Biosynthesis of Covalently Bound Flavin: Isolation and in Vitro Flavinylation of the Monomeric Sarcosine Oxidase Apoprotein†

Alshaimaa Hassan-Abdallah, Robert C. Bruckner, Guohua Zhao, and Marilyn Schuman Jorns*

Department of Biochemistry and Molecular Biology, College of Medicine, Drexel University, Philadelphia, Pennsylvania 19102

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ABSTRACT: The covalently bound FAD in native monomeric sarcosine oxidase (MSOX) is attached to the protein by a thioether bond between the 8α-methyl group of the flavin and Cys315. Large amounts of soluble apoenzyme are produced by controlled expression in a riboflavin-dependent *Escherichia coli* strain. A time-dependent increase in catalytic activity is observed upon incubation of apoMSOX with FAD, accompanied by the covalent incorporation of FAD to ∼80% of the level observed with the native enzyme. The spectral and catalytic properties of the reconstituted enzyme are otherwise indistinguishable from those of native MSOX. The reconstitution reaction exhibits apparent second-order kinetics ($k = 139\ M^{-1}\ min^{-1}$ at 23 °C) and is accompanied by the formation of a stoichiometric amount of hydrogen peroxide. A time-dependent reduction of FAD is observed when the reconstitution reaction is conducted under anaerobic conditions. The results provide definitive evidence for autoflavinylation in a reaction that proceeds via a reduced flavin intermediate and requires *only* apoMSOX and FAD. Flavinylation of apoMSOX is not observed with 5-deazaFAD or 1-deazaFAD, an outcome attributed to a decrease in the acidity of the 8α-methyl group protons. Covalent flavin attachment is observed with 8-nor-8-chloroFAD in an aromatic nucleophilic displacement reaction that proceeds via a quininoid intermediate but not a reduced flavin intermediate. The reconstituted enzyme contains a modified cysteine−flavin linkage (8-nor-8-*S*-cysteinyl) as compared with native MSOX (8α-*S*-cysteinyl), a difference that may account for its ∼10-fold lower catalytic activity.

Although flavins often function as noncovalently bound prosthetic groups, a growing number of flavoenzymes have been found to contain covalently bound flavin. This diverse group of enzymes perform a remarkable range of reactions, including ion pumping, antibiotic synthesis, and metabolism of biogenic amines (*1*−*3*). Monomeric sarcosine oxidase (MSOX)[1] is a prototypical member of a recently discovered family of amine-oxidizing enzymes that all contain covalently bound flavin (*4*−*7*). MSOX is an inducible bacterial enzyme that plays an important role in the catabolism of sarcosine (*N*-methylglycine), a common soil metabolite (*8*). The enzyme is widely used in the clinical evaluation of renal function (*9*). MSOX catalyzes the oxygen-dependent demethylation of sarcosine to yield glycine, formaldehyde, and hydrogen peroxide. High-resolution crystal structures are available for free MSOX and complexes of the enzyme with competitive inhibitors (*10*, *11*). MSOX contains 1 mol of FAD, attached to the protein via a thioether linkage between the 8α-methyl group of the isoalloxazine ring and Cys315 (8α-*S*-cysteinyl-FAD) (*12*) (see Table 1 for the structure). The same covalent linkage is found for FAD in monoamine oxidase A and B, mammalian enzymes whose sequences are 20% identical with that of MSOX. In other enzymes, tyrosine or histidine may replace cysteine as the site of flavin attachment, but the covalent linkage nearly always involves the 8α-methyl group of the flavin (*1*).

Covalent flavin attachment is thought to occur in a reaction catalyzed by the apoprotein itself (*1*, *13*−*15*). A molecular mechanism involving iminoquinone methide and reduced flavin intermediates (Scheme 1, intermediates I and II, respectively) has been proposed for MSOX and other enzymes (*1*, *10*), but *in vitro* studies to characterize the reaction have been limited by difficulties in isolating reconstitutable apoenzymes. A notable exception is provided by *p*-cresol methylhydroxylase, a hemoflavoenzyme ($\alpha_2\beta_2$) that contains separate flavin- and heme-binding subunits. In this perhaps unusual example, both subunits are required for covalent flavinylation (8α-*O*-tyrosyl-FAD). As a consequence, the reaction can be blocked by expressing the flavin-binding subunit by itself and then triggered by mixing the separately isolated subunits in the presence of FAD (*13*). Expression of apo-monoamine oxidase A and B was achieved by using a riboflavin-dependent strain of *Saccharomyces cerevisiae*. Isolation of the mitochondrial membrane-bound proteins was, however, thwarted by the extreme instability of the solubilized apoenzymes (*16*, *17*). An unstable apoenzyme form of 6-hydroxy-D-nicotine oxidase was isolated in low yield by expressing the recombinant enzyme in the presence of diphenylene iodonium (*14*). Analytical amounts of apo-succinate dehydrogenase have been prepared by using a reticulocyte lysate transcription−translation system (*15*).

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* To whom correspondence should be addressed. Phone: (215) 762-7495. Fax: (215) 762-4452. E-mail: marilyn.jorns@drexelmed.edu.

[1] Abbreviations: MSOX, monomeric sarcosine oxidase; FAD, flavin adenine dinucleotide; IPTG, isopropyl β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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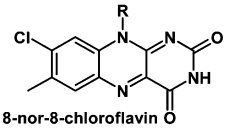
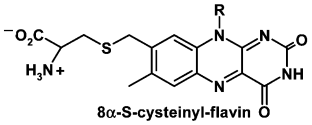
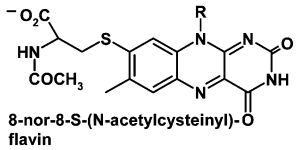
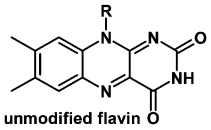
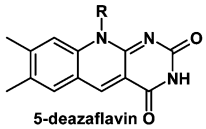
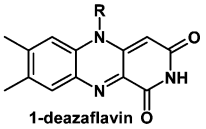
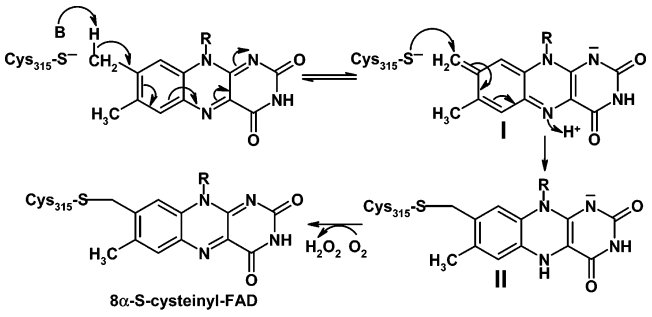
Table 1: Structure and Redox Potential of Selected Flavin Derivatives[a]

Flavin	$E_{m,7}$ (mV)	Ref.
 8-nor-8-chloroflavin	-144	(39)
 8α-S-cysteinyl-flavin	-169	(*40*)
 8-nor-8-S-(N-acetylcysteinyl)-flavin	-180	(*33*)
 unmodified flavin	-199	(*41*)
 5-deazaflavin	-273	(*42*)
 1-deazaflavin	-280	(*43*)

[a] Redox potentials are for the corresponding riboflavin derivatives. The carboxyl and amino groups in the structure shown for 8α-S-cysteinyl-flavin are replaced by peptide bonds in the 8α-S-cysteinyl-FAD covalently attached to Cys315 in MSOX.

Scheme 1: Postulated Mechanism for Covalent Flavinylation of MSOX with FAD



Flavinylation of both of these apoenzymes [8α-*N*(3)-histidyl-FAD] was markedly accelerated by small molecule effectors.

