Copper Amine Oxidase: Heterologous Expression, Purification, and Characterization of An Active Enzyme in *Saccharomyces cerevisiae*†

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ABSTRACT: A copper amine oxidase gene from a methylotrophic yeast *Hansenula polymorpha* has been expressed in *Saccharomyces cerevisiae* under the control of the *ADHI* promoter and the recombinant protein purified to near homogeneity. The recombinant enzyme is as active as the native enzyme in catalyzing methylamine oxidation. We demonstrate that it is a quinoprotein by redox-cycling staining and titrations with carbonyl reagents. The absorption spectral properties of the recombinant amine oxidase and its phenylhydrazine derivative are very similar to those of other copper amine oxidases. The cofactor in the enzyme is 2,4,5-trihydroxyphenylalanine (topa) quinone, as demonstrated by the pH-dependent shift in the λ_{max} of the *p*-nitrophenylhydrazone adduct. Alignment of an active-site peptide and DNA-derived protein sequences reveals a tyrosine residue as the precursor to topa quinone, consistent with findings with other copper amine oxidases. All evidence presented herein indicates that the heterologously expressed copper amine oxidase protein is processed posttranslationally in *S. cerevisiae* to form an active enzyme with an intact cofactor. This occurs despite an inability of *S. cerevisiae* to utilize amines as a nitrogen source. The implications of this study for the mechanism of topa quinone biogenesis are discussed.

Copper-containing amine oxidases catalyze the oxidative deamination of primary amines by dioxygen to form aldehydes, ammonia, and hydrogen peroxide. The active-site cofactor in these enzymes is the oxidized form of 2,4,5-trihydroxyphenylalanine, referred to as topa[1] quinone, or 6-hydroxydopa quinone. The structure of the cofactor was first demonstrated with bovine serum amine oxidase (Janes et al., 1990) and later with a range of copper amine oxidases (Brown et al., 1991; Janes et al., 1992; Mu et al., 1992), ending a long period of speculation regarding the nature of the cofactor in copper amine oxidases (cf. Klinman et al., 1991). These findings also led to an improved interpretation of enzymatic kinetic data (e.g., Janes & Klinman, 1991; Hartmann & Klinman, 1991; Hartmann et al., 1993) and to the design, synthesis, and characterization of model compounds for topa quinone (Mure & Klinman, 1993).

One important question which arises following the identification of topa quinone as the cofactor of amine oxidases is the mechanism of its biosynthesis *in vivo*. The cofactor of amine oxidases is unique in that it is part of the polypeptide chain and linked to adjacent amino acid residues through normal peptide bond linkages (Janes et al., 1990). Amino acid residues immediately flanking topa are conserved among all amine oxidases examined thus far with the consensus sequence being Asn-Topa-Asp/Glu (Janes et al., 1992). Alignment of topa-containing peptide sequences with protein sequences derived from available cDNA sequences has revealed a tyrosine codon in the position of topa quinone (Mu et al., 1992, 1994), implicating topa quinone formation via a posttranslational modification of the side chain of the tyrosyl residue. It has been proposed that this processing involves an initial hydroxylation of tyrosine to dopa, followed by oxidation to dopa quinone; hydration of dopa quinone is a likely next step, giving rise to topa, which would be readily oxidized in air to topa quinone (Mu et al., 1992).

Amine oxidases are generally abundant in living organisms under normal or induced conditions. The fact that these enzymes occur in both prokaryotes and eukaryotes, including bacteria, fungi, plants, and mammals (McIntire & Hartmann, 1993), implies that a pathway exists in each of these organisms for the modification of tyrosine to topa quinone. The pathway leading to the formation of topa quinone *in vivo* requires high specificity and careful regulation, due to the fact that topa quinone as a free amino acid is a highly neurotoxic and cytotoxic agent (Graham et al., 1978). We have now begun investigating the mechanism for topa quinone biogenesis in the copper amine oxidases. Our approach has been to express an amine oxidase gene in a heterologous host which lacks the ability to produce any endogenous amine oxidases. *Saccharomyces cerevisiae* was chosen as the host for expression because it does not appear to contain any copper amine oxidases (Large, 1986). de Hoop et al. (1992) had expressed in *S. cerevisiae* an amine oxidase gene isolated from *Hansenula polymorpha* and detected amine oxidase activity in crude cell extracts. In the present study, this recombinant enzyme has been purified, kinetically characterized, and studied for cofactor integrity. We report that it contains a fully processed topa quinone at the active site.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. S. cerevisiae wild-type strain X2180-1A and the transformable strain CG 379 (*α ade5 his7-2 leu2-3 leu2-112 trp1-289$_a$ ura3-52*) were purchased from the Yeast Genetic Stock Center (University of California, Berkeley). The amine oxidase gene from *H. polymorpha* inserted in pTZ19R (de Hoop et al., 1992) was kindly provided by Professor G. Ab (University of Groningen, The Netherlands). Yeast expression vector pDB20 was provided by Dr. L. Guarente (Massachusetts Institute of Technology).

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[1] Abbreviations: dopa, 3,4-dihydroxyphenylalanine; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; topa, 2,4,5-trihydroxyphenylalanine; URA, uracil.

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Plasmid Construction and Yeast Transformation. The amine oxidase gene was obtained as a 2.3 kb *Hin*dIII and *Bam*HI fragment by restriction enzyme digestion. The *Bam*HI site was converted to a *Hin*dIII site by filling in the 3′ overhang using the Klenow fragment, followed by ligation to a *Hin*dIII linker [d(pCCCAAGCTTGGG), from New England Biolabs]. This *Hin*dIII fragment was then inserted into the yeast expression vector pDB20, which has the alcohol dehydrogenase (*ADHI*) promoter and terminator (Becker et al., 1991). The *ADHI* promoter was considered to be constitutive in glucose-containing media (Schneider & Guarente, 1991). Plasmids pDB20 or pDB20 containing the insert were introduced into yeast CG 379 cells by lithium acetate transformation (Ito et al., 1983). Transformation mixtures were plated out on uracil (URA) dropout plates to select for URA^+ transformants. URA dropout media were prepared as the synthetic minimal media (Sherman, 1991), supplemented with adenine, histidine, and tryptophan to 50 mg/L each and leucine to 75 mg/L.

