8-Thiocyanatoflavins as Active-Site Probes for Flavoproteins†

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ABSTRACT: 8-Thiocyanatoflavins at the riboflavin, FMN, and FAD level were prepared via the diazonium salt of the corresponding 8-aminoflavin and some of the physical and chemical properties studied. 8-Thiocyanatoriboflavin has a UV–visible spectrum similar to that of the native flavin with absorbance maxima at 446 nm (ϵ = 14900 M^{-1} cm^{-1}) and 360 nm. Reaction with thiols such as dithiothreitol and mercaptoethanol gives rise to an 8-mercapto- and an 8-SR-flavin, whereas reaction with sulfide yields only the 8-mercaptoflavin. The 8-SCN-flavin binds to riboflavin-binding protein as the riboflavin derivative, to apoflavodoxin, apo-Old Yellow Enzyme, and apo-lactate oxidase as the FMN derivative, and to apo-D-amino acid oxidase, apo-*p*-hydroxybenzoate hydroxylase, apo-glucose oxidase, apo-anthranilate hydroxylase, and apo-general acyl-CoA dehydrogenase as the FAD derivative. In two cases, namely, with anthranilate hydroxylase and D-amino acid oxidase, the 8-SCN-FAD was spontaneously and completely converted to the 8-mercapto-FAD derivative, suggesting the presence of a nucleophile (most likely the thiol of a cysteine residue) in the vicinity of the 8-position. It was also found that flavodoxin stabilizes the neutral radical and Old Yellow Enzyme the anionic radical of 8-SCN-FMN. Further studies with Old Yellow Enzyme established that fully (two electron) reduced 8-SCN-FMN undergoes photoelimination of cyanide.

Recent studies described the synthesis (Ghisla et al., 1986) and use of 6-thiocyanatoflavins as active-site probes of flavoproteins (Massey et al., 1986). By virtue of the high reactivity of the thiocyanate group with nucleophiles the 6-thiocyanatoflavin can be used as a probe of solvent accessibility as well as a probe for protein functional groups near the flavin 6-position. The results obtained by this strategy contributed to our understanding in many flavoproteins of the nature of the flavin binding site and the role of protein residues in catalysis [for a review, see Ghisla and Massey (1986)]. Although the 8-position has been derivatized in many ways (SH, Cl, and S-alkyl, to mention only a few) and the solvent accessibility of the 8-position was intensively studied by Schopfer et al. (1981), it appeared promising to compare 8-thiocyanatoflavins with 6-thiocyanatoflavins with respect to their properties and reactivity. In this paper we describe the synthesis of 8-thiocyanatoflavins and selected physicochemical properties. We also present studies with nine flavoproteins reconstituted with the appropriate 8-thiocyanatoflavin and demonstrate the usefulness of the 8-thiocyanate group as a probe for active-site thiols.

Experimental Procedures

Materials

DTT,[1] mercaptoethanol, 5,5′,7-indigotrisulfonic acid, and *Naja naja* venom were from Sigma Co., St. Louis, MO. 5,5′-Indigodisulfonic acid was from K&K Laboratories, Inc. MMTS was from Aldrich and 8-amino-Rfl was a gift from Dr. Sandro Ghisla, Universität Konstanz, FRG. 8-Amino-FAD was prepared by using the general method described by Spencer et al. (1976). 6-Thiocyanato-FAD was prepared according to the procedure described by Ghisla et al. (1986); the 8-thiocyanatoflavins were prepared in a similar way, as described below.

Methods

Synthesis of 8-Thiocyanatoriboflavin. An aqueous solution of 8-aminoriboflavin (1.4 mL of an approximately 1 mM concentration) was brought to pH 1.5 with 6 N HCl and cooled to ice temperature. Diazotization was performed by addition of 30 μL of saturated $NaNO_2$ solution. Excess nitrite was removed after 1 min with 150 μL of saturated urea solution followed by the addition of 300 μL of saturated ammonium thiocyanate solution. The reaction mixture turned red and the pH was adjusted to 4 with a 4 M sodium acetate solution. The reaction mixture was kept at ice temperature for 0.5 h and was then brought to pH 7 with 2 N NaOH. The reaction product showed a bright yellow fluorescence (the starting material, 8-amino-Rfl, is nonfluorescent).

The crude reaction solution was loaded on a Sep-Pak C-18 cartridge (Waters Associates) and salt was removed by washing with 2 volumes of the applied volume with glass distilled water. The bound riboflavin was recovered with 10% acetonitrile. Solvent was removed in a Speed Vac concentrator. Further purification of the 8-thiocyanate-Rfl was achieved with an ISCO HPLC instrument equipped with an RP-18 reverse-phase column. Best results were obtained by running a gradient from 100% 10 mM sodium/potassium phosphate buffer (pH 7.4) to 30% methanol/70% 10 mM sodium/potassium phosphate buffer (pH 7.4) in 30 min (flow rate 0.5 mL/min). Elution time under these conditions for the 8-

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[1] Abbreviations: DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MMTS, methyl methanethiolsulfonate; TCA, trichloroacetic acid; Rfl, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; 5dRfl, 5-deaza-5-carbariboflavin; HPLC, high-performance liquid chromatography; GO, glucose oxidase; GAD, general acyl-CoA dehydrogenase; LAO, lactate oxidase; DAAO, D-amino acid oxidase; AH, anthranilate hydroxylase; pHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; RBP, riboflavin-binding protein; OYE, Old Yellow Enzyme.

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thiocyanato-Rfl was approximately 24 min.

Synthesis of 8-Thiocyanato-FAD and -FMN. An analogous procedure to the one described above was applied starting with 8-amino-FAD, but the diazotization was performed at a higher pH (2–2.5). 8-Thiocyanato-FMN was obtained from the FAD level by means of the phosphodiesterase activity of snake venom (*N. naja*).

Preparation of Flavoproteins and the Corresponding Apoproteins. Riboflavin-binding protein was isolated in the apoprotein form from hen egg white according to the procedure of Becvar and Palmer (1982). Flavodoxin from *Megasphera elsdenii* was prepared and converted to the apoprotein as described by Mayhew and Massey (1969) and Mayhew (1971). L-Lactate oxidase from *Mycobacterium smegmatis* was prepared as described by Sullivan et al. (1977) and the apoprotein preparation followed the procedure of Choong et al. (1975). Old Yellow Enzyme was prepared by affinity chromatography (Abramowitz & Massey, 1976a) and apoprotein was prepared according to Abramowitz and Massey (1976b). D-Amino acid oxidase from pig kidneys was prepared according to Curti et al. (1973) and apoprotein by the method of Massey & Curti (1966). The general acyl-CoA dehydrogenase from pig kidneys was a gift from Dr. C. Thorpe, University of Delaware, DE, and the apoprotein preparation was performed following the procedure of Mayer and Thorpe (1981). Glucose oxidase from *Aspergillus niger* was prepared according to Swoboda and Massey (1965). The apoprotein was prepared by a modification of the procedure of Mayer and Thorpe (1981). Anthranilate hydroxylase was a gift of Dr. J. Powlowski, University of Michigan, and the apoprotein was obtained according to Mayer and Thorpe (1981), using a resolving charcoal suspension adjusted to pH 3. *p*-Hydroxybenzoate hydroxylase was isolated according to the procedure of Müller et al. (1979) and the apoprotein was prepared following the description of Entsch et al. (1980).

