Structure of the Flavocoenzyme of Two Homologous Amine Oxidases: Monomeric Sarcosine Oxidase and *N*-Methyltryptophan Oxidase†

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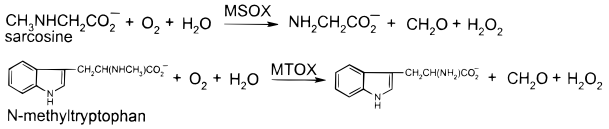
ABSTRACT: Monomeric sarcosine oxidase (MSOX) and *N*-methyltryptophan oxidase (MTOX) are homologous enzymes that catalyze the oxidative demethylation of sarcosine (*N*-methylglycine) and *N*-methyl-L-tryptophan, respectively. MSOX is induced in various bacteria upon growth on sarcosine. MTOX is an *E. coli* enzyme of unknown metabolic function. Both enzymes contain covalently bound flavin. The covalent flavin is at the FAD level as judged by electrospray mass spectrometry. The data provide the first evidence that MTOX is a flavoprotein. The following observations indicate that 8α-(*S*-cysteinyl)FAD is the covalent flavin in MSOX from *Bacillus sp. B-0618* and MTOX. FMN-containing peptides, prepared by digestion of MSOX or MTOX with trypsin, chymotrypsin, and phosphodiesterase, exhibited absorption and fluorescence properties characteristic of an 8α-(*S*-cysteinyl)flavin and could be bound to apo-flavodoxin. The thioether link in the FMN-containing peptides was converted to the sulfone by performic acid oxidation, as judged by characteristic absorbance changes and an increase in flavin fluorescence. The sulfone underwent a predicted reductive cleavage reaction upon treatment with dithionite, releasing unmodified FMN. Cys315 was identified as the covalent FAD attachment site in MSOX from *B. sp. B-0618*, as judged by the sequence obtained for a flavin-containing tryptic peptide (GAVCMYT). Cys315 aligns with a conserved cysteine in MSOX from other bacteria, MTOX (Cys308) and pipecolate oxidase, a homologous mammalian enzyme known to contain covalently bound flavin. There is only one conserved cysteine found among these enzymes, suggesting that Cys308 is the covalent flavin attachment site in MTOX.

Monomeric sarcosine oxidase (MSOX[1]) (*1*−*4*) and *N*-methyltryptophan oxidase (MTOX) (*5*) are members of a recently recognized family of eukaryotic and prokaryotic enzymes that catalyze similar oxidative reactions with various secondary or tertiary amino acids. Other family members include pipecolate oxidase (PIPOX) (*6*, *7*), heterotetrameric sarcosine oxidase (TSOX) (*4*), and possibly two mitochondrial dehydrogenases, sarcosine (SDH) and dimethylglycine dehydrogenase (DMGDH) (*4*, *8*, *9*).[2] MSOX (44 kDa) and MTOX (42 kDa) are the closest homologues in this family, exhibiting 43% sequence identity. Both enzymes catalyze the oxidative demethylation of *N*-methyl amino acids using oxygen as electron acceptor.



Despite the sequence similarity with MSOX, sarcosine is a very poor substrate for MTOX which exhibits a preference for aromatic or large aliphatic *N*-methylated amino acids (*5*). MSOX is induced in various soil microorganisms upon growth with sarcosine as the source of carbon and energy (*1*). MTOX is encoded by the *E. coli solA* gene which was isolated based on sequence homology with MSOX (*5*). Unlike sarcosine, the preferred substrates of MTOX are not common metabolites. The metabolic roles of MTOX in *E. coli* and its physiological substrate are unknown. Nevertheless, MTOX is more efficient in oxidizing *N*-methyltryptophan ($k_{cat}/K_m = 1.07 \times 10^7$ M^{-1} min^{-1})[3] than is MSOX in oxidizing sarcosine ($k_{cat}/K_m = 6.25 \times 10^5$ M^{-1} min^{-1})[4] under similar conditions (25 °C, air-saturated solution).

All members of this amine oxidoreductase family have previously been shown to contain covalently bound flavin, except for MTOX where no information was available regarding coenzyme content. The covalent FAD in SDH and

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[1] Abbreviations: MSOX, monomeric sarcosine oxidase; MTOX, *N*-methyltryptophan oxidase; PIPOX, pipecolate oxidase; TSOX, heterotetrameric sarcosine oxidase; SDH, sarcosine dehydrogenase; DMGDH, dimethylglycine dehydrogenase; FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MAO, monoamine oxidase; FCSD, flavocytochrome *c* sulfide dehydrogenase;

[2] M. Eschenbrenner and M. S. Jorns, unpublished results.

[3] P. Khanna and M. S. Jorns, unpublished results.

[4] M. A. Wagner and M. S. Jorns, unpublished results.

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DMGDH is attached to a histidyl residue near the NH_2-terminus via an 8α-N^3-histidyl linkage (*8*−*10*).[2] TSOX, the most complicated family member, contains four different subunits and two noncovalently bound coenzymes (FAD, NAD^+) (*4*, *11*). MSOX exhibits modest sequence homology with the β subunit of TSOX (βTSOX, 44 kDa) (23% identity). The covalent flavin in TSOX is at the FMN level and is attached to a histidyl residue near the middle of the β subunit via the same type of linkage found in SDH and DMGDH (*12*, *13*). PIPOX (44 kDa), a mammalian enzyme, exhibits 30% identity with MSOX. Its covalent flavin has not been characterized. Studies with MSOX from two microorganisms have shown that the covalent flavin is present at the FAD level but the nature of the covalent linkage was not determined (*13*). In this paper we show that the covalent flavin in MSOX from *Bacillus sp. B-0618* and *E. coli* MTOX is present at the FAD level and attached to a conserved cysteine near the COOH-terminus via an 8α-*S*-cysteinyl linkage.

