Accelerated Publication

The Catalytic Function of Bovine Lysyl Oxidase in the Absence of Copper*

Received for publication, March 21, 2001, and in revised form, May 22, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.C100138200

Chunlin Tang‡ and Judith P. Klinman§
From the Departments of Chemistry and Molecular and Cell Biology, University of California, Berkeley, California 94720

Bovine lysyl oxidase (BLO) contains two different cofactors, copper (Kagan, H. M. (1986) in Biology of Extracellular Matrix (Mecham, R. P., ed) Vol. 1, pp. 321–398, Academic Press, Orlando, FL) and lysine tyrosyl quinone (LTQ) (Wang, S. X., Mure, M., Medziradszky, K. F., Burlingame, A. L., Brown, D. E., Dooley, D. M., Smith, A. J., Kagan, H. M., and Klinman, J. P. (1996) Science 273, 1078–1084). By a combination of UV-visible spectroscopy, metal content analysis, and activity measurements, we find that copper-depleted BLO reacts in an irreversible manner with phenylhydrazine, an amine substrate analog, and catalyzes multiple turnovers of the substrate benzylamine. After removal of the major contaminating apolysyl oxidase, apoBLO exhibits a decrease in the LTQ content, as evidenced by the drop of the 510–520-nm absorbance, suggesting that the copper may play a structural role in stabilizing the LTQ. The remaining intact LTQ in the apoBLO reacted with phenylhydrazine, both in the presence and absence of the chelator, 10 mM 2,2′-dipyridyl. When benzylamine was used as the substrate, the apoBLO turned over at a rate of 50–60% of the native BLO (after correction for the residual copper and the change of LTQ content). Copper contamination from the assay buffer was ruled out by comparison of enzyme activity using different apoBLO concentrations. These studies demonstrate that the mature form of lysyl oxidase retains many of its functions in the absence of copper.

Protein-lysine 6-oxidase (lysyl oxidase, LO; EC 1.4.3.13) is an extracellular matrix protein. It has been assigned to the copper amine oxidase family based on the presence of copper and its catalysis of the oxidative deamination of amine to aldehyde (1, 2). It is well established that decreased LO activity in connective tissues is observed in diseases of impaired copper metabolism, such as the human type IX Ehlers-Danlos syndrome and Menke’s syndrome, both of which are chromosome X-linked, recessively inherited disorders (2, 3). In vitro assays of LO, using tritium-labeled elastin (prepared from chick embryonic aorta) as substrate, have led to the conclusion that lysyl oxidase requires copper for enzyme turnover (4–6). From the data of Gacheru et al. (6) obtained for purified bovine lysyl oxidase (BLO), it was proposed that a single copper atom in lysyl oxidase monomer is essential for the catalytic function and possibly for the structural integrity of the enzyme (6).

Recently, the lysine tyrosyl quinone (LTQ) has been identified as the active site carbonyl cofactor in bovine lysyl oxidase (7). Using the UV-visible spectra of LTQ as a probe, we now report the effects of copper removal on the reactivity of BLO with benzylamine and its analog, phenylhydrazine. For the first time, it is observed that BLO retains its ability to interact with amines in the absence of copper. These results are related to the studied role for copper in the analogous 2,4,5-trihydroxy-phenylalanine-containing copper amine oxidases (8, 9). We propose that the established physiological requirement for copper in LO (3) is a consequence of an essential role for copper in LTQ biogenesis, together with a role for copper in the maintenance of cofactor and/or protein structural integrity.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Activity Assay—Lysyl oxidase was purified from bovine aorta using a modified procedure.* The modified method yielded more reproducible results, which are less dependent on the tissue sources, as compared with the published method (10). The final purified lysyl oxidase is a two-banded material with the contaminant band ranging from 10 to 50% of the total protein concentration as estimated from the relative density of the Coomassie-stained bands in SDS-PAGE gels. Earlier studies showed that the contaminating band was free of LTQ (7). The specific activity of the BLO was assayed in a buffer of 1.2 M urea, 16 mM potassium Pi, pH 7.8, and 10 mM benzylamine at 37 °C. The production of benzaldehyde was monitored by the change in absorbance of 250 nm, using 12,800 M−1 cm−1 as the molar extinction coefficient. The BLO activity was typically 0.02–0.04 units/mg after correction for the contaminating band. Unless specified specifically that other methods were used, the protein concentration was measured with Bio-Rad protein assay (Bradford assay) using bovine serum albumin as a standard.

