Structural Characterization and Mapping of the Covalently Linked FAD Cofactor in Choline Oxidase from *Arthrobacter globiformis*

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ABSTRACT: The flavoenzyme choline oxidase catalyzes the oxidation of choline and betaine aldehyde to betaine. Earlier studies have shown that the choline oxidase from *Arthrobacter globiformis* contains FAD covalently linked to a histidine residue. To identify the exact type of flavin binding, the FAD-carrying amino acid residue was released by acid hydrolysis. The fluorescence excitation maxima of the isolated aminoacylriboflavin, showing a hypsochromic shift of the near-ultraviolet band relative to riboflavin, and the pH-dependent flavin fluorescence confirmed the presence of an 8α-substituted flavin linked to histidine. Similarly, MALDI-TOF mass spectrometry showed a molecular mass corresponding to histidylriboflavin. Classical experiments used to distinguish between the N(1) and N(3) isomers all indicated that the flavin was linked to the N(1) position of the histidine residue. The position of the FAD-carrying histidine residue in the choline oxidase polypeptide was identified by tryptic cleavage of the denatured enzyme, HPLC separation of the proteolytic peptide fragments, and characterization of the purified flavin-carrying peptide by mass spectrometry and spectroscopy. The FAD moiety was assigned to the tryptic peptide, His-Ala-Arg, corresponding to residues 87−89 in the open reading frame of the previously published cDNA sequence. Further analysis of the flavopeptide by collision-induced dissociation mass spectrometry confirmed that the flavin cofactor was attached to His^{87}. We conclude that this variant of choline oxidase contains 8α-[N(1)-histidyl]FAD at position 87 in the polypeptide chain.

Choline oxidase (COX,[1] choline:oxygen 1-oxidoreductase, EC 1.1.3.17) catalyzes the oxidation of choline and betaine aldehyde to betaine with concomitant reduction of molecular oxygen to hydrogen peroxide. The enzyme has been purified and characterized from extracts of *Alcaligenes* sp. (*1*) and *Arthrobacter globiformis* (*2*). The COX gene of *A. globiformis* was recently isolated, cloned, and sequenced (*3*), and the enzyme is currently of great interest to researchers trying to develop transgenic plants with enhanced stress tolerance (*3*, *4*). Structurally, COX belongs to the relatively small group of flavoenzymes in which the FAD coenzyme is covalently linked to the polypeptide chain, and the flavin-carrying amino acid residue in COX has been found to be histidine. Specifically, the *Alcaligenes* enzyme has been reported to contain 8α-[N(3)-histidyl]FAD (*1*), as opposed to the other isomer, 8α-[N(1)-histidyl]FAD, which has generally been found in only a few of the presently known enzymes with flavin linked to histidine (*5*). A preliminary report published previously suggested that *A. globiformis* COX also contains the N(3) isomer of 8α-(N-histidyl)FAD (*6*). The position of the flavin-carrying histidine residue has at present not been mapped in any of the two COX variants known, although partial amino acid sequence data for a flavopeptide have been presented for the *Alcaligenes* variant [D-N-P-N-(**H**,S,R) (*1*)].

To map the flavin-carrying amino acid residue in COX from *A. globiformis*, we isolated a flavinylated tryptic peptide, determined the structure of the peptide by mass spectrometry, and localized the flavin-carrying histidine by alignment with the cDNA-derived amino acid sequence. During the course of this work, preparations of 8α-(N-histidyl)riboflavin isolated from COX were found to be unstable during prolonged storage in an aqueous solution. This property was quite unexpected, since classical studies of the N(1) and (N3) isomers of histidylribolflavin have shown that the N(3) form is stable under these conditions whereas the N(1) form is not. Actually, stability under these conditions is one of the characteristic differences which are used to distinguish between the N(3) and N(1) isomers during structure elucidation of a flavin-carrying histidine (*7*). We have therefore carefully investigated the structure of 8α-(N-histidyl)riboflavin released from COX from *A. globiformis*, and our data show that the enzyme contains the N(1) isomer, in accordance with our initial observations of an unstable histidylriboflavin.

At present, only a few amino acid sequences surrounding a site of covalent flavin attachment have been elucidated, and the existence of common sequence motifs regulating covalent flavinylation is still obscure, as is the catalytic significance of covalent linkage of the FAD cofactor (*5*). The data presented in this paper may contribute to a better

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[1] Abbreviations: CID, collision-induced dissociation; COX, choline oxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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understanding of the structural and catalytic importance of covalently linked flavin coenzymes.

