Covalent Flavinylation of Monomeric Sarcosine Oxidase: Identification of a Residue Essential for Holoenzyme Biosynthesis†

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ABSTRACT: FAD in monomeric sarcosine oxidase (MSOX) is covalently linked to the protein by a thioether linkage between its 8α-methyl group and Cys315. Covalent flavinylation of apoMSOX has been shown to proceed via an autocatalytic reaction that requires only FAD and is blocked by a mutation of Cys315. His45 and Arg49 are located just above the *si*-face of the flavin ring, near the site of covalent attachment. His45Ala and His45Asn mutants contain covalently bound FAD and exhibit catalytic properties similar to wild-type MSOX. The results rule out a significant role for His45 in covalent flavinylation or sarcosine oxidation. In contrast, Arg49Ala and Arg49Gln mutants are isolated as catalytically inactive apoproteins. ApoArg49Ala forms a stable noncovalent complex with reduced 5-deazaFAD that exhibits properties similar to those observed for the corresponding complex with apoCys315Ala. The results show that elimination of a basic residue at position 49 blocks covalent flavinylation but does not prevent noncovalent flavin binding. The Arg49Lys mutant contains covalently bound FAD, but its flavin content is ∼4-fold lower than wild-type MSOX. However, most of the apoprotein in the Arg49Lys preparation is reconstitutable with FAD in a reaction that exhibits kinetic parameters similar to those observed for flavinylation of wild-type apoMSOX. Although covalent flavinylation is scarcely affected, the specific activity of the Arg49Lys mutant is only 4% of that observed with wild-type MSOX. The results show that a basic residue at position 49 is essential for covalent flavinylation of MSOX and suggest that Arg49 also plays an important role in sarcosine oxidation.

The discovery of a covalently bound flavin in mammalian succinate dehydrogenase (*1*, *2*) paved the way for the identification of covalent flavin linkages in diverse enzymes from a broad spectrum of organisms (*3*). These enzymes exhibit a wide range of biological functions, such as nonribosomal biosynthesis of peptidyl antibiotics (*4*), ion pumping (*5*), biosynthesis of anticancer drugs (*6*, *7*), and production of pharmaceutically important alkaloids (*8*). The covalent linkage is known to modulate the properties of the coenzyme as a redox catalyst and may also play an important structural role in some enzymes (*3*, *9*−*16*).

It is generally thought that covalent flavin incorporation is catalyzed by the apoprotein (*3*, *17*−*20*), but definitive mechanistic studies have been hampered by difficulties encountered in the preparation of reconstitutable apoenzymes. We recently overcame this obstacle and developed a robust in vitro system to probe the mechanism of covalent flavin attachment by using the stable and easily isolated apoenzyme of monomeric sarcosine oxidase (MSOX[1]) (*21*). MSOX catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide. The enzyme contains 1 mol of FAD that is covalently linked to the protein by a thioether linkage between the 8α-methyl group in the isoalloxazine ring of the coenzyme and the side chain of Cys315. The same linkage is also found in numerous other enzymes (e.g., monoamine oxidase). In general, the covalent linkage nearly always involves the 8α-methyl group of the flavin, but tyrosine or histidine may replace cysteine as the site of attachment to the protein (*3*).

Covalent flavinylation of apoMSOX is found to proceed via an autocatalytic reaction that requires only FAD and produces a reconstituted enzyme that exhibits catalytic properties identical to the native holoenzyme. The reaction involves the initial, rapid formation of a noncovalent enzyme•FAD complex. Attachment to the protein occurs in a slower process that generates covalently bound reduced FAD (1,5-dihydroFAD) as the first observable intermediate. This oxygen-sensitive species is produced in quantitative yield when the reaction is conducted in anaerobic buffer but is rapidly oxidized under aerobic conditions, accompanied by the formation of a stoichiometric amount of hydrogen peroxide (*10*, *21*).

The results are consistent with a flavinylation mechanism involving the initial abstraction of a proton from the 8α-methyl group of FAD to generate an iminoquinone methide intermediate (I) (Scheme 1, step 1). The protons on the 8α-methyl group of FAD are relatively acidic as judged by the exchange reaction observed with free riboflavin in D_2O ($k = 0.014$ min^{-1}, pH 6.9, 90 °C) (*22*). The thiolate side chain of Cys315 ($Cys_{315}S^-$) is the most likely candidate for the base required in this step, as judged by the crystal structure

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[1] Abbreviations: MSOX, monomeric sarcosine oxidase; FAD, flavin adenine dinucleotide.