Growth of the Wild-Type Yeast Cells and Protein Extraction. The wild-type X2180-1A strain was always maintained in synthetic minimal medium. To test for its ability to grow in various amines, ammonium sulfate in the minimal medium was replaced by 40 mM ethylamine, benzylamine, or diethylamine, or 4 mM ethylamine or diethylamine. For protein extract preparations, cells were grown in 1 L of the synthetic minimal medium at 30 °C with shaking to saturation (OD_{600} = 4). Cells were collected by centrifugation, washed twice with an equal volume of 0.1 M potassium phosphate buffer, pH 7.2, and disrupted by sonication (see below). The broken cells were extracted twice for soluble proteins with two volumes of 0.1 M potassium phosphate buffer, pH 7.2. The combined supernatants contained 145 mg of protein. One volume of 0.1 M potassium phosphate buffer containing 1.5% Triton X-100 and 0.3 M NaCl was added to the pellet and the solution was stirred for over 2 h at 0 °C. After centrifugation, the supernatant contained 20 mg of protein. All supernatants were stored at −20 °C.

Protein Gel Electrophoresis and Redox-Cycling Staining for Quinoproteins. Protein samples were analyzed in SDS–polyacrylamide (12% or 15%) gel under reducing conditions according to the method described (Laemmi, 1970). A Mini-Protean II electrophoresis cell (Bio-Rad) with 0.75- or 1.5-mm spacers was used. Following electrophoresis, gels were treated with a fixing solution (Sigma) for 15 min and stained with Brilliant Blue G-Colloidal (Sigma) for at least 2 h to visualize proteins. Gels were destained first in 10% acetic acid/25% methanol for 30 s, followed by 25% methanol solution until the background became clear. Isoelectric focusing gel electrophoresis was performed using a Phast Gel System (Pharmacia). Proteins were visualized by staining with Coomasie Blue according to a procedure described in the instrument manual.

For redox-cycling staining, proteins were first transferred to a 0.22-μm nitrocellulose or PVDF membrane (Bio-Rad) following electrophoresis by overnight electroblotting at 150 mA in 20% methanol containing 25 mM Tris and 192 mM glycine. The blot was then stained with 0.24 mM nitroblue tetrazolium in 0.1 M potassium glycine, pH 10, for 45 min (Paz et al., 1991).

Large-Scale Culture and Cell Extract Preparation. One colony of URA^+ transformants was picked and grown in 50 mL of URA dropout medium to saturation with shaking at 30 °C; 10 mL of this culture was transferred to 1 L of URA dropout medium and incubated overnight at 30 °C. This 1-L culture was then used as inoculum for large-scale cultures. Typically for one enzyme preparation, 12 L of culture were grown overnight in six flasks at 30 °C with shaking to an OD_{600} of ~3.5. Cells were collected by centrifugation at 4 °C (4000*g*, 10 min), washed twice with cold 0.1 M potassium phosphate buffer, pH 7.2, and stored at −70 °C. The yield of wet cell was about 45 g. Cells were resuspended in 2–3 volumes of the same buffer before lysis and disrupted at 0 °C by sonication using a Branson Sonifier 450. Several cycles of sonication were applied with a duration of 2–5 min/cycle. The temperature of the cell suspension was kept below 30 °C by controlling the length of time of each sonication cycle and by cooling the solution to below 10 °C before starting the next cycle. Sonication was continued until >80% of the cells appeared broken when examined under a light microscope. The solution was cleared by centrifugation at 4 °C (10000*g*, 30 min), and the pellet was resuspended in one volume of the buffer and sonicated briefly. Supernatants from both extractions were combined.

Enzyme Purification and Assay. Proteins in the crude extracts were precipitated by $(NH_4)_2SO_4$ (ultrapure, from ICN). Pellets from the 10–40% saturation fraction were redissolved in 5 mM potassum phosphate buffer, pH 7.2, and dialyzed against the same buffer. The dialyzed solution was loaded onto a DE52 DEAE cellulose (Whatman) column with a bed volume of about 400 mL and eluted by a linear phosphate gradient (5–100 mM potassium phosphate, pH 7.2). Active fractions were pooled and concentrated using a 50-mL Amicon concentrator with a PM30 membrane. The concentrated protein solution was then loaded onto a Sephacryl S-200 HR (Pharmacia) gel-filtration column (75 cm long, 2.5 cm inner diameter), which was pre-equilibrated with 10 mM potassium phosphate, pH 6.5. The most active fractions were pooled, concentrated using a 50-mL Amicon concentrator, and further purified using a Mono Q FPLC column (Pharmacia). Proteins were eluted with a linear gradient of potassium phosphate, pH 6.5, from 10 to 125 mM over 50 min at a flow rate of 2 mL/min.

Amine oxidase activity was assayed at 37 °C using benzylamine as the substrate. An aliquot containing 3–5 μg of the amine oxidase was added to 5 mM benzylamine hydrochloride (Sigma) in 0.1 M potassium phosphate, pH 7.2, and product benzaldehyde formation was monitored spectrophotometrically as an increase in absorbance at 250 nm ($\Delta\epsilon$ = 12.8 mM^{-1} cm^{-1}) (Neumann et al., 1975). Protein concentrations were determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin used as a standard. Copper content in the purified enzyme was determined by standard addition method. The protein samples were first dialyzed extensively against distilled, deionized water and diluted with the deionized water to 10 μg/mL with copper standard added to 0–0.035 μg of copper/mL. Total copper content of the solution was analyzed by an atomic absorption spectrophotometer Model 360 equipped with an HGA-2200 graphite furnace (Perkin-Elmer). Copper reference solution (containing 1 mg/mL of copper in 2% nitric acid) was purchased from Fisher.

