Evidence for a Self-catalytic Mechanism of
2,4,5-Trihydroxyphenylalanine Quinone Biogenesis in Yeast
Copper Amine Oxidase*

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Danying Cai and Judith P. Klinman
From the Department of Chemistry, University of California, Berkeley, Berkeley, California 94720-1460

Copper amine oxidases are representative of a new class of redox enzymes that contain a peptide-bound quinone cofactor, generated by posttranslational modification of amino acid side chains(s). We have investigated the mechanism for the biogenesis of 2,4,5-trihydroxyphenylalanine quinone (TPQ) in amine oxidase with two site-specific mutants of the yeast methylamine oxidase. Our results show that the capacity for TPQ formation in vivo is abolished when a putative ligand to copper, His-456, is changed to Asp; this H456D mutant binds copper at a low level (~4.1%), relative to the wild-type protein. In contrast, altering the active site consensus sequence that contains the precursor tyrosine does not affect TPQ production. The data implicate a self-catalysis mechanism for TPQ biogenesis, in which the protein-bound copper plays a key role. We propose that the minimal information required for TPQ biogenesis lies in a structural motif consisting of the copper site and the precursor tyrosine.

2,4,5-Trihydroxyphenylalanine quinone (topa quinone, or TPQ) is the redox cofactor for copper amine oxidases, that catalyze the oxidative deamination of primary amines (1). In the catalytic cycle of amine oxidases, TPQ is reduced by substrate amine to an aminophenol species, which is reoxidized back to TPQ by molecular oxygen (2). This novel cofactor represents a unique class of quinone cofactors that are derived by posttranslational modification of polypeptide-bound amino acid side chain(s) (3). It has been established firmly that the precursor for TPQ is a tyrosine residue contained in an active site consensus sequence which contains the precursor tyrosine in water, the standard addition method according to the procedures described for the WT protein (6) with the following modifications in the purification procedure. The DEAE resin was DEAE-Sepharose CL-4B from Pharmacia Biotech Inc. Following DEAE chromatography, the concentrated sample was separated further on a gel filtration column (75 cm long, 1.5 cm in diameter) packed with Sephacryl S-300 HR (Pharmacia Biotech Inc.). The column was pre-equilibrated with 10 mM potassium phosphate, pH 6.7, and washed with the same buffer to elute proteins. The most active fractions were pooled, concentrated, and stored at −20 °C. This pool of protein samples essentially appeared >90% pure, as judged on SDS-polyacrylamide gels. Less active fractions were concentrated and reloaded onto the gel filtration column. The fast protein liquid chromatography step described in the original procedure (6) was not included for the purification of E406N protein because we found that it did not increase the enzyme specific activity in the final purified protein preparations. In the case of H456D mutant where no enzyme activity was detected, fractions from each step and the protein purity were analyzed by SDS-polyacrylamide gel electrophoresis.

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† To whom correspondence should be addressed. Tel.: 510-642-2668; Fax: 510-643-6232.
‡ The abbreviations used are: TPQ, 2,4,5-trihydroxyphenylalanine (topa) quinone; WT, wild-type.

Copper and Protein Concentration Determination—Copper content was analyzed by the standard addition method according to the procedure described previously (6). The purified and dialyzed H456D protein was diluted with distilled and deionized water to a final concentration of 16.7 µg/ml with copper standard (Fisher) added to 0–28 ng/ml. Due to the instability of the mutant protein in water, the standard addition
was also performed in the following way: For each reading, 0–42 ng of copper standard prepared in 16 μl of deionized water or 0.05 M HNO₃ was injected into the graphite tube, followed by 10.5 μg of the purified H456D protein sample in 4 μl of 10 mM potassium phosphate, pH 6.7. The stoichiometry of protein-bound copper was calculated based on a molecular mass of 151.5 kDa for the dimer (see *Results and Discussion*) and protein concentrations determined using the Bio-Rad protein assay with bovine serum albumin as the standard.

