STRUCTURE OF THE FLAVIN SITE OF *CHROMATIUM* FLAVOCYTOCHROME c_{-552}*

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SUMMARY

Two flavin peptides have been isolated from *Chromatium* cytochrome c_{-552} by digestion with pepsin and with trypsin-chymotrypsin, respectively. Acid hydrolysis and aminopeptidase digestion of the peptic peptide shows the presence of 1 mole each of threonine and cysteine and 2 of tyrosine, per mole of FAD. Edman degradation gives the sequence: tyr-thr-cys (flavin)-tyr. Tryptic-chymotryptic digestion yields a flavin tripeptide of the structure: thr-cys (flavin)-tyr. The N-terminal tyrosine in the peptic tetrapeptide shows a strong interaction with the flavin, as judged by CD spectra, and this may account for the resistance of the bridge sulfur to oxidation by cold performic acid. Both peptides show abnormally low fluorescence in the pure state (1 to 5% relative to riboflavin) and positive iodoplatinic test; the peptic peptide yields a positive, the tryptic-chymotryptic peptide negative ninhydrin reaction. The electrophoretic mobility of the major product of aminopeptidase digestion (presumably the thiazolidine form of cysteinyl-8α-FAD thiohemiaceta') and other properties of the two peptides are in accord with previous suggestions that the linkage of the flavin to the peptide is a thiohemiacetal.

Bartsch *et al.* (1) reported in 1961 that *Chromatium* cytochrome c_{-552} contains covalently bound flavin. Hendricks and Cronin (2) suggested that the linkage to the FAD is by way of the 8α carbon, as in other enzymes containing covalently bound flavin (3, 4). In a collaborative study with Prof. Cronin's laboratory we have shown that the 8α carbon of the flavin is indeed the site of attachment, that the substituent amino acid is cysteine, but the linkage does not appear to be a thioether, as in monoamine oxidase, but probably a thiohemiacetal (5, 6). We have now isolated two pure FAD peptides by peptic and tryptic-chymotryptic digestion of the cytochrome. The present paper describes their properties and amino acid sequence. The chemical and physical properties of the peptides are in accord with the previously suggested thiohemiacetal structure and seem to rule out a thioether.

MATERIALS AND METHODS

Chromatium strain D cells were kindly supplied by Dr. J. Cronin and cytochrome

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c_{552} was purified as previously described (7). Peptic and tryptic-chymotryptic digestion and purification of the flavin peptides were as outlined in previous papers (5, 6), except that in the terminal purification step descending paper chromatography in n-butanol:acetic acid:water (4:2:2) was substituted for high voltage electrophoresis. In CD studies the material eluted from phosphocellulose was used directly. Sequential Edman degradations, acid hydrolysis and aminopeptidase digestion of peptides, and amino acid analyses were performed as before (8). Concentrations of flavin peptides were calculated assuming a molar extinction coefficient of 11,300 for dinucleotides. In fluorometry the Hitachi-Perkin Elmer MP-3 absolute fluorometer was used. Qualitative ninhydrin and sulfur tests were carried out as in the studies of Kearney et al. (4).

RESULTS AND DISCUSSION

Amino Acid Composition and Sequence - Homogeneous preparations of the peptic and tryptic-chymotryptic flavin peptides at the FAD level on acid hydrolysis gave the results shown in Table I. It may be seen that, per mole of flavin, 2 moles of tyrosine were

TABLE I

Amino Acid Composition of Flavin Peptides [a]

Amino Acid	Tryptic-chymotryptic Peptide (nmoles)	Peptic Peptide (nmoles)
Thr	9.5	9.4
Cys	10.4	8.0
Tyr	11.6	20.6
Flavin [b]	14.2	13.2

[a] Determined after hydrolysis at 110° in 6 N HCl for 24 hrs.
[b] Based on $\varepsilon_{450} = 11.3 \times 10^3$.

recovered in fair yield in the peptic and 1 mole in the tryptic-chymotryptic peptide, but the recovery of threonine and cysteine was lower. Loss of cysteine is to be expected, since it was determined as such, without prior oxidation to cysteic acid and the yield was, in fact, higher in other experiments where hydrolysis was continued for 48 hours. In addition, some glycine was found, which appears to arise from breakdown of the FAD moiety, as determined by acid hydrolysis of an equivalent amount of FAD. In amino-peptidase digests of the two peptides neither glycine nor cysteine were found, but threo-

