Sarcosine Oxidase Contains a Novel Covalently Bound FMN†

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ABSTRACT: Sarcosine oxidase from *Corynebacterium* sp. P-1 is a heterotetrameric protein containing three different coenzymes: noncovalent FAD, noncovalent NAD^+, and a covalently bound flavin which is released as 8α-(N^3-histidyl)riboflavin upon complete hydrolysis of the protein. The following results show that the covalent flavin is not at the FAD level, as previously proposed, but is rather an 8α-(N^3-histidyl)FMN coenzyme. First, no AMP is released when the protein moiety is treated with phosphodiesterase or subjected to mild acid hydrolysis. The enzyme contains a total of 5 mol of phosphate. Only one phosphate is covalently bound. The other four phosphates are noncovalent and attributed to noncovalently bound FAD and NAD^+. The ^{31}P NMR spectrum of native enzyme exhibits resonances due to a single phosphate monoester and two pyrophosphates. Only a resonance due to phosphate monoester is observed after removal of the noncovalent cofactors and proteolytic digestion of the protein moiety. The 8α-(N^3-histidyl)FMN found in corynebacterial sarcosine oxidase represents a novel type of covalent flavin. Studies with sarcosine oxidases from *Arthrobacter* sp. and *Pseudomonas* sp. show that these heterotetrameric enzymes also contain covalently bound FMN plus noncovalently bound FAD and NAD^+, similar to corynebacterial sarcosine oxidase. In contrast, two monomeric sarcosine oxidases (from *Bacillus* sp. and an unidentified microorganism) were found to contain only covalently bound FAD.

Sarcosine oxidase catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide. Enzyme expression in various bacteria is induced by growth with sarcosine as a source of carbon and energy. Two major classes of sarcosine oxidases have been described. Heterotetrameric enzymes ($\alpha = 96-103$ kDa, $\beta = 42-45$ kDa, $\gamma = 20-23$ kDa, $\delta = 6-14$ kDa) contain both covalent and noncovalent flavin, a feature which distinguishes these proteins from all other known flavoenzymes. Monomeric enzymes (40−45 kDa) contain only covalent flavin (Kvalnes-Krick & Jorns, 1991).

The best characterized sarcosine oxidase is the heterotetrameric enzyme from *Corynebacterium* sp. P-1 (Kvalnes-Krick & Jorns, 1986). The noncovalent flavin (FAD)[1] accepts electrons from sarcosine which are then transferred to the covalent flavin where oxygen is reduced to hydrogen peroxide (Ali et al., 1991; Zeller et al., 1989). Tetrahydrofolate does not affect the rate of sarcosine oxidation. However, when the reaction is conducted in the presence of tetrahydrofolate, 5,10-methylenetetrahydrofolate is formed instead of formaldehyde (Kvalnes-Krick & Jorns, 1987). Recent studies show that corynebacterial sarcosine oxidase also contains 1 mol of tightly bound NAD^+. The function of this coenzyme is unknown (Willie & Jorns, 1995). The genes encoding the enzyme's four subunits have been cloned, sequenced, and overexpressed in *Escherichia coli*. The β subunit exhibits about 25% identity and 45% similarity with various monomeric sarcosine oxidases (Chlumsky et al., 1993, 1995).

The covalent flavin in corynebacterial sarcosine oxidase is attached to a histidyl residue in the β subunit. It was identified in earlier studies as 8α-(N^3-histidyl)FAD (Kvalnes-Krick & Jorns, 1986), but the presence of a covalent flavin at the FAD level was not compatible with the molecular weight of the β subunit, as recently determined by electrospray mass spectrometry (Chlumsky et al., 1995). Instead, the observed molecular weight (44 314) matched a value calculated for the case where the β subunit contained covalently bound FMN (44 308). However, the mass spectral data are not definitive since the stability of a pyrophosphate linkage in FAD under the conditions of the mass spectral analysis is unknown. To resolve this apparent discrepancy, we sought to obtain evidence which would unambiguously discriminate between covalently bound FMN *versus* FAD.

In this paper, we show that the covalent flavin in corynebacterial sarcosine oxidase is a novel 8α-(N^3-histidyl)-FMN. All other known covalent flavins are found at the FAD level, except for 6-(*S*-cysteinyl)FMN in trimethylamine and dimethylamine dehydrogenase (Steenkamp et al., 1978; Steenkamp, 1979). Studies with two other heterotetrameric sarcosine oxidases show that these enzymes also contain covalently bound FMN, noncovalently bound FAD, and NAD^+. Intriguingly, two monomeric sarcosine oxidases contained only covalently bound FAD.

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; ribo, riboflavin; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide 2′-phosphate; NADH, 1,4-dihydronicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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EXPERIMENTAL PROCEDURES

Materials. Phosphodiesterase, ammonium molybdate, Fiske & Subbarow Reducer, phosphorus standard solution, 30% hydrogen peroxide, NADH, ADP, AMP, FAD, FMN, riboflavin, myokinase, pyruvate kinase, lactate dehydrogenase, trypsin, chymotrypsin, and sarcosine oxidases from *Pseudomonas* sp., *Bacillus* sp., and *Arthrobacter* sp. were obtained from Sigma. A recombinant sarcosine oxidase from an unspecified microorganism (monomeric enzyme, expressed in *E. coli*) was a generous gift from Boehringer Mannheim Corp. Guanidine hydrochloride was obtained from Heico Chemical, Inc. Microcon-3 and Centricon-10 microconcentrators were obtained from Amicon. Homogeneous 12.5% polyacrylamide PhastGels, PhastGel native buffer strips, and PhastGel Blue R stain were obtained from Pharmacia. The soluble form of succinate dehydrogenase from beef was a generous gift from Dr. Brian Ackrell.