Preparation of Copper-free Lysyl Oxidase (apo-lysyl oxidase)—The removal of copper from bovine lysyl oxidase was similar to that described by Gacheru et al. (6) with some changes. Specifically, the enzyme was dialyzed first in a chelating buffer containing 6 M urea, 16 mM potassium Pi, 10 mM 2,2′-dipyridyl, pH 7.8, at 4 °C overnight and then against the buffer of 6 M urea, 16 mM potassium Pi, pH 7.8, extensively for 24 h. Except for the 2,2′-dipyridyl-containing dialysis buffer, the buffers used for apoBLO preparation and assay were run through columns packed with chelating resin (Chelex 100, Sigma) to remove residual copper. Trace metal analysis was performed with a PerkinElmer Life Sciences 300DV inductively coupled plasma-atomic emission spectrometer, using commercially available metal standard solution. The copper content of buffers was found to be below 0.1 μM (<6 ppb), close to the detection limit. For the activity assay with two different apoBLO concentrations, the apoBLO was dialyzed against a buffer of 3 M urea, 16 mM potassium Pi, pH 7.8, after removal of copper by 2,2′-dipyridyl-containing buffer.

RESULTS AND DISCUSSION

Reaction of the Apo-Bovine Lysyl Oxidase with Phenylhydrazine—BLO has been shown to catalyze amine oxidation via a ping-pong mechanism (11). To examine the copper dependence of

* This work was supported in part by National Institutes of Health Grant GM 39296 (to J. P. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: Onyx Pharmaceuticals, 3031 Research Dr., Richmond, CA 94806.
§ To whom correspondence should be addressed. Tel.: 510-642-2668; Fax: 510-643-6232; E-mail: klinman@socrates.berkeley.edu.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org

30575
TABLE I

The LTQ content, activity, and metal content of native and apoBLO

| Enzyme                | LTQ contenta | Activityb | Metal contentc |
|-----------------------|--------------|-----------|----------------|
|                       | µM           | %         | Cu          | Zn          |
| Native BLO            | 6.28         | 100       | 11.3        | 1.70        |
| ApoBLO with chelator  | 4.68         | ND        | 0.52        | 0.71        |
| ApoBLO                | 4.11         | 39 (51)   | 0.76        | 1.28        |

a The LTQ content was calculated from the absorbance at 454 nm using an extinction coefficient of 15.4 mM⁻¹ cm⁻¹ for the phenylhydrazone adduct (7).

b The benzylamine activity assay was carried out in a buffer containing 1.2 M urea, 16 mM potassium F, (pH 7.8), 10 mM benzylamine at 37 °C by dilution of 20 µl of enzyme stock solution into 80 µl of assay buffer. The enzyme concentration in the stock solution was ~12 µM, after correction for the presence of the contaminating protein based on relative intensity of the band in SDS-PAGE, and using a molecular weight of 30,000 for BLO. The activity is expressed as the percentage of that of the native enzyme. The activity, corrected for the residual copper content and the change in LTQ concentration in the apoBLO, is in parenthesis. ND means not determined.

c The metal content was measured by an inductively coupled plasmaatomic emission spectrometer as described under "Experimental Procedures."

The reductive half-reaction, we first tested the accessibility of the LTQ cofactor in both the native and apoBLO to the substrate analog and irreversible inhibitor, phenylhydrazine. Table I shows that in the native BLO, the copper content was 11 µM, and the molar ratio of copper to enzyme was about 1:1 (Table I). The LTQ content of the native BLO was 6 µM, meaning that about 50% of the native enzyme was titratable by phenylhydrazine and was catalytically functional, consistent with previous reports (7, 12). As shown in Fig. 1A, in the presence of 10 mM 2,2'-dipyridyl, the apoBLO reacted with phenylhydrazine and formed a derivative, which absorbed at 454 nm. The copper and zinc contents in the reaction mixture were found to be 0.52 and 0.71 µM (Table I), respectively, which is much less than the amount of the LTQ (4.7 µM). Without the chelator, 2,2'-dipyridyl, the apoBLO also reacted well with phenylhydrazine (Fig. 1B). If the enzyme was denatured with 1% SDS before addition of phenylhydrazine, no appearance of the 454-nm peak was observed within 20 min (not shown). These results indicate that the reaction of phenylhydrazine with the LTQ in the apoBLO is conformation-specific and that transition metal ions such as copper or zinc are not required to keep the LTQ in the correct conformation(s) for reaction with phenylhydrazine. These findings contrast with those observed for the yeast amine oxidase from *Hansenula polymorpha* expressed in *Saccharomyces cerevisiae* (HPAO) (9). It was found that the apo-form of HPAO cannot react with phenylhydrazine until Cu²⁺, Zn²⁺, or Co²⁺ are added back to the enzyme. It has been suggested that the binding of a metal ion to the active site of HPAO alters the conformation such that it can react with phenylhydrazine. Because the LTQ cofactor has a cross-linked structure (between a tyrosine and a lysine side chain (7)), it is likely that the LTQ in BLO is more rigid than the TPQ in HPAO, such that metal ions are not required to orient the LTQ for the reaction with phenylhydrazine.