Kinetic Measurements. Methylamine, ethylamine, and benzylamine (all in hydrochloride form) were purchased from Sigma and recrystallized. [1,1-$^2H_2$]benzylamine hydrochloride was prepared as described (Bardlsey et al., 1973). Substrate stock solutions were prepared in 0.1 M potassium phosphate, pH 7.2. The oxidation of methylamine or ethylamine was determined by monitoring oxygen consumption using an oxygen electrode Model 53 (Yellow Springs Instrument). The reaction was initiated by the addition of the enzyme to 1 or 1.5 mL of air-saturated buffer containing

various amounts of substrate. Benzylamine oxidation was followed spectrophotometrically as described above. Steady-state kinetic parameters were calculated by fitting data to the Michaelis–Menten equation using nonlinear regression.

Derivatization with Phenylhydrazine and p-Nitrophenylhydrazine. The recombinant amine oxidase (5–40 μM) was dissolved in 1 mL of 0.1 M potassium phosphate buffer, pH 7.2. Two molar equivalents of phenylhydrazine hydrochloride (Fluka) was added to the enzyme solution and the mixture incubated at 37 °C for over 30 min for batch derivatization. During the incubation the absorption spectrum of the enzyme was monitored using a Hewlett-Packard 8452A diode-array spectrophotometer. The reaction was monitored until the absorbance at 448 nm reached a maximal value. Unreacted phenylhydrazine was removed by desalting through a DG-10 column (Bio-Rad), pre-equilibrated with 10 mM potassium phosphate, pH 7.2, or by dialysis against the same buffer. The same procedure was followed to derivatize the enzyme with *p*-nitrophenylhydrazine except that the incubation time was extended to 3 h due to the low reactivity of the enzyme with the inhibitor, and the desalting column was pre-equilibrated with 20 mM potassium phosphate, pH 7.2. To determine the labeling stoichiometry, aliquots of phenylhydrazine (in less than 1 μL) were titrated into the enzyme solution. The spectrum was recorded when absorbance at 448 nm reached a constant value following each addition.

Proteolysis and Isolation and Characterization of Topa-Containing Peptide. Phenylhydrazine-derivatized enzyme, prepared in 6 M guanidine hydrochloride and 50 mM potassium phosphate, pH 7.2, was reduced by β-mercaptoethanol (Sigma), followed by alkylation with iodoacetic acid (Sigma) according to a method described by Flannery et al. (1989). Unreacted reagents were removed by desalting. The reduced and carboxymethylated protein sample was prepared in 0.1 M NH_4HCO_3 and 2 M urea for proteolysis by trypsin. The digestion was initiated by the addition of trypsin (type I, Sigma) to a final concentration of 2.5% (w/w). The digestion mixture was incubated at 37 °C for 4–6 h and later stored at −20 °C.

Peptides resulting from the digestion were separated by HPLC. The digested protein sample was first injected onto a Dynamax reverse-phase C4 column (Rainin), which was pre-equilibrated with solvent A (5% acetonitrile containing 0.11% trifluoroacetic acid). Peptides were eluted with a linear gradient of 25–50% solvent B (80% acetonitrile and 0.1% trifluoroacetic acid) over 40 min at a flow rate of 1 mL/min. The elution was monitored at 214 nm for peptides and at 384 nm for phenylhydrazine-labeled peptides. The predominant peak at 384 nm was lyophilized to <100 μL and reinjected onto a Microsorb reverse-phase C18 column (Rainin), which was pre-equilibrated with solvent C (0.3% triethylammonium acetic acid, pH 6.5). Peptides were eluted with a linear gradient of 25–45% solvent D (60% acetonitrile and 0.3% triethylammonium acetic acid, pH 6.5) over 40 min at a flow rate of 1 mL/min. The elution was monitored at 214 nm for peptides and at 448 nm for phenylhydrazine-labeled peptides. The final fraction was concentrated by lyophilization to about 10 μL, and the amino acid sequence of the peptide was analyzed by Edman degradation. Both C4 and C18 columns were 4.6 mm in inner diameter and 25 cm in length.

RESULTS

Screening for Quinoproteins in S. cerevisiae. Unlike *H. polymorpha*, *S. cerevisiae* cannot utilize monoamines or diamines as a nitrogen source to support its growth (Barnett et al., 1983; Large, 1986). This was confirmed in the present study. No growth of the wild-type *S. cerevisiae* strain was observed in minimal media containing ethylamine, benzylamine, or diethylamine as the sole nitrogen source. The inability of *S. cerevisiae* to utilize amines is most likely correlated with the lack of enzyme(s) in this organism that are required for the oxidation of amines, namely amine oxidases. *S. cerevisiae* therefore provides an excellent background for the heterologous expression of amine oxidase genes. The ability of *S. cerevisiae* to produce quinoproteins was also tested. Proteins extracted by potassium phosphate buffer or by Triton X-100 and sodium chloride were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and stained for quinoproteins. No positive stain was observed with samples containing up to 370 mg of total protein (data not shown). We conclude either that *S. cerevisiae* does not produce any quinoproteins or that these quinoproteins are present at less than our present detection limit of 0.1% of total protein. It is also possible that an as yet undetermined set of growth conditions exists for the induction of quinoproteins in *S. cerevisiae*.

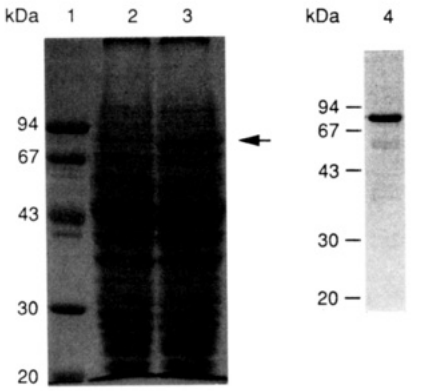


FIGURE 1: SDS–PAGE analysis of protein samples: Lane 1, protein molecular weight standards (Molecular weights in kDa are indicated to the left of the lane.); Lane 2, 75 μg of protein from the extract prepared from *S. cerevisiae* cells transformed with the vector pDB20; Lane 3, 78 μg of protein from the protein extract prepared from *S. cerevisiae* cells transformed with pDB20 containing the amine oxidase gene insert (The arrow indicates the position where the recombinant yeast amine oxidase protein is present.); Lane 4, 2.7 μg of the purified, recombinant yeast amine oxidase.

