobic 0.1 M potassium phosphate buffer, pH 7.0, containing 20 mM EDTA at 4 °C. Curve 2 shows the corresponding 1,5-dihydro form, obtained after reduction with a stoichiometric amount of dithionite. Curve 3 is the absorption spectrum of free 1,5-dihydro-8-(melthylsulfonyl)riboflavin in the same buffer. (B) Curve 1 is the absorption spectrum of native flavodoxin in anaerobic 70 mM sodium pyrophosphate buffer, pH 8.3, at 4 °C. Curve 2 is the spectrum of the fully reduced enzyme, obtained after reduction with a stoichiometric amount of dithionite. A similar spectrum has previously been reported (Mayhew, 1971). Curve 3 is the absorption spectrum of free 1,5-dihydro-FMN anion in 0.1 M pyrophosphate buffer, pH 8.5, as reported by Ghisla et al. (1974). Inset: Curve 2 is the absorption spectrum of the blue neutral FMN radical, obtained after reduction of native enzyme with 0.5 mol of dithionite per mole of enzyme. Curve 1 is the absorption spectrum observed with 8-$MeSO_2$FMN-reconstituted enzyme at the midpoint of the dithionite titration. The spectra in the inset have been normalized to the same initial concentration of oxidized enzyme.

was complete in 36 min. A similar displacement reaction was initiated upon aeration of the anaerobic reaction mixture.

Unlike flavins with more positive reduction potentials, 8-Cl-riboflavin is not readily reducible by thiols. In this case, the dihydro form was generated by reaction with a stoichiometric amount of dithionite under anaerobic conditions (20 mM sodium borate buffer, pH 9.0, at 15 °C). The reduced flavin was then mixed with 20 mM β-mercaptoethanol, incubated under anaerobic conditions for 4 h, and then made aerobic. The spectrum observed immediately after aeration was similar to the initial oxidized spectrum of 8-Cl-riboflavin and then slowly changed to a spectrum characteristic of 8-(alkylthio)-flavins (data not shown). The latter reaction parallels results obtained in a control experiment where an aerobic solution of oxidized 8-Cl-riboflavin was directly reacted with β-mercaptoethanol under otherwise similar conditions ($t_{1/2}$ = 50 min) (data not shown).

Spectral Properties of 8-(Methylsulfonyl)-FMN Bound to Flavodoxin in Various Redox States. Initial studies to evaluate 8-(methylsulfonyl)flavin as an active site probe were conducted with flavodoxin from *C. beijerinckii MP*. Crystallographic studies show that FMN is bound near the periphery of the protein with the isoalloxazine ring sandwiched between two hydrophobic residues, Met-56 and Trp-90. The average dielectric constant of the flavin binding site is less than that of water. Only the benzene ring is significantly exposed to solvent (Burnett et al., 1974). It has been suggested that electron transfer from the flavin to other redox proteins occurs through this region (Burnett et al., 1974; Mayhew & Tollin, 1992).

8-(Methylsulfonyl)-FMN (8-$MeSO_2$FMN) is rapidly bound to apoflavodoxin, as judged by the immediate quenching of the fluorescence of the free flavin (data not shown). The absorption spectrum of 8-$MeSO_2$FMN enzyme in the oxidized state is shown in Figure 6A (curve 1). Comparison of this spectrum (λ_{max} = 450, 338 nm, ϵ_{450} = 9.3 mM^{-1} cm^{-1}) with that observed for the corresponding free flavin (λ_{max} = 450, 333 nm, ϵ_{450} = 11.2 mM^{-1} cm^{-1}) shows that binding causes a 17% decrease in extinction at 450 nm, the appearance of a pronounced shoulder at 480 nm, and a 5-nm bathochromic shift of the near-UV band. Very similar differences are observed when the spectrum of native flavodoxin (Figure 6B, curve 1) (λ_{max} = 445, 376 nm, ϵ_{445} = 10.4 mM^{-1} cm^{-1}) (Mayhew, 1971) is compared with free FMN (λ_{max} = 445, 373 nm, ϵ_{445} = 12.5 mM^{-1} cm^{-1}). The resolution of the 450-nm band, observed with both native and reconstituted enzymes, suggests a more hydrophobic environment as compared with water, consistent with the crystallographic data (Burnett et al., 1974).

