Benzylamine oxidase from brain microvessels

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Copper-containing benzylamine oxidase with a specific activity of 200 units was isolated from bovine brain microvessels. It was shown that the content of the enzyme in microvessels was significantly higher as compared with large blood vessels such as heart aorta. Some physico-chemical properties of the enzyme were determined. The enzyme was inhibited by high concentrations of the substrate as well as thiol reagents and β-aminopropionitrile fumarate. On the basis of EPR and optical spectra of the enzyme its copper was considered to be 'non-blue' type.

Benzylamine oxidase *Brain microvessel* *Copper-containing enzyme*

1. INTRODUCTION

Two copper-containing enzymes have so far been isolated from large blood vessels such as aorta. One is the soluble enzyme, benzylamine oxidase [1], and the other lysyl oxidase which is solubilized using urea solutions or nonionic detergents [2–4]. These enzymes play a central role in regulation of vessel elasticity [5,6]. Recently, we were able to obtain benzylamine oxidase in a highly purified state from bovine aorta and some optical and magnetic properties of the enzyme were studied [7]. It should be noted, however, that there are many differences in functions of large and small blood vessels [8]. Thus, the role of microvessels as regulators of blood-tissue barriers is well known, whereas large vessels lack this property [9]. Therefore, it was of importance to compare properties of these copper-containing enzymes isolated from large and small blood vessels. However, to date no data on copper-containing proteins of microvessels have been published. Bearing this in mind the main aim of this study was to determine whether brain microvessels contain any soluble copper-containing protein and, if this is the case, to test its enzymatic properties and to compare it with copper-containing enzymes of the aortal tissue.

2. MATERIALS AND METHODS

Microvessels from grey matter of bovine brain hemispheres were prepared using filtration of the homogenate through nylon sieves and floating properties of microvessels as described in [10]. In typical experiments 1 kg portions of grey matter were used as a starting material from which about 1 g wet weight of microvessels was obtained. The purity of the vessel preparation was tested by light microscopy.

Benzylamine oxidase activity was followed spectrophotometrically as in [11]. The reaction mixture was in 0.04 M phosphate buffer, pH 7.7. One unit of activity was defined as the amount of the enzyme that catalyzed at 20°C an increase in absorbance at 250 nm of 0.001 per min. The specific activity of the enzyme was expressed as units per mg of protein as determined in [12] with bovine serum albumin as standard. Copper content in the enzyme preparation was determined chemically as in [13] as well as by the double integration technique of the EPR spectrum of the preparation. The content of thiol groups in the enzyme was assayed as in [14].

The M_r was evaluated by gel filtration through Sepharose 4B and Sephadex G-200 (superfine) using for calibration bovine serum albumin (67000),

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ceruloplasmin (140000), catalase (220000), dopamine-β-hydroxylase (300000), ferritin (500000) and Blue Dextran (2000000).

Optical spectra were obtained at 20°C in 10-mm cells on a Beckman Acta M-IV spectrophotometer. EPR spectra were recorded at 77 K using a Varian E-104 instrument operating at microwave power, 10 mW; microwave frequency, 9.08 GHz; and modulation amplitude, 6.3 G. Elution patterns were obtained with a 1 mm cell on a Uvicord-S (LKB) operating at 253 nm.

We used BAPN (β-aminopropionitrile fumarate), DTNB (5,5'-dithiobis-2-nitrobenzoic acid), p-CMB (*p*-chloromercuribenzoate) and benzylamine hydrochloride from Sigma. The latter was additionally purified by recrystallization from ethanol immediately before use. Sepharose 4B and Sephadex G-200 were from Pharmacia and poly(ethylene glycol) (PEG) of M_r 2000 from Loba-Chemie. The concentrating of the enzyme was carried out using ultrafiltration through Millipore type PSAC membranes.

