Articles

Identification of the Covalent Flavin Attachment Site in Sarcosine Oxidase†

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ABSTRACT: Sarcosine oxidase from *Corynebacterium sp. P-1* is a heterotetrameric enzyme ($\alpha\beta\gamma\delta$) that contains two noncovalently bound coenzymes (FAD, NAD^+) and covalently bound FMN [8α-(N^3-histidyl)-FMN] which is attached to the β subunit. Chlumsky et al. [(1995) *J. Biol. Chem. 270*, 18252−18259] tentatively identified His175 as the covalent FMN attachment site in the β subunit, based on an alignment of the sequence of *C. sp. P-1* β subunit with a highly homologous flavin-containing peptide from another corynebacterial sarcosine oxidase (*C. sp. U-96*). To test this hypothesis, His175 in the *C. sp. P-1* β subunit was mutated to an alanine. Unexpectedly, the mutant enzyme was found to contain 1 mol of covalently bound flavin and to exhibit catalytic activity similar to wild-type enzyme. Covalent flavin-containing peptides were isolated from wild-type and mutant enzymes and analyzed by electrospray mass spectrometry. The mass observed for the mutant peptide (1152.4 Da) matched that predicted for an FMN-containing hexapeptide, corresponding to residues 173−178 (1152.1 Da). In the mutant, this region (HDAVAW) contains a single histidine (His173) which must be the covalent flavin attachment site. The mass observed for the wild-type peptide (1218.6 Da) matched that predicted for an FMN-containing hexapeptide, also corresponding to residues 173−178 in the β subunit (1218.2 Da). This region in the wild-type enzyme includes two histidine residues (HDHVAW). Attempts to sequence the wild-type or mutant peptides by automated Edman degradation were unsuccessful. Instead, the peptide sequences were investigated by collisional-activated dissociation (CAD) and tandem mass spectrometry. The CAD mass spectral data with the mutant peptide confirmed the sequence deduced based on the mass of the intact peptide. The CAD mass spectral results with the wild-type peptide showed that FMN was covalently attached to the N-terminal histidine in the hexapeptide, which corresponds to His173 in the β subunit.

Sarcosine oxidase from *Corynebacterium sp. P-1* is a heterotetrameric enzyme ($\alpha\beta\gamma\delta$) which catalyzes the oxidative demethylation of sarcosine to yield glycine, formaldehyde, and hydrogen peroxide (eq 1).

$$CH_3NH_2^+CH_2CO_2^- + O_2 + H_2O \rightarrow H_3N^+CH_2CO_2^- + CH_2O + H_2O_2 \quad (1)$$

In the presence of tetrahydrofolate and sarcosine, the enzyme catalyzes the formation of 5,10-methylenetetrahydrofolate which replaces formaldehyde as a reaction product. The enzyme contains 1 mol of covalently bound FMN[1] which is present as 8α-(N^3-histidyl)FMN and is attached to the β subunit. The enzyme also contains 1 mol each of noncovalently bound FAD and NAD^+ *(1−5)*. During sarcosine oxidation, electrons are transferred from sarcosine to the noncovalently bound FAD. Electrons are subsequently transferred in one-electron steps from the reduced FAD to the covalently bound FMN where oxygen is reduced to hydrogen peroxide *(6−8)*. The role of the tightly bound NAD^+ in the structure and/or function of the enzyme is unknown *(3)*. Similar heterotetrameric sarcosine oxidases have been isolated from other bacteria and are found to contain the same three coenzymes and to exhibit the ability to use tetrahydrofolate as a substrate *(4, 5, 9)*. A second class of bacterial sarcosine oxidases are monomeric enzymes which contain 1 mol of covalently bound FAD as their only prosthetic group *(4, 9)*. These enzymes are unable to use

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide; TFA, trifluoroacetic acid; CAD, collisional-activated dissociation.

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FIGURE 1: Alignment of residues 161−186 in the β subunit from *C. sp. P-1* sarcosine oxidase with a flavin-containing peptide from the β subunit of *C. sp. U-96* sarcosine oxidase. His175 in the sequence of the *C. sp. P-1* β subunit aligns with the histidine identified as the covalent flavin attachment site in the *C. sp. U-96* peptide.

tetrahydrofolate as a substrate *(5)*.

