Tyrosine Codon Corresponds to Topa Quinone at the Active Site of Copper Amine Oxidases*

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The recently discovered organic cofactor of bovine serum amine oxidase, topa quinone, is an uncommon amino acid residue in the polypeptide backbone (Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., and Klinman, J. P. (1990) Science 248, 981–987). The amine oxidase gene from the yeast Hansenula polymorpha has been cloned and sequenced (Bruinenberg, P. G., Evers, M., Waterham, H. R., Kuipers, J., Arnberg, A. C., and Geert, A. B. (1989) Biochim. Biophys. Acta 1008, 157–167). In order to understand the incorporation of topa quinone in eukaryotes, we have isolated yeast amine oxidase from H. polymorpha. Following protocols established with bovine serum amine oxidase, yeast amine oxidase was derivatized with [14C]phenylhydrazine, followed by thermolytic digestion and isolation of a dominant radio-labeled peptide by high pressure liquid chromatography. Comparison of resonance Raman spectra for this peptide to spectra of a model compound demonstrates that topa quinone is the cofactor. By alignment of a DNA-derived yeast amine oxidase sequence with the topa quinone-containing peptide sequence, it is found that the tyrosine codon, UAC, corresponds to topa quinone in the mature protein. In a similar manner, alignment of a tryptic peptide from bovine serum amine oxidase implicates tyrosine as the precursor to topa quinone in mammals.

Unlike the majority of enzymatic redox cofactors, the recently discovered organic cofactor of bovine serum amine oxidase (BSAO), topa quinone, resides in the polypeptide backbone (1). As discussed previously, two hypotheses can be advanced for topa incorporation, involving either a post-translational modification of a phenylalanine or tyrosine precursor or a direct incorporation into the enzyme via a unique tRNA (1). To distinguish these possibilities, we have compared the sequence of an active site, topa quinone-containing peptide with its corresponding DNA-derived sequence. While the cloning of a BSAO gene has not yet been accomplished, the yeast amine oxidase (YAO) gene from Hansenula polymorpha has been cloned and sequenced (2). Based on the size of the open reading frame (692 amino acids), Bruinenberg et al. (2) proposed that YAO belongs to the same class of copper amine oxidases (EC 1.4.3.6) as BSAO (2).

As described herein, we have confirmed that YAO from H. polymorpha is copper-containing, at a stoichiometry of one copper atom per enzyme subunit. Inhibition of enzyme with [14C]phenylhydrazine, followed by proteolysis with thermolysin (1), has led to the isolation of the cofactor-containing peptide. Comparison of resonance Raman spectra for this peptide to a model compound indicates that topa quinone is the active site cofactor in YAO. Alignment of the amino acid sequence of the isolated peptide with the homologous segment in the YAO DNA allows us to conclude that tyrosine is the precursor to topa quinone in eukaryotes. As a result of these findings, post-translational modification pathways for the biogenesis of topa quinone can be postulated.

EXPERIMENTAL PROCEDURES

Cell Growth and Lysis—H. polymorpha CBS 4732 was purchased from the American Type Culture Collection. The expression of YAO in the methylotrophic yeast Hansenula polymorpha is sensitive to the growth conditions (3). Elevated activities of the amine oxidase are obtained in mineral media containing diethyamine as the sole nitrogen source and glucose as the carbon source (3). Yeast were grown in 4-liter Erlenmeyer flasks containing 1.5 liters of a medium with the following composition (4): KH2PO4, 4.5 g; MgSO4·7H2O, 0.3 g; trace element solution (5), 0.3 ml; yeast extract, 0.75 g; 4 M diethylamine, 15 ml; 25% glucose (w/v), 15 ml; and water, 1470 ml. Cells were harvested at midexponential phase (A600nm = 1.25). In the preparation of cell-free extracts, 1.5 liters of culture medium was centrifuged at 3000 × g for 30 min and washed twice with water. The final pellet was resuspended in 15 ml of 50 mM potassium phosphate buffer, pH 7.0. This suspension was sonicated at 20 KHz for 5 cycles (16 s/cycle) at 0 °C with an MSE 100W ultrasonic disintegrator (M.S.E. Ltd., London). Whole cells and debris were removed by centrifugation of the crude sonicated suspension at 5000 × g for 30 min. The supernatant, which contained 6–8 mg of protein/ml, was used for the assay of YAO activity and for further purification.

