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Active-Site Titration of Pig Plasma Benzylamine Oxidase with Phenylhydrazine

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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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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-^{14}C]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 280nm ($\varepsilon_{280} = 250 mM^{-1} \cdot cm^{-1}$), 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 225nm in Waddell's (1956) method are higher than at 280nm 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 1cm light-path and a thermostatically controlled (±0.1°C) cell holder, benzaldehyde production being followed at 250nm; (2) measurement of O_2 consumption with a Rank oxygen electrode (Dixon & Kleppe, 1965); (3) radioisotopic methods (McCaman *et al.*, 1965) using [^{14}C]benzylamine and measuring the benzaldehyde produced. The aldehyde was extracted with ethyl acetate, and 0.1–0.2ml of this solution was added to 15ml of a scintillation solution containing 4g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) and 200ml 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 (0.05ml) of 16.644μM-enzyme, dialysed against 0.05M-sodium–potassium phosphate buffer

Table 1. *Spectrophotometric titration of pig plasma benzylamine oxidase with phenylhydrazine*

Conditions of reaction were as follows: 0.05ml of enzyme solution (16.644μM), 1.10ml of 0.05M-phosphate buffer, pH7.0, 0.05ml of phenylhydrazine hydrochloride solution at different concentrations. A double-beam spectrophotometer was used. A 0.05ml portion of enzyme solution (16.644μM) and 1.15ml of the same buffer was used as blank for each reading at room temperature in a 1cm-light-path cell. The values are the means of two determinations.

Molar ratio of phenylhydrazine to enzyme	E_{430}
0	0
0.475	0.006
0.959	0.015
1.439	0.022
3.000	0.045
3.839	0.045
4.797	0.045

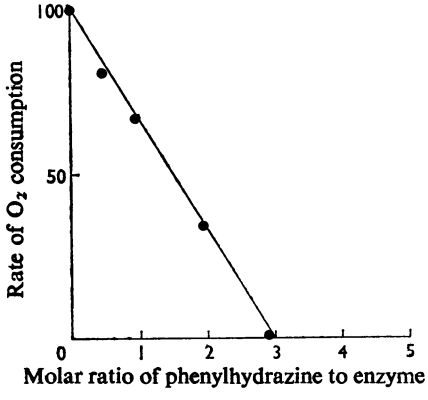

Fig. 1. *Inhibition of O_2 consumption of pig plasma benzylamine oxidase by phenylhydrazine*

The O_2 electrode contained 0.05ml of enzyme solution (16.644μM), 2.85ml of 0.05M-sodium–potassium phosphate buffer, pH7.0, and 0.05ml of phenylhydrazine at different concentrations. After 10min of incubation at 37°C 0.05ml of 0.6M-benzylamine hydrochloride was tipped in. The rate of consumption in the absence of phenylhydrazine (1.5μl/min) was set equal to 100.

(pH7.0), and 1.1ml of the buffer were titrated by addition of 0.05ml of a freshly prepared solution of phenylhydrazine in the buffer. The concentration of phenylhydrazine ranged from a fraction of the enzyme concentration to five times higher. The absorbance measurement was made at room temperature (20°C) 10min after the addition of the inhibitor to allow equilibration. Table 1 shows the results obtained by measurements at 430nm, the maximum of the peak observed. This titration shows that a fully active preparation of crystalline enzyme (specific activity

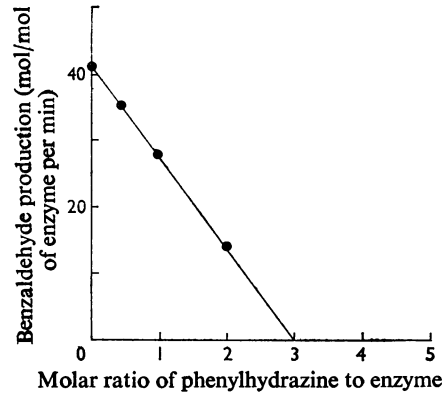

Fig. 2. *Active-site titration of pig plasma benzylamine oxidase with phenylhydrazine*

One mixture contained 0.05ml of enzyme solution (0.465μM), 0.05ml of 0.05M-sodium–potassium phosphate buffer, pH7.0, the others 0.05ml of enzyme solution (0.465μM), 0.05ml of phenylhydrazine hydrochloride (0.2325μM or 0.465μM or 0.930μM). They were incubated for 10min at 37°C in air with shaking. Then 0.05ml of 2mM-[7-^{14}C]benzylamine hydrochloride solution was added and incubation was continued for 10min and stopped by addition of 0.1ml of 3M-HCl before extraction with 0.5ml of ethyl acetate.

