# Identification of the quinone cofactor in a lysyl oxidase from *Pichia pastoris*

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Received 2 October 1996; revised version received 16 October 1996

**Abstract A copper amine oxidase from *Pichia pastoris* is the only known non-mammalian lysyl oxidase [Tur, S.S. and Lerch, K. (1988) FEBS Lett. 238, 74–76]. Recently, the cofactor in mammalian lysyl oxidase has been identified as a novel lysine tyrosylquinone moiety [Wang, S.X., Mure, M., Medzihradszky, K.F., Burlingame, A.L., Brown, D.E., Dooley, D.M., Smith, A.J., Kagan, H.M. and Klinman, J.P. (1996) Science 273, 1078–1084]. In order to identify the cofactor in *P. pastoris* lysyl oxidase, we have isolated the phenylhydrazone-derivative of the active-site peptide. This peptide has the active-site sequence conserved among topa quinone containing amine oxidases. The resonance Raman spectra of the phenylhydrazone derivatives of the enzyme, active-site peptide, and a topa quinone model compound are essentially identical. Collectively, these results establish that *P. pastoris* lysyl oxidase is a topa quinone enzyme.**

*Key words:* Amine oxidase; Lysyl oxidase; Topa quinone; Copper protein

## 1. Introduction

The organic cofactor in bovine serum amine oxidase was identified as 2,4,5-trihydroxyphenylalanine (topa) in 1990 [1] and was subsequently shown to be ubiquitous in copper amine oxidases from bacteria to humans [2–4]. In the resting state of the enzyme, the cofactor is in its oxidized form as topa quinone (TPQ, Fig. 1A). Several aspects of its role in the oxidative deamination reaction (Eq. 1) catalyzed by amine oxidases have been elucidated [2–4].

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2 \quad (1)$$

TPQ is produced by post-translational modification of a tyrosine residue in the fully conserved active-site consensus sequence, Asn-Tyr-Asp/Glu [5]. The available evidence indicates that the post-translational modification proceeds via a novel self-processing mechanism in both eukaryotic and prokaryotic organisms [6–8].

One member of the copper amine oxidase family, lysyl oxidase, catalyzes the oxidation of collagen and elastin lysyl residues during the biogenesis of connective tissue [9]. The cofactor in lysyl oxidase has been the subject of considerable speculation because the protein has much different properties than the known TPQ-containing enzymes (e.g. subunit molecular mass of 75–85 kDa compared to 32 kDa for lysyl oxidase). Moreover, mammalian lysyl oxidase does not contain the active-site consensus sequence for TPQ [10]. The lysyl oxidase cofactor has now been identified as a modified tyrosine covalently cross-linked to the ε-amino group of a lysyl residue and has been designated lysine tyrosylquinone (LTQ, Fig. 1B) [11].

A copper amine oxidase with a subunit molecular mass of approx. 116 kDa, which is relatively large for an amine oxidase, was isolated from *Pichia pastoris* by Green et al. [12] and originally identified as a benzylamine oxidase. Subsequently, Tur and Lerch reclassified the enzyme as a lysyl oxidase based on the preferential oxidation of lysine, lysine derivatives, and lysyl residues in synthetic peptides. In addition, fibrillar collagen was found to be a substrate for the *P. pastoris* enzyme [13]. The *P. pastoris* amine oxidase displayed a substrate specificity that was surprisingly similar to that of mammalian lysyl oxidase [14].

These observations raise several important questions about the relationship among amine oxidases and specifically whether lysyl oxidase activity is associated with the presence of LTQ as a cofactor. We have undertaken identification of the *P. pastoris* lysyl oxidase cofactor and have now shown that the cofactor is TPQ rather than LTQ which is present in mammalian lysyl oxidase.

## 2. Materials and methods

### 2.1. Protein purification

*P. pastoris* was obtained from Invitrogen or the ATCC (no. 28,485). Growth in minimal media with butylamine as the sole nitrogen source was carried out essentially as described [12]. Cells were lysed and collected according to Cai and Klinman [6] or by vortexing with glass beads. Purification of the protein for peptide isolation was carried out as described [12]. Protein that was sufficiently pure for resonance Raman was obtained via the following procedure. After an initial centrifugation to sediment large debris, the supernatant was centrifuged at 81 000×*g* prior to chromatography. The centrifuged cell extract was treated with DEAE fast-flow media equilibrated in 20 mM $KPO_4$ buffer, pH 7.0. *P. pastoris* lysyl oxidase was quantitatively absorbed under these conditions. The DEAE was then poured into a column and the enzyme was eluted with 500 mM $KPO_4$ buffer (pH 7.0) and concentrated. Gel-filtration chromatography was then carried out using a 42×1.0 cm column (33 ml gel volume) of Superose-12 at flow rate of 0.06 ml/min on a Pharmacia FLPC system. These steps provided an approx. 200-fold purification of the *P. pastoris* lysyl oxidase. Enzyme activity was measured with benzylamine as described [15]. Protein concentrations were estimated using the micro-Bradford assay with bovine serum albumin as a standard. Protein purity was assessed by SDS-PAGE.

