RESEARCH COMMUNICATION

Evidence for copper and 3,4,6-trihydroxyphenylalanine quinone cofactors in an amine oxidase from the Gram-negative bacterium *Escherichia coli* K-12

Ronald A. COOPER,* Peter F. KNOWLES,†§ Doreen E. BROWN,‡ Michele A. McGUIRL‡ and David M. DOOLEY‡

* Biochemistry Department, University of Leicester, Leicester LE1 7RH, U.K., † Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K., and ‡ Chemistry Department, Amherst College, Amherst, MA 01002, U.S.A.

The cofactors present in an amine oxidase induced in *Escherichia coli* K-12 by growth on 2-phenylethylamine have been studied by spectroscopic methods. E.s.r. spectroscopy establishes the presence of cupric copper while resonance Raman spectroscopy on the phenylhydrazine derivative of the enzyme provides strong evidence for the oxidized form of 3,4,6-trihydroxyphenylalanine (TOPA) quinone. The amine oxidase should accordingly be classified as EC 1.4.3.6. This is the first report of such an amine oxidase in a Gram-negative bacterium.

INTRODUCTION

It has been shown that growth of *Escherichia coli* K-12 on 2-phenylethylamine as sole carbon and energy source induces an amine oxidase (Parrott *et al.*, 1987) though it was not known whether the amine oxidase was in the flavin (EC 1.4.3.4) or the copper-containing class (EC 1.4.3.6). Both classes of amine oxidase are widespread in mammals, plants and both prokaryotic and eukaryotic micro-organisms, though there has been no report of a copper-containing amine oxidase in a Gram-negative bacterium.

Spectroscopic methods are useful for characterizing the nature of cofactors in amine oxidases. Flavin-containing amine oxidases have an easily identified visible absorption spectrum (Yamada, 1971; Yamada *et al.*, 1971). All copper-containing amine oxidases show a characteristic e.s.r. spectrum due to Cu(II) and have a peak at around 480 nm in the visible spectrum (Collison *et al.*, 1989). Recently the organic cofactor of amine oxidases from bovine serum and yeast has been identified as an oxidized form of 3,4,6-trihydroxyphenylalanine (TOPA) quinone (Janes *et al.*, 1990; Brown *et al.*, 1991) which gives rise to the 480 nm absorption band (Duine, 1991). Amine oxidases containing TOPA quinone react with phenylhydrazine to produce an absorption band at 430–440 nm and this derivative has a characteristic resonance Raman spectrum (Brown *et al.*, 1991; Klinman *et al.*, 1992).

Evidence is presented in this paper which establishes that the amine oxidase induced in *E. coli* K-12 by growth on 2-phenylethylamine has copper and TOPA quinone as cofactors.

substrate by the continuous spectrophotometric method (Parrott *et al.*, 1987). Protein was measured from the ratio of 280/260 nm absorbance values (Warburg & Christian, 1941).

