Active-Site Titration of Pig Plasma Benzylamine Oxidase with Phenylhydrazine

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(Received 2 July 1974)

Pig plasma benzylamine oxidase is a protein containing cupric copper and pyridoxal phosphate. The pyridoxal phosphate is stably linked to the enzyme. Discrepancies in the numbers of active sites per molecule of enzyme are reported in the literature. This paper shows that the fully active pure enzyme contains 3 mol of pyridoxal phosphate per mol, whereas enzymes with a lower specific activity are shown by titration with phenylhydrazine to have a lower pyridoxal phosphate content.

Recently a number of studies on the active site of pig plasma benzylamine oxidase have been reported. We have shown that a highly purified preparation of this enzyme contains 3 mol of pyridoxal phosphate/mol of enzyme (Blaschko & Buffoni, 1965). Lindström & Pettersson (1973) have reported that pig plasma amine oxidase contains only 1 mol of pyridoxal phosphate/mol of enzyme and 2 atoms of copper per protein molecule. On the other hand we have also shown that the enzyme readily loses copper on storage in the crystalline form in (NH₄)₂SO₄ solution (Buffoni & Della Corte, 1972). Because of these discrepancies in the quantitative determination of the active sites of pig plasma benzylamine oxidase we decided to titrate the active sites of pig plasma benzylamine oxidase against phenylhydrazine.

Materials and Methods

Materials

DEAE-cellulose DE-32 (microgranular form) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. DEAE-Sephadex A-50 was from Pharmacia, Uppsala, Sweden. Hydroxyapatite was prepared by the method of Tiselius et al. (1956). Aristar-grade (NH₄)₂SO₄ was used in the enzyme purification beyond stage III. General chemicals, all AnalaR grade, were either from British Drug Houses Chemicals, Poole, Dorset, U.K., or Merck, Darmstadt, Germany. [7-¹⁴C]Benzylamine (5.6mCi/mmol) was obtained from Mallinkrodt/Nuclear, Orlando, Fla., U.S.A.

Enzyme isolation

The enzyme was isolated by the method of Buffoni & Blaschko (1964, 1971).

Methods

Protein concentrations were usually determined by the method of Waddell (1956), but for the titration experiments we used the millimolar extinction coefficient at 280 nm (ε₂₈₀ = 250mM⁻¹·cm⁻¹), calculated gravimetrically by using a pure freeze-dried preparation and referred to dry weight (the water content of the freeze-dried preparation was 6.02%) and to the molecular weight of 195500 obtained by Archibald's method (see Blaschko & Buffoni, 1965; Buffoni & Blaschko, 1964). No differences were obtained between these two methods. The readings at 215 and 225 nm in Waddell's (1956) method are higher than at 280 nm and are therefore more accurate at low enzyme concentrations.

Enzymic activity was measured by three different methods: (1) spectrophotometric assay (Tabor et al., 1954) in a Beckman ACTA III instrument, with a silica cell of 1 cm light-path and a thermostatically controlled (±0.1°C) cell holder, benzaldehyde production being followed at 250 nm; (2) measurement of O₂ consumption with a Rank oxygen electrode (Dixon & Kleppe, 1965); (3) radioisotopic methods (McCaman et al., 1965) using [¹⁴C]benzylamine and measuring the benzaldehyde produced. The aldehyde was extracted with ethyl acetate, and 0.1–0.2 ml of this solution was added to 15 ml of a scintillation solution containing 4 g of 5-(4-biphenyl)-2-(4-t-butyl-phenyl)-1-oxa-3,4-diazole (butyl-PBD) and 200 ml of ethoxyethanol in 1 litre of toluene. A Packard liquid-scintillation spectrometer was used for the determination of radioactivity. The efficiency was 80.6%±0.15 (12 determinations). Quenching was measured with an external standard.