EXPERIMENTAL PROCEDURES

Materials. COX from *A. globiformis*, riboflavin, and FAD were purchased from Sigma-Aldrich. SDS−polyacrylamide gel electrophoresis of the COX sample showed a degree of purity which was estimated to be fully acceptable for the studies reported here (>90%). Bovine trypsin was obtained from Roche Molecular Biochemicals. A synthetic mixture of 8α-[N(1)-histidyl]riboflavin (∼25%) and 8α-[N(3)-histidyl]riboflavin (∼75%) was a gift from D. E. Edmondson (Department of Biochemistry, Emory University School of Medicine, Atlanta, GA). Iodoacetic acid recrystallized from toluene was a gift from K. Skriver (Department of Protein Chemistry, University of Copenhagen, Copenhagen, Denmark). 8-Formylriboflavin was synthesized as described previously (*8*). All other reagents were analytical grade or better.

Spectroscopy. Absorbance measurements were carried out at room temperature in a Unicam UV2 spectrometer (Unicam, Cambridge, U.K.). Fluorescence measurements were performed at room temperature in a Perkin-Elmer LS50B luminescence spectrometer using a flavin concentration corresponding to 0.42 μM riboflavin. An emission wavelength of 525 nm was used in all experiments. All fluorescence intensities are given relative to an equimolar amount of riboflavin.

Release and Purification of Histidylriboflavin from the Holoenzyme. Samples of the holoenzyme were hydrolyzed *in vacuo* in 6 N HCl at different temperatures (80, 95, or 110 °C) for 17 h in sealed glass ampules. The hydrolysis was performed in total darkness. After lyophilization, the samples were resuspended in water and fluorescence excitation spectra were recorded. Samples (1 μL) were analyzed in parallel in a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems) after the sample had been mixed with a surplus of matrix (α-cyano-4-hydroxycinnamic acid). The histidylriboflavin from COX was purified by TLC in an airtight system using a stationary phase of Kieselgel 60 (Merck, Darmstadt, Germany) and a mobile phase consisting of *n*-butanol, acetic acid, and water (2:1:1, v/v/v). The fluorescent spot corresponding to histidylriboflavin was scraped off and flavin extracted using four aliquots of 0.1 mL of 5% pyridine (v/v) followed by sonication and three cycles of lyophilization and resuspension in water.

Reduction of Histidylriboflavin with $NaBH_4$. Sodium borohydride was added to suspensions of histidylriboflavin (0.42 μM) to a final concentration of 0.66 μM with respect to the BH_4^- ion, and the reaction mixtures were incubated for 30 min in the dark at room temperature in open containers. Fluorescence excitation spectra were recorded before addition of $NaBH_4$ and after reduction for 30 min.

Determination of the pK_a Value for the pH-Dependent Fluorescence of Histidylriboflavin. Histidylriboflavin samples (0.42 μM) were diluted with a range of 0.05 M citrate-phosphate buffers adjusted to pH values between 3 and 8. The fluorescence emission intensity at 525 nm was recorded using an excitation wavelength of 450 nm. The values shown in Figure 3 represent an average of three independent determinations. Riboflavin was diluted using the same buffers and recorded as a reference.

Detection of 8-Formylriboflavin. Samples of riboflavin, the synthetic histidylriboflavin mixture, and histidylriboflavin isolated from COX were stored at room temperature in aqueous solutions for 30 days in sealed containers in absolute darkness. Samples (10 μg) were then lyophilized and resuspended in 6 N HCl. Excess $TiCl_3$ (∼1 mM) was added to a total volume of 1 mL. Absorbance spectra were recorded before and after the addition of $TiCl_3$. 8-Formylriboflavin, which was used as a standard, was quantitated using the absorbance maximum at 565 nm.