Preparation of Corynebacterial Sarcosine Oxidase. Recombinant sarcosine oxidase was isolated from an *E. coli* clone, XL1-Blue/pLJC305. The natural enzyme was obtained from *C.* sp. P-1 cells grown in the presence of sarcosine. Enzyme purification was conducted similar to that previously described (Kvalnes-Krick & Jorns, 1986; Chlumsky et al., 1993). Protein concentration and enzyme activity were determined as described by Chlumsky et al. (1993).

Chromatography and Spectroscopy. HPLC analyses were conducted using a Rainin gradient HPLC system equipped with a Rainin Microsorb C_{18} reversed-phase column (5 μm, 46 mm $\times$ 250 mm). The elution profile (flow rate = 0.8 mL/min) was adapted from procedures previously described (Sato et al., 1993; DuPlessis et al., 1994): 7 min isocratic elution with 0.1 M potassium phosphate buffer, pH 5.3; 40 min linear gradient to 50% methanol; 1 min linear gradient to 60% methanol. The column eluate was monitored by its absorbance at 260 nm.

Absorption spectra were recorded at 25 °C using a Perkin-Elmer Lambda 3B spectrometer. The concentration of free FAD ($\epsilon = 11.3$ mM^{-1} cm^{-1}) or FMN ($\epsilon = 12.2$ mM^{-1} cm^{-1}) was determined based on the absorbance at 450 nm. As will be described, corynebacterial sarcosine oxidase remains in solution after heat denaturation in dilute buffer. The concentration of heat-denatured enzyme was determined from the absorbance at 450 nm using an extinction coefficient ($\epsilon = 23.1$ mM^{-1} cm^{-1}) obtained by summing values for free FAD and 8α-(N^3-histidyl)riboflavin (11.8 mM^{-1} cm^{-1}) (Walker et al., 1972). The extinction coefficient of 8α-(N^3-histidyl)riboflavin was used to determine the concentration of covalently bound flavin in heat-denatured enzyme after microfiltration which removes free FAD. The same set of extinction coefficients were used in related studies with various other sarcosine oxidases (*vide infra*).

Attempts To Release AMP from the Covalent Flavin in Corynebacterial Sarcosine Oxidase. Sarcosine oxidase was denatured by heating the enzyme in 10 mM potassium phosphate, pH 8.0, for 5 min in a boiling water bath. Small molecules released from the denatured enzyme were removed using a Centricon-10 microconcentrator and repeated buffer washes of the retentate. The final washed protein fraction (200 μL, 76 μM with respect to covalent flavin in 10 mM potassium phosphate, pH 8) was treated with 0.5 mg of phosphodiesterase. After a 45 min incubation at room temperature, the sample was microfiltered (Microcon-3). The filtrate was assayed for AMP using the enzymatic procedure described by Bergmeyer (1974) or subjected to HPLC analysis as described above.

In a second approach, the washed protein fraction was dissolved in 0.1 M HCl (0.3 mL, 67 μM with respect to covalent flavin) and boiled for 10 min. The sample was neutralized by addition of 2 M dibasic potassium phosphate and microfiltered (Microcon-3). The filtrate was analyzed for the presence of AMP using the HPLC method. For control studies with succinate dehydrogenase, the enzyme was denatured with 3 M guanidine hydrochloride, microfiltered (Centricon-10), and washed repeatedly with the same solvent. The protein retentate was then subjected to acid hydrolysis.

In a third approach, the washed protein fraction (450 μL, 19 μM with respect to covalent flavin in 10 mM Tris-HCl, pH 8) was incubated with 0.21 μg of trypsin and 0.21 μg of chymotrypsin overnight at 37 °C. A second aliquot was incubated without proteases. The samples were then microfiltered (Microcon-3), and the filtrate was analyzed for AMP using the enzymatic assay.

Phosphate Analysis with Corynebacterial Sarcosine Oxidase. The enzyme was exchanged into phosphate-free buffer by passing a 1 mL aliquot (98 μM) over a Sephadex G-25 column (1 $\times$ 30 cm) equilibrated with 50 mM Tris-HCl, pH 8, followed by multiple washes with 1 mM Tris-HCl, pH 8.0, using a Centricon-10 microconcentrator. The enzyme was denatured by heating for 5 min in a boiling water bath. An aliquot was removed for analysis of the total phosphate content of the enzyme. The remainder of the sample was microfiltered (Microcon-3). The filtrate was analyzed to determine the amount of phosphate released from the enzyme during heat denaturation. (In the latter calculations, similar results were obtained when the enzyme concentration was based on the value observed prior to microfiltration or based on the amount of FAD in the filtrate, assuming 1 mol of FAD/mol of enzyme.) The retentate was washed with 1 mM Tris-HCl, pH 8.0, until the absorbance of the filtrate was less than 0.01 at 260 and 450 nm. The phosphate content of the retentate was then analyzed. Phosphate assays were carried out as described by Bartlett (1959).

