Biochemical and Biophysical Research Communications **281,** 193–199 (2001)
doi:10.1006/bbrc.2001.4315, available online at http://www.idealibrary.com on IDEAL®

Characterization of a Model Compound for the Lysine Tyrosylquinone Cofactor of Lysyl Oxidase

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Received January 3, 2001

We characterized a model compound for the lysine tyrosylquinone (LTQ) cofactor of lysyl oxidase which is one of the mammalian copper-dependent amine oxidases. The model compound, 4-butylamino-5-methyl-*o*-quinone, was prepared from *n*-butylamine and 4-methylcatechol by the oxidation with sodium iodate and characterized by spectroscopic analyses. The absorption maximum at 494 nm is consistent with that of lysyl oxidase. The model compound was capable of deaminating benzylamine to benzaldehyde at 37°C in buffered aqueous acetonitrile. The aldehyde production was markedly elevated in the presence of the Cu(II)-EDTA complex but inhibited by free Cu(II). The catalytic cycle was observed at pH 10 in the presence of Cu(II), and the pH activity profile showed a broad optimum at about pH 9.0. In the presence of β-aminopropionitrile and upon deoxygenation with N_2 aldelyde, production was decreased. The important features of the reaction were consistent with the enzymatic reaction. © 2001 Academic Press

Key Words: **lysyl oxidase; lysine tyrosylquinone; amino-*o*-quinone; β-aminopropionitrile; oxidative deamination.**

Lysyl oxidase (EC 1.4.3.13), a mammalian copper-dependent amine oxidase, catalyzes the oxidative deamination of the ϵ-amino group of lysine and/or hydroxy-lysine residue of elastin, collagen, and egg shell membrane, which are connective tissue proteins, according to the equation (1–3): Protein-CH_2-NH_2 + O_2 + H_2O → Protein-CHO + H_2O_2 + NH_3

The resulting aldehydes, α-aminoadipic-δ-semialdehyde (allysine), undergo Schiff's base formation or the aldol condensation to form intra- and intermolecular covalent crosslinks such as desmosine, isodesmosine, and pyridinoline (4–8). The polymeric elastin and collagen fibers perform essential mechanical functions, i.e., giving elasticity or extensibility, supporting the tissue, and anchoring the cell to the extracellular matrix (6, 9, 10). Therefore, lysyl oxidase plays an important role for the development and repair of the connective tissue (11). Moreover, inhibition of lysyl oxidase activity of growing animals by copper deficiency or intake of lathyrogen such as β-aminopropionitrile (BAPN) and phenylhydrazine derivatives decreases crosslinks and results in an increased fragility of tissues (6).

Recently, lysine tyrosylquinone (LTQ, Fig. 1) has been identified as the covalently-bound active site cofactor of lysyl oxidase (12, 13). The cofactor was identified by spectroscopic characterization of a derivative of a cofactor-containing peptide with phenylhydrazine to form a stable hydrazone adduct and intact enzyme through a comparison with the model compounds. The structure of the novel cofactor consisted of an amino *o*-quinone skeleton derived from the crosslinking of a lysyl side chain and modified tyrosine residue. It appears likely that LTQ non-enzymatically arise by copper-dependent posttranslational modification (12, 14). Among the known quinocofactors, 2,4,5-trihydroxyphenylalanine quinone (topaquinone, TPQ; Fig. 1), a cofactor of copper-containing amine oxidases, has been studied extensively (15–17). Furthermore, the non-enzymatic oxidative deamination of primary amines by TPQ analogs has been clarified and characterized in detail (18–23). Thus, the results of these studies have provided important insight into the mechanistic properties of TPQ at the active site of amine oxidases.

We have assumed that a model compound for LTQ (MLTQ) also acts as an amine oxidant. In this work, 4-butylamino-5-methyl-*o*-quinone (MLTQ) was synthesized from 4-methylcatechol and *n*-butylamine, and characterized by spectroscopic experiments. The oxidative-deamination activity of MLTQ under a *quasi*-physiological condition (at 37°C in buffered aqueous CH_3CN, pH 7.4) was observed. The activity

Abbreviations used: BAPN, β-aminopropionitrile; LTQ, lysine tyrosylquinone; MLTQ, a model compound for LTQ.

