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STRUCTURE OF THE COVALENTLY BOUND FLAVIN OF *CHLOROBIUM* CYTOCHROME c_{553}

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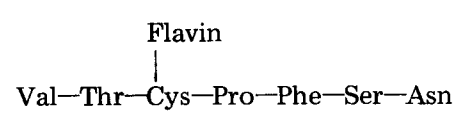
Summary

Cytochrome c_{553} (*Chlorobium thiosulfatophilum*), is known to contain both a cytochrome moiety and a flavin component, which is not extracted by denaturation with trichloroacetic acid. In the present study a peptic flavin peptide was isolated and characterized. This confirms the hypothesis that the flavin is covalently linked to the polypeptide chain.

Several lines of evidence suggest that the flavin is attached via its 8α position to the sulfur of a cysteinyl residue. These include a hypsochromic shift of the near ultraviolet maximum relative to riboflavin, an increase in fluorescence on oxidation with performic acid (from <6% to 70% that given by an equimolar concentration of riboflavin), with a further hypsochromic shift of the near ultraviolet maximum, and the presence of cysteine after acid hydrolysis but not after aminopeptidase M digestion. All of these properties have previously been observed in 8α-*S*-cysteinylriboflavin and its peptides. The cysteinyl flavin bond survives mild acid hydrolysis if the sulfur moiety is first oxidized to the sulfone prior to acid treatment. It was thus possible to obtain an amino acyl derivative by this method and show its identity to 8α-*S*-cysteinylsulfone-2′,5′-anhydro-riboflavin obtained via synthetic methods. The flavin occurs as a dinucleotide, presumably FAD, and from the many properties in common with cysteinyl-riboflavin and its peptides it is concluded that the flavin structure of *Chlorobium* cytochrome c_{553} is 8α-*S*-cysteinyl-FAD thioether.

The amino acid sequence around the flavin site was found to be:



Introduction

Flavocytochrome c_{553} of *Chlorobium thiosulfatophilum* (no EC number assigned) contains one molecule each of covalently bound flavin and heme *c* [1]. The cytochrome is composed to two non-identical subunits, which can be resolved into cytochrome and flavoprotein moieties by treatment with trichloroacetic acid or by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weights of the cytochrome moiety and flavoprotein moiety are 11 000 and 47 000, respectively [2,3].

Although the flavin could not be removed from the protein by acid ammonium sulfate or by extraction with 5% (w/v) trichloroacetic acid, flavin was liberated by incubation of urea-treated cytochrome c_{553} with a bacterial protease [1]. The flavoprotein moiety has absorption maxima in the visible region at 350 and 452 nm, similar to many flavoproteins [3].

These early studies suggest that the yellow chromophore of cytochrome c_{553} is a covalently bound flavin derivative, several types of which have now been identified in many other enzymes [4]. The nature of the linkage, including the site of attachment of the flavin and the amino acid involved in the linkage to the cytochrome, have not been investigated until now.

In this report we present evidence that the covalently bound flavin of *Chlorobium* cytochrome c_{553} is 8α-*S*-cysteinyl-FAD thioether, a flavin derivative also found in monoamine oxidase [5].

Materials and Methods

Materials. Chlorobium thiosulfatophilum cytochrome c_{553} was prepared as previously described [3]. DEAE-cellulose (DE-52) was obtained from W. & R. Balston, Ltd., England, phosphocellulose from Gallard-Schlesinger Chemical Manufacturing Corp., trypsin and α-chymotrypsin from Worthington Biochemical Corp., pepsin from Mann Research Laboratories, aminopeptidase M from Rohm and Haas, Darmstadt, Germany, and nucleotide pyrophosphatase (type II from *Crotalus adamenteus*) and intestinal alkaline phosphatase (type VII from calf mucosa), from Sigma. 8α-*S*-cysteinylriboflavin was synthesized as before [6].

Purification of flavin peptide. During the proteolytic digestion and purification of the flavin peptide, all operations were performed under an atmosphere of N_2.