Reconstitution of the Apoproteins with 8-Thiocyanatoflavins. In most cases the apoprotein was mixed directly after preparation in a 1.1–1.5-fold excess with 8-thiocyanato-FMN or 8-thiocyanato-FAD in a 1-mL cuvette at 4 °C, and the spectral changes associated with binding were followed. With most flavoproteins the reconstitution as judged by the spectral changes or by the disappearance of the fluorescence of the 8-thiocyanatoflavin was completed within 30 min. Apoprotein concentrations were estimated on the basis of published extinction coefficients at 280 nm or, in the case of the very stable apoproteins of riboflavin-binding protein and flavodoxin, standardized by titration of pure riboflavin and FMN, respectively.

Instrumentation

HPLC purification was performed with an ISCO, Model 2350, instrument equipped with an RP-18 reverse phase column (Regis Co., 5 μm). Absorbance spectra were taken either with a Varian spectrophotometer, Cary Model 219, or with a Hewlett-Packard diode array spectrophotometer, Model 8452A. Oxygen consumption was measured with a Yellow Springs oxygen monitor, Model 53 (Yellow Springs Instruments Co., Inc., OH).

Light irradiation was carried out with a sun gun (Smith Victor Corp., Griffith) with an intensity of 6×10^6 ergs s^{-1} cm^{-2}, if not stated otherwise.

Results and Discussion

Physicochemical Properties of 8-Thiocyanatoflavin. The absorbance spectrum of 8-thiocyanato-FMN is similar to that of FMN. The extinction coefficient determined by titration

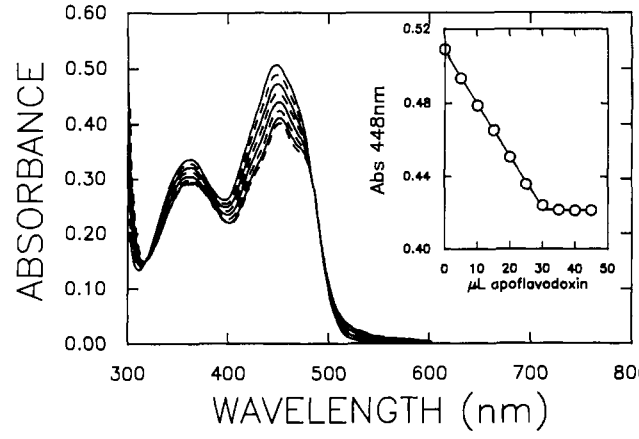


FIGURE 1: Titration of 8-SCN-FMN with apoflavodoxin. 8-SCN-FMN, 0.9 mL, in 0.1 M sodium acetate buffer, pH 5.6, was titrated with 5-μL aliquots of 1.01 mM apoflavodoxin (succeeding curves). The concentration of apoflavodoxin was determined by titration with pure FMN. The absorbance changes at 448 nm were corrected for dilution and plotted against the volume of apoflavodoxin added (see insert). The end point of the titration (30 μL) allows the calculation of the extinction coefficient for 8-SCN-FMN: ϵ_{max} = 14900 M^{-1} cm^{-1}.

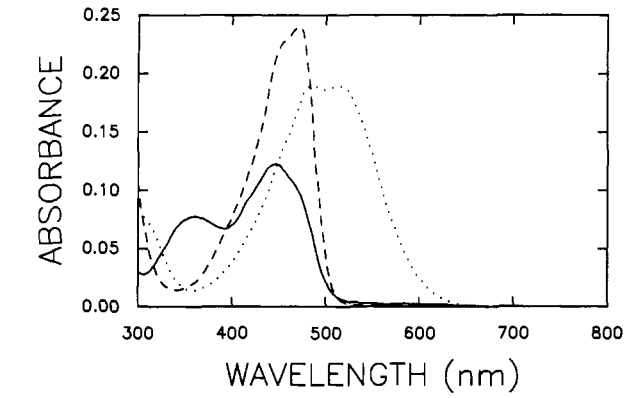
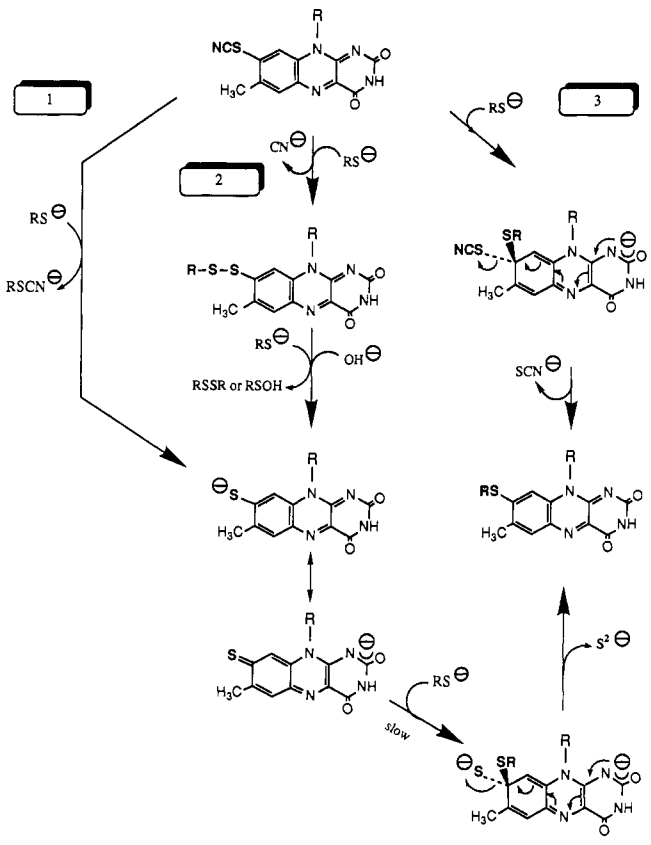


FIGURE 2: Reaction of 8-SCN-riboflavin with DTT and subsequent reaction with iodoacetamide. 8-SCN-riboflavin (7.4 μM, solid trace) in 0.1 M potassium phosphate buffer, pH 7.0 (volume = 0.8 mL), was reacted with 1 μL of 2 M DTT (final concentration = 2.5 mM) at 4 °C. Formation of the reaction products was complete within 8 min (dotted trace). Upon addition of an excess of iodoacetamide a slow conversion to a spectrum with an absorbance maximum at 476 nm occurred (dashed trace). On the basis of the extinction coefficient of 8-SCN-riboflavin (ϵ_{max} = 14900 M^{-1} cm^{-1}) this new species has an extinction coefficient at 476 of ϵ_{max} = 29 100 M^{-1} cm^{-1}.

with apoflavodoxin (Figure 1) and OYE is higher (ϵ_{max} at 446 nm = 14900 M^{-1} cm^{-1}) than that of FMN (ϵ_{max} at 450 nm = 12500 M^{-1} cm^{-1}). 8-Thiocyanato-Rfl has excitation maxima at 365 and 450 nm and a fluorescence emission maximum at 525 nm. Photoreduction of 8-thiocyanato-Rfl according to the method of Massey and Hemmerich (1978) resulted in complete conversion to the 8-mercapto derivative.