The genes encoding the four subunits of sarcosine oxidase from *C. sp. P-1* have been cloned, sequenced, and expressed in *E. coli (10, 11)*. Although gene sequence data are not available for any other heterotetrameric sarcosine oxidase, the sequences of peptide fragments encompassing about 50% of the β subunit from another corynebacterial enzyme (*C. sp. U-96*) have been reported *(12−14)*. These fragments are readily aligned with and exhibit greater than 80% identity with the sequence of the β subunit from the *C. sp. P-1* enzyme *(11)*. The alignment observed with a covalent flavin-containing peptide from *C. sp. U-96* suggested that His175 was the site of flavin attachment in the β subunit from *C. sp. P-1* (Figure 1). In this case, mutagenesis of His175 to a residue such as alanine should yield enzyme where the covalent flavin site is unoccupied or contains noncovalently bound FMN.

Contrary to expectations, it has been found that mutagenesis of His175 to alanine does not prevent covalent flavinylation. In this paper, we describe the properties of the mutant enzyme and the structure of flavin peptides from mutant and wild-type enzyme.

EXPERIMENTAL PROCEDURES

Materials. Flavodoxin from *Megasphaera elsdenii* was a gift from Dr. Stephen Mayhew. 8α-(N^3-Histidyl)riboflavin was a gift from Dr. Dale Edmondson. Trypsin and chymotrypsin were obtained from Sigma. CNBr-activated Sepharose 4B was obtained from Pharmacia. Concentrators (Microcon-3, Microcon-10, Microcon-30) and Micropure-EZ columns were obtained from Amicon. Alkaline phosphatase was obtained from Promega. T4 DNA ligase and the Klenow fragment of DNA polymerase I were obtained from New England Biolabs. Restriction enzymes were purchased from New England Biolabs and Promega. Oligonucleotides were purchased from Ransom Hill Bioscience, Inc. DEAE 45 membranes were obtained from Schleicher & Schuell. T7 Sequenase version 2.0 DNA sequencing kit was purchased from Amersham Life Science, and sequencing was performed using dITP mixes. The Geneclean II kit was obtained from BIO101. *Taq* DNA polymerase and *Pfu* DNA polymerase were purchased from Promega and Stratagene, respectively. Geneluter spin columns were purchased from Supelco.

Construction of Plasmids pLJC305ΔKpnI, pBluescript II SK(+)ΔKpnI, and pLJC400. All common DNA manipulations were performed by standard procedures *(15)*.

The expression plasmid for wild-type recombinant sarcosine oxidase (pLJC305) contains a 7.3 kb corynebacterial DNA insert, a vector-encoded *Kpn*I site at the 3′ end of the corynebacterial insert, and a second *Kpn*I site near the 3′ end of the gene (*sox*B) that codes for the β subunit of sarcosine oxidase *(10, 11)*. Plasmid pLJC305 was digested with *Kpn*I, precipitated using ethanol, and recovered by centrifugation. A portion of the sample was treated with T4 DNA ligase at low DNA concentration to favor self-ligation. The ligation mixture was used to transform *E. coli* strain JM101 to ampicillin resistance. Colonies were screened for the presence of a single *Kpn*I site in a plasmid of 4741 bp. One of these plasmids was designated pLJC305Δ*Kpn*I. This deletion eliminates a portion of the *sox*B gene that codes for the C-terminal 50 amino acids in the 405-amino acid β subunit, the genes coding for the α, γ, and δ subunits of sarcosine oxidase, and 2 downstream open reading frames. Plasmid pLJC305Δ*Kpn*I contains unique *Sal*I and *Pst*I sites that flank the His175 codon in the β subunit and are separated by 352 bp.

pBluescript II SK(+) (Stratagene) contains a single *Kpn*I site in the multicloning region. To eliminate this *Kpn*I site, the plasmid was digested with *Kpn*I and then treated with Klenow fragment (1.5 h at room temperature, followed by 0.5 h at 37 °C). The plasmid was precipitated using ethanol and recovered by centrifugation. The sample was treated with T4 DNA ligase at low DNA concentration to favor self-ligation. The ligation mixture was redigested with *Kpn*I and used to transform *E. coli* strain XL-1 Blue to ampicillin resistance. Plasmids were screened by restriction analysis with *Kpn*I. One of these plasmids was designated pBluescript II SK(+)Δ*Kpn*I.

To construct plasmid pLJC400, plasmid pLJC305 was digested with *Xba*I, *Xho*I, and *Sca*I. This reaction yields a *Xba*I−*Xho*I fragment corresponding to the complete corynebacterial DNA insert in pLJC305. A second *Xba*I−*Xho*I fragment, containing only vector DNA, is further cut by *Sca*I. In a separate reaction, pBluescript II SK(+)Δ*Kpn*I was digested with *Xba*I and *Xho*I. The fragments resulting from these reactions were separately precipitated using ethanol and recovered by centrifugation. The fragments were then mixed, treated with T4 DNA ligase, and used to transform XL-1 Blue competent cells to ampicillin resistance. Colonies were screened for the presence of a plasmid that was ~10 kb in size and contained a single *Kpn*I site. One of these plasmids was designated pLJC400.