Approximately 40 mg of *Chlorobium* cytochrome c_{553} in an ammonium sulfate suspension were sedimented at 4°C for 10 min at 33 000 × *g*. The precipitate was dissolved in H_2O and the protein denatured and freed of any acid-soluble flavins by the addition of 0.1 volume 55% (w/v) trichloroacetic acid at 0°C. The suspension was centrifuged as above and the precipitate washed with 1% (w/v) trichloroacetic acid. The resulting precipitate was suspended in a minimum volume of H_2O and the residual trichloroacetic acid extracted with 4 volumes ethyl ether. Residual ether was then removed by flushing with N_2. To the aqueous suspension formic acid was added to a final concentration of 5% (v/v) and the volume adjusted with 5% formic acid to a protein concentration of 15 mg/ml. Peptic digestion was performed in the dark

with 0.06 mg pepsin per mg cytochrome for 4 h at 38° C. The digest was then applied to a column (0.9 × 12 cm) of phosphocellulose (pyridinium form) equilibrated with 5% (v/v) formic acid and eluted with this solvent. The flavin fractions were pooled and the solvent removed by lyophilization. The flavin (ca. 250 nmol) was dissolved in 0.5 ml 5% (v/v) pyridine and applied to a column (0.9 × 8 cm) of DEAE-cellulose (acetate form). The column was then washed with 3 ml each of 5% pyridine and H_2O, and the flavin was eluted using a non-linear gradient of pyridinium acetate (equimolar in each) with 60 ml H_2O in a constant-volume mixing chamber and 0.1 M pyridinium acetate in the reservoir. A flavin peptide was eluted at 0.05 M pyridinium acetate. Amino acid analysis indicated that this peptide was homogeneous and it was thus used for sequence analysis.

Other methods. Absorption spectra were recorded with a Cary 14 spectrophotometer and fluorescence spectra with a Hitachi-Perkin Elmer Model MPF-3 spectrofluorometer, equipped with a self-correcting accessory. Flavin concentrations were estimated assuming extinction coefficients at their 445—450 nm maxima of 11 300 M^{-1} cm^{-1} for dinucleotides and 12 300 for mononucleotides. Amino acid analyses were performed on Hitachi Perkin-Elmer 034 Liquid Chromatograph equipped with a scale expansion accessory.

Two systems were used for thin-layer chromatography. In system A cellulose was the support and 1-butanol : acetic acid : water (12 : 3 : 5) the solvent; in system B silica gel was the support and 1-butanol : acetic acid : water (7 : 2 : 1) the solvent. High voltage electrophoresis was conducted at 50 V/cm at pH 1.6 (8% (v/v) formic acid), pH 5.0 (1% (v/v) pyridine titrated to pH 5.0 with glacial acetic acid), or at pH 6.25 (0.5% (v/v) acetic acid, 10% (v/v) pyridine).

Performic acid oxidation was performed as described by Walker et al. [5] and reductive zinc cleavage by a modification of their method, in that incubation was performed at 100° C in 9 : 1 (v/v) glacial acetic acid: trifluoroacetic acid. Phosphatase digestion was carried out at 38° C for 1 h with 0.5 mM flavin and 0.05 mg phosphatase per ml in 0.1 M ammonium bicarbonate, pH 9.0. Incubation with nucleotide pyrophosphatase was performed as in previous studies [6]. The conditions of acid hydrolysis, incubation with aminopeptidase M (36 h), and sequential Edman degradation were as described in a previous paper [7]. Total phosphorus was measured by Bartlett's method [8].

Results and Discussion

Purification of a flavin peptide. Mention should be made of the effectiveness of chromatography on phosphocellulose in 5% (v/v) formic acid, as described in Materials and Methods. At this pH most peptides remain bound to the support owing to the presence of the α-amino group, while flavin peptides devoid of basic amino acyl residues do not, since the phosphate group counterbalances the positive charge. It is thus possible to separate the flavin peptide from heme and other peptides in this first step of purification. Subsequent chromatography on DEAE-cellulose yielded a homogeneous peptide, as evidenced by amino acid analysis and sequence determination (cf. below).

Spectra properties of flavin peptide from Chlorobium cytochrome c_{553}. The absorption spectrum of the flavin peptide (Fig. 1) shows maxima at 449 and