Purification of the amine oxidase

The amine oxidase is present in the bacterial periplasm (S. Parrott & R. A. Cooper, unpublished work) which was prepared by a modification of the method described by Rassoulzadegan *et al.* (1982) for the preparation of spheroplasts. For this, cells from 1 litre of culture were resuspended at 0 °C in 10 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 20% (w/v) sucrose. Then 2 ml of lysozyme solution (10 mg/ml) was added with gentle mixing and incubated at 0 °C for 10 min. After addition, with gentle stirring, of 5 ml of 42 mM-EDTA, pH 8.0, the suspension was diluted by progressive addition of 10 ml of Tris/HCl buffer, pH 8.0, containing 9% (w/v) sucrose and 22 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 9% (w/v) sucrose and 15 mM-MgCl₂. The solution containing the periplasm (approx. 50 ml) was recovered after centrifugation at 9000 g at 4 °C for 5 min to sediment the spheroplasts. In a typical preparation of amine oxidase, bacteria from 12 litres of culture were used to produce approx. 600 ml of periplasm. The amine oxidase was purified from the periplasm using a Pharmacia f.p.l.c. system by a combination of ion-exchange and hydrophobic-interaction chromatography. In the first step, approx. 100 ml of periplasm was applied to a MonoQ 5/5 ion-exchange column equilibrated with 20 ml of Tris/HCl buffer, pH 7.0, and eluted with a 20 ml gradient of 0–0.35 M-NaCl at a flow rate of 1 ml/min. Fractions (1 ml) were collected and the pink-coloured fraction containing the amine oxidase eluted at approx. 0.13 M-NaCl. In a typical preparation, this step was carried out a total of six times and the fraction with the highest amine oxidase specific activity from each run was selected to give a total sample volume of 6 ml. For the second step, solid (NH₄)₂SO₄ was added to this sample to give a concentration of 1.7 M and the solution applied to a phenyl-Superose 5/5 hydrophobic-interaction column equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 1.7 M-(NH₄)₂SO₄. A 15 ml decreasing gradient of 1.7–0 M-

MATERIALS AND METHODS

Cell growth

Cells were grown on 2-phenylethylamine as described previously (Parrott *et al.*, 1987).

Enzyme assay

The amine oxidase was assayed with 2-phenylethylamine as

Abbreviation used: TOPA, 3,4,6-trihydroxyphenylalanine.

§ To whom correspondence should be addressed.

Table 1. Summary of amine oxidase purification

Purification step	Volume (ml)	Total protein (mg)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
1. Periplasm	594	457.0	243	0.5	100.0	1.0
2. MonoQ (pH 7.0)	6	24.5	95	3.9	39.0	7.8
3. Phenyl-Superose	2	9.6	50	5.2	20.5	10.4
4. MonoQ (pH 8.0)	1	5.5	31	5.6	12.8	11.2

$(\text{NH}_4)_2\text{SO}_4$ was applied at a flow rate of 0.5 ml/min and the amine oxidase eluted at approx. 0.34 M- $(\text{NH}_4)_2\text{SO}_4$. The fractions with the highest amine oxidase specific activity were pooled and analysed by SDS/PAGE (Laemmli, 1970). A single band of M_r 80000 accounted for 80–85 % of the protein present, with 10–20 weak slower- and faster-moving bands. To purify the amine oxidase further, the peak fractions from the phenyl-Superose column were diluted four times with 20 mM-Tris/HCl buffer, pH 8.0, and applied to a MonoQ column equilibrated with 20 mM-Tris/HCl buffer, pH 8.0. A 15 ml gradient of 0–1 M-NaCl was applied and the amine oxidase eluted at approx. 0.75 M-NaCl. SDS/PAGE analysis of the fraction with the highest amine oxidase specific activity indicated that it was at least 90 % pure, with 10 or more weak bands accounting for the remainder of the protein. A summary of the purification procedure is given in Table 1.

Spectral characterization of the enzyme

All glassware was acid-washed to remove extraneous metal ions. Enzyme was dialysed (two changes of 2 litres of Chelex-treated 0.1 M-potassium phosphate buffer, pH 7.2) to remove any extraneously bound copper ions, then concentrated to approx. 0.2 ml using a Micro-Pro apparatus (Bio-Molecular Dynamics, Beaverton, OR, U.S.A.). The sample was transferred to a quartz e.s.r. tube and the spectrum recorded at liquid nitrogen temperature (insert Dewar) on a Bruker 220D SRC 9 GHz spectrometer interfaced to an IBM PS/2 data acquisition system. The e.s.r. operating conditions are given in the legend to Fig. 1. The e.s.r. spectrum of a cupric ion standard (0.1 mM-copper perchlorate) was run under identical conditions and the cupric ion content of the enzyme sample determined by comparison of integrated intensities.

The sample from the e.s.r. study was transferred to a 0.1 ml capacity, 1.0 cm-light-path spectrophotometer cell and the visible spectrum recorded (300–600 nm) on a Cary 219 spectrophotometer interfaced to an IBM PS/2 data acquisition system.