**Titrations with Phenylhydrazine and Kinetic Measurements**—Methods for the titration with phenylhydrazine and for measuring ethylenediaminetetraacetic acid and benzamide oxidation were as reported (5). The quinone stoichiometry of the E406N mutant protein was calculated based on a molecular mass of 152 kDa for the dimer and corrected for protein purity. The steady-state kinetic parameters were calculated by fitting data to the Michaelis-Menten equation using nonlinear regression.

**RESULTS AND DISCUSSION**

**Mutation in the Copper Binding Site**—Three histidines have been implicated as ligands to the copper in copper amine oxidases from spectroscopic investigations (9–11). We have found recently that 3 histidine residues are highly conserved in the primary sequences of copper amine oxidases at a fixed distance from the active site TPQ (5). Two such histidines are present as His-X-His, a motif well known to contain copper ligands in several copper proteins (12). In the protein sequence of the yeast methylamine oxidase from *H. polymorpha*, this motif is contained in the sequence His-Asn-His-Gln-His. The middle histidine His-456 is most certainly one of the copper ligands, although without a three-dimensional structure we cannot be certain which of the other 2 histidines performs a similar role (5).

We decided first to change the copper ligand field by replacing His-456 with an aspartic acid residue by site-directed mutagenesis. The mutated gene was expressed in *S. cerevisiae* and the protein purified. We found that the mutant protein was expressed to a level comparable with that of the WT protein based on analyses by SDS-polyacrylamide gel electrophoresis. The H456D mutant protein was eluted from the anion exchange and gel filtration columns in a manner virtually identical to that of the WT enzyme throughout the purification process. The peaks for the purified mutant protein are highly conserved, indicative of similar folding of the mutant polypeptide and that the protein remains as a dimer analogous to the WT enzyme.

We found that H456D protein does not exhibit any amine oxidase activity and, furthermore, contains no quinone moiety detectable by the redox-cycling staining method (13). Copper analyses indicate a low level (~4.1% of wild type), suggesting a greatly reduced affinity for Cu(II) in this mutant protein. These results indicate that disruption of the native copper binding site generates a protein incapable of cofactor production. Consistent with the lack of quinone cofactor, the H456D protein lacks the characteristic pink color and absorption peak at 472 nm associated with the active WT enzyme (6) (Fig. 1).

To confirm the identity of the purified mutant protein, the N terminus of the protein was sequenced with a highly purified sample. The first 19 amino acid residues from the N terminus were identified as N-Ala-Pro-Ala-Arg-Pro-Ala-His-Pro-Leu-Asp-Pro-Leu-Ser-Thr-Ala-Glu-Ile-Lys-Ala.

The first residue corresponds to the residue at position 17 in the DNA-derived protein sequence of the yeast methylamine oxidase (7). This N-terminal sequence and the starting residue were identical to the sequence His-Asn-His-Gln-His contained in the sequence His-Asn-His-Gln-His. The middle histidine His-456 is most certainly one of the copper ligands, although without a three-dimensional structure we cannot be certain which of the other 2 histidines performs a similar role (5).

**Mutation in the Consensus Sequence**—The above conclusion is substantiated by findings with another mutant protein in which the active site consensus sequence is altered. The glutamic acid residue linked to the carboxyl group of the aspartate residue at the mutated site continues to serve as a ligand to copper in H456D protein is presently unknown. In any case, the most profound effect of the change in the copper ligand field is the absence of the redox cofactor TPQ in the protein. The fact that the redox-cycling staining assay did not detect any reactive quinone moiety on the H456D protein indicates that the modification of tyrosine did not occur at all, since the redox-cycling staining method could also detect 3,4-dihydroxyphenylalanine (dopa) (13), the first intermediate in the proposed scheme for TPQ biogenesis (4). It is clear that TPQ biogenesis requires the presence of a functional copper binding site.

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Origin of Topa Quinone in Yeast Copper Amine Oxidase

Table I
Steady-state kinetics of the wild-type and the mutant E406N yeast methylamine oxidase

| Enzyme               | $k_{m} \pm 0.07$ | $k_{m}/K_m$ | $K_m$ | $K_{m}/k_m$ |
|----------------------|------------------|--------------|-------|-------------|
| Ethylamine           |                  |              |       |             |
| Wild-type            | 5.33             | 0.244        | 0.12  | 2.18        |
| E406N                | 4.26             | 0.259        | 0.023 | 1.64        |
| Benzylamine          |                  |              |       |             |
| Wild-type*           | (8.85 ± 0.24) × 10^-4 | 0.682 | 0.058 | 130         |
| E406N                | (9.93 ± 0.33) × 10^-4 | 3.94  | 0.34  | 25.2        |

* Data have been reported previously (6).