However, as shown in Table I, the amount of the LTQ that can be titrated by phenylhydrazine decreases as the copper in the enzyme was removed. The LTQ content reached 4.7 µM, about 74% of that of the native BLO, after dialysis against chelating buffer. The amount of LTQ decreased further to 4 µM, 65% of the original after removal of the chelator. Under certain conditions such as lowering the pH (~7.0) of the chelating buffer, the final apoBLO showed little absorbance at 510–520 nm (Fig. 2A), and its reactivity with phenylhydrazine was down to the base-line level (Fig. 2B). Addition of Cu (II) ion to this apoBLO preparation did not restore the LTQ peak or its reactivity with phenylhydrazine (data not shown). Noticeably, a new peak at 350 nm appeared in all the apoBLO preparation. Although the exact nature of this new peak still remains to be explored, it is likely derived from the modification of the LTQ and probably represents an unreactive form of LTQ following copper removal at reduced pH levels. As shown in Fig. 3, the preparation of apoBLO that was no longer reactive toward phenylhydrazine was still able to conduct redox cycling, suggesting that the modified LTQ in this apoBLO preparation is either able to renature back to its native state during the electrophoresis process or is capable of catalyzing the redox-cycling reaction itself. These results imply that copper may
azine (PH) is shown as a control (H11002 open squares filled squares analysis; the reaction with phenylhydrazine (H11001 PH; (13), completely inhibited the apoBLO activity (data not shown). Incubation of apoBLO with 4 \( \mu M \) Cu\(^{2+} \), which was equivalent to the amount of reactive LTQ, at room temperature for 10 min did not increase the activity; however incubation at 4 °C for 48 h did increase the activity by 36% (data not shown), implying that the reincorporation of copper at its original site may be slow.

To rule out the possibility that a trace amount of copper from the assay buffer was contributing to the activity measured for the apoBLO, two different concentrations of the apoBLO were used to conduct the assays, and their activity was compared. As shown in Table II, when the LTQ content of the diluted apoBLO in the assay was 0.4 \( \mu M \), the activity was 54% of that of the native BLO after correction for the decrease of the LTQ and for the residual copper content in the apoBLO. A similar activity (51% of the native BLO activity) was obtained when 5-fold the amount of apoBLO (2.0 \( \mu M \) LTQ) was used in the assay. The level of trace Cu\(^{2+} \) in the assay buffer was <0.1 \( \mu M \).

TABLE II

| LTQ \( \mu M \) of Protein | Cu \( \mu M \) | Activity % | LTQ \( \mu M \) of Protein | Cu \( \mu M \) | Activity % |
|---------------------------|------------|-----------|---------------------------|------------|-----------|
| Native BLO                | 5.01       | 11.9      | 100                       | 1.00       | 2.38      |
| ApoBLO                    | 2.00       | 0.65\(^a\) | 27 (51)                   | 0.40       | 0.13\(^b\) | 28 (54)   |

\(^a\) A 5-fold dilution of the same enzyme stock studied at 77.9 \( \mu g \) of protein.

\(^b\) The level of trace Cu\(^{2+} \) in the assay buffer was <0.1 \( \mu M \).

The assay buffer contained 3 \( \mu M \) urea, 16 \( mM \) potassium Pi (pH 7.8), 5 \( mM \) benzylamine, and 77.9 or 15.6 \( \mu g \) of total protein in a final volume of 100 \( \mu l \) at 37 °C. The protein concentration in this study was estimated by using an extinction coefficient of 2.0 absorbance units at 280 nm for 1 \( \mu g/ml \) of protein (17). The activity was calculated from the average of duplicate measurements. The values in parentheses are the relative activity after correction for the LTQ content and the residual Cu\(^{2+} \) content in the apoBLO, assuming the residual Cu\(^{2+} \) gave full activity. LTQ and Cu were determined as described under "Experimental Procedures."

Comparison of apoBLO activity assayed with two different enzyme concentrations

The level of trace Cu\(^{2+} \) in the assay buffer was measured to be <0.1 \( \mu M \), which is 4-fold less than the LTQ when using 15.6 \( \mu g \) of protein and 20-fold below the LTQ level with the higher protein concentration. One would expect higher specific

play a structural role in stabilizing the BLO in a conformation that maintains the LTQ cofactor in its correct configuration. It is also possible that the copper-related conformation in BLO may be important in binding of its natural substrates, elastin and collagen molecules.