EXPERIMENTAL PROCEDURES

Materials. Ammonium carbonate, carbenicillin, ethyl ether, FMN, formic acid, hydrogen peroxide, TCA, Sephadex G-25, trypsin, α-chymotrypsin type II, *N*-methyl-L-tryptophan, *o*-dianisidine, and horseradish peroxidase were from Sigma. Sodium dithionite was obtained from Fisher. Sarcosine was purchased from Aldrich. IPTG was from USB. Tryptone and yeast extract were from Difco. YM30 membranes and microconcentrators (Microcon-3, Microcon-10, Centricon-3) were from Amicon. Slide-A-Lyzer cassettes (3 and 10 kDa MWCO) were from Pierce. DEAE Sepharose CL-6B and phenyl-Sepharose CL-4B were obtained from Pharmacia. Apoflavodoxin from *Desulfovibrio vulgaris* was a generous gift from Stephen G. Mayhew (University College Dublin).

Cell Strains and Plasmids. *E. coli* DH1 was obtained from the American Type Culture Collection (ATCC 33849). Plasmid pOXI103 is a derivative of pBR322 containing a 5.3 kb *Eco*RI−*Cla*I fragment of *Bacillus sp. B-0618* DNA. It was obtained as a gift from Dr. Masanori Sugiyama (Institute of Pharmaceutical Sciences, Faculty of Medicine, Hiroshima University). A 1.6 kb *Xho*I−*Bgl*II fragment from pOXI103 that contained the gene coding for MSOX (*sox*) (*3*) was cloned into the *Bam*HI/*Sal*I sites of pUC119. The resulting construct, pMAW, placed the *sox* gene under the control of the *lac* promoter and was used to transform *E. coli DH1*. Plasmid pESOX1 is a derivative of the vector pUTE200 with the *solA* gene inserted downstream from the *lacUV5* promoter (*5*). *E. coli* JM109 containing pESOX1 was obtained as a gift from Dr. Haruo Ohmori (Kyoto University).

Activity and Protein Assays. MSOX activity was determined in a discontinuous assay based on the amount of formaldehyde produced. MSOX (≤0.5 μg of pure enzyme) was added to assay mixtures containing 120 mM sarcosine and 60 mM potassium phosphate, pH 7.7, in a final volume of 0.5 mL. Reaction mixtures were incubated for 3 min at 37 °C and then quenched by addition of 0.5 mL of 0.5 N acetic acid. Total formaldehyde was determined by the method of Nash (*14*). A unit of MSOX activity is defined as the formation of 1 μmol of formaldehyde/min. *N*-Methyltryptophan oxidase activity was measured in a continuous coupled assay using horseradish peroxidase. Assays were initiated by adding MTOX (∼150 ng of pure enzyme) to 500 μL reaction mixtures containing 1 mM *N*-methyltryptophan, 320 μM *o*-dianisidine, and 2.6 units of horseradish peroxidase in 50 mM potassium phosphate buffer, pH 8. Reaction progress at 25 °C was monitored by following the increase in absorbance at 460 nm due to the formation of oxidized *o*-dianisidine ($\epsilon_{460} = 6770\ M^{-1}\ cm^{-1}$). A unit of MTOX activity is defined as the formation of 1 μmol of H_2O_2/min. During enzyme purification, MSOX and MTOX protein concentrations were generally determined using the Bio-Rad micro protein assay with bovine serum albumin as standard. In the case of MTOX, 1 unit of absorbance at 280 nm was found to be equivalent to 0.46 mg of protein as determined by the Bio-Rad assay. In some cases, this conversion factor was used to estimate protein concentration based on the absorbance at 280 nm. A different method to determine protein was used when estimating the stoichiometry of flavin incorporation (vide infra).

Purification of Recombinant MSOX. DH1/pMAW was grown at 37 °C in LB media containing carbenicillin (100 μg/mL) to maintain the plasmid; 5 mL overnight cultures, started from a single colony of freshly transformed cells, were used to innoculate 500 mL cultures in 2 L flasks. A total of 3 L of culture was grown in a shaking incubator at 37 °C and 250 rpm. After approximately 4 h, at an $A_{600} \sim 0.5$, expression of MSOX was induced by addition of IPTG (48 μg/mL). After 18−20 h, cells were harvested, washed, and lysed similar to methods described by Chlumsky et al. (*15*). All purification procedures were performed at 4 °C under yellow light by a procedure similar to the initial steps used to purify the natural enzyme from *Bacillus sp. B-0618* (*16*). The lysate was centrifuged for 30 min at 34860*g* to remove cell debris. The cleared lysate was subjected to ammonium sulfate fractionation. The yellow 50−70% ammonium sulfate pellet was redissolved in 10 mM potassium phosphate, pH 7.5 (buffer A), containing 50 mM KCl. The sample was desalted on a Sephadex G25 column (2.5 × 58 cm) equilibrated in buffer A containing 50 mM KCl. Fractions absorbing at 450 nm were pooled and applied to a 5 × 10.5 cm DEAE Sepharose CL-6B column equilibrated with the same buffer. After loading, the column was washed with 1 column volume of buffer A containing 50 mM KCl, followed by 1 column volume of buffer A containing 100 mM KCl. A 5 column volume linear gradient from 0.10 to 0.50 M KCl in buffer A was applied. MSOX eluted between 0.35 and 0.42 M KCl. Fractions with an A_{280}/A_{450} ratio less than 10 were pooled and concentrated on an Amicon concentrator using a YM30 membrane. The DEAE Sepharose CL-6B column was regenerated by washing with 2 M KCl, and then reequilibrated with buffer A containing 50 mM KCl. The concentrated enzyme pool was diluted with buffer A and reapplied to the regenerated DEAE Sepharose CL-6B. The column was developed, as described above. MSOX eluted at the same KCl concentration. Fractions with an A_{280}/A_{450} ratio less than 6.0 were pooled, concentrated, and dialyzed versus buffer A containing 50 mM KCl. Purified enzyme was stored at −20 °C.