Enzyme Purification and Assay—Yeast amine oxidase was isolated according to the method of Haywood and Large (6). Crude cell extracts (18 units, 0.004 units/mg) were purified 98-fold to yield 2.0 units of YAO (0.39 units/mg). Protein concentration was determined by the dye-binding method of Bradford (7). In initial studies, methyamine and benzylamine were found to be interchangeable in the assays of amine oxidase-containing fractions. Subsequent studies were restricted to benzylamine, which affords a rapid spectrophotometric assay for enzyme activity (8). SDS gel electrophoresis was performed on purified enzyme using 7.5% polyacrylamide containing dithialtartardiamide as cross-linker (9); Coomassie Blue staining was used to visualize protein bands. The incorporation of radioactivity into protein samples inactivated with [14C]phenylhydrazine was determined by solubilizing gel slices with 0.5 ml of 10% (w/v) periodic acid. After incubation at room temperature for 1–2 h and vigorous mixing, a water-miscible scintillation mixture was added and the samples counted (10). Copper analyses were performed by the method of standard addition, according to the procedures of Klinman et al. (11).

Peptide Isolation and Characterization—Using protocols established with BSAO (1), [14C]phenylhydrazine-labeled YAO was di-
ggested with thermolysin, followed by the purification and analysis of peptide fragments by HPLC. A tryptic peptide of BSA oxidase was also isolated, using conditions analogous to those published for thermolytic digestion of BSA oxidase. Gas-phase sequencing of peptides was carried out on an Applied Biosystems 470 with an on-line HPLC in the presence of Polybrene. Sample sizes were in the range of 0.2 nmol of peptide. Acquisition of resonance Raman spectra was as previously described in the literature (12).

Sequence Alignment—Alignment of the YAO active site peptide with the available DNA-derived protein sequence for H. polymorpha YAO (2) was accomplished on a Macintosh SE/30 computer using the program MacMolly (version 3.0). Test sequences by the active site peptide were Val-Ala-Asn-X-Glu-Tyr-Val (see "Results and Discussion"), where each of the 20 amino acids was inserted into the unknown position. Although no overlapping sequences could be found containing 0 or 1 mismatches, setting the number of mismatches to 2 led to a unique alignment.

RESULTS AND DISCUSSION

The results of SDS gel electrophoresis of purified YAO are shown in Fig. 1. Coomassie Blue staining indicated a dominant band at 80 kDa, which is very close to a mass of 77-78 kDa estimated by gene sequencing (2). In light of the presence of several trace protein bands, we felt it was essential to demonstrate that the reaction of [14C]phenylhydrazine with protein preparations was specific for YAO. SDS gel electrophoresis was therefore repeated on 14C-labeled protein. Following elution and counting of protein bands, it could be shown that 94% of the initial radioactivity was recovered at 80 kDa, with no detectable radioactivity in the minor bands.

Initial studies of YAO were directed at establishing that enzyme isolated from H. polymorpha belongs to the same class of copper amine oxidases (EC 1.4.3.6) as BSAO. Copper analyses were performed on purified protein. As shown in Fig. 2, the intercept of a copper standard curve was significantly elevated in the presence of YAO, relative to a standard curve in the absence of added protein (Fig. 2, inset). From these data, YAO can be calculated to contain 1.09 ± 0.10 mol of copper per enzyme subunit. Analogous to BSAO (13), active site titration of YAO with phenylhydrazine was found to be close to stoichiometric (0.80 ± 0.02) and to produce a new absorbance band at 447 nm (λmax = 448 nm for the phenylhydrazone of BSAO (1)). Thermolytic digestion of the [14C]phenylhydrazone of YAO was conducted according to protocols established for BSAO (1). The elution profile of a YAO digest is shown in Fig. 3, revealing a major chromophoric (Fig. 3A) and radiolabeled peak (Fig. 3C), which elutes near the end of the gradient. This peak was further purified by HPLC, prior to analysis. Peptide sequencing has indicated a heptapeptide of the following sequence, Val-Ala-Asn-X-Glu-Tyr-Val. In order to identify the nature of the unknown, a resonance Raman spectrum of the peptide was compared with a spectrum of a model compound, the hydantoin of topa quinone which has been derivatized with phenylhydrazine (1). As shown in Fig. 4, these spectra were found to be essentially identical with regard to peak frequencies and intensities, providing clear-cut evidence for the presence of topa quinone at the YAO active site.