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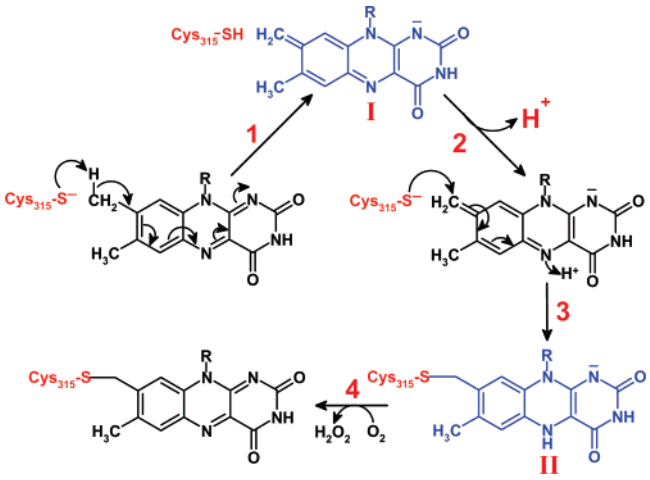
Scheme 1: Mechanism Proposed for Covalent Flavinylation of MSOX



observed for the native holoenzyme (*20*). Nucleophilic attack by $Cys_{315}S^-$ at the 8α-methenyl group of intermediate I (Scheme 1, step 3) yields covalently bound 1,5-dihydroFAD (II), a known intermediate that is converted to an oxidized holoenzyme upon reaction with molecular oxygen (Scheme 1, step 4).

Two steps in the postulated flavinylation mechanism require the thiolate form of Cys315. $Cys_{315}S^-$ may be stabilized by electrostatic and hydrogen bonding interactions with Arg49, a residue in van der Waals contact with the *si*-face of the flavin ring in the native holoenzyme (Figure 1). The postulated roles of $Cys_{315}S^-$ as a base (step 1 in Scheme 1) and a nucleophile (step 3 in Scheme 1) necessitate an intervening step (step 2 in Scheme 1) to regenerate the reactive thiolate group. Ionization of $Cys_{315}SH$ might be achieved by proton transfer to another active site base. The most likely candidate, His45, is located above the *si*-face of the flavin but is not optimally positioned to act as a proton acceptor, as judged by the distance between His45:ND1 and Cys315:SG in the native holoenzyme structure (4.7 Å) (Figure 1). However, a role for His45 cannot be excluded on this basis since a modest conformational change may bring His45 closer to Cys315 in the noncovalent enzyme·FAD complex. Alternatively, instead of proton transfer to another active site base, the proton from $Cys_{315}SH$ might be transported to bulk solvent by a proton relay system that has been detected above the *si*-face of the flavin. This putative proton shuttle begins with the guanidinium group of Arg49, followed by a pair of bridging waters: Lys265:NZ and Thr48:OG1 (Figure 1).

In this study, we investigated the potential role of His45 and Lys49 in covalent flavinylation of MSOX.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes *Nde I* and *Xho I* and T4 DNA ligase were purchased from New England Biolabs. Talon Metal Affinity Resin (Co^{2+} affinity matrix) was obtained from Clontech. Amplex Red was obtained from Molecular Probes. Horseradish peroxidase and *o*-dianisidine were purchased from Sigma. 5-Deazariboflavin was previously synthesized in this laboratory, according to the method of O'Brien et al. (*23*).

Expression and Purification of Wild-Type and Mutant MSOX. Wild-type MSOX holoenzyme was expressed and purified as previously described (*21*, *24*). The same procedure was used for the expression of mutant enzymes except that the cells were grown at 25 °C instead of 37 °C. The His45 and Arg49 mutants contained a carboxyl-terminal affinity tag, $(His)_6$, and were purified by using a Co^{2+} affinity matrix, similar to that previously described for the isolation of His-tagged wild-type MSOX apoenzyme (*21*). Unless otherwise specified, the catalytic activity was monitored by using a horseradish peroxidase-coupled assay with Amplex Red as the chromogenic substrate (*10*). Reactions were conducted at 25 °C in 50 mM Tris, pH 8.0, containing 100 μM Amplex Red and horseradish peroxidase (6 U/mL) and monitored at 563 nm ($\Delta\epsilon_{563} = 52\ 200\ M^{-1}\ cm^{-1}$) (*21*). The concentration of mutant holoenzymes (mg/mL) was estimated based on flavin absorbance by using the extinction coefficients listed in Table 1 and the known molecular weight of MSOX (44 000 Da) (*24*). Total protein concentration was calculated based on the absorbance at 280 nm ($\epsilon_{280} = 36\ 270\ M^{-1}\ cm^{-1}$) after correcting for the contribution due to FAD absorbance at 280 nm (when necessary), as previously described (*24*).

Mutation of His45 to Ala or Asn and Arg49 to Ala, Gln, or Lys. All PCR reactions were conducted using a Hybaid Touchdown Thermocycler. Unless otherwise noted, PCR products were purified by agarose gel (1.5%) electrophoresis and recovered using a QIAQuick Gel Extraction Kit (Qiagen). Sequencing was conducted by MWG Biotech. Mutations were generated by using the plasmid pGZ26 (*21*) as the template and the overlap extension PCR method described by Ho et al. (*25*). PCR reactions were performed by using *Pfu* DNA polymerase (Stratagene) and the following settings: 1 cycle of 94 °C for 2 min; 30 cycles of 94 °C for 40 s, 52 °C for 40 s, 72 °C for 2 min; 1 cycle, 72 °C for 30 min; and 1 cycle, 30 °C for 5 min. The left-hand fragment was generated using START (external primer) as the forward primer and an internal backward primer containing the desired mutation (see Table 2). The right-hand fragment was generated using an internal forward primer containing the desired mutation and END (external primer) as the backward primer. The purified left- and right-hand fragments were combined using START and END as the forward and backward primers, respectively. The final PCR product was purified by using a QIAquick PCR Purification Kit (Qiagen), digested with *NdeI* and *XhoI*, purified again, and then subcloned between the *NdeI* and the *XhoI* sites of plasmid pET23a. The resulting construct was used to transform *Escherichia coli* BL21(DE3) cells to ampicillin resistance. For screening, plasmid DNA was isolated from randomly selected clones using the QiaPrep Spin Miniprep kit (Qiagen) and digested with *NdeI* and *XhoI*. Plasmids that exhibited the expected insert size (pGZ51, pGZ52, pGZ53, pGZ54, and pAHA01 for the His45Ala, His45Asn, Arg49Lys, Arg49Gln, and Arg49Ala mutations, respectively) were isolated using the Qiagen Plasmid Midi kit (Qiagen) and sequenced across the entire insert.

