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Cloning of the *maoA* gene that encodes aromatic amine oxidase of *Escherichia coli* W3350 and characterization of the overexpressed enzyme

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The *mao* operon of *Escherichia coli* W3350, which comprises the genes *maoC* and *maoA*, was cloned and appeared to be similar to that of *Klebsiella aerogenes* [Sugino, H., Sasaki, M., Azakami, H., Yamashita, M. & Murooka, Y. (1992) *J. Bacteriol. 174*, 2485–2492]. The gene that encodes aromatic amine oxidase (*maoA*) was isolated, sequenced, and expressed in *E. coli* TG2.

The purified enzyme exhibited properties characteristic of a copper/topaquinone(TPQ)-containing amine oxidase with respect to the optical absorption and EPR spectra, the size of the subunits, and the optical absorption spectra obtained upon derivatization with hydrazines. However, high-resolution anion-exchange chromatography revealed that the preparation was heterogeneous. The enzyme preparation appeared to consist of at least four enzyme species with different specific activities, $A_{474\,nm}/A_{340\,nm}$ ratios and TPQ/subunit ratios. Since the overall properties of the overexpressed enzyme and the authentic enzyme were similar and the separated enzyme species had identical N-terminal amino acid sequences, the heterogeneity does not seem to be caused by improper expression of the gene in the recombinant strain but by factors that interfere with the processing of the specific tyrosine in the precursor enzyme to functional TPQ. Although other causes cannot be excluded, the spectral data and TPQ/subunit ratios reported in the literature for other amine oxidases suggest that suboptimal synthesis of functional TPQ also occurs in other organisms.

Keywords: amine oxidase; overexpression; enzyme characterization; topaquinone.

Copper/topaquinone(TPQ)-containing amine oxidase has a wide distribution among eukaryotic and prokaryotic organisms. It converts primary amines into the corresponding aldehydes, ammonia and hydrogen peroxide. Similarity exists among the members of this enzyme group with respect to the size of the protein (approximately 180 kDa), the number of subunits (two), and the copper content (one/subunit) (reviewed by McIntire and Hartmann, 1993; Klinman and Mu, 1994). Unresolved questions concern the TPQ/enzyme ratio [one (Morpurgo et al., 1992) or two (Janes and Klinman, 1991)], the formation of TPQ [which probably proceeds autocatalytically when copper is inserted into the proenzyme (Matsuzaki et al., 1994, 1995)], and the topology of the inorganic and organic cofactors (Greenaway et al., 1991; Turowski et al., 1993; Bossa et al., 1994). No three-dimensional structure of amine oxidase is available. Since microorganisms can be manipulated rather easily, their amine oxidases are useful for studies of these matters.

The first quinoprotein amine oxidase purified from a microorganism was that from *Aspergillus niger* (Yamada et al., 1965). The first bacterial one was methylamine oxidase from the gram-positive bacterium *Arthrobacter* P1 (van Iersel et al., 1986). Aromatic and aliphatic amine oxidases, probably of the quinoprotein type, have been detected in yeasts, and aromatic and aliphatic enzymes have been found in the same strains (Haywood and Large, 1981; Bruinenberg et al., 1989; Mu et al., 1992). Subsequently, amine oxidases have been isolated from the gram-negative bacterium *Escherichia coli* (Cooper et al., 1992) and the gram-positive bacterium *Arthrobacter globiformis* (Shimizu et al., 1990). Genes that encode amine oxidases of *Klebsiella aerogenes* (Sugino et al., 1992), *Arthrobacter* P1 (Zhang et al., 1993), *A. globiformis* (Tanizawa et al., 1994), *E. coli* (Azakami et al., 1994; Hanlon et al., 1995) and *Hansenula polymorpha* (Cai and Klinman, 1994) have been cloned, sequenced and expressed. However, in some studies (Roh et al., 1994; Hanlon et al., 1995) the purified overexpressed enzyme had lower specific activity than authentic enzyme, which suggests that the recombinant strains were less competent at expression of the structural gene or processing of the proenzyme.

To investigate an amine oxidase of which the synthesis could be manipulated during overexpression, we chose the enzyme from the genetically easily accessible organism *E. coli*. Short reports on the purification and partial characterization of this enzyme (Cooper et al., 1992) and on the cloning and expression of the structural gene (*maoA*) (Azakami et al., 1994; Roh et al., 1994; Hanlon et al., 1995) have been published. Here we report the cloning of the *mao* operon, the overexpression of the *maoA* gene, and the high-resolution purification and characterization of the overexpressed enzyme. Direct resonance Raman spectros-

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Abbreviations. ECAO, *Escherichia coli* amine oxidase; TPQ, topaquinone (6-hydroxydopaquinone).

Enzymes. DNA restriction endonucleases, *Bam*H1, *Bgl*II, *Hin*dIII, *Eco*R1, *Pst*I, *Cla*I, *Hin*cII, *Sau*3A, *Kpn*I, *Acc*I (EC 3.1.21.4); DNA polymerase I (EC 2.7.7.7); polynucleotide 5′-hydroxyl-kinase (EC 2.7.1.78); alkaline phosphatase (EC 3.1.3.1); DNA ligase (ATP) (EC 6.5.1.1); amine oxidase (EC 1.4.3.6); ribonucleotide reductase (EC 1.17.4.1).

copy of underivatized enzyme (Moënne-Loccoz et al., 1995), EPR spectroscopy of ^{63}Cu-substituted and ^{65}Cu-substituted enzyme, ^{19}F-NMR spectroscopy of enzyme derivatized with F-substituted hydrazines, and identification of TPQ in the enzyme (Steinebach et al., 1995) will be described elsewhere.

MATERIALS AND METHODS

Growth media, bacterial strains and plasmids. The media used were Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 50 μM $CuCl_2$, 0.5% NaCl) and mineral medium (medium A; 60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 250 μM $MgSO_4$, pH 7.0). Media were prepared with demineralized water. Media were solidified with 1.5% Bactoagar.

