Volume 238, number 1, 74–76 FEB 06338 September 1988

Unprecedented lysyloxidase activity of *Pichia pastoris* benzylamine oxidase

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Received 8 July 1988

Benzylamine oxidase (EC 1.4.3.6) from the yeast *Pichia pastoris* is a 106 kDa quinoprotein containing one copper atom per molecule. It has a broad substrate specificity ranging from butylamine to peptidyl lysine in collagen and elastin. The kinetic data obtained using lysine-containing model peptides as substrates indicate an astonishing similarity to mammalian lysyloxidase. This similarity is further supported by the inhibition of both enzymes with β-aminopropionitrile.

Amine oxidase; Pyrroloquinolinequinone; Lysyloxidase; (*Pichia pastoris*)

1. INTRODUCTION

Many yeasts are known to grow on amines as the sole nitrogen source, implying the presence of an amine oxidase [1]. In the yeast *Pichia pastoris* two amine oxidases have been isolated and partially characterized [2]. Both enzymes were proposed to contain an organic cofactor and copper as prosthetic groups. In a number of different amine oxidases (bovine serum amine oxidase [3,4], porcine kidney amine oxidase and others [5]), pyrroloquinone quinone (PQQ) has recently been identified as the organic cofactor. In the light of these findings the benzylamine oxidase from *P. pastoris* has been further investigated. The enzyme was identified as a quinoprotein and surprisingly was also found to oxidize peptidyl lysine in synthetic model peptides, elastin and collagen. This is the first report of a non-mammalian lysyloxidase.

2. MATERIALS AND METHODS

P. pastoris was obtained from CBS (Baarn, Netherlands). Induction with butylamine and the purification of the enzyme were performed as previously described [6]. The PQQ content was determined by derivatization with 2,4-dinitrophenylhydrazine [3]. Copper concentration was measured by atomic absorption spectroscopy on an Instrumentation Laboratory atomic absorption spectrophotometer 157. The protein concentrations were determined by standard methods [7]. The lysine derivatives lysine methylester, *N*-α-acetyllysine and *N*-α-acetyllysine methylester were obtained from Bachem (Bubendorf, Switzerland) and used without further purification. The synthetic hexapeptides were obtained by solid-phase peptide synthesis [8], and subsequently purified by HPLC. The peroxidase-coupled spectrophotometric assays were performed as described previously [6].

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3. RESULTS AND DISCUSSION

3.1. *Molecular properties*

Fig.1a depicts the absorption spectrum of freshly isolated *P. pastoris* benzylamine oxidase. The enzyme shows a broad absorption in the visible region (400–600 nm, $\epsilon_{480\,nm} = 2850\ M^{-1} \cdot cm^{-1}$) indicative of PQQ. To confirm the presence of PQQ as the organic cofactor in *P. pastoris* amine oxidase, the purified enzyme was treated with a 30-fold excess of 2,4-dinitrophenylhydrazine. The spectrum (fig.1b) of the resulting 2,4-dinitrophenylhydrazone derivative shows an absorption maximum at 365 nm. Based on the molar absorption coefficient for the hydrazone of 31 400 [3], the PQQ content of benzylamine oxidase was cal-

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00145793/88/$3.50

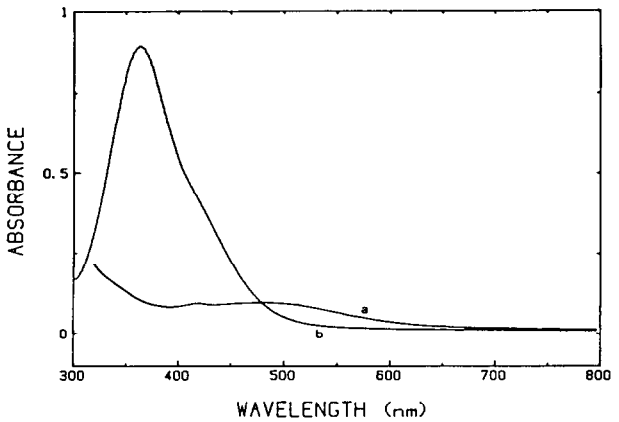

Fig.1. Absorption spectra of native (a) and 2,4-dinitrophenylhydrazine-treated (b) *Pichia pastoris* benzylamine oxidase. The concentration of the enzyme was 33 μM and a 30-fold molar excess of 2,4-dinitrophenylhydrazine was used. Spectrum (b) was recorded after 24 h incubation at 25°C under oxygen atmosphere.

