Effect of Metal on 2,4,5-Trihydroxyphenylalanine (Topa) Quinone Biogenesis in the Hansenula polymorpha Copper Amine Oxidase*

(Received for publication, February 19, 1997, and in revised form, May 2, 1997)

Danying Cai‡, Neal K. Williams§, and Judith P. Klinman¶

From the Departments of Chemistry and Molecular and Cell Biology, University of California, Berkeley, California 94720

Previous studies of wild-type and mutant forms of a recombinant copper amine oxidase from Hansenula polymorpha, expressed in Saccharomyces cerevisiae, have indicated a self-processing mechanism for 2,4,5-trihydroxyphenylalanine (topa) quinone biogenesis involving the active site copper (Cai, D., and Klinman, J. P. (1994) J. Biol. Chem. 269, 32039–32042). In contrast to prokaryotic copper amine oxidases, however, it has not been possible to initiate topa quinone formation by the addition of exogenous copper to precursor H. polymorpha amine oxidase lacking copper. Metal analysis of copper-depleted wild-type enzyme reveals 0.2–0.3 mol copper, together with 0.6 mol zinc. Despite changes in the zinc and copper levels in growth media, the level of zinc in purified enzyme remains fairly constant. Further, we have been unable to displace protein-bound zinc by exogenously added copper. The H. polymorpha amine oxidase gene was subsequently expressed in Escherichia coli and found to be almost completely free of copper and zinc. In vitro reconstitution of this apoprotein confirms that zinc binds to H. polymorpha amine oxidase and prevents reconstitution with copper. By contrast, addition of copper first to apoprotein leads to formation of topa quinone and stable activity in the presence of added zinc. These findings indicate efficient binding of either zinc or copper to a site that undergoes little or no change. The data confirm that topa quinone biogenesis in the H. polymorpha system is catalyzed by copper and occurs in the absence of added factors. We conclude that the mechanisms of cofactor biogenesis in pro- and eukaryotic systems are likely to be similar or identical. The results described herein imply different pathways for the in vivo assembly of heterologously expressed amine oxidases in S. cerevisiae and E. coli.

Copper amine oxidases from all sources, which include bacteria, yeast, plant, and mammals, contain 2,4,5-trihydroxyphenylalanine (topa) quinone as the redox cofactor (1). It has been well established that the precursor for topa quinone is a peptidyl tyrosine residue contained in an active site consensus sequence, Asn-Tyr-Asp/Glu, and that the precursor tyrosine is converted to topa quinone by a post-translational modification process (2–4). Heterologous gene expression of a copper amine oxidase from Hansenula polymorpha in Saccharomyces cerevisiae has produced a functional recombinant enzyme with an active site indistinguishable from the native enzyme (5). Based on the fact that S. cerevisiae is one of the few yeast species that does not have an endogenous amine oxidase (6), we have concluded that the modification of the precursor tyrosine to topa quinone occurs through post-translational autoprocessing (5). A mechanism to account for the oxidation of the tyrosyl side chain through the involvement of a bound copper in precursor protein has been proposed (cf. Ref. 5). Site-directed mutagenesis studies of either the active site consensus sequence or the copper binding site support an essential role for copper in topa biogenesis via a self-catalytic mechanism (7).

The biogenesis of topa quinone has also been studied with a bacterial system in which the gene for phenethylamine oxidase or a histamine oxidase from Arthrobacter globiformis was expressed in Escherichia coli (8–10). These studies show that the recombinant protein produced in a copper-free state lacks the topa quinone cofactor. Subsequent addition of exogenous cupric ion to the purified protein under aerobic conditions leads to rapid formation of the active site topa quinone, as indicated by its characteristic absorption in the visible region and the appearance of enzyme activity.

Using the wild-type yeast copper amine oxidase from H. polymorpha expressed in S. cerevisiae, our preliminary study on the effect of copper has yielded very different results from the bacterial enzyme; specifically, we have been unable to perform in vitro reconstitution studies by addition of copper to topa-free protein. This raised the possibility of fundamentally different biogenetic pathways for TPQ formation in the prokaryotic and eukaryotic systems. Reported herein are the metal binding properties of the recombinant H. polymorpha amine oxidase expressed in S. cerevisiae or E. coli under normal or copper-depleted conditions, together with results from the in vitro reconstitution of inactive enzymes with either copper or zinc. Major differences between enzyme isolated from S. cerevisiae and E. coli expression systems are observed, which we attribute to different pathways for the in vivo assembly of metalloproteins in these species. From the properties of in vitro reconstitution studies of the H. polymorpha amine oxidase expressed in E. coli, we conclude that the mechanisms of cofactor biogenesis in pro- and eukaryotic systems are likely to be similar or identical.

