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Distribution of amine oxidases and amine dehydrogenases in bacteria grown on primary amines and characterization of the amine oxidase from *Klebsiella oxytoca*

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The bacteria *Klebsiella oxytoca* LMD 72.65 (ATCC 8724), *Arthrobacter* P1 LMD 81.60 (NCIB 11625), *Paracoccus versutus* LMD 80.62 (ATCC 25364), *Escherichia coli* W LMD 50.28 (ATCC 9637), *E. coli* K12 LMD 93.68, *Pseudomonas aeruginosa* PAO1 LMD 89.1 (ATCC 17933) and *Pseudomonas putida* LMD 68.20 (ATCC 12633) utilized primary amines as a carbon and energy source, although the range of amines accepted varied from organism to organism. The Gram-negative bacteria *K. oxytoca* and *E. coli* as well as the Gram-positive methylotroph *Arthrobacter* P1 used an oxidase whereas the pseudomonads and the Gram-negative methylotroph *Paracoccus versutus* used a dehydrogenase for amine oxidation. *K. oxytoca* utilized several primary amines but showed a preference for those containing a phenyl group moiety. Only a single oxidase was used for oxidation of the amines. After purification, the following characteristics of the enzyme indicated that it belonged to the group of copper-quinoprotein amine oxidases (EC 1.4.3.6): the molecular mass (172000 Da) of the homodimeric protein; the UV/visible and EPR spectra of isolated and *p*-nitrophenylhydrazine-inhibited enzyme; the presence and the content of copper and topaquinone (TPQ). The amine oxidase appeared to be soluble and localized in the periplasm, but catalase and NAD-dependent aromatic aldehyde dehydrogenase, enzymes catalysing the conversion of its reaction products, were found in the cytoplasm. From the amino acid sequence of the N-terminal part as well as that of a purified peptide, it appears that *K. oxytoca* produces a copper-quinoprotein oxidase which is very similar to that found in other *Enterobacteriaceae*.

Keywords: amine oxidases, amine dehydrogenases, copper-quinoprotein amine oxidase, topaquinone, *Klebsiella oxytoca*

INTRODUCTION

Many micro-organisms convert primary amines via an oxidation step into products which can be utilized either as a carbon and/or energy source, as a nitrogen source or as both (Yamada *et al.*, 1965; Levering *et al.*, 1981):

$$R\text{-}CH_2NH_2 + H_2O \rightarrow RCHO + 2[H] + NH_3$$

Abbreviations: IEF, isoelectric focusing; *p*NPH, *para*-nitrophenylhydrazine; PEA, phenylethylamine; TPQ, topaquinone; TTQ, tryptophyl tryptophanquinone.

The EMBL accession number for the sequence reported in this paper is P80695.

Various enzymes with different cofactors and mechanisms of action can bring about this oxidation. These include quinoprotein amine dehydrogenase (EC 1.4.99.3), which contains TTQ [tryptophyl tryptophanquinone, the trivial name or 4-(2′-tryptophyl)tryptophan-6,7-dione]; a number of dehydrogenases with unidentified cofactors; flavoprotein amine oxidase (EC 1.4.3.4); and quinoprotein amine oxidase (EC 1.4.3.6), which contains copper and TPQ (topaquinone, the trivial name for 6-hydroxyphenylalanine-3,4-dione). In an attempt to determine which enzymes are used by various bacteria, we have investigated the bacteria's capacity to utilize a number of simple aliphatic and phenyl-group-containing pri-

mary amines as a carbon and energy source, and have identified the type of oxidoreductase involved.