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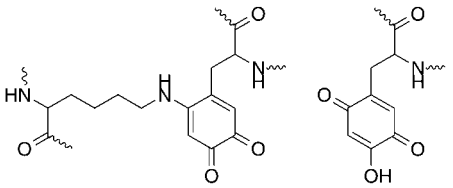
0006-291X/01 $35.00



LTQ TPQ

FIG. 1. Structure of LTQ and TPQ.

was determined by measuring the conversion of *n*-butylamine and benzylamine to the corresponding aldehydes. In this paper, the effects of pH, copper ion, inhibitor of lysyl oxidase, and molecular oxygen (O_2) on the catalytic oxidation are discussed in comparison with lysyl oxidase. These results provide important insight into the chemical properties of LTQ at the active site of lysyl oxidase.

MATERIALS AND METHODS

Materials. Catalase (1500 U/mg), BAPN, and 4-methylcatechol were from Tokyo Kasei Co. (Tokyo, Japan). Chloroform-*d* was from Sigma Chemical, Co. (St. Louis, MO). A precoated Kiesel gel 60 on an aluminum sheet (Art. 5553) for thin-layer chromatography (TLC) was from E. Merck (Darmstadt, Germany). Silica gel 60 for column chromatography and CH_3CN of HPLC grade were from Kanto Chemical Co. (Tokyo, Japan). All other chemicals were of analytical grade from Nacalai Tesque Co. (Kyoto, Japan).

Spectroscopy. The ultra violet and visible (UV-vis) spectra were obtained on an UV 2001 spectrophotometer (Hitachi, Tokyo, Japan). UV-vis spectra were taken at room temperature (path length 1 cm) after dilution. The electron ionization (EI) mass spectra (MS) were measured with a MS Tation JMS 700 (JOEL, Tokyo, Japan), and the sample was dissolved with methanol. The NMR spectra were obtained on a Varian Unity INOVA 500 or 600 spectrometers (Palo Alto, CA) at room temperature. Chemical shifts were referenced to tetramethylsilane.

Synthesis of MLTQ. 4-Butylamino-5-methyl-*o*-quinone (MLTQ) was prepared from 4-methylcatechol and *n*-butylamine by the procedure of Tanaka *et al.* as follows (24). Sodium iodate (6 g, 30 mmol) in 30 ml of distilled water was added dropwise to a stirred suspension of 4-methylcatechol (5 g, 40 mmol) and *n*-butylamine (3 g, 41 mmol) in 80 ml of acetic acid. The suspension was allowed to react in an ice-bath for 1 h. Then, the reaction mixture was slowly added to 500 ml of cold distilled water followed by filtration. The resin was dissolved in 200 ml of CH_3CN, and the solution was extracted three times with 300 ml of *n*-hexane. The filtrate was extracted three times with 500 ml of *n*-hexane. All the hexane layers were combined and concentrated under reduced pressure. The remaining sample was separated by silica gel column chromatography with a solvent of *n*-hexane/ethyl ether (8:2, v/v), and the orange fraction was collected. MLTQ was recrystallized from *n*-hexane/ethyl ether and characterized by UV-vis, EI-MS, ^{1}H-NMR, ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H detected single quantum coherence (HSQC), and ^{1}H detected multiple quantum coherence (HMQC) spectra. MLTQ: yield 207 mg (1.07 mmol); red needle; R_f = 0.15 (*n*-hexane/ethyl ether, 8:2, v/v); EI MS *m/z* 193 (M^+); NMR see Table 1.

Oxidative deamination of benzylamine catalyzed by MLTQ. Reaction mixtures (100 μl) in a micro test tube contained 10 mM benzylamine, 0.50 mM $CuSO_4$, and 0.1 M sodium phosphate buffer, pH 7.4. The reaction was initiated by the addition of 100 μl of MLTQ solution (0.50 mM in CH_3CN). The reaction mixtures were incubated at 37°C with shaking in the dark in an air atmosphere. After incubation, the reaction was terminated by the addition of acetic acid (100 μl). We confirmed that this procedure completely stopped the reaction. After centrifugation at 12,000 rpm for 10 min at room temperature, the samples (20-μl aliquots) were chromatographed with CH_3CN/distilled water (4:6, v/v) containing 0.2% phosphoric acid. The formation of benzaldehyde was monitored by HPLC (Perkin Elmer Liquid Chromatograph Integral 4000 system, Norwalk, CT) using reversed-phase HPLC column (Cosmosil 5C_{18}-AR-II, Nacalai Tesque Co.) with detection at 245 nm. Benzaldehyde was eluted at 5.2 min at a flow rate of 1.0 ml/min. *N*-Benzylidenebenzylamine, which was Schiff's base of benzylamine and benzaldehyde, was not observed. The results are shown as means ± SEM for single measurements of individual aliquots from three separate incubations. Error bars smaller than the size of the symbol are not shown in the figures.