EXPERIMENTAL PROCEDURES

S. cerevisiae Cell Culture and Protein Purification—Under normal growing conditions, S. cerevisiae strain CG179 bearing the expression vector for the H. polymorpha amine oxidase was maintained and cultured in synthetic minimal media supplemented with 50 mg/liter each adenine, histidine, and tryptophan and 75 mg/liter leucine (5). The synthetic minimal medium contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Fisher Scientific), 0.5% am-
Topa Quinone Biogenesis in a Eukaryotic Copper Amine Oxidase

Metal Analysis—The copper and zinc standard solutions were of atomic absorption grade purchased from Fisher Scientific. The copper content of the purified protein was determined by atomic absorption spectroscopy as described previously (5) and the zinc content by inducively coupled plasma (ICP) emission spectroscopy using a Perkin-Elmer Plasma 40 instrument. The copper line at 213.9 nm was used for the ICP measurement. The protein-bound copper was calculated by the standard addition method unless otherwise indicated. The zinc content was calculated against a zinc standard curve for 0–40 ppb of zinc.

In Vitro Reconstitution with Copper—The “Cu-free” H. polymorpha amine oxidase purified from S. cerevisiae was dialyzed against 5 mM sodium-HEPES, pH 7.0. For the direct incubation with copper, the Cu-free protein was further dialyzed against deionized water. Following 21 h of incubation at 25 °C with an equal molar concentration of CuSO$_4$ in deionized water, the protein solution was back-dialyzed against deionized water and analyzed for copper content, protein concentration, and the enzyme activity. The enzyme activity was assayed by monitoring the oxygen consumption rate at 25 °C in 3 mM ethylamine, 100 mM potassium phosphate, pH 7.2.

RESULTS AND DISCUSSION

Recombinant Protein Produced in S. cerevisiae under Copper-free Conditions—There was little difference in growth when S. cerevisiae cells were grown in the normal or the copper-free media, indicating that the trace amount of copper contained in the copper-free media was sufficient for the biosynthesis of essential copper enzymes, such as cytochrome c oxidase, to support the normal aerobic growth of the yeast. Consistent with this notion that the media contained trace copper, the purified H. polymorpha amine oxidase from the copper-free media was only partially copper-free. In general, based on the atomic absorption, 20–30% of purified protein was copper-bound, compared with near 100% for a normal enzyme preparation (Table I). The specific activity of such a protein preparation was also about 20% of the normal level. Although the protein-bound copper has been implicated in the catalytic cycle of copper amine oxidase (15, 16), the reduction in enzyme activity seen herein can be correlated with a reduction in topa quinone content. The absorption spectrum of a purified Cu-free enzyme sample, which was >85% pure as determined by SDS-polyacrylamide gel electrophoresis, displays a $A_{420}$ in the visible region identical to that of a normal enzyme (Fig. 1A; see Ref. 5). However, the absorbance is much less than that of the normal enzyme of similar purity, i.e. for a solution of 1 mg/ml, the absorbance is 0.007 for the Cu-free enzyme while it is 0.022 for the normal enzyme. Because the absorbance at 472 nm is proportional to the amount of topa quinone, a 70% reduction indicates a decrease of the topa quinone content in the Cu-free enzyme by ~70%. Reconstitution experiments were performed with the purified protein, produced in the copper-free medium. The results from the direct incubation (Table I) indicate that unlike the phenethylamine oxidase of A. globiformis (8), the incubation of the Cu-free protein with equimolar copper for up to 21 h did not increase the stoichiometry of the protein-bound copper or the enzyme-specific activity. In a step dialysis experiment with purified protein (see “Experimental Procedures”), the copper content was increased anomalously to greater than 1 copper/subunit when the protein was dialyzed in 0.1 μM or higher CuSO$_4$ solution (up to 4 Cu/subunit in 2.5 μM CuSO$_4$).
The concentration of metal ion per enzyme subunit was calculated based on the measured protein concentration without correction for purity. A direct comparison of the metal contents was possible because all samples had similar purity (>85%) as judged on SDS-polyacrylamide gels.

| Sample               | Metal content | Specific activity, units/mg (% of wild-type) |
|----------------------|---------------|---------------------------------------------|
| Cu-free wild-type    |               |                                             |
| Dialyzed in water    | 0.23          | 0.63 (22%)                                  |
| Incubated with Cu    | 0.20          | 0.42 (14%)                                  |
| Cu-free media        |               |                                             |
| From Cu-free media   | 0.20          | 0.0293 (26%)                                |
| From Cu-free and Zn-| 0.30          | 0.012 (11%)                                 |
| limited media        |               |                                             |
| From Cu-free and Zn-| 0.29          | NA                                          |
| rich media           |               |                                             |
| Normal wild-type     | 0.90          | 0.113 (100%)                                |
| H456D                | 0.065         | 0.085                                       |

The Cu-free wild-type protein was concentrated in 5 mM sodium-HEPES, pH 7.0. The Cu-free wild-type sample used for copper reconstitution (Preparation 1) was different from that analyzed for zinc (Preparation 2). The normal wild-type and H456D, obtained from regular culture media, were used in previous studies (5, 7).