Table 1

Kinetic constants for the oxidation of various substrates by *P. pastoris* benzylamine oxidase

Substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (10^{-3} min^{-1} · mM^{-1})
Butylamine	720	0.235	3.1
Ornithine	870	0.80	1.1
Lysine	1030	0.091	10.6
Lysine methylester	210	0.0047	44.6
N-α-Acetyllysine	690	2.97	0.2
N-α-Acetyllysine methylester	850	0.013	65.4

The assays were performed at 25°C, in a total volume of 3 ml [6]: 100 mol potassium phosphate buffer, pH 7.0, 2.5 μmol 2,2'-azino-di-[3-ethyl-benzthiazolinsulfonate (6)] disodium salt (Na_2ABTS), 15 units horseradish peroxidase, 5 μg of benzylamine oxidase and varying amounts of substrate (3–3000 μM)

culated to be 0.96 mol PQQ/mol enzyme. The copper content was found to be 0.9 gatoms/mol. This stoichiometry (1 Cu/PQQ) is different from those reported for plasma amine oxidases (2 Cu/PQQ) [4], possibly due to differences in the bioavailability of PQQ in the different species.

3.2. *Kinetic properties*

In a previous study [6] on *P. pastoris* benzylamine oxidase, the enzyme was found to oxidize a large number of different aliphatic and aromatic amines and diamines. In contrast to other microbial amine oxidases the *P. pastoris* enzyme also oxidizes the amino acids ornithine and lysine [6]. This unusual property prompted us to further investigate the substrate specificity of this amine oxidase. Table 1 shows the kinetic parameters (K_M and k_{cat}) for oxidation of butylamine, ornithine, lysine and N- or C-protected lysine derivatives. A comparison of ornithine with lysine shows that the sidechain length influences the binding appreciably. Lysine is bound nearly 10-fold more strongly than ornithine. The binding increases again about 20-fold in the C-protected substrate lysine methylester. On the other hand, the observed binding of the N-protected derivative *N*-α-acetyllysine is greatly diminished compared to free lysine. Therefore, it is reasonable to assume that the negative charge of free lysine hinders the formation of the enzyme-substrate complex. However the acetylated α-amino group of lysine, although not oxidized by the enzyme, seems to enhance binding as shown by the increased K_M after its acetylation. To date the best synthetic substrate was found to be *N*-α-acetyllysine methylester (table 1, column 3). To elucidate further the substrate specificity of benzylamine oxidase five synthetic hexapeptides were studied (table 3). The peptides were designed to contain a tyrosine as an internal concentration standard and lysine in position two. Table 2 shows that the kinetic parameters are dependent on the amino acids flanking the oxidizable lysine residue.

Table 2

Kinetic constants for the oxidation of synthetic hexapeptides by *P. pastoris* benzylamine oxidase

Substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (10^{-3} min^{-3} · mM^{-1})
Ala-Lys-Ala-Tyr-Asp-Val	1054	0.444	2.3
Ala-Lys-Glu-Tyr-Asp-Val	170	0.523	0.3
Glu-Lys-Glu-Tyr-Asp-Val	–	–	–
His-Lys-His-Tyr-Asp-Val	740	0.0024	308
Phe-Lys-Tyr-Tyr-Asp-Val	840	0.35	2.4

Assay conditions as in table 1

Table 3

Kinetic constants for the inhibition of *P. pastoris* benzylamine oxidase by β-aminopropionitrile

Substrate	K_I (M)
Lysine	0.171
N-α-Acetyllysine methylester	0.22
Ala-Lys-Ala-Tyr-Asp-Val	0.216

Assay conditions as in table 1

The peptide with the sequence Ala-Lys-Ala-, commonly found to be the major crosslinking site in elastin [9], shows the highest k_{cat} value. The introduction of one negative charge of the C-terminal site of the lysine residue (Ala-Lys-Glu-, table 3), leads to a 10-fold drop in k_{cat}. This effect is further amplified in the peptide containing two negative charges (Glu-Lys-Glu-, table 3). In fact this peptide was such a poor substrate that accurate determination of the kinetic parameters could not be carried out. On the other hand, the introduction of two positive charges (His-Lys-His-, table 3) decreased the K_M value more than two orders of magnitude. Side chains with aromatic character, as in Tyr-Lys-Phe- (table 3), hardly affected the turnover number or the K_M value, when compared to Ala-Lys-Ala-. Taken together, this sequence specificity is very similar to that described earlier for lysyloxidase [10].

In addition, the benzylamine oxidase from *P. pastoris* was found to act also upon elastin, collagen and histones. With these substrates, however, the photometric assay was not well suited due to their limited solubility. Using the more sensitive tritium release assay [11] it was unambiguously shown that peptidyllysine residues are oxidized in fibrillar collagen from chick endothelia. Furthermore *P. pastoris* benzylamine oxidase is inhibited by β-aminopropionitrile (table 3) which is considered to be a highly specific inhibitor of lysyloxidase [11].

In conclusion, the benzylamine oxidase from the yeast *P. pastoris* was found to be remarkably similar in many respects to mammalian lysyloxidase. However, the k_{cat} values for different substrates are two to three orders of magnitude higher in the microbial enzyme.

Acknowledgements: We thank B. Gutte, S. Klauser and H. Langen for the synthesis of model peptides, and P. Royce (Stoffwechsellabor, Kinderspital Zürich) for performing the tritium release assay. This work was supported by the Swiss National Science Foundation (Grant 3.236-0.85), the Geigy-Jubiläumsstiftung, the Hartmann-Müller Stiftung and the Kanton of Zürich.

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