Isolation of a Tryptic Flavopeptide from COX. Denatured COX, which had been reduced and carboxymethylated according to standard procedures, was digested with trypsin (1:100, w/w) in 0.1 M Tris-HCl and 1.5 M urea (pH 8.5) for 24 h at 37 °C in the dark. The tryptic peptides were separated by reversed phase HPLC on a SMART system equipped with a μRPC C2/C18 SC2.1/10 narrow-bore column (Pharmacia). The flow rate was 100 μL/min, and the eluting peptides were monitored by their absorbance at 220, 340, and 445 nm. Flavin-carrying peptides were identified and selected by their significant absorbance at 340 and 445 nm. After each chromatography step, a sample of the main fraction exhibiting the highest absorbance at 340 and 445 nm was scanned for flavin fluorescence. Samples with a spectral signature corresponding to that of synthetic histidylriboflavin were analyzed by MALDI-TOF mass spectroscopy, and peptides coeluting with or eluting in the vicinity of the flavin-carrying peptide were sequenced by Edman degradation in an automated sequencer (Applied Biosystems, model 494). The chromatographic protocol used for purification of the tryptic flavopeptide comprised three successive steps with different gradients of 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 0.1% TFA in acetonitrile (buffer B), followed by a fourth step with a gradient of buffer A and 0.1% TFA in methanol (buffer C). The gradient profile in step 1 was from 0 to 15% B (0−8 min) and from 15 to 45% B (8−38 min), in step 2 from 0 to 22% B (0−8 min) and from 22 to 38% B (8−38 min), in step 3 from 0 to 22% B (0−8 min) and from 22 to 25% B (8−22 min), and in step 4 from 0 to 20% C (0−8 min) and from 20 to 45% C (8−58 min). To optimize column resolution, only one-fifth of a 2.5 mg digest was applied at each injection. After each step, the fractions containing the flavopeptide were pooled, diluted 5-fold in buffer A, and divided into five aliquots to maintain the homogeneity.

Collision-Induced Dissociation (CID) Tandem Mass Spectrometry. The purified flavopeptide from COX was analyzed in a Q-TOF ESI-MS//MS mass spectrometer (Micromass).

RESULTS

Quantitative Determination of the FAD Content in COX. It has previously been reported that the covalently attached flavin coenzyme in COX from *A. globiformis* is at the dinucleotide level (*7*), but the molar ratio of FAD to polypeptide has not previously been determined for this variant of COX. Therefore, a sample of purified, commercially available COX was analyzed by amino acid analysis, and the exact, molar protein concentration, adjusted for the loss of serine, threonine, and tryptophan, was calculated. The molecular mass of the COX polypeptide was obtained from the cDNA-derived amino acid sequence (*3*).

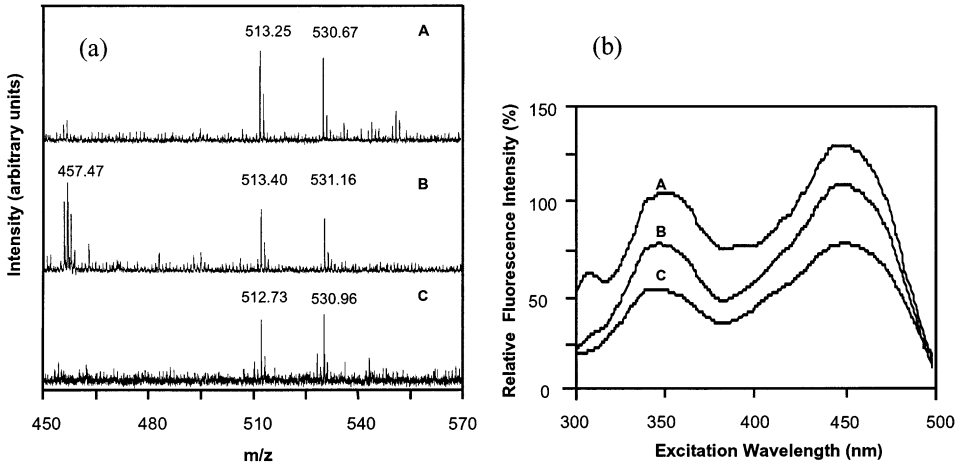

FIGURE 1: Analysis of aminoacylflavin released from COX by acid hydrolysis. MALDI-TOF mass spectra (a) and fluorescence excitation spectra (b) of equimolar amounts of aminoacylflavin ($\lambda_{em} = 525$ nm) were recorded after hydrolysis of COX samples for 17 h in 6 N HCl at 95 (A) or 80 °C (B). Corresponding spectra for synthetic histidylriboflavin, used as a reference compound, are shown in spectra C.