The concentrated enzyme stock (approx. 5 mg/ml; 90 μl) was reacted with 9 μl of a fresh solution of 1.5 mM-phenylhydrazine hydrochloride (recrystallized from ethanol), which corresponds to a 2-fold excess of reagent over the protein monomer (M_r 80000). The visible absorbance spectrum of this derivative was recorded, as was the resonance Raman spectrum, according to procedures described elsewhere (Brown *et al.*, 1991). The absorbance spectrum of the phenylhydrazine-derivatized enzyme (results not shown) is typical of TOPA-quinone-containing enzymes (Janes & Klinman 1991), with a λ_{max} of 435 nm.

Fig. 1. E.s.r. spectrum (9 GHz) of *E. coli* K-12 amine oxidase at 77 K

The protein concentration was approx. 5 mg/ml. Spectrometer settings: field centred at 0.3 T, scan 0.15 T; modulation amplitude, 2×10^{-3} T; scan time, 50 s; microwave frequency, 9.421 GHz; 20 mW microwave power.

RESULTS AND DISCUSSION

Much effort has been expended in optimizing the purification procedure for bovine serum amine oxidase to produce high-specific-activity enzyme for a variety of studies (Janes & Klinman, 1991). The purification procedure described here for the *E. coli* K-12 enzyme can be carried out in 5 days or less, and yields enzyme with a specific activity for 2-phenylethylamine oxidation more than 10 times greater than the best reported for the bovine serum enzyme with its optimal substrate, benzylamine. This purified enzyme should therefore be useful for investigating a number of ambiguities that still exist in relation to reactions of the enzyme, as well as the investigations reported here concerning the nature of its cofactors.

The e.s.r. spectrum (Fig. 1) of the sample indicates Cu(II) in a site of near axial symmetry and is typical of other copper-containing amine oxidases. The cupric ion concentration of the sample was calculated to be 30 μM .

Fig. 2. Visible absorption spectrum of *E. coli* K-12 amine oxidase

The protein concentration was approx. 5 mg/ml.

Fig. 3. Resonance Raman spectra from the phenylhydrazine derivatives of (a) *Arthrobacter P1* amine oxidase, (b) *E. coli* K-12 amine oxidase and (c) 3,4,6-trihydroxyphenylalanine quinone (TOPA)

The excitation wavelength was 457.9 nm at a laser power of 30 mW.

The visible absorbance spectrum of *E. coli* K-12 amine oxidase (Fig. 2) is similar to the absorption spectrum of the copper-containing methylamine oxidase from *Arthrobacter P1* (Dooley *et al.*, 1990) and clearly lacks the intense absorbance bands at 370–360 nm and 460–470 nm which are characteristic of bacterial flavin-containing amine oxidases (Yamada, 1971; Yamada *et al.*, 1971). Using the millimolar absorption coefficient for the TOPA absorption band at 480 nm in the native *Arthrobacter P1* enzyme of $4.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Dooley *et al.*, 1990), the TOPA concentration in the *E. coli* enzyme sample was found to be $23 \mu\text{M}$.

Fig. 3(a) shows the resonance Raman spectrum of the phenylhydrazine derivative of *E. coli* K-12 amine oxidase. The spectrum is virtually identical with those from the phenylhydrazine derivatives of *Arthrobacter P1* amine oxidase (Fig. 3b) and of TOPA quinone (Fig. 3c). It has been fully established (Brown *et al.*, 1991) that resonance Raman spectroscopy is a reliable monitor for the presence of TOPA quinone in amine oxidases, and thus it can be concluded that TOPA quinone is indeed the organic cofactor in *E. coli* K-12 amine oxidase.

