SEQUENCE AND STRUCTURE OF A CYSTEINYL FLAVIN PEPTIDE FROM MONOAMINE OXIDASE

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SUMMARY. Previous studies in this laboratory have shown that the active center of hepatic monoamine oxidase contains flavin dinucleotide covalently linked to the peptide chain via the 8α position of the flavin but that, unlike in succinate dehydrogenase, the linkage is not to histidine but to another amino acid. A pure flavin pentapeptide has now been isolated from monoamine oxidase which yields on acid hydrolysis or digestion with aminopeptidase M 1 mole each of serine and tyrosine and 2 of glycine and gives a positive test for sulfur. Oxidation of the peptide with performic acid yields, in addition to the amino acids mentioned, cysteic acid. The physical and chemical properties of the peptide are in accord with the conclusion that the amino acid substituted on the 8α group of the flavin is cysteine in thioether linkage. Edman degradation followed by dansylation revealed the sequence:

Ser-gly-gly-cys-tyr
|
Flavin

INTRODUCTION

Studies in the laboratories of Yasunobu (1) and Hellerman (2) indicated that, like succinate dehydrogenase (3), liver and kidney monoamine oxidase (MAO) contains FAD covalently linked to the protein. We have recently reported (4) the preparation and properties of a flavin peptide from MAO, which was isolated from outer membranes of liver mitochondria so as to eliminate contamination with flavin peptides originating from succinate dehydrogenase. The optical and ESR spectra of the flavin peptide showed that the peptide chain is linked to the 8α $-CH_2$ of the flavin, as in succinate dehydrogenase. Since the pH-fluorescence curve of the MAO flavin peptide showed no inflection in the pH range of 3.4 to 8, the flavin could not be bound to histidine as in succinate dehydrogenase (5, 6) in which the imidizole pK has a profound effect on the flavin fluorescence. The present paper describes evidence indicating

that cysteine is the immediate substituent in MAO, in thioether linkage with the flavin, and presents the amino acid sequence of the flavin pentapeptide.

MATERIALS AND METHODS

Membrane preparations and all methods, except as noted below, were as in previous work (4). The enzyme was purified from outer membranes of liver by sequential extraction (all w/v) with 0.3% digitonin, 0.2% Triton X-100, and 1.5% Triton X-100, all in 50 mM NaP_i buffer, pH 7.2. The final extract was concentrated by dialysis against polyethylene glycol 20 M and passed through a column of cellulose phosphate, Na^+ cycle, equilibrated with 50 mM NaP_i buffer, pH 7.2. The enzyme was in the effluent with a specific activity of 3620 (average of 8 preparations to be compared with 122 for the starting mitochondria). Flavin peptides were extracted as before (4), except that two digestions of 4 hr each were used, the first with 0.1 mg each of trypsin and chymotrypsin/mg of protein, the second with 0.03 mg/mg. A pure flavin peptide (monophosphate level) was isolated by chromatography on Florisil, cellulose phosphate, and paper (N-butanol:acetic acid:H_2O = 4:2:2, v/v).

RESULTS AND DISCUSSION

The major flavin band from the phosphocellulose column was resolved into 2 flavin components on paper chromatography. One of these was probably at the dinucleotide level (u.v spectrum and P analysis), the other was a monophosphate (1 mole P/mole of flavin, Table I) and was the material used for analytical work, since it was free from demonstrable peptide impurities. Its properties are summarized in Table I and its spectrum is shown in Fig. 1. The compound shows the usual 3-banded absorption spectrum, but the 375 mμ band of neutral, oxidized FMN is shifted to 367 mμ, higher than in succinate dehydrogenase flavin (neutral, 355 mμ; 345 mμ when the histidine is protonated).

Hydrolysis of the flavin peptide in 6 N HCl at 110° (15 hrs) followed by amino acid determination by analyzer revealed the presence of 1 mole each of serine and tyrosine and 2 of glycine. The same results were obtained on

TABLE I

Characteristics of the MAO Flavin Pentapeptide

Treatment	Result
Native peptide	
Ninhydrin[a]	yellow-brown
Mobility[a]	Rf = 0.6 relative to FMN
Mobility[b]	0.65 relative to FMN
Hydrolysis (6 N HCl, 15 hrs, 110°, under vacuum)	0.84 ser[c] 2.06 gly 0.84 tyr
Amino acids liberated by digestion with aminopeptidase M[d]	Ratio, ser:gly:tyr = 1:2:1
Fluorescence at pH 3.4 and pH 7.0	10% of equivalent riboflavin
Phosphorus analysis	1 mole
Folin test[a]	positive
Chloroplatinic test[a]	positive
Iodine-azide test[a]	negative
Absorption ratio 367 mμ/448 mμ	0.72
Performic acid oxidation product	
Absorption ratio, 354 mμ/448 mμ	0.84
Chloroplatinic test[a]	negative
Fluorescence at pH 3.4 and 7.0	70-80% of equivalent riboflavin
Amino acids liberated (6 N HCl, 15 hrs, 105°)	ser, gly, tyr, cysteic acid

a) On paper chromatograms, Whatman No. 1, descending, n-butanol:acetic acid: water, 4:2:2, v/v.
b) Paper electrophoresis, 250 mM, pyridinium acetate, pH 5.5.
c) By amino acid analyzer. Molar ratios are per mole of flavin based on absorption at 450 mμ, $\epsilon = 12 \times 10^3$.
d) 20 nmoles flavin peptide + 0.05 mg aminopeptidase M, in 0.03 ml of 0.14 M triethylamineacetate, pH 8.0; + 0.5 μl toluene; incubated for 24 hrs. at 38°. Hydrolysis was about 75% complete.

digestion with aminopeptidase M (Table I). Neither of these procedures, however, liberated free flavin but an 8α-substituted derivative thereof, as indicated by TLC, paper chromatography, and absorption spectrum.