The same enzyme sample was analyzed by absorption spectrophotometry at 445 nm. Using the assumption that the molar extinction coefficient of covalently bound FAD is comparable to that of nonassociated FAD [$\epsilon_{445(FAD)} = 11.3$ mM^{-1} cm^{-1} (*7*)], the molar ratio of FAD to COX polypeptide was determined to be 0.87:1. This result is in excellent agreement with the ratio of 0.81:1 which may be deduced from data reported earlier for the corresponding enzyme from *Alcaligenes* sp. (*1*). The presence of equimolar amounts of FAD and polypeptide was verified by MALDI-TOF mass spectrometry. The observed mass of the holoenzyme (59 852 Da) matched the predicted mass of FAD bound to the apoenzyme (59 648 Da, deviation of 0.34%). This result confirmed that one molecule of FAD (785 Da) is bound per polypeptide chain (58 865 Da).

Isolation of Aminoacylflavin from the Native Enzyme. All known 8α-substituted flavins show a hypsochromic shift of the second absorption or fluorescence band in the near-UV region, and the magnitude of this shift reflects the type of aminoacyl bond present (*9*). COX from *A. globiformis* exhibited a hypsochromic shift of the second fluorescence excitation maximum of 17−27 nm as compared to riboflavin (data not shown). This shift was dependent upon the acidity of the environment with the largest shift observed at pH 3 and the smallest shift observed at pH 7 (data not shown). To release the aminoacylriboflavin from the COX polypeptide, the pure enzyme was hydrolyzed in 6 N HCl at 95, 80, or 110 °C. MALDI-TOF mass spectrometry and fluorescence excitation spectroscopy were used to examine the hydrolyzed enzyme, to optimize the conditions for isolation of the aminoacylflavin. Hydrolysis at 95 and 80 °C was sufficiently mild to allow survival of the aminoacylriboflavin (Figure 1). The mass spectra indicated that no peptide bonds remained (Figure 1a), and the fluorescence excitation maxima of the aminoacylriboflavin hydrolysis product remained identical to those of the native enzyme (Figure 1b), showing that the aminoacyl−flavin bond was intact. The two components of approximately 530 and 513 Da observed by mass spectrometry exactly matched the molecular masses expected for histidylriboflavin (530 Da) and its acid-modified product, 2′,5′-anhydrohistidylriboflavin (513 Da), which has previously been described as a byproduct formed during acid hydrolysis of histidylriboflavin (*7*, *10*). Hydrolysis of free FAD at 95 and 80 °C resulted in extensive breakdown to riboflavin and FMN, in accordance with the expected loss of ADP generally observed under these conditions (data not shown). When a sample of COX was hydrolyzed at 110 °C, the aminoacyl−flavin bond was broken. This was observed (i) in the absence of the mass of the histidylriboflavin in the mass spectrum, (ii) in the release of free histidine, as seen by amino acid analysis, and (iii) in the fluorescence excitation spectrum, which exhibited a bathochromic shift to maxima at 372 and 445 nm, identical to those observed for nonassociated flavins (data not shown).

On the basis of the observations presented in Figure 1, it was concluded that a hydrolysis temperature of 95 °C favored the preservation of unmodified histidylriboflavin. This was also true for hydrolysis at 80 °C, but at that temperature, free FMN was apparently generated as a byproduct (Figure 1a, panel B, 457 Da), although the identity of this component was not investigated further. To avoid confusion due to this fluorescent byproduct in the subsequent isolation of the histidylriboflavin, a hydrolysis temperature of 95 °C was chosen. After hydrolysis, the histidylriboflavin was isolated by TLC by scraping off the fluorescent spot with an R_f value of 0.21 as compared with riboflavin. A sample of synthetic histidylriboflavin migrated at the same rate, and the spectral characteristics of the isolated histidylriboflavin were identical to those of the hydrolysis product.

Characterization of the Histidylriboflavin Isolated from the Choline Oxidase from A. globiformis. Three classical experiments were performed in an effort to compare the behavior of histidylriboflavin from COX with that of known N(1)- and N(3)-bound histidylriboflavin derivatives.

(i) It is well-known that sodium borohydride reduction of N(1)-histidylriboflavin irreversibly quenches the flavin fluorescence, whereas the N(3) isomer is unreactive toward $NaBH_4$ (*9*). Reduction of the histidylriboflavin isolated from COX with sodium borohydride almost completely bleached the absorption spectrum (data not shown). Likewise, the fluorescence excitation spectrum showed a low, residual excitation at 445 nm (Figure 2), and the remaining 445 nm fluorescence was independent of pH (data not shown). The two fluorescence excitation maxima at 408 and 338 nm, which appeared after addition of sodium borohydride, were identical to the maxima observed previously after $NaBH_4$