MSOX is a relatively small (44 kDa), stable, cytoplasmic protein containing a single prosthetic group. The recombinant enzyme is highly overexpressed in *Escherichia coli* and readily isolated in gram quantities. These features suggested that MSOX might provide a viable system for in vitro flavinylation studies. In this paper, we describe a system for expression and preparative-scale (40 mg/L of culture) isolation of a reasonably stable apoprotein that can be reconstituted with FAD and certain FAD derivatives.

EXPERIMENTAL PROCEDURES

Materials. Amplex Red was obtained from Molecular Probes. NADH peroxidase, horseradish peroxidase, and *o*-dianisidine were purchased from Sigma. 8-Nor-8-chlororiboflavin and 1-deazariboflavin were generous gifts from Dr. J. Lambooy and W. Ashton, respectively. 5-Deazariboflavin was previously synthesized in this laboratory according to the method of O'Brien et al. (*18*). Recombinant FAD synthetase from *Corynebacterium ammoniagenes* was kindly provided by Dr. B. McIntire. *Pfu* DNA polymerase was obtained from Stratagene. *Nde*I and *Xho*I restriction enzymes were purchased from New England Biolabs. QIAquick PCR Purification and QIAquick Gel Extraction Kits were obtained from Qiagen. Plasmid pET24a was supplied by Novagen. T4 DNA ligase was purchased from New England Biolabs. The Co^{2+} affinity matrix was obtained from BD Bioscience. Sep-Pak C18 cartridges were purchased from Waters.

Expression and Purification of the MSOX Holoenzyme. MSOX was expressed in *E. coli* and purified as previously described (*12*) except that the cells (*E. coli DH1*/pMAW) were grown in Terrific Broth (*19*) instead of LB. Enzyme activity was measured using the Nash assay (*12*) or a horseradish peroxidase assay with *o*-dianisidine as the chromogenic substrate (*20*). The level of protein was determined using the Bio-Rad micro protein assay (*12*). Enzyme concentrations were also estimated on the basis of flavin absorbance at 454 nm ($\epsilon_{454} = 12\ 200\ M^{-1}\ cm^{-1}$) (*12*). The isolated enzyme exhibited catalytic and spectral properties similar to those reported for the enzyme isolated from cells grown on LB (*12*) (see Table 2). However, the yield of the purified enzyme (3.97 g from 10 L of cell culture) was 10-fold higher.

Generation of a Riboflavin-Dependent Derivative of E. coli BL21(DE3). Chemical mutagenesis of *E. coli* BL21(DE3) was performed by exposing the cells to ethylmethane sulfonate for 15 min, according to the procedure described by Miller (*21*). The mutagenized cells were enriched for riboflavin auxotrophs using the ampicillin enrichment method described by Molholt (*22*). Five riboflavin-dependent colonies [*E. coli* BL21(DE3) M1a−M1e)] were isolated on the basis of their ability to grow on LB only when the medium was supplemented with riboflavin (100 μg/mL). *E. coli* BL21(DE3) M1b cells were selected for expression of the MSOX apoenzyme, as described below.

Construction of Plasmid pGZ26. Plasmid pMAW contains the cloned gene for MSOX from *Bacillus* sp. B-0618 (*12*). PCR was used to introduce an *Nde*I restriction site at the 5′ end of the gene, delete the stop codon, and introduce a *Xho*I restriction site at the 3′ end of the gene. Reactions were performed using plasmid pMAW as a template, 5′-AGGAATCATATGAGCACACATTTTGATG-3′ as the forward primer (*Nde*I site underlined), and 5′-AGACTACTCGAGGATAGTTGTTTTTTGTAACG-3′ as the backward primer (*Xho*I site underlined). PCR was conducted using *Pfu* DNA polymerase and a Hybaid Touchdown Thermocycler with the following cycle settings: 30 cycles of 94 °C for 40 s, 52 °C for 40 s, and 72 °C for 2 min, followed by a single cycle of 72 °C for 30 min. The PCR product was purified

Table 2: Spectral and Catalytic Properties of Native MSOX, ApoMSOX, and the Reconstituted Enzyme[a]

preparation	ϵ_{4XY} (M^{-1} cm^{-1})	A_{280}/A_{4XY}	mol of flavin/ mol of protein	specific activity (units/mg)	$k_{cat(app)}$ (min^{-1})	$K_{m(app)}$ (mM)
native MSOX	12200[b]	5.7 (5.5)[b]	0.80 (0.87)[b]	34.7	2510 ± 10 (2730 ± 32)[c]	5.6 ± 0.1 (4.5 ± 0.1)[c]
apoMSOX	nd	73	nd	1.1	nd	nd
FAD-reconstituted	12300	7.3	0.61	27.0	2330 ± 20	5.2 ± 0.1
8-nor-8-chloroFAD-reconstituted	25600	3.1	0.68	3.0	392 ± 7	13 ± 0.6

[a] Specific activity and apparent steady-state kinetic parameters were determined using a horseradish peroxidase assay with *o*-dianisidine as the chromogenic substrate (*20*). 4XY is 454 nm for FAD-reconstituted, native MSOX and apoMSOX; 4XY is 475 nm for 8-nor-8-chloroFAD-reconstituted MSOX. [b] Values previously reported by Wagner et al. (*12*). [c] Values previously reported by Wagner and Jorns (*20*).

using the QIAquick PCR Purification Kit, cut with *Nde*I and *Xho*I, purified by agarose gel (1.5%) electrophoresis, and recovered using the QIAquick Gel Extraction Kit. The desired *Nde*I−*Xho*I fragment (containing the MSOX gene without its stop codon) was then subcloned between the *Nde*I and *Xho*I sites of plasmid pET24a using T4 DNA ligase. The resulting construct (plasmid pGZ26) places MSOX expression under the control of the T7 promoter and adds eight amino acids [LeuGlu$(His)_6$] to the 3′ end of the recombinant protein. Plasmid pGZ26 was sequenced across the entire MSOX insert. Sequencing was conducted by the Nucleic Acid/Protein Research Core Facility at the Children's Hospital of Philadelphia (Philadelphia, PA). Plasmid pGZ26 was used to transform *E. coli* BL21(DE3) M1b cells to kanamycin resistance.

Expression and Purification of the MSOX Apoenzyme. E. coli BL21(DE3) M1b/pGZ26 cells were grown at 37 °C in LB containing riboflavin (4 μg/mL) and kanamycin (100 μg/mL) until the A_{595} reached 0.9. The cells were then collected by centrifugation and washed twice with riboflavin-free LB. The washed cells were resuspended in riboflavin-free LB at 15 °C. MSOX expression was induced with 1 mM IPTG. Cells were harvested after 3 days at 15 °C.

All steps of the purification were conducted at 4 °C. Cells were disrupted by sonication in the presence of protease inhibitors, as described for the isolation of the MSOX holoenzyme (*12*). The pellet obtained after centrifugation of the cell lysate was discarded. The supernatant was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol and 500 mM NaCl and then mixed with 15 mL of Co^{2+} affinity matrix. After being gently rocked for 45 min, the mixture was poured into a column. The column was washed with dialysis buffer until the eluate exhibited no detectable absorbance at 280 nm. ApoMSOX was then eluted using a 1 L linear gradient from 0 to 150 mM imidazole in dialysis buffer. The enzyme was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) containing 50 mM KCl, concentrated using a Centricon YM-30 ultrafiltration cell, and stored in aliquots at −80 °C. The yield from 3 L of culture was 123 mg. Activity and protein were measured as described for the holoenzyme. The concentration of the apoenzyme was also estimated on the basis of its absorbance at 280 nm ($\epsilon_{280} = 36\,270$ M^{-1} cm^{-1}) (*12*).