^{31}P NMR Studies with Corynebacterial Sarcosine Oxidase. The enzyme was exchanged into phosphate-free buffer by passing a 3 mL sample (~0.4 mM) through a Sephadex G-25 column (1 $\times$ 40 cm) equilibrated with 50 mM Tris-HCl, pH 8. The G-25 eluate was concentrated to 0.8 mM using a Centricon-10 microconcentrator, and EDTA was added to give a final concentration of 1 mM. (The filtrate was subjected to phosphate analysis and found to be phosphate-free.) Half of the sample was mixed with D_2O (20% v/v) and subjected to ^{31}P NMR analysis at 202.4 MHz using a GE 500 MHz spectrometer with trimethyl phosphate as the external standard. The other half of the sample (0.4 mL) was dialyzed against 50 mL of 50 mM Tris−acetate, pH 8, containing 4 M guanidine hydrochloride to remove noncovalently bound FAD and NAD^+. Spectrophotometric analysis of the dialysate for released flavin showed the expected level of FAD. The protein in the dialysis bag was still yellow due to the covalently bound flavin. Further inspection showed the protein had formed a gel. When the sample was dialyzed against Tris buffer to remove guanidine hydrochloride, the protein precipitated in the dialysis bag. The sample was subsequently dialyzed against 50 mM Tris−acetate, pH

8, containing 4 M urea. This caused the protein to form a gel again, as observed with guanidine hydrochloride. Addition of dithiothreitol did not result in any alteration of the gel state. In order to prepare a sample suitable for NMR analysis, the sample was dialyzed against several changes of distilled H_2O which caused the protein to precipitate. The sample was centrifuged, the yellow pellet was suspended in 4 mL of 50 mM NH_4HCO_3, and trypsin was added at a ratio of 1:10 w/w. The reaction was stirred overnight at room temperature in the dark. The sample was then centrifuged to remove any undigested insoluble material and the supernatant lyophilized to dryness. The residue was dissolved in H_2O (1 mL) and the solution redried in a Speed-vac. This treatment removed any remaining NH_4HCO_3. The sample was dissolved in 37 mM Tris−acetate, pH 8.0, containing 1 mM EDTA and 25% (v/v) D_2O and subjected to ^{31}P NMR analysis.

Studies with Other Heterotetrameric and Monomeric Sarcosine Oxidases. Sarcosine oxidases from *Bacillus* sp., *Pseudomonas* sp., *Arthrobacter* sp., and an unspecified microorganism were received as lyophilized powders. The enzymes were dissolved in 20 mM potassium phosphate, pH 7.0, and washed with the same buffer using a Centricon-10 microconcentrator. Protein purity was determined by native gel polyacrylamide gel electrophoresis using the Phast-System (Pharmacia). Proteins were visualized by staining with Phast Gel Blue R (Coomassie). Sarcosine oxidase activity was detected as a pink band when the gels were stained using 0.32 mM iodonitrotetrazolium violet as redox indicator (Kvalnes-Krick & Jorns, 1986). The samples were denatured by a 1:1 dilution with 20 mM potassium phosphate, pH 7, containing 6 M guanidine hydrochloride. Absorption spectra were recorded before and after denaturation. Small molecules released from the denatured enzymes were removed using a Microcon-3 microconcentrator. The absorption spectrum of the filtrate was recorded and the sample analyzed by HPLC as described above. The retentate was washed with 20 mM potassium phosphate, pH 7, containing 3 M guanidine hydrochloride until the absorbance of the filtrate at 260 nm was less than 0.003. The final washed protein fraction (7−12 μM with respect to covalent flavin) was subjected to acid hydrolysis and neutralized as described above. The samples were filtered through a Micron-3 microconcentrator, and the filtrate was analyzed for the presence of AMP by HPLC.

RESULTS

Attempt To Release AMP from the Covalent Flavin in Corynebacterial Sarcosine Oxidase by Phosphodiesterase Treatment. Corynebacterial sarcosine oxidase does not precipitate after heating for 5 min at 100 °C in dilute buffer (10 mM potassium phosphate or 1 mM Tris-HCl, pH 8.0). However, noncovalently bound FAD and NAD^+ are released into solution and can be removed from the denatured protein by microfiltration. The protein fraction retains the covalent flavin. Evidence to discriminate between covalently bound FMN *versus* FAD was sought by incubating the protein fraction with phosphodiesterase. The reaction mixture was microfiltered, and the filtrate was analyzed for the presence of AMP. The same results were obtained in studies conducted with recombinant sarcosine oxidase or the natural enzyme isolated from *Corynebacterium* sp. P-1. In each case, no AMP was detectable using an enzymatic assay or when the sample was analyzed by reversed-phase HPLC. Reaction of free FAD with phosphodiesterase under the same conditions resulted in complete hydrolysis to AMP and FMN (data not shown).

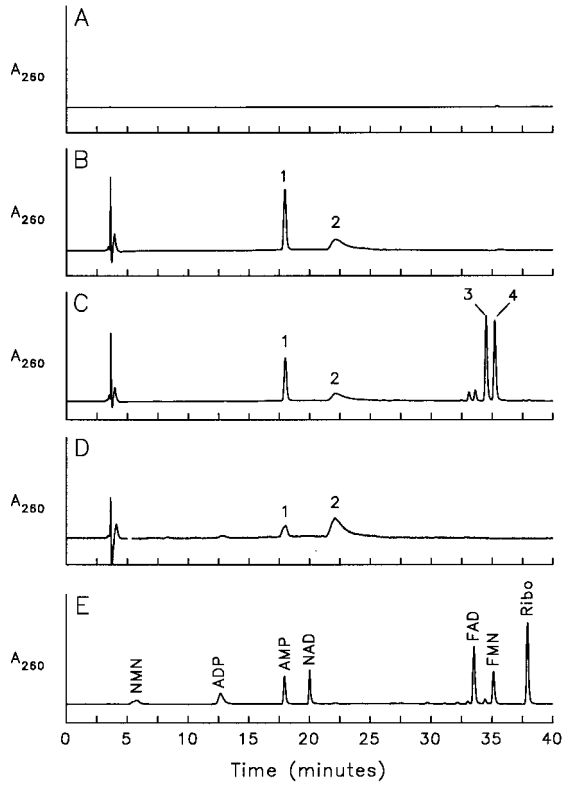

FIGURE 1: HPLC analysis of extracts obtained after boiling covalently bound flavin, AMP, or FAD in 0.1 M HCl for 10 min. The protein fraction from corynebacterial sarcosine oxidase (panel A) or succinate dehydrogenase (panel D) was microfiltered after acid treatment, and the filtrate was analyzed by HPLC. The elution profiles obtained for acid-treated AMP and FAD are shown in panels B and C, respectively. Panel E shows the profile obtained for a set of untreated standards.