Expression and Purification of the Recombinant Protein. Protein extracts were prepared from *S. cerevisiae* cells that had been transformed with pDB20 containing the yeast amine oxidase gene insertion or with the vector pDB20 alone to be used as a control and analyzed on SDS–polyacrylamide gels. A protein band at 78 kDa was visible in the expression extract (Figure 1, lane 3), which was absent in the control extract. The size of the protein corresponds to the expected size of the amine oxidase calculated from the protein sequence (Bruinenberg et al., 1989). We estimated that the recombinant protein was 3% of total soluble protein.

The recombinant protein in crude extract is capable of catalyzing the oxidation of benzylamine to benzaldehyde (Table 1) and stains positive for quinoprotein by the redox-cycling staining method (data not shown). These results indicate that the recombinant protein is an active amine oxidase and contains a functional quinone moiety. Our expression of an active amine oxidase in *S. cerevisiae* is consistent with a previous study by de Hoop et al. (1992) which demonstrated amine oxidase activity in crude cell extracts. We have further found that the expressed amine oxidase is active *in vivo*. When *S. cerevisiae* cells transformed with the amine oxidase gene are cultured in a URA dropout medium in which ammonium sulfate is substituted by 4 or 40 mM ethylamine, the cell density of cultures at saturation is typically 20–40% higher

Table 1: Purification of the Recombinant Amine Oxidase[a]

purification steps	total volume (mL)	total protein (mg)	total units[b]
crude extract	250	3788	21
dialysis of 10–40% $(NH_4)_2SO_4$ pellet	144	1526	24.6
DEAE chromatography			
pool A	12	139	5.8
pool B	4.2	54	2.6
Sephacryl S-200 gel filtration[c]			
pool A1	3.2	16.8	1.9
pool A2	3.0	20.6	1.7
pool A3	2.5	21.8	1.0
Mono Q FPLC[d]			
pool A11	0.5	8.3	1.1
pool A12	0.2	5.0	0.4

[a] An 87-g sample of wet cells was used for the purification. [b] Activity measured at 37 °C using benzylamine as substrate. [c] Only results with pool A from the preceding step are reported. [d] Only results with pool A1 are reported.

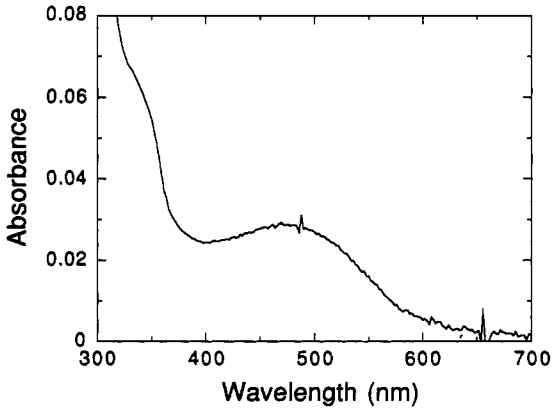


FIGURE 2: Absorption spectrum of the purified recombinant enzyme. The spectrum was taken with a protein sample of 1.3 mg/mL, prepared in 20 mM potassium phosphate, pH 7.2.

than that of control cultures lacking ammonium sulfate or ethylamine. Although the final level of cell density was less than that reached with ammonium sulfate, this result indicates a functional activity of the recombinant amine oxidase *in vivo* to release ammonium ion from ethylamine and support *S. cerevisiae* growth.

The recombinant enzyme was purified in several steps including ammonium sulfate fractionation, anion-exchange chromatography, gel filtration, and FPLC. Table 1 gives a summary of the purification results. The purest fraction had a specific activity of 0.13 unit/mg, determined at 37 °C using benzylamine as the substrate, and was >90% pure as judged on SDS–polyacrylamide gel (Figure 1, lane 4). We find that the enzyme is pink and has a λ_{max} at 472 nm (Figure 2), typical of a copper amine oxidase. Its p*I* is 5.55–5.67. As expected for a member of the copper amine oxidase class, the recombinant protein is found to contain 2.04 mol of copper/mole of enzyme dimer. de Hoop et al. (1992) had reported previously that recombinant amine oxidase in crude cell extracts behaved as a dimer.

Kinetic Properties of the Recombinant Amine Oxidase. In addition to benzylamine, the recombinant amine oxidase is capable of catalyzing the oxidation of methylamine and ethylamine (Table 2). V_{max} and K_m for methylamine oxidation at 25 °C, pH 7.2, are 4.71 s^{-1} and 0.146 mM, respectively. The reported specific activity for methylamine oxidation was 1.1 units/mg for the native enzyme purified from *H. polymorpha* (Bruinenberg et al., 1989). We obtained a value of 5.6 units/mg for the recombinant amine oxidase under the same assay conditions as for the native enzyme. This result

Table 2: Oxidation of Aromatic and Aliphatic Amines by Yeast Amine Oxidases

	benzylamine oxidation[a]				aliphatic amine oxidation[b]	
enzymes	V_m, units/mg	K_m, mM	$^D(V/K)$	$^D(V)$	methylamine	ethylamine
recombinant enzyme	0.0686	0.682	7.04	4.90	5.4	7.9
benzylamine oxidase[c]	1.73	0.017	4.02	1.17	0.14	1.48

[a] Assayed in 100 mM potassium phosphate, pH 7.2, at 25 °C with benzylamine concentrations in the range of 0.045–5.0 mM for the recombinant enzyme and 5.0–110 μM for the benzylamine oxidase. Concentrations of [1,1-$^2H_2$]benzylamine were in the range of 0.25–7.3 mM for the recombinant enzyme and 19–357 μM for the benzylamine oxidase. [b] Determined with 3 mM methylamine and 5 mM ethylamine in 100 mM potassium phosphate, pH 7.2, at 37 °C; expressed as units/mg. [c] Purified from *H. polymorpha* according to the method of Mu et al. (1992).

indicates that the recombinant enzyme is as active as the native enzyme; the difference in specific activity may be due to differences in protein purity and/or the presence of inactivated enzyme species in the native enzyme preparation (also see Discussion).