Reconstitution of Mutant MSOX Preparations with Oxidized FAD (His45Ala and Arg49Lys) or Reduced 5-deazaFAD (Arg49Ala). As will be described, the isolated preparations of His45Ala and Arg49Lys were found to contain holoenzyme with covalently bound FAD plus significant amounts of apoprotein. The preparation of the Arg49Ala mutant contained only apoprotein.

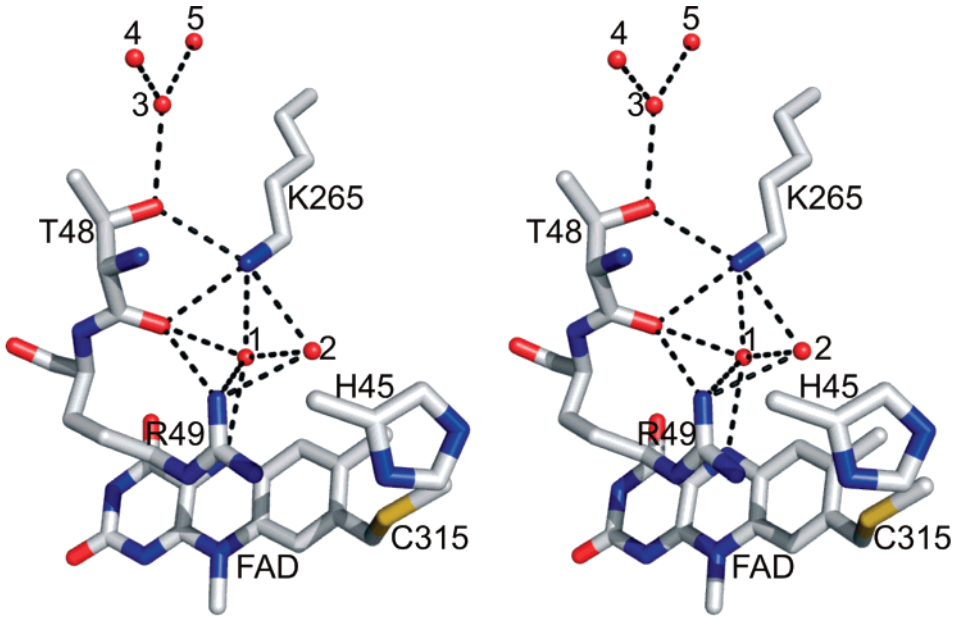


FIGURE 1: Stereoview of the region above the *si*-face of the flavin ring in wild-type MSOX (PDB code 2GBO). Carbon atoms are in white, oxygen atoms are in red, nitrogen atoms are in blue, and the sulfur is yellow. Active site waters are shown as balls. Waters 3−5 are in contact with bulk solvent. Hydrogen bonds are indicated by dotted lines. The diagram was rendered using PYMOL (http://www.pymol.org).

Table 1: Comparison of Spectral and Catalytic Properties of His45 and Arg49 Mutants with Wild-Type MSOX

		His45Ala			Arg49Lys		
	wild-type	untreated	reconstituted	His45Asn	untreated	reconstituted	Arg49Gln/Ala
mol of FAD/mol of protein	0.87[a]	0.25	0.56	0.20	0.24	0.59	none
λ_{max} (nm)[b]	454, 373	454, 379	454, 375	455, 373	450, 393	450, 392	
ϵ_{4xy} (M^{-1} cm^{-1})[c]	12,200[a]	13100	12800	12300	14100	13700	
A_{280}/A_{4xy}[c]	5.54[a]	13.7	7.33	17.5	13.6	6.92	
specific activity (units/mg)[d]	42.4 ± 0.4 (44.8 ± 0.4)	21.5 ± 0.7	nd[e]	22.1 ± 0.6	1.9 ± 0.1	1.73 ± 0.02	0

[a] Data previously reported (*24*). [b] Absorption spectra were recorded in 50 mM Tris-HCl, pH 8.0, at 25 °C. [c] 4xy = 454 nm (wild-type, His45Ala), 455 nm (His45Asn), or 450 nm (Arg49Lys). [d] Catalytic activity was monitored at 25 °C using a horseradish peroxidase-coupled assay with Amplex Red as the chromogenic substrate. The value shown in parentheses for wild-type MSOX was previously reported using a similar coupled assay but with *o*-dianisidine as the chromogenic substrate (*10*). Specific activities of the mutant enzymes are expressed as units per milligram of holoenzyme, as described in the Experimental Procedures, to correct for the presence of substantial amounts of apoenzyme in these preparations. [e] Not determined.