Attempt To Release AMP from the Covalent Flavin in Corynebacterial Sarcosine Oxidase by Acid Hydrolysis. Flavin covalently bound to the denatured sarcosine oxidase β subunit might not be accessible to phosphodiesterase. Evidence to evaluate this possibility was sought by using an alternate method for pyrophosphate hydrolysis. In this approach, the heat-denatured protein fraction was prepared as described above and then heated for an additional 10 min in 0.1 M HCl at 100 °C. No AMP was detected when the filtrate obtained after microfiltration was subjected to HPLC analysis (Figure 1A). The same results were obtained when the protein fraction was prepared by denaturation with 3 M guanidine hydrochloride (data not shown).

Control studies with free AMP showed that the absorbance of the nucleotide at 260 nm was not affected by boiling in HCl, but HPLC analysis (Figure 1B) showed that some decomposition had occurred, as judged by the appearance of a peak at 16.5 min (peak 2) in addition to the peak due to intact AMP at 12.8 min (peak 1). The results indicate that AMP and its decomposition product have similar extinction coefficients at 260 nm.

Four major peaks were observed after acid hydrolysis of FAD in the presence (Figure 1C) or absence of guanidine hydrochloride. (The peak at 4.0 min in Figure 1C is due to guanidine hydrochloride.) Peaks 1 and 2 coelute with AMP

and its decomposition product, respectively. Total AMP recovery was estimated based on the sum of the areas under peaks 1 and 2 and varied between 70 and 80% in different experiments. Peak 4 coelutes with FMN. Peak 3 coelutes with the major contaminant in commericial FMN and appears to be a phosphorylated riboflavin derivative since it is converted to riboflavin upon treatment with alkaline phosphatase (data not shown). The major contaminant in commercial FMN has previously been identified as riboflavin 4′-phosphate (Scola-Nagelschneider & Hemmerich, 1976). A 1:1 mixture of 4′- and 5′-phosphate esters of riboflavin is formed upon acid hydrolysis of the 4′,5′-cyclic phosphate of FMN (Scola-Nagelschneider & Hemmerich, 1976), suggesting that the latter may be an intermediate in the acid hydrolysis of FAD.

An additional control reaction was conducted with the soluble form of beef succinate dehydrogenase. This enzyme contains 8α-(*N* ^{3}-histidyl)FAD plus three iron−sulfur clusters (Ackrell et al., 1991). The iron−sulfur centers were removed by denaturation with 3 M guanidine hydrochloride, followed by microfiltration. The protein fraction was subjected to the same acid hydrolysis treatment used with sarcosine oxidase. HPLC analysis of the filtrate obtained after microfiltration showed that it contained AMP and its acid decomposition product (Figure 1D). Based on total AMP recovery (estimated as described above), acid hydrolysis of succinate dehydrogenase released 0.8 mol of AMP/mol of covalently bound FAD.

Attempt To Release AMP from the Covalent Flavin in Corynebacterial Sarcosine Oxidase by Proteolytic Digestion. Proteolytic digestion of the protein fraction from corynebacterial sarcosine oxidase was previously found to generate a peptide containing 8α-(*N* ^{3}-histidyl)FMN. However, AMP was detected in the digest, suggesting that the covalent flavin in the intact enzyme was at the FAD level but underwent hydrolysis during proteolysis (Kvalnes-Krick & Jorns, 1986). In an attempt to repeat this experiment, a sample of the protein fraction from sarcosine oxidase was incubated overnight at 37 °C with chymotrypsin and trypsin under the same conditions used in previous studies. A second sample was incubated at 37 °C without addition of proteases. The samples were microfiltered, and the filtrates were subjected to enzymatic analysis. No AMP was detected in either sample.

Analysis of the Phosphate Content of Corynebacterial Sarcosine Oxidase. The covalent flavin in corynebacterial sarcosine oxidase appeared to be at the FMN level, as judged by the failure to release AMP when the protein fraction was treated under conditions which hydrolyze the pyrophosphate link in FAD. Further evidence was sought by determining the phosphate content of the enzyme. For these studies, the enzyme was equilibrated with phosphate-free buffer (1 mM Tris-HCl, pH 8.0) and then denatured by heating for 5 min at 100 °C. An aliquot of the denatured enzyme was analyzed directly to determine the total phosphate content. The remainder of the sample was microfiltered. The filtrate was analyzed to determine the amount of noncovalently bound phosphate. The retentate was used to determine the amount of covalently bound phosphate. The results, summarized in Table 1, show that the enzyme contains a total of 5 mol of phosphate. The amount of noncovalently bound phosphate (4 mol/mol of enzyme) is consistent with other studies which show that the enzyme contains 1 mol each of noncovalently bound FAD and NAD^+ (Kvalnes-Krick & Jorns, 1986; Willie & Jorns, 1995). Only 1 mol of phosphate was found to be covalently attached to the enzyme. The results provide further evidence that the covalent flavin is at the FMN level.

Table 1: Phosphate Analysis of Corynebacterial Sarcosine Oxidase

sample	mol of phosphate/mol of enzyme or free flavin[a]
total phosphate	5.0 ± 0.20
noncovalent phosphate	3.9 ± 0.05
covalent phosphate	1.1 ± 0.05
FAD (control)	2.1
FMN (control)	1.1 ± 0.05

[a] For each sample, enzyme or free flavin concentration was determined based on the absorbance at 450 nm as detailed under Experimental Procedures. Phosphate analyses are the average of two determinations (±SE). The same value was obtained in both determinations with the FAD control sample.