### 2.2. Phenylhydrazine labeling

Two procedures were used. For monitoring the reaction optically, 15 μM purified amine oxidase was titrated with 2 μM aliquots of recrystallized phenylhydrazine hydrochloride. The increase in chromophore absorbance was followed at 448 nm after each addition until no

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*PII* S0014-5793(96)01245-8

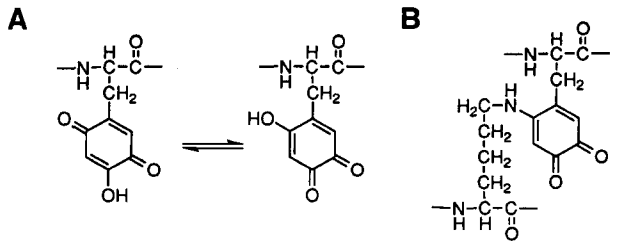


Fig. 1. Quinone cofactor structures. (A) 2,4,5-Trihydroxyphenylalanine quinone (TPQ); (B) lysine tyrosylquinone (LTQ).

further change was observed. Excess phenylhydrazine was removed using a Bio-Rad DG-10 desalting column pre-equilibrated with 100 mM $NH_4HCO_3$ (pH 8.0). Resonance Raman samples of the intact enzyme were prepared by incubation with a 10-fold excess of hydrazine reagent over protein concentration for 8–10 h at ambient temperature. Excess reagent was removed via exhaustive dialysis against 20 mM $KPO_4$ buffer (pH 7.0) using a flow, micro-dialysis unit. This procedure worked well for both phenylhydrazine and *p*-nitrophenylhydrazine.

### 2.3. Peptide isolation and characterization

Labeling with [$^{14}$C]phenylhydrazine hydrochloride (universally labeled, 5550 dpm/nmol, California Bionuclear Corp.) was performed as previously described [6]. Excess phenylhydrazine was removed with a desalting column as described. The sample was lyophilized and redissolved in 6 M guanidine hydrochloride and 50 mM $KPO_4$ buffer, pH 7.0. Reduction and carboxymethylation were carried out as previously described [16]. Reagents were removed using a desalting column as described.

The enzyme was again lyophilized and redissolved in 100 mM $NH_4HCO_3$ containing 2 M urea and shaken at 37°C. Proteolytic digestion was initiated by the addition of 2% (w/w) thermolysin (Sigma) and stabilized with 2 mM $CaCl_2$ [17]. After 8 h, the reaction was terminated by freezing at −70°C. Labeled peptide was isolated using a Vydac reverse-phase C18 column pre-equilibrated with solvent A (0.1% trifluoroacetic acid (TFA), 5% $CH_3CN$, $H_2O$) and eluted with a linear gradient from 30–50% solvent B (0.1% TFA, 80% $CH_3CN$, $H_2O$) over 40 min. The eluent was monitored at 214 and 440 nm. Yield for the digestion was 11.1% based on incorporation of radioactivity in the peptide peak relative to that in the initial digest.

The peptide recovered from the first digestion was lyophilized and redissolved in 50 mM potassium phosphate (pH 7.8) and 1 M urea to provide endoproteinase Glu-C (Sigma) specificity for cleavage at Asp

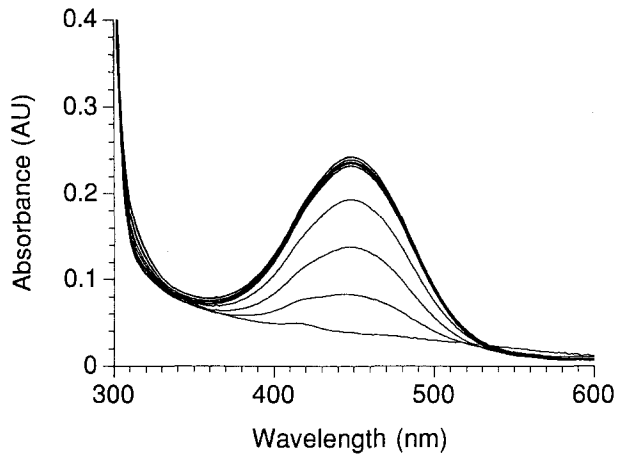


Fig. 2. *P. pastoris* lysyl oxidase phenylhydrazine titration.