Preparation of FAD Analogues. The amount of FAD synthetase required to achieve complete conversion of each riboflavin derivative to the FAD level (3.6−7.1 μg/mL) was determined in preliminary analytical trials under the conditions described below. Reaction progress was monitored by thin-layer chromatography on silica gel plates using *n*-butanol, acetic acid, and water (12:3:5). Riboflavin derivatives (200 μM) were incubated for 18 h at 37 °C with FAD synthetase in 50 mM potassium phosphate buffer (pH 7.6) containing 5 mM ATP and 20 mM magnesium chloride. Preparative-scale (200 mL) reaction mixtures were then filtered through an Amicon PM-10 membrane to remove protein. Except as noted below, the filtrate was applied to a 35 mL Sep-Pak C18 cartridge, previously activated by being washed with methanol and then equilibrated with water. The cartridge was washed with water. Elution conditions varied somewhat, depending on the FAD analogue. 5-DeazaFAD was eluted with 10% acetonitrile in water. 1-DeazaFAD was not eluted by a 1 L linear gradient from 0 to 5% acetonitrile in water. The compound began to slowly leach off the cartridge upon further washing with 5% acetonitrile. 1-DeazaFAD was eluted by switching to 10% acetonitrile in water. The Sep-Pak eluates were concentrated by lyophilization and stored at −20 °C. In the case of 8-nor-8-chloroFAD, the filtrate was applied to a 1 L DEAE-cellulose column previously equilibrated with 5 mM potassium phosphate buffer (pH 6.8). The column was washed successively with 5 and 10 mM potassium phosphate buffers (pH 6.8). 8-Nor-8-chloroFAD was eluted with a 12 L linear gradient from 20 to 75 mM potassium phosphate buffer (pH 6.8). The eluate was applied to a 35 mL Sep-Pak C18 cartridge, previously treated as described above. The cartridge was washed with water. 8-Nor-8-chloroFAD was eluted with methanol, rotoevaporated to dryness, dissolved in water, and stored at −20 °C.

The concentration of stock solutions of the flavin analogues was estimated using the following extinction coefficients: $\epsilon_{448} = 10\,600$ M^{-1} cm^{-1} for 8-nor-8-chloroFAD (*23*), $\epsilon_{399} = 11\,500$ M^{-1} cm^{-1} for 5-deazaFAD (*24*), and $\epsilon_{540} = 6800$ M^{-1} cm^{-1} for 1-deazaFAD (*25*).

Reconstitution of ApoMSOX with FAD or FAD Analogues. Reactions were conducted at 23 °C in 100 mM potassium phosphate buffer (pH 8.0) containing apoMSOX and excess flavin, as indicated in the text. Reactions were initiated by addition of apoMSOX. The kinetics of the reconstitution reaction with FAD were monitored by withdrawing small aliquots at various times to measure sarcosine oxidase activity using a horseradish peroxidase-coupled assay with *o*-dianisidine as the chromogenic substrate (*20*). Reaction progress was also monitored by measuring the extent of hydrogen peroxide formation during reconstitution of apoMSOX with FAD or 8-nor-8-chloroFAD. These reactions were conducted as described above except the reconstitution mixtures also contained horseradish peroxidase (18 units/mL), Amplex Red (80 μM), and dimethyl sulfoxide (0.4%). Hydrogen peroxide formation was monitored at 563 nm ($\Delta\epsilon_{563} = 52\,200$ M^{-1} cm^{-1}). The value for $\Delta\epsilon_{563}$ was determined in studies with known amounts of hydrogen peroxide. Hydrogen peroxide solutions for these studies were standardized using an NADH

peroxidase-coupled assay (*26*). Data obtained for reconstitution reactions were corrected for a small linear reagent blank (ΔA_{563}/min = 3.2×10^{-7}), observed in the presence of all components except apoMSOX. Because of the large spectral change observed upon reaction of apoMSOX with 8-nor-8-chloroFAD, the kinetics of covalent attachment could also be directly monitored at 499 nm, as described in the text.

Isolation and Characterization of the Reconstituted Enzyme. Unbound flavin was removed by ultrafiltration using a Centricon YM-30 concentrator. The absorption spectrum of the reconstituted enzyme was recorded after centrifugation. Extinction coefficients for covalently bound flavins were determined on the basis of absorbance changes observed after denaturation of the reconstituted enzyme with 3 M guanidine hydrochloride. Calculations with the FAD-reconstituted enzyme were made using the extinction coefficient reported for free FAD in the presence of guanidine hydrochloride (ϵ_{450} = 11 900 M^{-1} cm^{-1}) (*12*). The extinction coefficient reported for free 8-nor-8-*S*-(*N*-acetylcysteinyl)-FAD (ϵ_{480} = 25 200 M^{-1} cm^{-1}) (*23*) was used to calculate the extinction coefficient of the 8-nor-8-chloroFAD-reconstituted enzyme. To determine the stoichiometry of flavin incorporation, the protein concentration was estimated on the basis of the absorbance of the denatured protein at 280 nm (ϵ_{280} = 36 270 M^{-1} cm^{-1}) (*12*). The absorbance at 280 nm was corrected for the contribution from free FAD (ϵ_{280} = 22 900 M^{-1} cm^{-1}) (*12*) or free 8-nor-8-*S*-(*N*-acetylcysteinyl)-FAD (ϵ_{280} = 18 900 M^{-1} cm^{-1}) (*27*).

Spectroscopy and Data Analysis. Absorption spectra were recorded using an Agilent Technologies 8453 diode array spectrophotometer or a Perkin-Elmer Lambda 2S spectrometer. Horseradish peroxidase assays using Amplex Red were conducted using only the Perkin-Elmer Lambda 2S spectrometer because the bright light source in the diode array spectrophotometer caused significant photodecomposition of the chromogenic substrate. For anaerobic reconstitution experiments, a solution of apoMSOX in 100 mM potassium phosphate buffer (pH 8.0) containing 50 mM glucose was placed in the main compartment of a special cuvette with two sidearms. The first and second sidearms contained small aliquots of glucose oxidase and FAD or 8-nor-8-chloroFAD, respectively. The cuvette was made anaerobic by multiple cycles of evacuation, followed by filling with oxygen-free argon gas. Identical spectra were observed before and after tipping glucose oxidase (5 units/mL). Reconstitution was initiated by mixing apoMSOX (276 μM) with FAD or 8-nor-8-chloroFAD (120 μM). All spectra are corrected for dilution. Kinetic data were fitted using the curve fit function in Sigma Plot (SPSS Inc.).

RESULTS

Expression of ApoMSOX. A riboflavin-dependent derivative of *E. coli* BL21(DE3) was generated by chemical mutagenesis using ethylmethane sulfonate (*21*), followed by enrichment for riboflavin auxotrophs as described by Molholt (*22*). This strain [BL21(DE3) M1b] does not grow in LB unless the medium is supplemented with riboflavin. We reasoned that apoMSOX might be produced if riboflavin was removed from the growth medium prior to induction of enzyme expression. Native MSOX is typically expressed at 37 °C under the control of the leaky *lac* promoter and purified by a procedure involving several chromatography steps. Huge amounts of pure enzyme are obtained, especially when the cell density is increased by using Terrific Broth in place of LB (400 vs 40 mg of MSOX/L of culture). Two changes were, however, introduced to facilitate apoenzyme expression and isolation: (1) MSOX expression was placed under the control of the T7 promoter to achieve tightly regulated expression, and (2) a carboxyl-terminal affinity tag, $(His)_6$, was incorporated to streamline protein purification.

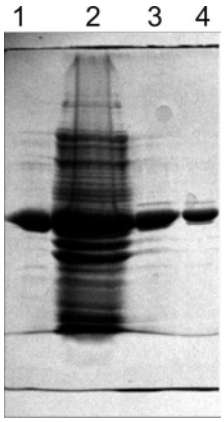


FIGURE 1: Expression of apoMSOX. The SDS−15% polyacrylamide gel was stained for protein with Coomassie blue: lane 1, native MSOX; lane 2, whole cell extract of *E. coli* BL21(DE3) M1b/pGZ26 cells, prepared as described in the text; and lanes 3 and 4, 10 and 5 μg, respectively, of purified apoMSOX.