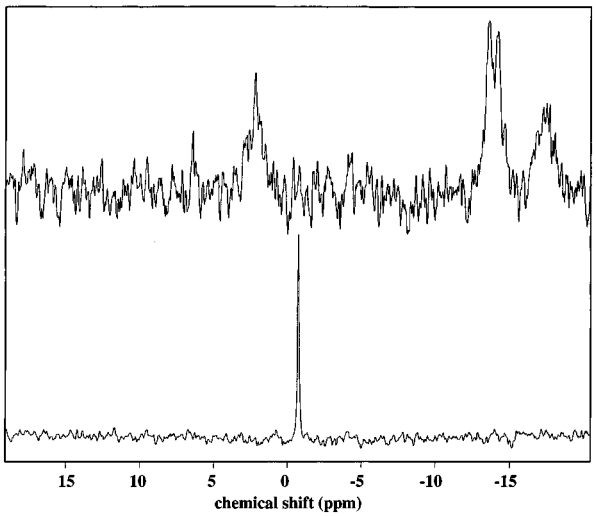

FIGURE 2: ^{31}P NMR spectrum of native sarcosine oxidase (top spectrum) was recorded using a 0.6 mM enzyme sample dissolved in 40 mM Tris-HCl, pH 8.0, containing 0.8 mM EDTA and 20% (v/v) D_2O. The bottom spectrum was obtained for a tryptic digest of the protein after removal of noncovalent cofactors by dialysis against 4 M guanidine hydrochloride, as detailed under Experimental Procedures. The sample (0.23 mM with respect to covalent flavin) was dissolved in 37 mM Tris−acetate, pH 8.0, containing 1 mM EDTA and 25% (v/v) D_2O. The spectra were recorded at 22 °C with proton decoupling. A 20 Hz line-broadening was employed before Fourier transformation of the free induction decays. Total acquisitions for each spectrum were 36 600. Chemical shifts are expressed relative to trimethyl phosphate which was used as an external standard.

^{31}P NMR Studies with Corynebacterial Sarcosine Oxidase. Chemical shift values in Figure 2 are shown relative to trimethyl phosphate, which was used as an external standard. In the following discussion and in Table 2, chemical shifts are expressed relative to 85% phosphoric acid to facilitate comparison with previous studies. Values (in ppm) relative to trimethyl phosphate are shown in parentheses.

The top curve in Figure 2 shows the ^{31}P NMR spectrum obtained for native corynebacterial sarcosine oxidase. A broad resonance at 5.7 ppm (2.2) is due to protein-bound monophosphate ester. The observed chemical shift is similar to that observed for FMN in various flavodoxins where the phosphate is probably in the dianionic state (Table 2). The upfield region of the sarcosine oxidase spectrum includes a pair of relatively sharp signals at −10.1 ppm (−13.6) and −10.7 ppm (−14.2) and a broad resonance at −14.0 ppm

Table 2: ^{31}P Chemical Shifts of Various Free and Enzyme-Bound Nucleotides[a]

	monophosphate ester	pyrophosphate
sarcosine oxidase		
native	5.7	−10.1, −10.7, −14.0 (broad)
flavin peptide	2.8	
flavodoxins		
A. vinelandii	5.6,[b] 5.6,[c] 6.3[d]	
D. vulgaris	4.99,[e] 5.4[d]	
M. elsdenii	4.8 [f] 5.3[d]	
Clostridium M.P.	5.7[d]	
D-OH-L-nic oxidase[g]		−12.2 (broad)
glucose oxidase[h]		−10.8, −12.9
xanthine oxidase[i]		−8.8, −13.5
P45OR [j]	4.1 (FMN)	−7.4, −11.3 (FAD)[k]
	3.69, 5.08 (FMN)	−7.7, −11.7 (FAD)[l]
	4.40, 3.98 (FMN)	−7.33, −11.25 (FAD)[m]
P450R·NADP$^+$ [j]	4.33, 3.97 (FMN)	−7.25, −11.05 (FAD)
	1.57 (NADP$^+$)	−9.40, −12.60 (NADP$^+$)[m]
DHFR·NADP$^+$ [n]	6.02	−11.02, −12.93
free FMN		
monoanion	0.9 [f]	
dianion	4.7,[f] 4.3,[c] 5.1[d]	
free FAD		−9.87, −10.55 (pH 7.65)[m]
		−10.8, −11.3 (pH 9.3)[h]
free NADP$^+$	4.08	−10.61, −10.95 (pH 7.65)[m]
	3.08	−10.85, −11.17 (pH 6.9)[o]

[a] All chemical shifts are expressed in parts per million (ppm) relative to 85% H_3PO_4. Data obtained with conventional *versus* superconducting magnets are not corrected for an expected 0.8 ppm difference in chemical shift values (Brauer & Sykes, 1984). Upfield shifts are negative. [b] From Edmondson and James (1982). [c] From Edmondson and James (1979). [d] From Vervoort et al. (1986). [e] From Favaudon et al. (1980). [f] From Moonen and Muller (1982). [g] Data on D-hydroxy-L-nicotine oxidase (D-OH-L-nic oxidase) from Pust et al. (1989). [h] From James et al. (1981). [i] From Davis et al. (1984). [j] Two peaks for FMN have been detected in preparations of free NADPH−cytochrome P450 reductase (P450R) or the complex of the enzyme with NADP$^+$ (P450R·NADP$^+$), a feature attributed to the presence of a proteolyzed enzyme form. [k] From Bonants et al. (1990). [l] From Nisimoto and Edmondson (1992). [m] From Otvos et al. (1986). [n] Data for the dihydrofolate reductase complex with NADP$^+$ (DHFR·NADP$^+$) from Feeney et al. (1975). [o] From Feeney et al. (1975).