Site-Directed Mutagenesis. The histidine at position 175 in the β subunit was converted to alanine using PCR and the overlap extension method described by Ho et al. *(16)*. All reactions were performed in a Hybaid Touchdown Thermocycler using 2.5 units of *Taq* DNA polymerase in 1.5 mM $MgCl_2$ and all other conditions as described by the manufacturer. The reactions were cycled 30 times at 94 °C (30 s), 63 °C (30 s), 72 °C (30 s); then 1 cycle at 72 °C (10 min). A 172 bp fragment (*Sal*I fragment) containing a *Sal*I site at one end and the βH175A mutation at the opposite end was synthesized using *Sal*I end primer (5′-GCGC-CGCGTGGAGGCC-3′) and βH175A#1 primer (5′-CCAG-GCCACGGCGTCGTGCTTGGCG-3′). An overlapping 254 bp fragment (*Pst*I fragment) containing a *Pst*I site at one end and the βH175A mutation at the opposite end was synthesized using *Pst*I end primer (5′-GCTCGGAGAC-CAGGGCC-3′) and βH175A#2 primer (5′-CGCCAAG-CACGACGCCGTGGCCTGG-3′). (In the βH175A#1 and βH175A#2 primers, the codon for Ala175 is underlined, and the base changes are double-underlined.) Both PCR reactions

were conducted using plasmid pLJC305 as template. The *Sal*I and *Pst*I fragments were purified from 4% agarose gels using Geneclean II. DNA concentration was estimated by densitometric analysis of an agarose gel containing various amounts of the purified fragments. The *Sal*I and *Pst*I fragments were spliced together in a third PCR reaction containing equal amounts of each fragment (100, 10, or 1 ng), *Sal*I end primer, and *Pst*I end primer. Two major products, one slightly larger than the other, were purified as described above. The incorporation of the βH175A mutation in each product was confirmed by the absence of a *Pml*I site. Both products yielded a 352 bp fragment, as expected, upon digestion with *Sal*I and *Pst*I. The 352 bp βH175A *Sal*I−*Pst*I fragment from the slightly larger product was used for further studies.

Plasmid pLJC305Δ*Kpn*I was digested with *Sal*I and *Pst*I, and a 4389 bp *Sal*I−*Pst*I fragment was purified from a 1% agarose gel using DEAE 45 membrane. The purified fragment was mixed, in various amounts, with the 352 bp βH175A *Sal*I−*Pst*I fragment. The mixture was ethanol-precipitated, treated with T4 DNA ligase, and used to transform XL-1 Blue competent cells to ampicillin resistance. Clones containing the βH175A mutation were identified by screening for the expected deletion of a *Pml*I site. The identified clones were sequenced between the *Sal*I and *Pst*I sites to confirm the correct sequence. One of these plasmids was designated pLJC305Δ*Kpn*I(βH175A).

In separate reactions, plasmids pLJC305Δ*Kpn*I(βH175A) and pLJC400 were digested with *Kpn*I. The DNA digests were purified using Geneclean II and then treated with *Xba*I. The 1862 bp *Xba*I−*Kpn*I fragment from pLJC305Δ*Kpn*I-(βH175A) and the 8380 bp *Xba*I−*Kpn*I fragment from pLJC400 were purified from 1% agarose gels using Geneclean II. The purified fragments were mixed, ethanol-precipitated, treated with T4 DNA ligase, and then used to transform XL-1 Blue competent cells to ampicillin resistance. Plasmids from several colonies were checked by restriction analysis with *Pml*I and sequencing between the *Sal*I and *Pst*I sites. One of these plasmids was designated pβH175A.

Enzyme Preparation. E. coli cells (XL-1 Blue) containing pLJC305 or βH175A were grown as previously described *(10)*. Purification of recombinant wild-type sarcosine oxidase or the βH175A mutant was conducted similar to that previously described *(1, 10)*. Protein concentration was determined from the absorbance at 280 nm using the extinction coefficient ($E^{1\%} = 13.1$) as reported by Suzuki *(17)*. Enzyme activity was determined as described by Chlumsky et al. *(10)*.

Spectroscopy and Reversed-Phase Chromatography. Absorption spectra were recorded at 25 °C using a Perkin-Elmer Lambda 3B spectrophotometer. Purification of FMN peptides was performed using a Rainin gradient HPLC system equipped with a Rainin Microsorb (C_{18}, 5 μm, 46 × 250 mm) reversed-phase column or a Vydac 218TP54 (C_{18}, 5 μm, 46 × 250 mm) reversed-phase column (The Separation Group).