Table 2: Primers Used for Mutagenesis[a]

primer type	forward	backward
External	START 5-GTGAGCGGATAACAATTCCCCTCTAG-3	END 5-TTTCGGGCTTTGTTAGCAGCCGGATC-3
Internal		
His45Ala	5-CGGAAGCCATgcCGGTGATACTC-3	5-GAGTATCACCGgcATGGCTTCCG-3
His45Asn	5-CGGAAGCCATaACGGTGATACTC-3	5-GAGTATCACCGTtATGGCTTCCG-5
Arg49Ala	5-CGGTGATACTgcTATCATCCGCC-3	5-GGCGGATGATAgcAGTATCACCG-3
Arg49Lys	5-CGGTGATACTaaaATCATCCGCC-3	5-GGCGGATGATtttAGTATCACCG-3
Arg49Gln	5-CGGTGATACTCaaATCATCCGCC-3	5-GGCGGATGATttGAGTATCACCG-3

[a] Mutagenic sites in the primers are shown in lower case; the codon targeted for mutagenesis is underlined.

Preparative-scale reconstitution reactions of the His45Ala or Arg49Lys mutant with FAD were conducted by incubating the preparations in 50 mM Tris-HCl buffer, pH 8.0, containing 500 μM FAD for 3 h at room temperature. The reconstituted preparations were then dialyzed against the same buffer or subjected to ultrafiltration to remove unbound FAD.

For kinetics studies, reconstitution of the Arg49Lys mutant with FAD was conducted by incubating the isolated preparation at 23 °C in 50 mM Tris-HCl buffer, pH 8.0, containing 20−125 μM FAD. Reaction progress was monitored by following the increase in catalytic activity using a horseradish peroxidase-coupled assay with *o*-dianisidine as the chromogenic substrate, as previously described (*10*).

Reconstitution of the Arg49Ala mutant with reduced 5-deazaFAD was performed by incubating the mutant apoprotein (480 μM) for 30 min at room temperature with a 4-fold excess of the reduced flavin in anaerobic buffer (90 mM potassium phosphate containing 20 mM Tris-HCl, pH 8), followed by aerobic dialysis versus 50 mM Tris-HCl buffer, pH 8.0. Reduced 5-deazaFAD was prepared by reduction of the oxidized coenzyme with sodium borohydride. Oxidized 5-deazaFAD was prepared from 5-deazariboflavin, as previously described (*21*).

Spectroscopy and Flavin Analysis. Absorption spectra were recorded using an Agilent Technologies 8453 diode

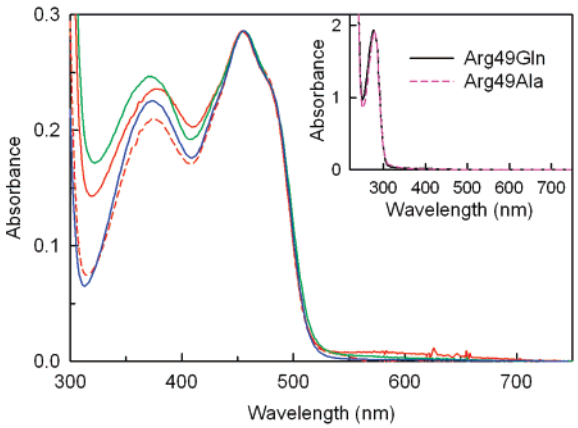


FIGURE 2: Comparison of absorption spectra of the isolated His45Ala and His45Asn mutants (solid red and green curves, respectively) with that observed for wild-type MSOX (blue curve). The spectrum of the His45Ala mutant after reconstitution with FAD is shown by the red dashed curve. Spectra were recorded in 50 mM Tris-HCl buffer, pH 8.0, at 25 °C and are normalized to the same absorbance at 454 nm. The inset shows absorption spectra of the Arg49Gln and Arg49Ala mutants in 50 mM Tris-HCl buffer, pH 8.0, at 25 °C.

array or a PerkinElmer Lambda 25 spectrophotometer. Extinction coefficients and the stoichiometry of FAD incorporation into His45Ala, His45Asn, and Arg49Lys were determined after denaturation of the mutant enzyme preparations with guanidine hydrochloride, as previously described (*24*). The stoichiometry of reduced 5-deazaFAD incorporation into apoArg49Ala was determined after oxidizing the reconstituted preparation with sarcosine imine, as described by Hassan-Abdallah et al. (*10*). To determine whether 5-deazaFAD was covalently bound to the protein, mutant holoenzymes were denatured with 3.0 M guanidine hydrochloride and then subjected to ultrafiltration (Centricon YM-30 concentrator) or gel filtration on a G-25 column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 3.0 M guanidine hydrochloride, as indicated in the text.