At the same time, the enzyme-specific activity decreased by up to 92% in 2.5 μM CuSO₄. We attribute the excess copper to nonspecific binding of copper to the protein, which in turn is the cause of the observed enzyme inhibition.

Although the *H. polymorpha* amine oxidase expressed in *S. cerevisiae* was not truly copper-free, the overall effect of copper depletion on topa quinone formation agrees with results obtained with the phenethylamine and histamine oxidases of *A. globiformis* expressed in *E. coli*, *i.e.*, that copper is required for topa quinone formation in *vivo* (8, 10). However, the *in vitro* reconstitution appeared to be very different. While the generation of topa quinone is spontaneous with the bacterial enzyme following the addition of exogenous copper, it was not possible to carry out a similar experiment with *H. polymorpha* enzyme expressed in *S. cerevisiae*. This suggested the possibility of fundamental differences between the bacterial and the yeast enzyme with regard to the mechanism of *in vitro* reconstitution. As an alternative explanation, we examined the possibility that the metal sites of the Cu-free *H. polymorpha* amine oxidase were occupied by other transition metals.

The Zinc Content—There are a number of documented examples of the ability of zinc to bind to native copper sites (*e.g.*, in superoxide dismutase (17) and in azurin, (18)). In the present study, we used plasma emission spectroscopy to test for zinc in the Cu-free *H. polymorpha* amine oxidase samples, finding a significant level (~57%, Table 1) of zinc-bound protein.

Since all efforts to reconstitute *H. polymorpha* amine oxidase activity by addition of copper to crude cell extracts were unsuccessful, we concluded that zinc incorporation had occurred during protein production in *S. cerevisiae*. Subsequent alteration of zinc levels in the growth media was carried out in an effort to obtain zinc-free enzyme. These experiments showed a more critical dependence on zinc than copper for growth of *S. cerevisiae*. While the yeast was not compromised when copper was depleted in the media, growth was minimal when zinc was present at 1:200 of the normal concentration in the culture medium. The yeast required the presence of ≥ 10% of the normal zinc concentration, as defined in the yeast nitrogen base (11), to show some healthy growth. Only when zinc was 20% or more of the normal level did the optical density of the overnight culture approach that produced in the normal culture media (5, 7). Because of the essential role for zinc in the growth of *S. cerevisiae*, it has not been possible to study a form of *H. polymorpha* amine oxidase obtained from *S. cerevisiae* grown on a medium severely depleted in both copper and zinc. However, as shown in Table I, either a reduction of the zinc concentration in the medium to 20% of the normal level or an increase in zinc to 10 times its normal level had little effect on the binding of zinc by the *H. polymorpha* amine oxidase. Although zinc homeostasis has not been examined in any detail in *S. cerevisiae*, we consider it unlikely that these changes in extracellular zinc levels had any impact on the intracellular zinc concentration.

As controls, the zinc content was also analyzed for the wild-type *H. polymorpha* amine oxidase and for a mutant with reduced copper binding (H456D), indicating levels of 16 and 8%
of the subunit concentration, respectively (Table I). The failure of H456D to bind either zinc or copper at a significant level argues that zinc is binding to the copper site, as opposed to a second, adventitious site. Our inability to reconstitute the Cu-free *H. polymorpha* enzyme is thus attributed to pre-binding of zinc in vivo, which prevents the *in vitro* incorporation of copper and the formation of TPQ.