Measurement of Flavin Content. Samples of sarcosine oxidase in 10 mM potassium phosphate buffer, pH 8, were heated at 100 °C for 4 min to denature the enzyme. The denatured protein remains in solution when prepared under these conditions. The absorbance of the denatured sample at 450 nm was used to determine the total flavin content of the sample. The sample was then filtered using microfiltration (Micron-3). The absorbance of the filtrate at 450 nm was used to determine the amount of noncovalently bound flavin which was released upon denaturation. The extinction coefficient of the intact enzyme was determined based on the absorbance change observed at 450 nm upon denaturation, as previously described *(10)*.

Preparation of Apoflavodoxin Affinity Column. An apoflavodoxin affinity column was prepared using a modification of the method reported by Mayhew and Strating *(18)*. Activated Sepharose 4B (1 g) was suspended in approximately 10 mL of 1 mM HCl. The resin was placed in a sintered glass filter and washed with approximately 200 mL of 0.1 mM HCl followed by approximately 30 mL of coupling buffer (0.1 M sodium carbonate/0.2 M sodium chloride, pH 10.5). The resin was suspended in approximately 6 mL of coupling buffer containing 0.1 mM flavodoxin, and the suspension was allowed to sit at room temperature for 16 h. The resin was then washed with approximately 45 mL of coupling buffer followed by approximately 45 mL of 0.1 M Tris-HCl, pH 8. A 3 mL column was prepared with the resin. Prior to use, the column was washed with 5% (w/v) trichloroacetic acid containing 0.3 mM EDTA to remove FMN from flavodoxin and then reequilibrated with 0.1 M Tris-HCl, pH 8.

Proteolytic Digestion of Wild-Type or Mutant Enzyme and Isolation of the FMN Peptide. A 1 mL sample of recombinant wild-type sarcosine oxidase (8 mg/mL) was digested with chymotrypsin and trypsin (0.05 mg of each protease/mg of sarcosine oxidase) in 0.3 M potassium phosphate, pH 8.5, at 37 °C for approximately 20 h. A chymotrypsin/trypsin digest of the βH175A sarcosine oxidase mutant (5 mg/mL) was prepared using similar conditions.

The proteolytic digest from wild-type or the βH175A mutant enzyme was applied to the apoflavodoxin affinity column. The column was washed with 0.1 M Tris-HCl, pH 8, until the absorbance of the eluent at 210 nm was less than 0.01. FMN peptide was then eluted from the column with 5% (w/v) trichloroacetic acid containing 0.3 mM EDTA. Trichloroacetic acid was removed by extracting 6 times with 4 volumes of ether.

The FMN peptide from wild-type or mutant enzyme was applied to a Microsorb C_{18} reversed-phase HPLC column. Elution profile (flow rate = 0.8 mL/min): 7 min isocratic elution with 0.1 M potassium phosphate buffer, pH 5.3; 33 min linear gradient to 50% methanol; 5 min linear gradient to 60% methanol. The column eluate was monitored by its absorbance at 210 and 450 nm. Fractions containing the FMN peptide were pooled and dried in vacuo. The FMN peptide was redissolved in a minimum volume of 5% acetonitrile containing 0.1% TFA and applied to a Vydac C_{18} reversed-phase column. Elution profile (flow rate = 1 mL/min): 5 min isocratic elution with 5% acetonitrile containing 0.1% TFA; 35 min linear gradient from 5% to 70% acetonitrile in the presence of 0.1% TFA; 4 min isocratic elution with 70% acetonitrile in the presence of 0.1% TFA. The column eluate was monitored by its absorbance at 210 and 450 nm.

In the preparation of a second batch of FMN peptide from wild-type enzyme, difficulties were encountered with the apoflavodoxin affinity column. This step was omitted. The FMN peptide was purified by reversed-phase chromatogra-

phy, as described above. The same peptide was isolated by this procedure, as judged by mass spectral analysis.

Acid Hydrolysis of FMN Peptide Isolated from Mutant Enzyme. FMN peptide isolated from the βH175A sarcosine oxidase mutant (9.9 μM) was first incubated for 30 min at 37 °C in 38.5 mM Tris-HCl buffer, pH 9.3, containing 0.77 mM $MgCl_2$, 77 μM $ZnCl_2$, 0.77 mM spermidine, and alkaline phosphatase (0.058 unit/μL). An aliquot of the dephosphorylated flavin peptide (0.2 nmol) was diluted 1:10 with 6 N HCl and placed in a glass ampule purged with argon. The ampule was sealed and then heated overnight at 115 °C. An aliquot of 8α-(N^3-histidyl)riboflavin was treated identically. The hydrolyzed samples were analyzed using reverse-phase chromatography (Vydac C_{18} column), as described above.