Fully reduced 8-$MeSO_2$FMN enzyme is formed upon reduction with a stoichiometric amount of dithionite (Figure 6A, curve 2). The fully reduced enzyme exhibits two well-defined absorption bands at wavelengths above 300 nm (λ_{max} = 382, 323 nm), which is similar to the spectrum observed for the corresponding free flavin (Figure 6A, curve 3), except that a significant bathochromic shift is observed for the protein-bound flavin. A blue neutral flavin radical is formed during reduction of the 8-$MeSO_2$FMN enzyme, as judged by the appearance of absorption in the long-wavelength region (λ_{max} = 600, 640 nm) which reaches a maximum at the midpoint of the dithionite titration (Figure 6B, inset, curve 1). As shown in previous studies (Mayhew, 1971), quantitative formation of a blue neutral radical is observed upon reduction of native enzyme with 0.5 mol of dithionite (Figure 6B, inset, curve 2). A considerably lower radical yield is obtained with 8-$MeSO_2$FMN enzyme, as judged by the relative intensity of the long-wavelength absorption band and by the presence of bands at 450 and 378 nm that are probably due to fully oxidized and fully reduced flavin, respectively. The results suggest that there may be a smaller separation of the two 1-electron potentials for flavin reduction in 8-$MeSO_2$FMN enzyme than with native enzyme.

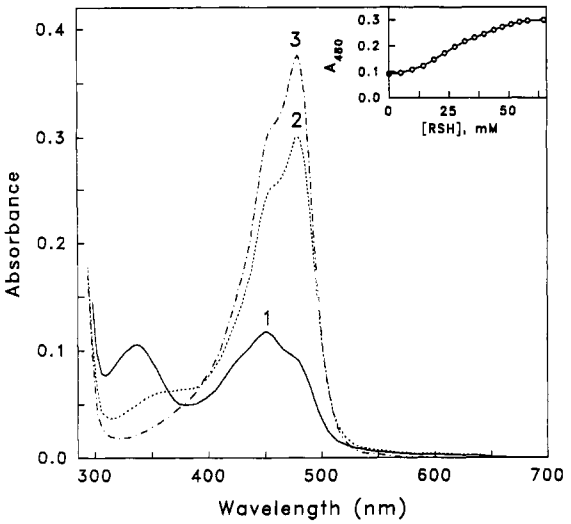


FIGURE 7: Reaction of thiol with 8-(methylsulfonyl)-FMN bound to flavodoxin. Curve 1 is the absorption spectrum of 8-$MeSO_2$FMN flavodoxin in 0.1 M potassium phosphate buffer, pH 7.0, containing 20 mM EDTA at 4 °C. Increases in absorbance at 480 nm, observed 10 min after the addition of successive aliquots of β-mercaptoethanol, are shown in the inset. Curve 2 was recorded 10 min after the addition of the last aliquot. Curve 3 was recorded after incubation of the sample for 5.5 days at 4 °C.

Several observations suggest that the bathochromic shift observed upon binding of fully reduced 8-$MeSO_2$FMN to flavodoxin at pH 7 reflects a change in the polarity of the flavin environment and is not due to a change in the ionization state of the reduced flavin. A substantial bathochromic shift is observed with free 1,5-dihydro-8-(methylsulfonyl)riboflavin at pH 7 when the solvent dielectric is decreased by addition of DMSO (λ_{max} = 376, 314 nm at pH 7.0 in 80% DMSO). Studies with native flavodoxin show that the fully reduced flavin is bound as an anion, probably because steric factors suppress protonation at N(1) (Vervoort et al., 1986; Ludwig & Luschinsky, 1992; Ludwig et al., 1990). 1,5-Dihydro-8-(methylsulfonyl)riboflavin is largely ionized when free in aqueous solution at pH 7.0 since absorption maxima (λ_{max} = 362, 310 nm) are unaffected when the pH is increased to 10.5 but shift closer together when the pH is decreased to 5.0 (λ_{max} = 355, 314 nm). Similar properties have been reported for the anionic (λ_{max} = 370, 308 nm) and neutral (λ_{max} = 358, 314 nm) forms of reduced 8-cyano-*N*(3)-methylisoalloxazine (pK_a = 5.9) (Bruice et al., 1977).

Reaction of Nucleophiles with 8-(Methylsulfonyl)-FMN Bound to Flavodoxin. No evidence for sulfite complex formation was observed upon reaction of 8-$MeSO_2$FMN flavodoxin with excess sulfite (79 mM sodium sulfite in 0.1 M potassium phosphate, pH 7.0, containing 20 mM EDTA, 4 °C). The results are similar to that obtained with native enzyme (Massey et al., 1969), although free 8-$MeSO_2$-flavin is nearly 3 orders of magnitude more reactive with sulfite than normal flavin.

