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CORYNEBACTERIUM SARCOSINE OXIDASE : A UNIQUE ENZYME HAVING COVALENTLY-BOUND AND NONCOVALENTLY-BOUND FLAVINS

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Summary : A sarcosine oxidase (sarcosine : oxygen oxidoreductase (demethylating), EC, 1.5.3.1) was purified to homogeneity from *Corynebacterium* sp. U-96 by the use of ionic exchange chromatographies and gel filtrations. The enzyme contained two mol FAD per mol enzyme (one covalently-bound and one noncovalently-bound; mol. wt., 174,000). The "semiapoenzyme", which contains the covalently-bound FAD alone, was prepared by the acid-ammonium sulfate treatment. The semiapoenzyme had practically no activity for sarcosine oxidation, but retained intact back-bone structure judging from the circular dichroic spectrum in the far ultraviolet region. On the contrary, the circular dichroic spectrum of the semiapoenzyme in the visible region (a large negative band around 443 nm) was quite distinct from that of the holoenzyme (positive bands at 387, 456 and 489 nm).

Sarcosine dehydrogenase (sarcosine : (acceptor) oxidoreductase (demethylating), EC, 1.5.99.1) and sarcosine oxidase (sarcosine : oxygen oxidoreductase (demethylating), EC, 1.5.3.1) have been purified from mammalian livers [1-3] or from microbes [4-6], and these enzymes were reported to contain covalently-bound flavins [3,4,6]. A detailed study on the flavin-bound peptide of bacterial sarcosine dehydrogenase has been performed [4].

In the course of studies on the sarcosine oxidase purified from Corynebacterium species, it was found that this enzyme contained both covalently-bound and noncovalently-bound flavins, each of which was identified as FAD. It was also found that the noncovalently-bound FAD was easily released from the enzyme by an

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Abbreviation used: CD, circular dichroism.

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acid-ammonium sulfate treatment to yield a protein with the covalently-bound FAD alone, which was tentatively named as the "semiapoenzyme".

Some properties of the native holoenzyme and the semiapoenzyme are described.

Materials and Methods

Sarcosine oxidase was purified from *Corynebacterium* sp. U-96, which was grown with sarcosine as the sources of carbon and nitrogen, by the use of DEAE-cellulose, QAE-Sephadex and Ultrogel chromatographies.* The molecular weight of the enzyme was estimated by the method of Yphantis [7]. The enzyme concentration was estimated spectrophotometrically by determining the enzyme-bound flavin, assuming that the extinction coefficient of the bound flavin is the same as free FAD. Identification of the flavin moiety was done by the use of snake venom phosphodiesterase [6], or thin-layer chromatography. The disc gel electrophoresis was performed as described by Davis [8]. The catalytic activity of sarcosine oxidase was determined by the oxygen-electrode method at 30°, pH 8.0, in the presence of 5.9 mM sarcosine. The CD measurements were made with a Union Giken Dichrograph, Mark III-J, which was interfaced to a computer. Spectrophotometer used was a Hitachi double-beam spectrophotometer, Model 124. Reagents were of the highest purity commercially available.

Results

The purified sarcosine oxidase sample was homogeneous by ultracentrifugation and disc gel electrophoresis at pH 8.0 (Fig. 1, upper). The molecular weight of the enzyme was estimated to be 174,000 by the method of Yphantis. Since the minimum molecular weight in terms of the bound flavin was calculated to be about 100,000 on the basis of the P_2O_5-dried weight, it was concluded that two mol flavin were contained per mol enzyme. The flavin moiety of the enzyme was found to be partially released upon treatment by heat (100° for 30 min), 10% trichloroacetic acid, or 80% ammonium sulfate under moderately acidic conditions (pH 4.0). The results are summarized in Table I. Since the molar ratio of the released flavin to the retained flavin was approximately unity in each case, it is highly likely that the enzyme contains the covalently-bound flavin and noncovalently-bound flavin with the

* The details on the purification and properties of the enzyme will be published elsewhere (Suzuki, M., J. Biochem., submitted).