Among the bacteria tested, *Klebsiella oxytoca* was able to utilize 2-phenylethylamine (PEA), and preliminary characterization of the oxidase involved revealed properties reminiscent of a soluble, copper-TPQ-containing amine oxidase. In these respects, the enzyme seemed to be different from that induced during growth on tyramine of a closely related organism, *Klebsiella aerogenes* (*K. pneumoniae*), originally reported to be a membrane-integrated monoamine oxidase (Okamura *et al*., 1976). This name is usually reserved for flavoprotein amine oxidases (EC 1.4.3.4), such an enzyme, for example, occurring in tyramine-grown *Sarcina lutea* (Kumagai *et al*., 1969). Later on (Yamashita *et al*., 1993), a gene was cloned from this bacterium, the expression of which yielded a soluble amine oxidase. Since this discrepancy has not been resolved and the nature of the enzymes has not been established (the authors did not discuss the possibility that the soluble enzyme might be of the copper-quinoprotein type), we have attempted to characterize the *K. oxytoca* amino oxidase and to investigate whether growth on different amines induces production of different amine oxidoreductases in this organism.

METHODS

Materials. Mono Q HR 5/5 and 10/10, Phenyl-Superose 5/5 and Superose 12 HR 10/30 chromatography columns, HPLC reversed phase column Superpac Pep-S 5 μm C_2/C_{18}, Phast system electrophoresis apparatus and kits with standard proteins and isoelectric focusing equipment, and PD 10 desalting columns were from Pharmacia; trifluoracetic acid, reversed phase 5 μm C_{18} and 5 μm C_8 HPLC columns, and Pronase E were from Merck; Sep-Pak C_{18} cartridges were from Waters/Millipore; *p*-nitrophenylhydrazine.HCl (*p*NPH) was from Fluka.

Cultivation of bacteria. *Klebsiella oxytoca* LMD 72.65 (ATCC 8724), *Arthrobacter* P1 LMD 81.60 (NCIB 11625), *Paracoccus versutus* LMD 80.62 (ATCC 25364), *Escherichia coli* W LMD 50.28 (ATCC 9637), *E. coli* K12 LMD 93.68, *Pseudomonas aeruginosa* PAO1 LMD 89.1 (ATCC 17933) and *Pseudomonas putida* LMD 68.20 (ATCC 12633) were obtained from the Laboratory of Microbiology Delft culture collection. The ability to utilize amines was studied by transferring the bacteria to a mineral medium supplemented with Luria broth (0·25 g l^{-1}), thiamin (50 mg l^{-1}) and the amine (5 mM). The mineral medium, brought to pH 7·0, contained 4·5 g KH_2PO_4, 11·7 g K_2HPO_4 and 3 g $(NH_4)_2SO_4$ per litre. The trace element solution (Vishniac & Santer, 1957), and the amines (as their HCl salts) were filter-sterilized and added separately to the heat-sterilized mineral medium. The trace element solution for *Paracoccus versutus* contained a fivefold higher concentration of copper salt. Thiamin was not incorporated in the media for the *Pseudomonas aeruginosa* and *P. putida* strains. The inoculated media (500 ml) were shaken in Erlenmeyer flasks (2 l) at 200 r.p.m. at 30 °C. The ability to utilize the amine was judged from the turbidity of the medium as compared to that in which the amine was lacking. Experiments were discontinued after 7 d. To determine the type of amine oxidoreductase involved, cells were harvested in the mid-exponential growth phase (OD_{600} approx. 0·6–0·8). To provide cells for isolating the amine oxidase and to perform localization studies of the enzymes, *K. oxytoca* was grown aerobically in a 100 l fermenter at 30 °C in the mineral medium supplemented with 5 mM PEA. The cells were harvested in the mid-exponential growth phase.

Localization of enzyme activities. The periplasmic fraction of *K. oxytoca* was prepared according to Rassoulzadegan *et al*. (1982) and Cooper *et al*. (1992).

Enzyme assays. The specific activities of *K. oxytoca* amine oxidase were determined spectrophotometrically using PEA as a substrate and measuring formation of phenylacetaldehyde in the form of its semicarbazone by including semicarbazide in the assay mixture. Although semicarbazide is a weak inhibitor of amine oxidase, inhibition does not occur in the presence of PEA (checked with the polarographic assay, see below), presumably because the substrate reacts more effectively with TPQ than the inhibitor. The assay mixture (1 ml) consisted of 75 mM sodium/potassium phosphate buffer, pH 6·1, 0·5 mM PEA-HCl and 1 mM semicarbazide.HCl. The reaction was started by adding enzyme and the increase in A_{230} was monitored against a blank containing all components except the enzyme. The molar absorption coefficient for phenylacetaldehyde semicarbazone used was $1{\cdot}6 \times 10^4$ M^{-1} cm^{-1} (Parrot *et al*., 1987). All assays were carried out at 30 °C. One unit of activity (U) is the amount of enzyme catalysing the formation of 1 μmol semicarbazone min^{-1}.