The riboflavin-dependent strain was transformed with the new expression plasmid (pGZ26) and grown at 37 °C in riboflavin-supplemented LB until a desired cell density was attained (A_{595} = 0.9). The cells were then transferred to unsupplemented LB, and MSOX expression was induced at 15 °C. Extracts from cells grown under these conditions exhibit a prominent 44 kDa protein band that comigrates with native MSOX on SDS−PAGE gels (Figure 1, lanes 2 and 1, respectively). Nearly all of the protein in the whole cell extract was present in the soluble fraction, as judged by SDS−PAGE analysis of supernatant and pellet fractions (data not shown). In contrast, apoenzyme expression at 37 °C generated inclusion bodies that resisted various renaturation attempts.

Isolation of a Reconstitutable Apoenzyme. The soluble 44 kDa protein produced at 15 °C was isolated by using a Co^{2+} affinity matrix. This single-step procedure yields pure protein (Figure 1, lanes 3 and 4) in amounts (40 mg/L of culture) similar to that observed for the isolation of the native enzyme from cells grown in LB. The preparation was predominantly apoMSOX, as judged by the low catalytic activity and minimal absorbance in the visible region, as compared with native enzyme (Figure 2 and Table 2). The residual FAD in the apoenzyme preparation may be due to a small amount of flavin in unsupplemented LB or the cytosol of washed cells. Attempts to eliminate the latter source by incubation of washed cells in unsupplemented LB for 1 or 4 h at 15 °C prior to enzyme induction reduced the yield but did not affect the properties of the isolated apoprotein.

To test for reconstitutability, the apoprotein was incubated with excess FAD (500 μM) at 23 °C and aliquots were removed at various times and assayed for catalytic activity. A time-dependent increase in activity was observed in a

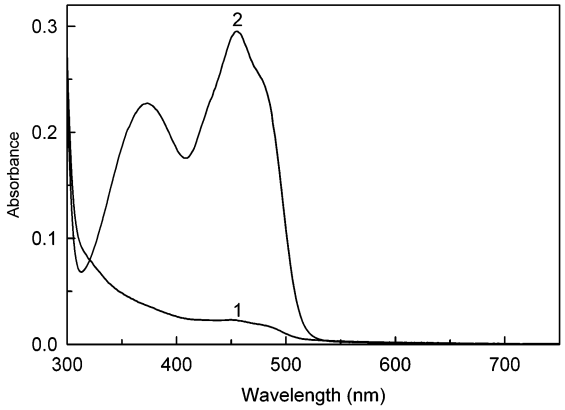


FIGURE 2: Comparison of the visible absorption spectrum of apoMSOX (curve 1) with that observed for the native enzyme (curve 2). Spectra were recorded in 100 mM potassium phosphate buffer (pH 8.0) at 23 °C and are normalized to the same absorbance at 280 nm.

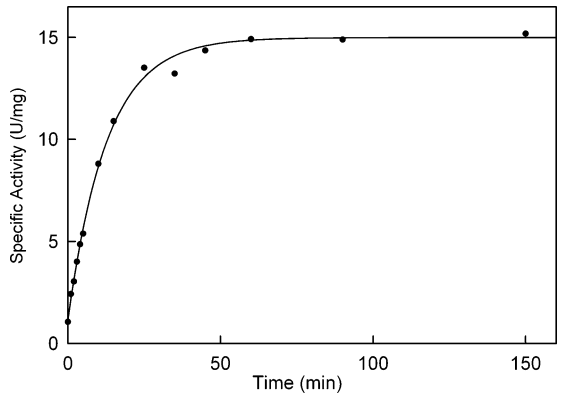


FIGURE 3: Reconstitution of apoMSOX with FAD. The reaction was initiated by adding apoMSOX (11.3 μM) to 100 mM potassium phosphate buffer (pH 8.0) containing 500 μM FAD at 23 °C. Aliquots were withdrawn at the indicated times and assayed for MSOX activity using a horseradish peroxidase assay (*20*). The solid line is a fit of the data (●) to a single-exponential equation, $A = A_o + \Delta A(1 - e^{-kt})$, where A is the observed activity, A_o is the activity at time zero, ΔA is the total increase in activity, and k is the apparent first-order rate constant. Each data point is the average of three duplicate runs.

reaction that exhibits apparent first-order kinetics ($k_{obs} = 0.078 \pm 0.004$ min^{-1}) (Figure 3). The final specific activity attained in this experiment (SA = 15 units/mg) was ~45% of that observed with the native enzyme. Somewhat lower values (~12 units/mL) were obtained after long-term storage. The results show that apoMSOX could be reconstituted with FAD.

Isolation and Properties of FAD-Reconstituted MSOX. A larger-scale reconstitution reaction was conducted. Unbound flavin was removed by ultrafiltration, and the preparation was then centrifuged to remove any insoluble protein. The reconstituted enzyme was isolated in ~50% yield and exhibited a specific activity (27.0 units/mL) that was 78% of that observed for the native enzyme (Table 2). The higher specific activity of the isolated reconstituted enzyme suggests that inactive protein is removed by centrifugation. The absorption spectrum of reconstituted MSOX exhibits maxima at 454, 373, and 275 nm with a pronounced shoulder at 475 nm (Figure 4, curve 1), similar to that observed for the native enzyme.

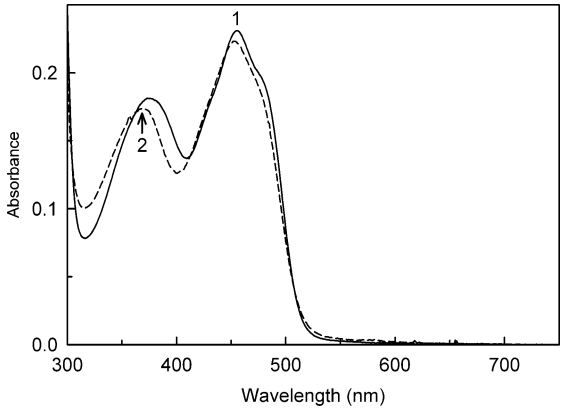


FIGURE 4: Spectral properties of MSOX reconstituted with FAD. Curve 1 is the absorption spectrum of the reconstituted enzyme in 100 mM potassium phosphate buffer (pH 8.0) at 23 °C. Curve 2 was recorded after denaturation with 3 M guanidine hydrochloride.

The reconstituted enzyme was denatured with guanidine hydrochloride to determine whether the flavin was covalently attached to the protein. Denaturation results in a hypsochromic shift of both visible absorption maxima and the loss of the shoulder at 475 nm (Figure 4, curve 2). A colorless filtrate was obtained upon ultrafiltration of the denatured reconstituted enzyme. Both protein and flavin were found in the yellow-colored retentate which exhibited a characteristic flavin absorption spectrum (data not shown). The results show that the FAD in reconstituted MSOX is covalently attached to the protein. The data provide definitive evidence for autoflavinylation in a reaction that requires only the apoenzyme and FAD.

The spectral changes observed after denaturation of reconstituted MSOX were used to estimate the extinction coefficient of the intact reconstituted enzyme at 454 nm. The calculated value ($\epsilon_{454} = 12\,300$ M^{-1} cm^{-1}) is virtually identical to that previously reported for native MSOX (Table 2). The flavin content of the reconstituted enzyme is ~80% of that observed for native MSOX, as judged by values obtained for the A_{280}/A_{454} ratio or the estimated molar ratio of flavin to protein (Table 2). The somewhat lower flavin content in the reconstituted enzyme is consistent with the observed difference in its specific activity as compared with that of native MSOX (Table 2).