Reactivity with Thiol Reagents. Thiols such as dithiothreitol or mercaptoethanol are good nucleophilic reagents and were used in a recent investigation (Massey et al., 1986) to displace cyanide from 6-thiocyanatoflavins and convert the thiocyanate group to a mercapto group. The reaction of DTT or mercaptoethanol with an 8-thiocyanatoflavin does not yield the 8-mercaptoflavin as sole product, as indicated by the spectral changes during the reaction (see Figure 2). The reaction proceeds with a set of isosbestic points at 326 and 450 nm and the final spectrum has absorbance maxima at 485 and 520 nm. 8-Mercaptoflavin has an absorption maximum at 520 nm (Moore et al., 1979) and we conclude that it is one of the products on the basis of the disappearance of the 520-nm peak in the reaction mixture upon addition of thiol reagents such

Scheme I: Reaction of 8-SCN-flavin with Thiol Reagents



as iodoacetamide (Figure 2). The second product of the reaction is likely to be an 8-SR-flavin, which typically have absorbance maxima around 476 nm (Moore et al., 1979). This conclusion is supported by the 2-fold increase of absorbance in the region from 470 to 520 nm, since both compounds have a much higher extinction coefficient than normal flavin (Moore et al., 1979). As judged by HPLC analysis the ratio of products is approximately 70% 8-mercaptoflavin to 30% 8-SR-flavin derivative at pH 7 and with DTT as thiol reagent.

When stoichiometric amounts of DTT and 8-thiocyanatoriboflavin were reacted, both products were created simultaneously, suggesting that the putative 8-SR-flavin is not produced from the 8-SH-flavin in a sequential reaction path. Further evidence for a parallel reaction path is provided by the finding of only one set of isosbestic points. Scheme I summarizes the observations obtained with DTT as a thiol reagent.

The reactivity of 8-thiocyanatoflavins with nucleophiles such as DTT or mercaptoethanol establishes a major difference from 6-SCN-flavins, which react with thiols only according to pathway 2 (Massey et al., 1986). An obvious explanation for this difference is that the carbon atom in position 8 is more electrophilic and reaction path 3 becomes more favorable for 8-SCN-flavins than for 6-SCN-flavins. In keeping with this explanation is the observed pH dependency of product formation. Above the pK_a of the flavin N3-deprotonation ($\sim$10.3) one observes only formation of 8-mercaptoflavin. Deprotonation of N3 causes an increase of electron density at the C-8-position and hence decreases the electrophilicity of C-8, thus favoring nucleophilic attack at the sulfur (reaction path 2) and/or the carbon (reaction path 1) of the thiocyanate group. The bimolecular reaction rate for the conversion of 8-SCN-flavin (either as the FMN or FAD form) to 8-mercaptoflavin using DTT was determined by monitoring the absorbance increase at 520 nm and found to be k = 550 M^{-1} min^{-1} at 4 °C and pH 7. This reaction is thus slightly faster than that with 6-SCN-riboflavin, where a rate of 1000 M^{-1} min^{-1} was found for the conversion to the corresponding mercapto derivative at 20 °C. The second-order rate constant for reaction of 8-SCN-FAD with mercaptoethanol under the same conditions was determined to be 120 M^{-1} min^{-1}.

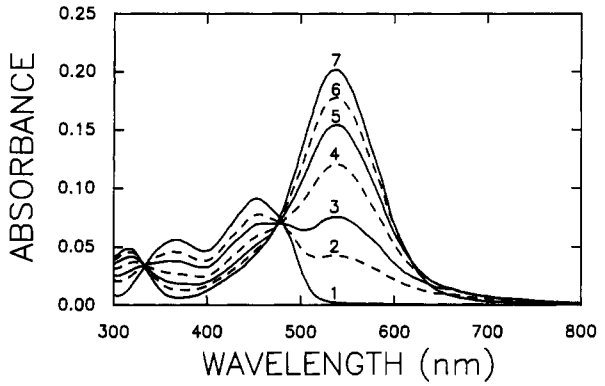


FIGURE 3: Reaction of 8-SCN-FAD with sodium sulfide. 8-SCN-FAD (7.4 μM) in 0.05 M potassium phosphate, pH 7.8 (1-mL volume), was mixed with sodium sulfide (final concentration = 2 mM) at 25 °C. Curve 1 shows the spectrum of the starting material and curves 2–7 show the course of the reaction as a function of time (spectra were taken after 1, 2, 4, 6, 9, and 21 min for curves 2–7, respectively). The reaction product, 8-mercapto-FAD, has a λ_{max} at 535 nm and from the known extinction coefficient (ϵ_{max} = 29 700 M^{-1} cm^{-1}) we can calculate the extinction coefficient of 8-SCN-FAD: 13 700 M^{-1} cm^{-1}.

The reaction of 8-SCN-FAD with sodium sulfide (see Figure 3) gives rise to 8-mercapto-FAD. This reaction allowed us to calculate the ϵ_{max} for the 8-thiocyanato-FAD by virtue of the known extinction coefficient for the product, i.e., 8-mercapto-FAD (ϵ_{536} = 29 700 M^{-1} cm^{-1}; Massey et al., 1979). The calculated ϵ_{max} of 13 700 M^{-1} cm^{-1} is in reasonable agreement with the value of 14 200 M^{-1} cm^{-1} based on the ϵ_{max} of 14 900 M^{-1} cm^{-1} determined for 8-SCN-FMN and the spectral changes accompanying the conversion of the FAD to the FMN form.

The photolability of 8-SCN-flavins excluded the use of photoreduction in the determination of the redox potential. Similarly the reactivity of sulfite, an oxidation product of dithionite, made dithionite an unsatisfactory reductant, since small amounts of 8-sulfonylflavin were formed as a result of anaerobic titration with dithionite. Hence the redox potential of 8-SCN-FAD was determined by equilibration at pH 7.0 with 5,5′-indigodisulfonic acid (E_o = −115 mV) or 5,5′,7-indigotrisulfonic acid (E_o = −81 mV) under anaerobic conditions, with xanthine and a catalytic amount of xanthine oxidase as reductant, by the method described by Massey (manuscript in preparation). The value of E_o (pH 7.0), 25 °C, so determined, was −98 + 3 mV.

8-Thiocyanatoflavins as Active-Site Probes. Several flavoproteins were reconstituted with 8-thiocyanatoflavins in order to probe the vicinity of the 8-position for reactive nucleophilic groups, such as the mercapto function of a cysteine residue. As a consequence of the high reactivity of 8-SCN-flavins with thiols, we frequently encountered some spontaneous conversion to the 8-mercaptoflavin form. An obvious question is whether this reaction occurs in the active site of the protein or rather takes place between released flavin and any accessible nucleophile on the surface of the protein. Subsequent rebinding of 8-mercaptoflavin formed in this way would essentially yield the same product, i.e., the 8-mercaptoflavoprotein. Conversion of an 8-SCN-flavin to 8-mercaptoflavin by reaction with a surface nucleophile followed by subsequent binding at the

active site may occur experimentally if the apoprotein is titrated in small aliquots into a solution of the 8-SCN-flavin. Under these conditions, during most of the experiment, there is an excess of flavin over the concentration of active sites, so that 8-mercaptoflavin formed by reaction with protein surface nucleophiles could be bound at the active sites of the next aliquot of apoprotein added. A simple test of such conversion is to compare the result obtained where the apoprotein is added directly in excess of the flavin. Another valuable strategy is to incubate native flavoprotein (or the apoprotein) with an 8-thiocyanatoflavin that does not bind to the protein, e.g., 8-SCN-riboflavin for most of the proteins. If there is no or a very slow conversion (compared to the reaction rate of bound 8-SCN-FAD or 8-SCN-FMN) it can be concluded that the conversion of the 8-thiocyanatoflavin coenzyme bound to a flavoprotein occurs in the flavin-binding pocket. On the other hand a fast reaction would indicate that the conversion of 8-thiocyanato- to the 8-mercaptoflavoprotein could be attributed, at least in part, to the reaction of free flavin with a surface nucleophile. The presence in the apoprotein preparation of damaged protein molecules, no longer capable of reforming viable holoprotein but possessing reactive nucleophiles, could be a source of 8-mercaptoflavin formation, which could subsequently bind to native apoprotein. In the following section we will discuss the studied flavoproteins in greater detail and we will revert to this problem in some cases.