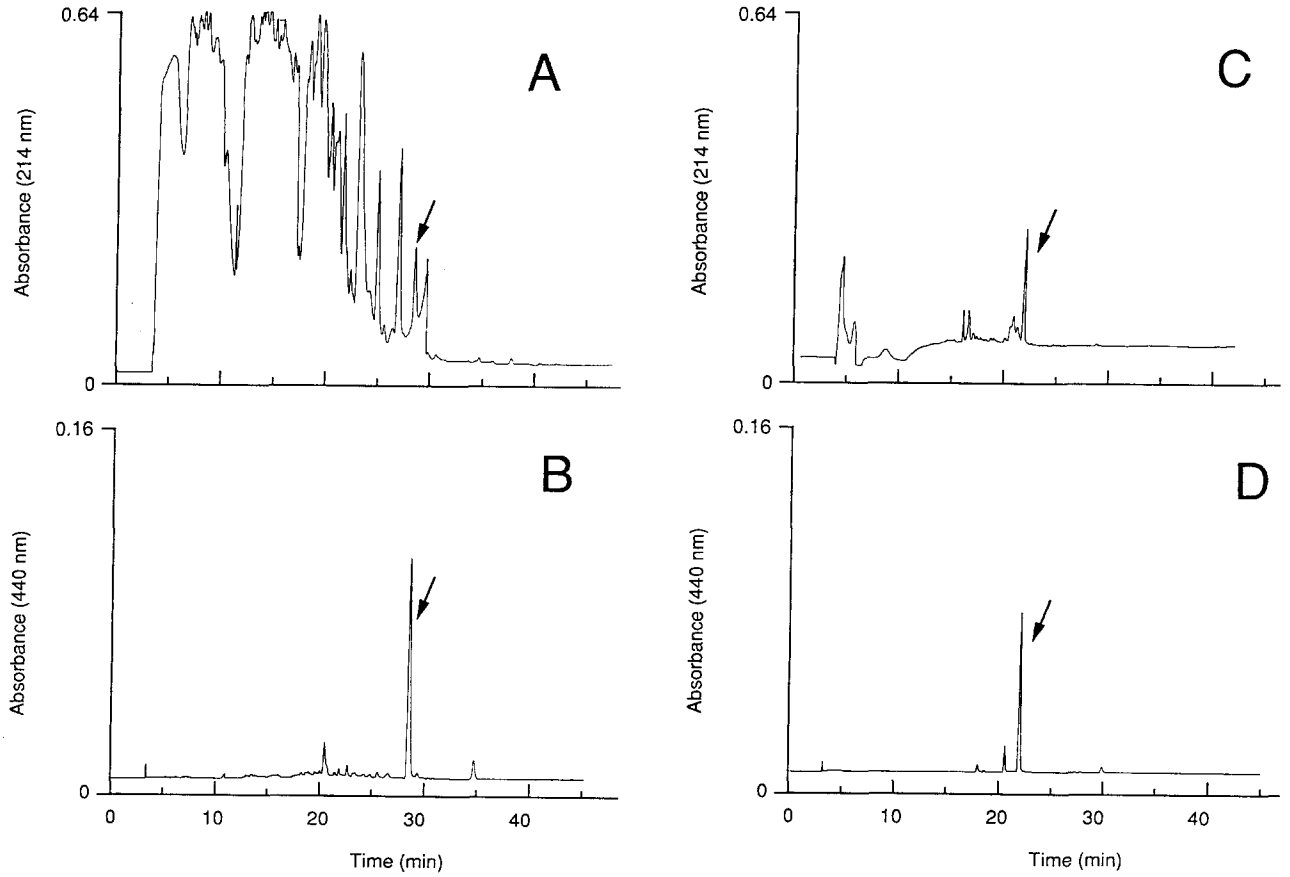


Fig. 3. HPLC purification profile of active-site peptide. Elution of the labeled peptide following thermolysin digestion was monitored at (A) 214 nm and (B) 440 nm. Elution following the Glu-C digestion was monitored at (C) 214 nm and (D) 440 nm.

and Glu residues [18]. Digestion was initiated by the addition of 2% (w/w) Glu-C at 37°C with shaking [18], terminated after 6 h as described above, and separated as before using a linear gradient from 35–55% solvent B over 40 min. Yield was 42% for the subdigestion based on radioactivity in the peptide peak relative to that injected on the HPLC column.