Apparent steady-state kinetic parameters for reconstituted MSOX and the native enzyme were determined by monitoring the rate of hydrogen peroxide formation at various sarcosine concentrations in air-saturated buffer. In these studies, the enzyme concentration was estimated on the basis of the absorption at 454 nm to compensate for differences in flavin content. Importantly, reconstituted MSOX exhibits apparent k_{cat} and K_m values that are virtually identical to those observed with the native enzyme (Table 2).

Effect of FAD Concentration on the Rate of Covalent Flavinylation. In addition to the covalent flavin linkage, there are numerous noncovalent interactions between FAD and the protein, as judged by the crystal structure of native MSOX (*10*). The observed noncovalent flavin contacts suggested that apoMSOX might form an initial noncovalent complex with FAD prior to covalent attachment. In this case, the rate of covalent flavinylation should exhibit saturation kinetics with respect to FAD. To evaluate this hypothesis, apoMSOX was incubated with various concentrations of FAD in the

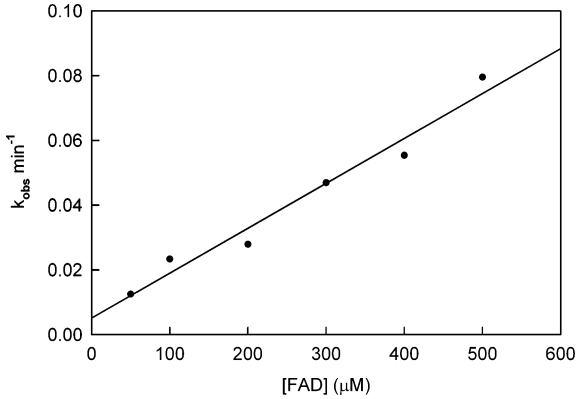


FIGURE 5: Effect of FAD concentration on the rate of reconstitution. The observed rate of reconstitution was estimated on the basis of the increase in catalytic activity, as described in the legend of Figure 3, except that the FAD concentration was varied in the range from 50 to 500 μM.

range from 50 to 500 μM. The rate of reconstitution was measured by monitoring the time-dependent increase in catalytic activity. Contrary to our expectation, the observed apparent first-order rate constants exhibited a linear (r^2 = 0.97) dependence on the FAD concentration (Figure 5). However, the plot does not go through the origin, suggesting that covalent flavin attachment may involve initial formation of an unstable apoMSOX·FAD complex. An apparent second-order rate constant of 139 M^{-1} min^{-1} was estimated from the slope of the plot in Figure 5.

Catalytic activity, albeit at reduced rates, is observed with mutant forms of other enzymes that bind flavin noncovalently when the normal flavin attachment site is eliminated by mutagenesis (*28*−*32*). These studies suggested that addition of FAD to the catalytic assay mixture might stimulate sarcosine oxidation by apoMSOX. FAD concentrations in the range from 25 to 1000 μM were tested. No effect on the initial rate of sarcosine oxidation was detected, consistent with very weak noncovalent binding of FAD to apoMSOX.

Is a Reduced Flavin Intermediate Formed during Reconstitution of ApoMSOX with FAD? Covalent flavin attachment is postulated to involve the formation of a reduced flavin intermediate (Scheme 1, intermediate II). We reasoned that rapid oxidation of the putative reduced flavin intermediate should occur under aerobic conditions, accompanied by the reduction of oxygen to hydrogen peroxide. Control studies showed that the amount of hydrogen peroxide could be measured in the presence of excess FAD by using a horseradish peroxide-coupled assay with Amplex Red as the chromogenic substrate. Oxidation of Amplex Red to resorufin results in a large increase in absorbance at 563 nm ($\Delta\epsilon_{563}$ = 52 200 M^{-1} cm^{-1}), a wavelength where FAD exhibits minimal absorbance.

Significantly, hydrogen peroxide formation was initiated by the addition of apoMSOX to reaction mixtures containing excess FAD, horseradish peroxidase, and Amplex Red, as judged by the time-dependent increase in absorbance at 563 nm. The reaction exhibited biphasic kinetics with 85% of the observed change occurring in the initial fast phase (k_{fast} = 0.142 ± 0.002 min^{-1}), followed by a 20-fold slower phase [k_{slow} = (7.3 ± 0.1) × 10^{-3} min^{-1}]. The slow phase is largely attributable to a blank reaction observed when apoMSOX is added to hydrogen peroxide assay mixtures in the absence

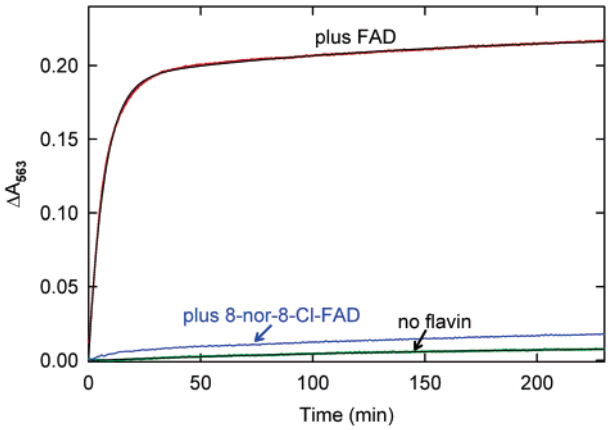


FIGURE 6: Formation of hydrogen peroxide during reconstitution of apoMSOX with FAD or 8-nor-8-chloroFAD. Reactions was initiated by adding apoMSOX (11.3 μM) to 100 mM potassium phosphate buffer (pH 8.0) containing 0 or 500 μM FAD or 500 μM 8-nor-8-chloroFAD, horseradish peroxidase (18 units/mL), Amplex Red (80 μM), and 0.4% dimethyl sulfoxide at 23 °C. Hydrogen peroxide formation was monitored by following the oxidation of Amplex Red at 563 nm. The spectral change observed in the presence of FAD (red line) was fitted to a biphasic exponential equation (black line). The spectral change observed in the absence of FAD (green line) was fitted to a single-exponential equation (black line). The blue line shows the spectral change observed with 8-nor-8-chloroFAD.

of FAD [k = (5.8 ± 0.2) × 10^{-3} min^{-1}] (Figure 6). The fast phase of hydrogen peroxide formation was 1.8-fold faster than the rate of reconstitution, as estimated on the basis of the increase in enzyme activity observed under nearly the same conditions (k = 0.078 ± 0.004 min^{-1} at 500 μM FAD). However, hydrogen peroxide assay mixtures included a small amount of dimethyl sulfoxide (0.4%), the solvent used to prepare stock solutions of Amplex Red. The rate of reconstitution was, therefore, remeasured in the presence of 0.4% dimethyl sulfoxide by monitoring the increase in enzyme activity. The rate observed under these conditions (k = 0.120 ± 0.006 min^{-1}) was in good agreement with the value obtained for the fast phase of hydrogen peroxide formation. The amount of hydrogen peroxide produced in the fast phase (3.6 nmol/mL) was stoichiometric with the amount of reconstituted MSOX formed under the same conditions (3.5 nmol/mL), as estimated on the basis of the increase in catalytic activity.