(−17.5). These resonances are attributed to the pyrophosphate groups in the enzyme's noncovalently bound FAD and NAD$^+$. Two resonances are typically observed for a pyrophosphate group. The single broad band at −14.0 ppm in the sarcosine oxidase spectrum may represent two unresolved resonances, similar to that observed for the pyrophosphate in FAD bound to D-hydroxy-L-nicotine oxidase (Table 2). Alternatively, a second resonance may be hidden under the set of peaks due to the other pyrophosphate. It is not possible to assign which resonances are due to the pyrophosphate in FAD *versus* NAD$^+$ without additional information since the chemical shift values of the free coenzymes are similar and can undergo significant shifts when bound to different proteins (Table 2).

We hoped to obtain an ^{31}P NMR spectrum of denatured sarcosine oxidase after removal of the enzyme's noncovalently bound FAD and NAD$^+$. However, the protein formed a gel when a concentrated sample of enzyme (0.6 mM) was dialyzed against 4 M guanidine hydrochloride or 4 M urea. In order to prepare a sample suitable for NMR analysis, the denatured protein was solubilized by digestion with trypsin, as detailed under Experimental Procedures. The bottom curve in Figure 2 shows the ^{31}P NMR spectrum obtained after removal of the noncovalent cofactors and trypsin digestion. As expected, this treatment resulted in the loss of the pyrophosphate resonances seen with the native enzyme. The sample exhibits a single sharp resonance at 2.8 ppm (−0.73) which is assigned to a monophosphate ester. (A much sharper signal at 3.3 ppm would be expected for free phosphate.) That the observed signal is shifted as compared with that seen with free FMN at pH 8.0 probably reflects interaction of the phosphate in the covalent FMN with the peptide.

Coenzyme Composition of Other Heterotetrameric and Monomeric Sarcosine Oxidases. The combined results obtained in AMP, phosphate, and ^{31}P NMR analyses clearly show that corynebacterial sarcosine oxidase contains 8α-(*N* ^{3}-histidyl)FMN. The only other known example of a covalently bound FMN is 6-(*S*-cysteinyl)FMN, found in trimethylamine and dimethylamine dehydrogenase (Steenkamp et al., 1978; Steenkamp, 1979). To determine whether covalent FMN is a general feature of sarcosine oxidases, we examined the coenzyme composition of four sarcosine oxidases obtained from commercial sources. These enzymes included two other heterotetramers (from *Arthrobacter* sp. and *Pseudomonas* sp.), a monomeric enzyme from *Bacillus* sp., and a recombinant monomeric enzyme from an unspecified microorganism. The enzymes were subjected to native gel polyacrylamide electrophoresis. A single band was observed when the gels were stained for sarcosine oxidase activity or protein, except for a single impurity (∼10%) in the *Bacillus* sp. preparation (data not shown). The approximate molecular weights of the enzymes, estimated by comparison with a set of molecular weight standards, were consistent with values provided by the commercial suppliers (Table 3). All of the preparations exhibited typical flavoprotein absorption spectra with two absorption bands in the visible region, except for the enzyme from *Arthrobactor* sp. which exhibited an additional band at 400 nm. The heterotetramers exhibited unresolved absorption bands at 450 nm (Figure 3A), similar to free flavins in polar solvents (Harbury et al., 1959). The 450 nm band in spectra of the monomers exhibited a pronounced shoulder around 470 nm (Figure 3B), similar to the resolved 450 nm band observed for free flavins in nonpolar solvents (Harbury et al., 1959).

The various sarcosine oxidase preparations were denatured with 3 M guanidine hydrochloride and then subjected to microfiltration. (The peak at 400 nm seen with the intact enzyme from *Arthrobactor* sp. was barely detectable after denaturation.) The monomeric enzymes contained only covalently bound flavin, as judged by the fact that all of the flavin absorbance was found in the retentates. With the heterotetramers, flavin absorbance was found in both the filtrates and the retentates. The amount of covalent flavin in the heterotetramers was estimated based on absorption of the denatured enzymes at 450 nm, after correcting for the contribution due to noncovalent flavin. The absorbance of the filtrates obtained after microfiltration of the denatured enzymes was used to estimate the amount of noncovalent flavin. The preparations contained slightly less than 1 mol of noncovalent flavin/mol of covalent flavin (Table 3).

The filtrates obtained after microfiltration of the denatured enzymes were also subjected to HPLC analysis. The upper traces in panels A and B in Figure 4 show the elution profiles obtained for the filtrates from the heterotetrameric enzymes (*Arthrobacter* sp. and *Pseudomonas* sp., respectively). In each case, the chromatograms exhibited peaks at 20.1 and 33.6 min which were identified as NAD$^+$ and FAD, respectively, by comparison with the set of standards shown

Table 3: Coenzymes in Various Monomeric and Heterotetrameric Sarcosine Oxidases

source	type (mol wt)	noncovalent FAD	covalent flavin	NAD^+	mol of FAD/ mol of covalent FMN	mol of FAD/ mol of NAD^+
Corynebacterium sp. P-1	heterotetramer (180 602)[a]	yes	FMN	yes	1.0	1.0
Arthrobacter sp.	heterotetramer (160 000)[b]	yes	FMN	yes	0.71	0.77
Pseudomonas sp.	heterotetramer (174 000)[b]	yes	FMN	yes	0.83	1.0
Bacillus sp.	monomer (40 000)[b]	no	FAD	no		
microorganism (recombinant)	monomer (40 000)[b]	no	FAD	no		

[a] Calculated based on the sequence of the genes (Chlumsky et al., 1995) and the coenzyme content. [b] Data provided by the commercial supplier.