Mass Spectral Analysis. Mass spectral analysis was performed on a API-III triple-quadrupole mass spectrometer (PE-SCIEX, Ontario, Canada) using the SCIEX IonSpray interface with nitrogen as the nebulizer gas and an ionspray voltage of ~3600 V. An orifice voltage of 75 V along with a difference of 3 V between R0 and R1 was used. The sample was infused into the mass spectrometer at 2 μL/min using a Harvard Apparatus syringe pump after dissolving in 50% acetonitrile/H_2O + 0.1% TFA. Collisional-activated dissociation (CAD) was performed using argon as the collision gas at a collision gas thickness of $\sim 1 \times 10^{10}$ molecules/cm^2 and a collision energy of 30−35 eV. Under these CAD conditions, product ion spectra of the selected precursor ions were obtained.

RESULTS AND DISCUSSION

Mutagenesis. The expression plasmid for wild-type enzyme (pLJC305) contains a 7.3 kb corynebacterial DNA insert with a G/C content of about 70% *(10, 11)*. For mutagenesis experiments, a portion of the sarcosine oxidase operon containing most of the gene coding for the β subunit was subcloned to yield a construct (pLJC305Δ*Kpn*I) with unique *Sal*I and *Pst*I sites that flanked the βHis175 codon and were separated by 352 bp. The PCR overlap extension method described by Ho et al. *(16)* was used to generate a 352 bp *Sal*I−*Pst*I fragment containing the βH175A mutation. The mutant fragment was used to replace the corresponding wild-type sequence in pLJC305Δ*Kpn*I. The corynebacterial DNA in the resulting construct [pLJC305Δ*Kpn*I(βH175A)] was cut out and used to replace the corresponding region in the wild-type operon. The final construct, pβH175A, was transformed into *E. coli XL-1* Blue cells. Sarcosine oxidase constitutes about ~30% of the soluble protein in crude cell extracts when the genes for the wild-type enzyme are expressed in *E. coli XL-1* Blue cells *(10)*. The expression level of the βH175A mutant enzyme was similar to that observed with wild-type recombinant enzyme, and the mutant enzyme was isolated by the same procedure.

Activity and Flavin Content of βH175A Mutant Enzyme. Wild-type recombinant enzyme contains 1 mol each of 8α-(N^3-histidyl)FMN, FAD, and NAD^+ and exhibits the same activity as observed for the natural enzyme isolated from *C. sp. P-1 (3, 4, 10)*. However, the spectral properties of wild-type recombinant enzyme differ significantly from those observed for the natural enzyme isolated from *C. sp. P-1* (see Table 1). These differences are due to the fact that about half of the covalent flavin in the recombinant wild-type enzyme forms a reversible covalent 4a-adduct with a cysteine residue in a reaction accompanied by bleaching of the 450 nm absorption band of the uncomplexed flavin *(10)*.

Table 1: Properties of the βH175A Mutant versus Wild-Type Recombinant Sarcosine Oxidase

property	enzyme preparation: βH175A mutant	enzyme preparation: wild type
specific activity	8.4	8.6
A_{368}/A_{450}	0.96	1.0 (0.83)[a]
A_{280}/A_{450}	15.5	16.9 (12.2)[a]
ϵ_{450} (mM^{-1} cm^{-1})	10.7	9.7 (12.7)[a]
mol of covalent FMN/mol of noncovalent FAD	1.0	1.0

[a] Shown in parentheses are values obtained for the natural enzyme, isolated from *Corynebacterium sp. P-1 (10)*.

The isolated βH175A mutant enzyme exhibited the same specific activity as the wild-type enzyme (Table 1). Except for a small decrease in the A_{368}/A_{450} ratio (Table 1), the visible absorption spectrum of the mutant enzyme nearly superimposed with that observed for wild-type recombinant enzyme. The A_{280}/A_{450} ratio of the mutant enzyme was somewhat lower than observed for wild-type recombinant enzyme (15.5 versus 16.9) but considerably higher than observed for the natural enzyme isolated from *C. sp. P-1* (15.5 versus 12.2). The difference in the values observed for the A_{280}/A_{450} ratio with mutant versus recombinant wild-type enzyme is attributable to the somewhat higher value of the extinction coefficient obtained for the mutant enzyme at 450 nm (10.7 versus 9.7 mM^{-1} cm^{-1}). The results indicate that the flavin content of the mutant enzyme is similar to wild-type recombinant enzyme. The observed differences in spectral properties suggest that the amount of the 4a-thiolate flavin adduct in the mutant enzyme is about 60% of that observed with wild-type recombinant enzyme.

