A NOVEL FORM OF COVALENTLY BOUND FLAVIN FROM THIAMINE DEHYDROGENASE

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Received January 14,1974

SUMMARY

Thiamine dehydrogenase contains 1 mole of FAD per mole of enzyme covalently linked to the protein. A pure flavin peptide has been obtained from tryptic-chymotryptic digests of the enzyme. The hypsochromic shift of the second absorption maximum shows that the peptide is substituted at the 8α position of the flavin. The pH-fluorescence curve of the flavin peptide is similar to that of flavin peptides containing 8α-[N(3)-histidyl]-flavin but differs from the latter in having a pK_a of fluorescence quenching over 1 pH unit higher. The amino acyl riboflavin derived from this peptide by acid hydrolysis is readily distinguished from both the N(3) and N(1) isomers of 8α-histidyl riboflavin by its pK_a of fluorescence quenching and mobility in high voltage electrophoresis. Drastic acid hydrolysis, however, liberated 1 mole of histidine per mole of flavin, showing that this is the amino acid substituted at the 8α position. Attachment of the flavin appears to be to the imidazole ring but not via either of the ring nitrogens. These results indicate that a novel type of covalently bound flavin exists in thiamine dehydrogenase.

Until a few years ago succinate dehydrogenase was the only enzyme known to contain covalently bound flavin. By now, however, at least 8 flavoenzymes containing covalently bound FAD have been described. (For a review see ref. 1). While the 8α position in the isoalloxazine ring is the site of attachment of the peptide in each known case, the nature of the substituent and the type of linkage vary considerably. So far three types of covalently linked flavin have been described: 8α-[N(3)-histidyl]-FAD, as in succinate dehydrogenase (2,3) and D-6-hydroxynicotine oxidase (4), 8α-cysteinyl-FAD thioether, as in monoamine oxidase (5,6), and 8α-cysteinyl-FAD thiohemiacetal, as in Chromatium cytochrome c_{552} (7). The purpose of this note is to describe a fourth type of covalently linked flavin, isolated from thiamine dehydrogenase.

Neal (8) purified an enzyme from an unidentified soil bacterium which oxidizes thiamine to thiamine acetic acid and named it thiamine dehydrogenase.

The absorption spectrum clearly suggested that this was a flavoprotein, but conventional methods of denaturation failed to liberate free flavin. These results suggested the flavin to be covalently bound to the enzyme, although, the nature of the linkage, the level of phosphorylation and the amino acid substituent remained to be determined. Moreover, although the enzyme was homogeneous, calculations based on the absorbance at 450 nm indicated the presence of only 0.37 mole flavin per mole of enzyme (8).

As shown in the present paper, the enzyme contains 1 mole of flavin per mole of protein in the form of covalently bound FAD. A pure flavin peptide has been isolated and shown to contain 8α-histidyl-FAD, but the nature of the linkage between the flavin and the histidine is different from that occurring in other enzymes containing histidyl flavin hitherto described.

MATERIALS AND METHODS

Thiamine dehydrogenase was isolated from an unidentified soil bacterium (ATCC # 25589) grown on thiamine chloride as the sole carbon and nitrogen source (9). It was isolated in 85-90% pure state by a modification of Neal's (8) procedure to be described in a later publication. From the absorbance of the enzyme at 450 nm the FAD content was found to be 1 mole per mole of biuret protein. Previous data in the literature (8) showing a flavin content of 0.37 mole/mole were due to the use of the Lowry (10) method for protein determination which overestimates the protein by a factor of 2.7 relative to the biuret method in the case of this enzyme. In the present study the biuret method (11) was used for protein determination.