Determinations of amine oxidase activity in general and of the substrate specificity of *K. oxytoca* amine oxidase were carried out polarographically. Initial rates of oxygen uptake were measured with a Clark oxygen electrode. The assay mixture (3 ml) consisted of 0·1 M sodium/potassium phosphate buffer, pH 7·0, containing 1 mM amine.

Amine dehydrogenase activity was determined spectrophotometrically at 600 nm by phenazine methosulphate (PMS)-mediated reduction of 2,6-dichlorophenolindophenol (DCPIP) or by direct reduction of DCPIP (Eady & Large, 1968).

Catalase activity was determined by monitoring the decomposition of H_2O_2 spectrophotometrically. The reaction mixture (1 ml) consisted of 80 mM sodium/potassium phosphate buffer, pH 7·0, containing 15 mM H_2O_2. A molar absorption coefficient for H_2O_2 of 40 M^{-1} cm^{-1} at 240 nm was used.

Phenylacetaldehyde dehydrogenase activity was assayed by measuring the formation of NADH spectrophotometrically. The reaction mixture (1 ml) consisted of 93 mM sodium/potassium phosphate buffer, pH 8·0, containing 0·75 mM NAD^+, and 10 mM phenylacetaldehyde. A molar absorption coefficient for NADH of 6220 M^{-1} cm^{-1} at 340 nm was used.

Malate dehydrogenase activity was determined spectrophotometrically by measuring the initial rate of its reverse reaction (Kitto, 1969).

Purification of *K. oxytoca* amine oxidase. Crude extract was prepared according to Haywood & Large (1981). Ammonium sulphate was added to the crude extract (26 ml, kept in an ice-water bath) while stirring. The precipitate formed between 60% and 90% saturation was collected by centrifugation (30 min at 20000 ***g***, 4 °C). The pellet was dissolved in 50 ml 20 mM Tris/HCl buffer, pH 7·0, and the solution was dialysed against the same buffer at 4 °C. All subsequent steps were performed at room temperature. The dialysate was applied to a Mono Q 10/10 anion-exchange column equilibrated with the same buffer and eluted with a 160 ml linear gradient of 0–0·5 M NaCl in the buffer at a flow rate of 2 ml min^{-1}. The enzyme eluted at about 0·1 M NaCl. Solid ammonium sulphate was added to the pooled active fractions to a conc-

entration of 1·7 M and the solution was applied to a Phenyl-Superose 5/5 hydrophobic interaction chromatography column equilibrated with 50 mM sodium phosphate buffer, pH 7·0, containing 1·7 M ammonium sulphate. A 15 ml linear gradient of 1·7–0 M ammonium sulphate was applied at a flow rate of 0·5 ml min^{-1}. Amine oxidase activity eluted at about 0·34 M ammonium sulphate. Active fractions were pooled and stored at −80 °C. Ammonium sulphate was removed by gel filtration chromatography on a PD 10 column when required.

Isolation of the cofactor-*p*-nitrophenylhydrazine product. The purified amine oxidase (149 nmol) in 50 mM sodium phosphate buffer, pH 7·0, was incubated with *p*NPH (450 nmol) at room temperature for 30 min. Excess *p*NPH was removed on a PD 10 column which was equilibrated with 50 mM sodium phosphate buffer, pH 7·0. The *p*NPH-derivatized amine oxidase was digested with Pronase E (16 mg) for 24 h at 37 °C. The cofactor-*p*-nitrophenylhydrazine product was isolated according to Steinebach *et al.* (1995).