Purification of Recombinant MTOX. JM109/pESOX1 were grown in LB medium containing carbenicillin (100 mg/mL) at 37 °C. Single colonies of JM109 cells, freshly transformed with the plasmid pESOX1, were used to inoculate a 100 mL

starter culture. After overnight growth, 10 mL of starter culture was added to 500 mL of medium in each of six 2-L flasks. The cultures were incubated with shaking (250 rpm). IPTG (125 mg/mL) was added to induce MTOX expression when the cells achieved a density $A_{600} \sim 0.6$. Incubation was continued overnight to a final $A_{600} \sim 6.0$. Cells were harvested by centrifugation at 8200*g* for 15 min at 4 °C. The cells were washed with phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.115% NaH_2PO_4, 0.02% KH_2PO_4) and recentrifuged, and the bright yellow cell pellet (~11 g) was stored at −20 °C. Enzyme purification was conducted at 4 °C under yellow light. Cells were thawed in lysis buffer and disrupted by sonication as previously described (*15*). Cell debris was removed by centrifugation (32000*g*) to yield a cell lysate which was subjected to stepwise ammonium sulfate fractionation. Most of the enzyme was recovered in the 50−75% ammonium sulfate precipitate which exhibited a characteristic flavin spectrum when dissolved in 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA (buffer B). This fraction was desalted on a Sephadex G-25 column (2.8 × 64 cm) equilibrated with buffer B and then applied to a DEAE Sepharose CL-6B (5 × 13 cm) column equilibrated with buffer B. The column was washed with 1 column volume each of 50 and 100 mM KCl in buffer B and then 2 column volumes of 150 mM KCl in buffer B. MTOX was eluted with a linear KCl gradient (200−500 mM KCl in buffer B, 6 column volumes). Fractions were pooled based on the A_{280}/A_{450} ratio, concentrated on an Amicon assembly using a YM-30 membrane, and dialyzed versus buffer B. In some preparations, pure MTOX was obtained at this stage, exhibiting an A_{280}/A_{450} ratio ≤7.5 and a single band on 12% SDS−PAGE. If not pure, ammonium sulfate was added to a final concentration of 30% by mixing the enzyme in buffer B with a concentrated ammonium sulfate solution in buffer B. The sample was then applied to a phenyl-Sepharose CL-4B column (2.8 × 13 cm) equilibrated with buffer B containing 30% ammonium sulfate. The column was washed with a 30−0% linear gradient of ammonium sulfate in buffer B (5 column volumes). MTOX was eluted with a 0−50% linear gradient of ethylene glycol in buffer B (5 column volumes). Fractions were pooled, concentrated, dialyzed versus buffer B, and stored at −80 °C.

Spectroscopy. Absorption spectra were recorded using a Perkin-Elmer Lambda 2S spectrometer. Fluorescence measurements were made using a Perkin-Elmer LS 50 luminescence spectrophotometer with excitation at 445 nm and emission at 523 nm. The instruments were equipped with thermostated cell holders maintained at 25 °C.

The extinction coefficient of the flavin in MSOX was determined based on absorbance changes observed after denaturation at 25 °C in 5 mM potassium phosphate buffer, pH 7.5, containing 25 mM KCl plus 3.0 M guanidine hydrochloride or 4.5 M urea. A similar method was used with MTOX except that complete denaturation required heating for 5 min at 100 °C in buffer B containing 3 M guanidine hydrochloride or 4.5 M urea. Calculations were made using values determined for the extinction coefficient of free FAD in the presence of guanidine hydrochloride or urea (ϵ_{450} = 11 900 or 11 700 M^{-1} cm^{-1}, respectively). These values are slightly higher than observed for free FAD in the absence of denaturants (ϵ_{450} = 11 300 M^{-1} cm^{-1}) (*17*).

Extinction coefficients at 280 nm of denatured MSOX and MTOX in guanidine hydrochloride at neutral pH (ϵ_{280} = 36 270 and 50 780 M^{-1} cm^{-1}, respectively) were calculated based on the tryptophan and tyrosine content of the proteins as described by Gill and von Hippel (*18*) using the ProParam tool (http://www.expasy.ch/www/tools.html). To determine the stoichiometry of FAD incorporation in MSOX and MTOX, protein concentration was determined based on the absorbance at 280 nm after enzyme denaturation with 3.0 M guanidine hydrochloride as described above. The absorbance at 280 nm was corrected for the contribution expected for free FAD in guanidine hydrochloride (ϵ_{280} = 22 900 M^{-1} cm^{-1}).

Preparation and Analysis of a Tryptic Digest of MSOX. A 5 μL aliquot of a solution containing 10.3 mg/mL MSOX in 10 mM potassium phosphate buffer, pH 7.5, was mixed with 20 μL of 50 mM ammonium bicarbonate, pH 8.0 (AmBi). A 5 μL aliquot of 10 mM DTT in AmBi was added, and the sample was heated at 56 °C for 20 min in the dark. A 5 μL aliquot of 50 mM iodoacetamide in AmBi was added and the sample incubated at room temperature for 20 min in the dark. Promega-modified sequencing-grade trypsin (0.1 μg/μL) was added to the reduced and alkylated sample, and the mixture was digested overnight at 37 °C. The resulting peptides were separated using an HP 1090 HPLC system equipped with a Zorbax C_{18} reversed-phase column (1 mm × 150 mm) using the following elution program at 0.345 mL/min: 0−130 min, linear gradient from 100% solvent A, 0% solvent B to 67.5% solvent A, 32.5% solvent B; 130−158.7 min, 67.5% solvent A, 32.5% solvent B; 158.7−160.0 min, 44.5% solvent A, 55.5% solvent B (flow rate changed to 0.433 mL/min). Solvent A is 0.060% TFA in water. Solvent B is 0.055% TFA in acetonitrile. Two peaks absorbing at 454 nm were sequenced by Edman degradation on a PE/Applied Biosystems Procise 494HT protein sequencer. Sample digestion and analysis were conducted at the Harvard Microchemistry Laboratory.