Riboflavin-Binding Protein. The binding of 8-SCN-riboflavin to hen egg white riboflavin-binding protein is associated with a shift of λ_{max} from 446 to 460 nm (ϵ_{max} = 12 300 M^{-1} cm^{-1}). These changes are typical for those observed for binding of riboflavin and riboflavin derivatives (Becvar & Palmer, 1982). However, the spectral changes observed upon titration of 8-SCN-riboflavin with aporiboflavin-binding protein were not linearly dependent on the concentration of protein added, indicating weak binding to the protein.

No spontaneous conversion of the bound 8-SCN-riboflavin occurs, even on prolonged incubation, indicating the absence of any reactive nucleophiles in the vicinity of the flavin 8-position. The 8-SCN-riboflavin is converted to 8-mercaptoriboflavin and an 8-SR-flavin derivative by reaction with DTT. However, this was shown to be due to reaction of DTT with free 8-SCN-riboflavin in dynamic equilibrium with the apoprotein, since the reaction rate was markedly decreased with increasing concentrations of apoprotein. From this dependency we estimated the dissociation rate constant of 8-SCN-riboflavin from the holoprotein to be 0.4 min^{-1}, using the equations of Schopfer et al. (1981). In addition, the reaction of the protein-bound flavin with DTT gave rise to the same mixture of reaction products as found with free 8-thiocyanatoriboflavin (see previous section).

Flavodoxin. Binding of 8-thiocyanato-FMN to apoflavodoxin is accompanied by a slight shift of λ_{max} from 446 to 450 nm, a decrease of ϵ_{max} to 12 400 M^{-1} cm^{-1}, and complete quenching of the 8-thiocyanato-FMN fluorescence. The second-order rate at which the 8-thiocyanato-FMN flavodoxin reacts with DTT was found to be 320 M^{-1} min^{-1} at 4 °C. This observation confirms earlier studies (Schopfer et al., 1981) showing the good accessibility of the 8-position in flavodoxin. Unlike in free solution the sole product of the reaction between DTT and 8-SCN-FMN flavodoxin is the 8-mercaptoflavin species. A possible explanation could be steric constraints that impede the reaction via pathway 3 (Scheme I), since the nucleophilic displacement of the thiocyanate group involves a tetrahedral transition state. This explanation is consistent with previous studies with 8-Cl-FMN flavodoxin, where the nucleophilic displacement of Cl^- by thiophenol, also involving a tetrahedral intermediate, was severely impeded (Schopfer et al., 1981).

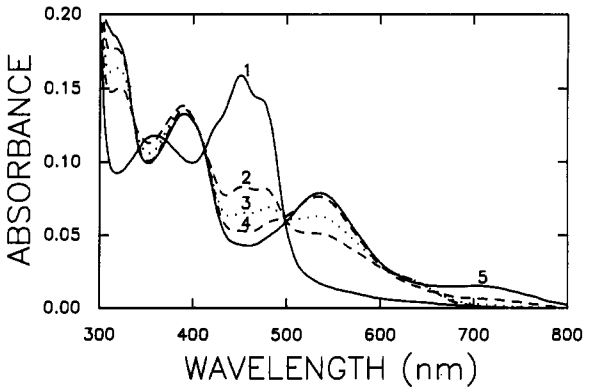


FIGURE 4: Photoreduction of 8-SCN-flavodoxin according to the method of Massey and Hemmerich (1978). 8-SCN-FMN and apoflavodoxin were mixed in an anaerobic cuvette to give a 14 μM 8-SCN-flavodoxin solution (in 0.1 M potassium phosphate, pH 7) (solid trace, 1). Twenty microliters of a 0.3 mM EDTA solution and 2 μL of a 10^{-3} M 5dRfl solution were mixed from a side arm after anaerobiosis was established. The spectra shown are taken after 10 s (dashed trace, 2), 15 s (dotted trace, 3), 20 s (dashed trace, 4), and 25 s (solid trace, 5) of irradiation. All operations were performed at 4 °C.

Preparations of 8-thiocyanato-FMN flavodoxin generally gave some spontaneous conversion to the 8-mercapto-FMN form with a characteristic increase of absorbance at 535 nm and with a half-life of approximately 3 h. However, in general, the extent of formation of 8-mercapto-FMN flavodoxin was small (typically 0–35%) and depended on the age of the apoprotein and on the pH of reconstitution. Thus fresh samples of apoprotein gave less spontaneous conversion than old samples, and the extent of conversion increased with increasing pH. Flavodoxin from *M. elsdenii* contains two cysteines (Tanaka et al., 1973) that are, according to the crystal structure of the closely related flavodoxin from *Clostridium MP* (Smith et al., 1977), not in the vicinity of the isoalloxazine ring or its ribityl phosphate side chain. This leaves us with the possible explanation that reaction of the 8-thiocyanate group occurs between the free flavin and the apoflavodoxin. On the other hand the cysteines play an essential role in binding the flavin (Mayhew, 1971) and it was therefore not surprising to find that MMTS-treated apoflavodoxin lost its capability to bind 8-thiocyanato-FMN as well as to bring about conversion to 8-mercapto-FMN. Despite the formation of some 8-mercapto-FMN enzyme, we were able to demonstrate by means of photoreduction that flavodoxin stabilizes the 8-SCN-FMN neutral radical with absorbance maxima at 322, 388, and 535 nm (Figure 4). Reduction to the radical species proceeds with isosbestic points at 335, 406, and 498 nm (Figure 4). Further reduction to the fully reduced flavin species causes an increase of absorption at 710 nm, indicating formation of the 8-mercaptoflavin neutral radical. Since this formation does not occur until the two electron reduced flavin is produced, we conclude that the radical species does not eliminate cyanide (see also discussion of OYE in the following section). Complete reduction and subsequent reoxidation by molecular oxygen yielded the spectrum of oxidized 8-mercapto-FMN flavodoxin.

Old Yellow Enzyme. Upon reconstitution of apo-OYE the absorbance maximum of 8-thiocyanato-FMN was shifted by 18 nm from 446 to 464 nm (ϵ_{max} = 13 900 M^{-1} cm^{-1}). OYE stabilizes the anionic radical of FMN (Nakamura et al. 1965) and of artificial flavins in general (Stewart & Massey, 1985).

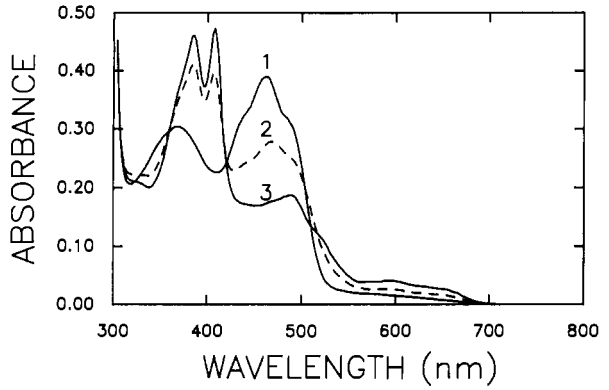


FIGURE 5: Photoreduction of 8-SCN-FMN OYE according to the method of Massey and Hemmerich (1978). One milliliter of a 33 μM 8-SCN-FMN OYE solution in 0.1 M potassium phosphate, pH 7, was irradiated in the presence of 1 μM 5dRfl and 5 mM EDTA at 4 °C under anaerobic conditions. Spectrum 1 shows the 8-SCN-FMN OYE before irradiation and spectra 2 (dashed trace) and 3 (solid trace) were recorded after 5 and 15 s of irradiation, respectively.