Polyacrylamide gel electrophoresis and isoelectric focusing. Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) were performed on commercially available gels (8–25 % gradient gel for native PAGE; 12·5 % homogeneous gels for SDS-PAGE; and IEF 5–8 gels for IEF) using the Pharmacia Phast system according to the instructions provided by the manufacturer. The high molecular kit for native PAGE and the low molecular kit for SDS-PAGE were used for calibration.

Isolation of the CNBr cleavage fragment. Purified enzyme (0·2 ml; 0·24 mg protein) was added to 1 ml 70 % formic acid containing 0·17 g CNBr and incubated for 24 h at room temperature in the dark. Formic acid was evaporated in a vacuum desiccator over NaOH. The S–S bridges in the peptides were split by dissolving the residue in 0·1 M Tris/HCl buffer, pH 8·5, containing 6 M guanidine.HCl, and 0·1 M dithiothreitol. The solution was centrifuged and applied to a reversed phase column (Superpac Pep-S 5 μm C_2/C_{18}) equilibrated with 0·2 % trifluoroacetic acid and the peptides were eluted with a 90 min linear gradient from 0 to 90 % acetonitrile in 0·2 % trifluoroacetic acid at a flow rate of 0·5 ml min^{-1}. The purity of the collected fractions was checked by SDS-PAGE using a 20 % gel. A fraction (retention time 48 min) containing a peptide which showed a single band on the SDS-PAGE was chosen for amino acid sequencing. The acetonitrile was removed under vacuum.

Analytical methods. Protein concentrations were determined according to Bradford (1976) using desalted bovine serum albumin as a standard. The specific absorption coefficient of the enzyme at 280 nm was determined by monitoring the eluting peak with a photodiode-array detector at 280 and 205 nm (van Iersel *et al.*, 1985). Copper was determined by both flame atomic absorption spectroscopy and a chemical assay (Felsenfeld, 1960). ^{1}H-NMR spectroscopy was performed on a Bruker 600 MHZ spectrometer and EPR spectroscopy on a Varian E-9 spectrometer. Amino acid sequencing was performed by automatic Edman degradation.

RESULTS AND DISCUSSION

Utilization of amines and type of oxidoreductase involved in their conversion

All of the bacteria investigated were able to utilize at least one of the amines tested (benzylamine, phenylethylamine, tyramine, methylamine, ethylamine and butylamine) as a carbon and energy source. However, there was substantial variation in the broadness of the range that could be utilized (only one, tyramine, for *Pseudomonas aeruginosa*; all except benzylamine for *Arthrobacter* P1) and type of amine that could be utilized (aromatic amines for *E. coli*, aliphatic amines for *Paracoccus versutus*). There was also variation in the type of amine oxidoreductase activity involved in the conversion, with extracts of the *Enterobacteriaceae* and the Gram-positive methylotrophs *Arthrobacter* P1 and *Amycolatopsis methanolica* (not shown) exhibiting oxidase activity and those of the pseudomonads and the Gram-negative methylotroph *P. versutus* exhibiting dehydrogenase activity. Given that the oxidase activity was found in extracts after ultracentrifugation and that KCN was not an inhibitor, it seems most likely that the activity results from a real oxidase and not a dehydrogenase transferring the reducing equivalents to the respiratory chain. The amine dehydrogenase activity of *P. aeruginosa* was found to be attached to membrane particles (obtained by ultracentrifugation of the extract at 50000 ***g*** for 2 h), in agreement with others (Cuskey *et al.*, 1987). Either an oxidase or a dehydrogenase was involved in the conversion of all amines utilized by a particular bacterium. Apparently it is the species rather than the substrate that determines which type of oxidoreductase is used for the conversion. In this respect, it is interesting to note that amine dehydrogenase activity was found only in Gram-negative bacteria. However, amine dehydrogenases do not have an exclusive role in this type of bacteria since *Enterobacteriaceae* possess an amine oxidase, implying that amine utilization by the latter requires an aerobic environment. Amine oxidase activities induced in PEA- and tyramine-grown *K. oxytoca* derive from the same enzyme, as judged from the identical behaviour of the purified enzymes on SDS-PAGE and the identical substrate specificities (not shown), the latter mimicking the growth pattern of the organism (good growth on PEA and tyramine, poor growth on butylamine, no growth on methylamine, ethylamine or benzylamine).