E. coli TG2 *supE hsdΔ5 thi Δ(lac-proAB) Δ(srb-recA)*306::Tn*10* F′[*traΔ*36 *proAB*$^+$ *lacI*^{q} *lacZΔ*M15] (Maniatis et al., 1989) was used as a host for cloning and expression. *E. coli* CAG12026 [*trg*-2::Tn*10*] and *E. coli* CAG12081 [*zcj-3061*::Tn*10*] were used for expression and were kindly provided by C. A. Gross. *E. coli* PPA207 [*sup0 lac gal pea*$^+$], a mutant that grows better on 2-phenylethylamine, was derived from *E. coli* W3350 [*sup0 lac gal*] by random mutagenesis with diethyl sulfate, as follows. A fully grown culture of *E. coli* W3350 in Luria-Bertani medium was diluted 100-fold in medium A and 100 μl was spread on solidified medium A with 0.1% (mass/vol.) 2-phenylethylamine as a sole source of carbon and energy. A small paper filter moistened with a drop of diethyl sulfate was placed in the middle of the plate. After several days of incubation at 37 °C, a few colonies appeared at some distance from the filter, one of which was designated as PPA207.

Vectors used were pUC18 and M13mp19 (Yannish-Perron et al., 1985), pJF119EH (Fürste et al., 1986) and pBlueScript II (Stratagene Inc.).

General materials. Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase and *E. coli* DNA polymerase I (Klenow fragment) were from Pharmacia. The Sequenase version II sequence kit was obtained from USB. Calf intestinal phosphatase and blocking reagent were purchased from Boehringer. [α-^{32}P]dATP and [γ-^{32}P]dATP (3000 Ci/mmol; 11.1 TBq/mmol) were from New England Nuclear. Genescreen Plus was obtained from Du Pont. GeneClean was purchased from Bio101. Goat anti-rabbit IgG conjugated to alkaline phosphatase was obtained from BioRad. Antiserum against the purified amine oxidase was raised in a New Zealand White rabbit. All enzymes and materials were used according to the manufacturers' recommendations.

Mops, phenylhydrazine/HCl, 4-nitrophenylhydrazine/HCl, and 2-phenylethylamine/HCl were from Fluka. PD10, DEAE-Sepharose FastFlow, phenyl-Sepharose FastFlow, FPLC MonoQ HR 5/5 and FPLC Superose 12 HR 10/30 columns were from Pharmacia. All reagents were of the highest purity commercially available and were used without further purification. All solutions were prepared with demineralized water, further deionized by passage through a Waters Milli-Q system to a resistance greater than 17 mΩ/cm.

General methods. Amine oxidase from *E. coli* K12 (ECAO) was induced and purified according to Cooper et al. (1992). Protein sequence analysis was performed by Edman degradation with an automated sequenator (Model 477A, Applied Biosystems), equipped with an on-line phenylthiohydantoin analyzer (Model 120A, Applied Biosystems).

Methods for DNA manipulation were essentially as described by Maniatis et al. (1989). SDS/PAGE (12%) and native homogeneous PAGE (9%) was performed with a Bio Rad Mini-Protean II. Native gradient (from 10% to 15%) PAGE was performed with the Pharmacia Phastsystem equipment. Pharmacia low-molecular-mass and high-molecular-mass markers were used for calibration in molecular-mass determinations. Gels were stained with Coomassie brilliant blue G-250.

Protein content was quantitated by the method of Bradford (1976) with desalted bovine serum as a standard. Amine oxidase solutions were concentrated with Filtron Microsep (7000×*g*) or Macrosep (at 5000×*g*) centrifugal concentrators equipped with 30-kDa cut-off membranes.

For semi-preparative HPLC a Hitachi L-6210 HPLC pump was used. The eluate was monitored with a Hewlett Packard HP1040 II photodiode array detector.

Cloning of the gene that encodes aromatic amine oxidase (*maoA*). Chromosomal DNA was isolated from *E. coli* PPA207 and digested with several restriction endonucleases. Digested DNA was separated on a 0.8% agarose gel in 0.04 M Tris/acetate, pH 8, 1 mM EDTA (buffer A) and transferred to a Genescreen Plus membrane by means of vacuum blotting. The blot was prehybridized in 6×NaCl/Cit (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 0.1% SDS and 1% blocking reagent at 65 °C. A 23-nucleotide probe was used, whose sequence was based on the sequence of the N-terminus of the purified enzyme from *E. coli* K12 (amino acid sequence, Asp-Lys-Thr-Leu-Lys-Glu-Phe-Gly; nucleotide sequence, $GA^T_CAA^A_GACIITICA^G_ATT^T_CGC$). The probe was labelled with T4 polynucleotide kinase and [γ-^{32}P]dATP. Hybridization was carried out at 65 °C for 16 h. The filter was washed twice with 6×NaCl/Cit, 0.1% SDS at room temperature for 0.5 h. The autoradiograph showed signals at 6 kb after digestion with *Bgl*II and at 0.8 kb after digestion with *Hin*dIII (data not shown). Chromosomal DNA was digested with *Bgl*II. After separation of the fragments on a 0.8% agarose gel in buffer A, 5–7-kb fragments were excised from the gel and purified with GeneClean followed by ligation of the fragments into *Bam*H1-digested pJF119EH. The resulting plasmids were used to transform *E. coli* TG2. Positive clones were identified by colony/filter hybridization with the labelled oligonucleotide as a probe. One such clone, pBCP468, was used for the isolation of DNA, which was digested with *Hin*dIII. After separation of the fragments by gel electrophoresis and transfer to a membrane followed by screening with the labelled oligonucleotide, plasmid pBCP468 was shown to contain the 0.8-kb positive fragment. The 0.8-kb *Hin*dIII fragment was isolated and ligated into the *Hin*dIII site of M13mp19 and sequenced from both directions by means of the Sequenase kit. In a similar way, a 7-kb *Eco*R1–*Pst*I fragment was cloned into pUC18 digested with *Eco*R1/*Pst*I and the 0.8-kb *Hin*dIII fragment (labelled by treatment of the recessed ends with Klenow polymerase, dTTP, dGTP, dCTP and [γ-^{32}P]dATP) was used to identify positive clones, which yielded plasmid pBCP467. pBCP467 was purified and used for sequence determination of the *maoA* gene by the dideoxynucleotide chain-termination method (Sanger et al., 1977) with sequence-specific primers.