**E. coli Expression System**—The two prokaryotic amine oxidases that have been reconstituted *in vitro* from inactive precursors have each been expressed in *E. coli* (8, 10). To investigate whether the observed zinc incorporation into the *H. polymorpha* amine oxidase was enzyme- or expression host-specific, an *E. coli* expression system was developed for the *H. polymorpha* amine oxidase. Cells were transformed with pKw3, and exogenous protein expression was induced with 1 mg IPTG (in M9ZB plus 100 μg/ml ampicillin medium not depleted in copper). SDS-polyacrylamide gels of the crude cell lysate showed a new protein band with a molecular mass of 76 kDa, as expected for *H. polymorpha* amine oxidase. Purification of this 76-kDa species based on the method developed for *H. polymorpha* amine oxidase expressed in *S. cerevisiae* yielded a protein >90% homogeneous, as judged on SDS-polyacrylamide gels, with a specific activity for benzylamine of 0.013 units/mg at 37 °C. This specific activity is approximately 10% of that reported for the fully active yeast amine oxidase expressed in *S. cerevisiae* (5). Metal analysis revealed copper and zinc contents of 0.13 and 0.07 mol/mol enzyme subunit, respectively. N-terminal amino acid sequencing of the purified product gave the sequence NH$_2$-Ala-Ala-Pro-Ala-Arg-Pro. This corresponds to the *H. polymorpha* amine oxidase sequence commencing at residue 16 (13) and indicates that the fMet plus 3 additional residues are removed from the N terminus of the recombinant protein during expression or purification. Thus, the *H. polymorpha* amine oxidase expressed in *E. coli* is a single residue longer than the processed enzyme recovered from *S. cerevisiae* (5, 7).

**Copper Depletion and in Vitro Reconstitution of E. coli-expressed H. polymorpha Amine Oxidase—** *E. coli* cells expressing *H. polymorpha* amine oxidase were then cultured in copper-depleted medium and the recombinant protein purified in the presence of copper chelators. Despite a yield for purified protein (3 mg/liter of cell culture) similar to that seen for enzyme expressed in *S. cerevisiae* (5, 7), the specific activity of 40.5 mM IPTG (in M9ZB plus 100 μg/ml ampicillin medium not depleted in copper) was 1 cm$^2$/min for holo-enzyme produced incubation at 4°C (3 weeks), a specific activity of 0.066 unit/mg was obtained; this can be compared with a specific activity of 0.13 unit/mg for holo-enzyme produced *in vitro* in yeast. Reaction of this product with phenylhaldazine yielded 0.6 mol phenylhaldazine/mol enzyme subunit, using an extinction coefficient of 40.5 mm$^{-1}$ cm$^{-1}$ at 448 nm calculated for the phenylhaldazine adduct of the *S. cerevisiae*-expressed *H. polymorpha* amine oxidase (5).

Incubation of the *E. coli*-expressed *H. polymorpha* amine oxidase with both copper and zinc added at various times was conducted to investigate the origin of the observations made in *S. cerevisiae* expression. As shown, topa biogenesis is depend-ent on the order of metal addition (Table II). Incubation with zinc prior to copper inhibits the reconstitution reaction, although some activity (3%) can be seen following addition of copper up to 1 h. In the reciprocal experiment, incubation with zinc subsequent to copper-induced biogenesis may lead to a very slight reduction in active enzyme. Overall, the data in Table II confirm the observations first seen with expression in *S. cerevisiae*, i.e., that zinc binds tightly to the *H. polymorpha* amine oxidase and that copper and zinc are in competition for a single site.

**Conclusions**—We have shown that expression of *H. polymorpha* amine oxidase in copper-depleted *S. cerevisiae* leads to a protein that is enriched in Zn$^{2+}$ and is incompetent toward Cu$^{2+}$-induced biogenesis of topa quinone. By contrast, expression of the *H. polymorpha* gene in copper-depleted *E. coli* produces a metal-free apoprotein. The differential behavior of *S. cerevisiae* and *E. coli*, with regard to insertion of zinc into an empty copper site, may reflect intrinsic differences between the two organisms in the cellular pathway for metalloprotein assembly, or it may reflect a lower availability of intracellular zinc levels in *E. coli*. The apo-form of *H. polymorpha* amine oxidase appears to have an unusually high avidity for zinc. Importantly, addition of copper alone to the apo-form of this eukaryotic amine oxidase leads to cofactor biogenesis at a level approximately 50% of that seen with recombinant protein isolated from *S. cerevisiae*. This indicates that oxidative activation of the precursor tyrosine (3) occurs in the absence of an exogenous source of reducing equivalents. Although the initial studies of topa quinone biogenesis in the prokaryotic system were carried out in the presence of dithiothreitol, recent studies indicate that biogenesis proceeds when this reducing agent has been removed (20). The ability of copper to initiate tyrosine oxidation in the absence of an external electron source contrasts with other well characterized copper-dependent enzymes such as dopamine β-monooxygenase (21) and tyrosinase (22). Although unprecedented thus far in enzymology, one possible mechanism for cofactor biogenesis in copper amine oxidases would involve an oxidation of the precursor tyrosine to a tyrosyl radical, concomitant with the conversion of active site Cu$^{2+}$ to Cu$^{3+}$ (cf. Refs. 20 and 23). Efforts are currently under way to detect and characterize the reactive intermediates along the topa quinone biogenetic pathway.