0.20μmol/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 (0.066mM), whereas preincubation with phenylhydrazine (10min) 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 $(NH_4)_2SO_4$ solution loses copper and enzymic activity. Old preparations of crystalline pig plasma benzylamine oxidase that have lost enzymic activity

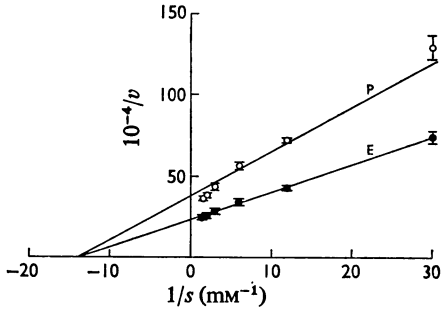

Fig. 3. *Inhibition of pig plasma benzylamine oxidase by phenylhydrazine*

The mixtures (E) contained 0.05ml of enzyme solution (0.465μM) and 0.05ml of 0.05M-sodium–potassium phosphate buffer, pH7.0. The mixtures (P) contained 0.05ml of enzyme solution (0.465μM) and 0.05ml of phenylhydrazine hydrochloride (0.465μM). They were incubated for 10min at 37°C in air with shaking, then 0.05ml of benzylamine hydrochloride solution at different concentrations was added and incubation was continued for 10min and stopped by addition of 0.1ml of 3M-HCl before extraction with 0.5ml of ethyl acetate. v, mol/mol of enzyme per min.

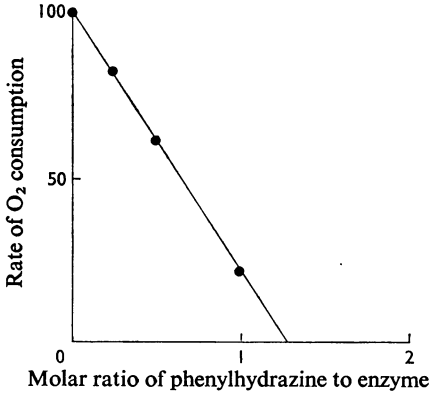

Fig. 4. *Inhibition of the O_2 consumption of an aged pig plasma benzylamine oxidase by phenylhydrazine*

The oxygen electrode contained 0.05ml of 30.98μM solution of the enzyme stored for 7 months in $(NH_4)_2SO_4$, 2.85ml of 0.05M-sodium–potassium phosphate buffer, pH7.0, and 0.05ml of phenylhydrazine at different concentrations. After 10min of incubation at 37°C 0.05ml of 0.06M-benzylamine hydrochloride was tipped in. The rate of consumption in the absence of phenylhydrazine (1.29μl/min) was set equal to 100. The specific activity of this enzyme was 43% of that of the enzyme of Fig. 1.

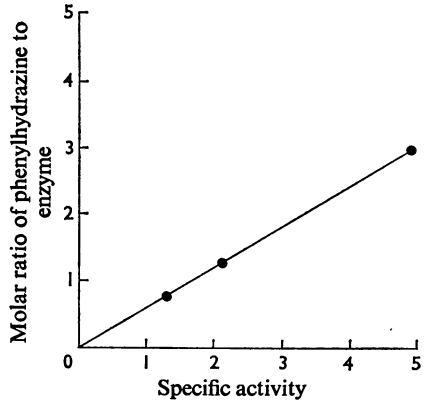

Fig. 5. *Active-site titration of some samples of pig plasma benzylamine oxidase with different specific activities*

The activity is expressed as E_{250}/min per mg of protein at 37°C and was obtained by the spectrophotometric method.

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 430nm. With a partially inactive enzyme, giving a maximum titration value for pyridoxal phosphate of 1mol/mol of enzyme, the millimolar extinction coefficient at 430nm is 23mM^{-1}·cm^{-1} (two determinations), and with the fully active enzyme, giving a maximum titration value of 3mol of pyridoxal phosphate/mol of enzyme, the extension coefficient is 63mM^{-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 $(NH_4)_2SO_4$ solution was obtained with an excitation maximum of 303nm and an emission maximum at 420nm.

Discussion

Treatment of benzylamine oxidase (Blaschko & Buffoni, 1965), spermine oxidase (Yamada & Yasunobu, 1963), diamine 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 430nm, 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 430nm of the chromophore formed in the reaction between the enzyme and phenylhydrazine is 63 $mM^{-1} \cdot cm^{-1}$. This value is higher than the value reported by Lindström & Pettersson (1973) for the same enzyme (35 $mM^{-1} \cdot cm^{-1}$). 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 $mM^{-1} \cdot cm^{-1}$, 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 4M-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 430nm 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 $(NH_4)_2SO_4$.

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