Role of the Covalent Flavin Linkage in Monomeric Sarcosine Oxidase†

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ABSTRACT: Monomeric sarcosine oxidase (MSOX) is a prototypical member of a recently recognized family of amine-oxidizing enzymes that all contain covalently bound flavin. Mutation of the covalent flavin attachment site in MSOX produces a catalytically inactive apoprotein (apoCys315Ala) that forms an unstable complex with FAD ($K_d = 100\ \mu M$), similar to that observed with wild-type apoMSOX where the complex is formed as an intermediate during covalent flavin attachment. In situ reconstitution of sarcosine oxidase activity is achieved by assaying apoCys315Ala in the presence of FAD or 8-nor-8-chloroFAD, an analogue with an ∼55 mV higher reduction potential. After correction for an estimated 65% reconstitutable apoprotein, the specific activity of apoCys315Ala in the presence of excess FAD or 8-nor-8-chloroFAD is 14% or 80%, respectively, of that observed with wild-type MSOX. Unlike oxidized flavin, apoCys315Ala exhibits a high affinity for reduced flavin, as judged by results obtained with reduced 5-deazaFAD (5-deazaFADH$_2$) where the estimated binding stoichiometry is unaffected by dialysis. The Cys315Ala·5-deazaFADH$_2$ complex is also air-stable but is readily oxidized by sarcosine imine, a reaction accompanied by release of weakly bound oxidized 5-deazaFAD. The dramatic difference in the binding affinity of apoCys315Ala for oxidized and reduced flavin indicates that the protein environment must induce a sizable increase in the reduction potential of noncovalently bound flavin ($\Delta E_m \sim 120$ mV). The covalent flavin linkage prevents loss of weakly bound oxidized FAD and also modulates the flavin reduction potential in conjunction with the protein environment.

Two broad classes of flavoenzymes can be distinguished on the basis of the mode of flavin binding. More commonly, the prosthetic group is bound noncovalently. However, an ever-expanding number of enzymes are being discovered that contain covalently bound flavin. These enzymes exhibit a wide range of biological functions, including the nonribosomal biosynthesis of peptidyl antibiotics, ion pumping, and metabolism of biogenic amines (*1*−*3*). Monomeric sarcosine oxidase (MSOX)[1] is arguably the best characterized member of a family of enzymes that all contain covalently bound flavin and catalyze oxidation reactions with various amine substrates (*4*−*9*). MSOX is an inducible enzyme found in many bacteria where it plays an important role in the catabolism of sarcosine (*N*-methylglycine), a common soil metabolite that can act as sole source of carbon and energy for many microorganisms (*10*). The enzyme catalyzes the oxygen-dependent conversion of sarcosine to glycine, formaldehyde, and hydrogen peroxide. MSOX is a key component in a creatinine biosensor used in the evaluation of renal function and muscle damage (*11, 12*).

High-resolution crystal structures are available for free MSOX from *Bacillus* sp. B-0618 and complexes of the enzyme with various inhibitors (*4*, *13*−*15*). MSOX is a two-domain, 44 kDa protein. The enzyme contains 1 mol of FAD, attached to the protein via a thioether linkage between the 8α-methyl group of the isoalloxazine ring and the sulfur of Cys315 [8α-(*S*-cysteinyl)FAD] (*16*). FAD is bound in an extended conformation with its ADP moiety bound to the flavin domain. The isoalloxazine ring of FAD is bound at the bottom of a cleft between the flavin and catalytic domains. In addition to the covalent linkage, there are numerous hydrogen-bonding and electrostatic interactions between FAD and the protein.

Biosynthesis of an apoprotein form of MSOX was recently achieved by tightly regulated expression of the wild-type enzyme using a riboflavin-dependent strain of *Escherichia coli* as host cell (*17*). The isolated apoprotein could be reconstituted with FAD in a reaction that proceeded via a reduced flavin intermediate and resulted in the covalent incorporation of FAD, accompanied by the formation of a stoichiometric amount of hydrogen peroxide. The spectral and catalytic properties of the reconstituted enzyme were virtually indistinguishable from those observed with native enzyme. These studies show that the covalent flavin linkage is formed in an autocatalytic reaction that requires only apoMSOX and FAD.

Little is known about the role of the covalent linkage in MSOX catalysis. 8α-*S*-Cysteinyl- and other aminoacyl flavin derivatives exhibit reduction potentials that are 30−50 mV higher than unmodified flavin (*18*−*21*), suggesting that the covalent flavin linkage might play a role in modulating the

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

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redox properties of MSOX. Direct evidence for such a function has been obtained for *p*-cresol methylhydroxylase (*22*), cholesterol oxidase (*23*), and vanillyl-alcohol oxidase (*24*). A role in redox modulation is also consistent with results obtained with monoamine oxidase A (*25*) and fumarate reductase (*26*). In contrast, a major role in raising the flavin reduction potential is assigned to the protein environment in trimethylamine dehydrogenase where the covalent flavin linkage is thought to function primarily in substrate binding and protecting the enzyme against inactivation during turnover (*27*). In monoamine oxidase A the covalent flavin linkage is also found to be important for protein stability (*25*). An additional function has been observed in multisubunit proteins such as *p*-cresol methylhydroxylase (*22*) and heterotetrameric sarcosine oxidase (*9*), where the covalent flavin linkage promotes tight association of the subunits. In this paper, we address the role of the covalent flavin linkage in MSOX from *Bacillus* sp. B-0618 in studies with a mutant form where covalent attachment cannot occur. The results provide insight regarding the factors that modulate the redox properties of the enzyme and evidence for a novel function of the covalent flavin linkage.

EXPERIMENTAL PROCEDURES

Materials. Horseradish peroxidase and *o*-dianisidine were purchased from Sigma. 8-Nor-8-chlororiboflavin was a generous gift from Dr. John Lambooy. 5-Deazariboflavin was previously synthesized in this laboratory according to the method of O'Brien et al. (*28*). Recombinant FAD synthetase from *Corynebacterium ammoniagenes* was kindly provided by Dr. Bill McIntire. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. The Co^{2+} affinity matrix was obtained from BD Bioscience.

Expression and Purification of Wild-Type Holoenzyme and Apoenzyme. Wild-type holoenzyme and apoenzyme were expressed and purified as previously described (*16*, *17*). Enzyme activity was measured at 25 °C using a horseradish peroxidase coupled assay with *o*-dianisidine as the chromogenic substrate, as previously described (*5*), except that the assay buffer was changed to 50 mM Tris-HCl, pH 8.0, unless otherwise indicated. Protein was determined using the Bio-Rad microprotein assay (*16*). The concentration of holoenzyme or apoenzyme was also estimated on the basis of flavin absorbance at 454 nm ($\epsilon_{454} = 12200\ M^{-1}\ cm^{-1}$) or protein absorbance at 280 nm ($\epsilon_{280} = 36270\ M^{-1}\ cm^{-1}$), respectively (*16*).

Mutation of Cys315 to Ala or Ser and Introduction of a C-Terminal His Tag. All PCR reactions were conducted using a Hybaid Touchdown thermocycler. PCR products and other DNA fragments were purified by agarose gel (1.5%) electrophoresis and recovered using a GeneClean column purification kit (Bio 101 Inc.) or a QIAQuick gel extraction kit (Qiagen). Sequencing was conducted by the Nucleic Acid/Protein Research Core Facility (NAPCORE) at the Children's Hospital of Philadelphia.