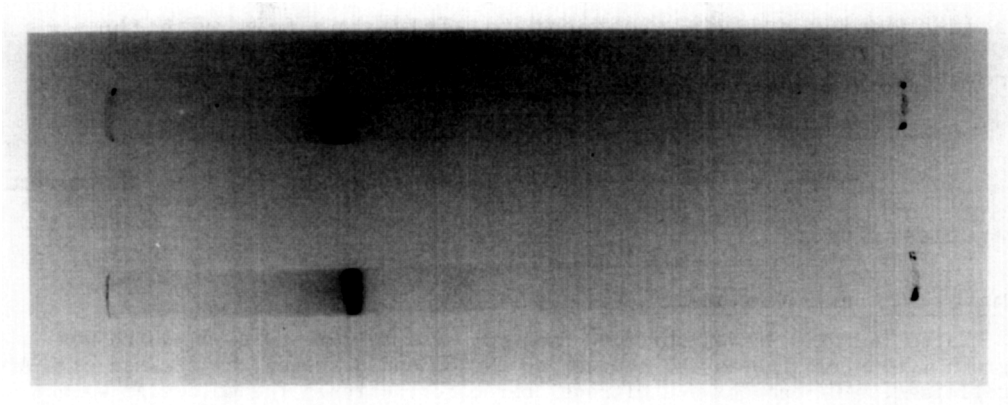

Fig. 1. Disc gel electrophoretic patterns of the holoenzyme (upper) and the semiapoenzyme (lower).
Stained with Coomassie brilliant blue G-250. Acrylamide concentration : 7.5%. Amounts of proteins : holoenzyme, 38 µg; semiapoenzyme, 31 µg. Electrophoresis was performed at pH 8.0 with the current of 3 mA per tube.

molar ratio of 1 : 1. This was verified by the phosphodiesterase analyses of the flavin moiety. The precipitate and the supernatant of the heat-treated enzyme sample were each incubated with phosphodiesterase for 30 min at 37°, and the released AMP was estimated by the use of myokinase [9]. As seen in Table II, an equimolar relationship was evident between the covalently-bound

Table I Relationship between the released flavin and the retained flavin after various treatments.
Flavin contents were determined spectrophotometrically on the assumption that the extinction coefficient of the bound falvin is the same as that of free FAD.

Treatment	Flavin content (nmol)		
	Holoenzyme	Precipitate	Supernatant
Heat (100° for 30 min)	8.73 (1.00)	N.D.*	3.66 (0.42)
10% Trichloroacetic acid	126.3 (1.00)	73.1 (0.58)	N.D.
80% Ammonium sulfate at pH 4.0	76.5 (1.00)	30.9 (0.40)	38.2 (0.50)

* N.D. : Not determined.

Table II Analyses of the covalently-bound and noncovalently-bound flavins by the phosphodiesterase method. The precipitate and the supernatant of the heat-treated enzyme sample were each incubated with 100 μg of snake venom phosphodiesterase for 30 min at 37°, and released AMP was estimated by the use of myokinase [9].

Substrate	Substrate used (nmol)	AMP recovered (nmol)
FAD (Control)	50.1	53.2
Precipitate (Semiapoenzyme)	50.5	42.5
Supernatant	——	44.5

flavin and noncovalently-bound flavin moieties, since the same amount of AMP was detected with both of the precipitate and the supernatant. The results also lead to a conclusion that FAD is the only flavin which is bound to the enzyme. The identity of the noncovalently-bound flavin with FAD was also confirmed by the thin-layer chromatography (BuOH: AcOH: H_2O = 4: 1: 5); the neutralized supernatant of the trichloroacetic acid-treated sample was applied on a thin-layer plate, and the observed RF-value agreed well with that of the authentic FAD.

The semiapoenzyme was prepared by repeated treatment of the acid-ammonium sulfate precipitation, and the prepared sample was revealed to be practically homogeneous by the disc gel electrophoresis at pH 8.0 (Fig. 1, lower).

The absorption spectra of the holoenzyme and the semiapoenzyme were shown in Fig. 2. The absorption maxima of the holoenzyme were at 278, 367 and 454 nm, and those of the semiapoenzyme were at 278, 367 and 447 nm. The absorption peak in the visible region

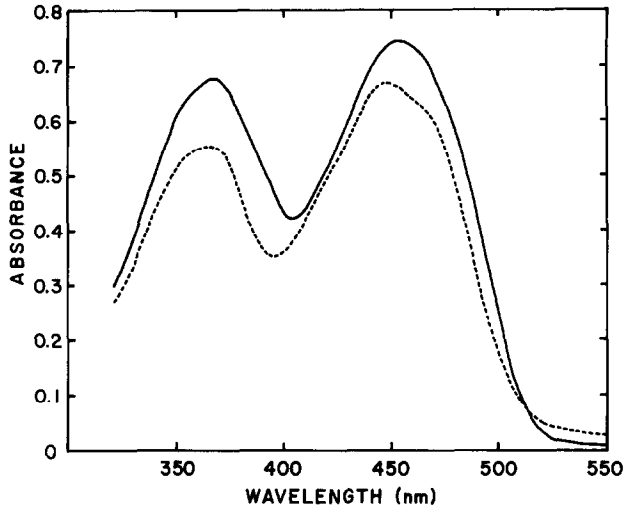

Fig. 2. Absorption spectra of the holoenzyme (solid line) and the semiapoenzyme (dotted line) measured at room temperature in 50 mM phosphate buffer, pH 8.0.

was shifted significantly toward shorter wavelength region in the case of the semiapoenzyme as compared with that of the holoenzyme.