The identity of the cofactors in *E. coli* K-12 phenylethylamine oxidase is established by the present results. Accordingly this enzyme should be classified with other copper/TOPA quinone enzymes as EC 1.4.3.6. The results further indicate that the TOPA/Cu(II) ratio is approx. 1. The calculated monomer concentration for the protein samples corresponding to Figs. 1 and 2 was approx. $20 \mu\text{M}$, as determined by the Bradford assay (Bradford, 1976). Thus the TOPA/monomer ratio is also approx. 1, but further work will be necessary to establish this point with confidence. In fact, the stoichiometry of cofactors/protein remains a controversial point for the amine oxidases (Janes & Klinman, 1991; Morpurgo *et al.*, 1992; McIntire & Hartmann, 1992).

The presence of TOPA quinone as the organic cofactor of the *E. coli* amine oxidase raises the question of how it is formed. The present view for bovine serum and yeast amine oxidases is that a post-translational modification of a tyrosine residue is involved (Mu *et al.*, 1992). The translated nucleotide sequence of the *E.*

coli amine oxidase gene includes the sequence Asn-Tyr-Asp-Tyr (R. A. Cooper, unpublished work) which can be compared with the consensus sequence Asn-TOPA-Asp/Glu in other amine oxidases (Klinman *et al.*, 1992). For example, the amino-acid sequences around the TOPA quinone are Asn-TOPA-Asp-Tyr for bovine serum amine oxidase and Asn-TOPA-Glu-Tyr for the yeast amine oxidase. Collectively, the results strongly suggest that for *E. coli* K-12 amine oxidase also, TOPA quinone is produced by a post-translational modification of tyrosine.

In summary, this is the first report of a Cu(II)/TOPA-quinone-containing amine oxidase in a Gram-negative bacterium, indicating that this class of amine oxidase is ubiquitous in nature.

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REFERENCES

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brown, D. E., McGuirl, M. A., Dooley, D. M., Janes, S. M., Mu, D. & Klinman, J. P. (1991) *J. Biol. Chem.* **266**, 4049–4051
- Collison, D., Knowles, P. F., Mabbs, F. E., Ruis, F. X., Singh, I., Dooley, D. M., Cote, C. E. & McGuirl, M. A. (1989) *Biochem. J.* **264**, 663–669
- Dooley, D. M., McIntire, W. S., McGuirl, M. A., Cote, C. E. & Bates, J. L. (1990) *J. Am. Chem. Soc.* **112**, 2782–2789
- Duine, J. A. (1991) *Eur. J. Biochem.* **200**, 271–284
- Janes, S. M. & Klinman, J. P. (1991) *Biochemistry* **30**, 4599–4605
- Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kauer, S., Maltby, D., Burlingame, A. L. & Klinman, J. P. (1990) *Science* **248**, 981–987
- Klinman, J. P., Palcic, M., Smith, A. J., Brown, D. E., Dooley, D. M. & Janes, S. M. (1992) *Biochemistry*, in the press
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- McIntire, W. S. & Hartmann, C. (1992) in *Principles and Applications of Quinoproteins* (Davison, V. L., ed.), Marcel Dekker, New York, in the press

- Morpurgo, L., Agostinelli, E., Mondovi, B., Avigliano, L., Silvestri, R., Stefancich, G. & Artico, M. (1992) *Biochemistry* **31**, 2615–2621
- Mu, D., Janes, S. M., Smith, A. J., Brown, D. E., Dooley, D. M. & Klinman, J. P. (1992) *J. Biol. Chem.* **267**, 7979–7982
- Parrott, S., Jones, S. & Cooper, R. A. (1987) *J. Gen. Microbiol.* **133**, 347–351
- Rassoulzadegan, M., Binetruy, B. & Cuzin, F. (1982) *Nature (London)* **295**, 257–259
- Warburg, O. & Christian, W. (1941) *Biochem. Zeit.* **310**, 384–421
- Yamada, H. (1971) *Methods Enzymol.* **17B**, 726–750
- Yamada, H., Kumagai, H. & Uwajima, T. (1971) *Methods Enzymol.* **17B**, 722–726

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