**Acknowledgments**—We thank Paul Brooks for assistance in using the ICP instrument and Kevin T. Huang for technical assistance.

**REFERENCES**

1. Klinman, J. P. & Mu, D. (1994) *Anal. Biochem.* **222**, 399–414.
2. Janes, S. M., Palcic, M. M., Scaman, C. H., Smith, A. J., Brown, D. E., Dooley, D. M., Mure, M. & Klinman, J. P. (1999) *Biochemistry* **38**, 12147–12154.
3. Mu, D., Janes, S. M., Smith, A. J., Brown, D. E., Dooley, D. M. & Klinman, J. P. (1992) *J. Biol. Chem.* **267**, 7979–7982.

**TABLE II**

| Incubation$^a$ | First addition | Second addition | Specific activity$^b$ | units/mg |
|---------------|----------------|-----------------|----------------------|---------|
| a             | Zn$^{2+}$      | Cu$^{2+}$       |                      | 1.5 × 10$^{-3}$ |
| b             | Zn$^{2+}$      | Cu$^{2+}$       |                      | 1.7 × 10$^{-3}$ |
| c             | Cu$^{2+}$      | Zn$^{2+}$       |                      | 5.1 × 10$^{-2}$ |
| d             | Cu$^{2+}$      | Zn$^{2+}$       |                      | 4.8 × 10$^{-2}$ |

$^a$ The final composition of each enzyme solution was 43 mM HEPES, pH 7.2, 19 mM protein, 19 mM CuCl$_2$, and 19 mM ZnSO$_4$. Incubation conditions: a) at 30 °C with zinc, 2 h, copper then added on ice just prior to assay; b) at 30 °C with zinc, 1 h, copper then added and incubation continued for 1 h; c) at 30 °C with copper, 2 h, zinc then added on ice just prior to assay; and d) incubated at 30 °C with copper, 1 h, zinc then added and incubation continued for 1 h.

$^b$ Assayed for benzylamine (5 mM) in 0.1 M potassium phosphate, pH 7.2, at 37 °C.
4. 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
5. Cai, D. & Klinman, J. P. (1994) Biochemistry 33, 7674–7683
6. Large, P. J. (1996) Yeast 2, 1–34
7. Cai, D. & Klinman, J. P. (1994) J. Biol. Chem. 269, 32039–32042
8. Matsuzaki, R., Fukui, T., Sato, H., Ozaki, Y. & Tanizawa, K. (1994) FEBS Lett. 351, 360–364
9. Matsuzaki, R., Suzuki, S., Yamaguchi, K., Fukui, T. & Tanizawa, K. (1995) Biochemistry 34, 4524–4530
10. Choi, Y.-H., Matsuzaki, R., Fukui, T., Shimizu, E., Yorifuji, T., Sato, H., Ozaki, Y. & Tanizawa, K. (1995) J. Biol. Chem. 270, 4712–4720
11. Sherman, F. (1991) Methods Enzymol. 194, 3–21
12. de Hoop, M. J., Valkema, R., Kienhuis, C. B. M., Hoyer, M. A., and AB, G. (1992) Yeast 8, 243–252
13. Bruinenberg, P. G., Evers, M., Waterham, H. R., Kuipers, J., Arnberg, A. C., and AB, G. (1989) Biochem. Biophys. Acta 1008, 157–167
14. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
15. Dooley, D. M., McGuirk, M. A., Brown, D. E., Turowski, P. N., McIntyre, W. S. & Knowles, P. F. (1991) Nature 349, 262–264
16. McCracken, J., Peisach, J., Cote, C. E., McGuirk, M. A. & Dooley, D. M. (1992) J. Am. Chem. Soc. 114, 3715–3720
17. Lu, Y. Gralla, E. B., Roe, J. A. & Valentine, J. S. (1992) J. Am. Chem. Soc. 114, 3560–3562
18. Lowery, M. D. & Solomon, E. I. (1992) Inorg. Chim. Acta 233, 198–200
19. 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
20. Ruggiero, C. E., Smith, J. A., Tanizawa, K. & Dooley, D. M. (1997) Biochemistry 36, 1953–1959
21. Klinman, J. P. (1996) Chem. Rev. 96, 2541–2563
22. Solomon, E. I., Sundaram, V. M. & Machonkin, T. E. (1996) Chem. Rev. 96, 2563–2607
23. Fontecave, M. & Eklund, H. (1995) Structure 15, 1127–1129