Mutations were generated by using the plasmid pMAW (*16*) as template and the overlap extension PCR method described by Ho et al. (*29*). PCR reactions to produce the left- and right-hand fragments were performed by using *Pfu* DNA polymerase (Stratagene) and the following settings: 30 cycles of 94 °C for 30 s and 53 °C for 30 s, followed by 2 min at 72 °C. The same conditions were used in the PCR reaction to join the left- and right-hand fragments for the Cys315Ser mutant, except for the inclusion of a 30 s preincubation step at 94 °C. The PCR reaction to join the left- and right-hand fragments for the Cys315Ala mutant was performed by using an Advantage-GC cDNA PCR kit (Clontech) and the following settings: preincubation for 1 min at 94 °C; 25 cycles of 94 °C for 30 s, 68 °C for 3 min; 68 °C for 3 min. The left-hand fragments were generated using 5′-ATGACCATGATTACGCCAAGC-3′ (start) as forward primer and 5′-TTCGTGTACATGgcGACTGC-3′ (Cys315Ala) or 5′-TTCGTGTACATGCtGACTGC-3′ (Cys315Ser) as backward primer. The right-hand fragments were generated using 5′-GCAGTCgcCATGTACACGAA-3′ (Cys315Ala) or 5′-GCAGTCaGCATGTACACGAA-3′ (Cys315Ser) as forward primer and 5′-TTAAGTTGGGTAACGCCAGG-3′ (end) as backward primer. The left- and right-hand fragments were combined using start and end as forward and backward primers, respectively. (Mutagenic sites in the primers are shown in lower case; codon 315 is underlined.) The two final PCR products were digested with *Eco*RI and *Pst*I, purified, and subcloned between the *Eco*RI and *Pst*I sites of plasmid pUC119. The resulting constructs were used to transform *E. coli* DH1 cells to ampicillin resistance. For screening, plasmid DNA was isolated from randomly selected clones using the QiaPrep spin miniprep kit (Qiagen) and digested with *Eco*RI and *Pst*I. For each mutation, a plasmid (pUSer, pUAla) that exhibited the expected insert size was isolated using the Qiagen plasmid midi kit (Qiagen) and sequenced across the entire insert.

Preliminary studies indicated that neither mutant protein exhibited catalytic activity. To facilitate isolation, vectors were constructed for expression of the mutant proteins with a carboxyl-terminal $(His)_6$ tag. PCR was used to introduce a *Nde*I restriction site at the 5′ end of the mutant genes, delete the stop codon, and introduce a *Xho*I restriction site at the 3′ end of the mutant genes. Reactions were performed using plasmid pUSer or pUAla as template, 5′-CATATGAGCACACATTTTGATGTCATC-3′ (*Nde*I site is underlined) as forward primer, 5′-CTCGAGGATAGTTGTTTTTTGTAACG-3′ as backward primer (*Xho*I site is underlined), and *Taq* DNA polymerase (Qiagen) with the following cycle settings: preincubation at 94 °C for 2 min; 30 cycles of 94 °C for 40 s, 52 °C for 40 s, 72 °C for 2 min; 72 °C for 30 min. The PCR products were purified and inserted into the TA cloning vector pCR2.1 (Invitrogen). The resulting intermediate plasmids were used to transform competent *E. coli* cells (INVαF′) (Invitrogen). The transformed cells were screened by growth on plates containing kanamycin and X-gal. The intermediate plasmids were then isolated and digested with *Nde*I and *Xho*I. *Nde*I−*Xho*I restriction fragments containing the desired mutant genes were then subcloned between the *Nde*I and *Xho*I sites of plasmid pET23a (Novagen). The newly formed plasmids, pGZ02 (Cys315Ser) and pGZ07 (Cys315Ala), were used to transform *E. coli* BL21(DE3) cells to ampicillin resistance, screened, and then sequenced across the entire insert.

Expression and Purification of Cys315Ala MSOX. *E. coli* BL21(DE3)/pGZ07 cells were grown at 25 °C in LB medium containing 100 μg/mL ampicillin until the A_{595} reached 0.6.

Cys315Ala expression was then induced with 0.5 mM IPTG. Cells were harvested approximately 12 h after induction. 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 wild-type holoenzyme (*16*). The supernatant obtained after centrifugation was dialyzed against 50 mM potassium phosphate, pH 8.0, containing 500 mM sodium chloride and 20% glycerol (buffer A). The lysate was mixed with 20 mL of Co^{2+} affinity matrix. After being gently rocked for 45 min, the mixture was poured into a column containing a bed formed from 5 mL of the Co^{2+} affinity matrix. The column was washed with buffer A until the eluate exhibited no detectable absorbance at 280 nm. The Cys315Ala mutant protein was then eluted using a 1 L linear gradient from 20 to 150 mM imidazole in buffer A. Enzyme activity was assayed as described above for wild-type MSOX except for the inclusion of FAD, as detailed in Results. Protein concentration was determined using the Bio-Rad microprotein assay (*16*). The concentration of apoenzyme was also estimated on the basis of its absorbance at 280 nm (ϵ_{280} = 36270 M^{-1} cm^{-1}) (*16*).

Preparation of FAD Analogues. 8-Nor-8-chlororiboflavin and 5-deazariboflavin were converted to the corresponding FAD derivatives using FAD synthetase, similar to that previously described (*17*). The concentration of stock solutions of the flavin analogues was estimated using the following extinction coefficients: 8-nor-8-chloroFAD, ϵ_{448} = 10600 M^{-1} cm^{-1} (*30*); 5-deazaFAD, ϵ_{399} = 11500 M^{-1} cm^{-1} (*31*).

Reconstitution of Wild-Type Apoenzyme with FAD. Reactions were conducted at 23 °C in 50 mM Tris-HCl, pH 8.0, containing apoMSOX and various amounts of FAD, as indicated. Reactions were initiated by addition of apoMSOX. The kinetics of the reconstitution reaction were monitored by withdrawing small aliquots at various times to measure sarcosine oxidase activity.

Inhibition of Covalent Flavinylation of Wild-Type Apoenzyme by Oxidized 5-DeazaFAD. Reactions were conducted as described above in the presence of 0, 137.3, or 228.8 μM 5-deazaFAD. A value for the inhibition constant (K_i) was obtained by fitting the following equation to the data: $k_{obs} = k_{lim}[FAD]/([FAD] + K_d(1 + [5\text{-deazaFAD}]/K_i))$, where k_{lim} is the limiting rate of covalent flavinylation and K_d is the dissociation constant of the noncovalent apoenzyme complex with FAD.

Reconstitution of ApoCys315Ala with Reduced 5-DeazaFAD. Reduced 5-deazaFAD was prepared by reduction with sodium borohydride, similar to that previously described (*31*). ApoCys315Ala (145 μM) was incubated with reduced 5-deazaFAD (185 μM) for 30 min at room temperature in anaerobic 7.4 mM potassium phosphate buffer, pH 7.5, containing 37 mM potassium chloride. The sample was then dialyzed overnight against a 300-fold excess of 50 mM Tris-HCl, pH 8.0, at 4 °C.