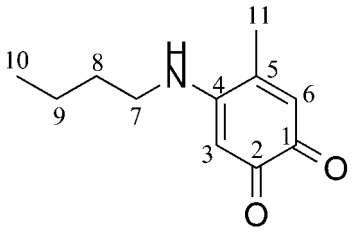
Determination of the production of aldehyde by the reaction of MLTQ with n-butylamine and BAPN. MLTQ (0.5 mM) in 1.0 ml of CH_3CN was added to 1.0 ml of 0.1 M sodium phosphate buffer, pH

TABLE 1

^{1}H and ^{13}C NMR Data for MLTQH in $CDCl_3$

					H coupled with	
Carbon no.*	δH	H coupled with H	δC	H coupled with C ($^1J_{C\text{-}H}$)	C ($^2J_{C\text{-}H}$)	C ($^3J_{C\text{-}H}$)
1			185.6		H_6	H_3
2			183.7		H_3	
3	5.45		97.9	H_3		NH
4			146.7		H_3	H_6, H_7
5			150.3		H_{11}	H_3
6	6.45	H_{11}	128.0	H_6		H_{11}
7	3.09	H_8, NH	42.1	H_7	H_8	H_9
8	1.63	H_7, H_9	30.2	H_8	H_7, H_9	H_{10}
9	1.40	H_8, H_{10}	20.2	H_9	H_8, H_{10}	H_7
10	0.95	H_9	13.7	H_{10}	H_9	H_8
11	2.07	H_6	16.8	H_{11}		H_6
NH	5.58	H_7	—	—	—	—

* For numbering system, see structure of MLTQH of Fig. 2.



MLTQH

FIG. 2. Structure of MLTQ.

7.4, containing 10 mM *n*-butylamine or BAPN. The reaction mixtures were incubated at 37°C with shaking in the dark in an air atmosphere, and the reaction was monitored by UV-vis spectroscopic analysis. After incubation for 96 h, 200 μl of a solution of 2.5 mM 2,4-dinitrophenylhydrazine in 4 M HCl-methanol or 200 μl of 4 M HCl-methanol for blank experiments was added to each reaction mixture. The mixtures were allowed to react for 6 h at room temperature. Then the samples were analyzed by HPLC using CH_3CN/distilled water (7:3, v/v) as solvent. The 2,4-dinitrophenylhydrazone of *n*-butyraldehyde was eluted at 6.1 min at a flow rate of 1.0 ml/min with detection at 358 nm.

Anaerobic and aerobic studies. The Pyrex test tube was tightly fitted with a rubber cap in which two hypodermic needles were inserted to serve as inlet/outlet ports for the introduction of N_2 and O_2, for anaerobic and aerobic experiments, respectively. Gas was passed through the incubation mixture for 1 min and charged until the pressure of 5.0 kgf/cm^2 inside the tube was reached. Then the reaction was started by addition of MLTQ solution using a hypodermic syringe.

RESULTS AND DISCUSSION

Characterization of MLTQ

The model compound (MLTQ) was isolated and determined as shown in Fig. 2, and showed poor stability, especially in a high alkaline solution. The structure was assigned by 1D and 2D NMR experiments such as HSQC and HMQC (Table 1). The ^{13}C NMR spectrum of the amino-*o*-quinone showed two signals with the typical chemical shift of the quinonoid carbonyl carbon at about 180 ppm, and methyl and *n*-butyl groups were determined. The broad proton resonance at 5.58 ppm was assigned as an NH to be coupled with H7 and exchangeable with D_2O. An HSQC experiment of MLTQ establishes the connectivity of a proton directly bound to carbon between C3 and H3, and C6 and H6 as shown in Table 1. In an HMQC experiments, the carbonyl signals at 185.6 and 183.7 ppm showed a $^2J_{C-H}$ correlation with H6 and H3 and hence were assigned C1 and C2, respectively. In addition, C4 was also assigned from a $^2J_{C-H}$ coupling to H3 and a $^3J_{C-H}$ coupling to H7. Thus the ring connectivity was determined.