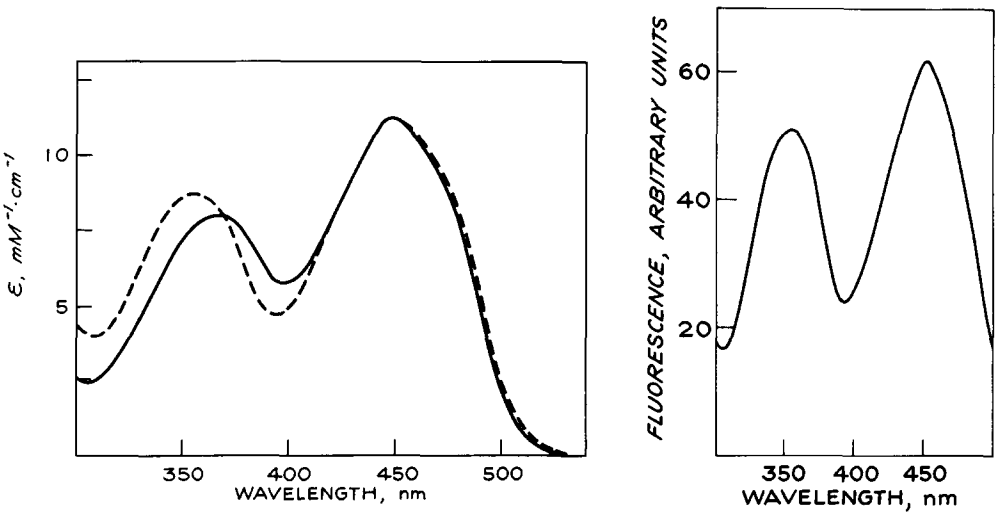


Fig. 1. Absorption spectrum of *Chlorobium* cytochrome c_{553} peptic flavin peptide in water (pH ca. 5). ———, native peptide; - - - - -, same after oxidation with performic acid.

Fig. 2. Corrected fluorescence excitation spectrum of cytochrome c_{553} peptic flavin peptide after performic acid oxidation. Emission wavelength of 525 nm, pH = 3.4.

366 nm. After incubation with performic acid, maxima at 352 and 452 nm are observed. The hypsochromic shift of the near ultraviolet absorption maximum (373 nm for FAD) is suggestive of substitution at the 8α position of the isoalloxazine ring, since it is characteristic of all naturally occurring and chemically synthesized 8α-substituted flavins [9].

The fluorescence of the flavin peptide is extensively quenched (<6% relative to riboflavin). On oxidation with performic acid, however, the fluorescence increases to 70% that of riboflavin and the fluorescence excitation spectrum exhibits maxima at 354 and 452 nm (Fig. 2). * The fluorescence yield is the same at pH 7.0 as at 3.4, indicating that histidine is not involved in the linkage as in succinate dehydrogenase [10] and several other enzymes [4]. On the other hand, the increase in fluorescence on performic acid oxidation and concommitant hypsochromic shift of the near ultraviolet maximum (from 366 to 354 nm) are characteristic of covalently bound flavins containing cysteine substituted in the 8α position of the flavin [5,6,11].

Amino acid composition and sequence of flavin peptide. The peptic flavin peptide was subjected to acid hydrolysis (6 M HCl, 105° C, 14 h, in vacuo) and to digestion with aminopeptidase M and hydrolysates were analyzed with an amino acid analyzer (Table I). The results indicate that, per mol of flavin, the peptic flavin peptide contains one mol each of asparagine, threonine, serine, proline, valine, phenylalanine. In addition, 0.69 mol of cysteine was recovered, in part as cysteic acid, if the hydrolysis was carried out without prior oxidation of the sulfur. Incomplete recovery of cysteine and partial recovery as cysteic acid is not unexpected of course. In order to improve the yield of cysteine, the

* The slight fluorescence observed prior to performic acid oxidation may in part reflect air oxidation of a small fraction of the flavin peptide during purification. Since this possibility remained, the fluorescence spectrum observed for the native peptide is not included.

TABLE I

AMINO ACID COMPOSITION OF FLAVIN PEPTIDE OF *CHLOROBIUM* CYTOCHROME c_{533}

All values are based on flavin = 1.0 assuming $\epsilon_{450} = 11\ 300\ M^{-1}\ cm^{-1}$.

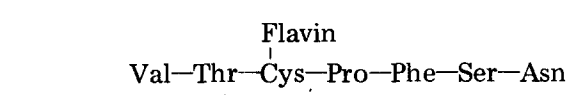
Amino acid	Acid hydrolysis	Performic acid then acid hydrolysis	Aminopeptidase M digestion
Aspartate	0.98	0.88	0.17
Asparagine			0.46
Threonine	1.03	0.88	0.99
Serine	1.12	1.28	0.77
Proline	0.89	N.D. [a]	0
Valine	1.08	0.89	1.07
Phenylalanine	1.03	N.D. [a]	N.D. [a]
Half-cystine	0.40		
Cysteic acid	0.29	1.01	0

[a] N.D., not determined.

preparation was oxidized with performic acid and acid hydrolysis was repeated (Table I). Under these conditions one mol of cysteic acid was recovered per mol of flavin in amino acid analysis. It is clear, therefore, that cysteine is a constituent of the peptide.