Results

Spectrophotometric titration

Samples (0.05 ml) of 16.644 μm-enzyme, dialysed against 0.05 m-sodium-potassium phosphate buffer...
Table 1. Spectrophotometric titration of pig plasma benzylamine oxidase with phenylhydrazine

Conditions of reaction were as follows: 0.05 ml of enzyme solution (16.644 μM), 1.10 ml of 0.05 M-phosphate buffer, pH 7.0, 0.05 ml of phenylhydrazine hydrochloride solution at different concentrations. A double-beam spectrophotometer was used. A 0.05 ml portion of enzyme solution (16.644 μM) and 1.15 ml of the same buffer was used as blank for each reading at room temperature in a 1 cm-light-path cell. The values are the means of two determinations.

<table>
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<th>Molar ratio of phenylhydrazine to enzyme</th>
<th>$E_{430}$</th>
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<tr>
<td>0</td>
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</table>

Fig. 1. Inhibition of $O_2$ consumption of pig plasma benzylamine oxidase by phenylhydrazine

The $O_2$ electrode contained 0.05 ml of enzyme solution (16.644 μM), 2.85 ml of 0.05 M-sodium-potassium phosphate buffer, pH 7.0, and 0.05 ml of phenylhydrazine at different concentrations. After 10 min of incubation at 37°C 0.05 ml of 0.6 M-benzylamine hydrochloride was tipped in. The rate of consumption in the absence of phenylhydrazine (1.5 μl/min) was set equal to 100.

Fig. 2. Active-site titration of pig plasma benzylamine oxidase with phenylhydrazine

One mixture contained 0.05 ml of enzyme solution (0.465 μM), 0.05 ml of 0.05 M-sodium-potassium phosphate buffer, pH 7.0, the others 0.05 ml of enzyme solution (0.465 μM), 0.05 ml of phenylhydrazine hydrochloride (0.2325 μM or 0.465 μM or 0.930 μM). They were incubated for 10 min at 37°C in air with shaking. Then 0.05 ml of 2 M-[7-14C]benzylamine hydrochloride solution was added and incubation was continued for 10 min and stopped by addition of 0.1 ml of 3 M-HCl before extraction with 0.5 ml of ethyl acetate.

0.20 μmol/min per mg of protein at 25°C, pure $O_2$ as gas phase and benzylamine at saturating concentration contains 3 mol of pyridoxal phosphate/mol.

Titration based on the enzymic activity

The decrease in enzymic activity obtained by preincubation of the enzyme with different concentrations of phenylhydrazine was measured with an oxygen electrode. This titration gives the same results as obtained by the spectrophotometric method, as shown in Fig. 1. More accurate measurements were obtained with the radioisotopic method, as shown in Fig. 2. With this method it was shown that the inhibition by phenylhydrazine is completely removed by the presence of benzylamine at relatively low concentration (0.066 mM), whereas preincubation with phenylhydrazine (10 min) before the addition of substrate inhibits without apparent change of $K_m$ (Fig. 3).

Effect of aging of the enzyme

In previous experiments Buffoni & Della Corte (1972) showed that enzyme stored in crystalline form in (NH$_4$)$_2$SO$_4$ solution loses copper and enzymic activity. Old preparations of crystalline pig plasma benzylamine oxidase that have lost enzymic activity...
The mixtures (E) contained 0.05 ml of enzyme solution (0.465 \( \mu \text{M} \)) and 0.05 ml of 0.05M-sodium–potassium phosphate buffer, pH 7.0. The mixtures (P) contained 0.05 ml of enzyme solution (0.465 \( \mu \text{M} \)) and 0.05 ml of phenylhydrazine hydrochloride (0.465 \( \mu \text{M} \)). They were incubated for 10 min at 37°C in air with shaking, then 0.05 ml of benzylamine hydrochloride solution at different concentrations was added and incubation was continued for 10 min and stopped by addition of 0.1 ml of 3M-HCl before extraction with 0.5 ml of ethyl acetate. \( v \), mol/mol of enzyme per min.