Characteristics of purified *K. oxytoca* amine oxidase

Amine oxidase purified from PEA-grown *K. oxytoca* (Table 1) migrated as a single protein band when subjected to SDS-PAGE. The specific activity of the pure enzyme with 0·5 mM tyramine or PEA as substrate was 5·6 U (mg protein)$^{-1}$. High activity was observed with tyramine (100 %), PEA (100 %) and tryptamine (100 %), moderate activity with butylamine (65 %) but no activity with benzylamine, methylamine, cadaverine or putrescine. Very low apparent K_m values (lower than 5 μM) were observed for the aromatic amines under air saturation. Apparently, the enzyme has a preference for primary amines (with more than one carbon atom) containing a phenyl moiety.

The absorption spectrum of the purified amine oxidase (Fig. 1) showed the characteristic features of copper-quinoprotein amine oxidases with a 470 nm absorption

Table 1. Purification of amine oxidase from *K. oxytoca*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Purification (-fold)
Crude extract	343	56	0·16	100	1
$(NH_4)_2SO_4$ precipitation	247	50	0·2	89	1·2
Anion-exchange (Mono Q) chromatography	9·3	38	4·1	68	25
Hydrophobic interaction chromatography	5·0	31	5·6	54	35

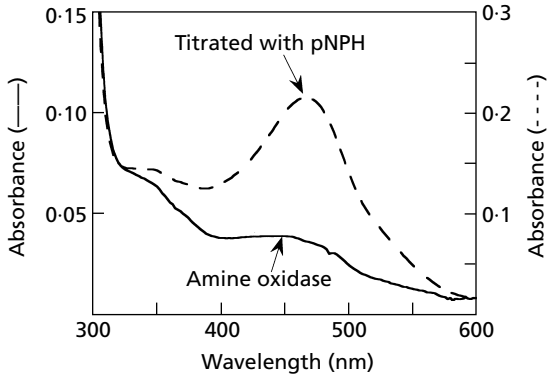

Fig. 1. Absorption spectra of untreated and *p*NPH-treated *K. oxytoca* amine oxidase. Absorption spectra of the enzyme (3·5 nmol, 0·56 mg protein ml^{-1}) were measured in 50 mM potassium phosphate buffer, pH 7·0. ——, Untreated enzyme; – – – – –, enzyme derivatized with 3·5 nmol *p*NPH.

band (Steinebach *et al*., 1996). Isoelectric focusing revealed a single band which corresponded to an isoelectric point of 5·7, indicating that the amine oxidase is an acidic protein. The molecular masses of the native enzyme and its subunit, as determined with PAGE and SDS-PAGE, were 172 kDa and 86 kDa, respectively, indicating that the enzyme is a dimeric protein. One enzyme molecule contained 2·0 copper atoms according to chemical assay and 1·7 according to atomic absorption spectrophotometry. The purified enzyme also showed the characteristic EPR spectrum of copper-quinoprotein amine oxidases (not shown). It is concluded, therefore, that the enzyme contains two Cu^{2+} ions per molecule, a value typical for quinoprotein amine oxidases (Knowles & Dooley, 1994). The amine oxidase was sensitive to carbonyl group reagents such as phenylhydrazine and semicarbazide. Semicarbazide at a concentration of 10 μM gave about 30% inhibition, whereas 55% inhibition was observed with 100 nM phenylhydrazine (2 min incubation at room temperature prior to the assay). *p*NPH reacted rapidly with the amine oxidase, as judged from the inhibition and concomitant spectral changes induced (Fig. 1). Since the ^{1}H-NMR spectrum of the isolated cofactor-*p*NPH product (Fig. 2) is virtually identical to that for the cofactor-*p*NPH peptide from bovine serum amine oxidase (Janes *et al*., 1990) and the cofactor-*p*NPH product isolated from *E. coli* amine oxidase or pig kidney diamine oxidase (Steinebach *et al*., 1995), we conclude that TPQ is present in this enzyme.