8-$MeSO_2$FMN flavodoxin reacts readily with exogenous thiols. A rapid, small increase in absorbance at 480 nm was observed upon addition of 4.9 mM β-mercaptoethanol at pH 7.0. Although the reagent was in excess and the reaction was complete in less than 10 min, additional rapid increases in absorbance were observed upon addition of successive aliquots of thiol (Figure 7, inset). The reaction appeared to be complete when the amount of thiol added reached 64.8 mM. (The actual thiol concentration at the end of the reaction was not measured, but the titration-like behavior suggests that thiol may have been consumed in a competing reaction, possibly involving a redox cycling of bound 8-$MeSO_2$FMN.) The spectrum recorded after the addition of the last aliquot of β-mercaptoethanol (Figure 7, curve 2) exhibits a peak at 480 nm, which is similar to that observed for the corresponding reaction with free 8-$MeSO_2$-riboflavin (λ_{max} = 474 nm) (see Figure 4), except for a 6-nm difference in absorption maxima and the presence of pronounced shoulders at 435 and 350 nm in the flavodoxin spectrum. Prolonged incubation of the sample at 4 °C (5.5 days) resulted in a loss of the shoulder at 350 nm, accompanied by a modest increase in absorbance at 480 nm (Figure 7, curve 3). The resolved 480-nm band plus filtration studies (Centricon 3 microconcentrator) showed that the flavin was still bound to flavodoxin at the end of incubation, but this does not rule out a reaction involving slow flavin dissociation followed by rapid rebinding. The nature of the slow secondary phase of the reaction is unclear. Similar behavior was observed with other thiols (DTT, methanethiol), except that the shoulder at 350 nm, observed at the end of the titration-like phase with β-mercaptoethanol, was not apparent in the reaction with methanethiol and appeared as a distinct peak at 355 nm in the reaction with DTT (data not shown).

DISCUSSION

Replacement of the methyl group at position 8 in riboflavin with a methylsulfonyl moiety increases the reduction potential by more than 150 mV (Moore et al., 1979). This is accompanied by a substantial increase in reactivity with various reducing reagents and an increase of nearly 3 orders of magnitude in susceptibility toward nucleophilic attack by sulfite. The results are consistent with linear free energy relationships previously observed for these parameters and the reduction potential of the flavin (Walsh et al., 1978; Muller & Massey, 1969). Reaction of enzyme-bound flavin with sulfite can provide a measure of the accessibility of the N(5)-position to solvent but failure to react with sulfite may also reflect a decreased reactivity of the bound flavin toward nucleophilic attack at N(5) (Massey et al., 1969). The increased stability of the sulfite complex formed with free 8-$MeSO_2$-flavin may enhance its sensitivity as a probe of solvent accessibility at the N(5)-position of bound flavin. A sulfite complex was not formed with 8-$MeSO_2$FMN bound to flavodoxin. In this case, the enhanced reactivity of the free flavin with sulfite is apparently not sufficient to overcome steric interference from Trp-90, coupled with electrostatic repulsion by carboxylates (Asp-58, Glu-59) near the N(5)-position of the flavin (Burnett et al., 1974).

8-$MeSO_2$-riboflavin exhibits two intense absorption bands above 300 nm in the reduced state (λ_{max} = 310, 362 nm), whereas most other reduced flavins exhibit weak, characterless absorption in this region. The dramatic effect of an 8-methylsulfonyl substituent on the spectral properties of the 1,5-dihydro form is attributable to a shift of π-electron density from the N(5) atom into the benzene ring (eq 1). The effect is observed with flavins bearing a strongly electronegative substituent at the 8-position (CN, $MeSO_2$) capable of resonance interaction with the isoalloxazine ring (see Table II). Replacement of the hydrogen at N(5) in reduced flavins with a bulky substituent, like sulfite, interferes with sp^2 hybridization at N(5) and therefore blocks delocalization of the lone pair of electron from N(5) into the benzene ring (Hemmerich et al., 1971; Moonen et al., 1984; Dudley et al., 1964). The sulfite complex formed with 8-$MeSO_2$-riboflavin or an 8-cyano-substituted flavin exhibits spectral properties very different from the corresponding 1,5-dihydro forms but similar to sulfite complexes formed with other flavins. [Reduction of 8-formylflavin yields a species with spectral properties (λ_{max} = 392, 520 nm) that are drastically different

from all known 1,5-dihydroflavin derivatives, including other high-potential flavins bearing electronegative substituents at position 8. This feature and the observed chemical reactivity of the 8-formyl substituent (Edmondson, 1974) suggest that further studies may be necessary to determine whether a monomeric 1,5-dihydro derivative is formed with 8-formylflavin.]