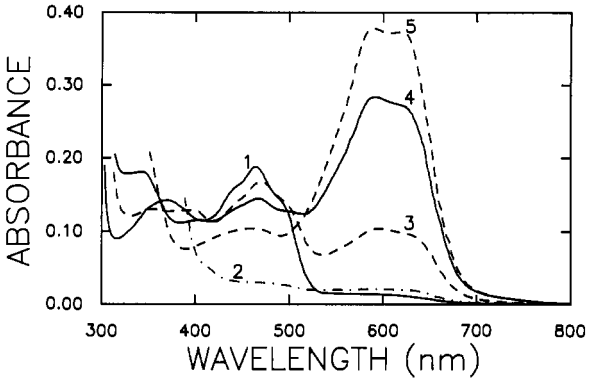


FIGURE 6: Formation of 8-mercapto-FMN OYE by means of light irradiation of reduced 8-thiocyanato-FMN OYE. 8-Thiocyanato-FMN OYE (spectrum 1) was anaerobically reduced with 5 μL of 10 mM NADPH (spectrum 2, dotted–dashed trace) and irradiated for 3 min. After reoxidation, spectrum 3 was recorded. Two more cycles of reduction and light irradiation (for 15 and 20 min) were performed and spectra 4 and 5, respectively, recorded after reoxidation.

It was therefore not surprising that photoreduction of 8-thiocyanato-FMN OYE gave rise to a radical species with absorbance maxima at 388 (ϵ = 16 100 M^{-1} cm^{-1}) and 410 nm (ϵ = 16 550 M^{-1} cm^{-1}), and a shoulder at 492 nm (ϵ = 6950 M^{-1} cm^{-1}) (see Figure 5). The anionic radical of OYE did not eliminate CN^-. Reoxidation of 8-SCN-FMN anion radical by oxygen—which was unusually slow ($t_{1/2}$ in the hour range)—yields the starting material, 8-SCN-FMN OYE, and not the corresponding 8-mercapto-FMN OYE. This again supports the conclusion that photoelimination of CN^- occurs only from the two electron reduced state. In order to demonstrate the requirement of light for the elimination of CN^-, 8-SCN-FMN OYE was reduced by NADPH, anaerobically and in the dark. After reoxidation the spectrum of 8-SCN-FMN OYE was regained without any indication of formation of 8-mercapto-FMN OYE. However, irradiation of the NADPH-reduced enzyme with visible light from a Smith-Victor Sun Gun (40 min) resulted in the formation of 8-mercapto-FMN OYE (Figure 6).

Earlier studies (Schopfer et al., 1981) established the solvent accessibility of the 8-position of the flavin in OYE. Reaction with Na_2S yielded 8-mercapto-FMN OYE, whereas reaction with DTT led to a mixture of 85% 8-mercapto-FMN OYE and 15% 8-SR-FMN OYE. This observation implies that unlike in flavodoxin, a tetrahedral transition state can be accommodated in OYE, allowing reaction pathways 3 and 1 (or 2) depicted in Scheme I.

The reaction of 8-SCN-FMN OYE with DTT is unusually fast and biphasic. Analysis over a range of DTT concentrations shows that the reaction is first order with DTT, with approximately equal extent of reaction in each phase. At pH 7.0, 4 °C, the second-order rate constants for the two phases were determined as 15 000 and 4300 M^{-1} min^{-1}. These values should be compared with the value of 550 M^{-1} min^{-1} obtained under the same conditions with 8-SCN-FMN (see earlier section). Experiments carried out with a Hewlett-Packard diode array spectrophotometer showed that the same spectral changes occurred in the two phases of the reaction. Hence it is likely that the biphasic kinetics reflect the existence of two different populations of enzyme molecules. The existence of several isozyme forms of OYE has been demonstrated previously (Miura et al., 1986; Beinert et al., 1985) as well as multiphasic kinetics behavior (Massey & Schopfer, 1986).

OYE forms long-wavelength-absorbing charge-transfer complexes with a wide variety of phenols (Abramowitz & Massey, 1976b). Similar behavior is shown with 8-SCN-FMN OYE, except that in all cases the wavelength maximum of the charge-transfer band is at longer wavelengths (lower energy) than with normal enzyme. For example, *p*-chlorophenol binds with considerable avidity to 8-SCN-FMN OYE (K_d = 2.6 μM at pH 7.0, 4 °C) with the development of an intense charge-transfer band with λ_{max} = 720 nm (ϵ = 5300 M^{-1} cm^{-1}). Comparable values with native enzyme under the same conditions are K_d = 1 μM, λ_{max} = 650 nm, and ϵ = 4000 M^{-1} cm^{-1} (Massey & Schopfer, 1986). A linear correlation has been observed between the redox potential of the enzyme-bound flavin at pH 7.0 and the energy of the long-wavelength transition with *p*-chlorophenol and *p*-methoxyphenol as ligands (Stewart & Massey, 1985). Applying this correlation to 8-SCN-FMN OYE predicts a value of E_m, pH 7.0, of approximately −140 mV (the λ_{max} of the complex of 8-SCN-FMN OYE with *p*-methoxyphenol is at 780 nm versus 700 nm for native enzyme). This is in keeping with the observation that the potential of the bound flavin in OYE is consistently lower than that of the free flavin, indicating a preferential binding of the oxidized flavin to the apoprotein over that of the reduced flavin.

Complex formation of 8-SCN-FMN OYE with phenolic ligands results in a dramatic change in the reactivity of the bound flavin with DTT. Again the reactions are biphasic, but the proportions of the reaction occurring in the two phases depends on the ligand. In addition, with some ligands, a mixture of 8-mercapto-FMN and 8-SR-FMN forms is produced, as with unliganded enzyme, while with others, the sole detected product is the 8-mercapto-FMN enzyme. For example, with the *p*-chlorophenol complex, the rate ocnstants were determined as 66 M^{-1} min^{-1} and 9.5 M^{-1} min^{-1}, with ~63% of the reaction occurring in the faster phase and ~37% in the slower phase and with approximately the same distribution of products as with unliganded enzyme. However, with *p*-hydroxybenzaldehyde as ligand, the sole detectable product was the 8-mercapto-FMN enzyme, formed in approximately equal size phases with rate constants of 160 and 25 M^{-1} min^{-1}. These observations are probably also explicable in terms of isozyme forms, to a certain extent interconvertible depending on the presence and nature of the phenolic ligand as was evidenced by a recent study (Macheroux et al., 1990). The results obviously imply a considerable change in accessibility to nucleophilic attack of the bound 8-SCN-FMN on binding of the phenolic ligand.