have a lower content of pyridoxal phosphate, as shown by titration with phenylhydrazine. An example is reported in Fig. 4. A linear relationship seems to exist between the enzymic activity and the maximum titration value obtained with phenylhydrazine (Fig. 5). Similarly a relationship exists between the maximum phenylhydrazine/enzyme molar ratio and the millimolar extinction coefficient at 430 nm. With a partially inactive enzyme, giving a maximum titration value for pyridoxal phosphate of 1 mol/mol of enzyme, the millimolar extinction coefficient at 430 nm is 23 mm\(^{-1}\) cm\(^{-1}\) (two determinations), and with the fully active enzyme, giving a maximum titration value of 3 mol of pyridoxal phosphate/mol of enzyme, the extension coefficient is 63 mm\(^{-1}\) cm\(^{-1}\) (values of Table 1). The mother liquor containing the crystals of enzyme preparations showing lower specific activity was analysed after centrifugation. No positive colorimetric reactions for pyridoxal phosphate were obtained; if pyridoxal phosphate was present it was undetectable by these methods. Nevertheless, a fluorescence spectrum like that of free pyridoxal phosphate in \((\text{NH}_4)_2\text{SO}_4\) solution was obtained with an excitation maximum of 303 nm and an emission maximum at 420 nm.

**Discussion**

Treatment of benzylamine oxidase (Blaschko & Buffoni, 1965), spermine oxidase (Yamada & Yasunobu, 1963), diamin oxidase (Goryachenkova et al., 1968) and amine oxidase of _Aspergillus niger_ (Yamada et al., 1972) with phenylhydrazine leads to the appearance of an absorption peak around 430 nm, which is close to the absorption maximum of
the phenylhydrazine of free pyridoxal phosphate. In previous experiments (Buffoni, 1968) we have shown that pig plasma benzylamine oxidase links 3 mol of $[^{14}C]$histamine/mol of enzyme in the form of a Schiff base.

The titration of the enzyme with phenylhydrazine is in agreement with our previous results. Since the enzyme has a molecular weight of 195 500 (Buffoni & Blaschko, 1964), we suggest that this enzyme is made up of three subunits and three active sites each formed by one molecule of pyridoxal phosphate and one atom of cupric copper. The 1:1 correspondence between copper and pyridoxal phosphate is in full agreement with the proposed mechanism of reaction (Buffoni & Della Corte, 1972; Taylor et al., 1972).

The millimolar extinction coefficient at 430 nm of the chromophore formed in the reaction between the enzyme and phenylhydrazine is 63 $\text{mm}^{-1} \cdot \text{cm}^{-1}$. This value is higher than the value reported by Lindström & Pettersson (1973) for the same enzyme (35 $\text{mm}^{-1} \cdot \text{cm}^{-1}$). However, these authors found only 1 mol of pyridoxal phosphate/mol of enzyme. With an aged enzyme preparation having a pyridoxal phosphate content of 1 mol/mol of enzyme we obtained a millimolar extinction coefficient of 23 $\text{mm}^{-1} \cdot \text{cm}^{-1}$, which is in good agreement with the extinction coefficient reported for the phenylhydrazone of free pyridoxal phosphate (Wada & Snell, 1961) and with the value obtained by Lindström & Pettersson (1973) in the presence of 4 M-urea. It is difficult to understand the differences between our results and those of Lindström & Pettersson (1973). They cannot be ascribed to the method used for the protein determination. A linear relationship seems to exist between the pyridoxal phosphate content and the enzymic activity, whereas such results (63 instead of 69) would seem to preclude a linear relationship between the millimolar extinction coefficient at 430 nm and the pyridoxal phosphate content of the enzyme.

The lower content of pyridoxal phosphate and copper reported in the literature for this enzyme can be explained by the observation that the enzyme loses pyridoxal phosphate and copper with storage under $(\text{NH}_4)_2\text{SO}_4$.

This work was supported by the Italian National Research Council, Grant no. C173.00488.04.

References