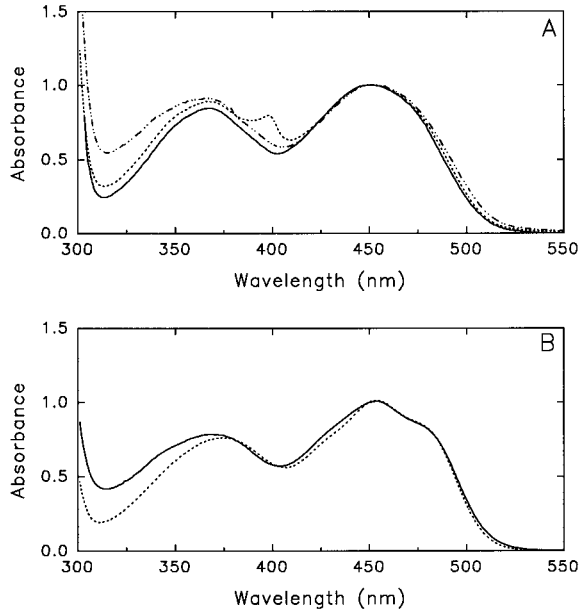

FIGURE 3: Absorption spectra of various sarcosine oxidases. Panel A shows spectra obtained for heterotetrameric sarcosine oxidases from *Corynebacterium* sp. P-1 (solid line), *Arthrobacter* sp. (dashed line), and *Pseudomonas* sp. (dash−dot−dot line). Panel B shows spectra for monomeric sarcosine oxidases from *Bacillus* sp. (solid line) and an unspecified microorganism (dashed line). Spectra were recorded at 25 °C in 20 mM potassium phosphate buffer, pH 7.0, except for the corynebacterial enzyme where the buffer was 10 mM potassium phosphate, pH 8.0.

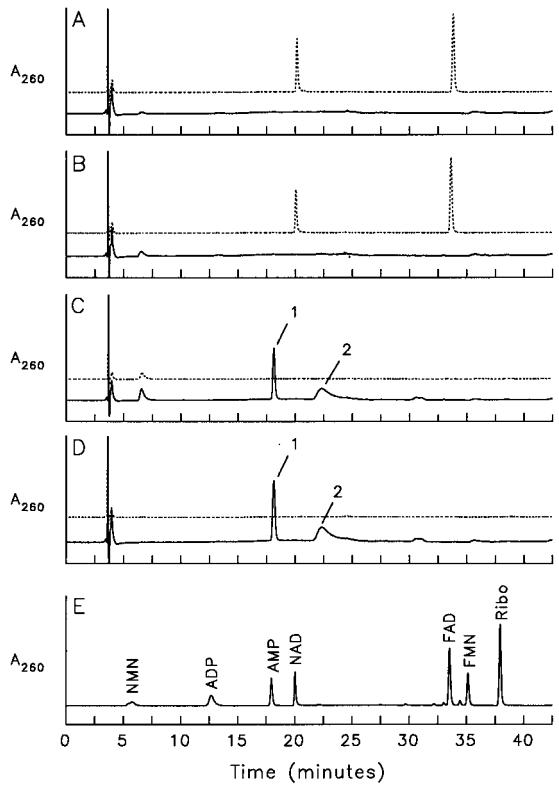

FIGURE 4: HPLC analysis of extracts obtained from various heterotetrameric and monomeric sarcosine oxidases. The enzymes were denatured in 20 mM potassium phosphate, pH 7.0, containing 3.0 M guanidine hydrochloride. The elution profiles of the filtrates obtained after microfiltration are shown by the upper traces (dashed lines) in panels A−D. The protein retentates were boiled for 10 min in 0.1 M HCl. The elution profiles of the filtrates obtained after acid hydrolysis are shown by the lower traces (solid lines) in panels A−D. The peak eluting at 4.0 min is due to guanidine hydrochloride. The peak eluting at 6.6 min, seen in some of the samples, was not identified. Data for the enzymes from *Arthrobacter* sp., *Pseudomonas* sp., *Bacillus* sp., and an unidentified microorganism are shown in panels A−D, respectively. Panel E shows the elution profile obtained for a set of standards.

in panel E. Based on the integrated areas under these peaks, the enzyme filtrates contained nearly equimolar amounts of FAD and NAD^+, similar to that observed for corynebacterial sarcosine oxidase (Table 3). In contrast, neither of these coenzymes was detected in the filtrates obtained from the monomeric sarcosine oxidases (see upper traces in panels C and D in Figure 4).

The retentates obtained after microfiltration of the denatured enzymes were boiled in 0.1 M HCl for 10 min and then filtered again to determine whether AMP was released from the covalent flavin. The HPLC elution profiles obtained for the filtrates from the acid-treated monomeric enzymes are shown in the lower traces in panels C and D in Figure 4. Both monomeric enzyme samples exhibited peaks which comigrated with AMP (peak 1) and its decomposition product (peak 2). Based on total AMP recovery, acid hydrolysis released 0.6 mol of AMP/mol of covalently bound flavin in the enzyme from *Bacillus* sp. and 1.0 mol of AMP/mol of covalently bound flavin in the recombinant monomeric enzyme. The results show that the covalent flavin is at the FAD level in the monomeric sarcosine oxidases. In contrast, no AMP was released from the covalent flavin in the heterotetrameric enzymes (see lower traces in panels A and B in Figure 4). The results strongly suggest that the heterotetrameric enzymes contain covalently bound FMN, as observed for corynebacterial sarcosine oxidase.