L-*Lactate Oxidase.* When freshly prepared apoprotein of lactate oxidase was mixed with 8-thiocyanato-FMN at pH 7

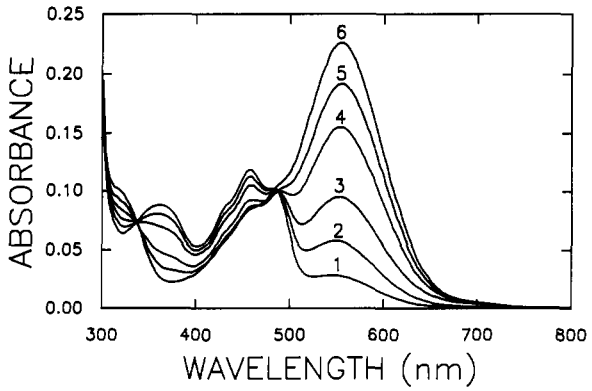


FIGURE 7: Spontaneous conversion of 8-SCN-FAD anthranilate hydroxylase to 8-mercapto-FAD anthranilate hydroxylase. The reaction of 1 mL of freshly prepared 8-SCN-FAD anthranilate hydroxylase (10 μM) in 0.1 M Tris buffer, pH 8.6, was followed in the spectrophotometer at 4 °C. The spectra were recorded after 4 min, 90 min, 5.6 h, 15 h, 29 h, and 69 h for traces 1–6, respectively.

a spontaneous conversion to 8-mercapto-FMN LAO was observed. However, this conversion did not go to completion but stopped at 20–30% conversion of the thiocyanate group. Such conversion could be largely avoided by carrying out the reconstitution at pH 6. After holoenzyme was formed in this way, the pH could be adjusted to 7 without conversion to 8-mercapto-FMN enzyme. Addition of DTT to 8-thiocyanato-FMN LAO did not result in formation of 8-mercapto-FMN enzyme, even over a period of days, in agreement with earlier studies by Schopfer et al. (1981) which indicated that the flavin 8-position was not accessible to solvent.

Anthranilate Hydroxylase. Binding of 8-thiocyanato-FAD to anthranilate hydroxylase is associated with a bathochromic shift from 360 to 364 nm and 451 to 459 nm, respectively. In addition to these shifts a pronounced shoulder at 484 nm appears (see Figure 7). The resulting resolved flavin spectrum is typical for FAD bound to anthranilate hydroxylase and was obtained after a 4-min incubation with apoenzyme.

8-Thiocyanato-FAD enzyme is converted to 8-mercapto-FAD enzyme in a monophasic spontaneous reaction with a half-life of 11 h at pH 8.6, 4 °C. The final spectrum (see Figure 7) has a maximum at 555 nm, which is typical for 8-mercapto-FAD hydroxylases (Massey et al., 1979) and shows no indication of formation of an 8-SR-FAD form. In a control experiment 8-thiocyanatoriboflavin was added to a slight excess (10%) of native anthranilate hydroxylase. Spontaneous conversion to the 8-mercapto species occurred but was extremly slow ($t_{1/2} \gg 30$ h at 4 °C and pH 7.8) and not complete. This result supports the conclusion that the reaction between the 8-thiocyanato-FAD and a nucleophile occurs in the flavin-binding site.

The accessibility of the flavin 8-position was probed by reaction of 8-thiocyanato-FAD anthranilate hydroxylase with DTT. The reaction products were the 8-mercapto-FAD enzyme and to a small extent 8-SR-FAD enzyme (<10%). A bimolecular reaction rate of 95 M^{-1} min^{-1} at 4 °C was found. This compares with a more than five times higher value for free flavin (see previous section), indicating limited accessibility as was recently suggested by Powlowski et al. (1989). Binding of anthranilate to 8-SCN-FAD anthranilate hydroxylase increases the reaction rate by a factor of 10, supporting the conclusion drawn by Powlowski et al. (1989) that binding of substrate causes a conformational change of the protein, resulting in greater accessibility of the 8-position. Further evidence was provided by reacting 8-mercapto-FAD anthranilate hydroxylase with iodoacetamide. Conversion to the 8-SCH_2CONH_2-flavin occurred with a bimolecular reaction rate of 10.6 M^{-1} min^{-1} without anthranilate and 13.4 M^{-1} min^{-1} in the presence of anthranilate (saturating concentration), respectively. Both rates are smaller than for the free flavin (46.3 M^{-1} min^{-1}; Schopfer et al., 1981), again indicating limited accessibility. However, the presence of anthranilate increased the rate of conversion only slightly, unlike the dramatic increase in the reaction rate observed when 8-chloro-FAD enzyme was treated with thiophenol (Powlowski et al., 1989) or when 8-SCN-FAD enzyme was treated with DTT as described above.

p-Hydroxybenzoate Hydroxylase. Although some spontaneous conversion of the 8-thiocyanato-FAD pHBH occurs, the reaction is very slow and generally does not go to more than 10% completion. The extent of conversion appears to be correlated with the presence of soluble but denatured apoprotein. Therefore formation of the 8-mercapto-FAD is likely to be due to free 8-thiocyanato-FAD reacting with a nucleophile of denatured protein followed by binding of 8-mercapto-FAD to native apoprotein. This interpretation is supported by the three-dimensional structure of pHBH, which shows that no nucleophilic amino acid residue is close to the flavin 8-position.

Reaction of 8-SCN-FAD-pHBH with DTT is extremely fast with second-order rate constants of 3×10^4 M^{-1} min^{-1} in the absence and 6×10^5 M^{-1} min^{-1} in the presence of pOHB. In both cases 8-mercapto-FAD-pHBH is the sole product of the reaction. These rates are 55 and 1100 times faster than with free flavin. Reaction with mercaptoethanol is also very fast, with second-order rate constants of 7.4×10^3 M^{-1} min^{-1} in the absence and 3.2×10^4 M^{-1} min^{-1} in the presence of pOHB, some 60 and 260 times greater than for the free flavin. Similar observations were made earlier for the reaction of 8-mercapto-FAD-pHBH with iodoacetamide (Schopfer et al., 1981). In general, the nucleophilicity of an anion can be enhanced by desolvation, which leads to a dramatic increase of the reaction rate. For example, the rate of reaction is increased when a polar but nonprotic solvent is used (DMF, DMSO) instead of a polar and protic solvent (water, methanol). Although the 8-position is clearly accessible to solvent-borne reagents, the crystal structure of pHBH (Schreuder et al., 1989) reveals that the nucleophile would have to travel through a cavity in the protein in order to reach the 8-position of the flavin ring. When DTT (or mercaptoethanol) enters this cavity it will experience a different—conceivably less protic—environment (several proline and arginine residues are in the vicinity of the 8-position), which will cause an at least partial desolvation and hence an increase of nucleophilicity. Another contribution to the enhanced reaction rate may stem from a lowering of the pK of the thiol group. According to the crystal structure, two arginines (R44 and R220) are positioned 6.3 and 8.8 Å (distance between the guanidinyl carbon and the 8α-carbon of the flavin ring system) from the 8α-carbon. This basic microenvironment may cause a drop of the pK and that in turn would give rise to a higher concentration of reactive thiolate.

p-Hydroxybenzoate hydroxylase is a well-documented example of a protein undergoing considerable conformational changes upon binding substrate or substrate analogues. These changes also affect the environment around the 8-position. It was therefore not surprising that the rates of reaction with DTT and with mercaptoethanol were also altered. The even higher rate in the presence of substrate can be accounted for by a conformational change that favors desolvation and/or increases the polarity of the cavity. Likewise the arrangement

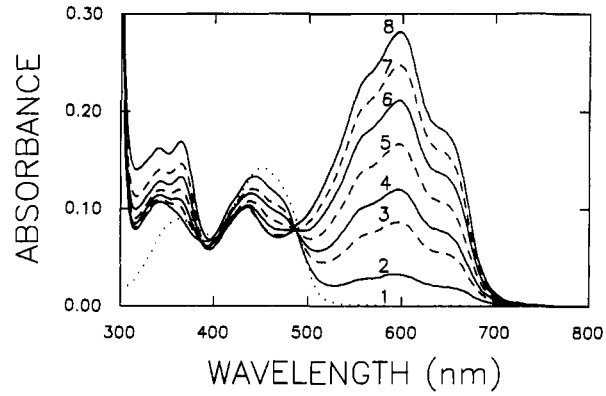


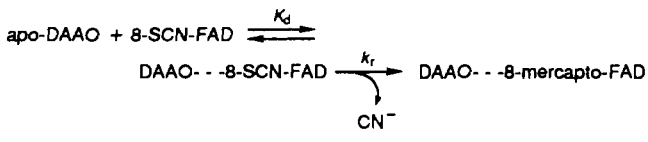
FIGURE 8: Spontaneous conversion of 8-SCN-FAD DAAO to 8-mercapto-FAD DAAO. The reaction was followed in the spectrophotometer at 25 °C in 0.02 M pyrophosphate buffer, pH 8.5. Spectrum 1 (dotted trace) was that of 11 μM 8-SCN-FAD recorded before addition of apo-DAAO. Spectra 2–8 were recorded at 3, 27, 53, 96, 212, and 312 min after addition of 34 μM apo-DAAO. The spectral changes proceeded with an isosbestic point at 484 nm.

of the two arginines mentioned earlier may change such that the pK value of the thiol group is further lowered.