In the oxidized state, a comparable spectral perturbation is observed upon binding FMN or 8-$MeSO_2$FMN to apoflavodoxin. In the fully reduced state, 8-$MeSO_2$FMN flavodoxin exhibits spectral properties similar to those of the corresponding free flavin, except for a bathochromic shift due to a change in the polarity of the flavin environment. In the fully reduced state, native flavodoxin exhibits two fairly well-resolved absorption bands at wavelengths above 300 nm (λ_{max} = 311, 362 nm) (Figure 6B, curve 2). With respect to peak resolution and intensity, the spectrum of reduced native enzyme appears to lie about halfway between the rather featureless spectrum observed for free reduced normal flavin (Figure 6B, curve 3) versus the intense, highly resolved spectrum observed for free reduced 8-$MeSO_2$-flavin (λ_{max} = 310, 362 nm) (Figure 6A, curve 3). This feature prompts speculation that the protein environment in native enzyme may favor enhanced resonance delocalization of π-electron density from the N(5) atom into the benzene ring, as compared with free reduced FMN. Although other models have been suggested (Ghisla et al., 1974), this hypothesis is consistent with ^{13}C and ^{19}F NMR studies of fully reduced native and 8-fluoro-FMN flavodoxin, respectively, which indicate unusually high electron density in the benzene ring, as compared with the corresponding free flavins (Vervoort et al., 1986; Macheroux et al., 1990). The strong hydrogen bond between N(5) and the carbonyl oxygen of Gly-57 plus unfavorable electrostatic interactions between the reduced flavin anion with negatively charged protein residues clustered near the pyrimidine ring (Vervoort et al., 1986; Ludwig & Luschinsky, 1992) should favor a shift in electron density into the benzene ring. Enhanced electron density in the benzene ring is also compatible with the proposal that electron transfer from reduced flavodoxin to other redox proteins occurs through this region of the flavin ring (Mayhew & Tollin, 1992; Burnett et al., 1974).

Nucleophilic displacement of $MeSO_2^-$ occurs upon reaction of 8-$MeSO_2$-riboflavin with thiols. The reaction appears to proceed via an aromatic nucleophilic substitution mechanism with thiolate anion as the reactive species. Nucleophilic displacement of halide has previously been observed upon reaction of thiols with 8-Cl-riboflavin or 8-F-riboflavin (Moore et al., 1978; Kasai et al., 1983). The reaction of 8-$MeSO_2$-riboflavin with thiols is more than 3 orders of magnitude faster than 8-Cl-riboflavin. 8-F-riboflavin reacts 23-fold faster than 8-Cl-riboflavin (Kasai et al., 1983). The high reactivity of 8-$MeSO_2$-riboflavin with thiols is unusual compared with other aromatic nucleophilic substitution reactions, where the approximate order of leaving group ability is F > Cl > RSO_2 (Bunnett et al., 1957; March, 1977). Formation of the Meisenheimer intermediate in an aromatic substitution reaction requires attack of the incoming nucleophile perpendicular to the plane of the benzene ring and formation of a tetrahedral carbon at C(8) (see eq 2). This step disrupts the aromatic π-system and is dependent on the presence of electron-withdrawing substituents (Bunnett et al., 1957; March, 1977). Unlike the oxidized flavins, 8-$MeSO_2$-riboflavin or 8-Cl-riboflavin in the fully reduced state does not react with thiols. This ability of the flavin ring to act as a redox switch in controlling the electrophilicity of the 8-position is attributed to a tricyclic resonance stabilization of the Meisenheimer intermediate that is possible only in the oxidized state.

Most of the spectral change observed upon reaction of thiols with 8-$MeSO_2$FMN flavodoxin occurs in a rapid, titration-like phase and is clearly due to the reaction of the bound flavin with exogenous reagent. Crystallographic data show that the 8-position of FMN in native enzyme is accessible to solvent (Burnett et al., 1974). With respect to chemical reactivity, previous studies indicate that access to the 8-position is unhindered in the flavin plane but suggest that perpendicular access may be restricted, as judged by the failure of 8-ClFMN flavodoxin to react with thiophenol (Schopfer et al., 1981). No evidence for restricted access was apparent in the reactions of 8-$MeSO_2$FMN flavodoxin with thiols, but this may reflect the smaller size of the thiol reagents used in these studies. Also, the nature of a very slow secondary phase in these reactions is unclear.

Covalent attachment of 8-$MeSO_2$FMN to flavodoxin by reaction with a protein thiol was not detected during preparation or storage of the reconstituted enzyme at pH 7.0. None of the three cysteine residues in flavodoxin is in direct contact with the flavin. Cys-108 is buried, but Cys-53 and Cys-128 appear somewhat accessible to solvent (Burnett et al., 1974). Reaction of 8-$MeSO_2$FMN with the latter residues is probably not competitive with its rapid binding at the flavin site, especially since only a small excess of flavin was used in reconstitution experiments. However, the high reactivity observed with the free flavin and thiols suggests that 8-$MeSO_2$-flavin may be useful as a covalent probe for those flavin binding sites that do contain a suitably oriented cysteine residue. In fact, quantitative, covalent attachment of 8-$MeSO_2$FAD at flavin binding sites has been observed in studies with two other flavoproteins (A. A. Raibekas and M. S. Jorns, unpublished observations).

After submission of this manuscript, Eckstein et al. (1993) briefly reported the use of 8-$MeSO_2$FMN in studies with bacterial luciferase, but the preparation of the flavin analogue was not described.

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