Expression of the *maoA* gene. In *E. coli* TG2 that harboured plasmid pBCP467, the *maoA* gene was expressed during growth in Luria-Bertani medium containing 100 μg/ml ampicillin or on medium A supplemented with 20 mg/l thiamin, 0.2% (mass/vol.) glucose, 0.4% (mass/vol.) amino acids (acid hydrolysate of casein), 50 μM $CuCl_2$, 100 μg/ml ampicillin. 100 μM isopropyl-thio-β-D-galactopyranoside was included in the media. Routinely, *E. coli* TG2 cells were freshly transformed with plasmid pBCP467 to produce the amine oxidase. 10-ml cultures of supplemented medium A or Luria-Bertani medium containing 100 μg/ml ampicillin were inocculated with a transformant and allowed to grow at 37 °C for 7 h. Cells were harvested by centrifugation, washed once to remove β-lactamase and used to inoculate 500 ml medium (Luria-Bertani or medium A) supple-

mented with 100 μM isopropyl-thio-β-D-galactopyranoside in 2-l flasks. Cultures were grown overnight at 37 °C with vigorous shaking.

Enzyme assay. Enzyme activity was routinely determined with a biological-oxygen monitor at 37 °C. The assay buffer (1.6 ml) contained 0.1 M potassium phosphate, pH 7.0, ECAO (greater than 1 μg/ml), catalase (2 μg/ml) and 2-phenylethylamine (10 mM). Specific activities were calculated based on an initial oxygen concentration of 0.199 mM.

Enzyme purification. All purification steps, except for MonoQ chromatography, were carried out at 4 °C. Cells were harvested, washed once with 10 mM Tris/HCl, pH 8.0, and disrupted by passage of the mixture twice through a cooled French pressure cell at 110 MPa. The suspension was centrifuged for 30 min at 28000×*g* to produce the cell-free extract.

The cell-free extract (100 ml) was applied to a DEAE-Sepharose FastFlow column (2.5 cm×25 cm) equilibrated with 5 mM potassium phosphate, pH 7.5 (buffer B). After washing the column with buffer B, proteins were eluted with a linear gradient from 0 to 1 M NaCl in buffer A over 60 min at a flow rate of 2.0 ml/min. The fractions that contained enzyme activity were pooled and concentrated.

The concentrate was adjusted to 1.5 M ammonium sulfate by addition of powdered ammonium sulfate and applied to a phenyl-Sepharose FastFlow column (2.5 cm×7 cm) equilibrated with 1.6 M ammonium sulfate, 15 mM potassium phosphate, pH 6.6 (buffer C). After washing the column with buffer C, proteins were eluted with a linear gradient from buffer C to buffer B over 60 min at a flow rate of 2.0 ml/min. The active fractions were pooled and concentrated. The buffer was exchanged by gel filtration on a PD10 column equilibrated with buffer B. The enzyme was further purified in six portions on a MonoQ 5/5 column equilibrated with buffer B. After application of the sample and washing of the column with buffer A, proteins were eluted with a linear gradient from buffer A to 0.15 M potassium phosphate, pH 7.0, over 20 min at a flow rate of 0.5 ml/min. Enzyme fractions 1, 2, 3 and 4 (Fig. 2A) were manually collected and stored at −80 °C.

Titration of ECAO with phenylhydrazines. Phenylhydrazine/HCl and 4-nitrophenylhydrazine/HCl solutions (approximately 10 mM in methanol) were prepared daily and kept at −20 °C in the dark. The phenylhydrazine and 4-nitrophenylhydrazine solutions were diluted 10−50-fold with water before titration. ECAO (in 10 mM Mops, pH 7.0, 20 mM NaCl) was titrated with small aliquots (1−10 μl) of phenylhydrazine or 4-nitrophenylhydrazine. The course of the titration was monitored by means of ultraviolet/visible spectra recorded with a HP8452A photodiode array spectrophotometer. Complete titration was determined independently by activity measurements. The molar absorption coefficients of the adducts in the enzyme were determined by application of a linear line-fitting routine (provided by the program Igor Pro) to the titration data obtained.

EPR spectroscopy. ECAO preparations (in 50 mM potassium phosphate, pH 7.0) were concentrated to approximately 60 mg/ml. Approximately 200 μl of these concentrated enzyme solutions were transferred to quartz EPR tubes and the spectra were recorded at 60 K on a Varian E9 EPR spectrometer at 9.23 GHz.

RESULTS

Cloning and expression of *maoA*. *E. coli* W3350 grows very poorly on 2-phenylethylamine. Therefore a faster-growing variant strain, PPA207, was selected to obtain a higher cell yield. The cells probably express the amine oxidase enzyme (or other enzymes in the degradation pathway) at an increased level due to a promoter mutation. *maoA* was cloned from *E. coli* PPA207. As a probe we used an oligonucleotide based on the N-terminal amino acid sequence of the aromatic amine oxidase purified from an *E. coli* K12 strain. A Southern blot of several digests of PPA207 chromosomal DNA revealed that hybridisation occurred either with large fragments (greater than 10 kb; *Eco*R1, *Pst*I, *Bam*H1, *Acc*I) or small fragments (less than 1 kb; *Hinc*II, *Hin*dIII). Digestion with *Bgl*II and *Kpn*I yielded fragments of 6 kb and 8 kb, respectively. Cloning the *Kpn*I fragment was unsuccessful but we cloned the *Bgl*II fragment, which yielded plasmid pBCP468 (Fig. 1A).