RESULTS

Effect of His45 Mutations on Covalent Flavinylation and the Catalytic Properties of MSOX. The His45Ala and His45Asn mutants exhibited typical flavoprotein absorption spectra with two maxima in the visible region (Figure 2). With both mutants, the lower energy absorption band at 454 nm is virtually identical to that observed with wild-type MSOX. Differences are, however, observed in the vicinity of the higher energy band, especially at $\lambda < 350$ nm.

The His45 mutants were denatured with guanidine hydrochloride and then subjected to ultrafiltration to determine whether the flavin was covalently attached. In each case, flavin absorption was found exclusively in the retentate, an outcome indicative of covalently bound flavin. The His45Ala and His45Asn mutants exhibit specific activity values (calculated based on holoenzyme content) that are about 50% of that observed with wild-type MSOX (Table 1). The results show that His45 is not required for covalent flavinylation of MSOX. The data also indicate that His45 is not a catalytically essential residue.

Reconstitution of the Apoprotein Present in the Isolated Preparation of His45Ala. Quantitative analysis showed that the FAD content in the preparations of the His45Ala or His45Asn mutant was about 4-fold lower than observed with wild-type MSOX (Table 1). We have shown that the wild-type apoprotein readily undergoes covalent flavinylation upon incubation with FAD in an autocatalytic reaction that can be monitored by the appearance of catalytic activity (*21*). This suggested that the covalent flavin content of the isolated preparations of His45Ala or His45Asn might be augmented by in vitro reconstitution with FAD.

A 2-fold increase in activity was observed after incubation of the His45Ala mutant with 500 μM FAD for 3 h at room temperature. To determine whether the observed increase in activity was attributable to covalent flavinylation, a larger amount of the mutant protein was incubated with FAD, dialyzed, and then subjected to flavin analysis. In fact, the reconstituted preparation of the His45Ala mutant was found to contain about twice the amount of covalently bound FAD (0.56 mol/mol protein) as compared to the original (untreated) preparation (0.25 mol of FAD/mol of protein) (Table 1). Unlike His45Ala, the apoprotein present in preparations of the His45Asn mutant could not be reconstituted with FAD, an outcome that is attributed to misfolding and/or the instability of the mutant apoprotein.

The absorption spectrum of the FAD-reconstituted His45Ala preparation is very similar to that observed with wild-type MSOX (Figure 2). This outcome suggests that spectral differences observed before reconstitution, especially at $\lambda < 350$ nm, are probably associated with the substantial amount of apoprotein in the untreated preparation.

Mutation of Arg49 to Ala or Gln Blocks Covalent Flavin Incorporation. Mutation of Arg49 to Ala or Gln resulted in the isolation of catalytically inactive preparations that did not contain FAD, as judged by the absence of absorption in the visible region (Figure 2, inset). No activity was observed after various attempts to reconstitute the apoArg49Ala or apoArg49Gln preparations by incubation with FAD. The results indicate that Arg49 plays a critical role in the covalent flavinylation of wild-type MSOX.

We have previously shown that covalent flavinylation of MSOX can be blocked by mutation of Cys315, the covalent attachment site in the wild-type enyzme. The Cys315Ala mutant is isolated as a catalytically inactive apoprotein but is able to form a weak noncovalent complex with FAD ($K_d = 100$ μM). The noncovalent Cys315Ala·FAD complex can be generated in situ by including excess FAD in the catalytic assay and is found to exhibit ~14% of the activity observed with wild-type MSOX (*10*). To determine whether an analogous complex might be formed with apoArg49Ala or apoArg49Gln, the mutant apoproteins were assayed in the presence of 500 μM FAD. No activity was, however, detectable with either of these mutant apoproteins, unlike results obtained with apoCys315Ala under the same conditions.

Reconstitution of apoArg49Ala with Reduced 5-DeazaFAD. Two possible reasons might account for the failure of FAD to stimulate activity when added to assays with apoArg49Ala or apoArg49Gln: (i) replacement of Arg49 with a neutral residue not only blocks covalent flavinylation but also prevents noncovalent binding of FAD; (ii) the Arg49 mutant apoproteins do bind FAD, but the resulting noncovalent complexes exhibit little or no sarcosine oxidase activity. A method to discriminate between these two

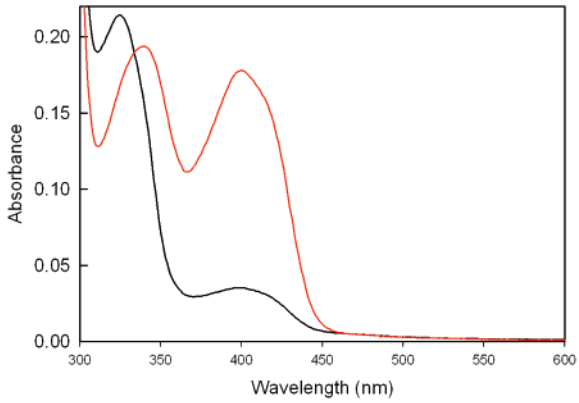


FIGURE 3: Reconstitution of apoArg49Ala with reduced 5-deazaFAD and reaction of the reconstituted enzyme with sarcosine imine. The black curve is the absorption spectrum of the reconstituted enzyme in 50 mM Tris-HCl buffer, pH 8.0, at 25 °C. The red curve was recorded after mixing the enzyme (350 μL) with 10 μL of an imine stock solution, prepared by incubating 2.7 M glycine with 370 mM formaldehyde for 5 min at 25 °C.