Efforts to identify the topa quinone codon began with a

Fig. 1. Electrophoretic analysis of yeast amine oxidase purified from H. polymorpha. Lane 1, a molecular mass marker composed of carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), fructose-6-phosphate kinase (84 kDa); lane 2, purified YAO (with an arrow).

Fig. 2. Determination of copper contents of YAO by the standard addition method. 20 μl of 0.11 μM YAO in 0–0.787 μM copper nitrate solutions was analyzed for total copper content on a Perkin-Elmer atomic absorption spectrophotometer, model 360 equipped with an HGA-2200 graphite furnace. Protein concentration was determined by the dye binding assay of Bradford (7). Inset, correspondence between standard solutions of copper nitrate and absorbance.

3 On a Vydac C18 HPLC column, with a trifluoroacetic acid gradient consisting of solvent A (0.11% trifluoroacetic acid and 5% v/v acetonitrile) and solvent B (0.10% trifluoroacetic acid and 80% v/v acetonitrile), solvent B was increased to 12.5% at 10 min, 50% at 70 min, and 100% at 80 min. Four nmol of pure, radiolabeled peptide were isolated for a final yield of 15%.
Codon Identification for Topa Quinone

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Table I

Comparison of the amino acid sequence of topa quinone-containing peptides from BSAO and YAO with the homologous DNA-derived YAO sequence

| Peptide isolated from tryptic digest of BSAO | DNA-derived YAO sequence | Topa quinone-containing peptide isolated from YAO |
|--------------------------------------------|--------------------------|--------------------------------------------------|
| Ser                                       | Gly                      | Ser                                               |
| Val                                       | Ile                      | Val                                               |
| Ser                                       | Phe                      | Ser                                               |
| Asp                                       | Thr                      | Asp                                               |
| Met                                       | Ala                      | Met                                               |
| Leu                                       | Ala                      | Leu                                               |
| Asn                                       | Asn                      | Asn                                               |
| Topa                                      | Tyr                      | Topa                                             |
| Asp                                       | Glu                      | Asp                                               |
| Tyr                                       | Tyr                      | Tyr                                               |
| Val                                       | Cys                      | Val                                               |
| Unknown                                   | Leu                      | Unknown                                           |
| Asp                                       | Tyr                      | Asp                                               |
| Met                                       | Try                      | Met                                               |
| Val                                       | Val                      | Val                                               |
| Phe                                       | Phe                      | Phe                                               |
| Tyr                                       | Met                      | Tyr                                               |
| Pro                                       | Gln                      | Pro                                               |
| Asn                                       | Asp                      | Asn                                               |
| Gly                                       | Gly                      | Gly                                               |
| Ala                                       | Ala                      | Ala                                               |
| Ile                                       | Ile                      | Ile                                               |
| Glu                                       | Arg                      | Glu                                               |

* Enzyme which had been derivatized with [14C]phenylhydrazine, was brought to a m urea and placed on a shaker bath at 37°C. Protein was initiated by the addition of 2% w/w trypsin. The course of proteolysis was monitored by small scale HPLC injections of digest. When the reaction was judged to be complete, the digest was filtered with phenylmethylsulfonyl fluoride and large scale purification initiated. Digested enzyme was injected onto a Dynamax-Cs column equilibrated with 0.1% trifluoroacetic acid containing 80% v/v acetonitrile (solvent A). Peptides were eluted using a linear gradient to 75% solvent B (0.1% trifluoroacetic acid containing 80% v/v acetonitrile). A single, dominant radiolabeled peak was observed at 43 min which contained 17% of the original counts. In micro-Edman sequencing of this peptide, the average yield per residue was 70% of the expected value based on carbon-14 recovery.

† The numbers refer to the DNA-derived sequence of YAO (2).

‡ The yield of Val, Ala, Asn, Glu, Tyr, and Val was 36, 80, 109, 37, 41, and 35 pmol, respectively. 35–40% of the total radioactivity released on sequencing the [14C]phenylhydrazine derivative of peptide was found at round four, which also released an unidentified phenylthiohydantoin derivative.