3. RESULTS AND DISCUSSION

3.1. *Isolation of benzylamine oxidase from microvessels*

One g of microvessels was suspended in 4 ml of 0.2 M phosphate buffer (pH 7.7) and homogenized in a hand-held homogenizer for 5–10 min. The residue obtained after centrifugation at 15000 × *g* for 15 min was again homogenized with a new portion of the buffer. Supernatants of both centrifugations were combined and solid PEG was added to a final concentration of 24%. The residue formed after 30 min incubation of the mixture at 5°C was collected by centrifugation (15000 × *g*, 30 min) and dissolved in 200 ml of 0.2 M phosphate buffer (pH 7.7). This solution was then gel filtered through Sepharose 4B. Fractions with benzylamine oxidase activity were collected, concentrated by ultrafiltration, and gel filtration through Sepharose 4B was repeated. Fig.1 shows elution patterns obtained during two gel-filtration procedures. In table 1 the protocol of the typical purification procedure of benzylamine oxidase from cerebral cortex microvessels is presented. As can be seen, more than 400-fold purification of the enzyme was achieved with high yield. The final preparation was found to have a specific activity of about 200 units. When yields of the enzyme from brain microvessels and heart aorta [7] were compared it was noted that microvessels were significantly richer (by a factor 10) in this enzyme than large vessels. This probably reflects the lesser content of collagenous proteins in the microvessels as compared with large vessels.

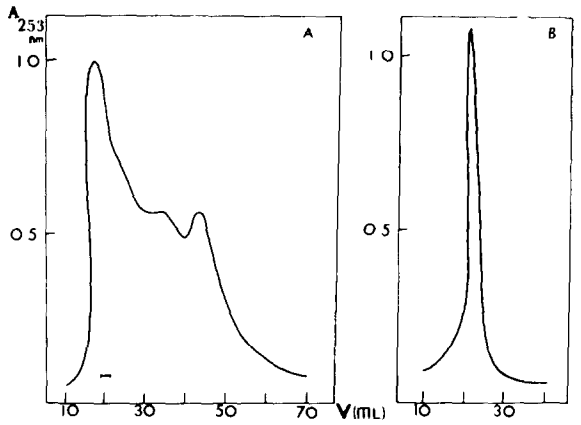

Fig.1. The typical elution patterns obtained in the course of purification of benzylamine oxidase through first (A) and second (B) columns with Sepharose 4B. For both gel filtrations the same column (1.2 × 56) was used.

3.2. *Properties of the enzyme from microvessels*

The M_r of the enzyme determined by gel filtration in 0.04 M phosphate buffer (pH 7.7) was found to be 800000. Assays of the copper content in the enzyme by EPR and chemical methods led to a value of 1 copper atom per 60000 Da. Thus, the enzyme from microvessels is a multicopper protein consisting of many subunits. A similar conclusion was drawn for benzylamine oxidase from bovine

Table 1

Basic steps of the purification of benzylamine oxidase from brain microvessels

Step	Total protein (mg)	Specific activity
Homogenate of microvessels		0.5
PEG fractionation	40	30
Second gel filtration through Sepharose 4B	5	200–210

heart aorta [7]. On the other hand, the M_r of benzylamine oxidase from large blood vessels and microvessels was significantly higher than that of blood plasma [15].

Results of kinetic studies of benzylamine oxidation by the enzyme are shown in fig.2. We found further that BAPN, which is known to be an effective inhibitor of benzylamine oxidase and lysyl oxidase of aorta [3], also inhibits benzylamine oxidase of brain microvessels. Optical and EPR spectra of benzylamine oxidase of brain microvessels are presented in fig.3. It should be noted that no band was present in the visible region of the optical spectrum. As can be seen, the optical spectrum in the UV region of the enzyme is very specific. Proteins rarely have a UV spectrum of this type. Besides benzylamine oxidase from aorta [7], the same spectrum has been observed for metallothionein, a small protein lacking aromatic amino acids but very rich in cysteines [16]. In this connection it is of importance to note that the content of thiol groups in benzylamine oxidase was significantly lower than that of metallothionein. Thus, only 3–4 groups per 60000 Da were found for the enzyme from microvessels whereas in typical metallothioneins there are more than 20 cysteine residues per molecule of 7000 Da [16]. Benzylamine oxidase