These characteristics indicate that *K. oxytoca* utilizes a copper-quinoprotein amine oxidase for growth on PEA or tyramine. In this respect, the organism is comparable to other *Enterobacteriaceae* like *E. coli* (Cooper *et al*., 1992; Steinebach *et al*., 1995) and most probably (see below) *K. aerogenes* (*K. pneumoniae*) (Yamashita *et al*., 1993). Surprisingly, other Gram-negative bacteria convert these substrates via quite different types of oxidoreductases: *Alcaligenes faecalis* with a periplasmic, TTQ-containing amine dehydrogenase (Govindaraj *et al*., 1994); *Pseudomonas aeruginosa* with a membrane-bound, uncharacterized amine dehydrogenase (Cuskey *et al*., 1987); *Pseudomonas putida* with a soluble haem-containing amine dehydrogenase (Durham & Perry, 1978); *Sarcina lutea* with a flavoprotein amine oxidase (Kumagai *et al*., 1969). At present, the reason for this diversity of enzymes catalysing the same reaction, and having similar substrate specificity, is unknown. However, it is certainly not related to the type of substrates which have to be converted since enzymes involved in aliphatic primary amine oxidation are similarly diverse. Neither is it due to a special type of organism, at least with respect to copper-TPQ-containing amine oxidase, since this type of amine oxidase occurs not only in Gram-negative, Gram-positive, and methylotrophic bacteria but also in eukaryotes such as yeasts (Large & Haywood, 1990) and fungi (Yamada & Adachi, 1971).

As shown in Table 2, 83% of the amine oxidase activity of *K. oxytoca* was detected in the periplasmid fraction and only 17% in the cytoplasmic/membrane fraction. In contrast, catalase and phenylacetaldehyde dehydrogenase activity were nearly absent from the periplasmic fraction. As shown by the distribution values of the marker enzyme, cytoplasmic malate dehydrogenase, adequate preparation of the cellular fractions was achieved. Thus, the distribution and the properties show that the *K. oxytoca* amine oxidase is, like the *E. coli* enzyme (Cooper *et al*., 1992), not a membrane-integrated enzyme but a soluble periplasmic enzyme. Whether the location, periplasmic in Gram-negatives and probably cytoplasmic or membrane-bound in