The CD spectra of the holoenzyme and the semiapoenzyme are shown in Fig. 3. The spectra in the far ultraviolet region (Fig.

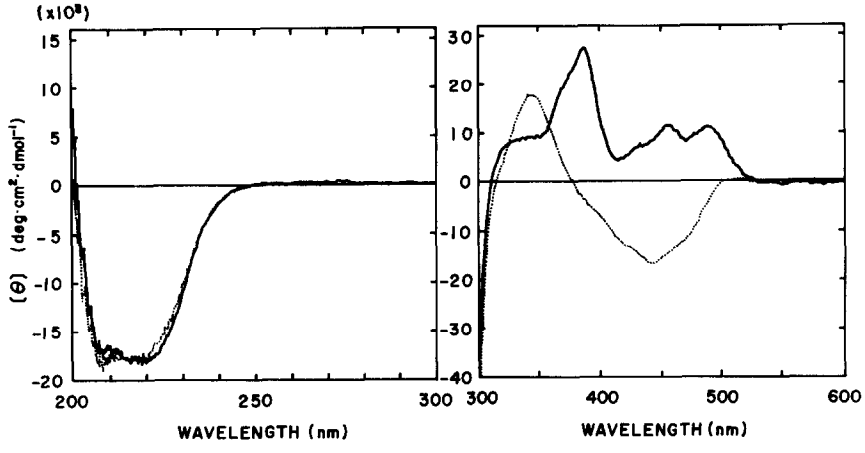

Fig. 3. CD spectra of the holoenzyme (solid lines) and the semiapoenzyme (dotted lines) in the far ultraviolet region (left) and in the near ultraviolet to visible region (right). Measurements were made in 50 mM phosphate buffer, pH 8.0, at room temperature by using a 1-mm cuvette for the far ultraviolet region and a 10-mm cuvette for the visible region. The spectra are of the average of 9 scans.

3, left) were essentially identical with both of the enzymes, indicating that the release of the noncovalently-bound FAD did not cause any significant alteration in the secondary structures of the protein moiety. The helical contents of these enzymes were known to be relatively high (about 45%). The CD sepctrum of the holoenzyme in the visible region (Fig. 3, right) is characterized by the positive dichroic bands at 387, 456 and 489 nm, which may correspond, respectively, to the absorption peaks at 367 and 454 nm, and a hidden shoulder around 480 nm which is often observable apparently with various flavoproteins. The CD profile of the present enzyme is somewhat reminiscent of that of choline oxidase of Alcaligenes species (an enzyme with a covalently-bound flavin) [10]. The CD spectrum of the semiapoenzyme is devoid of the positive bands in the visible region in a striking contrast to that of the holoenzyme; instead, a large negative band at 443 nm and a small positive band in the near ultraviolet region (345 nm) are observable.

The overall catalytic activity of the holoenzyme was 696 min^{-1}, while that of the semiapoenzyme was 23.9 min^{-1}, some 3.5% of the holoenzyme. This may possibly be due to contamination of the holoenzyme.

Discussion

The present paper may be the first one which describes a unique enzyme containing both covalently-bound and noncovalently-bound FAD's. The noncovalently-bound FAD was easily released from the protein moiety by relatively mild treatment, yielding the semiapoenzyme, a protein which has solely the covalently-bound FAD.

Since the CD spectrum of the semiapoenzyme was essentially identical with that of the holoenzyme in the far ultraviolet region, it was conceivable that the release of the noncovalently-

bound FAD from the holoenzyme caused practically no alteration in the polypeptide back-bone structure, but caused quite a localized modification probably in the flavin-binding region. The alteration was accompanied by the complete loss in the catalytic activity.

The observed lack of the catalyzing ability in the semiapoenzyme may be explained by one of the following possibilities: 1) the substrate-binding site was modulated by the subtle conformational changes resulted from the release of the noncovalently-bound FAD, or 2) the noncovalently-bound FAD accepts electrons directly from the substrate, while the covalently-bound FAD accepts electrons only from the reduced noncovalently-bound FAD, so that the semiapoenzyme could not be reduced by sarcosine directly.

Further studies are in progress.

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