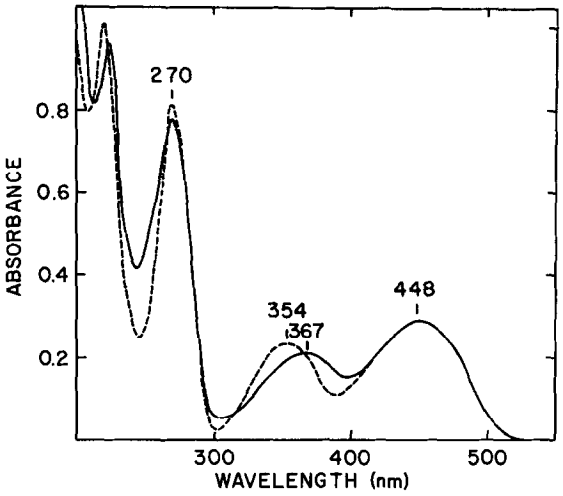

Fig. 1 Optical spectrum of flavin peptide from MAO in H_2O (1 cm light path). Solid line, before oxidation; dashed line, after oxidation with performic acid.

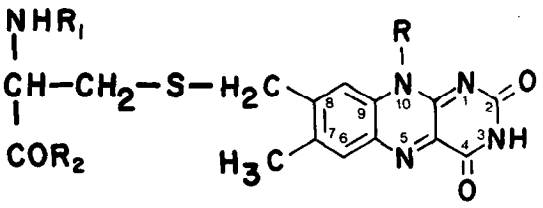

Fig. 2 Structure of the flavin peptide from MAO. R=rest of FAD in native enzyme or rest of FMN in pure peptide; R_1=serylglycylglycine; R_2=tyrosine.

Edman degradation (7) combined with the dansylation technique (8) revealed that a fifth amino acid must be present and that the sequence is ser-gly-gly-X-tyr. A clue to the nature of the unknown amino acid was provided by the observation that the native flavin peptide gives a positive chloroplatinic acid test for sulfur but a negative iodine-azide reaction (Table I). These tests suggested that X could be cysteine in thioether linkage with the 8α-CH_2 group of the flavin. On performic acid oxidation of the flavin peptide

the chloroplatinic acid test became negative, although the peptide was still attached to the flavin. Acid hydrolysis (6 N HCl, 15 hr, 105°) of the performic acid oxidation product, however, revealed the presence of cysteic acid, as determined by TLC, paper chromatography, and amino acid analyzer, in accord with the postulated cysteine thioether linkage.

The thioether is readily cleaved upon chemical reduction (Zn in glacial acetic acid + trifluoroacetic acid, 80°) and cysteic acid was identified after performic acid oxidation and 6 N HCl hydrolysis by electrophoresis and amino acid analyzer. By this procedure a better yield of cysteic acid was obtained (95%) than by direct performic acid oxidation (33%).

The flavin structure is given in Fig. 2 and the full sequence is then:

ser-gly-gly-cys-tyr
|
Flavin

Performic acid oxidation of the thioether to the sulfone also induced a spectral shift, the 367 mμ maximum being changed to 354 mμ (Fig. 1) and with an increased absorption relative to the 448 mμ peak (Table I). Further, the fluorescence of the flavin peptide increased from 10% of that of riboflavin at the thioether stage to 70-80% after oxidation to the sulfone, without any characteristic dependence on pH between 3.4 and 8. This is as expected, since the Π-electron donor properties of the thioether decrease on oxidation to the sulfone and thus the fluorescence increases. A similar effect is found in histidyl flavin (3,5), in which protonation of the imidazole leads to an increase in fluorescence from 10 to 100%.

Similar changes in spectrum and fluorescence occur very readily, suggesting that this type of thioether is exceedingly subject to oxidation: e.g., 10 min exposure of the flavin peptide to 1 N HCl at 100° in air or 24 hrs at 38°, pH 8 lead to major (3 to 4-fold) increases in fluorescence and to a hypsochromic shift of the second absorption band. This lability probably accounts for the finding of the second absorption peak in the 340-350 mμ region in previous reports (4,9), although, besides oxidation, the presence of u.v. absorbing impurities may have contributed to this observation.

Another point of interest is that the flavin thioether at the active center of MAO is reminiscent of mammalian cytochrome c, in which a thioether of cysteine to porphyrin occurs, so that on performic acid oxidation and acid hydrolysis cysteic acid is recovered, as in this case (10,11).

As will be reported elsewhere, Dr. S. Ghisla and Prof. P. Hemmerich (U. of Konstanz) have recently synthesized 8α-substituted flavin thioethers, including cysteinyl 8α-riboflavin, the properties of which are in excellent agreement with those of the natural compound described here.

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