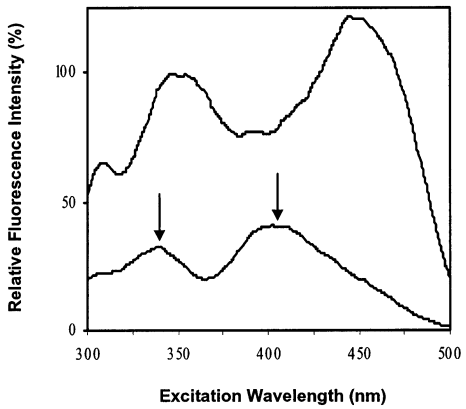

FIGURE 2: Reduction of histidylriboflavin from COX with $NaBH_4$. Fluorescence excitation spectra were recorded before addition of $NaBH_4$ (top spectrum) and 30 min after the addition (bottom spectrum). Arrows indicate new excitation maxima.

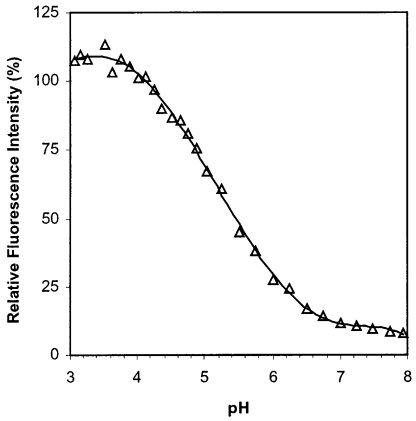

FIGURE 3: pH-dependent fluorescence of histidylriboflavin isolated from COX. The fluorescence intensity levels are shown as a percentage of the intensity of an equimolar amount of riboflavin.

reduction of the flavin coenzyme in D-amino acid oxidase (*11*), and this characteristic flavin derivative has been identified as 3,4-dihydroflavin (*12*).

(ii) Earlier studies have shown that the pH-dependent deprotonation of the imidazole nitrogen in histidylriboflavin quenches the fluorescence yield, and the pK_a for the imidazole ionization has been estimated to be 4.7 for N(3)-histidylriboflavin and 5.2 for the N(1) isomer (*9*). The pH-dependent fluorescence of histidylriboflavin isolated from COX exhibited a pK_a of 5.23 (Figure 3).

(iii) The N(3) and N(1) isomers of N-histidylriboflavin differ with respect to stability during prolonged storage in aqueous solution at room temperature. While the N(3) form is stable under these conditions, the N(1) isomer undergoes decomposition to 8-formylriboflavin, which may be identified by its characteristic cationic flavohydroquinone absorption spectrum showing four unique maxima at 388, 410, 565, and 605 nm (*13*). The stability of the N-histidylriboflavin isolated from COX was examined by storage and $TiCl_3$ reduction as described in Experimental Procedures. The absorption spectrum obtained after this treatment clearly indicated the presence of the cationic flavohydroquinone form of 8-formylriboflavin (data not shown). A reference sample of synthetic histidylriboflavin, known to contain the N(3) and N(1) isomers in a ratio of approximately 3:1, was treated similarly, and the amount of 8-formylriboflavin generated in this sample confirmed the composition of the synthetic mixture.

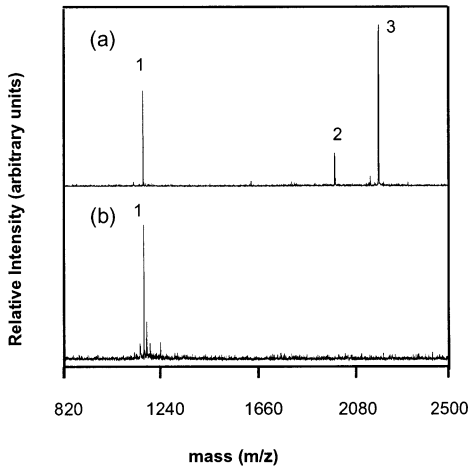

FIGURE 4: Isolation of a tryptic flavopeptide from COX. An apparently pure peptide peak (according to the absorbance at 220 and 450 nm) obtained by reversed phase HPLC with a gradient of acetonitrile in 0.1% TFA (a) and an apparently pure peptide peak obtained after a subsequent purification step using a gradient of methanol in 0.1% TFA (b) were analyzed by MALDI-TOF mass spectrometry. Peptide numbers refer to Table 1.

