Recent Advances in the Chemistry of Covalently Bound Flavin Coenzymes

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Following elucidation of the structures of the flavin components of succinate dehydrogenase (SD) as N(3)-histidyl-8α-FAD and of monoamine oxidase (MAO) as cysteinyl-8α-FAD and determination of the peptide sequences around the flavin sites of these enzymes, attention has been focused on the covalently bound FAD of *Chromatium* cytochrome *c*-552. As documented in preliminary communications, the FAD moiety of this enzyme is also substituted at the 8α-position, as judged from ESR hyderfine structure of the free radical cation and the characteristic hypsochromic shift of the second absorption band of the neutral flavoquinone in purified preparations of the flavin. Definite proof has come from the liberation of 8-carbxyriboflavin on performic acid treatment of the enzyme. In regard to ESR and optical spectra and the tendency of the purified flavin (liberated by proteolysis) to undergo autooxidation with a further hypsochromic shift of the second absorption band and increased fluorescence, the flavin resembles the MAO flavin. The fact that fluorescence is >90% quenched at all pH values even at the FMN level and doees not vary with pH between 3.2 and 8 also suggests a thioether linkage as in cysteinyl riboflavin. In many respects, however, the *Chromatium* flavin differs from cysteinyl riboflavin. Highly purified preparations from tryptic-chymotryptic digests give a positive chloroplatinic test. Electrophoresis clearly shows the presence of carboxyl and amino groups but the peptide gives no characteristic ninhydrin reaction and amino acid analysis of performic acid oxidized samples yields cysteic acid and threonine in amounts less than equimolar to the flavin. The amino acid environment around the flavin may account for these results although a linkage other than a thioether remains a possibility.

Abbreviations: MAO, monoamine oxidase; SD, succinate dehydrogenase.

During the past two years the structures of the covalently bound flavin coenzymes of succinate dehydrogenase (SD) and of monoamine oxidase (MAO) have been elucidated. The former enzyme has been shown [1–3] to contain N(3) histidyl-8α-FAD while the latter cysteinyl-8α-FAD [4–6]. Both histidyl and cysteinyl riboflavin have been synthesized [3, 7, 8] and the amino acid sequences around the flavin sites of SD and MAO have been determined [3, 6, 9]. As yet unpublished results [10] indicate that the covalently bound flavin of D-6-hydroxynicotine oxidase from Arthrobacter oxidans is also histidyl-8α-FAD.

Another enzyme to be added to the list of dehydrogenases which contain flavin covalently bound at the 8α position is the cytochrome *c*-552 of *Chromatium*. BARTSCH et al. [11, 12] reported some years ago that the flavin component of this enzyme is not released by acid denaturation but is extracted after proteolysis, treatment with mercurials, exposure to alkaline pH, or long incubation with saturated urea. HENDRIKS and CRONIN [13] subsequently suggested that the flavin is 8α-substituted FAD because of the hypsochromic shift of the second absorption band of purified preparations of the flavin and recently we [14] proved this assignment by ESR techniques and by isolating 8-carboxyriboflavin from the performic acid oxidized enzyme. The present report describes some properties of *Chromatium* flavin and provides evidence that a sulfur linkage is involved between the 8α-position of the isoalloxazine system and the peptide chain.

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Materials and Methods

The flavin was liberated from purified cytochrome *c*-552 by trypsin and chymotrypsin and purified as in previous work [14] except that no thiol was present during digestion and the sample was kept anaerobic

during purification whenever possible. A peptic peptide was obtained by 4 hours digestion with crystalline pepsin (0.1 mg/mg enzyme) in 0.1 N acetic acid at 38° under N_2. The peptide was purified essentially as in the case of the tryptic-chymotryptic peptide. Dephosphorylation with acid phosphatase (potato) was conducted with 2 μg enzyme/nmole of flavin in 0.1 M acetate, pH 5, at 38° under N_2 for 15 hours.

Results and Discussion

Evidence for a Sulfur Linkage

The following observations [14] indicated that the flavin in *Chromatium* cytochrome *c*-552 is bridged to the peptide chain by way of a reduced sulfur atom. 1) The hyperfine ESR spectrum of the radical cation is essentially indistinguishable from that of the flavin pentapeptide isolated from MAO or of synthetic cysteinyl riboflavin. 2) The flavin, isolated from proteolytic digests under anaerobic conditions, shows a fluorescence excitation peak at 365 nm, representing the same small hypsochromic shift relative to riboflavin as had been observed in the case of MAO [4, 5] but this compound is readily autooxidized, with a further downward shift of the second fluorescence excitation peak to 360 nm (Table I). This ease of autooxidation and the characteristic spectral shift accompanying it and the quenched fluorescence (>90%) between pH 3.2 and 8 which increases 7 to 8 fold on performic acid oxidation are features previously seen in the MAO flavin [4, 5].

The purified flavin, isolated from tryptic-chymotryptic digests, gives a positive chloroplatinic acid test for reduced sulfur. A disulfide or thioester linkage between the flavin and the peptide chain appear to be ruled out since neither reducing agents nor hydroxylamine liberate the flavin from the protein [14]. Thus a thioether or a thiohemiacetal remain possibilities, as discussed below.