Figure 3A shows the UV-vis spectra of MLTQ in CH_3CN. This compound exhibited three absorption maxima: MLTQ, 209 (ϵ 5310), 272 (ϵ 2370), and 472 nm (ϵ 520). In Fig. 3B, the absorption spectra of MLTQ in buffered aqueous CH_3CN at pH 7.4 are shown. This solution caused a significant red shift of the absorption maximum observed at 472 nm in CH_3CN, presumably reflecting the electronic environment of a double bond conjugated to the quinone ring. The absorption maximum at 494 nm, which is an n to π^* transition band, is consistent with that of lysyl oxidase (12).

Oxidative Deamination by MLTQ

Lysyl oxidase catalyzes primary amine oxidation *via* a ping pong *bi ter* kinetic mechanism (11, 25). Figure 4 shows the putative mechanism of the catalytic cycle of LTQ. Primary amine reacts with LTQ, I, to form quinone ketimine, II, followed by the trans Schiff's base reaction, giving quinolaldimine, III. Then, the adduct progressively releases aldehyde through hydrolysis, thereby yielding aminophenol, IV. The O_2-dependent oxidation of IV catalyzed by Cu(II) can generate quinoneimine, V, accompanying the release of H_2O_2. Then LTQ regenerates from V by hydrolysis concomitant with NH_3 and undergoes the subsequent catalytic reactions. To elucidate the chemical function of the cofactor, we investigated the oxidative deamination of primary amine by MLTQ. In Fig. 5, the UV-vis spectral monitoring of the reaction of MLTQ (0.125 mM) and

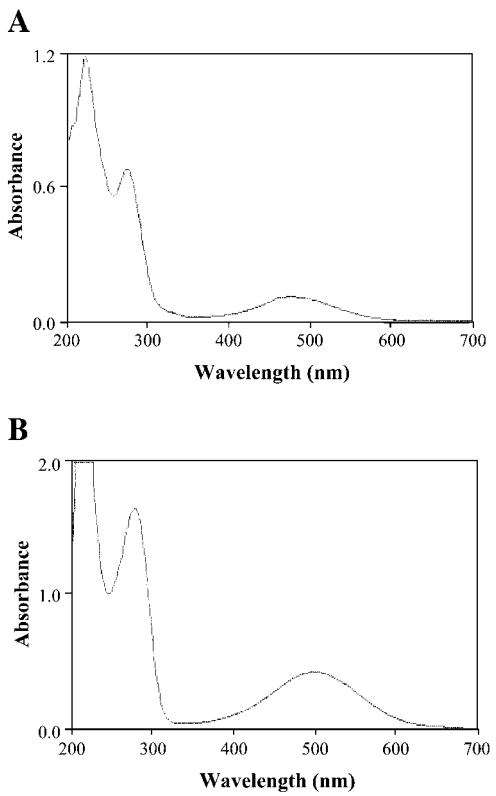


FIG. 3. UV-vis spectra of MLTQ in CH_3CN (A) and in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, v/v) (B).