The rapidity of the reaction of DTT and mercaptoethanol with 8-SCN-FAD-pHBH, especially that in the presence of substrate, allowed us to determine the stoichiometry of the reactions. With DTT, 0.83 mol of DTT was found to react with 1 mol of 8-SCN-FAD enzyme, indicating that reaction path 2 of Scheme I was at least partially operating, possibly with intramolecular attack of the second thiolate on the intermediate disulfide. With mercaptoethanol, 1.0 mol of thiol was required per mole of 8-SCN-FAD enzyme, indicating that in this case the 8-mercaptoflavin was formed by attack on the carbon atom of the thiocyanate function as shown in Scheme I, path 1.

General Acyl-CoA Dehydrogenase. Binding of 8-thiocyanato-FAD to GAD causes a hypsochromic shift of both absorbance peaks (365 to 340 nm and 449 to 442 nm). No spontaneous conversion was observed and reactions with thiol reagents were very slow ($t_{1/2}$ > days), in agreement with previous results (Thorpe & Massey, 1983), which had indicated solvent inaccessibility of the flavin 8-position. Complexation of 8-thiocyanato-FAD-GAD with acetoacetyl-CoA results in a charge-transfer band at 560 nm (ϵ_{max} = 3000 M^{-1} cm^{-1}), analogous to similar results with native enzyme and enzyme reconstituted with other artificial flavins (Thorpe & Massey, 1983).

D-*Amino Acid Oxidase.* As shown in Figure 8, when 8-SCN-FAD is bound to the apoprotein of D-amino acid oxidase, the 8-mercapto-FAD enzyme is formed quantitatively with a final spectrum identical with that reported previously for the binding of 8-mercapto-FAD to D-amino acid oxidase (Massey et al., 1979). After a small initial burst, the reaction proceeds to completion following first-order kinetics (see Figure 8) with half-times that decrease as the concentration of apoprotein is increased. For example, when 4 μM 8-SCN-FAD was reacted with 9 μM apoprotein, the half-life for conversion was 135 min at pH 8.5, 25 °C, whereas when reacted with 23 μM apoenzyme, the half-life was 75 min. Such behavior is consistent only with the chemical conversion occurring with enzyme-bound flavin, in equilibrium with apoprotein and free 8-SCN-FAD:



Analysis of the results of eight such experiments by the graphical method of Strickland et al. (1975), with apoprotein concentrations up to 42 μM, yielded a value for K_d of 7.5 μM and a value of k_r of 1.1 × 10^{-2} min^{-1}. Thus, the binding of 8-SCN-FAD to apo-DAAO is rather weak, approximately 10 times poorer than that of FAD itself, where K_d values of 0.5–0.9 μM have been reported under similar conditions (Massey et al., 1966). The weak binding of 8-SCN-FAD was confirmed by ultrafiltration experiments. For example, 8.6 μM 8-SCN-FAD was incubated with 14.8 μM apo-DAAO for 20 min and centrifuged through a Centricon 30 ultrafiltration membrane and the filtrate was found to contain 37.5% the 8-SCN-FAD initially added. After correction for the 20% conversion to 8-mercapto-FAD enzyme that had occurred during the experiment, a K_d of 8.5 μM could be estimated.

Although not studied in detail, the spontaneous conversion was inhibited by benzoate. At a concentration of 2 × 10^{-4} M benzoate the reaction rate was decreased to 30% that in the absence of benzoate, and at 3 × 10^{-3} M benzoate the reaction rate was decreased to less than 10%. These results indicate that the enzyme–benzoate complex is much less reactive than the 8-SCN-FAD enzyme alone and imply a conformational change in the protein on binding of benzoate, which makes it much more difficult for the protein nucleophile to attack the SCN substitutent. Previous studies with 8-chloro-FAD enzyme (Schopfer et al., 1981) and 4-thio-FAD enzyme (Massey et al., 1984) have provided strong evidence that a conformational change in the protein occurs on binding benzoate, since chemical reactions with both sulfur substituents were slowed dramatically in the presence of benzoate.

A similar spontaneous conversion of 6-SCN-FAD DAAO to the 6-mercapto-FAD enzyme has also been reported (Massey et al., 1986). Since DAAO contains five cysteine residues per subunit (Curti et al, 1973), it is possible that two separate cysteine residues are located in the flavin-binding site, close to the 6- and 8-positions. It is, however, conceivable that a single cysteine residue may be positioned in such a way as to attack either the 6-SCN or the 8-SCN group.

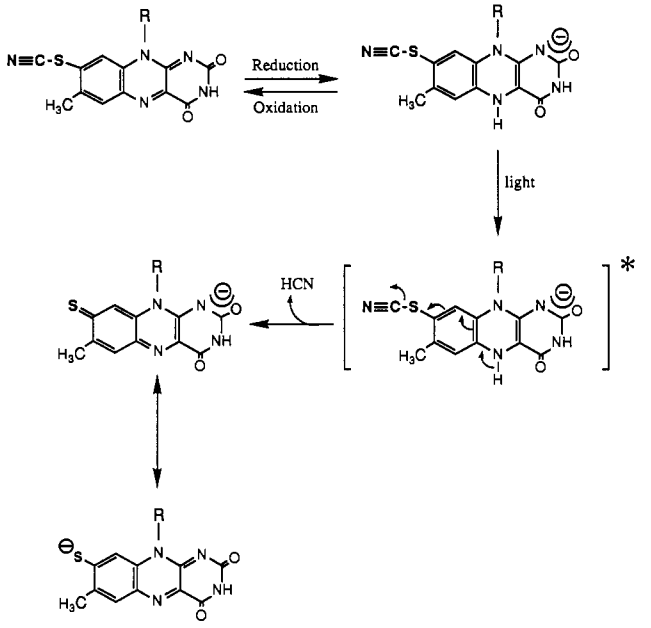
Glucose Oxidase. 8-Thiocyanato-FAD GO was a stable active holoenzyme (40% activity compared to native GO as judged by oxygen consumption). Addition of DTT did not result in a conversion of the 8-thiocyanate group to the corresponding mercapto species nor did catalytic turnover. The spectral characteristics of 8-thiocyanato-FAD GO are similar to those of free 8-thiocyanato-FAD with absorbance maxima at 362 and 454 nm.