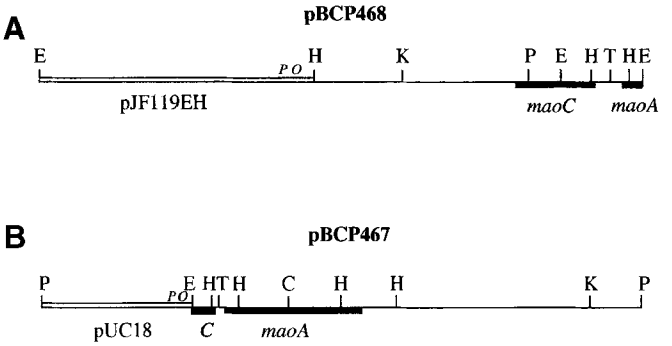

Fig. 1. Schematic representation of plasmids pBCP468 and pBCP467. (A), a 6-kb *Bgl*II fragment in *Bam*H1-digested pJF119EH; (B), a 7-kb *Eco*R1−*Pst*I fragment in *Eco*R1/*Pst*I-digested pUC18. Thick lines indicate coding regions. *P* and *O* indicate the promoter and operator of the *lacZ* α-peptide coding region, respectively. C, *Cla*I; E, *Eco*R1; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I, T, *Tth*III.

Table 1. Purification of *E. coli* amine oxidase.

Purification step	Specific activity	Total protein	Total activity	Yield	Purification factor
	U/mg	mg	U	%	-fold
Cell-free extract	0.59	663	391	100	1
DEAE-Sepharose	2.06	180	371	95	3.5
Phenyl-Sepharose	4.84	70	339	87	8.2
MonoQ 5/5	6.98[a]	24	167	42	11.8

[a] Species B.

A 0.8-kb *Hin*dIII fragment was subcloned from plasmid pBCP468 in phage M13mp19 and sequenced. Comparison of its translated sequence with the amino acid sequence of the N-terminus of the purified enzyme and the published sequence of the *mao* operon from *K. aerogenes* (Sugino et al., 1992) showed that the cloned *Hin*dIII fragment coded for the last 20 amino acids of the C-terminal part of *maoC* (whose function is unknown), the intergenic region between *maoC* and *maoA*, and the N-terminal part of *maoA*. Further restriction-enzyme analysis revealed that the 6-kb *Bgl*II fragment of pBCP468 contained the upstream region of the *mao* operon, the complete *maoC* gene, but only 0.4 kb of the *maoA* gene.

Screening of blots of doubly digested *E. coli* PPA207 chromosomal DNA with the 0.8-kb *Hin*dIII fragment from plasmid pBCP468 as a probe identified a 7-kb *Eco*R1−*Pst*I fragment that could contain the complete *maoA* gene. Four identical clones were obtained with the desired insert, which yielded plasmid pBCP467 (Fig. 1B). The inserts contained part of the *maoC* gene, the entire *maoA* gene and 4 kb of downstream sequence. Sequencing of the *Hin*dIII fragment, isolated from plasmid pBCP467, demonstrated that the plasmid contained the *maoA*