Oxidation of benzylamine by copper amine oxidases is believed to entail the formation of a substrate Schiff base between benzylamine and topa quinone in the active site. Abstraction of a proton from the Cα position on the substrate generates a product Schiff base, which is hydrolyzed to form a reduced cofactor and benzaldehyde (Hartmann & Klinman, 1991). Kinetic isotope effects on benzylamine oxidation were determined (Table 2), to characterize further the recombinant enzyme. For comparison, similar experiments were performed with a yeast amine oxidase, purified from *H. polymorpha* (Mu et al., 1992). The results show that both $^D(V/K)$ and $^D(V)$ are larger for the recombinant enzyme, implying that the proton abstraction is more rate-limiting with this substrate. The recombinant enzyme is also different in substrate specificity from the yeast amine oxidase purified by Mu et al. (1992), with aliphatic amines being the preferred substrates (Table 2). Based on these observations we conclude that these amine oxidases are different gene products: the amine oxidase encoded by the cloned gene (Bruinenberg et al., 1989) is thus designated as yeast methylamine oxidase and the enzyme purified by Mu et al. (1992) as yeast benzylamine oxidase.

Titration with Phenylhydrazine. The presence of a quinone moiety in the recombinant methylamine oxidase, as evidenced in the redox-cycling staining experiment, was further investigated by reaction with the carbonyl reagent phenylhydrazine. Substoichiometric amounts of phenylhydrazine were titrated into an enzyme solution, and the spectrum of the enzyme was recorded after a 30–45-min incubation at 37 °C. As shown in Figure 3, the titration of phenylhydrazine was accompanied by the formation of a yellow adduct with an absorption λ_{max} at 448 nm, very similar to that observed with bovine serum amine oxidase (Janes & Klinman, 1991). The increase in absorbance was proportional to the amount of phenylhydrazine titrant and reached an end point (Figure 3, inset). No change in the enzyme absorption spectrum was observed after the enzyme sample was desalted, indicative of the formation of a covalent adduct. Covalent labeling of the recombinant yeast methylamine oxidase with phenylhydrazine was confirmed by titrating with [^{14}C]phenylhydrazine.

With an enzyme sample of specific activity 0.097 unit/mg (determined at 37 °C using benzylamine as the substrate), the titration yielded 1.2 mol of phenylhydrazine/mol of enzyme dimer. An increased stoichiometry of 1.5 mol of phenyl-

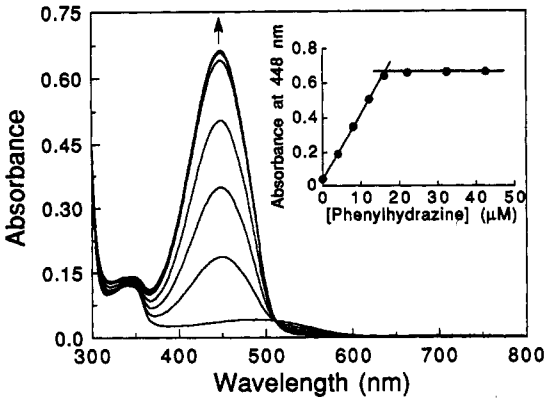


FIGURE 3: Stoichiometric titration of the purified recombinant amine oxidase with phenylhydrazine. Enzyme (2.1 mg; specific activity, 0.097 units/mg) was prepared in 1 mL of 0.1 M potassium phosphate, pH 7.2. Phenylhydrazine was titrated into the solution to final concentrations of 0, 4.06, 8.13, 12.2, 16.3, 22.4, 32.5, and 42.7 μM, respectively. Each spectrum was recorded after a 30–45-min incubation at 37 °C following each addition. The arrow indicates the direction of spectral change. The titration curve is shown in the inset.

hydrazine/mol of enzyme dimer was subsequently obtained with an enzyme sample of 0.117 unit/mg specific activity. An extinction coefficient of 40.5 mM^{-1} cm^{-1} at 448 nm was calculated for the yellow phenylhydrazone adduct based on the radioactivity and the absorbance of the adduct after desalting.

Isolation and Characterization of an Active-Site Peptide. Phenylhydrazine-derivatized recombinant methylamine oxidase was digested with trypsin following reduction and alkylation, and the resulting peptides were subjected to reverse-phase HPLC. Figure 4A,B shows the elution pattern monitored at 214 and 384 nm, respectively. Two peptides, I and II, with respective retention times of 25.0 and 26.7 min, were found to be the major phenylhydrazine-containing peaks accounting for about 40% of the total labeled peptides (based on the radioactivity). Fractions containing peptide I were concentrated and further purified by reinjection onto a reverse-phase C18 column. This procedure resolved peptide I (retention time, 30.2 min) from at least two contaminating peptides, as shown in Figure 4C,D.

The amino acid sequence of peptide I, determined by Edman degradation, is given in Table 3. The sequence of the peptide matches exactly with that derived from the DNA sequence (Bruinenberg et al., 1989) with the exception of the blank residue. The blank residue is aligned with a tyrosine residue, as expected from previous findings implicating tyrosine as the precursor to topa quinone (Mu et al., 1992, 1994). The lack of complete identity between the DNA-derived sequence and the active site peptide from benzylamine oxidase isolated from *H. polymorpha* (Mu et al., 1992 and Table 3) can now be attributed to isozyme variants with different active-site sequences. We note that the last amino acid residue of peptide I does not match the normal cleavage site for trypsin, namely a Lys or Arg. The presence of such an apparent cleavage site for chymotrypsin has been attributed to the presence of a small amount of ψ-trypsin in the trypsin preparation (Wilkinson, 1986).