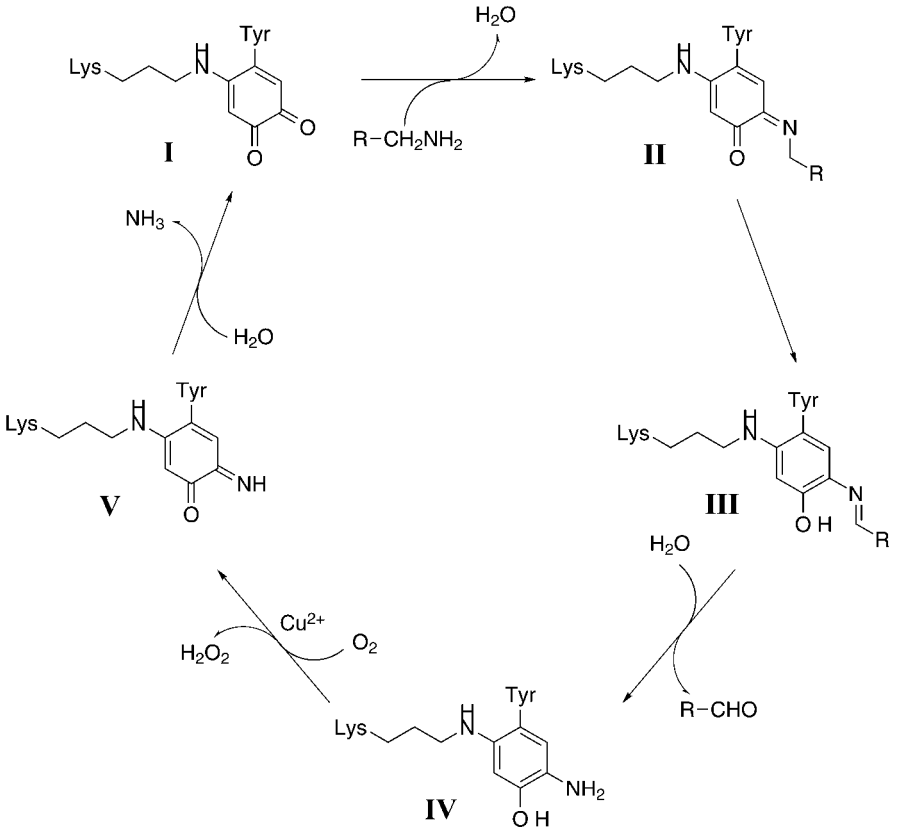


FIG. 4. Proposed mechanism of action of LTQ.

n-butylamine (5.0 mM) in buffered aqueous CH_3CN, pH 7.4, containing 0.25 mM Cu(II) at 37°C is shown. The characteristic absorption band of MLTQ measured at 494 nm was gradually bleached with the appearance of new bands at 310 and 393 nm, which may be attributed to the formation of ketimine, II. Thus, *n*-butyraldehyde generated in the incubation was converted to 2,4-dinitrophenylhydrazone and then determined by HPLC as described under Materials and Methods.

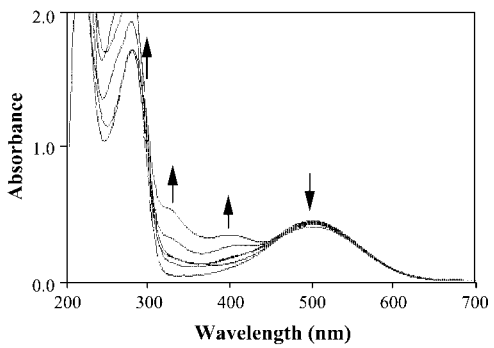


FIG. 5. UV-vis spectrum of the reaction of MLTQ with *n*-butylamine as a function of time. MLTQ (0.125 mM) was incubated with *n*-butylamine (5.0 mM) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, v/v) at 37°C. UV-vis spectra were recorded at 0, 1, 3, 6, 12, 24, and 48 h.

The time course of the oxidation of benzylamine by MLTQ was monitored by the formation of benzaldehyde (Fig. 6). At 168 h, the production of benzaldehyde (0.27 mM) at pH 10 in the presence of Cu(II) was larger than the amount of the added MLTQ (0.25 mM), indicating that the catalytic cycle occurs. Moreover, pH and Cu(II) apparently influenced the catalytic activity.

We also examined the effect of pH on the oxidative deamination. Reaction mixtures, containing 5.0 mM benzylamine and 0.25 mM MLTQ were incubated at 37°C for 24 h. As shown in Fig. 7, the pH activity profile showed a broad optimum at about pH 9.0. The production of benzaldehyde was rarely observed in acidic solutions. The accelerating catalytic activity at alkaline pH is consistent with the fact that lysyl oxidase shows a broad optimum of activity at about pH 8–9 (26). The effective oxidation by basic medium can be explained as follows. The anionic form of the quinone and the unprotonated amine act as an efficient electrophile and nucleophile, respectively, to form quinone ketimine, II, at the initial step. Moreover, basic medium is required for the abstraction of α-proton of II to form quinolaldimine, III (27). In lysyl oxidase, a histidine residue has been implicated to function as the general base (28).