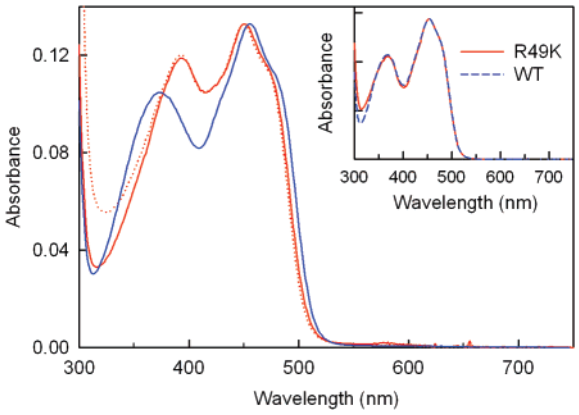


FIGURE 4: Spectral properties of the Arg49Lys mutant. Absorption spectra recorded for the mutant as originally isolated and after reconstitution with FAD are shown by the dotted and solid red lines, respectively. Spectra of the mutant preparations and wild-type MSOX (blue curve) are normalized to the same absorbance at their respective maxima (λ_{max} = 450 and 454 nm, respectively). All spectra were measured in 50 mM Tris-HCl buffer, pH 8.0, at 25 °C. The inset shows absorption spectra obtained after denaturation of the reconstituted preparation of the Arg49Lys mutant or wild-type MSOX with 3 M guanidine hydrochloride.

hypotheses was suggested by results obtained with the Cys315Ala mutant. Unlike for oxidized FAD, apoCys315Ala exhibits a high affinity for reduced flavin and forms a stable complex with reduced 5-deazaFAD (Cys315Ala·dFADH$_2$, $K_d \leq \sim$10 nM) (*10*).

Accordingly, we sought to determine whether a similar complex might be formed with apoArg49Ala and reduced 5-deazaFAD. Indeed, a stable complex was formed with apoArg49Ala, as judged by the absorption spectrum of the isolated reconstituted enzyme (Figure 3). The Arg49Ala·dFADH$_2$ complex exhibits an intense band at 325 nm, a feature characteristic of reduced 5-deazaFAD. The preparation also exhibited a second weaker band at 400 nm due to the presence of a small amount of oxidized 5-deazaFAD. Reaction of the reconstituted enzyme with sarcosine imine resulted in a dramatic increase in absorbance at 400 nm and a bathochromic shift of the 325 nm band to 340 nm (Figure 3). The results show that the Arg49Ala·dFADH$_2$ complex is readily oxidized by sarcosine imine and therefore functions as a sarcosine imine reductase, similar to that observed with Cys315Ala·5-dFADH$_2$ (*10*).

The total 5-deazaFAD content of Arg49Ala·dFADH$_2$ was determined after oxidation of the reconstituted enzyme with sarcosine imine, followed by denaturation with guanidine hydrochloride. The observed stoichiometry of 5-deazaflavin incorporation (0.33 mol of 5-deazaFAD/mol of protein) is about 50% of that reported with apoCys315Ala (0.63 mol of 5-deazaFAD/mol of protein) (*10*). The preparation of Arg49Ala·dFADH$_2$ contained about 20% oxidized 5-deazaFAD, as judged by comparison of the absorbance observed at 400 nm before and after reaction with sarcosine imine. On the basis of the total 5-deazaFAD content, this corresponds to about 0.07 mol of oxidized 5-deazaFAD per mol of protein.

To determine whether the coenzyme was covalently attached, Arg49Ala·dFADH$_2$ was oxidized with sarcosine imine, denatured, and then subjected to gel filtration on a G-25 column in the presence of guanidine hydrochloride. The protein eluate did not contain any 5-deazaFAD, indicating the absence of a covalent linkage between the coenzyme and the protein, as observed with Cys315Ala·5-dFADH$_2$ (*10*). In a separate experiment, a second aliquot of Arg49Ala·dFADH$_2$ was reacted with sarcosine imine, and the intact protein was subjected to gel filtration on a G-25 column under nondenaturing conditions. Under these conditions, only a small amount of oxidized 5-deazaFAD coeluted with the protein (0.07 mol of 5-deazaFAD/mol of protein). The results indicate that the oxidized 5-deazaFAD generated by the reaction of Arg49Ala·dFADH$_2$ with sarcosine imine is weakly bound to apoArg49Ala, similar to results obtained with the Cys315Ala mutant (*10*). However, a small fraction of the apoprotein in the Arg49Ala preparation appears to have formed a stable complex with oxidized 5-deazaFAD. The basis for the microheterogenity observed with the apoArg49Ala preparation is unclear.