Spectroscopic Characterization of the Recombinant Amine Oxidase. *p*-Nitrophenylhydrazine has been used to identify topa quinone in copper amine oxidases (Janes et al., 1992). Reaction of the recombinant yeast methylamine oxidase with *p*-nitrophenylhydrazine was much slower than the reaction with phenylhydrazine, yielding a final yellow adduct with a λ_{max} at 472 nm at neutral pH (Figure 5). Addition of KOH

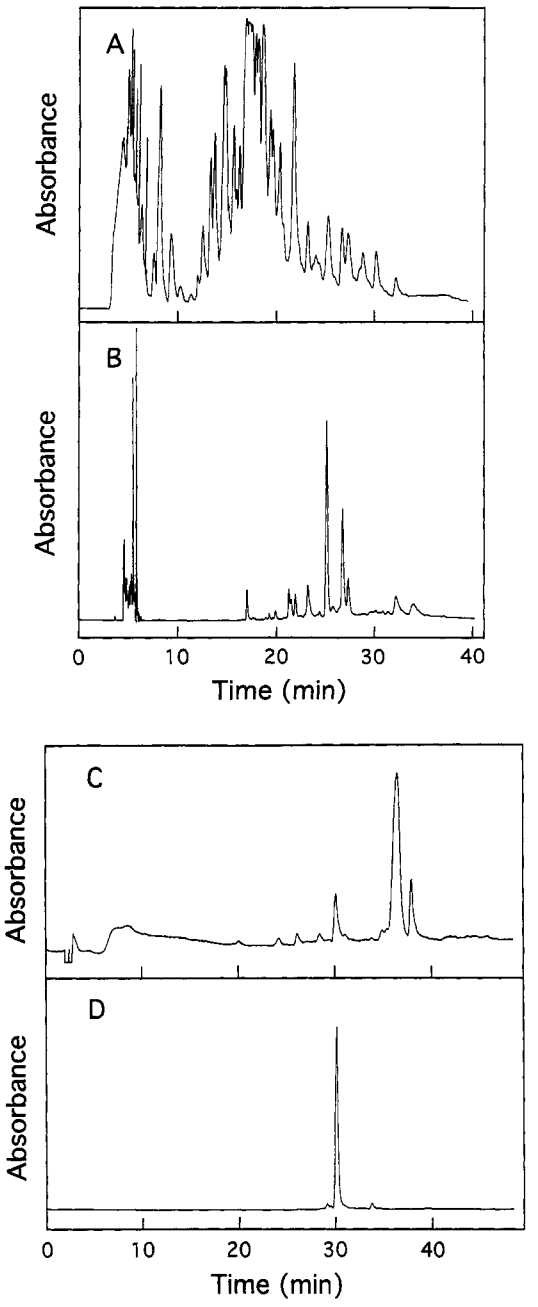


FIGURE 4: Purification of the cofactor-containing peptide using reverse-phase HPLC. A 16 nmol sample of trypsin-digested phenylhydrazine derivative was injected onto a C4 column and the elution was monitored at 214 nm (A) and 384 nm (B). The peak with a retention time of 25.0 min was collected, concentrated, and reinjected onto a C18 column. The elution was monitored at 214 nm (C) and 448 nm (D).

to 2.8 M resulted in a 116-nm red shift in the λ_{max} (Figure 5) as the color of the solution changed from yellow to purple. A 120-nm red-shift is very typical of and specific for the adduct of topa quinone with *p*-nitrophenylhydrazine upon basification (Janes et al., 1992). This confirms topa quinone formation in the expressed amine oxidase.

DISCUSSION

A gene encoding a copper-containing amine oxidase from *H. polymorpha* has been expressed in *S. cerevisiae* under the control of the *ADHI* promoter. In the present study we report the purification of this recombinant protein to near homogeneity and its characterization with regard to enzymatic activity, the active-site amino acid sequence, and the identity of the cofactor.

Table 3: Topa Quinone-Containing Peptide Sequences and DNA-Derived Yeast Methylamine Oxidase Protein Sequence

peptide I[a]	DNA-derived sequence[b]	peptide from the yeast benzylamine oxidase[c]
Thr	Thr[401]	
Ala	Ala	Val
Ala	Ala	Ala
Asn	Asn	Asn
blank	Tyr	Topa
Glu	Glu	Glu
Tyr	Tyr	Tyr
CmCys	Cys	Val
Leu	Leu	
Tyr	Tyr	

[a] Blank is an unidentifiable phenylthiohydantoin derivative, and CmCys is a carboxymethylated cysteine residue. [b] From Bruinenberg et al., 1989. The number is the residue number of the protein sequence. [c] From Mu et al., 1992.

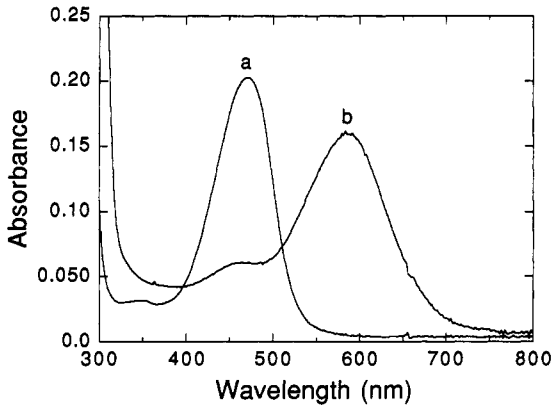


FIGURE 5: Absorption spectra of the *p*-nitrophenylhydrazone adduct of the recombinant yeast methylamine oxidase. A 1.0-mg sample of the adduct was prepared in 1.3 mL of 20 mM potassium phosphate, pH 7.2, before (spectrum a) and after (spectrum b) the addition of KOH to 2.8 M.

Nature of the Active-Site Cofactor and Relation to Enzyme Activity. We have demonstrated that the recombinant enzyme has the same level of activity as the native enzyme purified from *H. polymorpha*. Aliphatic amines are better substrates for this enzyme than aromatic amines (Table 2), which has led us to name this enzyme yeast methylamine oxidase in order to distinguish it from yeast benzylamine oxidase purified from the same source by Mu et al. (1992). Formation of an active amine oxidase indicates the presence of a functional organic cofactor in the enzyme active site. The visible spectrum of the resting enzyme (Figure 2), titration with carbonyl reagent phenylhydrazine (Figure 3), and the spectral properties of its adduct with *p*-nitrophenylhydrazine (Figure 5), together with the resonance Raman spectral properties of the phenylhydrazine-labeled active-site peptide (data not shown), all agree with those of other amine oxidases that have been characterized (Janes et al., 1991; Brown et al., 1991; Mu et al., 1992; Cooper et al., 1992; Janes et al., 1992). These results indicate that 6-hydroxydopa, or topa quinone, is formed properly in the active site of the recombinant methylamine oxidase.