Tryptic−Chymotryptic Flavin Peptide Preparations from MSOX and MTOX. All procedures were performed under yellow light. *D. vulgaris* apoflavodoxin was prepared according to the method described by Wassink and Mayhew (*19*). An aliquot of MSOX or MTOX (11 mg, ~250 nmol of flavin) was precipitated with 5% TCA and centrifuged at 10000*g* for 10 min. The yellow pellet was washed with 1% TCA and then resuspended in 1.2 mL of water. Residual TCA was extracted with ether (3 × 4 mL). Residual ether was blown off under argon. The sample was transferred to a glass ampule, and a concentrated aliquot of $(NH_4)_2CO_3$, pH 8.5, was added to a final concentration of 100 mM. The sample was purged with argon. Chymotrypsin and trypsin (0.1 mg of each/mg of flavoprotein) were added. The ampule was purged with argon again and flame-sealed. The sample was digested for 20 h at 37 °C and then stored at −20 °C overnight. Upon thawing, proteases were removed using a Centricon-3 microconcentrator. Phosphodiesterase (0.95 mg) was added to the filtrate, and the mixture was incubated at room temperature for 1.5 h. Phosphodiesterase was removed using a Centricon-3 microconcentrator. The filtrate was lyophilized using a Savant Speed-Vac concentrator. The glassy residue was dissolved in a minimum volume of 100 mM potassium phosphate, pH 6.0, containing 0.3 mM EDTA. Aliquots (4) containing ~10 nmol of flavin were

Table 1: Purification of Recombinant MSOX from *E. coli*

step	total activity (units)[a]	protein (mg)	specific activity (units/mg)	yield (%)	purification (*x*-fold)
lysate[b]	10800	535	20.2	100	1
$(NH_4)_2SO_4$	10100	380	26.5	94	1.3
Sephadex G-25	10700	378	28.3	99	1.4
DEAE #1	10200	246	41.4	94	2.1
DEAE #2	6160	132	46.7	57	2.3

[a] A unit of activity is defined as the formation of 1 μmol of formaldehyde/min at 37 °C under standard assay conditions. [b] The lysate was prepared from cells harvested from 3 L of culture.

mixed with apoflavodoxin (3.15 mg per aliquot), and the volume of each aliquot was adjusted to 663 μL by adding 100 mM potassium phosphate, pH 6.0, containing 0.3 mM EDTA. After 30 min at room temperature, the samples were cooled on ice. Unbound peptides were removed using a Microcon-10 microconcentrator and water washes (3 × 500 μL) of the retentate. The four retentates were combined. Apoflavodoxin was removed by precipitation with 5% TCA, and centrifugation at 10000*g* for 10 min. The yellow supernatant contained the purified flavin peptide preparation. TCA was removed by extraction with ether (3 × 2 mL), and residual ether was blown off under argon.

Performic Acid Oxidation and Reductive Cleavage of the Tryptic−Chymotryptic Flavin Peptide Preparations. Performic acid (4 μL of performic acid/nmol of flavin) was added to an aliquot of the flavin peptide preparation from MSOX (6.3 nmol) or MTOX (11.5 nmol). Samples were incubated on ice for 2 h, lyophilized using a Speed-Vac, and then redissolved in 350 μL of water, similar to that described by Walker et al. (*20*). Absorbance and fluorescence properties were recorded before and after performic acid oxidation. Anaerobic dithionite reduction of the performic acid oxidized peptides was conducted in 100 mM potassium phosphate, pH 7.0, as described in Edmondson and Singer (*21*). Samples were subjected to thin-layer chromatography before and after dithionite reduction using systems described by Jorns et al. (*22*).

Mass Spectrometry. Electrospray mass spectral analysis was performed by the Laboratory for Macromolecular Analysis at the Albert Einstein College of Medicine.

RESULTS AND DISCUSSION

Enzyme Purification. MSOX from *Bacillus sp. B-0618*, expressed in *E. coli* under the control of the *lac* promoter, constitutes about 50% of the protein in crude cell extracts which are yellow due to the large amount of recombinant flavoprotein. MSOX was purified by ammonium sulfate fractionation and ion exchange chromatography (Table 1). The purified enzyme exhibited a single band on SDS−PAGE and a characteristic flavoprotein absorption spectrum with maxima at 454, 372, and 275 nm and a shoulder around 475 nm ($A_{280}/A_{454} = 5.54$, $\epsilon_{454} = 12\,200$ M^{-1} cm^{-1}) (Figure 1, curve 1). The observed absorption maxima differ somewhat from values previously reported for the natural enzyme isolated from *B. sp. B-0618* (453, 360, and 276 nm) (*16*) or recombinant enzyme (445 and 366 nm) (*3*).

Yellow cell extracts were also obtained when MTOX was expressed in *E. coli* under the control of the *lacUV5* promoter (JM109/pESOX1). Homogeneous enzyme was generated

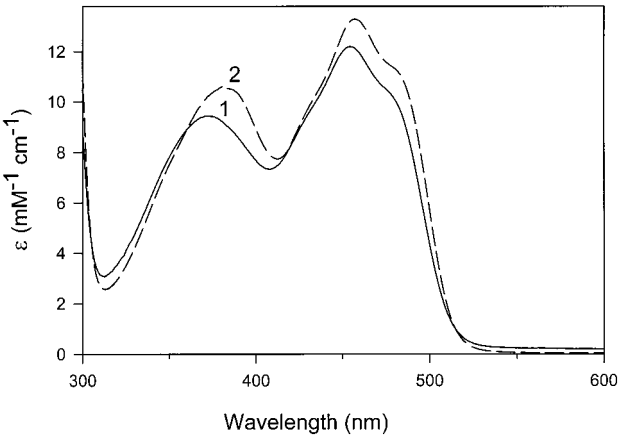


FIGURE 1: Absorption spectra of MSOX (curve 1) and MTOX (curve 2). Spectra were recorded at 25 °C in 50 mM potassium phosphate buffer, pH 8.0 (MSOX), or in the same buffer containing 1 mM EDTA (MTOX).