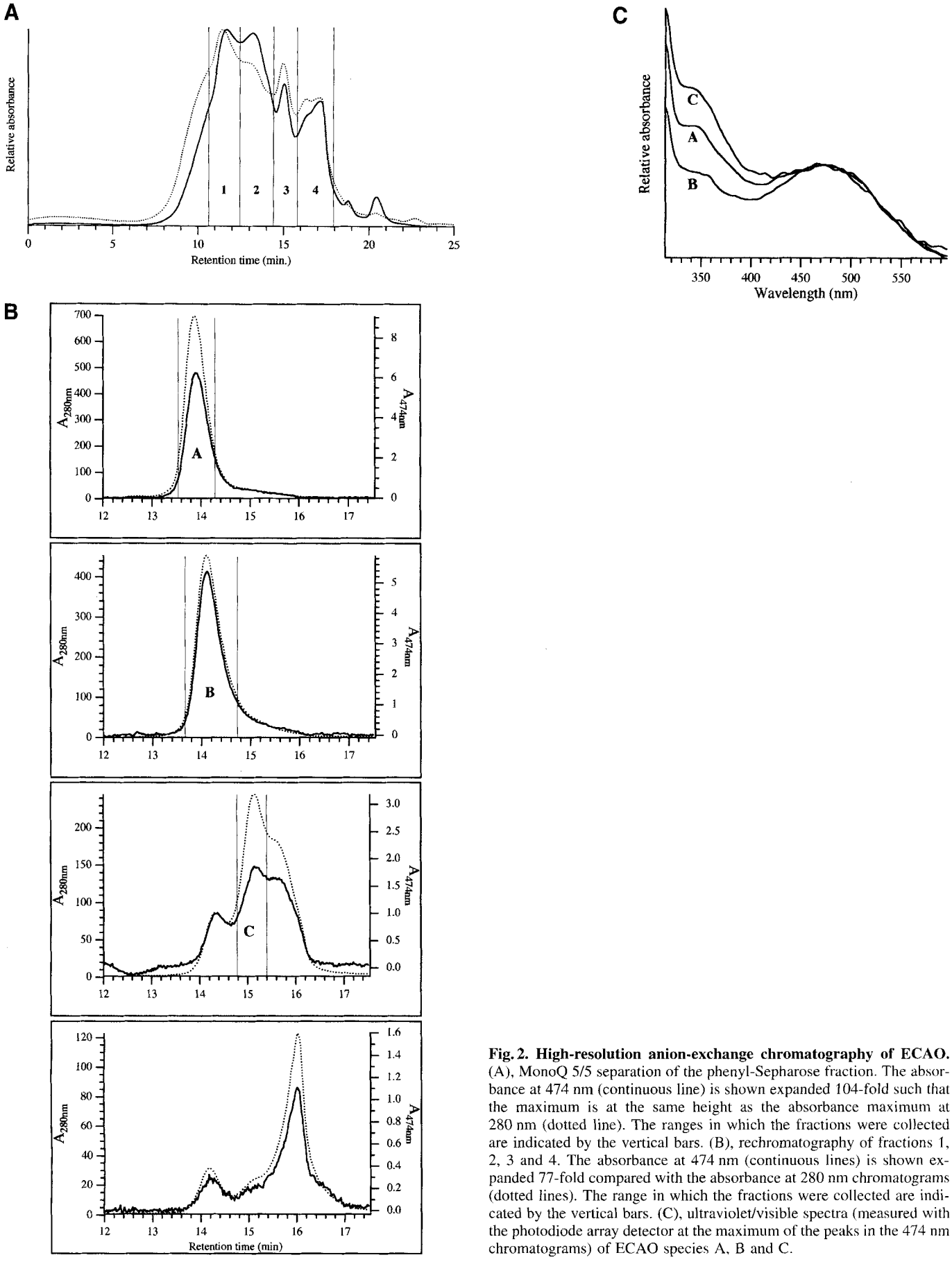

Fig. 2. High-resolution anion-exchange chromatography of ECAO. (A), MonoQ 5/5 separation of the phenyl-Sepharose fraction. The absorbance at 474 nm (continuous line) is shown expanded 104-fold such that the maximum is at the same height as the absorbance maximum at 280 nm (dotted line). The ranges in which the fractions were collected are indicated by the vertical bars. (B), rechromatography of fractions 1, 2, 3 and 4. The absorbance at 474 nm (continuous lines) is shown expanded 77-fold compared with the absorbance at 280 nm chromatograms (dotted lines). The range in which the fractions were collected are indicated by the vertical bars. (C), ultraviolet/visible spectra (measured with the photodiode array detector at the maximum of the peaks in the 474 nm chromatograms) of ECAO species A, B and C.

gene. Based on restriction-enzyme analysis, a map was constructed which indicated that the complete *maoA* gene should be located on three *Hin*dIII fragments. Subcloning of these fragments into M13mp19 and partial sequencing of the inserts from both orientations demonstrated this. The complete *maoA* sequence was determined by means of pBCP467 and specific oligonucleotide sequence primers designed on the basis of sequence data already obtained. Comparison of the nucleotide-sequence-derived amino acid sequence published by Azakami et al. (1994) revealed amino acid substitutions at six positions (the histidine residue of the mature enzyme was regarded as position 1): Glu218→Lys; Val228→Gly; Ile229→Tyr; Ile260→Pro; Pro426→Ala; and Asp629→His. The isoleucine residue at position 246 was absent and an alanine residue was found at residue 258 (between tryptophan and histidine residues).

When *E. coli* TG2 (pBCP467) was grown in Luria-Bertani medium, expression of *maoA* was constitutive and high (590 mU/mg protein in cells that harboured pBCP467 versus 0 mU/mg protein in cells that harboured pUC18). SDS/PAGE confirmed this (data not shown) because protein staining revealed a band at 80 kDa (which migrated with the purified amine oxidase) only in cells that harboured pBCP467. The expression of *maoA* by *E. coli* TG2 (pBCP467) is approximately 29-fold higher than in the wild-type strain (Cooper et al., 1992).

Inspection of the sequence of the *K. aerogenes mao* operon (Sugino et al., 1992) and our sequence data of the *E. coli mao* operon did not reveal the presence of a promoter. Expression of the *E. coli maoA* gene must, therefore, depend on the promoter of the plasmid, i.e., the *lac* promoter of pUC18. This was confirmed by the finding that when the insert was cloned in the opposite direction in pUC19, the *maoA* gene was not expressed, either in a constitutive way or after addition of isopropyl-thio-β-D-galactopyranoside. The finding that the expression was constitutive is probably due to leakiness of the *lac* promoter in *E. coli* TG2. To ensure high expression of the *maoA* gene, 100 μM isopropyl-thio-β-D-galactopyranoside was included in the growth media.

Enzyme purification and characterization. ECAO was purified from cell-free extracts of strain TG2 (pBCP467), grown in Luria-Bertani medium, with a yield of 42% (Table 1). The enzyme appeared (data not shown) to prefer aromatic amines (2-phenylethylamine, tyramine, tryptamine) to aliphatic amines (methylamine, putrescine), as has been found by others (Roh et al., 1994). Therefore, the enzyme is an aromatic amine oxidase. The preparation after the phenyl-Sepharose purification step was homogeneous, as judged by SDS/PAGE and native gradient PAGE, which showed one protein band on each gel with relative molecular masses of 77 kDa and 174 kDa, respectively. Anion-exchange chromatography (MonoQ 5/5 column) of this preparation revealed, however, that it was not homogeneous (Fig. 2A), and (overlapping) peaks with different A_{280}/A_{474} ratios were obtained. Fractions 1–4, which corresponded to the main peaks, were collected as indicated. To verify whether the collected fractions contained stable entities, rechromatography was carried out. This showed that chromatographic (Fig. 2B) and spectral (Fig. 2A,C) behaviour was rather constant, which suggests that the chromatographic fractions contain distinct enzyme species. For this reason, and to distinguish them from fractions 1–4, the fractions collected after rechromatography (Fig. 2B) were named ECAO species A–D (the latter fraction was not collected because the amount of TPQ was too low). The enzyme species differ in chromatographic, enzymatic and spectral properties (Table 2; Fig. 2C). High activity appears to correspond to high A_{474}/A_{340} and TPQ/subunit ratios. Since species B (termed ECAO-B hereafter) was optimal in these respects, it was used for further investigations. Its $A_{280}^{0.1\%}$ value was determined (van Iersel et al., 1985) to be 1.67. Based on this value and an estimated molecular mass of 160 kDa, the molar absorption coefficient of this enzyme species at 474 nm was calculated as 3100 $M^{-1} \cdot cm^{-1}$.