To determine whether the H175A mutation in the β subunit blocked flavin covalent attachment, the mutant enzyme was denatured by heating under conditions where the denatured protein remains in solution. Total flavin content was determined based on the absorbance of the denatured sample at 450 nm. The sample was microfiltered, and the absorbance of the filtrate at 450 nm was used to determine the amount of noncovalently bound flavin released upon denaturation. It was found that 49% of the flavin absorbance was released upon denaturation of the mutant enzyme, similar to that observed in a control experiment with wild-type recombinant enzyme. The results show that the βH175A mutant contains equimolar amounts of covalently and noncovalently bound flavin.

Identification of the Covalent Flavin Attachment Site in βH175A Mutant and Wild-Type Enzyme. Samples of the βH175A mutant and wild-type recombinant enzymes were subjected to proteolytic digestion with chymotrypsin and trypsin. A flavin-containing peptide was isolated from each digest, as detailed under Experimental Procedures. An aliquot of the flavin peptide from the mutant enzyme was treated with alkaline phosphatase and then subjected to acid hydrolysis in 6 N HCl. The flavin elution profile observed

Table 2: Electrospray Mass Spectral Analysis of Flavin Peptides from βH175A Mutant and Wild-Type Recombinant Sarcosine Oxidase

Peptide Source	Molecular Weight $(M+H)^+$ (m/z)		Sequence
	Observed	Predicted	
Wild type	1218.6	1218.2	HDHVAW (FMN) or HDHVAW (FMN)
βH175A Mutant	1152.4	1152.1	HDAVAW (FMN)

upon chromatography on a Vydac C_{18} HPLC column matched that observed for a similarly treated control sample of 8α-(N^3-histidyl)riboflavin. The results indicate that the covalent flavin in mutant enzyme is attached to a histidine residue.

Mass values for the flavin-containing peptides from mutant or wild-type enzyme were determined by electrospray mass spectrometry. The mass observed for the wild-type peptide matched that predicted for an FMN-containing hexapeptide, corresponding to residues 173−178 in the β subunit. This region includes two histidine residues, His173 and His175 (Table 2). No other peptide of similar mass containing an FMN moiety and at least one histidine is expected upon proteolysis of the wild-type enzyme, as judged based on the specificity of the proteases used for the digestion and the sequence of the β subunit. The mass observed for the mutant peptide matched that predicted for an FMN-containing hexapeptide corresponding to residues 173−178, the same region observed for the wild-type peptide (Table 2). Since the mutant enzyme contains only a single histidine in this region, the results indicate that His173 is the site of covalent flavin attachment. In the case of the wild-type peptide, the data cannot distinguish between attachment at His173 or His175.

Attempts to sequence the wild-type or mutant peptides by automated Edman degradation were unsuccessful. Instead, the peptide sequences were investigated by collisional-activated dissociation (CAD) and tandem mass spectrometry. In this approach, the molecular ion formed in the initial ionization process is selected (precursor ion) in the first quadrupole and fragmented by collisions with a neutral gas in the second quadrupole, producing product ions. The product ions are then analyzed in a third quadrupole. The end result is a mass spectrum containing product ions characteristic of the sequence of the amino acids present in the intact peptide *(19, 20)*.

The CAD mass spectrum obtained for the mutant peptide is shown in Figure 2. Amino acid immonium ions ($NH_2{=}CHR^+$) appear at the low mass region of the CAD spectrum and are named using the single-letter code of the particular amino acid. Although seldom observed for all residues, the immonium ions that are observed provide partial information about the amino acid composition of the peptide *(20)*. The three immonium ions observed for the mutant peptide (A, V, W) are consistent with those predicted (A, V, W, D) based on the mass of the intact peptide.

Cleavage of a single peptide bond in the parent molecular ion is accompanied by the transfer of the positive charge from the parent ion to *either* the N-terminal or the C-terminal fragment. (Only charged fragments are detected in the CAD mass spectrum.) The CAD mass spectrum of the mutant peptide shows a somewhat larger number of N-terminal fragments (a_n, b_n, or c_n) than C-terminal fragments (x_n, y_n, or z_n). This is expected since formation of charged N-terminal fragments is favored by the presence of a basic amino acid residue at or near the N-terminus of the intact peptide *(20)* and the mutant peptide contains a substituted N-terminal histidine residue. The numeric subscript, n, in a