Aminopeptidase M digestion (Table I) has provided evidence that asparagine rather than aspartate is present in the peptide and that cysteine is involved in the linkage to the flavin since it is found after acid hydrolysis (in part as cysteic acid) but is absent after incubation with the enzyme. The absence of proline in the peptidase digest may be a consequence of diketopiperazine formation [12], but this possibility was not investigated further.

The sequence of the flavin peptide was found to be:



No dansyl (5-dimethylaminonaphthalenesulfonyl-) amino acid was observed at the third position. Since all other amino acids present in the peptide could be assigned to a particular position, it may be assumed that the undetected residue is the cysteinyl flavin moiety.

Comparison of flavin derivative of Chlorobium cytochrome c_{553} with 8α-S-cysteinylriboflavin. The results above indicate that cysteine is involved in the linkage via the sulfur moiety of the peptide to the 8α position of the isoalloxazine ring of cysteine. Confirmation of this assignment came from further comparison of the properties of the flavin peptide with those of 8α-*S*-cysteinylriboflavin. Both compounds are readily oxidized in air, resulting in an increase in fluorescence, and both are destroyed by acid hydrolysis, yielding cysteine and 8-formyl flavin as products. Reductive zinc cleavage, which converts 8α-*S*-cysteinylriboflavin to riboflavin [5]. gave rise to FMN when applied to the flavin peptide, as ascertained by high voltage electrophoreses at pH 6.25 and 1.6, by thin-layer chromatography in system A, and by fluorescence excitation maxima at 370 and 445 nm at neutral pH. Dithionite reduction of 8α-*S*-cysteinylsulfoneriboflavin results in the cleavage of the cysteinyl flavin bond with the formation of riboflavin [13]. Dithionite reduction (3-fold excess) of

the flavin peptide, following oxidation to the sulfone by performic acid, resulted in the liberation of flavin and a shift in the absorption maxima from 352 and 452 nm to 369 and 446 nm, respectively. The flavin component co-migrated with FMN on high voltage electrophoreses at pH 1.6 and pH 5.0. An aliquot of the reaction mixture following dithionite reduction was incubated with alkaline phosphatase (see Materials and Methods) resulting in the liberation of authentic riboflavin as judged by spectral properties (absorption maxima of 371 and 445 nm), and by co-migration with riboflavin in thin-layer chromatographic systems A and B. The behavior of the peptide on oxidation by performic acid followed by reduction with a small excess of dithionite thus parallels the behavior of cysteinyl flavins in which linkage to the 8α position is via a thioether [13].

As mentioned earlier, cysteinylriboflavin and its peptides do not survive incubation under conditions which hydrolyze peptide bonds. If cysteinylriboflavin is oxidized to the sulfone with performic acid prior to acid hydrolysis (6 M HCl at 95° C in vacuo for 16 h), however, little cleavage of the amino acyl flavin bond occurs. The peptic flavin peptide was therefore oxidized with performic acid and subjected to the acid treatment above. High voltage electrophoresis at pH 1.6 yielded a cationic flavin component, indicating that an amino acid was still attached to the flavin, which migrated identically with 8α-*S*-cysteinylsulfone-2′,5′-anhydroriboflavin. Formation of the anhydroflavin under acidic conditions has previously been shown to occur, particularly with phosphorylated flavin derivatives [14,15].

In past work both on the flavin components of hepatic monoamine oxidase [5] and *Chromatium* cytochrome c_{552} [6] the assignment of structure was based on comparison of a flavin peptide isolated from natural sources with a synthetic amino acyl flavin. The reasons for this were the instability of the amino acyl flavin on acid hydrolysis or on aminopeptidase M digestion and the ease of aerobic oxidation of the sulfur moiety. Conditions have now been found for stabilizing the cysteinyl flavin bond during the hydrolysis step by prior oxidation to the sulfone. This has permitted for the first time direct comparison of the oxidized amino acyl flavin derivative obtained by acid hydrolysis of the natural peptide with the synthetic compound of known structure obtained by an unambiguous route. The demonstration of their identity makes it possible to conclude that the flavin is linked to the polypeptide backbone via a thioether linkage between a cysteinyl residue and the 8α position of the isoalloxazine ring system.

Evidence for flavin dinucleotide. The conclusion that the flavin component of cytochrome c_{553} is a dinucleotide rests on two lines of evidence. First, the mobility on high voltage electrophoresis at pH 6.25 of the flavin peptide decreased slightly after incubation with pyrophosphatase. A decrease in mobility is also observed when FAD is converted to FMN. Second, approximately 2 mol (2.4 mol/mol) of phosphate were found per mol of flavin. Although the presence of adenosine has not been demonstrated, it appears very likely that the dinucleotide occurs as FAD. The structure of the covalently bound flavin of *Chlorobium* cytochrome c_{553} is then 8α-*S*-cysteinyl-FAD (Fig. 3).