One problem we encountered reconstituting apo-GO with 8-thiocyanato-FAD was the spontaneous appearance of free 8-mercapto-FAD, which can be separated by means of molecular sieve chromatography (Sephadex G-25). Native holo-GO has no titratable thiol groups but the apoprotein has a single thiol group per monomer, as judged by reaction with DTNB (Swoboda & Massey, 1965). The observed 8-mercapto-FAD may then be the reaction product of 8-thiocyanato-FAD with this mercapto function. To investigate this possibility we removed the accessible thiol group with MMTS prior to the addition of 8-thiocyanato-FAD. This procedure yielded fully active 8-thiocyanato-FAD GO without any traces of 8-mercapto-FAD. Since the 8-mercapto-FAD produced in the absence of MMTS treatment is not protein bound, it follows that 8-SCN-FAD must be bound to the apoprotein considerably more tightly than is 8-mercapto-FAD.

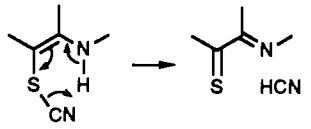
CONCLUSIONS

While 6-SCN-flavins react with thiols such as DTT to yield 6-mercaptoflavins as the sole primary product (Ghisla et al.,

Scheme II: Possible Mechanism for the Reductive Photoelimination of Cyanide



1986), 8-SCN flavins yield either or both 8-mercaptoflavins and 8-SR-flavins, depending on the reaction conditions. Another interesting difference between the two thiocyanato flavins is the elimination of cyanide. Whereas free 6-SCN-flavins eliminate cyanide from the two electron reduced form in a dark reaction (Ghisla et al., 1980), the corresponding reduced 8-thiocyanato flavin requires irradiation with visible light (see Scheme II). The facile elimination of cyanide from the reduced 6-thiocyanato flavin can be rationalized as a (concerted?) rearrangement as shown below:



However, it is important to note that such elimination of cyanide from the reduced flavin has not yet been observed with any protein-bound 6-SCN-flavin (Massey et al., 1986). This reaction scheme is not feasible in the case of 8-thiocyanatoflavins and therefore a different reaction mechanism has to be postulated for the elimination of cyanide. A probable mechanism is shown in Scheme II. This mechanism involves an excited reduced 8-thiocyanatoflavin, which decomposes to the oxidized 8-mercaptoflavin and HCN. A somewhat similar photochemical elimination of chloride from reduced 7- and 8-chloroflavins has been reported previously (Massey et al., 1980). The photochemical elimination reactions reported here and previously occur with both free and protein-bound flavin.

The nine investigated flavoproteins can be divided in three groups: (1) flavoproteins that bind the 8-SCN-flavins without spontaneous conversion to the 8-mercaptoflavin (riboflavin-binding protein, OYE, glucose oxidase, general acyl-CoA dehydrogenase); (2) flavoproteins that bind the 8-SCN-flavin and convert it spontaneously to the 8-mercapto derivative (D-amino acid oxidase and anthranilate hydroxylase); (3) flavoproteins that bind the 8-SCN-flavin and partially convert it to the 8-mercapto derivative (flavodoxin, lactate oxidase, *p*-hydroxybenzoate hydroxylase). For the second group of proteins we have provided evidence that the complete and spontaneous conversion is due to a reaction with a nucleophile in the flavin-binding site. In contrast, the third group of proteins shows a variable degree of conversion that depends on the history of the protein and other conditions detailed in earlier sections. Therefore we suggest that conversion in these cases is rather due to the reaction with an accessible nucleophile on the surface of the protein or to reaction with denatured apoprotein.

Registry No. 8-SCN-FAD, 130574-31-1; 8-SCN-FMN, 130574-32-2; DTT, 3483-12-3; SCN^-, 302-04-5; 8-aminoriboflavin, 5178-05-2; 8-amino-FAD, 76598-24-8; 8-thiocyantoriboflavin, 130574-30-0; Old Yellow Enzyme, 9001-68-7; lactate dehydrogenase, 9001-60-9; D-amino acid oxidase, 9000-88-8; *p*-hydroxybenzoate hydroxylase, 9059-23-8; glucose oxidase, 9001-37-0; anthranilate hydroxylase, 37340-81-1; general acyl-CoA dehydrogenase, 9027-65-0; 2-mercaptoethanol, 60-24-2; sulfide, 18496-25-8; iodoacetamide, 144-48-9; anthranylic acid, 118-92-3.

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Identification of a Membrane-Bound, Glycol-Stimulated Phospholipase A_2 Located in the Secretory Granules of the Adrenal Medulla†

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ABSTRACT: Chromaffin granule membranes prepared from bovine adrenal medullae showed Ca^{2+}-stimulated phospholipase A_2 (PLA_2) activity when assayed at pH 9.0 with phosphatidylcholine containing an [^{14}C]-arachidonyl group in the 2-position. However, the activity occurred in both soluble and particulate subcellular fractions, and did not codistribute with markers for the secretory granule. PLA_2 activity in the granule membrane preparation was stimulated dramatically by addition of glycerol, ethylene glycol, or poly(ethylene glycol). This glycol-stimulated PLA_2 activity codistributed with membrane-bound dopamine β-hydroxylase, a marker for the granule membranes, through the sequence of differential centrifugation steps employed to prepare the granule membrane fraction, as well as on a sucrose density gradient which resolved the granules from mitochondria, lysosomes, and plasma membrane. The glycol-stimulated PLA_2 of the chromaffin granule was membrane-bound, exhibited a pH optimum of 7.8, retained activity in the presence of EDTA, and was inactivated by *p*-bromophenacyl bromide. When different ^{14}C-labeled phospholipids were incorporated into diarachidonylphosphatidylcholine liposomes, 1-palmitoyl-2-arachidonylphosphatidylcholine was a better substrate for this enzyme than 1-palmitoyl-2-oleylphosphatidylcholine or 1-acyl-2-arachidonyl-phosphatidylethanolamine, and distearoylphosphatidylcholine was not hydrolyzed.

The secretion of peptides, hormones, and most neurotransmitters occurs by a similar exocytotic mechanism in a variety of endocrine, exocrine, and neurosecretory cell types (Kelly, 1985), including the well-characterized chromaffin cells of the adrenal medulla (Burgoyne, 1984; Pollard et al., 1985; Strittmatter, 1988; Winkler, 1988). Stimulation of chromaffin cells leads to an increase in the intracellular Ca^{2+} concentration (Knight & Kesteven, 1983), which triggers secretion (Baker & Knight, 1978; Dunn & Holz, 1983; Wilson & Kirshner, 1983). Bilayer fusion between the secretory vesicle membrane and the plasmalemma is believed to represent a kinetically difficult step, and appears to be Ca^{2+}-dependent (Winkler, 1988; Plattner, 1989). The regulation of secretory vesicle transport, cytoskeletal rearrangements, and other requisite steps in the overall exocytotic process by Ca^{2+} or other factors has not yet been clearly defined (Gomperts, 1986; Sarafian et al., 1987; Aunis & Bader, 1988; Strittmatter, 1988; Holz et al., 1989).

A number of lines of evidence suggest the participation of a phospholipase A_2 (PLA_2)[1] in exocytosis in chromaffin and other neuroendocrine tissue. Enzymes in the PLA_2 family catalyze hydrolysis of the *sn*-2 acyl ester bonds in phospholipids, to release 1-acyllysophospholipid and free fatty acid. Phospholipids occurring in membranes of chromaffin cells, as in most cells, have an acyl chain composition such that the fatty acids liberated by PLA_2 action consist primarily of arachidonic acid and other cis-unsaturated fatty acids (Winkler & Smith, 1968; Balzer & Khan, 1975). The important roles of arachidonic acid in cellular signalling are well recognized, acting both as a precursor in eicosanoid biosynthesis and as

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[1] Abbreviations: PLA_2, phospholipase A_2; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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