Fluorescence Changes on Oxidation and Reduction

Autooxidation of the purified flavin results in a 10-fold increase in fluorescence, accompanied by a 5 nm blue shift of the second excitation peak (Table I). The oxidation product (possibly a sulfoxide) is reduced by dithionite with a return of the second fluorescence excitation peak to 365 nm, but the increased fluorescence of the autooxidized flavin is not quenched thereby (Table I). It is possible that dithionite causes both reduction and cleavage of the sulfur bond, since the hyperconjugation responsible for the stability of the 8α-thioether bond is absent in the reduced flavin. The fact that dithionite does not liberate the flavin from the holoenzyme [14] may reflect the influence of amino acid groups surrounding the flavin in the protein.

Table I. Effect of dithionite on fluorescence emission of *Chromatium* flavin peptide.

Component	λ_{max}, nm excitation	Fluorescence yield, percent[a]
C-flavin, anaerobic	365	5
C-flavin + performic acid	354	50
C-flavin, aerobic	360	50
same + dithionite	365	>40
S-cysteinyl riboflavin	365	10
same + performic acid	354	80

[a] Relative to $A_{450\,nm}$.

Table I also shows that oxidation of the purified flavin with performic acid causes a large increase in molar fluorescence and a shift of the 365 nm band to 354 nm. Under these conditions an oxidation to the level of the sulfone may take place, since dithionite does not reduce the product and since no negative charge is acquired by the flavin, as would be expected if cleavage to the sulfonic acid or to carboxyriboflavin occurred. The difference in products arising on performic acid oxidation of the holoenzyme (8-carboxyriboflavin [14]) and of the purified flavin is again thought to reflect the influence of the protein environment on the course of oxidation.

Evidence for Peptide

It has been implied in the foregoing that the 8α-position of the flavin is substituted by thiol amino acid and the use of trypsin-chymotrypsin to liberate the flavin further implies that product is a flavin peptide. Nevertheless, the purified flavin gives a negative ninhydrin reaction, while cysteinyl riboflavin and the flavin peptide from MAO both give a positive, though uncharacteristic, ninhydrin test [6]. Oxidation by performic acid, followed by hydrolysis with 6 N HCl at 105° for 18 hours gave on amino acid analysis approximately 0.5 mole each of threonine and a strongly acidic amino acid with the behavior of cysteic acid, in addition to traces of other amino acids. While substantial loss of cysteic acid in this procedure is not unexpected, the low

yield of threonine remains unexplained but it could be an impurity.

The tryptic-chymotryptic product must be nevertheless a peptide containing carboxyl and amino groups, as judged by high voltage electrophoresis at various pH values (Table II). At pH 1.6 the de-

Table II. Electrophoretic mobility of *Chromatium* flavin peptide. Migration of FMN relative to riboflavin = +1.0.

Component	pH = 1.6	pH = 3.4
C-flavin, monophosphate	0	+ 0.47
C-flavin + phosphatase	− 0.8	− 0.24
MAO-flavin, monophosphate		+ 0.46
S-cysteinyl riboflavin	− 0.7	0

phosphorylated form of the *Chromatium* flavin shows strong cationic mobility, indicating the presence of a cationic group, most likely an $-NH_3^+$ group. The cationic mobility is still evident at pH 3.4, although it is much decreased at this pH, while cysteinyl riboflavin has zero mobility at pH 3.4. This shows that in going from pH 1.6 to 3.4 deprotonation of a group occurs, which is most likely a COOH group, but one with a higher pK value than the carboxyl in cysteinyl rifloflavin but similar to MAO flavin peptide. All this is compatible with a flavin peptide structure. The peptide, however, is likely to be small (of the order of 2 to 3 amino acids), as judged by electrophoretic mobility at pH 6.5.

It is noteworthy that a small flavin peptide has recently been isolated from peptic digests of the cytochrome and purified by the same procedure as has been used with the tryptic-chymotryptic peptide. The isolated product, which appears to be homogeneous, gives a positive ninhydrin reaction and contains 1 mole each of tyrosine and of a S-amino acid.

Differences between MAO and Chromatium Flavins

Despite the similarities mentioned between the flavins of *Chromatium* cytochrome *c*-552 and MAO, several important differences also exist. First, liberation of 8-carboxyriboflavin by performic acid oxidation of the MAO holoenzyme with significant yield has not been observed. Second, the tryptic-chymotryptic peptide from *Chromatium* is considerably more prone to autooxidation than is the MAO flavin peptide. Third, oxidation of the latter by performic acid yields 80% of the molar fluorescence of riboflavin, while the former gives only 50% fluorescence (Table I). Fourth, on dithionite reduction of the autooxidation product of the *Chromatium* flavin, cleavage occurs, as judged by the high fluorescence, while cysteinyl riboflavin subjected to this treatment does not show a significant enhancement of fluorescence. To this list may be added the negative ninhydrin reaction and the apparent low yield of amino acids on acid hydrolysis.

The experiments presented are compatible either with a thioether or thiohemiacetal linkage in the tryptic-chymotryptic peptide. The former would explain the different oxidation states of the sulfur (Table I), but not the low molar fluorescence relative to MAO flavin peptide. A thiohemiacetal linkage would explain most of the differences from the MAO peptide, assuming that a thiazolidine is formed to account for the negative ninhydrin reaction.

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