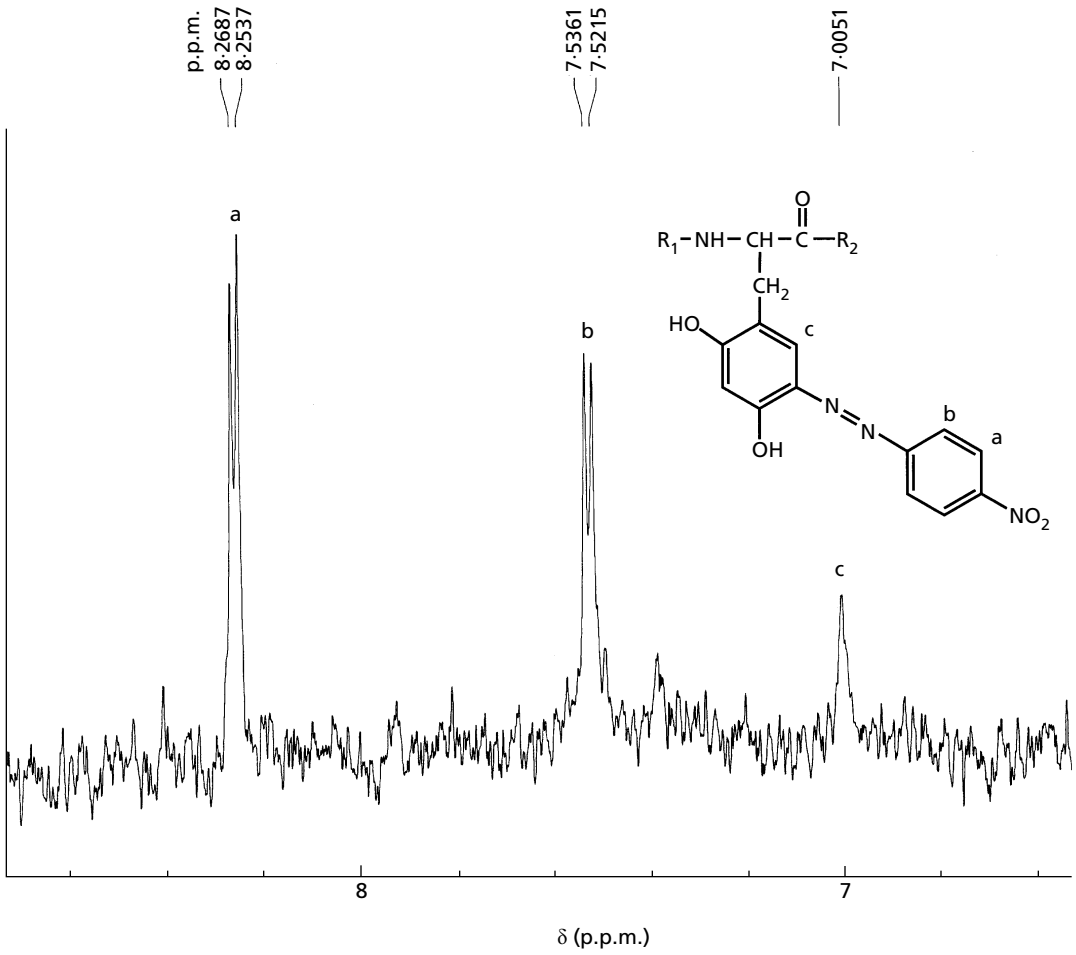

Fig. 2. ^{1}H-NMR spectrum of cofactor-*p*-nitrophenylhydrazine product isolated from *K. oxytoca* amine oxidase. The spectrum of the purified cofactor adduct was taken in D_2O, pH 7·5. The deduced structure of the cofactor (TPQ) is shown in an inset.

Table 2. Distribution of enzyme activity in cellular fractions of *K. oxytoca*

Enzyme	Activity (percentage of activity of crude extract) in:	
	Cytoplasm/ membrane	Periplasm
Amine oxidase	17	83
Catalase	99	1
Phenylacetaldehyde dehydrogenase	85	15
Malate dehydrogenase	93	7

Gram-positives (Levering *et al.*, 1981) and peroxisomal in yeasts (Bruinenberg *et al.*, 1989), is related to structural differences of the enzymes (see below) is presently unknown. Although the products of the reaction, H_2O_2 and aldehyde, are very toxic, apparently *K. oxytoca* is able to cope with them by transforming these compounds into less toxic products by the cytoplasmic catalase and NAD-dependent phenylacetaldehyde dehydrogenase activities. The latter has been purified and appears to be an aromatic aldehyde dehydrogenase (D. M. A. M. Luykx, M. Chettou, J. A. E. Benen, P. W. Postma, S. de Vries & J. A. Duine, unpublished observations).

Comparison with other copper-quinoprotein amine oxidases

The alignment of the sequence of the N-terminal region and the purified peptide from *K. oxytoca* amine oxidase with similar stretches from other copper-quinoprotein amine oxidases shows high similarity with those of *E. coli* and *K. aerogenes* (*K. pneumoniae*) but much less with those of the Gram-positive bacteria *Arthrobacter* P1, *A. globiformis* and the yeast *Hansenula polymorpha*

Enzyme from:	Amino acid sequences	
	N-terminal	Peptide
K.oxytoca	HGGEAHMVPMDKTLQDFGAD	DKQLWVTRYHPTERFPEGKYPNRSIHDTGLGQYAKD
K. aerogenes	HGSEAHMVPLDKTLKEFGAD	[665] DKQLWVTRYHDTERYPEGKYPNRSAHDTGLGQYAKD
E. coli	HGGEAHMVPMDKTLQDFGAD	[665] DKQLWVTRYHPGERFPEGKYPNRSTHDTGLGQYSKD
Arthrobacter P1		[539] RNNLWVTAYDRTERFAAGEYPNQATGGADDGLHIWT
A. globiformis		[541] TKDLWVTRYADDERYPTGDFVNQHSGGAGLPSYIAQ
H. polymorpha		[570] SHSVNVVPYKDNRLYPSGDHVPQWSGDGVRGMREWI
Bovine serum		[628] RYQLAITQRKETEPSSSSVFNQNDPWTPTVDFSDFI
Pea seedling		[549] NYNVWVTAYNRTEKWAGGLYVDHSRGDDTLAVWTKQ
Lentil seedling		[549] NYNVWVTPYNRTEKWAGGLYVDHSRGDDTLAVWTKK