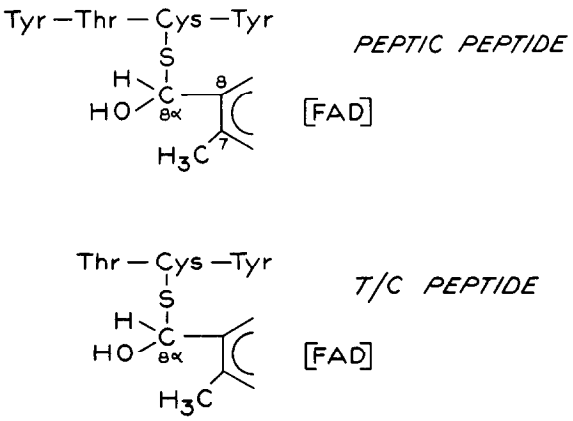

Fig. 1. Proposed structures of flavin peptides isolated from Chromatium cytochrome c_{552}.

nine (1 mole) and tyrosine (1 mole in the tryptic-chymotryptic, 2 moles in the peptic peptide) were recovered in excellent yield. The reason for the absence of cysteine from enzymatic hydrolyzates is that during aminopeptidase digestion the cysteine-flavin linkage remains intact. Taken together, these data indicate that the amino acid composition of the tryptic-chymotryptic peptide is Flavin:1 Thr:1 Cys:1 Tyr and that of the peptic peptide Flavin:1 Thr:1 Cys:2 Tyr.

The amino acid sequence, determined by Edman degradation, gave the structures shown in Fig. 1. The N-terminal tyrosine present in the peptic peptide may be responsible for some unusual properties of this compound to be discussed below.

Properties of the Flavin Peptides - Both peptides gave positive iodoplatinic and negative I_2-azide tests for reduced sulfur. The ninhydrin reaction was positive, though atypical, in the peptic peptide but negative in the tryptic-chymotryptic peptide (Table II). Both compounds exhibited a very low fluorescence which was pH-independent in the range pH 3.2 to 7.

The extremely low fluorescence of the peptic peptide distinguishes this compound from cysteinyl flavin thioethers and the flavin thioether pentapeptide isolated from monoamine oxidase (4) and is not due to internal quenching by the adenylate, because the value in Table II was determined at pH 3.4. It should be also mentioned that the fluorescence gradually declines in the course of purification of the peptic peptide from about 10% of that given by riboflavin to about 1%.

Oxidation of the tryptic-chymotryptic peptide with cold performic acid increases the fluorescence to 50% of the value given by equimolar riboflavin with a shift of the second excitation maximum from 365 nm to 354 nm. Oxidation of flavin thioethers under these con-

TABLE II

Properties of Flavin Peptides Isolated from Chromatium Cytochrome c_{552}

Test	Result	
	Tryptic-chymotryptic Peptide	Peptic Peptide
Molar Fluorescence [a]	0.05	0.01
Same [a] after performic acid oxidation at 0°	0.50	0.05
Same [a] after performic acid oxidation at 40°		0.50
Ninhydrin reaction	—	+
Iodine-azide test	—	—
Iodoplatinic test	+	+

[a] Relative to equimolar solution of riboflavin

ditions gives 80% of the molar fluorescence of riboflavin (4). Interestingly, cold performic acid does not attack the peptic peptide, since it causes only a trivial increase in fluorescence. At elevated temperatures performic acid does attack the peptic peptide, however, yielding the same 50% maximum fluorescence relative to riboflavin as given by the tryptic-chymotryptic peptide on oxidation with cold performic acid.

The resistance of the peptic peptide to oxidation by performic acid in the cold may be the consequence of an interaction of the N-terminal tyrosine with the cysteine-flavin moiety and such interaction may also be responsible for the low fluorescence of this compound as compared with the tryptic-chymotryptic peptide, which lacks this tyrosine. The presence of large amounts of extraneous material in crude preparations may minimize such interaction, accounting for the higher fluorescence of the peptic peptide in crude preparations.