Proteolytic Digestion of COX and Subsequent Isolation of a Tryptic Peptide with Covalently Attached FAD. During the initial experiments with proteolytic digestion of COX, it became evident that reduction and carboxymethylation of the enzyme was essential to obtain a highly specific cleavage of the enzyme. The reducing conditions did not affect the oxidation state of the flavin moiety, as measured from the absorbance at 450 nm (data not shown). The reduction, carboxymethylation, and consequent unfolding of the enzyme led to extensive precipitation of the alkylated polypeptide. Therefore, a buffer containing 1.5 M urea was required to dissolve the protein before digestion with trypsin. The proteolytic cleavage was monitored by SDS−polyacrylamide gel electrophoresis, and it was found that the digestion was complete after 18 h (data not shown). The tryptic peptide carrying covalently linked flavin was purified by reversed phase HPLC with absorbance monitoring of the eluting peptides at 220, 340, and 445 nm. After elution with a relatively steep gradient of acetonitrile, the flavin-containing fractions were pooled, reinjected, and reeluted twice using different gradient profiles, as described in Experimental Procedures. This procedure resulted in an apparently pure peptide peak with fluorescence excitation maxima corresponding to those of synthetic, histidine-bound flavin (data not shown). Analysis by MALDI-TOF mass spectrometry, however, revealed that the peak contained three peptide fragments coeluting at an acetonitrile concentration of ~25% (Figure 4a).

The three peptides were finally separated by elution with a gradient of methanol, and the purified peptides were analyzed by mass spectrometry, spectroscopy, and amino acid sequencing. Two of the peptides were successfully sequenced, and their amino acid sequences, as well as their molecular masses, exactly matched two of the tryptic peptide fragments predicted from the cDNA sequence of COX (Table 1). Attempts to sequence the third unidentified peptide, with a molecular mass of 1168 Da (Figure 4b), by automated Edman degradation were unsuccessful. Likewise, C-terminal sequencing by digestion with carboxypeptidase B or Y followed by mass spectrometry showed no sign of cleavage

Table 1: Peptide Fragments Generated by Trypsin Digestion of Choline Oxidase from *A. globiformis*

peptide no.[a]	observed molecular mass (Da)	theoretical molecular mass + H^+ (Da)	theoretical molecular mass + FAD + H^+ (Da)	observed absorbance ratio (A_{450}/A_{220})	position in the amino acid sequence as deduced from the cDNA sequence	amino acid sequence determined by Edman degradation
1	1168.65	–	1167.99	4.40	$H^{87}-R^{89}$	–
2	2000.66	2000.19	–	0.08	$F^{184}-R^{201}$	FNTGTTVVNGANFFQINR
3	2191.95	2192.31	–	0.09	$E^{441}-R^{459}$	ELSPGVEAQTDEELQDYIR

[a] Peptide numbers refer to Figure 4.