Table 2: Purification of Recombinant MTOX from *E. coli*

step	total activity (units)[a]	protein (mg)	specific activity (units/mg)	yield (%)	purification (*x*-fold)
lysate[b]	16000	1500	10.6	100	1
$(NH_4)_2SO_4$	8940	662	13.5	56	1.3
Sephadex G-25	8570	702	12.2	54	1.2
DEAE	6920	373	18.5	43	1.7
phenyl-Sepharose[c]	5900	163	36.2	37	3.4

[a] A unit of activity is defined as the formation of 1 μmol of H_2O_2/min at 25 °C under standard assay conditions. [b] The lysate was prepared from cells harvested from 3 L of culture. [c] A portion of the DEAE eluate (36%) was subjected to phenyl-Sepharose chromatography. Total protein and activity are normalized to those expected for the entire DEAE eluate.

after an approximate 3-fold purification procedure which involved ammonium sulfate fractionation and chromatography on DEAE and, when needed, phenyl-Sepharose (Table 2). (In some preparations, pure enzyme was obtained after the DEAE step, and the phenyl-Sepharose step was omitted). A characteristic flavoprotein spectrum was observed for purified MTOX which exhibited absorption maxima at 457, 382, and 275 nm and a shoulder at 480 nm ($\epsilon_{457} = 13\,300$ M^{-1} cm^{-1}, $A_{280}/A_{450} = 7.3$) (Figure 1, curve 2). The enzyme has not been previously isolated from any source. Our results provide the first evidence that MTOX is a flavoprotein, as expected based on the observed sequence homology of MTOX and MSOX and the similar amine oxidation reactions catalyzed by these enzymes.

Flavin Content of MSOX and MTOX. The flavin prosthetic groups in MSOX and MTOX were not released into solution after denaturation with 5% trichloroacetic acid. In each case, a yellow protein precipitate was obtained. The results show that the flavin in MTOX is covalently bound, similar to MSOX and other members of this amine oxidoreductase family. The absorbance of the denatured enzymes at 450 and 280 nm in 3 M guanidine hydrochloride was used to determine the flavin content, as detailed under Experimental Procedures. On this basis, MSOX and MTOX were found to contain 0.87 and 0.85 mol of covalently bound flavin, respectively, per mole of protein.

The molecular weights of MSOX and MTOX, determined by electrospray mass spectrometry, were more than predicted

Table 3: Electrospray Mass Spectral Analysis of MSOX and MTOX

	molecular weight $(M+H)^+$ (m/z)		
		predicted	
enzyme	observed	apoenzyme	FAD holoenzyme
MSOX	43839	43051	43834
MTOX	41684	40902	41688

based on the gene sequence alone but in excellent agreement with calculated values which include a contribution due to covalently bound FAD (Table 3). The presence of covalently bound FAD in MSOX from *B. sp. B-0618* is consistent with previous studies with MSOX from other bacteria where the covalent flavin was also shown to be present at the FAD level as judged by the release of AMP upon mild acid hydrolysis (*13*). The mass spectrometry data provided no evidence for the presence of apoenzyme in either preparation although a small amount was expected based on the observed flavin/protein stoichiometry values. The latter were determined by using the extinction coefficient of free FAD to estimate the concentration of covalently bound flavin in denatured samples of MSOX or MTOX. This assumption may not be strictly correct although previous studies have shown that the extinction coefficient of riboflavin is not affected by the introduction of a substituent similar to that contributed by the covalent flavin linkage in MSOX and MTOX (vide infra).

Identification of the Covalent Flavin Attachment Site in MSOX. MSOX, MTOX, and PIPOX exhibit about 20% identity with the NH_2-terminal half of SDH and DMGDH and contain a conserved histidine (His45 in MSOX) that aligns with the covalent FAD attachment site in these dehydrogenases (*8*−*10*).[2] To determine whether His45 was the site of flavin attachment in MSOX, an enzyme sample was reductively alkylated with iodoacetamide in the presence of DTT and then subjected to an overnight digestion with trypsin at 37 °C. The tryptic digest was chromatographed on a Zorbax C_{18} HPLC column. Elution profiles, obtained by monitoring the absorbance at 205 and 454 nm, revealed a number of peaks absorbing at 454 nm (Figure 2). Peak 70 was selected for sequencing since it exhibited a high value for the ratio A_{454}/A_{205}. The amino acid sequence obtained for peak 70 coincides with residues 312−318 in the sequence of MSOX (Table 4). No amino acid derivative was detected in the fourth cycle of the sequence which coincides with Cys315. The same sequence was found as a secondary component upon analysis of peak 71. (The other peaks absorbing at 454 nm were not analyzed but may reflect incomplete proteolysis, partial hydrolysis of the FAD phosphodiester linkage, and/or partial oxidation of the sulfur in Cys315.) The results show that the covalent flavin in MSOX from *B. sp. B-0618* is attached to a cysteinyl residue, Cys315, rather than the predicted histidyl linkage. Cys315 is conserved in MSOX from other microorganisms, MTOX and PIPOX (Figure 3), and is the only conserved cysteine found among these enzymes.

Identification of the Covalent Flavin Linkage in MSOX and MTOX. MSOX and MTOX were digested with trypsin and chymotrypsin under anaerobic conditions and then treated with phosphodiesterase to convert the covalent flavin to the FMN level. FMN-containing peptide(s) in the MSOX

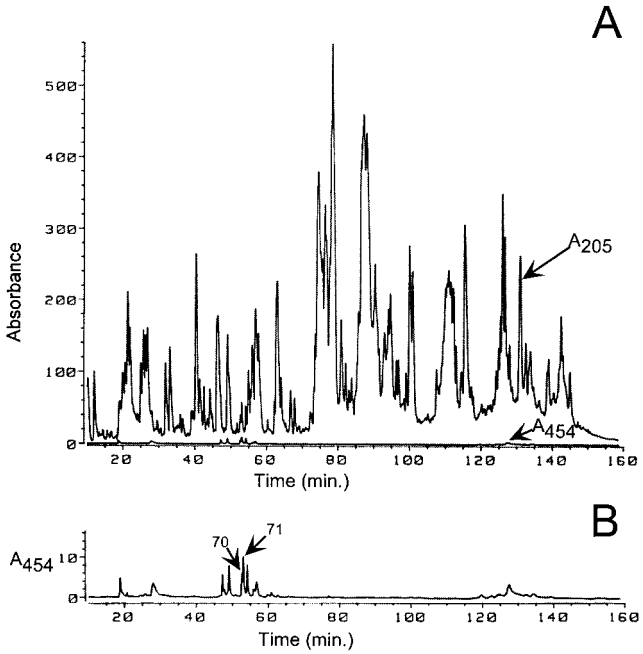


FIGURE 2: Reversed-phase chromatography of a tryptic digest of MSOX. Panel A shows the elution profiles obtained by monitoring absorbance at 205 and 454 nm. The latter profile is shown expanded in panel B.