We have observed that the recombinant enzyme loses part of its enzymatic activity relatively quickly following purification and storage (the degree of activity loss differs from preparation to preparation). The stoichiometry of titration with phenylhydrazine reflects the loss in activity, varying in a wide range between 0.8 and 1.5 mol of titrated quinone/mol of enzyme dimer. High stoichiometry could only be obtained with freshly prepared enzyme prior to freezing. In general, the stoichiometry had a linear correlation with the specific activity of the enzyme sample, very similar to what was observed with bovine serum amine oxidase (Janes & Klinman, 1991). We estimate that an enzyme sample of 0.164 units/mg (using benzylamine as substrate) would yield a stoichiometry of 2 mol titrated quinone/enzyme dimer. Protein samples used for this study usually appeared >80% pure as judged by SDS–PAGE. It is thus apparent that low specific activity is not due solely to the presence of impurities in protein preparations. These variations most likely result from enzyme inactivation which alters the ability of the cofactor to undergo active-site titration (Janes & Klinman, 1991).

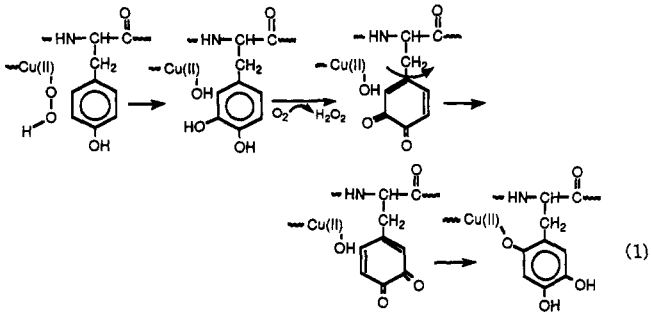
In a previous study, alignment of an active-site peptide from a yeast amine oxidase purified from *H. polymorpha* with a DNA-derived protein sequence led to the assignment of tyrosine as the precursor amino acid to topa quinone (Mu et al., 1992). However, the alignment did not give a perfect match between the peptide and the DNA-derived sequences (also see Table 3). In the present study we show an exact match between the sequence of a cofactor-containing active site peptide and the DNA-derived sequence. It has become clear through this work that earlier discrepancies were the result of the existence of different amine oxidase isozymes in *H. polymorpha*. Although both enzymes were isolated from *H. polymorpha* cultures grown under the same induction conditions, different substrates were used for the enzyme activity assay: methylamine in the case of Bruinenberg et al. (1989) and benzylamine in the case of Mu et al. (1992). It has been shown that multiple amine oxidases can be induced by a single type of amine in cultures of yeast (including *H. polymorpha*) that are capable of utilizing amines as the sole nitrogen source (Green et al., 1982).

Implications for Topa Quinone Biogenesis. It is now well-established that the tyrosine precursor to topa quinone is contained in a similar consensus sequence Asn-Tyr-Asp/Glu for all organisms studied (Klinman & Mu, 1994). The modification is proposed to occur in four steps (Mu et al., 1992): hydroxylation of tyrosine to dopa, oxidation of dopa to dopa quinone, hydration of dopa quinone to topa, and oxidation of topa to topa quinone.

Conversion of peptidyl tyrosine to dopa has been well-documented in several naturally occurring proteins, such as the eggshell protein from a liver fluke (Waite & Rice-Ficht, 1987, 1989) and the adhesive protein of a mussel (Waite, 1983). This process is generally believed to occur through posttranslational modification of tyrosine residues catalyzed by a tyrosine hydroxylase-like or a tyrosinase-like activity. Although some sequence similarity is seen in dopa-containing peptides isolated from the eggshell protein (Waite & Rice-Ficht, 1987) or the mussel glue protein (Waite et al., 1985), there does not appear to be interspecies conservation of sequence. If the modification of tyrosine in amine oxidases is enzyme-catalyzed, the enzyme(s) would therefore be expected to have higher specificity than the enzymes specific for dopa formation. We have noticed that *two* Asn-Tyr-Glu sequences are present in the gene of the yeast methylamine oxidase (Bruinenberg et al., 1989), implying that the amino acid sequence alone is not sufficient to determine the specificity of recognition. This conclusion is further supported by the finding that the consensus sequence Asn-Tyr-Asp/Glu is present in a large number of proteins which are unlikely to contain quinone moieties in their mature polypeptide chains (Mu, 1993).

As an alternative to the use of a class of enzymes to convert protein-bound tyrosine to topa, Mu et al. (1992) have proposed that this process may be catalyzed by copper bound near the active site of copper amine oxidases. In this later mechanism,

referred to as self-processing, ring hydroxylation would be achieved by a bound copper-hydroperoxide in the folded protein precursor (eq 1). Oxidation of dopa to dopa quinone, followed



(1)

by bond rotation about the β-carbon, would place copper hydroxide adjacent to C2 of the mature cofactor. This proximity would be expected to greatly facilitate a nucleophilic attack by copper hydroxide to the intermediate dopa quinone, yielding topa. These postulated functions for copper require the folding of the polypeptide to a correct conformation and the positioning of copper near C2 of the final topa. In fact, such a proximity of copper to the cofactor has been implicated by McGuirl et al. (1991) on the basis of NMR (Williams & Falk, 1986) and EPR studies (Greenaway et al., 1991). It is noteworthy that the formation of dopa from a tyrosine residue has been shown to occur in a ribonucleotide reductase mutant F208Y (Ormö et al., 1992; Åberg et al., 1983); the observed chemistry has been proposed to arise from a ferric hydroperoxide dependent hydroxylation of the tyrosine residue located in the closest proximity to the metal center. In the present study we demonstrate clearly that topa quinone forms at the correct position of the sequence of the recombinant amine oxidase. The fact that this occurs in *S. cerevisiae*, which itself is unable to metabolize amines and appears to lack quinoproteins altogether, points toward a self-processing mechanism for topa quinone. Studies are in progress to address this intriguing process in greater detail.