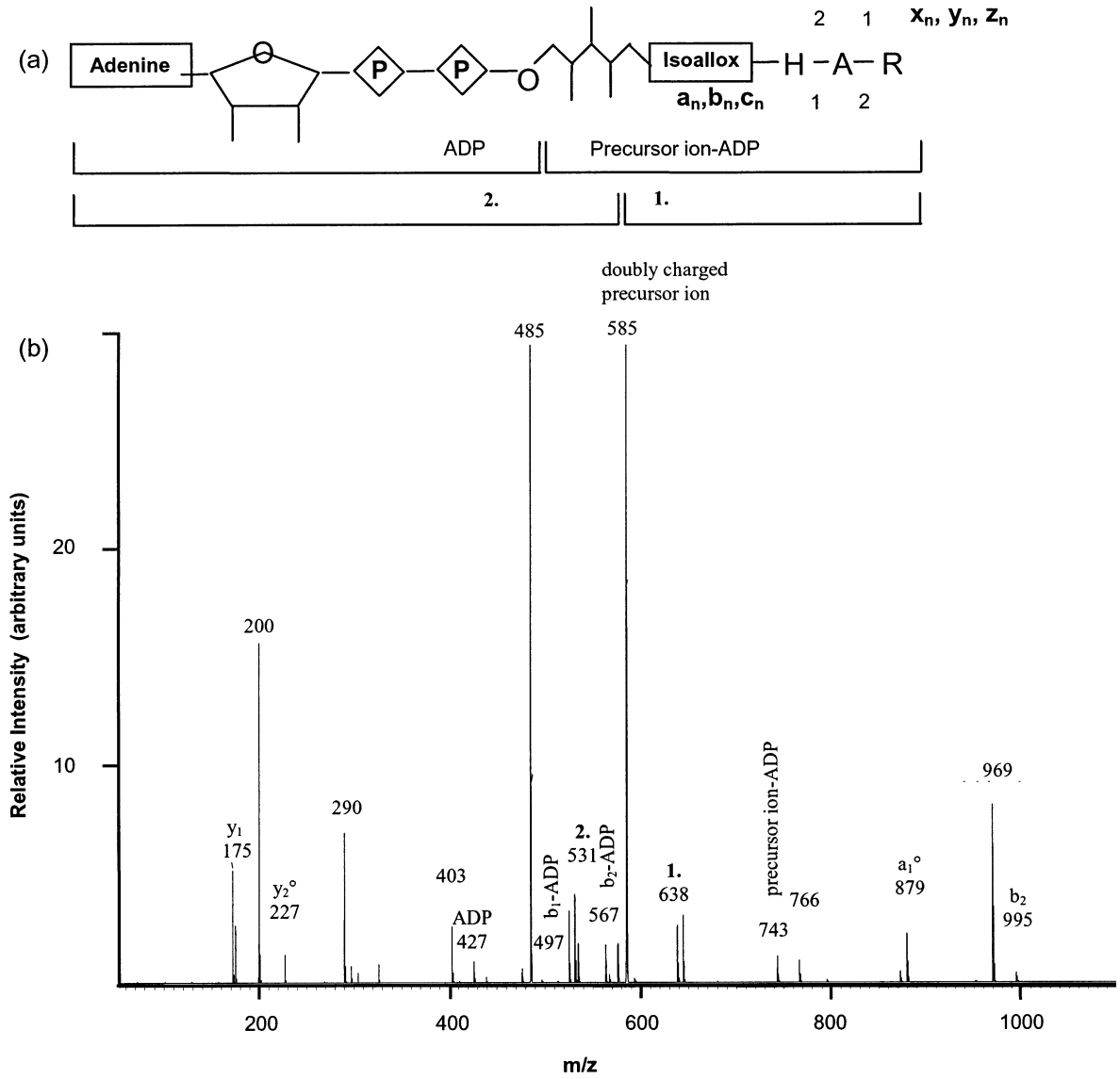

FIGURE 5: Structural analysis of a COX-derived flavopeptide by CID mass spectrometry. The mass spectrum was obtained using the doubly charged ion of the flavopeptide (585 Da) as the selected precursor ion. N-Terminal (a_n-c_n) and C-terminal (x_n-z_n) fragments are indicated according to the conventional nomenclature (*22*, *23*). The three subtypes of N- and C-terminal fragments indicate whether the peptide bond was cleaved between C(α) and C═O (a_n and x_n), between C═O and NH (b_n and y_n), or between NH and C(α) (c_n and z_n). The degree sign denotes loss of water from that particular ion, and −ADP signifies that the fragment has lost a mass equal to that of ADP. The structures of fragments 1 and 2 are schematically shown in panel a.

(data not shown). Among the three peptides, only this one exhibited the characteristic spectral properties of a flavin. When the molecular mass of FAD (785 Da) was subtracted from the observed peptide mass, the resulting mass of 383 Da corresponded to one of the tryptic peptides (plus H^+), predicted from the COX sequence, His-Ala-Arg at positions 87−89.

Because of the failure to sequence the peptide from either the N-terminal or the C-terminal end, CID tandem mass spectrometry was employed to obtain supporting evidence for the structure of the flavopeptide. A doubly charged precursor ion at *m*/*z* 585 in the first quadrupole was selected for collision. The fragments produced by collision in the second quadrupole exhibited a CID spectrum reflecting the ionized fragments of the flavopeptide (Figure 5b). The observed N-terminal (b_2 and a_1°) and C-terminal fragments (y_1 and y_2°), indicating the presence of unmodified arginine and alanine residues, showed that FAD was covalently bound

to the N-terminal histidine residue of the peptide, as predicted above. ADP and the precursor ion without ADP were detected, consistent with the presence of a covalently bound FAD moiety undergoing cleavage at the ribityl part. It was somewhat surprising, however, that neither AMP nor adenine appeared as a cleavage product. The absence of free histidine is in agreement with the tandem mass spectrometry data reported by other groups who similarly did not observe any cleavage of the covalent linkage to FAD (*14*, *15*). The fragments denoted 1 and 2 (Figure 5a) were found to represent internal cleavage of the flavin molecule between C_1 and C_2 of the ribityl part.