Table 4: Identification of the Covalent Flavin Attachment Site in MSOX

sample	sequence
peptide[a]	
peak 70	GAV-mYs
peak 71	GAV-MYt
protein	^{310}KRGAVCMYTKT^{320}

[a] Amino acid residues shown in lower case letters indicate data assigned with low confidence. The secondary component in peak 71 exhibited the sequence shown. The sequence obtained for the primary component (SINRPALK) coincides with residues 374−381 in the sequence of MSOX.

and MTOX tryptic−chymotryptic digests were bound to apoflavodoxin, as judged by the quenching of flavin fluorescence (data not shown). After binding to apoflavodoxin, the yellow FMN-containing peptides were purified from colorless, unbound peptides by microfiltration. Apoflavodoxin was removed by precipitation with 5% TCA which released the FMN-containing peptides into solution. After removal of TCA by ether extraction, the resulting FMN peptide preparation from MSOX exhibited absorption maxima at 357 and 451 nm (Figure 4A, curve 1). Similar spectral properties were observed for the MTOX peptide preparation (λ_{max} = 363 and 450 nm) (Figure 4b, curve 1). In each case, a hypsochromic shift of the near-UV band is observed as compared with riboflavin (372 nm), a feature characteristic of 8α-substituted flavins (*20*, *23*−*25*).

There are two known types of covalent flavin linkages involving a cysteinyl residue. 6-*S*-Cysteinyl flavins, found in trimethylamine and dimethylamine dehydrogenase (*26*, *27*), are readily identified by their unusual absorption spectra. These flavins exhibit a single absorption band at λ >300 nm [λ_{max} = 437 nm for 6-(*S*-cysteinyl)riboflavin)] (*26*), unlike the typical two-banded flavin absorption spectrum in this region. A 6-*S*-cysteinyl linkage can be ruled out for MSOX and MTOX since the intact enzymes and the FMN

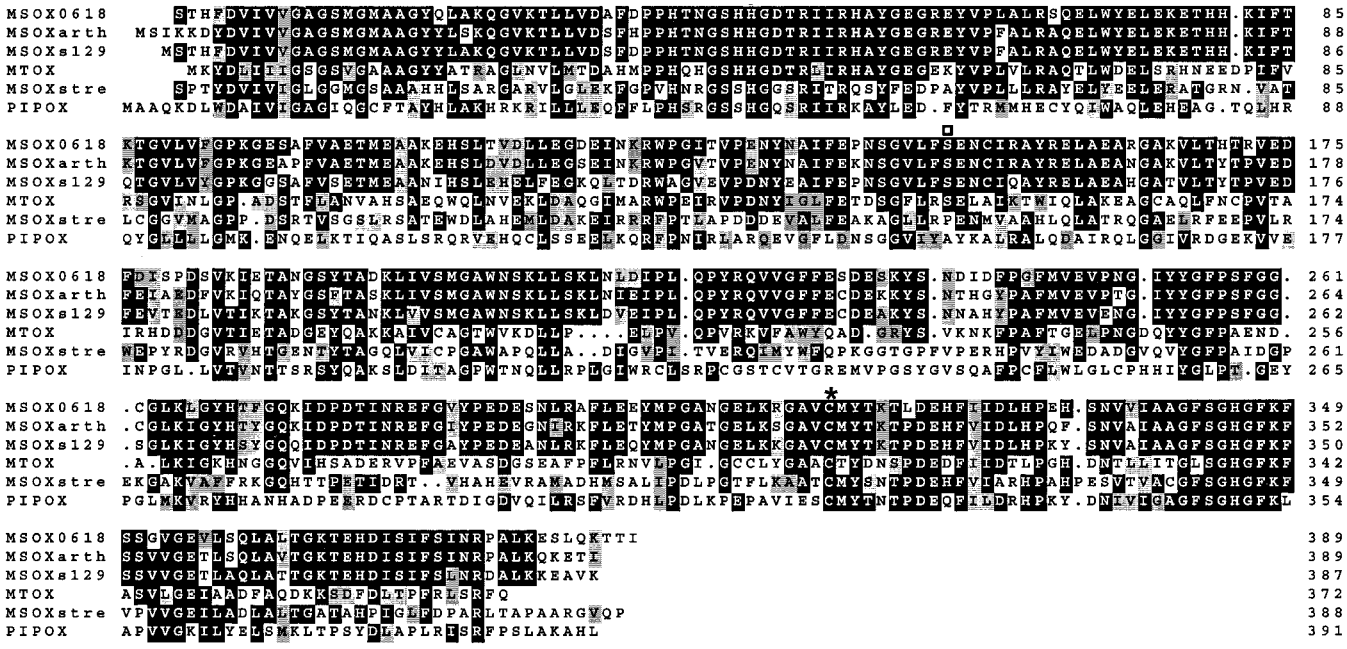


FIGURE 3: Multiple sequence alignment of MSOX from various sources [*Bacillus sp. B-0618* (MSOX0618), *Arthrobacter TE-1826* (MSOXarth), *Bacillus sp. NS-129* (MSOXs129), *Streptomyces sp. KB210-8SY* (MSOXstre)], MTOX from *E. coli*, and PIPOX from rabbit. The alignment was generated using the PILEUP program (*45*). The conserved cysteine (Cys315), identified as the covalent flavin attachment site in MSOX0618, is marked by an asterisk. The conserved histidine that aligns with the histidine covalent flavin attachment site in SDH and DMGDH is marked by an open circle (○). The conserved serine in MSOX0618 and MTOX that aligns with the histidine covalent flavin attachment site in βTSOX is marked by an open square (□).