Fig. 3. Alignment of the amino acid sequence of the purified peptide and N-terminal part of *K. oxytoca* amine oxidase with similar stretches from other amine oxidases. Amino acids identical with *K. oxytoca* amine oxidase are indicated in bold. Comparisons were made with published sequences for: overexpressed monoamine oxidase from *K. aerogenes* (*K. pneumoniae*) (Yamashita *et al.*, 1993); tyramine oxidase from *E. coli* W3110 (Azakami *et al.*, 1994); methylamine oxidase from *Arthrobacter* P1 (Zhang *et al.*, 1993); PEA oxidase from *A. globiformis* (Tanizawa *et al.*, 1994); methylamine oxidase from the yeast *H. polymorpha* (Bruinenberg *et al.*, 1989); bovine serum amine oxidase (Mu *et al.*, 1994); pea and lentil seedling amine oxidases (Tipping & McPherson, 1995).

(Fig. 3). With respect to the N-terminal region, this is not surprising since a stretch of about 100 amino acids is lacking in the N-terminal region in the amine oxidases of Gram-positive bacteria (Roh *et al.*, 1994).

Okamura *et al.* (1976) and Sugino *et al.* (1991) reported an amine oxidase in *K. aerogenes* (*K. pneumoniae*) which was a membrane-integrated, hydrophobic enzyme, but they were unable to isolate it. However, overexpression of the cloned gene in the same strain yielded a soluble enzyme which could be purified (Yamashita *et al.*, 1993); this observation was supported by the fact that the sequence of the structural gene did not predict a hydrophobic protein (Sugino *et al.*, 1992). The discrepancy could be explained by assuming that this *K. aerogenes* strain can produce two different amine oxidases, a membrane-integrated one and a soluble one, also supported by the observation that the former did not oxidize PEA (Okamura *et al.*, 1976) whereas the latter did (Yamashita *et al.*, 1993). From the sequence comparison (Fig. 3), it appears that the soluble one is very similar to the enzyme described here. On the other hand, the specific activity and the copper content of the *K. aerogenes* enzyme were much lower (Yamashita *et al.*, 1993) than those observed here for the *K. oxytoca* enzyme. However, since the copper catalyses the conversion of the precursor tyrosyl residue into TPQ (Matsuzaki *et al.*, 1994, 1995) and addition of copper to the recombinant enzyme increased the activity by 83% (Yamashita *et al.*, 1993), the lower values may be partly due to suboptimal processing of the pro-enzyme in the overexpression system of the host. Therefore, all the available evidence indicates now that amine oxidation by *Enterobacteriaceae*, including *Klebsiella* species, can be catalysed by a copper-TPQ-containing amine oxidase. On the other hand, the ability of these organisms to produce another amine oxidase cannot be discounted. Apart from the indications for the *K. aerogenes* strain, it has been found that amine oxidases with different substrate specificity are produced by the same organism grown on different amines; this is the case for the mould *Aspergillus niger* (Frebort *et al.*, 1996), the yeast *Hansenula polymorpha* (Bruinenberg *et al.*, 1989; Mu *et al.*, 1992), and the Gram-positive bacterium *Arthrobacter globiformis* (Tanizawa *et al.*, 1994; Shimizu *et al.*, 1994). Although the results presented here for *K. oxytoca* indicate that a single amine oxidase is involved in the conversion of all amines utilized, it remains possible that another enzyme could be induced under different growth conditions. In this connection, it would be interesting to study the type of amine oxidoreductase induced when the amines serve only as a nitrogen source.

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