Lysyl oxidase contains one tightly bound Cu(II) per mole of enzyme (29). The Cu(II) cofactor is bound in a tetragonally distorted, octahedrally coordinated ligand

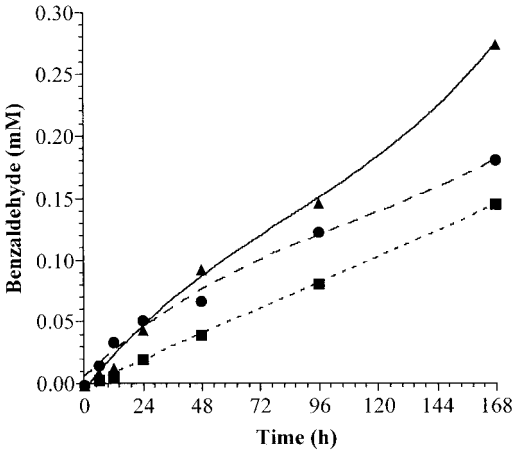


FIG. 6. Time course of the oxidation of benzylamine catalyzed by MLTQ. MLTQ (0.25 mM) was incubated with benzylamine (5.0 mM) in 0.1 M sodium phosphate buffer/CH_3CN (1:1, v/v) at 37°C: (J) at pH 7.4, (B) at pH 7.4 in the presence of 0.25 mM $CuSO_4$, (H) at pH 10.0 in the presence of 0.25 mM $CuSO_4$.

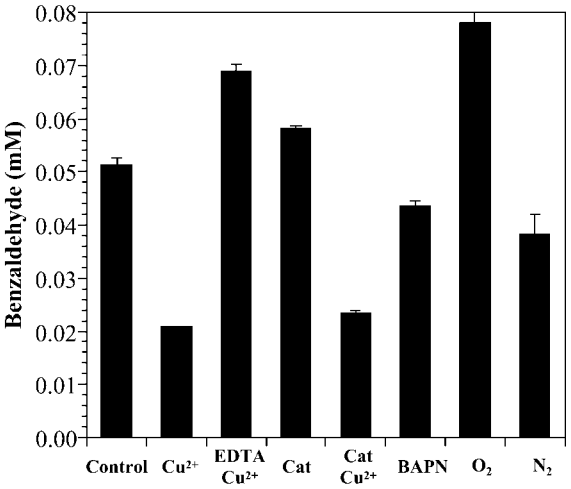


FIG. 8. Effect of BAPN, EDTA, and O_2 on the oxidation of benzylamine catalyzed by MLTQ. MLTQ (0.25 mM) was incubated with benzylamine (5.0 mM) in 0.1 M sodium phosphate buffer/CH_3CN (1:1, v/v) at 37°C for 24 h: Cu^{2+}, in the presence of 0.25 mM $CuSO_4$; EDTA, in the presence of 0.25 mM EDTA; Cat, in the presence of 200 U/ml catalase; O_2, under O_2 atmosphere; N_2, under N_2 atmosphere; BAPN, in the presence of 0.25 mM BAPN.