DISCUSSION

Corynebacterial sarcosine oxidase is a heterotetrameric protein containing three different coenzymes: noncovalent FAD, noncovalent NAD^+ (function unknown), and a covalently bound flavin which is released as 8α-(N^3-histidyl)-riboflavin upon complete hydrolysis of the protein (Kvalnes-Krick & Jorns, 1986; Willie & Jorns, 1995). In this paper, we show that the covalent flavin is not at the FAD level, as previously proposed (Kvalnes-Krick & Jorns, 1986), but is

rather an 8α-(N^3-histidyl)FMN coenzyme. Consistent with this structure, no AMP is released when the protein moiety is treated with phosphodiesterase or subjected to mild acid hydrolysis. The enzyme contains a total of 5 mol of phosphate. Only one phosphate is covalently bound. The other four phosphates are noncovalent and attributed to noncovalently bound FAD and NAD^+. The ^{31}P NMR spectrum of native enzyme exhibits resonances due to a single phosphate monoester and two pyrophosphates. Only a resonance due to phosphate monoester is observed after removal of the noncovalent cofactors and proteolytic digestion of the protein moiety.

Prior to the present study, there were five known types of covalently bound flavin (Decker, 1991). In most enzymes containing covalent flavin, a link is found between the 8α-methyl group of FAD and the protein. This category encompasses four types of covalent flavin: 8α-(N^3-histidyl)-FAD, 8α-(N^1-histidyl)FAD, 8α-(*O*-tyrosyl)FAD, and 8α-(*S*-cysteinyl)FAD. A fifth type of covalent flavin has thus far been found only in trimethylamine and dimethylamine dehydrogenase (Steenkamp et al., 1978; Steenkamp, 1979). In these enzymes, the flavin [6-(*S*-cysteinyl)FMN] is present at the FMN level and linked to a cysteine residue in the protein via carbon 6 of the isoalloxazine ring.

The 8α-(N^3-histidyl)FMN found in corynebacterial sarcosine oxidase represents a novel, sixth type of covalent flavin. This flavin may also be present in other heterotetrameric sarcosine oxidases. Our studies with sarcosine oxidases from *Arthrobacter* sp. and *Pseudomonas* sp. show that these heterotetrameric enzymes also contain covalently bound FMN plus noncovalently bound FAD and NAD^+, similar to corynebacterial sarcosine oxidase. 8α-Substituted flavins exhibit two absorption maxima in the 300−600 nm region, similar to unsubstituted flavin, except for a hypsochromic shift of the near-UV peak (Edmondson et al., 1976; McIntire et al., 1981; Walker et al., 1971, 1972). Flavins bearing a 6-(*S*-cysteinyl) substituent exhibit a single absorption maximum around 440 nm (Steenkamp et al., 1978). Although the nature of the covalent flavin linkage in the *Arthrobacter* sp. and *Pseudomonas* sp. enzymes was not determined, the spectral properties observed after denaturation and removal of noncovalent cofactors rule out 6-(*S*-cysteinyl)FMN but are consistent with a linkage via the 8α-methyl group of the flavin (Willie and Jorns, unpublished data). It is interesting to note that adenine nucleotides of unknown function are found in enzymes containing covalent FMN: NAD^+ in heterotetrameric sarcosine oxidases (Willie & Jorns, 1995; this study) and ADP in trimethylamine dehydrogenase (Lim et al., 1988).

The coenzyme composition of the heterotetrameric enzymes differs from that observed in parallel studies with two monomeric sarcosine oxidases (from *Bacillus* sp. and an unidentified microorganism) which were found to contain only covalently bound FAD. The spectral properties of the monomeric enzymes are consistent with a linkage involving the 8α-methyl group of the flavin. The sequences of the monomeric enzymes used in these studies are not known. However, sequence data are available for four other monomeric enzymes which were found to exhibit sequence homology (∼25% identity, ∼45% similarity) with the β subunit from corynebacterial sarcosine oxidase and with the NH_2-terminal half of rat liver dimethylglycine dehydrogenase (Chlumsky et al., 1995). Dimethylglycine dehydrogenase, a protein twice the size of the monomeric enzymes or the β subunit, contains covalently bound 8α-(N^3-histidyl)FAD as its only coenzyme. The covalent flavin attachment site in dimethylglycine dehydrogenase aligns with a histidine residue that is conserved in the monomeric sarcosine oxidases but not in the corynebacterial β subunit where an alanine is found (Ala^{63}). (The covalent flavin attachment site in the β subunit is tentatively identified as His^{175} based on sequence homology with another heterotetrameric enzyme. This possibility is currently under investigation.) Further studies are clearly needed to elucidate the intriguing similarities and differences among these proteins.

With respect to the presence of flavins at the FAD and FMN levels, the heterotetrameric sarcosine oxidases are similar to several other enzymes, including NADPH−cytochrome P450 reductase (Vermillion et al., 1981; Oprian & Coon, 1982), NO synthase (Marletta, 1993), NADPH−sulfite reductase (Ostrowski et al., 1989), glutamate synthase (Vanoni et al., 1992), and dihydroorotate dehydrogenase (Aleman & Handler, 1967), except that the FAD and FMN in these enzymes are both noncovalently bound. The direction of electron flow through the flavins has been determined in the case of NADPH−cytochrome P450 reductase and NADPH−sulfite reductase. In both cases, FAD serves as the entry port for electrons which are then transferred to FMN which acts as an exit port, analogous to that observed for corynebacterial sarcosine oxidase (Zeller et al., 1989; Ali et al., 1991).

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