Spectroscopy. Absorption spectra were recorded using an Agilent Technologies 8453 diode array spectrophotometer. To determine the stoichiometry of flavin incorporation, apoCys315Ala was reconstituted with reduced 5-deazaFAD as described above, oxidized by reaction with sarcosine imine as described in Results, and then denatured with 3.0 M guanidine hydrochloride. The concentration of oxidized 5-deazaFAD was estimated by using an extinction coefficient reported for the free flavin (ϵ_{399} = 11500 M^{-1} cm^{-1}) (*31*). Protein concentration was determined on the basis of the absorbance observed for the denatured enzyme at 280 nm (ϵ_{280} = 36270 M^{-1} cm^{-1}) (*16*). The absorbance at 280 nm was corrected for the contribution due to free 5-deazaFAD (ϵ_{280} = 22600 M^{-1} cm^{-1}). The stoichiometry of 5-deazaFADH_2 formation in the presence of apoCys321Ala, 5-deazaFAD, and sarcosine was estimated on the basis of the observed decrease in absorbance at 401 nm using the extinction coefficient of oxidized 5-deazaFAD since the reduced form does not absorb at this wavelength (*31*).

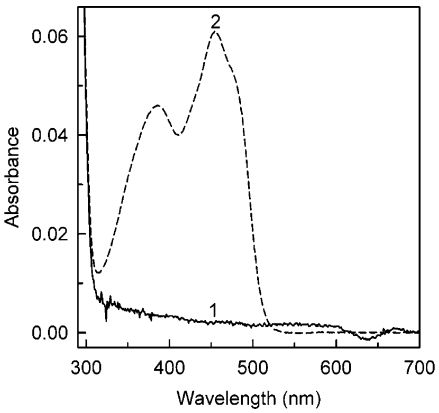


FIGURE 1: Comparison of the visible absorption spectrum of the Cys315Ala mutant (curve 1) with that observed for wild-type MSOX (curve 2). Spectra were recorded in 10 mM potassium phosphate buffer, pH 7.5, containing 50 mM potassium chloride at 25 °C and are normalized to the same absorbance at 280 nm.

RESULTS

Mutation of Cys315 and General Properties of the Cys315Ala Mutant. Vectors for expression of Cys315Ala or Cys315Ser mutant proteins containing a carboxyl-terminal $(His)_6$ tag were constructed to facilitate isolation since preliminary studies indicated that both mutant proteins were catalytically inactive. A 44 kDa band, that comigrated with native MSOX on SDS−PAGE, was detected in soluble cell extracts from cells expressing the His-tagged Cys315Ala or Cys315Ser mutant protein. The band was observed when expression was induced at 25 °C but not at 37 °C, the temperature typically used to induce expression of wild-type MSOX. The Cys315Ala mutant exhibited a prominent 44 kDa band at the lower induction temperature, similar to that observed with wild-type enzyme at 37 °C (*16*). However, a much lower expression level was observed for the Cys315Ser mutant. Further efforts were, therefore, focused on characterization of the highly expressed Cys315Ala mutant.

The Cys315Ala mutant was isolated from cells induced at 25 °C and purified to apparent homogeneity by using a Co^{2+} affinity matrix, as judged by SDS−PAGE. We obtained 275 mg from 5 L of cell culture, a yield comparable to that observed with wild-type MSOX (*16*). The isolated mutant protein did not contain flavin, as judged by the absence of absorbance in the visible region (Figure 1, curve 1), nor did it exhibit sarcosine oxidase activity. This flavin-free form of the mutant protein will be referred to as apoCys315Ala.

Assay of ApoCys315Ala in the Presence of FAD. MSOX activity is measured by monitoring hydrogen peroxide formation in a horseradish peroxidase coupled assay with *o*-dianisidine as the chromogenic substrate. Although no

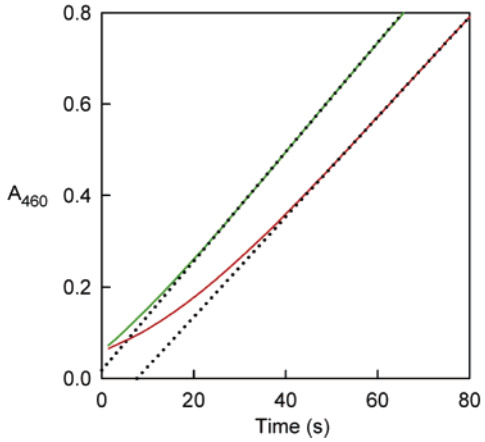


FIGURE 2: Effect of preincubation of apoCys315Ala in catalytic assay mixtures containing FAD. Horseradish peroxidase coupled assays containing 25 μM FAD were initiated by addition of apoCys315Ala (red curve) or sarcosine (green curve). In the latter case, the apoenzyme was added to the temperature-equilibrated reaction mixture 60 s prior to sarcosine addition. The dotted straight lines are drawn through the linear region observed after the initial lag. Results shown are the average of duplicate assays.

activity was observed with apoCys315Ala in a standard assay, the mutant protein did exhibit detectable sarcosine oxidase activity provided that FAD was included in the assay. This effect is illustrated in Figure 2 (red trace) for results obtained with the mutant protein in the presence of 25 μM FAD. These assays exhibit a distinct lag, lasting about 30−60 s, followed by a constant rate. In contrast, assays initiated with wild-type enzyme are linear with no evidence for an initial lag. To investigate the basis for the lag, apoCys315Ala was added to assay mixtures containing FAD but not sarcosine. After a 60 s incubation, the assay was initiated by addition of sarcosine. The lag was virtually eliminated by prior incubation of apoCys315Ala with FAD (Figure 2, green trace); the observed linear rate was only slightly faster (9%) than that attained in assays initiated by addition of apoCys315Ala. The results indicate that the lag reflects binding of FAD to apoCys315Ala.

The results strongly suggested that elimination of the covalent flavin attachment site in MSOX yields a mutant protein that binds FAD weakly but can be reconstituted in situ by including FAD in the assay mixture. Further evidence to evaluate this hypothesis was sought by monitoring the activity of apoCys315Ala over a wide range of FAD concentrations (25−1000 μM). The observed specific activity was found to exhibit a hyperbolic dependence on the concentration of FAD (Figure 3, curve 1). Values for the limiting specific activity in the presence of excess FAD and the apparent dissociation constant of the Cys315Ala·FAD complex were obtained by fitting eq 1 (SA = specific actvity)

$$SA_{obs} = SA_{lim}[FAD]/(K_d + [FAD]) \quad (1)$$

to the data. The specific activity estimated for the mutant in the presence of excess FAD ($SA_{lim} = 4.1 \pm 0.1$ units/mg) is about 10% of the specific activity observed with wild-type MSOX (Table 1). The value obtained for the apparent dissociation constant of the Cys315Ala·FAD complex ($K_d = 100 \pm 8$ μM) indicates that FAD is indeed weakly bound to the mutant protein. The results obtained with the MSOX mutant are particularly striking in comparison with the stable