The resulting peptide was sequenced by automated Edman degradation. Resonance Raman spectra were obtained using methods and instruments as described [19,20].

## 3. Results and discussion

Purified *P. pastoris* lysyl oxidase was titrated with phenylhydrazine hydrochloride to form the corresponding phenylhydrazone with maximal absorbance at 448 nm as shown in Fig. 2. This spectrum is similar to those observed for phenylhydrazone derivatives of other TPQ-containing enzymes (see [2,3]).

[$^{14}$C]Phenylhydrazine-labeled enzyme was reduced and carboxymethylated [16] and subsequently digested with thermolysin. The active-site peptide was purified by reverse-phase HPLC. Fig. 3A,B shows a representative HPLC elution profile monitored at 214 and 440 nm. The majority of the chromophore eluted in one peak, was collected, and was quantified by measuring the incorporation of radioactivity. To reduce the size and increase the purity of the active-site peptide, the thermolysin peptide fragment was further digested with endoproteinase Glu-C. Fig. 3C,D shows a typical elution profile for the Glu-C digestion followed at 214 and 440 nm. Again, the chromophore eluted in one major peak which was collected and quantified by measuring $^{14}$C incorporation.

Table 1
Edman sequencing of the peptide from the Glu-C digestion of *P. pastoris* lysyl oxidase

| Cycle number | Glu-C peptide | | AGAO[b] | Lysyl oxidase[c] |
|---|---|---|---|---|
| | Amino acid | Yield (pmol) | | |
| 1 | Ile | 23 | Ile | Tyr |
| 2 | Gly | 44 | Gly | Asp |
| 3 | Asn | 38 | Asn | Thr |
| 4 | blank[a] | | TPQ | LTQ |
| 5 | Asp | 28 | Asp | Gly |
| 6 | Tyr | 15 | Tyr | Ala |
| 7 | Asn | 23 | Gly | Asp |

[a]No amino acid was identified at this position.
[b]See [21].

The sequence of the active-site peptide was determined by standard Edman degradation. For comparison, Table 1 shows the active-site sequence of *P. pastoris* lysyl oxidase is aligned with active-site sequences from *Arthrobactor globiformis* phenethylamine oxidase (AGAO), a TPQ-containing enzyme [21], and LTQ-containing lysyl oxidase [11]. A blank in the amino acid sequencing data was seen at the site of the modified