An unidentified phenylthiohydantoin derivative (without radioactivity) was released after this round of the micro-Edman sequencing.

YAO sequence using the computer program MacMolly. As indicated in Table I, there is identity of residues at positions 403, 404, 406, and 407 (shown as underlined). The difference observed at position 402, Val versus Ala, has been confirmed in several preparations of the YAO peptide and suggests the existence of YAO isozymes with modest differences. The difference at position 406, Val versus Cys, is more puzzling, since all available copper amine oxidase peptides indicate Val in this position. This property initially suggested to us an error in the DNA sequence; however, re-examination of DNA sequencing gels clearly indicates Cys at position 408. Since the DNA sequence for YAO is derived from a genomic clone, an error in reverse transcriptase action can also be eliminated. More work will be needed to clarify this discrepancy.

Turning to position 405 of the DNA-derived YAO sequence,

4 S. M. Janes, M. Palcic, A. J. Smith, D. E. Brown, D. M. Dooley, and J. P. Klinman, manuscript in preparation.

5 A. B. Geert, personal communication.
the presence of Tyr implicates this residue as the precursor to topa quinone in the mature form of eukaryotic amine oxidases. To our knowledge, this represents the first example of an enzyme cofactor arising from functional group additions to an existing peptide-bound amino acid precursor in higher organisms. In early studies of prokaryotes, Snell and coworkers (13) demonstrated the generation of protein-bound pyruvoyl residues, arising from the dehydroxylation of an active site serine to dehydroalanine and subsequent strand scission. More recently, pyruvoyl residues have been implicated in mammalian decarboxylases (cf. Ref. 14). Two reports have appeared in the very recent literature documenting the presence of a derivatized tyrosine (as a thioether to a cysteine) in galactose oxidase from the fungus D. rottendorfii (15) and a derivatized tryptophan (as a quinone, joined to a second tryptophan) in melamine dehydrogenase from several methylotrophic bacteria (16).

The exact pathway for topa quinone biogenesis is of considerable interest, in particular whether this is catalyzed by a new class of oxidative enzymes or whether the copper amine oxidases are capable of self-processing. As shown in Scheme I, a likely pathway for the generation of topa quinone from Tyr involves initial hydroxylation of Tyr to dopa, followed by oxidation of dopa to dopaquinone. The $\alpha,\beta$-unsaturated ketone structure of dopaquinone would be expected to promote a hydrolysis reaction at position 6 of dopa, producing topa. Further oxidation of topa to topaquinone is expected to occur rapidly under aerobic conditions. Several enzymes have been described which catalyze the hydroxylation of Tyr to dopa, tyrosine hydroxylase (17), and tyrosinase (18). In the case of tyrosine hydroxylase, activity is believed to be restricted to free tyrosine, making it an unlikely candidate for step 1 in Scheme I. By contrast, tyrosinasases have been shown (i) to act on peptide-bound tyrosine and (ii) to catalyze both initial dopa formation and its subsequent oxidation to dopaquinone. However, tyrosinasases are reported to be restricted to melanocytes, whereas topa quinone-containing enzymes are likely to arise in a range of tissues. This raises the possibility of a new class of oxidative enzymes, performing the catalytic functions outlined in Scheme I.

Alternatively, the copper amine oxidases may be capable of self-processing. The presence of copper in the active site of amine oxidases is well documented (cf. Ref. 19 and data herein), with distance-mapping experiments supporting a placement of copper near the C-2 position of topa quinone (20, 21). It is conceivable that a binding of hydroperoxide to copper in unmodified protein would support an initial ring hydroxylation of Tyr to dopa. Subsequent oxidation of dopa to dopaquinone, followed by C-C bond rotation would place the C-2 ring carbon in close proximity to a copper-bound hydroxide ion. Given the predicted susceptibility of dopaquinone to nucleophilic attack, dopa hydration by Cu(OH) would be expected to lead readily to topa quinone.

From the above discussion, it is clear that at least two post-translational modification pathways can be considered as viable alternatives for topa quinone biogenesis. A major challenge for the future will be the design of experimental protocols for the identification and characterization of the physiologically relevant pathway.

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