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REFERENCES

Åberg, A., Ormö, M., Nordlund, P., Sjöberg, B.-M. (1993) *Biochemistry 32*, 9845–9850.

Bardlsey, W. G., Crabbe, M. J. C., & Schindzer, J. S. (1973) *Biochem. J. 131*, 459–469.

Barnett, J. A., Payne, R. W., & Yarrow, D. (1983) in *Yeasts: Characteristics and Identification*, Cambridge University Press.

Becker, D. M., Fikes, J. D., & Guarente, L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1968–1972.

Brown, D. E., McGuirl, M. A., Dooley, D. M., Janes, S. M., Mu, D., & Klinman, J. P. (1991) *J. Biol. Chem. 266*, 4049–4051.

Bruinenberg, P. G., Evers, M., Waterham, H. R., Kuipers, J., Arnberg, A. C., & Ab, G. (1989) *Biochim. Biophys. Acta 1008*, 157–167.

Cooper, R. A., Knowles, P. F., Brown, D. E., McGuirl, M. A., & Dooley, D. M. (1992) *Biochem. J. 288*, 337–340.

de Hoop, M. J., Valkema, R., Kienhuis, C. B. M., Hoyer, M. A., & Ab, G. (1992) *Yeast 8*, 243–252.

Flannery, A. V., Beynon, R. J., & Bond, J. S. (1989) in *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J., & Bond, J. S., Eds.) pp 145–162, IRL Press, Oxford, England.

Graham, D. G., Tiffany, S. M., Bell, W. R., & Gutknecht, W. F. (1978) *Mol. Pharmocol. 14*, 644–653.

Green, J., Haywood, G. W., & Large, P. J. (1982) *J. Gen. Microbiol. 128*, 991–996.

Greenaway, F. T., O'Gara, C. Y., Marchena, J. M., Poku, J. W., Urtiaga, J. G., & Zou, Y. (1991) *Arch. Biochem. Biochys. 285*, 291–296.

Hartmann, C., & Klinman, J. P. (1991) *Biochemistry 30*, 4605–4611.

Hartmann, C., Brzovic, P., & Klinman, J. P. (1993) *Biochemistry 32*, 2234–2241.

Ito, H., Fukada, Y., Murata, K., & Kimura, A. (1983) *J. Bacteriol. 153*, 163–168.

Janes, S. M., & Klinman, J. P. (1991) *Biochemistry 30*, 4599–4605.

Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., & Klinman, J. P. (1990) *Science 248*, 981–987.

Janes, S. M., Palcic, M. M., Scaman, C. H., Smith, A. J., Brown, D. E., Dooley, D. M., Mure, M., & Klinman, J. P. (1992) *Biochemistry 31*, 12147–12154.

Klinman, J. P., & Mu, D. (1994) *Annu. Rev. Biochem.* (in press).

Klinman, J. P., Dooley, D. M., Duine, J. A., Knowles, P. F., Mondovi, B., & Villafranca, J. J. (1991) *FEBS Lett. 282*, 1–4.

Laemmi, U. K. (1970) *Nature 227*, 680–685.

Large, P. J. (1986) *Yeast 2*, 1–34.

McGuirl, M. A., Brown, D. E., McCahon, C. D., Turawski, P. N., & Dooley, D. M. (1991) *J. Inorg. Biochem. 43*, 186.

McIntire, W. S., & Hartmann, C. (1993) in *Principles and Applications of Quinoproteins* (Davidson, V. L., Ed.) pp 97–171, Marcel Dekker, Inc., New York.

Mu, D. (1993) Ph.D. Thesis, University of California, Berkeley.

Mu, D., Janes, S. M., Smith, A. J., Brown, D. E., Dooley, D. M., & Klinman, J. P. (1992) *J. Biol. Chem. 267*, 7979–7982.

Mu, D., Medzihradszky, K. F., Adams, G. W., Mayer, P., Hines, W. M., Burlingame, A. L., Smith, A. J., Cai, D., & Klinman, J. P. (1994) *J. Biol. Chem. 269*, 9926–9932.

Mure, M., & Klinman, J. P. (1993) *J. Am. Chem. Soc. 115*, 7117–7127.

Neumann, R., Harvey, R., & Abeles, R. H. (1975) *J. Biol. Chem. 250*, 6362–6367.

Ormö, M., deMaré, F., Regnström, K., Aberg, A., Sahlin, M., Ling, J., Loehr, T. M., Sanders-Loehr, J., & Sjöberg, B.-M. (1992) *J. Biol. Chem. 267*, 8711–8714.

Paz, M. A., Flückiger, R., Boak, A., Kagan, H. M., & Gallop, P. M. (1991) *J. Biol. Chem. 266*, 689–692.

Schneider, J. C., & Guarente, L. (1991) *Methods Enzymol. 192*, 373–388.

Sherman, F. (1991) *Methods Enzymol. 194*, 3–21.

Waite, J. H. (1983) *J. Biol. Chem. 258*, 2911–2915.

Waite, J. H., & Rice-Ficht, A. C. (1987) *Biochemistry 26*, 7819–7825.

Waite, J. H., & Rice-Ficht, A. C. (1989) *Biochemistry 28*, 6104–6110.

Waite, J. H., Housley, T. J., & Tanzer, M. L. (1985) *Biochemistry 24*, 5010–5014.

Wilkinson, J. M. (1986) in *Practical Protein Chemistry—A Handbook* (Darbre, A., Ed) pp 121–148, John Wiley & Sons Ltd., New York.

Williams, T. J., & Falk, M. C. (1986) *J. Biol. Chem. 261*, 15949–15954.