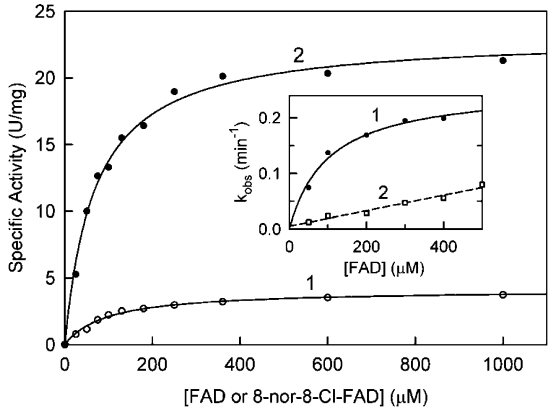


FIGURE 3: Effect of FAD or 8-nor-8-chloroFAD concentration on the activity observed with apoCys315Ala. Horesradish peroxidase coupled assays containing FAD (open circles) or 8-nor-8-chloro-FAD (closed circles) were initiated by addition of apoCys315Ala; reaction rates were measured on the basis of the linear region observed after the lag. The solid lines were obtained by fitting a theoretical binding curve (eq 1) to the data. The inset shows the effect of FAD concentration on the observed rate of covalent flavinylation of wild-type apoMSOX. Incubations of apoMSOX with FAD were conducted in 50 mM Tris-HCl buffer, pH 8 (closed circles), or 100 mM potassium phosphate buffer, pH 8.0 (open squares), at 23 °C. The solid line was obtained by fitting a theoretical curve (eq 4) to the data obtained in Tris-HCl buffer. The dashed line shows a linear regression analysis of the data obtained in phosphate buffer. The phosphate data were taken from ref 17.

Table 1: Properties of Noncovalent Complexes of Mutant or Wild-Type ApoMSOX with Oxidized Flavin[a]

MSOX preparation	K_d (μM)	specific activity (units/mg)
noncovalent complexes		
Cys315Ala·FAD	100 ± 8	4.1 ± 0.1
		(6.3 ± 0.2)[b]
Cys315Ala·8-nor-8-Cl-FAD	70 ± 5	23.2 ± 0.5
		(35.7 ± 0.8)[b]
wild-type MSOX·FAD	100 ± 20	
wild-type MSOX·5-deazaFAD	150 ± 20	
native MSOX		44.8 ± 0.4

[a] All measurements were conducted in 50 mM Tris-HCl buffer, pH 8.0, at 25 °C, except as indicated. Parameters for Cys315Ala·flavin complexes were estimated using a horseradish peroxidase coupled assay containing the indicated flavin, as described in the text. The same assay, without added flavin, was used to monitor the extent of covalent flavinylation of wild-type apoMSOX and to measure the activity of native MSOX. Incubations of wild-type apoMSOX with FAD were conducted at 23 °C; the K_d for the noncovalent complex with FAD was estimated on the basis of the dependence of the observed rate of covalent flavinylation on the concentration of FAD, as described in the text. 5-DeazaFAD competitively inhibits covalent flavinylation of wild-type apoMSOX; the K_d for the noncovalent complex with 5-deaza-FAD was estimated on the basis of the observed value for the competitive inhibition constant, as described in the text. [b] Values shown in parentheses are corrected for an estimated 65% reconstitutable apoprotein in the apoCys315Ala preparation, as discussed in the text.

noncovalent flavin complexes observed with other flavoproteins, including analogous mutant forms of enzymes that normally contain covalently bound flavin (*22*−*27*). The phosphate buffers routinely used during MSOX isolation are likely to further destabilize the Cys315Ala·FAD complex, as judged by the 3-fold larger value obtained for the apparent dissociation constant when the 50 mM Tris-HCl assay buffer, pH 8.0, was switched to 50 mM potassium phosphate, pH 8.0 ($K_d = 290 \pm 30$ μM, $SA_{lim} = 6.1 \pm 0.3$ units/mg).

Effect of 8-Nor-8-chloroFAD on the Activity Observed with ApoCys315Ala. The 10-fold lower specific activity observed for the Cys315Ala·FAD complex as compared with wild-type MSOX might reflect the lower reduction potential of FAD as compared with the 8α-substituted flavin [8α-(*S*-cysteinyl)FAD] found in wild-type MSOX [$\Delta E \sim 30$ mV, as estimated by the reduction potentials of the corresponding riboflavin derivatives (*18*, *19*)]. Evidence to evaluate this hypothesis was sought by replacing FAD in the assay with an analogue exhibiting a higher reduction potential, 8-nor-8-chloroFAD [$\Delta E \sim 55$ mV, as estimated by the reduction potentials of the corresponding FMN derivatives (*18*, *32*)].

An initial lag was observed in assays containing 8-nor-8-chloroFAD, followed by a linear rate, similar to that observed with FAD. The specific activity observed with apoCys315Ala in the presence of 8-nor-8-chloroFAD was found to exhibit a hyperbolic dependence on the flavin concentration (Figure 3, curve 2), as observed with FAD. The value estimated for the apparent dissociation constant of 8-nor-8-chloroFAD ($K_d = 70 \pm 5$ μM) is fairly similar to that obtained with FAD. In contrast, replacing the 8-methyl group of FAD with an 8-chloro substituent resulted in a 5.7-fold increase in the limiting value of the specific activity. The specific activity estimated for the Cys315Ala·8-nor-8-chloroFAD complex ($SA_{lim} = 23.2 \pm 0.5$) is more than 50% of that observed with wild-type enzyme (Table 1). This represents a minimum estimate since the specific activity of the mutant complex is calculated by assuming that the apoCys315Ala preparation is 100% reconstitutable. In fact, results described below suggest that this value is probably about 65%.

Noncovalent Binding of FAD to Wild-Type ApoMSOX. Our previous studies show that the wild-type apoenzyme can be produced by controlled MSOX expression in a riboflavin-dependent strain of *E. coli*. A time-dependent increase in catalytic activity was observed upon incubation of wild-type apoMSOX with FAD, accompanied by the covalent incorporation of FAD (*17*). It seemed reasonable to expect that the flavinylation reaction would involve initial formation of a noncovalent complex of wild-type apoMSOX with FAD (eq 2) and that this complex would exhibit a dissociation

$$\mathrm{E} + \mathrm{FAD} \overset{K_d}{\rightleftharpoons} \mathrm{E{\cdot}FAD} \quad (2)$$

$$\mathrm{E{\cdot}FAD} \xrightarrow{k_{lim}} \mathrm{E{\cdot}FAD\ (covalent)} \quad (3)$$

constant similar to that observed for the Cys315Ala·FAD complex. In this case, the reconstitution rate observed with wild-type apoMSOX should exhibit a hyperbolic dependence on the FAD concentration within the range previously tested (50−500 μM). However, a linear dependence was observed with a finite *y*-intercept, suggesting that the putative noncovalent FAD complex with wild-type MSOX was much less stable than with the mutant protein. We reasoned that this apparent anomaly might be attributable to the phosphate buffer (100 mM, pH 8.0) used in previous reconstitution studies with wild-type apoMSOX, as judged by the destabilizing effect of phosphate buffer (50 mM, pH 8.0) on the Cys315Ala·FAD complex, as described above. To test this hypothesis, the kinetics of reconstitution of wild-type apoMSOX were measured in 50 mM Tris-HCl buffer, pH 8.0. Indeed, the observed rate of reconstitution exhibited an expected hyperbolic dependence on the FAD concentration (Figure 3, inset, curve 1). Values for the apparent dissociation constant of the wild-type enzyme·FAD complex ($K_d = 100 \pm 20$ μM) and the limiting rate of covalent flavinylation ($k_{lim} = 0.26 \pm 0.02$ min^{-1}) were estimated by fitting eq 4 to the data. The value obtained for the apparent

$$k_{obs} = k_{lim}[\mathrm{FAD}]/(K_d + [\mathrm{FAD}]) \quad (4)$$

dissociation constant of the noncovalent FAD complex with wild-type MSOX is, in fact, identical to that observed with the Cys315Ala mutant (Table 1).