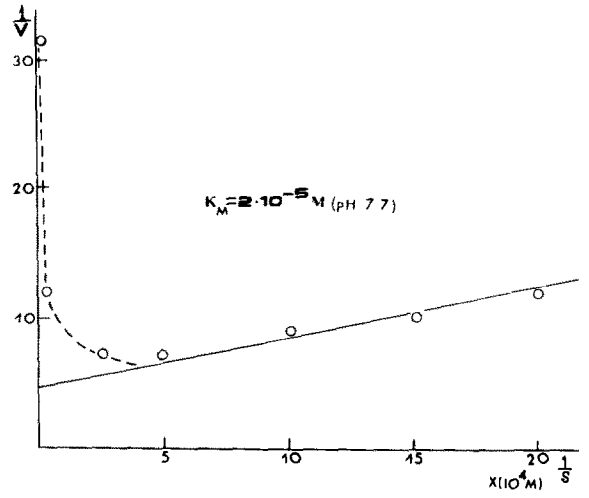

Fig.2. Reciprocal plot for benzylamine oxidation by the enzyme from brain microvessels. The dotted line indicates the region of substrate concentration where inhibition by the substrate was observed.

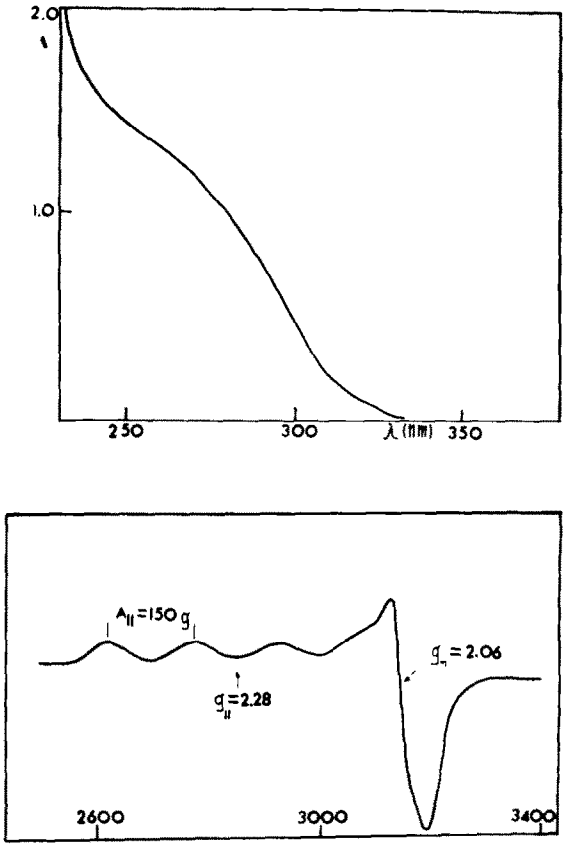

Fig.3. Optical (above) and EPR (below) spectra of the native preparation of benzylamine oxidase from microvessels taken at pH 7.4 (phosphate buffer).

from microvessels should be considered as a thiol-dependent enzyme because thiol reagents such as p-CMB and DTNB inhibit the activity of the enzyme.

EPR spectral parameters of the enzyme, in particular the value of $A_{\parallel}$ = 150 G, show that this enzyme should be referred to the class of so-called 'non-blue' copper-containing oxidases. We found further, in accordance with previous data on benzylamine oxidase from aorta, that in the presence of cyanide or BAPN the EPR signal of Cu^{2+} disappears. This phenomenon has also been observed with other copper-containing proteins [17]. Thus, there are many analogies, if not identity, between benzylamine oxidases from large and small blood vessels, however the content of the enzyme in small vessels appears to be higher than in large vessels.

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