Does a Conservative Mutation of Arg49 Block Covalent Flavinylation of MSOX? Replacement of Arg49 with Lys yields a yellow, flavin-containing preparation that exhibits two absorption bands in the visible region (Figure 4), unlike the apoproteins obtained upon mutation of Arg49 to Gln or Ala (Figure 2, inset). The lower energy band observed with the Arg49Lys mutant (λ_{max} = 450 nm) is only slightly shifted as compared to wild-type MSOX (λ_{max} = 454 nm). However, the higher energy band in the mutant spectrum (λ_{max} = 393 nm) exhibits a dramatic 20 nm bathochromic shift as compared to wild-type MSOX (λ_{max} = 373 nm). Interestingly, the absorption spectrum obtained after denaturation of the Arg49Lys mutant with guanidine hydrochloride is virtually identical to that observed with denatured wild-type MSOX (Figure 4, inset). The results indicate that the striking spectral difference observed under nondenaturing conditions is attributable to a change in the active site environment in the mutant enzyme. The yellow color of the Arg49Lys mutant was found in the retentate when the sample was denatured and then subjected to ultrafiltration. The results show that the Arg49Lys mutant contains covalently bound flavin. We conclude that a basic residue, such as Arg or Lys, at position 49 is required for autocatalytic flavinylation of apoMSOX. Although the Arg49 to Lys mutation did not prevent flavin incorporation, the mutation did result in a substantial decrease in catalytic activity. The specific activity

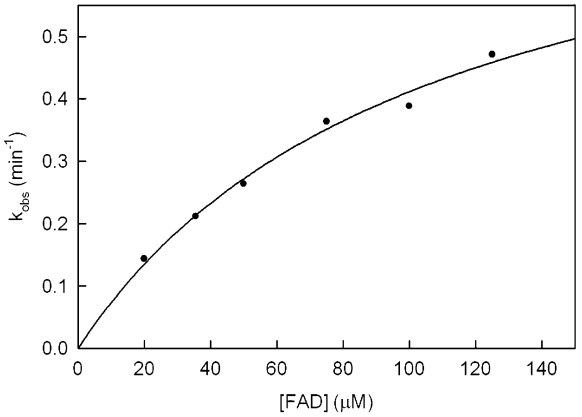


FIGURE 5: Effect of FAD concentration on the observed rate of covalent flavinylation of the apoenzyme present in the isolated preparation of Arg49Lys. The reactions were conducted in 50 mM Tris HCl buffer, pH 8.0, at 23 °C. Values for k_{obs} (solid circles) were determined by monitoring the time-dependent increase in catalytic activity. Enzyme activity was measured at 23 °C by using a horseradish peroxidase-coupled assay with *o*-dianisidine as the chromogenic substrate, as previously described (*31*). The solid line was obtained by fitting a theoretical curve (eq 3) to the data.

of the Arg49Lys mutant, calculated based on the holoenzyme content, is 4% of that observed with wild-type enzyme (Table 1).

Reconstitution of the Apoprotein Present in the Isolated Preparation of Arg49Lys. The extent of FAD incorporation into the Arg49Lys mutant (0.24 mol/mol of protein) is about 4-fold lower than wild-type MSOX. To determine whether the apoprotein in the Arg49Lys preparation was able to undergo autocatalytic flavinylation, the protein was incubated with excess FAD for 3 h at room temperature. Indeed, in vitro reconstitution was observed in a reaction that increased the covalent flavin content of the Arg49Lys preparation to a value that is nearly 70% of that observed with wild-type MSOX (Table 1). Reconstitution with FAD had virtually no effect on the visible absorption spectrum (Figure 4) or specific activity (calculated based on the holoenzyme content) of the mutant enzyme (Table 1).

The kinetics of flavinylation of the apoprotein in the Arg49Lys preparation were monitored by following the increase in catalytic activity that was observed upon incubating the protein with various concentrations of FAD at 23 °C. The reactions observed with 20−125 μM FAD were all found to exhibit apparent first-order kinetics. The observed rate of reconstitution exhibits a hyperbolic dependence on FAD concentration (Figure 5). The results are consistent with a mechanism involving the initial rapid binding of FAD to form a noncovalent complex (eq 1), followed by formation of a covalent linkage at Cys315 (eq 2), as observed with wild-type apoMSOX (*10*)

$$E + FAD \overset{K_d}{\rightleftharpoons} E{\cdot}FAD \quad (1)$$

$$E{\cdot}FAD \xrightarrow{k_{lim}} E{-}FAD\ (covalent) \quad (2)$$

Values for the apparent dissociation constant of the noncovalent complex of the Arg49Lys mutant with FAD (K_d = 106 ± 20 μM) and the limiting rate of covalent flavinylation (k_{lim} = 0.85 ± 0.09 min^{-1}) were estimated by fitting the data to eq 3

$$k_{obs} = k_{lim}[FAD]/(K_d + [FAD]) \quad (3)$$

These values must, however, be regarded as approximations because the reaction was too fast to monitor at higher concentrations of FAD and most of the experimental points are below the estimated K_d value, a problem not encountered with the wild-type apoprotein (*10*). With this caveat in mind, the stability of the noncovalent FAD complex with apoArg49Lys appears to be identical to that observed with wild-type apoMSOX. The limiting rate of covalent flavinylation appears to be about 3-fold faster with the mutant apoprotein (Table 3).