Scheme 1: Stoichiometry of 5-DeazaFAD (5-dFAD) Reduction Mediated by ApoCys315Ala (E) in the Presence of Sarcosine (S)[a]

$$\mathrm{E} + \text{5-dFAD} \rightleftharpoons \mathrm{E{\bullet}}\text{5-dFAD} \quad (1)$$

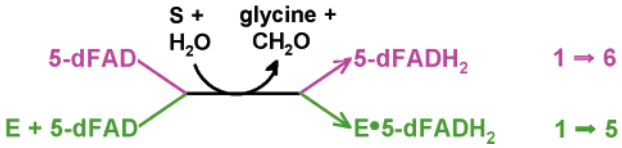
$$\mathrm{E{\bullet}}\text{5-dFAD} + \mathrm{S} \rightleftharpoons \mathrm{E{\bullet}}\text{5-dFAD{\bullet}S} \quad (2)$$

$$\mathrm{E{\bullet}}\text{5-dFAD{\bullet}S} \rightleftharpoons \mathrm{E{\bullet}}\text{5-dFADH}_2\mathrm{{\bullet}IA} \quad (3)$$

$$\mathrm{E{\bullet}}\text{5-dFADH}_2\mathrm{{\bullet}IA} \rightleftharpoons \mathrm{E{\bullet}}\text{5-dFADH}_2 + \mathrm{IA} \quad (4)$$

$$\mathrm{IA} + \mathrm{H_2O} \longrightarrow \text{glycine} + \mathrm{CH_2O} \quad (5)$$

$$\mathrm{E{\bullet}}\text{5-dFADH}_2 \overset{?}{\rightleftharpoons} \mathrm{E} + \text{5-dFADH}_2 \quad (6)$$



[a] For reactions 1 and 3, the equilibria are expected to favor the reverse reaction, as indicated. Hydrolysis of the labile sarcosine imine (IA) (reaction 5) is essentially irreversible in the absence of excess glycine and/or formaldehyde. The predicted stoichiometry of 5-deazaFAD reduction depends on whether 5-deazaFADH$_2$ (5-dFADH$_2$) is weakly or tightly bound to apoCys315Ala, as indicated by the net equations for reactions 1 → 6 or 1 → 5, respectively.

Reaction of ApoCys315Ala with 5-DeazaFAD in the Presence of Sarcosine. 5-DeazaFAD is a good structural analogue for FAD, as judged by results obtained with numerous other flavoenzymes (*33*). It was therefore likely that 5-deazaFAD would bind to apoCys315Ala, forming a relatively weak complex, similar to that observed with FAD or 8-nor-8-chloroFAD. Although sarcosine might reduce the Cys315Ala·5-deazaFAD complex, we expected that turnover would be thwarted by the known poor reactivity of reduced 5-deazaflavin toward molecular oxygen. Consistent with this hypothesis, no activity was observed with apoCys315Ala when 5-deazaFAD (500 μM) was added to the catalytic assay.

Nevertheless, we reasoned that 5-deazaFAD might act as a "co-substrate" for apoCys315Ala in a series of reactions that would result in a net conversion of the oxidized cofactor to reduced 5-deazaFAD (5-deazaFADH$_2$). Two assumptions are implicit in the postulated reaction sequence shown by eqs 1−6 in Scheme 1: (1) the Cys315Ala·5-deazaFAD complex is reducible by sarcosine; (2) the mutant protein exhibits a weak affinity for the reduced coenzyme, as observed with oxidized flavin. Reduction of wild-type MSOX by sarcosine is essentially irreversible (*34*). However, due to the lower reduction potential of 5-deazaflavin, the reverse

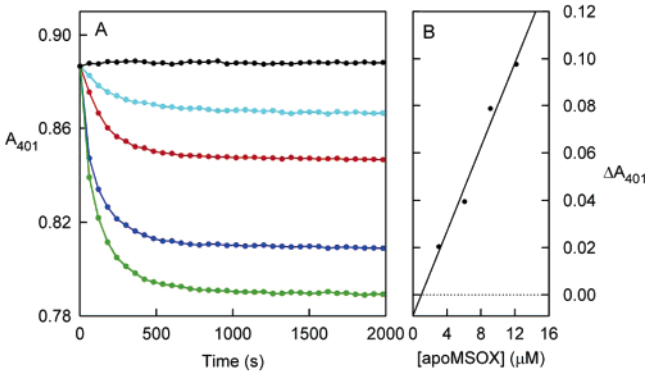


FIGURE 4: Reduction of 5-deazaFAD in the presence of apoCys315Ala and sarcosine. Reactions were conducted at 25 °C in 50 mM Tris-HCl buffer, pH 8.0, containing 77.1 μM 5-deazaFAD and 120 mM sarcosine. Panel A: The black, cyan, red, blue, and green traces were obtained after addition of 0, 3.0, 6.1, 9.1, and 12.2 μM apoCys315Ala, respectively. Panel B: The observed absorbance change at 401 nm (circles) is plotted versus the concentration of apoCys315Ala in the assay. The line was generated by linear regression analysis ($r^2 = 0.978$; y-intercept = 8.91×10^{-3}).

reaction would be favored with Cys315Ala·5-deazaFAD and might prevent accumulation of 5-deazaFADH$_2$. Fortunately, oxidation of Cys315Ala·5-deazaFADH$_2$ by sarcosine imine would probably be short-circuited by rapid hydrolysis of the imine, a reaction that is effectively irreversible in the absence of excess glycine and/or formaldehyde.

To evaluate the postulated reaction sequence, separate aliquots of a solution containing 77.1 μM 5-deazaFAD and 120 mM sarcosine were mixed with 3.0, 6.1, 9.1, or 12.2 μM apoCys315Ala. In each case, reduction of 5-deazaFAD was observed, as judged by the decrease in absorbance at 401 nm. No spectral change was detected in a control reaction initiated by addition of buffer instead of protein (Figure 4A). The reactions observed in the presence of apoCys315Ala were complete in about 10 min but, unexpectedly, reached an apparent end point in the presence of considerable amounts of residual oxidized 5-deazaFAD. Interestingly, the magnitude of the observed absorbance change was found to be proportional to the amount of apoCys315Ala added to the reaction mixture (Figure 4B). Indeed, the yield of 5-deazaFADH$_2$ was approximately stoichiometric with respect to the amount of apoprotein added to the assay ([5-deazaFADH$_2$]/[apoCys315Ala] = 0.65 ± 0.05). Formation of a stoichiometric amount of 5-deazaFADH$_2$ would be consistent with a reaction sequence that terminates in the formation of a *stable* complex of the reduced flavin with apoCys315Ala (Scheme 1, eqs 1−5), contrary to our original assumption.

Reconstitution of ApoCys315Ala with 5-DeazaFADH$_2$. To directly evaluate the stability of the Cys315Ala·5-deazaFADH$_2$ complex, the apoprotein was incubated with a modest excess (~30%) of reduced 5-deazaFAD and then dialyzed overnight against a 300-fold excess of flavin-free buffer. The dialyzed enzyme exhibited an absorption spectrum with a maximum at 322 nm (Figure 5, magenta curve). The observed spectral properties suggested that the reconstituted enzyme contained reduced 5-deazaFAD, as judged by comparison with those reported for the neutral or anionic form of free reduced 5-deazariboflavin (λ_{max} = 323 or 319 nm, respectively; pK_a = 7.2) (*31*).

