ISOLATION OF ACID-NONEXTRACTABLE FLAVINS FROM A BACTERIAL SARCOSINE OXIDASE

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SUMMARY

An acid-nonextractable flavin has been isolated from a sarcosine oxidase of a strain of Pseudomonas capable of growing on sarcosine as its sole source of C and N. When purified by successive chromatography on Florisil, Dowex 1, CM-Sephadex and DEAE-Sephadex, the flavin exhibits the acid-lability of a dinucleotide and is spectrally similar to the acid-nonextractable flavin of the mammalian sarcosine and succinate dehydrogenases. The E_m of the purified flavin is -160 mv at pH 7.10.

The flavin of the sarcosine dehydrogenase of mammalian liver mitochondria is acid-nonextractable, but can be released in peptide form by proteolysis (1,2). A similar type of flavin has been demonstrated in a strain of Pseudomonas capable of growing on sarcosine as its sole source of C and N. Moreover, induction of sarcosine oxidase in the organism results in a concomitant increase in the quantity of the flavin (3). The present paper is the first report of the isolation and purification of such a bacterial flavin.

METHODS

Cells were grown 24 hours in media containing either serine or sarcosine as the sole source of C and N (3). The acid-nonextractable flavins were solubilized by treating acid-denatured cells with "1-300" trypsin, crystalline trypsin, and chymotrypsin (4). Following their isolation on Florisil (3-5), the flavins were chromatographed on Dowex 1, CM-Sephadex, and DEAE-Sephadex as described below. Fluorescence was

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monitored at 523 mμ by activating at 455 mμ, employing the Aminco-Bowman recording spectrophotofluorometer with the slits set at 3 mm.

Materials. Florisil, 30-60 mesh, was supplied by Matheson Scientific, Inc. Dowex 1 (analytical grade, 1-200 mesh) in the chloride cycle was purchased from Bio-Rad Laboratories. CM-Sephadex C50 (40-120μ) in the sodium cycle and DEAE-Sephadex A50 (40-120μ) in the chloride cycle were obtained from Pharmacia Fine Chemicals. All of the other materials were of the highest quality available commercially.

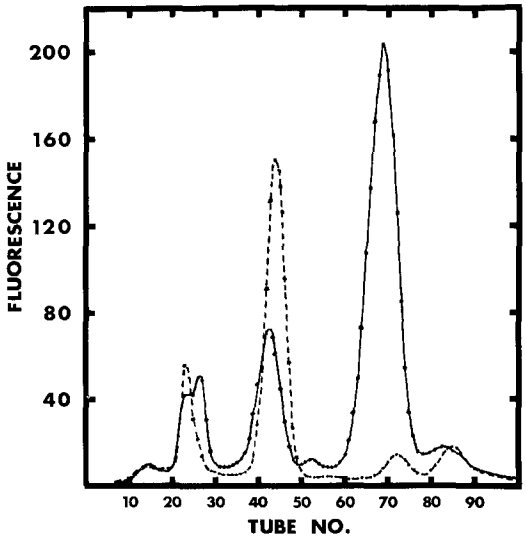

Fig. 1 Dowex 1 chromatography of acid-nonextractable flavins isolated from sarcosine and serine-cultured bacteria. The acid-nonextractable flavins from identical quantities of cells (equivalent to 0.65 g of protein) grown on either sarcosine or serine were isolated on Florisil (3). The flavins were then chromatographed on 104 x 1 cm columns of Dowex 1 (acetate cycle) and were eluted with 0.6 N pyridine acetate, pH 4.8. ------ flavin from serine-grown cells; ——— flavin from sarcosine-grown cells.

RESULTS AND DISCUSSION

As shown in Fig. 1, the flavins from cells grown on sarcosine were separated into three fractions on Dowex 1, the dominant peak appearing in fraction 70. With the serine-cultured bacteria, the flavin in fraction 70 was only 7% as great while that in number 44 was doubled in quantity. From previous results, it was possible to conclude that the flavin in fraction 70 is derived from sarcosine oxidase and that isolated in fraction 44 is from the succinate oxidase of the cells. It is of interest that the latter

peak coincides with the flavin isolated chromatographically under the same conditions from the membrane-bound succinate dehydrogenase of liver mitochondria.*

Further evidence that the flavin in fraction 70 on the Dowex column (Fig. 1) is derived from sarcosine dehydrogenase was obtained from studies on the purification of the enzyme. A phenazine-dependent sarcosine oxidase can be precipitated from the supernatant fraction of sonically-irradiated cells with $(NH_4)_2SO_4$ between 30 and 50% of saturation (3), and can be purified 4-6 fold by sedimentation at 100,000 x g for 2 hours

Table I

Purification of Phenazine-Dependent Sarcosine Oxidase by Ultracentrifugation

The 30-50% $(NH_4)_2SO_4$ fraction of the supernatant solution of sonically-irradiated cells (1) was dissolved in 0.075 M potassium phosphate, pH 7.5 (25 ml). After centrifugation at 100,000 x g for 2 hours, the resulting pellet was dissolved in 25 ml of water.

