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Covalently Bound Flavin as Prosthetic Group of Choline Oxidase

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SUMMARY

Highly purified choline oxidase of Arthrobacter globiformis fluoresced as a yellow band on SDS gel in 7% acetic acid. The absorption spectrum of the enzyme showed marked hypsochromic shift of the second absorption band. Aminoacyl flavin obtained from this enzyme was identified with 8α-[N(3)-histidyl]FAD.

INTRODUCTION

Choline oxidase of Arthrobacter globiformis B-0577 (NRRL B-11097)* catalyzes the oxidation of choline to betaine via betaine aldehyde (1). The enzyme has been shown to posses a flavin as prosthetic group (1). During the course of the study on the properties of this prosthetic group, we found that it linked to the apoprotein through the covalent bond between the 8α-methylene group of flavin and N(3) of a histidine residue of the protein. This paper deals with briefly with the data obtained in this study.

MATERIALS AND METHODS

The sample of choline oxidase prepared from Arthrobacter globiformis was kindly donated by Toyo Jozo Co., Ltd., Shizuoka. The sample was further purified to a single band on a SDS gel electrophoretogram by using Sephacryl S 200 column chromatography. Trypsin and nucleotide pyrophosphatase were obtained from Sigma Chemical Co., St. Louis, and α-chymotrypsin from Miles-Servac (PTY) Ltd., Maidenhead. The authentic 8α-[N(3)-histidyl]riboflavin was synthesized according to Walker et al. (2), and purified by high voltage electrophoresis. All other chemicals were of analytical grade.

Digestion of the enzyme with trypsin and α-chymotrypsin, hydrolysis with nucleotide pyrophosphatase, and purification of flavin peptide were made according to Kenney et al. (3).

The aminoacyl flavin was obtained by hydrolyzing the flavin peptide with 6 N HCl at 95°C for 16 hr in vacuo. Its purification was carried out by high voltage electrophoresis.

* This strain can be obtained from Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Illinois, U. S. A.

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Polyacrylamide disc gel electrophoresis was carried out by the method of Davis (4) in Tris (0.025 M)-glycine (0.18 M) buffer (pH 8.2). Disc gel electrophoresis in the presence of SDS was carried out by a slightly modified method (5) of Dunker and Rueckert (6). The gels consisted of 1% SDS and 5% acrylamide in 0.1 M sodium phosphate buffer (pH 7.2).

After electrophoresis, the gels were stained with Coomassie Blue for the detection of protein and with choline (0.1 M)-phenazine methosulfate (20 mg%)-2,3,4-triphenyltetrazolium chloride (20 mg%) for the demonstration of enzyme activity. The gels were also checked under ultraviolet illumination (λ 365 nm) after soaking the gels in 7% acetic acid to detect the intrinsic fluorescence of the covalently bound flavin (7).

Absorption spectra were recorded on a Union Giken SM-401 Spectrophotometer. Corrected fluorescence spectra were measured in a Shimadzu spectrofluorophotometer RF 502.

RESULTS AND DISCUSSION

As shown in Fig. 1, a purified preparation of choline oxidase was found to fluoresce as a yellow band on SDS-gel which had been soaked in 7% acetic acid. This fluorescent band corresponded to the protein band, and its molecular weight was estimated to be 59,000 by co-electrophoresis with marker proteins. In polyacrylamide disc gel electrophoresis, the migration distance of the fluorescent band also coincided to those of the bands stained for protein and enzymatic activity.

In absorption spectrum of this enzyme, marked hypsochromic shift of the second absorption band was observed.

Since these observations showed the possibility that the prosthetic group of choline oxidase was linked to the apoprotein covalently, the protein was treated with 5% trichloroacetic acid. It was found that all of the flavin precipitated with the denatured protein. From the precipitates, the peptide fraction containing flavin was isolated according to the procedure reported by Kenney et al. (3), viz., trypsin-chymotrypsin digestion, followed by purification with Florisil and phosphate-cellulose column chromatography.

The absorption spectrum of this purified flavin peptide also showed blue shift of the second absorption band as compared with normal flavins such as FMN and FAD. After hydrolysis with nucleotide pyrophosphatase, the fluorescence intensity at pH 3 increased about 2 times, and the release of AMP was found on a TLC plate, indicating that the prosthetic group was of dinucleotide type.