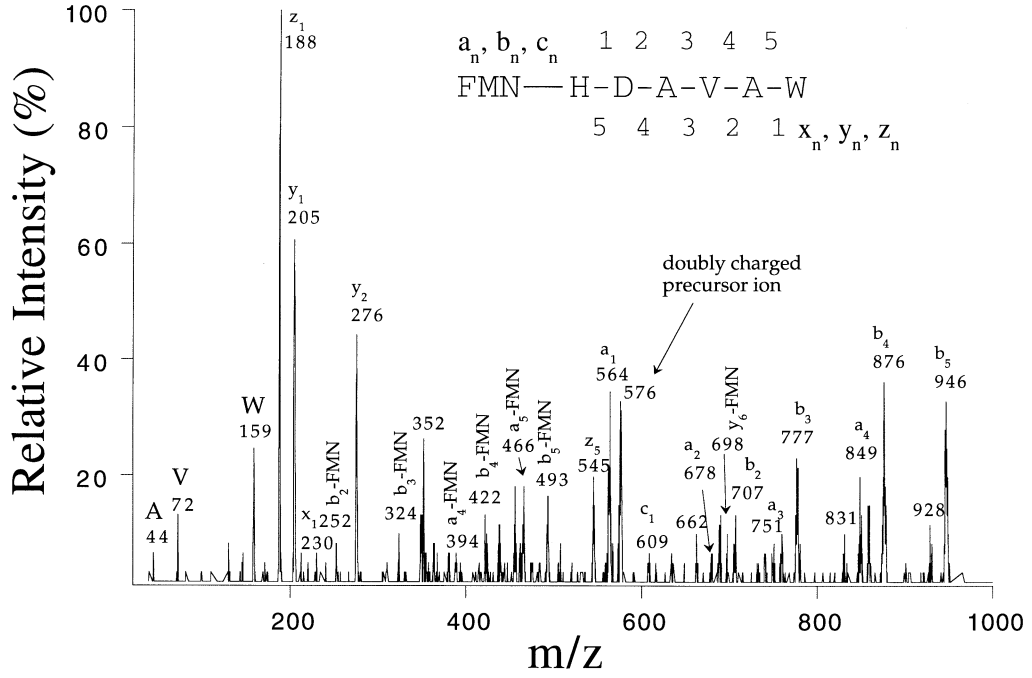

FIGURE 2: CAD mass spectrum of the FMN peptide isolated from βH175A mutant enzyme. The CAD mass spectrum was obtained using the doubly charged ion of the mutant flavin peptide (576.8 Da) as the selected precursor ion. Immonium ions are named using the single-letter code of the amino acid. The numbering system for N-terminal (a_n, b_n, c_n) and C-terminal (x_n, y_n, z_n) fragments is indicated in the figure and described in the text. The three subtypes of N- and C-terminal fragments indicate whether the peptide bond was cleaved between C(α) and C═O (a_n, x_n), C═O and NH (b_n, y_n), or NH and C(α) (c_n, z_n) *(19, 20)*.