Preparation	Protein	Sarcosine Oxidase Activity	Recovery of Activity
	mg	μatoms O/20 min/mg protein	%
(1) Initial 30-50% $(NH_4)_2SO_4$ Fraction	319	1.05	--
(2) Pellet from (1), sedimented at 100,000 x g, 2 hours	56	6.37	94

in 0.075 M potassium phosphate, pH 7.5 (Table I). The increase in enzyme activity is accompanied by a 3-4 fold increase in the amount of acid-nonextractable flavin per unit weight of protein isolated in fractions 63-74 on the Dowex column (Fig. 2). Moreover, the much smaller amount of the flavin identified with succinate dehydrogenase (fraction 45) is decreased more than 10-fold by the sedimentation procedure. Consistent with these results, it is found that the purified sarcosine oxidase is devoid of succinate dehydrogenase activity.

* D. R. Patek and W. R. Frisell, manuscript in preparation.

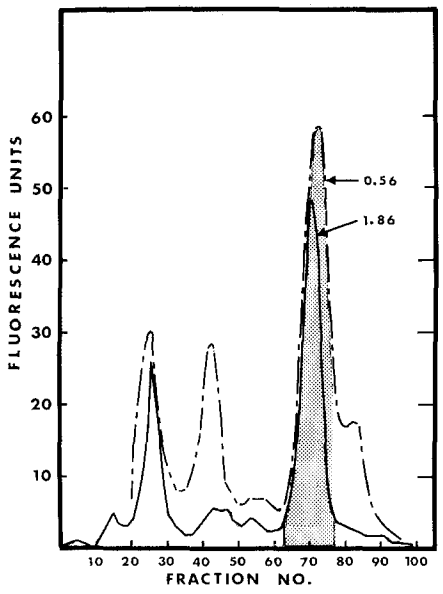

Fig. 2 Dowex 1 chromatography of acid-nonextractable flavins of the phenazine-dependent sarcosine oxidase. — — —, flavin isolated from the oxidase preparation before ultracentrifugation (267.8 mg protein); ———, flavin from the same oxidase preparation sedimented in 2 hours at 100,000 x g (47.0 mg). Fluorescence units per mg protein in combined fractions 63-77: — — —, 0.56; ———, 1.86.

The flavin in fractions 63-74 from the Dowex column, indicated by the shaded area in Figs. 1 and 2, can be purified further by successive chromatography on CM-

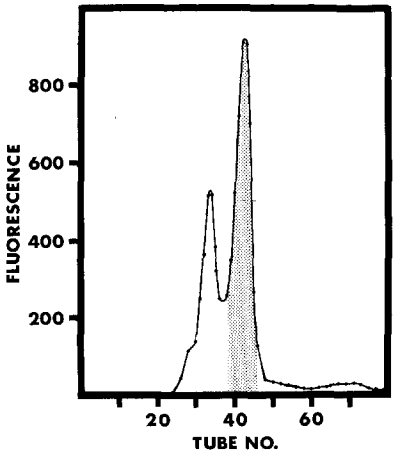

Fig. 3 Chromatography of acid-nonextractable flavin on CM-Sephadex. The flavin in fractions 63-74, combined from two identical Dowex 1 columns (Fig. 1), was dried under vacuum and redissolved in 3.0 ml of 20 mM acetate buffer, pH 4.8. A portion containing 165 mμ moles of flavin was chromatographed on a 2.5 x 55 cm column of CM-Sephadex (pyridinium cycle).

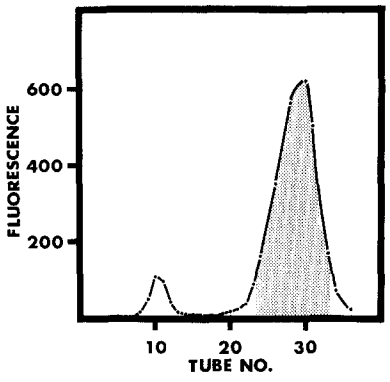

Fig. 4 Chromatography of acid-nonextractable flavin on DEAE-Sephadex. The flavin in fractions 39-46 from the CM-Sephadex columns (Fig. 2), containing 65 mμ moles of flavin, was dried under vacuum, redissolved in 3.0 ml of 18 mM potassium formate, pH 3.0, and chromatographed on a 2.5 x 55 cm column of DEAE-Sephadex (formate cycle).