Table 2. Characteristics of the *E. coli* amine oxidase species. Specific activities, TPQ/subunit ratios (based on phenylhydrazine titrations) and A_{474}/A_{340} ratios were determined for the rechromatographed fractions (Fig. 2B).

ECAO species	Specific activity	TPQ/subunit	A_{474}/A_{340}
	U/mg		
A	4.18	0.35	0.74
B	6.98	0.5	1.03
C	2.42	0.2	0.68

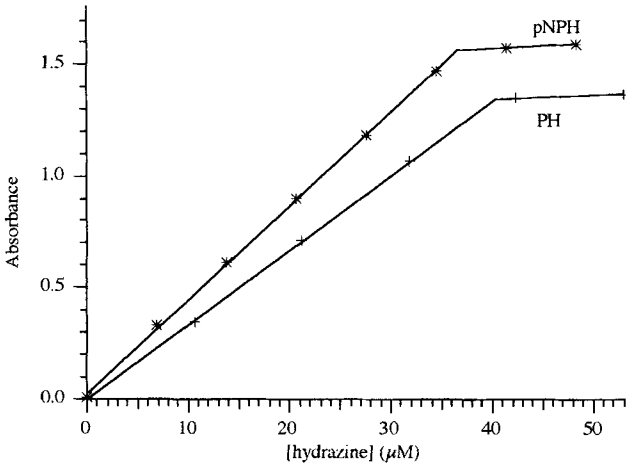

Fig. 4. Titration curves for ECAO-B with hydrazines. 35 μM ECAO-B (in 10 mM Mops, pH 7.0, 20 mM NaCl) was titrated with phenylhydrazine and 4-nitrophenylhydrazine. The course of the titration was followed by monitoring the absorbance at the absorption maxima of the reaction products (436 nm for the TPQ-phenylhydrazine and 466 nm for the TPQ-4-nitrophenylhydrazine adduct).

N-terminal amino acid sequencing (five amino acids) of species A, B and C revealed identical sequences that lacked the leader sequence. Therefore, the steps of protein synthesis and transport to the periplasm seem not to be reponsible for the heterogeneity observed.

The absorption spectrum of ECAO-B is similar in shape to that of *E. coli* K12 amine oxidase (Cooper et al., 1992) but has a higher A_{474}/A_{340} ratio (Figs 2C and 3). The specific activity of our enzyme, determined under similar conditions as those for the *E. coli* K12 enzyme (Cooper et al., 1992), was higher [8 U/mg protein versus 5.8 U/mg protein (at 5 mM 2-phenylethylamine)]. Substrate inhibition occurred, such that the optimal 2-phenylethylamine concentration for the assay was approximately 200 μM, which yielded a specific activity of 17.6 U/mg ECAO-B. Due to the sensitivity limit of the biological-oxygen monitor and the high affinity of the enzyme for 2-phenylethylamine, no reliable K_m value could be determined for this substrate (the value should be below 100 μM).

Titration of amine oxidase with phenylhydrazines. The addition of phenylhydrazine and 4-nitrophenylhydrazine to ECAO-B induced a new absorbance maxima at 436 nm and 466 nm, respectively (Fig. 3), with a concomitant disappearance of the

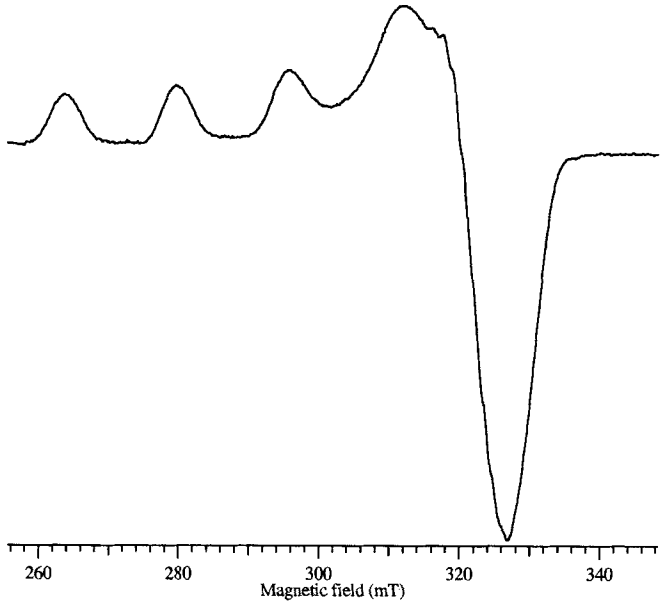

Fig. 5. Low-temperature EPR spectrum of ECAO-B. The EPR spectrum of approximately 200 µl ECAO-B (60 mg/ml in 50 mM potassium phosphate, pH 7.0) was measured at 60 K with the following settings: microwave power, 2 mW; frequency, 9.2316 GHz; modulation amplitude, 1 mT.