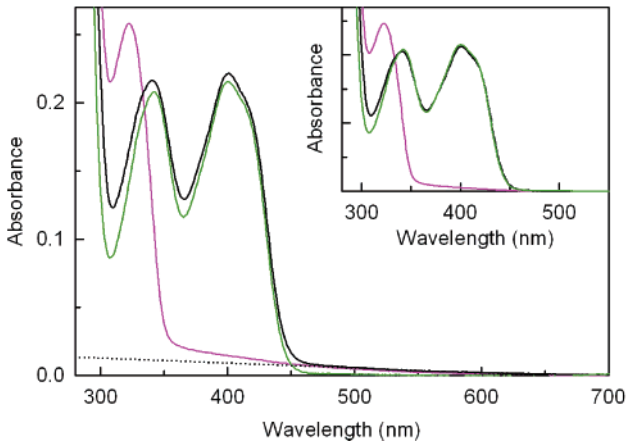


FIGURE 5: Reaction of Cys315Ala·5-deazaFADH$_2$ with sarcosine imine at 25 °C. The magenta curve is the absorption spectrum of the reconstituted enzyme in 50 mM potassium phosphate buffer, pH 8.0. The black curve was recorded immediately after mixing the enzyme (350 μL) with 10 μL of an imine stock solution, prepared as described in the text. The green curve was recorded after denaturation of the oxidized sample with 3 M guanidine hydrochloride. The dotted black line is the estimated contribution due to light scattering in spectra recorded in the absence of denaturant. The line was generated by linear regression analysis of the 500−600 nm region in the absorption spectrum of the oxidized sample (black curve). Inset: The magenta and black curves were obtained after correcting absorption spectra, recorded before and after reaction of Cys315Ala·5-deazaFADH$_2$ with sarcosine imine, respectively, for the estimated contribution due to light scattering. For comparison, the inset also shows the observed absorption spectrum of the denatured oxidized sample (green curve).

We surmised that the putative Cys315Ala·5-deazaFADH$_2$ complex would act as a sarcosine imine reductase in a reaction that should result in the oxidation of the bound flavin. To test this hypothesis, a stock solution containing sarcosine imine was prepared by incubating 3.0 M glycine with 375 mM formaldehyde for 5 min at 25 °C. A 10 μL aliquot of the imine stock solution was then mixed with a 350 μL sample of reconstituted enzyme. Immediate oxidation of the reduced flavin was observed, as judged by the appearance of an absorption spectrum characteristic of oxidized 5-deazaFAD with maxima at 340 and 401 nm (Figure 5, black curve). No reaction was observed when only glycine or formaldehyde was added to a sample of reconstituted enzyme.

The stoichiometry of flavin incorporation into 5-deazaFADH$_2$-reconstituted enzyme was estimated by denaturation of the imine-oxidized enzyme with 3 M guanidine hydrochloride, as detailed in Experimental Procedures. We found that the reconstituted enzyme contained 0.63 mol of reduced 5-deazaFAD. The results are in excellent agreement with the percentage of reconstitutable apoprotein (65%), estimated on the basis of the stoichiometry of reduced flavin formation observed upon reaction apoCys315Ala with oxidized 5-deazaFAD and sarcosine (see Figure 4B).

Spectra recorded before or after oxidation of Cys315Ala·5-deazaFADH$_2$ with sarcosine imine exhibit weak absorbance at wavelengths greater than 470 nm, a feature that is eliminated upon addition of guanidine hydrochloride (Figure 5, green curve). The spectral effect is attributed to the elimination of light scattering due to a small amount of aggregated protein that is solubilized in the presence of

guanidine hydrochloride. The absorption spectrum of the oxidized sample was corrected for the contribution due to light scattering, as described in the legend to Figure 5. The corrected absorption spectrum is virtually identical to that observed after denaturation (Figure 5, inset).

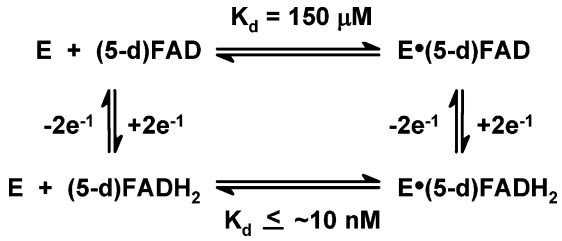
The results suggested that reaction of the reconstituted enzyme with sarcosine imine resulted in the release of oxidized 5-deazaFAD into solution, as predicted on the basis of the observed instability of Cys315Ala complexes with other oxidized flavins. To test this hypothesis, the Cys315Ala·5-deazaFADH$_2$ complex was oxidized by reaction with sarcosine imine and then dialyzed against flavin-free buffer. This resulted in complete loss of the oxidized coenzyme as judged by the absence of absorbance of the dialyzed sample in the visible region, as observed for the original apoprotein preparation. ApoCys315Ala and wild-type apoMSOX exhibit similar dissociation constants for oxidized flavin, as judged by results obtained with FAD. Oxidized 5-deazaFAD does not covalently attach to wild-type apoMSOX (*17*) but is found to competitively inhibit the covalent attachment of FAD. The observed inhibition constant ($K_i = 150 \pm 20$ μM) indicates that oxidized 5-deazaFAD is weakly bound to apoMSOX, as observed with other oxidized flavins (Table 1).

An extinction coefficient for enzyme-bound 5-deazaFADH$_2$ at 322 nm was calculated by using the corrected absorption spectra for reduced and imine-oxidized enzyme, as shown in the inset to Figure 5 ($\epsilon_{322} = 13300$ M^{-1} cm^{-1}). A similar value was obtained on the basis of the absorbance observed for the imine-oxidized sample after denaturation with guanidine hydrochloride ($\epsilon_{322} = 13100$ M^{-1} cm^{-1}). The value obtained for enzyme-bound 5-deazaFADH$_2$ falls within the range expected based on extinction coefficients reported for the neutral and anionic forms of free reduced 5-deazariboflavin ($\epsilon_{323} = 12400$ M^{-1} cm^{-1} and $\epsilon_{319} = 12000$ M^{-1} cm^{-1}, respectively) (*31*), assuming a modest perturbation by the protein environment.

DISCUSSION

A catalytically inactive apoprotein is produced when the covalent flavin attachment site in MSOX from *Bacillus* sp. B-0618 (Cys315) is mutated to Ala. However, the mutant protein (apoCys315Ala) forms weak noncovalent complexes with FAD or 8-nor-8-chloroFAD ($K_d = 100$ and 70 μM, respectively) and does exhibit sarcosine oxidase activity when assayed in the presence of free flavin. A similar, weak noncovalent complex with FAD is observed with wild-type apoMSOX ($K_d = 100$ μM) where the complex is formed as an intermediate during covalent flavin attachment. Unlike results obtained with oxidized flavins, reconstitution of apoCys315Ala with reduced 5-deazaFAD produced a remarkably stable Cys315Ala·5-deazaFADH$_2$ complex. The estimated stoichiometry of reduced flavin binding (0.63−0.65 mol of 5-deazaFADH$_2$/mol of protein) was unaffected by dialysis. The results indicate that the apoCys315Ala preparation probably contained about 65% reconstitutable apoprotein. The Cys315Ala·5-deazaFADH$_2$ complex is air-stable but readily oxidized by reaction with sarcosine imine. Complete loss of oxidized 5-deazaFAD is observed upon dialysis of the imine-treated sample. The results show that the covalent flavin link in wild-type MSOX plays a critical role in preventing loss of oxidized FAD.