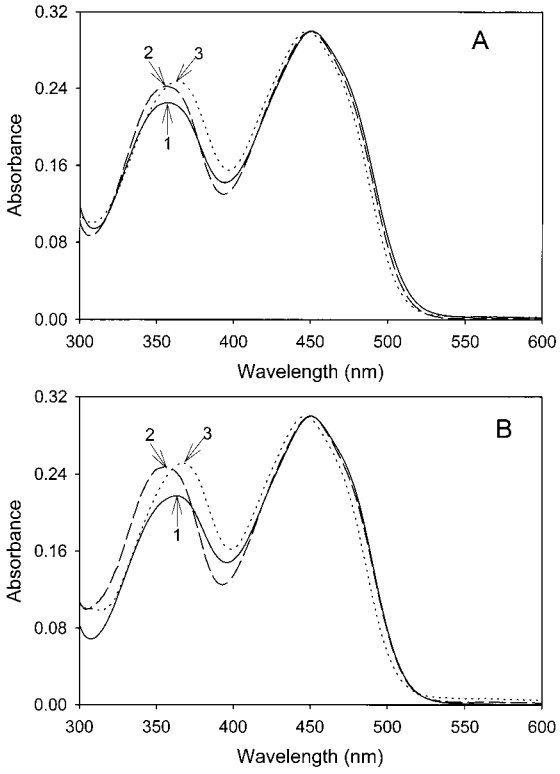
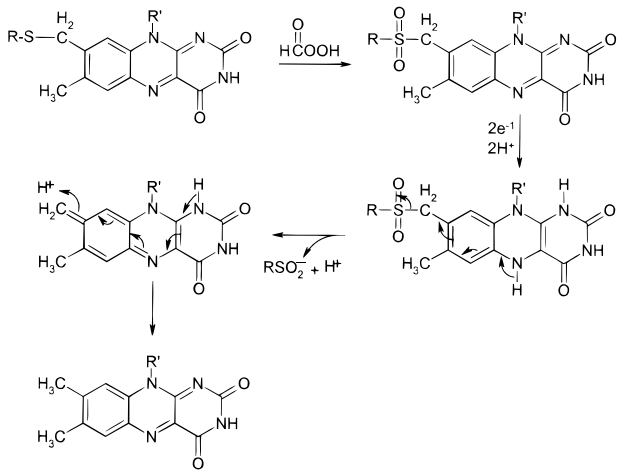


FIGURE 4: Effect of performic acid oxidation and dithionite on absorption spectra observed for the flavin peptide preparations from MSOX (panel A) and MTOX (panel B). Unless otherwise noted, spectra were recorded in water. Curve 1 (solid line), untreated peptide; curve 2 (dashed line), after performic acid oxidation; curve 3 (dotted line), after reaction of performic acid oxidized peptide with dithionite in 100 mM potassium phosphate, pH 7.0.

peptide preparations exhibit two absorption maxima at $\lambda > 300$ nm. 8α-*S*-Cysteinyl flavins are found in monoamine oxidase (MAO) A and B (*20*, *28*, *29*) and in flavocytochrome

Scheme 1



c sulfide dehydrogenase (FCSD) (*30*−*33*). These flavins exhibit two-banded visible absorption spectra and weak pH-independent fluorescence which can be increased upon performic acid oxidation of the thioether linkage to the sulfone (see Scheme 1).

The flavin fluorescence observed for the FMN peptide preparations from MSOX and MTOX was the same at pH 7.0 and 3.4, consistent with an 8α-*S*-cysteinyl linkage (Table 5). In contrast, covalent flavins with a histidyl linkage at the 8α position [8α-(N^3-histidyl) or 8α-(N^1-histidyl)] exhibit a characteristic increase in fluorescence below pH 7 (*23*, *24*). The FMN peptide preparations from MSOX and MTOX exhibited 30% and 17%, respectively, of the fluorescence observed with riboflavin. The results obtained with the MTOX peptide preparation agree fairly well with other 8α-*S*-cysteinyl flavin-containing peptides which typically exhibit ≤10% of the fluorescence observed with riboflavin (Table

Table 5: Spectral Properties of the Flavin Peptides from MSOX and MTOX and Other 8α-*S*-Cysteinyl-Substituted Flavins[a]

	fluorescence (%)[b]		absorbance				
			untreated		performic acid oxidized		
flavin	untreated	performic acid oxidized	λ_{max} (nm)	ratio[d]	λ_{max} (nm)	ratio[d]	reference
MSOX peptide	29.4 (30.8)[c]	53.7 (61.2)[c]	357, 451	0.75	353, 450	0.83	this paper
MTOX peptide	17.5 (17.4)[c]	51.8 (53.0)[c]	363, 450	0.72	353, 450	0.84	this paper
8α-*S*-cysteinylriboflavin	10	90	367, 448	0.71	354, 448	0.83	(*20*)
MAO peptide							
SGG**C**Y	10	70−80	367, 448	0.72	354, 448	0.84	(*20, 28*)
Chromatium FCSD peptides							
T**C**T	5	50	365, 451	0.72			(*30, 31*)
YT**C**T	1	5	366, 454	0.69			
Chlorobium FCSD peptide							
VT**C**PFSN	<6	70	366, 449	0.72	352, 452	0.77	(*33*)

[a] At neutral pH, unless otherwise indicated. [b] Relative to riboflavin. [c] Values in parentheses were obtained a pH 3.4. [d] Ratio = $\epsilon(\lambda_{max3xx})/\epsilon(\lambda_{max4xx})$.

5). The higher fluorescence observed for the MSOX peptide preparation and the hypsochromic shift of the near-UV absorption maximum as compared with other 8α-*S*-cysteinylflavins (Table 5) are probably due to partial oxidation during the aerobic apoflavodoxin purification step. A similar air sensitivity has been observed for the MAO (*20*) and FCSD (*30*) flavin peptides.