The physical and kinetic properties of the E406N mutant was compared with the WT enzyme. The results show that consistent with the WT enzyme, E406N mutant is more active in catalyzing amine oxidation than benzylamine (Table I). The mutation appears to have no effect on the kinetics of ethylamine oxidation but increases the $K_m$ for benzylamine, resulting in a 5-fold reduction in $k_{m}/K_m$ for benzylamine. The most dramatic difference between these two enzymes is in the oxidation of methylamine. E406N enzyme is inactivated after 19–21 turnovers, although the initial velocity and $K_m$ for methylamine appear to be similar to that of the WT enzyme under the same conditions. We presently do not know the mechanism for such an inactivation. Nonetheless, the kinetic data indicate that the glutamate residue in the consensus sequence is not required for the amine oxidase activity, ruling out the possibility that the carboxyl side chain of this residue functions as the active site base to catalyze proton abstraction from the substrate Schiff base to form a product Schiff base in amine oxidation (17). This conclusion most likely holds true for those amine oxidases that have an aspartate in the active site consensus sequence (18).

The table provides data on the kinetic parameters of the wild-type and E406N mutant enzymes for different substrates. The mutant enzyme shows a 5-fold reduction in $k_{m}/K_m$ for benzylamine compared to the wild-type enzyme, indicating a more efficient catalytic process.

Table II
Active site peptide sequences of the wild-type yeast methylamine oxidase and the E406N mutant protein and the E406N DNA-derived mutant protein sequence

| Wild-type peptide* | Peptide DNA-derived* |
|--------------------|----------------------|
| Thr                | Thr                 |
| Ala                | Ala                 |
| Ala                | Ala                 |
| Asn                | Asn                 |
| blank*             | blank*              |
| Blank*             | Blank*              |
| Glu                | Asn                 |
| Tyr                | Tyr                 |
| CmCys*             | CmCys*              |
| Leu                | Leu                 |
| Tyr                | Tyr                 |
| Tyr                | Tyr                 |

* The sequence has been reported previously (6).

The numbers are the residue number on the cDNA-derived protein sequence (7).

An unidentified phenylthiohydantoin-derivative.

CmCys is a carboxymethylated cysteine residue.

A possible modification site is 51 amino acid residues from the precursor. These considerations argue strongly against the enzymatic mechanism in which a separate class of enzyme(s) catalyze the posttransla-
tional modification of tyrosine in the copper amine oxidase.

The importance of the protein-bound copper for TPQ biogenesis is supported further by the demonstration that the H456D mutant, with its altered copper ligand field, is devoid of TPQ. This observation supports the previously proposed function for protein-bound copper in a self-catalytic mechanism for TPQ biogenesis (6). It is known that the protein-bound copper is absolutely required for the activity of copper amine oxidases (20, 21).

It has been proposed that copper is involved in the reoxidation of the reduced TPQ cofactor in the amine oxidase catalytic cycle, by mediating electron transfer between the reduced cofactor and molecular oxygen (22, 23). Here, a new role is implicated for the protein-bound copper, i.e. as a catalyst for tyrosine oxidation in the active site of the copper amine oxidases.

In all of the known sequences of copper amine oxidases, the 3 histidines that are ligands to copper and the TPQ precursor tyrosine residue are located invariantly on a stretch of sequence of less than 100 amino acid residues in the carboxyl half of the protein sequence, and the spacing between these histidines and the tyrosine is highly conserved (3). These findings suggest that the structure of the copper site relative to the TPQ has been preserved through evolution. In the present study, we have shown that it is essential to maintain a functional copper binding site in order to form TPQ. We therefore propose that the sequence containing all of the histidine ligands to copper and the TPQ precursor comprises a three-dimensional structural motif, which is essential for TPQ formation in the active site of copper amine oxidases.

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