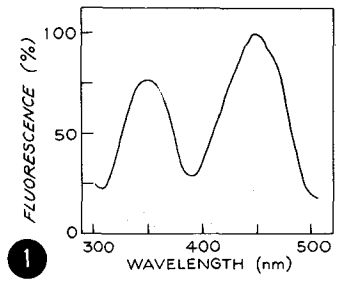
Flavin peptides were isolated by a procedure involving trichloroacetic acid treatment, trypsin-chymotrypsin digestion, and column chromatography on Florisil, Sephadex G-15, phosphocellulose, and DEAE-cellulose. The final purification step was preparative thin-layer chromatography on silica gel using n-butanol, acetic acid, water 4:2:2 (v/v).

The amino acyl flavin was obtained following the procedure established for the histidyl flavin of succinate dehydrogenase (2). The purified peptide was hydrolyzed anaerobically in 6 N HCl at 95° for 16 hrs. The amino acyl flavin was purified by thin-layer chromatography on cellulose sheets (Eastman #13255), using n-butanol: acetic acid: water (4:1:5 (v/v), upper phase). Drastic acid hydrolysis was performed in 6 N HCl at 125° for 16 hrs (3).

Corrected fluorescence excitation spectra were measured using a Perkin-Elmer MPF-3 spectrofluorometer and absorption spectra were measured on a Cary 14 spectrophotometer. Concentrations of flavin peptides were calculated assuming a molar extinction coefficient of 11,300 for dinucleotides and 12,000 for mononucleotides.

RESULTS AND DISCUSSION

In agreement with Neal's report (8), precipitation of the enzyme with 5% (w/v) trichloroacetic acid resulted in recovery of essentially all of the flavin in the denatured protein fraction. The flavin could be liberated in



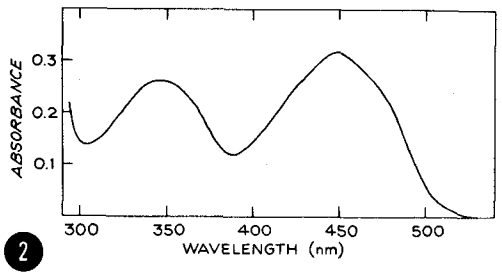


Fig. 1. Corrected fluorescence excitation spectrum of purified flavin peptide (FMN level) in 1 mM citrate, pH 3.65

Fig. 2. Absorption spectrum of flavin peptide (FAD level) in 0.02 M pyridine acetate, pH 4.0.

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acid-soluble form by subsequent digestion with trypsin plus chymotrypsin (0.1 mg of each per mg of protein), in the form of a flavin peptide. The latter was obtained in pure form by a series of chromatographic procedures.

Throughout the purification of the peptide and at the terminal stage the maxima of the fluorescence excitation spectrum were at 448 and 345 nm at pH 3.6 (Fig. 1). The hypsochromic shift of the latter band, as compared with riboflavin (372 nm) is characteristic of substitution at the 8α position of the flavin (1-3). Thus, as in all other known covalently bound flavins, also in the flavin component of thiamine dehydrogenase, an 8α linkage appears to be involved. The absorption spectrum (Fig. 2) shows maxima at 448 and 345 nm, in close agreement with the fluorescence excitation spectrum.

The dinucleotide nature of the flavin is shown by the increase in fluorescence on hydrolysis of the flavin peptide with nucleotide pyrophosphatase. As seen in Fig. 3, the quantum yield of fluorescence of the flavin peptide, measured in the vicinity of pH 3.5, is only 50% of that of riboflavin, but becomes equal to that of riboflavin after digestion with nucleotide pyrophosphatase to the FMN level. No further increase in the quantum yield of fluorescence at pH 3.5 occurs on acid hydrolysis (Fig. 3), which removes the 5'-phosphate group.