CD Spectra - Fig. 2 compares the CD spectra of FAD and of the two FAD peptides isolated from Chromatium. The peptic peptide exhibits a positive, broad Cotton effect with a maximum at 484-490 nm and negative bands at 375 and 305 nm. The negative 375 nm band of the peptic peptide has a two-fold greater intensity than that of FAD. The absence of the

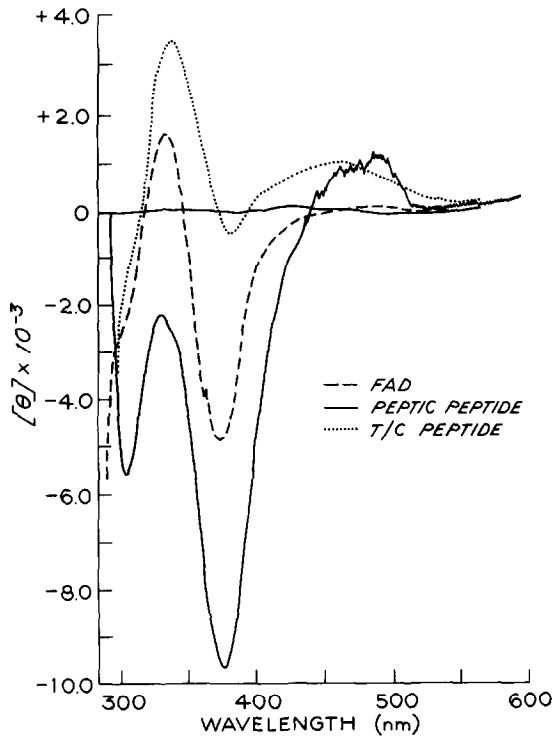

Fig. 2. CD spectra of FAD and of flavin peptides from Chromatium cytochrome c-552. T/C = trypsin-chymotrypsin. The spectra were measured at 1.65×10^{-4} M concentration at pH 6.8 at 25° in 10 mm cylindrical quartz cells, using a JASCO UV 5 instrument with a Sproul Scientific SS 10 modification.

N-terminal tyrosine in tryptic-chymotryptic peptide dramatically alters the Cotton effects of the flavin, resulting in a positive band at 340 nm, as in free FAD. The long wavelength positive band with a maximum at 460 nm is still present but only a small negative band at 380 nm is observed.

The results are consistent with a direct tyrosin-flavin interaction in the peptic peptide, as suggested above. It is of interest that the general features of the CD spectrum of this peptic peptide closely resemble those reported for flavodoxin (9, 10), an enzyme in which recent X-ray crystallographic studies revealed the presence of a tyrosine residue parallel in a "stacking" arrangement with the flavin (11, 12). These results indicate a planar "stacking" of the N-terminal tyrosine with the flavin in the peptic flavin tetrapeptide.

Linkage of the Flavin to the Peptide - In previous papers (5, 6) we have cited evidence ruling out a thioether or disulfide linkage and pointed out that the properties of both the tryptic-chymotryptic and peptic peptides differed from known flavin thioethers so that a cysteinyl flavin thioether is unlikely. On the other hand, most of the properties

were compatible with a thiohemiacetal structure, as visualized in Fig. 1. Recent observations add to the list of differences between the Chromatium flavin and synthetic or naturally occurring flavin thioethers and enhance the probability that the linkage is indeed a thiohemiacetal, without proving it conclusively. Thus the product of aminopeptidase digestion of either Chromatium peptide, before or after dephosphorylation, on performic acid oxidation gives considerably lower molar fluorescence than does cysteinyl riboflavin thioether. This cysteinyl-flavin compound is also considerably more labile on standing than authentic cysteinyl riboflavin. Moreover, when the cysteine-FAD adduct, derived from aminopeptidase digestion, was subjected to high voltage electrophoresis at pH 6.5, two flavin components were detected in about equal amounts. One had about 95% of the negative mobility of FAD, as expected for a linear thiohemiacetal, the other a much greater mobility than free FAD, suggesting that the - NH_2 group of cysteine was blocked, as would be expected if cyclization to a thiazolidine had occurred at pH 8.2. On elution and reelectrophoresis of the latter component only the compound with 95% of the mobility of FAD was found, showing that the + charge of the -NH_2 group had been regained, as would be expected on ring opening to the thiohemiacetal form.

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