field. Once freed of copper, the resulting inactive apoenzyme is fully reactivated by reconstitution with Cu(II) but not by other divalent metal ions. Although the Cu(II)-free enzyme is almost completely inactivated, the Cu(II)-free reaction (control) of MLTQ gave a significant amount of aldehyde (Fig. 8). On the other hand, the addition of Cu(II) to the reaction mixture markedly inhibited the production of aldehyde to 40% of the control level. From our result, the process in releasing aldehyde (I → IV) by LTQ does not seem to necessarily need Cu(II). The steric change of enzyme by removal of Cu(II) might hinder the interaction between LTQ and substrate. However, because of its redox property, Cu(II) is suggested to participate in the O_2-mediated reoxidation of the reduced LTQ, IV, to the quinoneimine, V, as the electron acceptor (29). Interestingly, the addition of Cu(II) and ethylenediamine tetraacetic acid (EDTA) to the reaction mixture increased the aldehyde production to 133% of the control level (Fig. 8), suggesting that the Cu(II)-EDTA complex is effective for the catalytic reaction but not free Cu(II). Thus the Cu(II) complex may function as an electron acceptor in the reaction. Therefore, the reoxidation of LTQ may be promoted by the coordinated Cu(II) near at the active site. Incubation of lysyl oxidase with H_2O_2 has been reported to inactive the enzyme irreversibly, probably due to the participation of hydroxyl radical (30). Whether the inhibition of the MLTQ-catalyzed reaction by the addition of free Cu(II) is responsible for the hydroxyl radical produced by the Fenton reaction between copper ion and the released H_2O_2 was investigated. As shown in Fig. 8, the addition of catalase (200 U/ml) to the reaction mixture did not prevent the inhibition by Cu(II). Nevertheless, the production of aldehyde slightly increased by the addition of catalase in the presence and absence of Cu(II). This fact is thought to be due to the supply of O_2 converted from H_2O_2 by catalase. The inhibitory effect of Cu(II) may be attributed to decomposition or polymerization of MLTQ *via* the autoxidation catalyzed by free Cu(II).

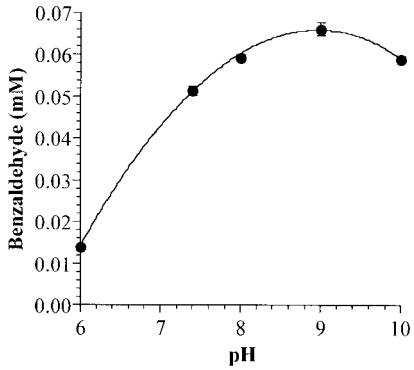


FIG. 7. Effect of pH on the oxidation of benzylamine catalyzed by MLTQ. MLTQ (0.25 mM) was incubated with benzylamine (5.0 mM) in 0.1 M sodium phosphate buffer/CH_3CN (1:1, v/v) in the presence of 0.25 mM $CuSO_4$ at 37°C for 24 h.

Lysyl oxidase releases aldehyde in an amount stoichiometric with the content at the functionally active site under anaerobic conditions according to the ping-pong kinetic pattern (25). To confirm this fact, MLTQ was tested by assaying for benzaldehyde production from benzylamine under an N_2 atmosphere (anaerobic condition). As shown in Fig. 8, although benzaldehyde was produced under the anaerobic condition, a significant amount decreased compared to the control (under an air atmosphere). On the other hand, the production of aldehyde rose to 152% of the control value under an

O_2 atmosphere (aerobic condition). These observations coincide with the result of the enzyme, and indicate that the aldehyde is produced and released by LTQ before the binding of O_2 as the second substrate. In addition, the decrease in the production of benzaldehyde under the anaerobic condition implies that the O_2-dependent reoxidation of MLTQ from aminophenol, IV, did not occur.

We also investigated the effect of inhibitor of lysyl oxidase on the oxidation by MLTQ. Micromolar concentrations of BAPN irreversibly inhibit the activity of lysyl oxidase *in vitro* (31). The mechanism of inhibition is postulated as the formation of a covalent bond between BAPN and LTQ (32). To assess this fact, MLTQ (0.25 mM) was incubated with benzylamine (5.0 mM) and BAPN (0.25 mM). The activity decreased to 83% of the control level in the presence of BAPN (Fig. 8). To further characterize the inhibition mechanism, MLTQ was incubated with BAPN in the absence of substrate. The absorbance at 494 nm was gradually decreased in the incubation, whereas a new band at λ_{max} 348 nm appeared. A possible aldehyde product generated by the reaction was converted to 2,4-dinitrophenylhydrazone and then analyzed by HPLC as described under Materials and Methods, however, no hydrazone derivative was detected. This observation agrees with the result obtained with the enzyme (32), implying that BAPN interacts with LTQ to form a covalent adduct. Nevertheless, an attempt to identify the adduct was unsuccessful.

In this work, the model compound for LTQ as the active site cofactor in lysyl oxidase was synthesized and characterized. Additionally, we demonstrated that the model compound catalyzes the oxidative deamination. The important features of the reaction were consistent with the enzymatic reaction. Although further studies will be needed to determine completely the unambiguous mechanism of the oxidation, the results presented herein provide a significant insight into the reaction of LTQ.

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