Throughout the purification of the flavin peptide its fluorescence, at

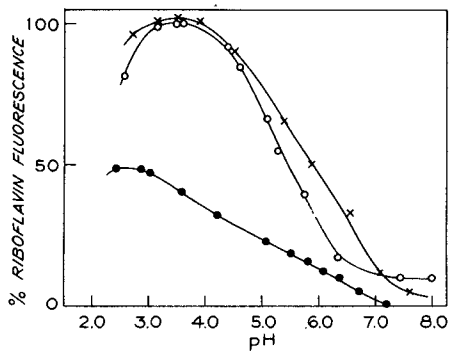

Fig. 3. pH-Fluorescence profile of flavin peptides. (•—•) at FAD level; (x—x) after treatment with nucleotide pyrophosphatase; (o—o) after hydrolysis in 6 N HCl at 95° for 16 hrs.

the mononucleotide level, showed a characteristic dependence on pH (Fig. 3). Thus, between pH 3.5 and >7 the fluorescence declined from 100% of that of riboflavin to ∿5%. The apparent pK_a for this fluorescence quenching is 5.8 ± 0.1 (Fig. 3). This is over one pH unit higher than that given by 8α-[N(3)-histidyl]-riboflavin isolated from succinate dehydrogenase or chemically synthesized (2,3). pH-Dependent fluorescence quenching in this region has been observed in those 8α-substituted synthetic flavins which have an ionizable secondary or tertiary nitrogen in the immediate vicinity of the 8α-carbon (2). It appeared likely,therefore, that the amino acid substituted at the 8α position would possess a secondary or tertiary nitrogen. An alternate possibility is that the amino acid responsible for the fluorescence quenching is not the direct 8α substituent but interacts with the flavin in the peptide. Such an interaction has been reported for the flavin peptide from Chromatium cytochrome c_{552} (12), where a tyrosine residue, although separated from the 8α-methylene by 2 amino acyl residues, interacts strongly with the flavin.

In order to eliminate this latter possibility, the flavin peptide was subjected to acid hydrolysis (6 N HCl, 95°) to remove all amino acids except that directly attached to the flavin. As shown in Fig. 3, the resulting amino acyl flavin adduct still showed extensive, pH-dependent fluorescence

quenching, showing that the nitrogenous compound responsible for this was the immediate substituent on the flavin. The fact that the pK_a of this acid-hydrolyzed flavin was some 0.7 pH unit lower (5.1 ± 0.1) than that of the peptide may be due to some additional interaction between the flavin and a residue on the peptide chain.

The amino acyl flavin was ninhydrin positive but gave a negative Pauly test. Drastic acid hydrolysis (6 N HCl, 125°) of this compound resulted in extensive destruction of the flavin and the liberation of one mole of histidine, as in the case of histidyl flavin from succinate dehydrogenase (3). Histidine was identified by its migration in TLC and quantitated by the Pauly reaction.

It is clear from these data that thiamine dehydrogenase contains 8α-histidyl-FAD at the flavin site, but the linkage between the 8α-carbon and the histidine cannot be a N-C bond for the following reasons. The histidyl riboflavin derived from thiamine dehydrogenase has a pK_a of fluorescence quenching of 5.1, whereas N(3)- and N(1)-histidyl riboflavin have pK_a values of 4.5 and 4.7, respectively (3). Further, the compound from thiamine dehydrogenase is readily separated from both the N(1) and N(3) isomers by high voltage electrophoresis at pH 5.0 by virtue of its higher mobility.

The histidyl residue cannot be linked via the α-NH_2 group to the flavin, because if this were the case the flavin peptide would give a positive Pauly reaction and, being N-terminal, the flavin would be expected to be liberated by aminopeptidase. Leucine aminopeptidase, however, does not cleave the flavin from the denatured enzyme,although an N-terminal amino acid (either leucine or isoleucine) is liberated. Further, since the flavin peptide gives a negative Pauly reaction, the imidazole ring appears to be substituted. Since a compound with coincident properties has now been obtained synthetically, hopefully the exact structure may become known in the near future.

ACKNOWLEDGMENTS

The authors are grateful to Dr. R.A. Neal for a culture of the microorganism used and to Mr. R.L. Seng and Mrs. N. Lysenko for their skilled

technical assistance. This study was supported by the National Heart Institute (Program Project No. 1 PO 1 HL 16251-01), the National Science Foundation (No. GB 36570X), and the American Heart Association (73-674).

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