Scheme 2: Thermodynamic Cycle for Binding and Reduction of FAD or 5-DeazaFAD [(5-d)FAD] to ApoMSOX (E)



This outcome was not anticipated on the basis of the crystal structure of wild-type MSOX which shows numerous hydrogen-bonding and electrostatic interactions between FAD and the protein (*13*). Unlike MSOX, elimination of the covalent flavin attachment site in other enzymes typically results in the production of mutant holenzymes that contain tightly bound noncovalent flavin in the oxidized state. Enzymes where this feature has been observed include *p*-cresol methylhydroxylase (*22*), cholesterol oxidase (*23*), vanillyl-alcohol oxidase (*24*), trimethylamine dehydrogenase (*27*), monoamine oxidase A (*25*), and fumarate reductase (*26*). Interestingly, an apparent exception is provided by results obtained upon mutation of the covalent flavin attachment site in MSOX from *Arthrobacter* sp. TE1826 (*35*). Although the flavin content was not reported, the mutant was probably isolated as an apoprotein as judged by the observed FAD requirement for catalytic activity.

The dramatic difference in affinity observed with apoCys315Ala for the oxidized and reduced forms of 5-deazaFAD means that the protein environment must induce a significant increase in the reduction potential of the bound coenzyme, as required by the thermodynamic cycle shown in Scheme 2. The dissociation constant of apoCys315Ala for oxidized 5-deazaFAD is likely to be about 150 μM, as judged by results obtained with wild-type apoMSOX. The dissociation constant for reduced 5-deazaFAD is not known, but a value of about 10 nM is probably a reasonable upper limit, as judged by the observed retention of the reduced coenzyme after dialysis against a 300-fold excess of flavin-free buffer. Using values of 150 μM and 10 nM for the oxidized and reduced coenzyme, respectively, we estimate that the protein environment in MSOX induces an ~120 mV increase in the reduction potential of noncovalently bound flavin. In trimethylamine dehydrogenase an ~200 mV increase in flavin reduction potential is attributed to a protein-stabilized butterfly bend of the oxidized flavin ring (*27*). In MSOX, redox modulation is probably mediated by the highly electropositive active site environment where a number of basic, but no acidic, residues are found near the flavin ring. Flavin O(2) is hydrogen bonded to the ϵ-amino group of Lys348. The N-terminus of helix αF4 points toward the N(1)−O(2) portion of the flavin ring. The side chain of Arg49 is in van der Waals contact with the flavin ring. A water molecule is hydrogen bonded to both flavin N(5) and the ϵ-amino group of Lys265 (*13*). Interestingly, a single positive charge near flavin N(1) in choline oxidase is estimated to cause a 30 mV increase in flavin reduction potential (*36*).

The 30−50 mV higher potentials observed for amino acylflavin derivatives as compared with unmodified flavin (*18*−*21*) suggested that the covalent flavin linkage in wild-

type MSOX would also play a role in modulating the flavin reduction potential. This prediction is consistent with the lower specific activity observed for the noncovalent Cys315Ala·FAD complex and the 5.7-fold rate enhancement obtained when FAD was replaced with 8-nor-8-chloroFAD, a derivative exhibiting an ~55 mV higher reduction potential. The specific activity of the Cys315Ala·8-nor-8-chloroFAD complex is about 80% of that observed with wild-type MSOX when corrected for the estimated fraction of reconstitutable apoprotein (0.65) in the preparation of apoCys315Ala.

The results indicate that the flavin reduction potential in MSOX is modulated by the protein environment and the presence of a covalent flavin linkage. An approximate value was calculated for the two-electron reduction potential of the covalent flavin in wild-type MSOX by assuming that the protein environment mediates an ~120 mV increase in the potential of 8α-*S*-cysteinylflavin ($E_{m\ free} = -169$ mV) (*19*). Is the estimated reduction potential ($E_m = \sim -50$ mV) consistent with the known redox properties of the enzyme? MSOX forms a thermodynamically stable anionic flavin radical. Quantitative radical formation is observed upon reaction of the enzyme with various one-electron reductants (*4*, *5*). The one-electron potential for the $EFAD_{ox}/EFAD_{rad}$ couple has been determined ($E_1 = 79.5$ mV) (*14*). However, a reliable value for the $EFAD_{rad}/EFAD_{red}$ couple (E_2) could not be obtained, despite considerable effort.[2] An approximate value for E_2 was calculated ($E_2 = \sim -180$ mV) using the estimated and observed values for E_m and E_1, respectively. The difference in potential between the observed value for E_1 and that estimated for E_2 is quite large ($\Delta E = \sim 0.3$ V), consistent with the observed thermodynamic stability of the MSOX radical.

In summary, a significant protein-mediated increase in flavin reduction potential is achieved by preferential binding of reduced flavin to apoMSOX in a double-edged sword strategy that requires formation of a covalent linkage in order to prevent loss of the weakly bound oxidized flavin. A further increase in flavin reduction potential is attained by covalent flavin attachment in a reaction that requires ionization of the 8α-methyl group in the oxidized flavin (*17*). Interestingly, the elevated reduction potential of the noncovalently bound flavin is likely to facilitate covalent attachment because the increased electrophilicity of the flavin ring will enhance the acidity of the 8α-methyl group protons. A delicate interplay of forces has apparently evolved to generate a MSOX holoenzyme that is highly stable and also a potent sarcosine oxidant. It will be interesting to see whether a similar strategy is utilized by other members of the MSOX family.