Table 3: Kinetics of Reconstitution of apoMSOX Preparations with FAD[a]

	K_d (μM)	k_{lim} (min^{-1})
apoArg49Lys	106 ± 20	0.85 ± 0.09
wild-type apoMSOX	100 ± 20	0.26 ± 0.02

[a] Dissociation constant of the initial noncovalent complex with FAD (K_d) and the limiting rate of covalent flavinylation (k_{lim}) were determined as described in the Figure 5 caption. The data for wild-type apoMSOX were taken from Hassan-Abdallah et al. (*10*).

DISCUSSION

In this investigation, we examined the potential role of His45 and Arg49 in covalent flavin attachment to Cys315 in MSOX. Mutation of His45 to Ala or Asn results in the production of mutant holoenzymes that contain covalently bound FAD and exhibit catalytic properties similar to wild-type MSOX. The results rule out a significant role for His45 in covalent flavinylation or sarcosine oxidation. In contrast, mutation of Arg49 to Ala or Gln results in the isolation of catalytically inactive preparations that do not contain FAD. A stable noncovalent complex is, however, formed upon reconstitution of apoArg49Ala with reduced 5-deazaFAD and exhibits properties similar to those previously observed for the complex of the reduced flavin with apoCys315Ala (*10*). The results show that the elimination of a basic residue at position 49 blocks covalent flavinylation but does not prevent noncovalent flavin binding.

Conservative mutation of Arg49 to Lys results in the production of an enzyme that contains covalently bound FAD. The FAD content in the isolated mutant enzyme is about 4-fold lower than observed with wild-type MSOX. However, substantial reconstitution of the apoprotein in the Arg49Lys preparation could be achieved in a reaction that increased the covalent flavin content to a value that is 70% of that observed with the wild-type enzyme. In vitro reconstitution of apoArg49Lys proceeds via the same mechanism and exhibits kinetic parameters similar to those observed for flavinylation of wild-type apoMSOX. Although covalent flavinylation is scarcely affected, the specific activity of the Arg49Lys mutant is only 4% of that observed with wild-type MSOX. The results show that a basic residue at position 49 is essential for covalent flavinylation of MSOX and suggest that Arg49 plays an important role in sarcosine oxidation as well. The catalytic role of Arg49 will be addressed in a separate communication.

How Does Arg49 Function in Covalent Flavinylation? Atom NH_2 of Arg49 is within a hydrogen bonding distance of the sulfur of Cys315 (3.6 Å). Hydrogen bond and electrostatic interactions between side chains of Arg49 and Cys315 are expected to raise the pK_a value for ionization of the guanidinium moiety of Arg49 and to lower the pK_a value for ionization of the sufhydryl group of Cys315. These interactions will thus shift the equilibrium in favor of the thiolate form of Cys315 ($Cys_{315}S^-$) and the positively charged guanidinium form of Arg49. Thus, interaction with Arg49 serves to stabilize $Cys_{315}S^-$, the form required for Cys315 to function as a base that abstracts a proton from the 8α-methyl group of FAD and a nucleophile that attacks the 8α-methenyl group in the iminoquinine intermediate (Scheme 1, steps 1 and 3, respectively). The van der Waals contact of the positively charged side chain of Arg49 with the *si*-face of the flavin ring is also expected to lower the pK_a value for ionization of the flavin 8α-methyl group by electrostatic interaction with the anionic iminoquininone intermediate. Finally, the positively charged guanidinium moiety of Arg49 may help to regenerate $Cys_{315}S^-$ (Scheme 1, step 2) by acting as the first component of a relay system that transports a proton from $Cys_{315}SH$ to bulk solvent (Figure 1).

The postulated roles for Arg49 in MSOX flavinylation are consistent with the total absence of covalent flavin incorporation when the residue is mutated to a neutral amino acid. On the other hand, covalent flavinylation is scarcely affected when Arg49 is conservatively mutated to Lys. The crystal structure determined for the Arg49Lys mutant shows that its overall structure is very similar to wild-type MSOX, the side chain of Lys49 occupies a position similar to that observed for Arg49 in wild-type MSOX, and the postulated proton relay system on the *si*-face of the flavin has not been disrupted (Jorns, M. S., and Mathews, F. S, unpublished results). The latter feature is compatible with a role for Lys49 in regenerating the thiolate form of Cys315, as proposed for Arg49 in the wild-type enzyme. Interaction between Lys49:NZ and Cys315:SG in the noncovalent Lys49Arg49·FAD complex is expected to raise and lower the pK_a values of the ionizable groups in Lys49 and Cys, respectively, stabilizing $Cys_{315}S^-$ and the protonated ϵ-amino group of Lys49. The van der Waals contact between the flavin ring and the positively charged side chain of Lys49 is likely to stabilize the anionic iminoquininone intermediate in the noncovalent complex, as proposed for Arg49 in wild-type MSOX.

Arg49 is highly conserved in other members of the MSOX family of amine oxidases that contain covalent flavin. These family members include nikD (Arg50) (*26*), *N*-methyltryptophan oxidase (Arg48) (*27*, *28*), and pipecolate oxidase (Arg53) (*29*). Interestingly, Arg49 is not conserved in the complex heterotetrameric sarcosine oxidase ($\alpha\beta\gamma\delta$), where FAD is bound noncovalently to the enzyme's β-subunit (*9*, *30*).

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