Sephadex and DEAE-Sephadex as demonstrated by the data in Figs. 3 and 4. The purified flavin eluted from the DEAE-Sephadex in fractions 23-33 is acid labile, suggesting that it exists in the dinucleotide form (5). This preparation is also devoid of tryptophane fluorescence.

The spectrum of the dinucleotide flavin of the sarcosine oxidase is shown in Fig. 5. Comparable to the flavin from the mammalian succinate dehydrogenase (5), the usual absorption band of FMN or FAD at 373 mμ is shifted toward the ultraviolet and is found at 348 mμ. The broadening of this band when the pH is raised from 3.0 to 7.0 is also similar to that observed for the flavin of succinate dehydrogenase (5). This effect is not obtained with FMN.

The oxidation-reduction potential of the flavin was measured by titration of the reduced species with oxygen, employing a saturated calomel reference electrode and a platinum wire indicator electrode. For reduction prior to titration, 3.0 ml of 12 μM bacterial flavin (dinucleotide form) in 50 mM potassium phosphate, pH 7.10, was added to a 0.2 ml mixture of $NaBH_4$ - HSO_3^-. The latter was prepared immediately before use by expressing 1.0 ml of 1 mM HSO_3^- (in the phosphate buffer) into a beaker containing 1 mg of dry $NaBH_4$.

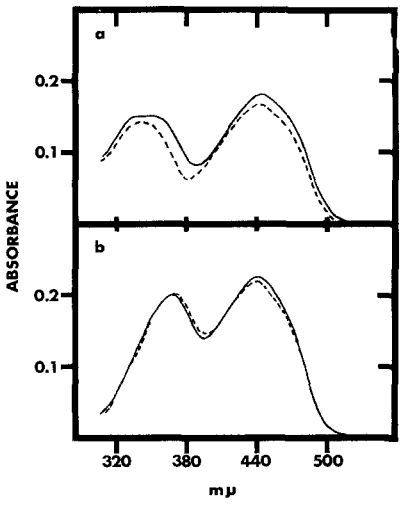

Fig. 5 Absorption spectra of acid-nonextractable flavin and FMN. (a) The dinucleotide form of acid-nonextractable flavin was purified chromatographically on Florisil, Dowex 1 and CM-Sephadex as described in Figs. 1-3, starting with 1.3 grams of bacterial protein. The fractions containing the flavins were dried under vacuum and redissolved in 6.0 ml of water. A 3.0 ml aliquot was acidified to pH 3.0 with 0.020 ml of 3.0 M potassium formate, pH 2.8. The remaining 3.0 ml of flavin solution was redried and dissolved in 3.0 ml of 0.05 M potassium phosphate, pH 7.0. The pH was adjusted to 7.0 with addition of a small quantity of solid KOH. (b) 18.5 μM FMN in 0.025 M potassium phosphate, pH 7.0, or in 0.025 M potassium phosphate, pH 7.0, or in 0.025 M potassium citrate, pH 3.0. The spectra, ------, pH 3.0, and ———, pH 7.0, were measured in a Cary 14 recording spectrophotometer.

Under conditions in which the rate of oxidation was constant, the apparent midpoint potential could be determined from the following relationship*:

$$E' = \frac{RT}{2F} \ln\left(\frac{C}{kt} - 1\right) - E_m + E_{SCE} + E_{junc}$$

A plot of E' versus t gives an inflection point at the titration midpoint, $t = \frac{C}{2k}$. At this point, $E' = (E_{SCE} + E_{junc}) - E_m$. Applying the foregoing analyses to FMN and 5,5'-indigodisulfonate as standards, a relationship between their values of E' and E_m (6,7) could be established. From these data and its observed value of E', the E_m of the dinucleotide form of the bacterial flavin was determined to be -160 millivolts, at pH 7.10. This value is almost identical with the E_m, -167 millivolts, of the

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* Abbreviations: t, time from start of flavin oxidation; C, concentration of $flavin_{ox}$ + $flavin_{red}$; E', observed potential; E_m, midpoint potential of the flavin couple, relative to the standard H electrode potential; E_{junc}, junction potential of the saturated calomel electrode; E_{SCE}, potential of saturated calomel electrode relative to the standard H electrode.

purified acid-nonextractable flavin isolated from the mitochondrial sarcosine dehydrogenase. From these results it may be concluded that the E_m for the derivatives of acid-nonextractable flavins isolated from sarcosine oxidase is 50-60 millivolts more positive than for FMN and FAD (7).

ACKNOWLEDGEMENTS

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