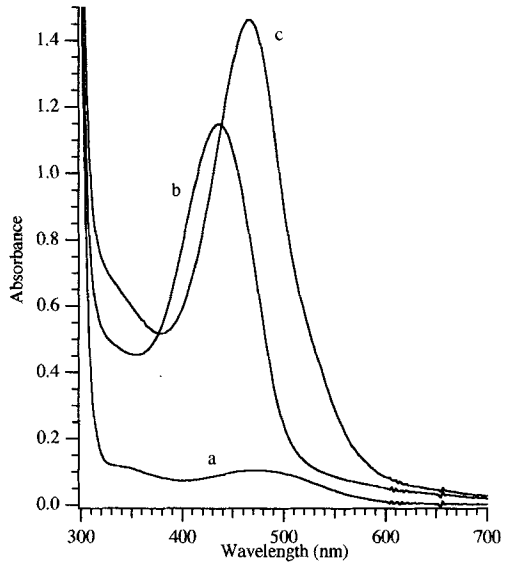

Fig. 3. Absorption spectra of untreated and hydrazine-treated ECAO species B. The absorption spectrum of ECAO-B [5.6 mg/ml (a)] was measured in 5 mM potassium phosphate, pH 7.5. Titration of ECAO-B (5.6 mg/ml in 10 mM Mops, pH 7.0, 20 mM NaCl) to nearly full saturation was carried out with phenylhydrazine (b) and 4-nitrophenylhydrazine (c).

activity. The molar absorption coefficients of the two hydrazine adducts were calculated as 33429 $M^{-1} \cdot cm^{-1}$ for the TPQ-phenylhydrazine product and 42005 $M^{-1} \cdot cm^{-1}$ for the TPQ-4-nitrophenylhydrazine product. The ratios between the amount of hydrazine required for complete inactivation or adduct formation and the amount of amine oxidase subunits were 0.57 and 0.52 for phenylhydrazine and 4-nitrophenylhydrazine, respectively (Fig. 4).

EPR spectroscopy. The EPR spectrum of ECAO-B showed the characteristic spectrum of Cu(II) in a tetragonal structure, with g_x, g_y and g_z values of 2.027, 2.094 and 2.299, respectively (Fig. 5). The Cu site seems to be homogeneous since the EPR spectrum could be interpreted if the presence of one Cu(II) species was assumed (data not shown).

DISCUSSION

To obtain large amounts of ECAO, we set out to clone and overexpress the gene that encoded this enzyme. The amino-acid-sequence-derived DNA probe, though degenerate, yielded unique signals that, after cloning of the corresponding fragments and verification by sequence analysis, appeared to contain the *maoA* gene, as judged from the following results. The deduced N-terminal amino acid sequence of the gene was identical to the N-terminal amino acid sequence of the enzyme. A database search with the complete DNA sequence revealed similarity with the *K. aerogenes* (Sugino et al., 1992) and the *E. coli maoA* genes (Azakami et al., 1994). However, as mentioned in Results, six amino acid substitutions, one deletion and one insertion were found with respect to the published *E. coli maoA* sequence. Whether these changes are relevant to the overall structure or catalytic function of the enzyme is doubtful since the enzyme is very well expressed and not truncated, and has a specific activity that is equal to, or even higher than, those reported previously (Cooper et al., 1992; Roh et al., 1994). It is also unlikely that a second *maoA* gene with sufficient sequence similarity to the one described in this report exists in *E. coli* since Southern blot analysis with the 0.8-kb *Hin*dIII fragment revealed only one copy of the gene (data not shown). SDS/PAGE of cell-free extract from *E. coli* (pBCP467) showed a band that migrated with the purified authentic enzyme. After purification, the enzyme showed the typical physicochemical and enzymatic features of a copper-quinoprotein amine oxidase.

When the pUC18-based plasmid pBCP467, containing the *maoA* gene, was used to transform *E. coli* TG2 cells, expression of the gene appeared to be constitutive and high, even in the absence of isopropyl-thio-β-D-galactopyranoside. By reversal of the insert, it was shown that the expression was directed by the *lac* promoter. High expression of a cloned gene under the direction of the *lac* promoter in *E. coli* TG2 cells in the absence of isopropyl-thio-β-D-galactopyranoside has been reported before (Benen et al., 1989).

Our sequence data demonstrate that in *E. coli* W3350 there is a sequence, upstream of the *maoA* gene, with very high similarity to the reported *maoC* gene of *K. aerogenes* (Sugino et al., 1992). This is in contrast with results obtained by Azakami et al. (1994) who reported that there is no *maoC* gene present upstream of the *maoA* gene in the Kohara clone 5F1 (Kohara et al., 1987), from which they obtained the *maoA* gene, but rather a gene that encodes another amine oxidase. The restriction map of the *mao* operon and its upstream and downstream regions, as obtained for our strain, is identical to the Kohara map at 31.1 min of the *E. coli* chromosome, as compiled by Rudd (1992) in the Ecoseq6 library. It is different from the one reported by Azakami et al. (1994). Therefore we conclude that we have cloned the *maoA* gene of *E. coli* and that it is preceded by the *maoC* gene, as in *K. aerogenes*. Despite the deviation from the published *E. coli* map and genetic organization, the sequence data of the *maoA* gene, as published by Azakami et al. (1994), are nearly identical to the sequence data of the *E. coli maoA* gene obtained here.

The isolated preparations (Fig. 2B, species B) appeared to consist of the expected ECAO for two reasons. Firstly, they showed the characteristics of copper-quinone amine oxidases with respect to the size of the subunits, the absorption spectra

of underivatized and hydrazine-derivatized enzyme (Fig. 3), the EPR spectrum (Fig. 5), and the presence of TPQ and the consensus sequence that surrounds it (Gly-Asn-TPQ-Asp), as shown with electrospray mass spectrometry and ^{1}H-NMR spectroscopy (Steinebach et al., 1995). Secondly, compared with authentic enzyme, they showed similar substrate specificities, chromatographic and electrophoretic behaviours, and N-terminal amino acid sequences.

The preparations were homogeneous with respect to their subunit composition (SDS/PAGE and native gradient PAGE showed only one band) and N-terminal amino acid sequence (only one dominant amino acid was observed in each of the six sequencing steps). Homogeneity also applied to the Cu(II) ions in the protein because the EPR spectrum (Fig. 5) could be fitted assuming the presence of one single type of Cu(II). However, slight heterogeneity was observed upon homogeneous PAGE of *native enzyme*, which revealed one diffuse band. High-resolution anion-exchange chromatography also indicated that the preparations were heterogeneous (Fig. 2A).