The Turnover of Benzylamine with the Apo-bovine Lysyl Oxidase—Table I shows that apoBLO containing 7% of its copper retained 51% of reactive LTQ after correction for the residual copper content and the decrease of LTQ content. The presence of \( \beta \)-aminopropionitrile, an active site inhibitor of native LO (13), completely inhibited the apoBLO activity (data not shown).
activity to be associated with the diluted apoBLO if trace copper from the buffer contributed significantly to the activity measured. Thus, it is unlikely that benzylamine activity observed with apoBLO arises from trace copper contamination in the assay solution.

The data presented here argue against the requirement for copper in the oxidation of benzylamine by BLO. In an earlier study of HPAO, Su and Klinman (8) presented evidence that active site Cu$^{2+}$ need not change its valence state during turnover of amine substrates. This view is supported by recent studies of Co$^{2+}$-substituted HPAO (9). For this TPQ-containing system, it has been proposed that the active site metal plays a role as electrostatic catalyst in dioxygen reduction to hydrogen peroxide. Evidence against an essential role for metal in the oxidation of reduced quino-cofactors comes from model studies of TPQ analogs, together with the fact that the tryptophan tryptophylquinone-containing methylamine dehydrogenase can function catalytically in the absence of active site metal (14). We have not yet tested the catalytic ability of apoBLO using elastin or collagen as substrate, and more detailed kinetic and spectroscopic studies will be needed to establish that the mechanism of apoBLO is the same as the native enzyme. However, the data presented within establish the ability of BLO to function in the absence of copper using a small substrate or its analog. The well established physiological requirement for copper in LO (3) is attributed to an essential role for copper in the biogenesis of the LTQ cofactor and to a role for copper in the maintenance of cofactor and/or protein structural integrity.

REFERENCES
1. Pinnell, S. R., and Martin, G. R. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 708–716
2. Kagan, H. M. (1986) in Biology of Extracellular Matrix (Mecham, R. P., ed) Vol. 1, pp. 321–398, Academic Press, Orlando, FL
3. Kuivaniemi, H., Peltonen, L., Pakstie, A., Kaatila, I., and Kivirikko, K. I. (1982) J. Clin. Invest. 69, 730–734
4. Siegel, R. C., Pinnell, S. R., and Martin, G. R. (1970) Biochemistry 9, 4486–4492
5. Harris, E. D., Gonnerman, W. A., Savage, J. E., and O’Dell, B. L. (1974) Biochim. Biophys. Acta 341, 332–344
6. Gacheru, S. N., Trackman, P. C., Shah, M. A., O’Gara, C. Y., Spaccapoli, P., Greenaway, F. T., and Kagan, H. M. (1990) J. Biol. Chem. 265, 19022–19027
7. Wang, S. C., Mure, M., Medzhuradsky, K. F., Burlingame, A. L., Brown, D. E., Donley, D. M., Smith, A. J., Kagan, H. M., and Klinman, J. P. (1996) Science 273, 1078–1084
8. Su, Q., and Klinman, J. P. (1998) Biochemistry 37, 1251–12525
9. Mills, S. A., and Klinman, J. P. (2000) J. Am. Chem. Soc. 122, 9897–9904
10. Cai, P., and Kagan, H. M. (1995) Methods Enzymol. 258, 122–132
11. Williamson, P. R., and Kagan, H. M. (1986) J. Biol. Chem. 261, 9477–9482
12. Liu, G., Nellaiappan, K., and Kagan, H. M. (1997) J. Biol. Chem. 272, 32570–32577
13. Tang S.-S., Trackman, P. C., and Kagan, H. M. (1983) J. Biol. Chem. 258, 4331–4338
14. Zhu, Z., and Davidson, V. L. (1998) Biochim. Biophys. Acta 1364, 287–300
15. Faz, M. A., Fiebig, R., Beak, A., Kagan, H. M., and Gallop, P. M. (1991) J. Biol. Chem. 266, 689–692
16. Cronshaw, A. D., MacBeath, J. R. E., Shackleton, D. R., Collins, J. F., Fothergill-Gilmore, L. A., and Hulmes, D. J. S. (1993) Matrix 13, 255–266
17. Williamson, P. R., and Kagan, H. M. (1987) J. Biol. Chem. 262, 8196–8201
The Catalytic Function of Bovine Lysyl Oxidase in the Absence of Copper
Chunlin Tang and Judith P. Klinman

J. Biol. Chem. 2001, 276:30575-30578.
doi: 10.1074/jbc.C100138200 originally published online June 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100138200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 17 references, 8 of which can be accessed free at
http://www.jbc.org/content/276/33/30575.full.html#ref-list-1