THE COVALENTLY BOUND FLAVIN PROSTHETIC GROUP OF β-CYCLOPIAZONATE OXIDOCYCLASE

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Received 11 February 1974

1. Introduction

It has been reported that β-cyclopiazonate oxidocyclase from *Penicillium cyclopium*, an enzyme which catalyzes the dehydrogenation and cyclization of β-cyclopiazonic to α-cyclopiazonic acid, contains covalently bound flavin [1]. A flavin peptide, released by digestion with pronase has been partially purified and preliminary results suggested that the flavin is at the dinucleotide level. The present paper is the first report of a collaborative study between the laboratories in South Africa and in San Francisco, aimed at defining the structure of the flavin site of this enzyme. It has been found that the flavin is attached to the protein by way of the 8 α group, as in other enzymes containing covalently bound flavin [2] and that the amino acid substituent is histidine, as in succinate dehydrogenase [3,4] but the nature of the linkage of the flavin to the imidazole is different from that in succinate dehydrogenase. The unusual properties of the flavin peptide from β-cyclopiazonate oxidocyclase appear to be in part due to the covalently bound histidine, in part to non-covalent interaction between the flavin and other aminoacyl residues within the peptide.

2. Materials and methods

β-Cyclopiazonate oxidocyclase was purified as in previous work [5]. After removal of acid-extractable flavins with trichloroacetic acid, flavin peptides were released by digestion with tripsin–chymotrypsin (0.1 mg each per mg of protein, 4 hr, 38°C, pH 8). A tryptic–chymotryptic peptide was then purified by chromatography on Florisil and cellulose phosphate columns. Corrected fluorescence spectra were recorded with a Hitachi–Perkin Elmer MPF-3 spectrofluorometer.

3. Results and discussion

The fluorescence excitation spectrum of the

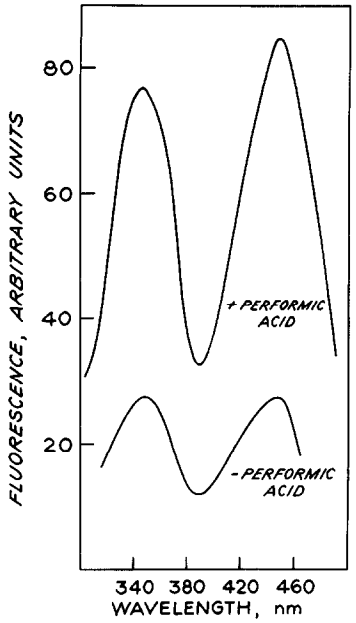

Fig. 1. Corrected fluorescence excitation spectra of tryptic–chymotryptic flavin peptide at pH 3.4 before and after oxidation with performic acid at 0°C.

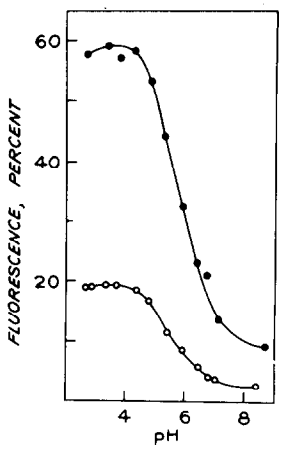

Fig. 2. pH-fluorescence profile of tryptic–chymotryptic flavin peptide (FMN level). (O), before performic acid oxidation; (●), after performic acid oxidation at 0°C. Fluorescence is percent relative to riboflavin assuming Σ_{450} = 12 000 for flavin peptide.

purified flavin peptide from β-cyclopiazonate oxidocyclase, at the monophosphate level (fig. 1), differs from the corresponding spectrum of FMN in two important respects. First, the 372 nm band is hypsochromically shifted to 348 nm, indicative of 8α substitution, as in other flavin peptides [2,6–8]. Second, the fluorescence is extensively quenched at pH 3.4, relative to FMN, but increases nearly threefold on oxidation with performic acid at 0° (table 1, fig. 2). Similar increase in fluorescence upon oxidation with performic acid has been seen in other flavin peptides, which contain an oxidizable group substituted on, or interacting with, the flavin, as in the case of cysteinyl flavin thioether from monoamine oxidase [9] and cysteinyl flavin thiohemiacetal from *Chromatium* cytochrome c_{552} [10,11].

Unlike in those flavin peptides, however, the second fluorescence excitation maximum does not shift significantly on oxidation with performic acid (fig. 1).