The spectral course of the reconstitution of apoMSOX with FAD was monitored under anaerobic conditions in an attempt to directly detect the postulated reduced flavin intermediate. A very high concentration of apoMSOX (276 μM) was used in this experiment to achieve an appreciable reaction rate at a moderate FAD concentration (120 μM) that would be compatible with spectral measurements using a cuvette with a light path of 1 cm. Indeed, the anaerobic reaction of apoMSOX with FAD resulted a time-dependent reduction of the flavin (Figure 7). The amount of reduced flavin formed (55.8 μM) was estimated on the basis of the observed decrease in absorbance at 450 nm and the difference in the extinction coefficient between free oxidized FAD and MSOX-bound reduced FAD ($\Delta\epsilon_{450}$ = 10 300 M^{-1} cm^{-1}). This value is ~50% of the value calculated for the amount of reconstituted MSOX (124.6 μM), as estimated on the basis of the increase in catalytic activity. This discrepancy may be due to difficulties in removing oxygen from a very

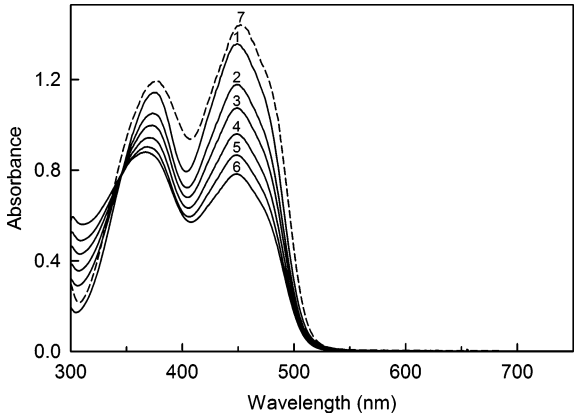


FIGURE 7: Anaerobic reconstitution of apoMSOX with FAD. Curve 1 is the absorption spectrum recorded immediately after mixing 276 μM apoMSOX with 120 μM FAD in 100 mM potassium phosphate buffer (pH 8.0) containing 50 mM glucose and glucose oxidase (0.5 unit/mL) at 23 °C. Curves 2−6 were recorded 2.5, 5.0, 10.0, 20.0, and 144 min, respectively, after mixing. Curve 7 was recorded after the cuvette was opened to air. All spectra are corrected for the residual absorbance of apoMSOX (see curve 1 of Figure 2).

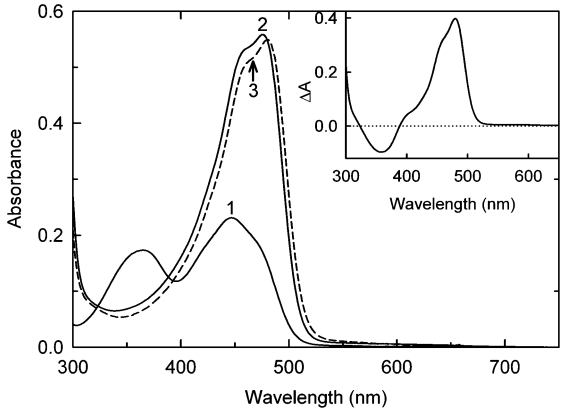


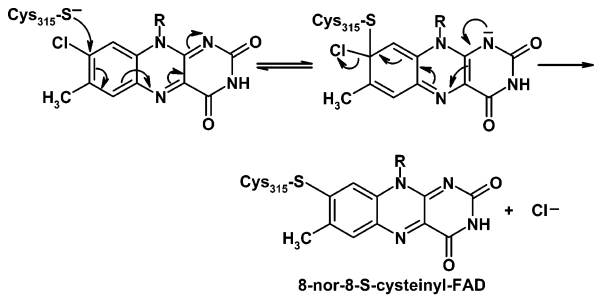
FIGURE 8: Comparison of the spectral properties of free 8-nor-8-chloroFAD (curve 1) with those of 8-nor-8-chloroFAD-reconstituted MSOX (curve 2). Curve 3 was obtained after denaturation of the reconstituted enzyme with 3 M guanidine hydrochloride. Spectra were recorded in 100 mM potassium phosphate buffer (pH 8.0) at 23 °C and are normalized to the same flavin concentration (21.8 μM). The inset shows the difference spectrum obtained by subtracting the spectrum of free 8-nor-8-chloroFAD from that observed for the intact, reconstituted enzyme.

concentrated protein solution that could not be overcome by including glucose and glucose oxidase as an oxygen scavenger. Immediate oxidation of the reduced flavin was observed upon aeration of the sample. Comparison of the initial spectrum (Figure 7, curve 1) with that observed after aeration (Figure 7, curve 7) shows that covalent flavinylation is accompanied by a small bathochromic shift and a small increase in the intensity of the 450 nm absorption band of free FAD. The observed change is consistent with differences in the spectral properties of free versus MSOX-bound FAD (λ_{max} = 450 and 454 nm, respectively; ϵ_{450} = 11 300 M^{-1} cm^{-1}, and ϵ_{454} = 12 200 M^{-1} cm^{-1}). The magnitude of the observed spectral change ($A_{454\ reoxidized} - A_{450\ initial} = 0.082$) is 73% of the value calculated on the basis of the estimated concentration of reconstituted MSOX.

Attempted Reconstitution of ApoMSOX with 5-DeazaFAD or 1-DeazaFAD. 5-DeazaFAD and 1-deazaFAD contain carbon in place of nitrogen at positions 5 and 1 of the flavin ring, respectively (see Table 1 for structures). No evidence for incorporation of 5-deazaFAD or 1-deazaFAD into apoMSOX was obtained even after prolonged incubation (24 h) under conditions [500 μM flavin in 100 mM potassium phosphate (pH 8.0) at 23 °C] where flavinylation is readily observed with FAD. Failure to observe flavinylation with 5-deazaFAD or 1-deazaFAD might reflect the inability of these derivatives to bind noncovalently to apoMSOX. This possibility was investigated by determining whether the analogues could inhibit covalent attachment of FAD. In these experiments, the rate of apoMSOX reconstitution with 100 μM FAD was monitored by measuring the increase in catalytic activity in the absence or presence of 500 μM 5-deazaFAD or 1-deazaFAD. The rates of reconstitution in the presence of 5-deazaFAD and 1-deazaFAD were 45 and 62%, respectively, of that observed with FAD alone. The observed competititon clearly indicates that the analogues can bind noncovalently to apoMSOX.

Reconstitution of ApoMSOX with 8-Nor-8-chloroFAD. The 8α-methyl group of FAD is replaced with a chlorine atom in 8-nor-8-chloroFAD (see Table 1 for the structure). This

Scheme 2: Covalent Attachment of 8-Nor-8-chloroFAD to MSOX via a Mechanism Involving Nucleophilic Aromatic Substitution



FAD analogue exhibits a typical flavin absorption spectrum with two maxima in the visible region (λ_{max} = 448 and 365 nm) (Figure 8, curve 1). Dramatically different spectral properties are, however, observed after reconstitution of apoMSOX with 8-nor-8-chloroFAD. The reconstituted enzyme exhibited a single maximum in the visible region at 475 nm (Figure 8, curve 2) with a shoulder around 460 nm. Denaturation with guanidine hydrochloride shifted the absorption maximun to 480 nm, resulting in a more pronounced shoulder at 460 nm (Figure 8, curve 3). The absorption spectrum of the denatured enzyme is virtually identical to that reported for 8-nor-8-*S*-(*N*-acetylcysteinyl)-FAD (*23*) (see Table 1 for the structure). The results suggested that flavinylation of the apoprotein had occurred in a reaction involving nucleophilic displacement of the 8-chloro substituent with a cysteine residue (Scheme 2). Consistent with this hypothesis, a colorless filtrate was obtained after ultrafiltration of the denatured enzyme. The 480 nm-absorbing chromophore was found in the retentate, along with the MSOX protein. Unlike flavinylation with FAD, the postulated nucleophilic displacment reaction with 8-nor-8-chloroFAD is not expected to generate a reduced flavin intermediate. Indeed, little or no hydrogen peroxide was detected during reconstitution of apoMSOX with 8-nor-8-chloroFAD (Figure 6). Furthermore, flavin reduction was not observed when the reaction with 8-nor-8-chloroFAD was

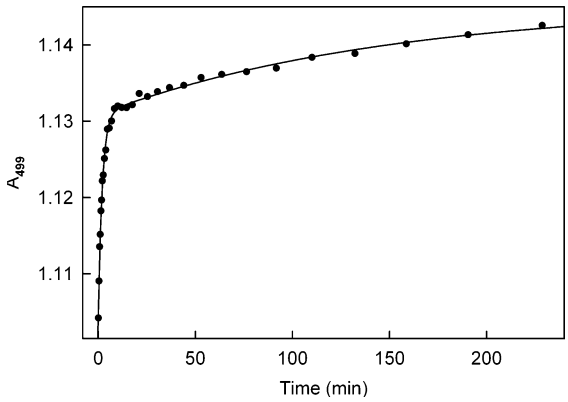


FIGURE 9: Kinetics of the reaction of apoMSOX with 8-nor-8-chloroFAD. The reaction was initiated by adding apoMSOX (11.3 μM) to 100 mM potassium phosphate buffer (pH 8.0) containing 500 μM 8-nor-8-chloroFAD at 23 °C and monitored at 499 nm. The solid line is a fit of the data (●) to a biphasic exponential equation.