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REFERENCES

1. Mewies, M., McIntire, W. S., and Scrutton, N. S. (1998) Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes—The current state of affairs, *Protein Sci. 7*, 7−20.
2. Bruckner, R. C., Zhao, G. H., Venci, D., and Jorns, M. S. (2004) Nikkomycin biosynthesis: Formation of a 4-electron oxidation product during turnover of nikD with its physiological substrate, *Biochemistry 43*, 9160−9167.
3. Barquera, B., Hase, C. C., and Gennis, R. B. (2001) Expression and mutagenesis of the NqrC subunit of the NQR respiratory Na^+ pump from *Vibrio cholerae* with covalently attached FMN, *FEBS Lett. 492*, 45−49.
4. Wagner, M. A., Trickey, P., Chen, Z., Mathews, F. S., and Jorns, M. S. (2000) Monomeric sarcosine oxidase: 1. Flavin reactivity and active site binding determinants, *Biochemistry 39*, 8813−8824.
5. Wagner, M. A., and Jorns, M. S. (2000) Monomeric sarcosine oxidase: 2. Kinetic studies with sarcosine, alternate substrates and substrate analogues, *Biochemistry 39*, 8825−8829.
6. Khanna, P., and Jorns, M. S. (2001) Characterization of the FAD-containing *N*-methyltryptophan oxidase from *Escherichia coli*, *Biochemistry 40*, 1441−1450.
7. Dodt, G., Kim, D. G., Reimann, S. A., Reuber, B. E., McCabe, K., Gould, S. J., and Mihalik, S. J. (2000) L-pipecolic acid oxidase, a human enzyme essential for the degradation of L-pipecolic acid, is most similar to the monomeric sarcosine oxidases, *Biochem. J. 345*, 487−494.
8. Venci, D., Zhao, G., and Jorns, M. S. (2002) Molecular characterization of nikD, a new flavoenzyme important in the biosynthesis of nikkomycin antibiotics, *Biochemistry 41*, 15795−15802.
9. Eschenbrenner, M., Chlumsky, L. J., Khanna, P., Strasser, F., and Jorns, M. S. (2001) Organization of the multiple coenzymes and subunits and role of the covalent flavin link in the complex heterotetrameric sarcosine oxidase, *Biochemistry 40*, 5352−5367.
10. Kvalnes-Krick, K., and Jorns, M. S. (1991) Role of the covalent and noncovalent flavins in sarcosine oxidase, in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) pp 425−435, CRC Press, Boca Raton, FL.
11. Fossati, P., Prencipe, L., and Berti, G. (1983) Enzymic creatinine assay: A new colorimetric method based on hydrogen peroxide measurement, *Clin. Chem. 29*, 1494−1496.
12. Berberich, J. A., Yang, L. W., Madura, J., Bahar, I., and Russell, A. J. (2005) A stable three-enzyme creatinine biosensor. 1. Impact of structure, function and environment on PEGylated and immobilized sarcosine oxidase, *Acta Biomaterialia 1*, 173−181.
13. Trickey, P., Wagner, M. A., Jorns, M. S., and Mathews, F. S. (1999) Monomeric sarcosine oxidase: Structure of a covalently-flavinylated secondary amine oxidizing enzyme, *Structure 7*, 331−345.
14. Zhao, G., Song, H., Chen, Z., Mathews, F. S., and Jorns, M. S. (2002) Monomeric sarcosine oxidase: Role of histidine 269 in catalysis, *Biochemistry 41*, 9751−9764.
15. Chen, Z., Zhao, G., Martinovic, S., Jorns, M. S., and Mathews, F. S. (2005) Structure of the sodium borohydride-reduced *N*-(cyclopropyl)glycine adduct of the flavoenzyme monomeric sarcosine oxidase, *Biochemistry 44*, 15444−15450.
16. Wagner, M. A., Khanna, P., and Jorns, M. S. (1999) Structure of the flavocoenzyme of two homologous amine oxidases: Monomeric sarcosine oxidase and *N*-methyltryptophan oxidase, *Biochemistry 38*, 5588−5595.
17. Hassan-Abdallah, A., Bruckner, R. C., Zhao, G. H., and Jorns, M. S. (2005) Biosynthesis of covalently bound flavin: Isolation and in vitro flavinylation of the monomeric sarcosine oxidase apoprotein, *Biochemistry 44*, 6452−6462.
18. Draper, R. D., and Ingraham, L. L. (1968) A potentiometric study of the flavin semiquinone equilibrium, *Arch. Biochem. Biophys. 125*, 802−808.
19. Edmondson, D. E., and Singer, T. P. (1973) Oxidation−reduction properties of 8-α-substituted flavins, *J. Biol. Chem. 248*, 8144−8149.
20. Edmondson, D. E., Kenney, W. C., and Singer, T. P. (1976) Structural elucidation and properties of 8a-(N^1-histidyl)riboflavin: The flavin component of thiamine dehydrogenase and *β*-cyclopiazonate oxidocyclase, *Biochemistry 15*, 2937−2945.
21. Ghisla, S., Kenney, W. C., Knappe, W. R., McIntire, W., and Singer, T. P. (1980) Chemical synthesis and some properties of 6-substituted flavins, *Biochemistry 19*, 2537−2544.
22. Efimov, I., Cronin, C. N., and McIntire, W. S. (2001) Effects of noncovalent and covalent FAD binding on the redox and catalytic properties of *p*-cresol methylhydroxylase, *Biochemistry 40*, 2155−2166.

[2] H. Song and M. S. Jorns, unpublished observations.

23. Motteran, L., Pilone, M. S., Molla, G., Ghisla, S., and Pollegioni, L. (2001) Cholesterol oxidase from *Brevibacterium sterolicum*—The relationship between covalent flavinylation and redox properties, *J. Biol. Chem. 276*, 18024−18030.
24. Fraaije, M. W., van den Heuvel, R. H. H., Van Berkel, W. J. H., and Mattevi, A. (1999) Covalent flavinylation is essential for efficient redox catalysis in vanillyl-alcohol oxidase, *J. Biol. Chem. 274*, 35514−35520.
25. Nandigama, R. K., and Edmondson, D. E. (2000) Influence of FAD structure on its binding and activity with the C406A mutant of recombinant human liver monoamine oxidase A, *J. Biol. Chem. 275*, 20527−20532.
26. Blaut, M., Whittaker, K., Valdovinos, A., Ackrell, B. A. C., Gonsalus, R. P., and Cecchini, G. (1989) Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin, *J. Biol. Chem. 264*, 13599−13604.
27. Trickey, P., Basran, J., Lian, L. Y., Chen, Z. W., Barton, J. D., Sutcliffe, M. J., Scrutton, N. S., and Mathews, F. S. (2000) Structural and biochemical characterization of recombinant wild type and a C30A mutant of trimethylamine dehydrogenase from *Methylphilus methylotrophus* (sp W_3A_1), *Biochemistry 39*, 7678−7688.
28. O'Brien, D. E., Weinstock, L. T., and Cheng, C. C. (1970) Synthesis of 10-deazariboflavin and related 2,4-dioxopyrimido-[4,5-*b*]quinolines, *J. Heterocycl. Chem. 7*, 99−105.
29. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction, *Gene 77*, 51−59.
30. Moore, E. G., Cardemil, E., and Massey, V. (1978) Production of a covalent flavin linkage in lipoamide dehydrogenase, *J. Biol. Chem. 253*, 6413−6422.
31. Spencer, R., Fisher, J., and Walsh, C. (1976) Preparation, characterization, and chemical properties of the flavin coenzyme analogues of 5-deazariboflavin, 5-deazariboflavin 5′-phosphate, and 5-deazariboflavin 5′-diphosphate, 5′-5′-adenosine ester, *Biochemistry 15*, 1043−1053.
32. Steward, R. C., and Massey, V. (1985) Potentionmetric studies of native and flavin-substituted old yellow enzyme, *J. Biol. Chem. 260*, 13639−13647.
33. Ghisla, S., and Massey, V. (1986) New flavins for old: Artificial flavins as active site probes of flavoproteins, *Biochem. J. 239*, 1−12.
34. Zhao, G., and Jorns, M. S. (2006) Spectral and kinetic characterization of the Michaelis charge transfer complex in monomeric sarcosine oxidase, *Biochemistry 45*, 5985−5992.
35. Nishiya, Y. (2000) A mutant sarcosine oxidase in which activity depends on flavin adenine dinucleotide, *Protein Expression Purif. 20*, 95−97.
36. Ghanem, M., and Gadda, G. (2006) Effects of reversing the protein positive charge in the proximity of the flavin N(1) locus of choline oxidase, *Biochemistry 45*, 3437−3447.

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