A third difference from FMN and riboflavin is illustrated in fig. 2, which shows that the fluorescence is further quenched on neutralization of the flavin peptide, both before and after oxidation with performic acid. The pK_a of this fluorescence quenching (5.8, table 1), is more than one pH unit higher, however, than in *N*(3)-histidyl flavin peptides from succinate dehydrogenase [4] or D-6-hydroxynicotine oxidase [12].

Since the foregoing results bear evidence both for the presence of a substituent containing a secondary or tertiary nitrogen on the flavin and for a readily oxidizable group in the flavin peptide, the latter was

Table 1
pK_a of fluorescence quenching

Flavin	Fluorescence* (%)	pK_a
Tryptic–chymotryptic peptide after pyrophosphatase	19	5.4
Same after performic acid oxidation at 0°C	59	5.8
Amino acyl flavin**	100***	5.0
Same after performic acid oxidation at 0°C	100***	4.9

* Relative to riboflavin = 100; pH 3.4.
** 6 N HCl, 95°C, 17 hr. Purification by thin-layer chromatography.
*** Estimated after consideration of loss due to acid hydrolysis and recovery from thin-layer chromatography. Actual yield = 48%.

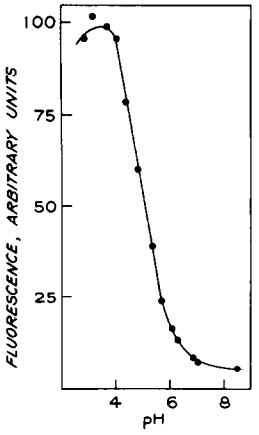

Fig. 3. pH-fluorescence curve of amino acyl flavin. The flavin peptide was hydrolyzed in 6 N HCl at 95°C for 17 hr and the aminoacyl flavin was isolated by thin-layer chromatography.

subjected to acid hydrolysis (6 N HCl, 95°C, 17 hr) in order to cleave all amino acids except that substituted on the flavin and thereby distinguish between effects involving direct substitution of the 8α group and those involving interaction between the flavin and amino acids in the peptide.

Fig. 3 presents the dependence of the fluorescence yield on pH of the acid-hydrolyzed tryptic-chymotryptic flavin peptide. The characteristic fluorescence quenching in the range of 3.2–8.0 is retained, with a pK_a of 5.0 ± 0.1 before (fig. 3) and 4.9 after (table 1) performic acid oxidation. The fluorescence yield, however, was not materially altered by performic acid oxidation of the aminoacyl flavin. It seems clear, therefore, that the pH dependence of the fluorescence is a function of the amino acid immediately substituted on the flavin, whereas the action of performic acid in increasing the fluorescence yield involves an interacting amino acid within the peptide.

The aminoacyl flavin, derived by acid hydrolysis of the tryptic–chymotryptic peptide, gives a positive ninhydrin test, a negative Pauly reaction and differs both in the pK_a of fluorescence quenching and in mobility on high voltage electrophoresis at pH 5.0 (being more cationic) from both *N*(1)- and *N*(3)-histidylriboflavin. Its properties appear to coincide, however, with those of the acid-hydrolyzed amino acyl flavin recently isolated from thiamine dehydrogenase [13]. As in the case of the latter, drastic acid hydrolysis (125°C, 6 N HCl, 16 hr) liberates histidine, which has been identified by its mobility in TLC (Eastman # 13255 cellulose plates, butanol: acetic acid: H_2O, 4:1:5, upper phase) and by a positive Pauly reaction. Thus, as in the amino acyl flavin from thiamine dehydrogenase, also in β-cyclopiazonate oxidocyclase the flavin appears to be linked to the imidazole ring of histidine, because the Pauly reaction is negative before drastic acid hydrolysis. Current studies [14] in this laboratory on a synthetic histidyl-flavin adduct, which appears to be identical with the amino acyl flavin derived from β-cyclopiazonate oxidocyclase, suggest that an imidazole nitrogen is the site of attachment but that it is not a condensation product of histidine with 8-hydroxyriboflavin as found in succinate dehydrogenase.

From analogy with previous studies on the peptic peptide of *Chromatium* cytochrome c_{552} [11], it is anticipated that an aromatic acyl residue in the tryptic–chymotryptic peptide is responsible for the major increase in fluorescence after oxidation with performic acid. Establishment of the nature of this effect will have to await further purification of the peptide and the determination of its amino acid composition and sequence.

Acknowledgements

The excellent technical assistance of Mr Richard Seng is gratefully appreciated. This work was supported from Program Project 1 PO 1 HL 16251 from the National Heart Lung Institute, NIH, and from grant # GB 36570X from the National Science Foundation.

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