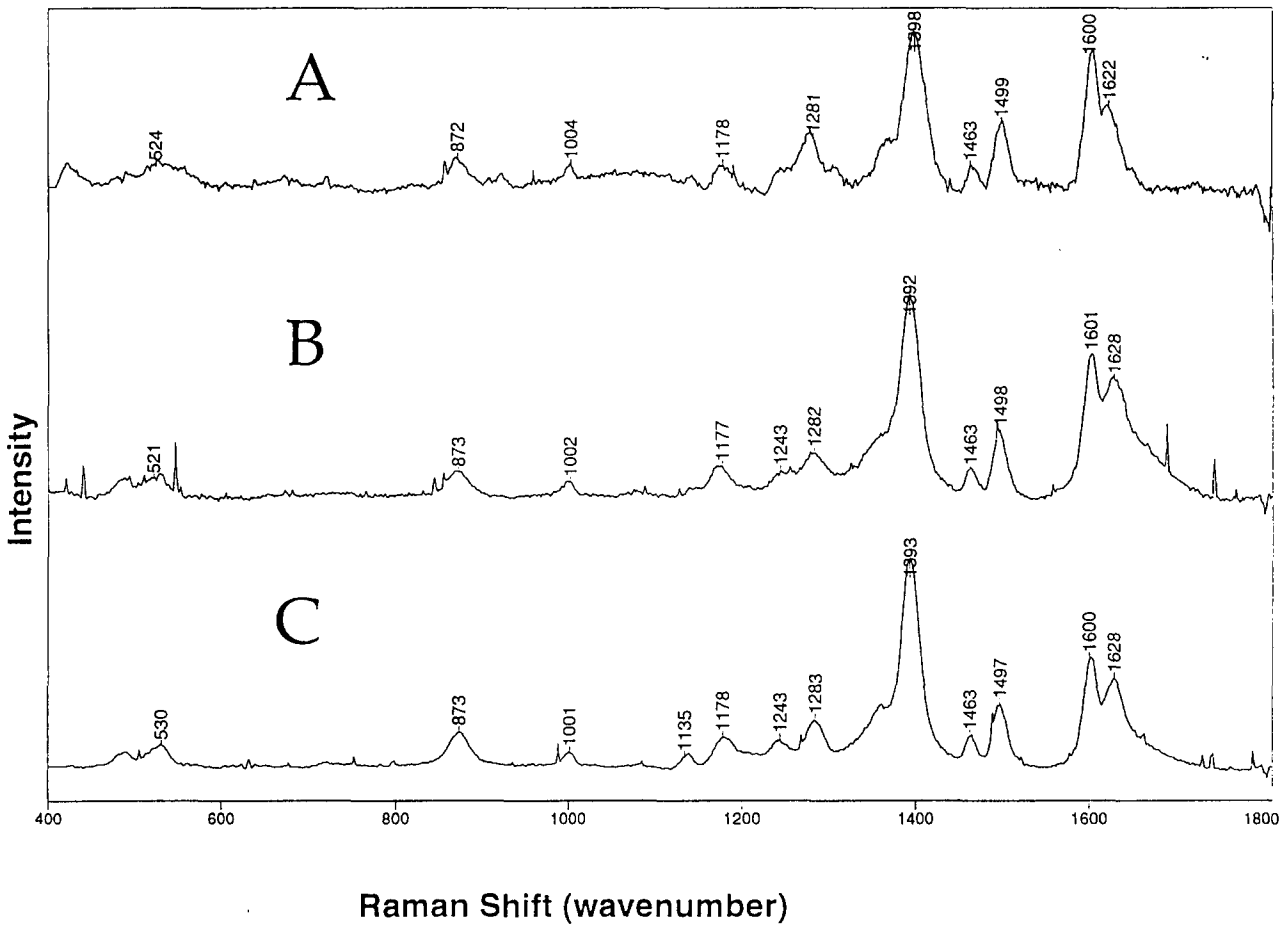


Fig. 4. Comparison of the resonance Raman of the *P. pastoris* lysyl oxidase phenylhydrazone, the derivatized active-site peptide, and the synthetic topa quinone-hydantoin derivative. (A) phenylhydrazone of the enzyme; (B) peptide phenylhydrazone; (C) phenylhydrazone of topa quinone hydantoin.

tyrosine for both AGAO and mammalian lysyl oxidase [11,21]. The *P. pastoris* peptide contains the Asn-BLANK-Asp/Glu consensus sequence for TPQ and is very similar to the AGAO active-site sequence. There is no apparent homology with the mammalian lysyl oxidase active-site sequence. Additionally, two amino acid residues would have been detected in each Edman round if *P. pastoris* lysyl oxidase contained a cross-linked cofactor like LTQ. The presence of the TPQ consensus sequence [22] strongly suggests the presence of this cofactor.

Resonance Raman spectroscopy has been used as a sensitive probe for the structure of quinone cofactors [2,3,20,23]. Active-site-derived peptides of mammalian lysyl oxidase exhibit resonance Raman spectra that are readily distinguished from TPQ-containing enzymes [11]. Resonance Raman spectra of the phenylhydrazone derivatives of *P. pastoris* lysyl oxidase and its active-site peptide are compared to the derivatized TPQ model compound in Fig. 4; these spectra are essentially superimposable. Further, the resonance Raman spectrum of the *p*-nitrophenylhydrazone derivative is also nearly identical to that of a corresponding derivative of a TPQ model (data not shown). These results provide very strong evidence for the presence of TPQ in *P. pastoris* lysyl oxidase.

The presence of TPQ in the *Pichia pastoris* lysyl oxidase is surprising considering the very similar substrate specificity between this amine oxidase and mammalian lysyl oxidase. It is clear, therefore, that the quinone cofactor is not involved in conferring substrate specificity. A complete amino acid sequence for *P. pastoris* lysyl oxidase would allow comparison to mammalian lysyl oxidase sequences and may reveal homology in those regions involved in substrate recognition. The failure thus far to find a non-mammalian LTQ-containing enzyme suggests a unique evolution of both structure and function among the family of lysyl oxidase enzymes in higher organisms.

*Acknowledgements:* Supported by grants from the National Institutes of Health (GM39296 to J.P.K.) and (GM27659 to D.M.D).

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