Performic acid oxidation of the MSOX or MTOX peptide preparations resulted in an approximately 2- or 3-fold increase in flavin fluorescence, respectively. Consequently, the oxidized MSOX and MTOX peptides exhibited similar fluorescence intensity relative to riboflavin (55−60%) (Table 5). The relative fluorescence observed for the oxidized peptides was somewhat lower than the sulfone derivative of 8α-S-cysteinylriboflavin (90%) (*20*). However, studies with synthetic (*34*) and naturally occurring peptides (Table 5) show that the fluorescence of the sulfone can vary depending on the structure of the peptide, particularly in the case of tyrosyl-containing peptides. Performic acid oxidation of the MSOX and MTOX peptides also resulted in an expected hypsochromic shift of the near-UV absorption band (curve 2 in Figure 4A,B). The absorption properties of the oxidized MSOX and MTOX peptide preparations are in good agreement with those reported for 8α-(*S*-cysteinylsulfone)riboflavin and various sulfone-containing peptides (Table 5).

A cleavage reaction is observed upon reduction of the sulfone derivative of 8α-*S*-cysteinylriboflavin with dithionite, yielding riboflavin and cysteine sulfinate as products (*21*) (see Scheme 1). Spectra observed after dithionite reduction of the performic acid-oxidized MSOX or MTOX peptides (curve 3 in Figure 4A,B) exhibited a 10 or 13 nm bathochromic shift, respectively, of the near-UV band and a 5 nm hypsochromic shift of the 450 nm band, as expected for release of unmodified FMN. Thin-layer chromatography showed that dithionite reduction generated a fluorescent compound which comigrated with authentic FMN.

Conclusions. MSOX from *Bacillus sp. B-0618* and MTOX from *E. coli* have been found to contain 1 mol of FAD, covalently bound via an 8α-*S*-cysteinyl linkage. The 8α-(*S*-cysteinyl)FAD in MSOX is attached to Cys315. Cys315 aligns with a conserved cysteine in MSOX from other bacteria, MTOX (Cys308) and PIPOX (Cys319), and is the only conserved cysteine found among these enzymes. The structure of the MSOX flavocoenzyme determined based on the biochemical studies described in this paper is in complete agreement with the very recently determined MSOX crystal structure (*35*). More than a dozen residues at or near the active site of MSOX, including a subset of five residues likely to bind sarcosine or assist in its oxidation, are conserved in MTOX and PIPOX. The results strongly suggest that Cys308 is the site of attachment of the 8α-(*S*-cysteinyl)-FAD identified in MTOX and that a similar linkage will be found for covalent flavin in PIPOX and MSOX from other bacteria.

The subset of five putative catalytic residues in MSOX are conserved in the β subunit of TSOX, a finding that might appear difficult to reconcile with the fact that the covalent flavin in TSOX is 8α-(N^3-histidyl)FMN (*13*) rather than 8α-(*S*-cysteinyl)FAD. However, the *noncovalently* bound FAD in TSOX is the flavin which accepts electrons from sarcosine which are then transferred to the covalent FMN where oxygen is reduced to hydrogen peroxide (*36*−*38*). Various observations suggest that the noncovalent FAD binds to βTSOX and acts as the structural equivalent of the covalent FAD in MSOX whereas the covalent FMN binds at an interface between βTSOX and another subunit (*35*).[2]

A conserved histidine in MSOX (His45), MTOX, and PIPOX aligns with the histidine that serves as the covalent FAD attachment site in SDH and DMGDH. Interestingly, although the conserved histidine in the oxidases does not serve the same function as in the dehydrogenases, His45 in MSOX is spatially near the covalent flavin linkage at Cys315 and may play a role in covalent flavinylation (*35*). An autocatalytic flavinylation mechanism with His45 acting as a proton acceptor has recently been proposed for MSOX (*35*), analogous to autocatalytic flavinylation mechanisms postulated for attachment of histidyl and tyrosyl residues to the 8α-methyl group of FAD in 6-hydroxynicotine oxidase and *p*-cresol methylhydroxylase, respectively (*39, 40*).

8α-(*S*-Cysteinyl)FAD has previously been found only in a few enzymes, mammalian MAO A and B (*20, 28, 29*) and the bacterial flavocytochrome FCSD (*30*−*33*). The presence of 8α-(*S*-cysteinyl)FAD in MSOX, MTOX, and probably PIPOX represents a significant increase in the number of enzymes containing this type of flavocoenzyme. FCSD exhibits an NH_2-terminal covalent flavin attachment site which is near an ADP-binding motif and catalyzes a different type of reaction, as compared with MSOX and its homologues (*41, 42*). In contrast, mammalian MAOs catalyze similar amine oxidation reactions and contain an NH_2-terminal ADP-binding motif and a COOH-terminal attachment site for 8α-(*S*-cysteinyl)FAD. Mammalian MAOs

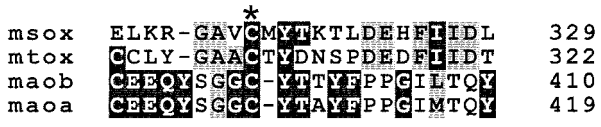


FIGURE 5: Multiple sequence alignment of MSOX from *Bacillus sp. B-0618*, MTOX from *E. coli*, human MAO B (maob), and human MAO A (maoa) was generated using the program Clustal W (*44*). The figure shows the region of the alignment surrounding the cysteine covalent flavin attachment site (indicated by an asterisk).

exhibit some sequence similarity with MSOX, as judged by alignment scores above the threshold value ($E = 0.001$) after iteration 4 of a PSI−Blast (*43*) search of the NCBI database. A multiple sequence comparison of MSOX, MTOX, human MAO A, and human MAO B using the program Clustal W (*44*) shows alignment of their ADP-binding motifs and cysteine covalent flavin attachment sites. Figure 5 shows the latter portion of the alignment. Arg49 and Arg52, residues near the 8α-(*S*-cysteinyl)FAD in MSOX (*35*), are conserved in MAO A and B.

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