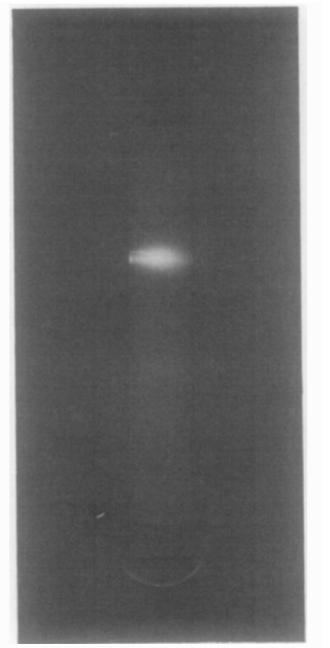

Fig. 1. SDS polyacrylamide gel electrophoresis of choline oxidase. Choline oxidase (14 μg protein) was subjected to electrophoresis as described in the text. The fluorescence band was made visible under UV illumination (λ 365 nm) after soaking the gel in 7% acetic acid.

The fluorescence of the flavin peptide of FMN level as well as that of FAD level at pH 3 was quenched at neutral and alkaline pH range. This shows that the amino acid linked to flavin ring is a histidyl residue.

The fluorescence excitation spectrum of aminoacyl riboflavin at pH 3.0 is shown in Fig. 2. The second excitation maximum of this spectrum was found to shift towards blue as compared with the fluorescence excitation spectrum of riboflavin (from 373 nm to 346 nm). This is also the case for the absorption spectra of the flavin peptide and the aminoacyl flavin. This hypsochromic shift indicates that covalent linkage occurs at the 8α-methylene group of the isoalloxazine ring.

To check whether N(1) or N(3) of the histidine residue of the protein was bound to the 8α-methylene group of flavin, pH-fluorescence profile and reduction with sodium borohydride were examined. The pK_a value of flavin peptide of FAD

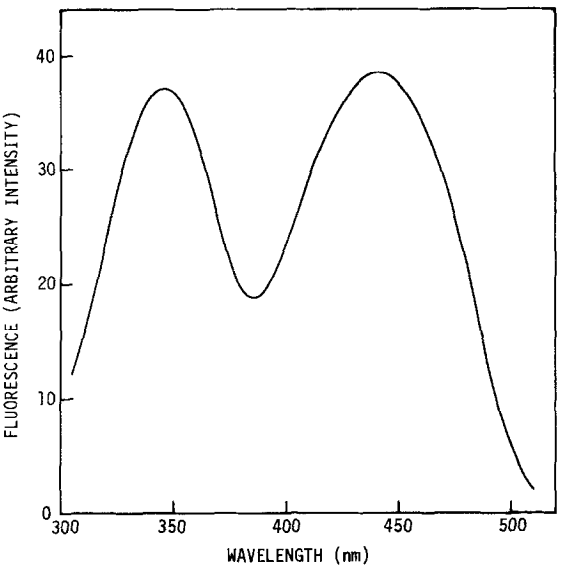

Fig. 2. Fluorescence excitation spectrum of the aminoacyl flavin of choline oxidase. Fluorescence excitation spectrum was monitored at 536 nm emission at pH 3.0. Concentration of the aminoacyl flavin was 6.4 μM.

Table 1 Mobility of aminoacyl flavin in high voltage electrophoresis

Sample	Migration (cm)	Mobility
Aminoacyl flavin	-11.1	-0.85
Flavin peptide (FMN level)	-2.1	-0.16
8α-[N(3)-histidyl]riboflavin	-11.1	-0.85
FMN	+13.0	1.0

High voltage electrophoresis (2000 V) was carried out at pH 5 for 90 min.

or FMN level was found to be 4.6, which is identical with that reported for succinate dehydrogenase (3,8) and that for bacterial sarcosine dehydrogenase (9). It was reported that the N(1) isomer of histidylflavin was reduced with BH_4^- and its fluorescence was quenched, while the N(3) isomer was not (10). The flavin peptide of FMN level and aminoacyl riboflavin of this enzyme were not reduced with BH_4^-.

Moreover, the possibility that the covalently bound flavin is 8α-[N(3)-histidyl]flavin is confirmed by simultaneous high voltage electrophoresis with

the authetic N(3) isomer. The result is shown in Table 1.

From all the results mentioned above, it is concluded that the covalently bound flavin isolated from choline oxidase of Arthrobacter globiformis is 8α-[N(3)-histidyl]FAD. In this respect, this enzyme belongs to the group of succinate dehydrogenase (3,8), bacterial sarcosine dehydrogenase (9) and D-6-hydroxynicotine oxidase (11).

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