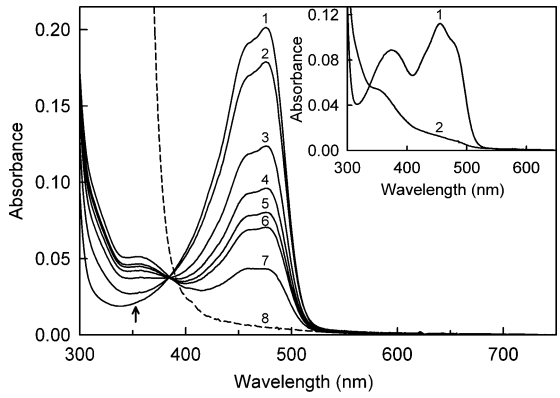


FIGURE 10: Reduction of reconstituted enzymes with sarcosine. Curve 1 is the absorption spectrum of the enzyme reconstituted with 8-nor-8-chloroFAD in 100 mM potassium phosphate buffer (pH 8.0) at 23 °C. Curves 2−7 were recorded 1, 2, 3, 4, 5, and 20 min, respectively after addition of 500 mM sarcosine to the aerobic sample in a tightly stoppered cuvette. No additional change was observed upon further incubation. (The arrow indicates the direction of absorbance change at wavelengths below 384 nm, the isosbestic point of the reaction.) Curve 8 was recorded immediately after adding solid dithionite. The inset shows absorption spectra obtained with the FAD-reconstituted enzyme under the same conditions. Curves 1 and 2 were recorded before and immediately after, respectively, adding 500 mM sarcosine. No further decrease in the 454 nm band was observed upon addition of solid dithionite (data not shown).

conducted under anaerobic conditions. The results provide strong evidence for the formation of an 8-nor-8-*S*-thioflavin linkage.

The absorbance change observed upon denaturation and the spectral properties reported for free 8-nor-8-*S*-(*N*-acetylcysteinyl)-FAD were used to calculate an extinction coefficient for the intact reconstituted enzyme at 475 nm (ϵ_{475} = 25 600 M^{-1} cm^{-1}). The enzyme reconstituted with 8-nor-8-chloroFAD exhibited a somewhat higher flavin-to-protein ratio than the FAD-reconstituted enzyme but was considerably less active as a catalyst (Table 2). The specific activity and apparent k_{cat} for sarcosine oxidation with the 8-nor-8-chloroFAD-reconstituted enzyme are ~1 order of magnitude lower and the apparent K_m for sarcosine is more than 2-fold larger than those observed with the native enzyme.

Kinetics of the Reaction of ApoMSOX with 8-Nor-8-chloroFAD. Formation of the 8-nor-8-*S*-thioflavin linkage is accompanied by a large spectral change. This suggested that it might be possible to directly monitor the kinetics of the flavinylation reaction at an appropriate wavelength, despite the presence of excess 8-nor-8-chloroFAD. The calculated difference spectrum for the reaction exhibits a maximum at 480 nm and a less intense minimum at 358 nm (Figure 8, inset). A wavelength as close as possible to the difference maximum at 480 nm was selected (λ = 499 nm) such that the initial absorbance due to 8-nor-8-chloroFAD (500 μM) was ~1. The flavinylation reaction, monitored at 499 nm, exhibited biphasic kinetics with 65% of the absorbance increase occurring in a fast initial phase (k_{fast} = 0.48 ± 0.02 min^{-1}), followed by a 70-fold slower phase [k_{slow} = (6.8 ± 1.4) × 10^{-3} min^{-1}] (Figure 9). The fast phase is ~6-fold faster than the rate of flavinylation observed with FAD at the same concentration.

MSOX contains three cysteine residues (Cys315, Cys152, and Cys262). Cys315 is the site of covalent flavin attachment in native MSOX (*12*) and the only cysteine residue at the active site (*10*). Cys315 is likely to be the most reactive cysteine in apoMSOX and responsible for the fast phase of the reaction observed with 8-nor-8-chloroFAD. Unlike FAD, free 8-nor-8-chloroFAD is known to react with thiols (*33*). This suggested that a non-active site cysteine residue might account for the slow phase of the MSOX reaction with 8-nor-8-chloroFAD. This hypothesis predicts that a portion of the 8-nor-8-*S*-cysteinyl-FAD in the reconstituted enzyme would not be reducible by substrate. To test this hypothesis, an aerobic sample of the reconstituted enzyme (0.27 mM oxygen) was mixed with a large excess of sarcosine (500 mM) in a stoppered cuvette. An isosbestic reduction of the covalently bound flavin was observed in a reaction that was complete within 20 min. However, further reduction occurred immediately after addition of solid dithionite (Figure 10). Reaction of FAD-reconstituted enzyme (Figure 10, inset) or native MSOX (data not shown) with sarcosine under the same conditions resulted in an immediate 89 or 90% bleaching, respectively, of the absorbance due to the oxidized enzyme at 454 nm. No further decrease at 454 nm was observed upon incubation or after addition of solid dithionite. The results show that all of the flavin in FAD-reconstituted and native MSOX can be reduced by substrate. In contrast, ~15% of the flavin in the 8-nor-8-chloroFAD-reconstituted enzyme cannot be reduced by sarcosine, as judged by the difference in absorbance at 475 nm observed with the substrate- versus dithionite-reduced enzyme.

DISCUSSION

Biosynthesis of a reconstitutable apoenzyme form of MSOX with a C-terminal affinity tag has been achieved by tightly regulated expression using a riboflavin-dependent strain of *E. coli* as the host cell. The transformed cells are grown to a desired density at 37 °C in LB medium supplemented with riboflavin, harvested, washed, and then transferred to unsupplemented LB at 15 °C for induction of MSOX expression. Large amounts of the soluble apoprotein are produced under these conditions (40 mg/L of culture), whereas inclusion bodies are formed at higher induction temperatures. The pure protein, isolated by a single affinity chromatography step, is predominantly apoMSOX, as judged by its low residual catalytic activity and visible absorbance.

A time-dependent increase in catalytic activity is observed upon incubation of the apoenzyme with FAD, accompanied by the covalent incorporation of FAD to ~80% of the level observed with the native enzyme. Except for a somewhat lower flavin content, the spectral and catalytic properties of the reconstituted enzyme are virtually indistinguishable from those observed with native MSOX. The results provide definitive evidence for autoflavinylation in a reaction that requires *only* apoMSOX and FAD. Surprisingly, the observed rate of flavinylation exhibits a linear, rather than an anticipated hyperbolic, dependence on the concentration of FAD ($k = 139\ M^{-1}\ min^{-1}$ at 23 °C). However, the plot does not intersect the origin, as would be expected for a second-order reaction, suggesting that covalent attachment may involve prior formation of an unstable noncovalent E·FAD complex. In contrast, the flavin-binding subunit from *p*-cresol methylhydroxylase exhibits only noncovalent binding with FAD (*34*). Covalent attachment of FAD is triggered by a chaperone-like action of the heme-binding subunit that induces a conformational change in the flavin-binding subunit (*13*).