Comparison of the covalently bound flavins of Chlorobium cytochrome c_{553}

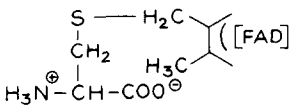


Fig. 3. Structure of amino acyl flavin from *Chlorobium* cytochrome c_{553}.

and Chromatium cytochrome c_{552}. The flavocytochromes from these two bacteria have many properties in common [1] and it was thus anticipated that the flavin structures might be similar as well. In the course of this investigation many differences in properties of these two flavins were demonstrated. Thus, performic acid oxidation of the protein releases the flavin from *Chromatium* cytochrome c_{552} [16] but not from *Chlorobium* cytochrome c_{553}. Performic acid oxidation of the flavin peptides gives a fluorescence excitation yield of 50% of that of riboflavin for cytochrome c_{552} [17] but 70% for cytochrome c_{553}. The amino acid sequences around the flavin sites of the two enzymes are also completely different. In view of these differences it would not be surprising if the linkage of the flavin to the cysteine were also different in the two flavocytochromes. The thioether structure demonstrated for the *Chlorobium* flavin differs, in fact, from the thiohemiacetal which was proposed for the flavin from *Chromatium* [6]. Whether such a difference in structure indeed exists between the two flavins is no longer probable, however. One of the key pieces of evidence for the suggestion that *Chromatium* cytochrome c_{552} contains a flavin thiohemiacetal was the finding that acid hydrolysis of the flavin peptide yielded 8-formylriboflavin [6]. In the course of the present investigation it was noted, however, that acid hydrolysis of cysteinylriboflavin thioether also gives rise to 8-formyl flavin. Thus, the finding of formyl flavin can no longer be taken as evidence that the carbon is at the oxidation level of a carbonyl in the parent compound. In the light of this recent observation it seems necessary to reevaluate the structure of the covalently bound flavin of *Chromatium* cytochrome c_{552}.

Acknowledgements

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References

1 Bartsch, R.G., Meyer, T.E. and Robinson, A.B. (1968) in Structure and Function of Cytochromes (Okunuki, K., Kamen, M.D. and Sekuzu, I., eds.), pp. 443—451, University of Tokyo Press, Tokyo
2 Yamanaka, T. and Kusai, A. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), pp. 292—301, Elsevier, Amsterdam
3 Yamanaka, T. (1976) J. Biochem. (Tokyo) 79, 655—660
4 Edmondson, D.E., Kenney, W.C. and Singer, T.P. (1977) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.) Academic Press, New York, in press
5 Walker, W.H., Kearney, E.B., Seng, R.L. and Singer, T.P. (1971) Eur. J. Biochem. 24, 328—331
6 Walker, W.H., Kenney, W.C., Edmondson, D.E., Singer, T.P., Cronin, J.R. and Hendriks, R. (1974) Eur. J. Biochem. 48, 439—448

7 Kenney, W.C., Walker, W.H. and Singer, T.P. (1972) J. Biol. Chem. 247, 4510—4513
8 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
9 Singer, T.P. and Kenney, W.C. (1974) in Vitamins and Hormones (Harris, R., Munson, P.L., Diczfalusy, E. and Glover, J., eds.), Vol. 32, pp. 1—45, Academic Press, New York
10 Walker, W.H. and Singer, T.P. (1970) J. Biol. Chem. 245, 4224—4225
11 Kearney, E.B., Salach, J.I., Walker, W.H., Seng, R.L., Kenney, W., Zeszotek, E. and Singer, T.P. (1971) Eur. J. Biochem. 24, 321—327
12 Takahashi, K., Stein, W.H. and Moore, S. (1967) J. Biol. Chem. 242, 4682—4690
13 Edmondson, D.E. and Singer, T.P. (1973) J. Biol. Chem. 248, 8144—8149
14 Edmondson, D.E. (1976) Fed. Proc. 35, 1542
15 Kenney, W.C., Edmondson, D.E., Singer, T.P., Steenkamp, D.J. and Schabort, J.C. (1976) Biochemistry 15, 4931—4935
16 Hendriks, R., Cronin, J.R., Walker, W.H. and Singer, T.P. (1972) Biochem. Biophys. Res. Commun. 46, 1262—1269
17 Kenney, W.C., Edmondson, D.E. and Singer, T.P. (1974) Eur. J. Biochem. 48, 449—453