DISCUSSION

The characterization of the flavin cofactor of COX from *A. globiformis*, presented in this paper, resulted in two major findings. The first part of the experiments, comprising the isolation and characterization of the aminoacylflavin found in COX, showed that this flavoenzyme contains covalently bound flavin of the type 8α-[N(1)-histidyl]FAD. The second part of the experiments presented here resulted in the identification of the attachment site of the FAD group as His^{87} in the cDNA-derived amino acid sequence of the enzyme.

The highly versatile nature of the flavin group and its ability to mirror its chemical changes spectroscopically enabled the investigation of the covalent binding site to be based almost solely on these characteristics. The major arguments for identification of the coenzyme as 8α-[N(1)-histidyl]FAD are as follows. (i) Treatment with $NaBH_4$ resulted in histidylriboflavin from COX being reduced and converted to 3,4-dihydroflavin, as previously observed for the N(1) isomer (*7*). (ii) The pK_a for imidazole ionization of histidylriboflavin from COX was found to be 5.23, in agreement with the value of 5.2 reported for synthetic 8α-[N(1)-histidyl]riboflavin (*7*). (iii) Finally, histidylriboflavin from COX was found to be unstable when stored for 30 days in an aqueous solution, and 8-formylriboflavin was identified as the breakdown product. In contrast, the N(3) isomer is known to be very stable during storage for longer periods of time (*7*, *10*).

Our results do not agree with the preliminary data published by Ohishi and Yagi (*6*), who concluded that COX from *A. globiformis* contains 8α-[N(3)-histidyl]FAD. This discrepancy may partially be ascribed to the fact that these authors determined the pK_a of the pH-dependent fluorescence quenching at the flavopeptide level. Other studies, however, have clearly shown that this may lead to an incorrect pK_a value, and that this experiment must be carried out with the isolated histidylriboflavin, to avoid any influence from the peptide part. The significant difference between pK_a values obtained for flavopeptide and histidylriboflavin was clearly presented in the previously published characterization of COX from *Alcaligenes* sp. which was unambiguously shown to contain 8α-[N(3)-histidyl]FAD (*1*).

The flavin-carrying amino acid residue in COX was identified by proteolytic cleavage of the protein followed by isolation and characterization of the flavopeptide. Attempts to analyze the flavopeptide by amino acid sequencing were unsuccessful, similar to the negative results reported previously for two hexapeptides with N-terminal flavin-carrying histidines (*14*). These observations suggest that Edman degradation of a peptide generally cannot proceed when an N-terminal histidine residue is the site of flavin attachment. The somewhat unexpected cleavage pattern observed by CID tandem mass spectrometry, as well as the inability to hydrolyze the flavopeptide with carboxypeptidases, may be attributed to the small size of the peptide relative to the FAD moiety.

The position of FAD attachment was identified as His^{87} in the COX polypeptide chain. This finding represents the first mapping of the flavin-carrying amino acid residue in any of the known variants of COX. The amino acid sequence around the flavinylated histidine (S-F-M-R-**H**-A-R-A-K) showed no similarity in sequence to the previously published, partial amino acid sequence of a flavopeptide from COX found in *Alcaligenes* sp. [D-N-P-N-(**H**,S,R) (*1*)]. Likewise, no sequence similarity was observed when the flavopeptide sequence of COX from *A. globiformis* was compared with the sequences in a newly discovered family of oxidoreductases that share a conserved FAD binding domain (*16*). Compared to other known FAD attachment sites which contain successive glycine residues in the immediate vicinity (*5*), His^{87} in COX is surrounded by bulky, positively charged residues (R-**H**-A-R-A-K). As opposed to glycine, these residues might serve to provide a more positively charged, rigid environment. The positively charged environment may serve to stabilize the two electrons taken up by oxidized flavin during reduction and thereby increase the redox potential of the enzyme. This concept has been put forward previously (*17*) and has been supported by crystallographic studies (*18*−*20*).

To our knowledge, COX is the first flavoenzyme with covalently linked FAD which has been shown to contain the N(1) isomer of N-histidylriboflavin in one organism (*A. globiformis*) and the N(3) isomer in another organism (*Alcaligenes* sp.). The covalent flavin attachment is currently regarded as an autocatalytic quinone−methide reaction (*21*), and therefore, the formation of isomeric histidylriboflavin structures may reflect differences in the folding of the protein and the geometry of the nucleophilic addition.

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