Strong evidence for a reduced flavin intermediate in the flavinylation reaction (Scheme 1, intermediate II) is provided by the similar reconstitution rates observed by measuring hydrogen peroxide formation or the increase in sarcosine oxidase activity, by the observed stoichiometric production of hydrogen peroxide and covalently bound FAD, and by the time-dependent reduction of FAD observed when reconstitution is conducted under anaerobic conditions. Since the reaction is facilitated by the apoprotein, flavinylation of apoMSOX can be regarded as an autocatalytic process. Of course, the apoprotein is not a true catalyst since it is modified during the reaction, analogous to single-turnover proteins such as O^6-methylguanine-DNA alkyltransferases that repair alkylated DNA by transferring the alkyl group to one of their own cysteine residues (35). It is nevertheless interesting that apoMSOX flavinylation and MSOX-catalyzed amine oxidation both involve formation of a reduced flavin intermediate that reacts rapidly with oxygen to generate oxidized flavin and hydrogen peroxide.

Further insight into the mechanism of covalent flavin attachment was sought in studies with FAD analogues. Flavinylation is not observed with 5-deazaFAD or 1-deazaFAD, even after prolonged incubation with apoMSOX, although the analogues do bind noncovalently to apoMSOX as judged by their ability to inhibit flavinylation with FAD. The same outcome was reported for 5-deazaFAD in *in vitro* studies with apo-*p*-cresol methylhydroxylase (*36*) and apo-6-hydroxy-D-nicotine oxidase (*14*). Similarly, no flavin incorporation was observed when monoamine oxidase (A or B) was expressed in a riboflavin-dependent yeast strain grown in the presence of 5-deazariboflavin (*16*). Reduced 5-deazaflavins exhibit greatly diminished reactivity with oxygen as compared with unmodified flavins. It has been suggested that the failure to observe covalent attachment of 5-deazaFAD to monoamine oxidase can be attributed to the inability of the reduced flavin intermediate to be reoxidized (*16*). This hypothesis cannot account for the results obtained with apoMSOX and 1-deazaFAD since reduced 1-deazaflavins exhibit high reactivity with oxygen, similar to that observed with unmodified flavin. 5-Deazaflavins and 1-deazaflavins do, however, have one property in common. Both substitutions result in a large decrease in redox potential as compared with that of unmodified flavin ($\Delta E_{m,7} \sim -80$ mV; see Table 1). Proton abstraction from the 8α-methyl group is required for formation of the iminoquinone methide intermediate (Scheme 1, intermediate I) in the mechanism postulated for covalent flavin attachment. The acidity of the 8α-methyl group protons is enhanced by electron delocalization from the 8α-CH_2^- carbanion into the flavin ring. This property can account for the *selective* exchange of the 8α-methyl group protons with solvent, as reported for free FMN in D_2O (*37*). Since 5-deazaflavins and 1-deazaflavins are much poorer electron acceptors, these substitutions are likely to decrease the acidity of the 8α-methyl group protons and therefore interfere with covalent flavin attachment. Consistent with this hypothesis, an enhanced rate of flavinylation of apoMSOX is observed when FAD is replaced by an analogue with a higher redox potential.[2]

The 8α-methyl group in FAD is replaced by a chlorine atom in 8-nor-8-chloroFAD, a substitution that blocks the normal mode of flavin attachment. Covalent flavin attachment is, however, observed in a reaction thought to involve displacement of the 8-chloro substitutent by a cysteine thiolate (Scheme 2), similar to the reaction observed with free 8-nor-8-chloroflavin and various thiolates (*33*). The aromatic nucleophilic displacement reaction proceeds via a quininoid intermediate but does not involve a reduced flavin intermediate, unlike the flavinylation reaction with FAD. Formation of the postulated 8-nor-8-S-thioflavin linkage in the apoMSOX reaction with 8-nor-8-chloroFAD is strongly supported by the dramatic change in the absorption spectrum of the flavin analogue, the absence of hydrogen peroxide formation, and the similar spectral properties observed for reconstituted MSOX and 8-nor-8-*S*-(*N*-acetylcysteinyl)-FAD. The covalent flavins in the 8-nor-8-chloroFAD-reconstituted enzyme and native MSOX are likely to exhibit similar redox potentials, as judged by data reported for the corresponding riboflavin derivatives (8-nor-8-*S*-cysteinyl- and 8α-*S*-cysteinyl-riboflavin, respectively; see Table 1). The ~10-fold lower catalytic activity observed with the 8-nor-8-chloroFAD-reconstituted enzyme may reflect a structural perturbation needed to accommodate the modified Cys315−flavin linkage at the active site.

Flavinylation of apoMSOX with 8-nor-8-chloroFAD occurs mainly at Cys315 but also involves a secondary reaction with a 70-fold less reactive, non-active site cysteine (Cys262 or Cys152). Evidence for a secondary reaction is provided by the observed biphasic reconstitution kinetics and the presence of flavin in the reconstituted enzyme that cannot be reduced by sarcosine. The fast phase of the reconstitution reaction is attributed to Cys315. The slow phase is probably due to Cys262 since this residue is partially accessible to solvent, whereas Cys152 is completely buried. The reconstituted enzyme contains only slightly more flavin than the enzyme reconstituted with FAD (0.68 and 0.61 mol of flavin/mol of protein, respectively). We estimate that 85% of the flavin in the enzyme reconstituted with 8-nor-8-chloroFAD can be reduced by substrate, as judged by the extent of bleaching of the flavin absorbance at 475 nm observed with the sarcosine- or dithionite-reduced enzyme. The fast phase

[2] A. Hassan-Abdallah, R. C. Bruckner, G. Zhao, and M. S. Jorns, unpublished results.

of the reconstitution reaction, however, accounts for 65% of the spectral change observed at 499 nm. A couple of factors might account for the modest discrepancy in these values. First, flavinylation at the active site may generate a more stable protein that is isolated in higher yield than protein flavinylated at a secondary site. Alternatively, covalently bound flavin at the active site and the secondary site may exhibit significantly different extinction coefficients at 475 and 499 nm. Indeed, model studies show that the spectral properties of 8-nor-8-*S*-alkylthio-flavins are extremely sensitive to solvent polarity (*27*).

Covalent attachment to the native cysteinyl flavin attachment site in monoamine oxidase A is observed when the protein is expressed in a riboflavin-dependent yeast strain grown in the presence of 8-nor-8-chlororiboflavin (*16*). In contrast, covalent flavin attachment of 8-nor-8-chloroFAD is not observed with apoenzymes in which tyrosyl or histidyl residues serve as the normal flavin attachment site, as judged by results obtained with apo-*p*-cresol methylhydroxylase (*36*) or apo-6-hydroxy-D-nicotine oxidase (*14*), respectively. Significantly, apoenzyme flavinylation is observed with 8-nor-8-chloroFAD when the native histidyl attachment site in 6-hydroxy-D-nicotine oxidase is mutated to cysteine (*38*). The results suggest that a more potent thiolate nucleophile is required for the nucleophilic aromatic substitution reactions with chloride as the leaving group as compared with nucleophilic addition to the iminoquinone methide intermediate in the normal flavinylation pathway.

A diverse and expanding group of enzymes, including numerous drug targets, have been found to contain covalently bound flavin. Experimental support for postulated intermediates and a detailed understanding of the role of the protein moiety in covalent flavin biosynthesis have been limited by the lack of a robust system for *in vitro* studies. This obstacle has been overcome by the development of an expression system that allows for the isolation of substantial amounts of a reconstitutable apoenzyme. Further study of the autocatalytic flavinylation reaction observed with apoMSOX may serve as a paradigm for other members of this important category of enzymes.

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