Rechromatography of the separately collected fractions under the same conditions showed their persistent chromatographic behaviour and A_{474}/A_{280} ratio (Fig. 2B), which suggests that different enzyme species were present in the preparations. This proposal was confirmed when the properties of the nearly homogeneous fractions, as judged from their peak symmetry (Fig. 2B), were compared (Table 2), which showed differences in specific activity, hydrazine-titratable TPQ (Fig. 4) and peak ratios in the absorption spectrum, as reflected by the A_{474}/A_{340} value. Thus the preparation must consist of at least four distinct enzyme species, the TPQ content of which (as estimated from the A_{474}/A_{340} ratio and the amount of hydrazine required for complete inhibition) correlates with the specific activity. The results suggest that proper expression of the gene (homogeneity in protein size and N-terminal amino acids) but improper processing of the precursor enzyme to holoenzyme occurred, as illustrated by the different TPQ contents of the ECAO species. Whether the organism, the overexpression system or the growth conditions used for the recombinant strain were responsible for the suboptimal synthesis of functional TPQ is unknown.

The specific activity of another overexpressed *E. coli* amine oxidase (Roh et al., 1994) was much lower than that of authentic enzyme. The specific activity of our preparation is the highest value reported for *E. coli* amine oxidase [17.6 U/mg (at 200 μM 2-phenylethylamine) versus 1.2 U/mg protein (at 100 μM tyramine) for overexpressed enzyme (Roh et al., 1994) and 5.6 U/mg protein (at 5 mM 2-phenylethylamine) for authentic enzyme (Cooper et al., 1992)] and the A_{474}/A_{340} ratio is higher than the value derived from the spectrum of authentic enzyme [1.0 versus 0.8 (Cooper et al., 1992)]. Recently, it was shown (Hanlon et al., 1995) that the specific activity of overexpressed *E. coli* amine oxidase varies with the recombinant strain and growth conditions used. Thus, although the activity of the overexpressed enzyme varies, the overexpression system *per se* is not responsible but the processing system present in the recombinant strain and the conditions during overexpression, are. However, is this inherent to *E. coli* or do other organisms exhibit the same inadequacy?

Direct quantification of TPQ in amine oxidases is not possible since the losses that occur during isolation by means of existing methods (Janes et al., 1990; Steinebach et al., 1995) preclude this. It is common, therefore, to estimate the amount in an indirect way by titration of the enzymes with hydrazines. Upon comparison of the TPQ/subunit ratio for a number of amine oxidases (Table 3), it appears that the values are in the range 0.82−0.50. It could be reasoned that the variability derives from analytical problems, e.g. from incorrect protein concentration or molecular mass estimates. However, this seems very unlikely since the values calculated for the molar absorption coefficient of the TPQ-hydrazine adducts in the enzymes (the slope of the plot of absorbance versus amount of added hydrazine; Fig. 4) are very similar (except for one case; Table 3) and the value for the Cu(II)/subunit ratio is nearly always close to one. Thus the data suggest that the values found reflect the presence of substoichiometric amounts of TPQ in the enzymes. There are two possible explanations for these data. Firstly, not all the TPQ present is available for the reaction with hydrazine or the substrate, e.g., since the enzyme has half-of-the-site reactivity (Morpurgo et al., 1992) or some active sites in the preparation are not properly formed, which prevents reaction of TPQ to the common product or keeps it in a state (protonated, reduced) unsuited for reaction. Secondly, TPQ may be absent from some of the active sites due to a defect in the TPQ-formation route. At present, insufficient knowledge exists on amine oxidases to discriminate between the two possibilities. The work presented here shows, however, that the ECAO preparation is composed of enzyme molecules with different activities, which can be separated from each other. This finding eliminates the possibility that the effects are caused by half-of-the-site reactivity.

Table 3. TPQ/subunit ratios and molar absorption coefficients of TPQ-phenylhydrazine adducts in amine oxidases. BSAO, bovine serum amine oxidase; DAO, pig kidney diamine oxidase; ECAO, *E. coli* amine oxidase. TPQ/subunit ratios were determined from the complete-titration experiments (mol hydrazine/mol amine oxidase subunit).

Phenylhydrazine adduct in	ε	TPQ/ subunit	References
	$M^{-1} \cdot cm^{-1}$		
BSAO	41 500	0.53	Morpurgo et al., 1992
BSAO	32 400	0.82	Janes et al., 1991
DAO	31 700	0.74	Steinebach et al., 1995
ECAO species B	33 200	0.5	this work

Since enzyme activity seems to correlate with the A_{474}/A_{340} ratio, it could be reasoned that a precursor in the biosynthesis of TPQ or a non-reactive form of TPQ in the active site, has an absorption band at 340 nm. In the simplest situation, three enzyme species can be imagined: one that contains two TPQ/enzyme molecule; one that contains one TPQ and one product that absorbs at 340 nm; and one that contains no TPQ and two products that absorb at 340 nm. The finding that at least four enzyme species are present and that the TPQ/subunit ratios of the three investigated species deviate from 1, 0.5 and 0, indicate that the situation is more complex than supposed.

The imperfect conversion of a particular tyrosine in the proenzyme into a cofactor, proposed here to be the cause of the heterogeneity of ECAO, is not unique. Expression of the gene for ribonucleotide reductase occurs properly [as judged from identical subunits observed in the three-dimensional structure (Nordlund et al., 1990)] but processing of the specific Tyr in the precursor enzyme to Tyr•, catalyzed by Fe(III), does not, since suboptimal values of the Tyr•/subunit ratio (in the range 0.15−0.30) are found (Ehrenberg and Reichard, 1972).

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