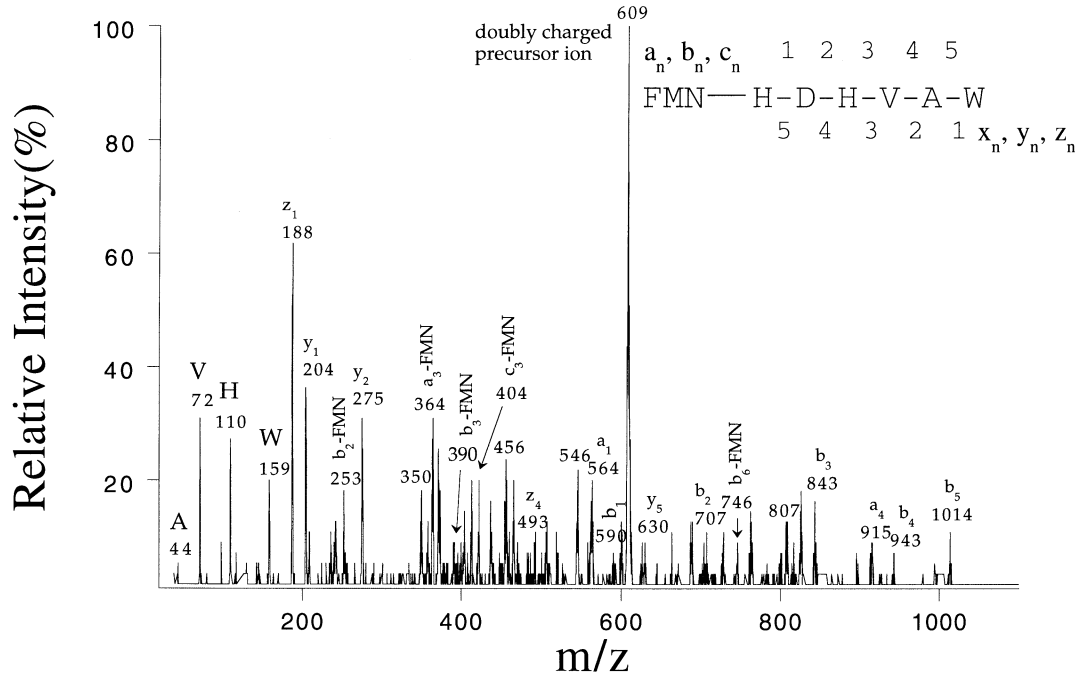

FIGURE 3: CAD mass spectrum of the FMN peptide isolated from wild-type recombinant enzyme. The CAD mass spectrum was obtained using the doubly charged ion of the wild-type flavin peptide (609.8 Da) as the selected precursor ion. Fragments are named as indicated in the legend to Figure 2. Owing to lack of space, labels were omitted for fragments x_3 (401) and z_3 (358).

fragment name indicates the site of peptide bond cleavage. (For N-terminal fragments, peptide bonds are numbered beginning at the N-terminus of the intact peptide. The opposite rule applies for C-terminal fragments where peptide bonds are numbered starting from the C-terminus.) The set of N-terminal fragments observed with the mutant peptide (a_1, c_1, a_2, b_2, a_3, b_3, a_4, b_4, b_5) constitutes a complete series with respect to the presence of fragments derived from cleavage at each of the five peptide bonds in the intact peptide. The CAD mass spectral data provide strong support for the sequence deduced based on the mass of the intact mutant peptide.

The CAD mass spectrum obtained for the wild-type flavin peptide is shown in Figure 3. The four immonium ions observed (A, V, H, W) are consistent with those predicted (A, V, H, W, D) based on the mass of the intact wild-type peptide. As with the mutant peptide, an immonium ion due to aspartate is not detected.

A subset of the predicted fragments for the native peptide is different depending on whether FMN is attached to the N-terminal (His173) or the internal (His175) histidine in the hexapeptide. This subset includes N-terminal fragments where $n = 1$ or 2 and C-terminal fragments where $n = 4$ or 5. Within this subset, the N-terminal fragments contain FMN only in the case where flavin is attached to the N-terminal histidine in the hexapeptide. Conversely, the C-terminal fragments in this subset contain FMN only when the internal histidine in the hexpeptide is the site of flavin attachment. It should be noted that the CAD mass spectra of both mutant and wild-type peptides contain peptide fragments that have also undergone loss of the FMN moiety. Therefore, unambiguous evidence to discriminate between the two possible FMN attachment sites in the hexapeptide was sought by focusing on FMN-containing fragments. Several FMN-containing N-terminal fragments where $n = 1$ or 2 could be identified in the CAD mass spectrum of the wild-type peptide, as judged by the agreement between the observed masses (in daltons) with predicted values (shown in parentheses): a_1, 564 (563.5); b_1, 590 (591.5); b_2, 707 (706.6). In contrast, the CAD mass spectral data provided no evidence for FMN-containing C-terminal fragments where $n = 4$ or 5. The results indicate that FMN is attached to the N-terminal histidine in the hexapeptide. Further evidence in support of the proposed sequence and flavin attachment site is provided by the observed sets of N-terminal (a_1, b_1, b_2, b_3, a_4, b_4, b_5) and C-terminal (y_1, z_1, y_2, x_3, z_3, z_4, y_5) fragments, each of which constitutes a complete series with respect to the presence of fragments derived from cleavage at each of the five peptide bonds in the intact peptide.

Conclusions. Histidine at position 175 in the β subunit of sarcosine oxidase from *C. sp. P-1* was tentatively proposed as the site of covalent FMN attachment *(11)*, based on an alignment of the β subunit with a highly homologous flavin-containing peptide from the β subunit of another coryne-bacterial sarcosine oxidase (Figure 1). The following results indicate that this assignment is incorrect and that His173 serves as the attachment site. Mutation of His175 to alanine in the β subunit of the *C. sp. P-1* enzyme yields a mutant enzyme which exhibits the same specific activity as wild-type enzyme and contains stoichiometric amounts of covalently bound flavin. Mass spectral analysis of flavin-containing peptides from wild-type and βH175A mutant enzyme shows that FMN is covalently attached to His173 in both enzyme preparations. In contrast to the βH175A mutation, recent studies show that the βH173A mutation abolishes catalytic activity and interferes with subunit assembly and expression.[2] This suggests that attachment at His175 cannot be promoted simply by preventing attachment at His173.

[2] M. Eschenbrenner, L. J. Chlumsky, and M. S. Jorns, unpublished observations.

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