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Generation of the topa quinone cofactor in bacterial monoamine oxidase by cupric ion-dependent autooxidation of a specific tyrosyl residue

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Abstract The quinone of 2,4,5-trihydroxyphenylalanine (topa), recently identified as the covalently bound redox cofactor in copper amine oxidases, is encoded by a specific tyrosine codon. To elucidate the mechanism of its formation, the recombinant phenylethylamine oxidase of *Arthrobacter globiformis* has been overproduced in *Escherichia coli* and purified in a Cu^{2+}-deficient form. The inactive precursor enzyme thus obtained was dramatically activated upon incubation with Cu^{2+}, concomitantly with the formation of the topa quinone at the position corresponding to Tyr^{382}, occurring in the tetrapeptide sequence highly conserved in this class of enzymes. The topa quinone was produced only under aerobic conditions, but its formation required no external enzymatic systems. These findings demonstrate the Cu^{2+}-dependent autooxidation of a specific tyrosyl residue to generate the topa quinone cofactor.

Key words: Monoamine oxidase; Copper ion; Topa quinone; Autogeneration

1. Introduction

Copper-containing monoamine oxidase (EC 1.4.3.6) catalyzes the oxidative deamination of various biogenic primary amines to the corresponding aldehydes, ammonia, and hydrogen peroxide [1]. The covalently bound organic cofactor of the enzyme from bovine plasma was recently identified by Klinman and her co-workers [2] as the quinone of 3-(2,4,5-trihydroxyphenyl)-L-alanine (6-hydroxydopa; topa). The novel quinone cofactor of topa has subsequently been shown to be ubiquitous in the enzymes of both eukaryotes [3–5] and prokaryotes [3,6]. Comparison of the quinone-containing peptide sequences with those of the enzymes deduced from the coding genes (cDNAs) of the yeast *Hansenula polymorpha* [7], the Gram-positive methylotroph *Arthrobacter* strain P1 [8], and mammalian tissues and serum [9,10] revealed that the precursor to the covalently bound topa quinone is a specific tyrosyl residue occurring in a highly conserved sequence, Asn-Tyr(topa quinone)-Asp/Glu-Tyr [5]. Thus, it became evident that the tyrosyl residue is converted co- or post-translationally into the topa quinone cofactor [4], although the mechanism of this conversion is as yet unknown.

Aiming at elucidation of the generation mechanism of the topa quinone cofactor in copper-containing amine oxidases, we have recently cloned and sequenced the gene encoding phenylethylamine oxidase from a Coryneform bacterium, *Arthrobacter globiformis* [11]. In the deduced amino acid sequence was found the consensus tetrapeptide sequence [5], containing a tyrosyl residue (Tyr^{382}) supposed to be the precursor to the topa quinone cofactor. Furthermore, we have constructed an overexpression plasmid for the recombinant phenylethylamine oxidase. Interestingly, the expression of the active, quinone-containing enzyme in *Escherichia coli* was markedly dependent on the presence of Cu^{2+} in the culture medium. The inactive, Cu^{2+}-deficient enzyme produced in the absence of Cu^{2+} could be converted in vitro into the active, quinone-containing form by subsequent reconstitution with Cu^{2+} of the crude extract [11]. We now purified the overproduced enzyme in a Cu^{2+}-deficient precursor form and herein report unequivocal evidence for the Cu^{2+}-dependent autooxidation of Tyr^{382} to generate the topa quinone cofactor.

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Abbreviations: topa, 3-(2,4,5-trihydroxyphenyl)-L-alanine; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

2. Experimental

2.1. Culture conditions and purification of precursor enzyme

Water used in the preparation of culture media and other solutions was obtained from a NANOpure II (Barnstead) system and had resistance greater than 17.6 MΩ·cm. Solutions of chemicals were all passed through Chelex chelating ion-exchange filters (Bio-Rex ion-exchange membrane, Bio-Rad), and all glassware was washed with 0.1 M sulfuric acid to remove traces of metal ions. *E. coli* BL21(DE3) cells transformed with pPEAO2 or pPEAO2-Y382F [11] were grown at 37°C in an LB medium supplemented with 50 μg/ml sodium ampicillin. After cultivation until the cell density reached A_{600} = 0.6 (about 3 h), 0.4 mM IPTG was added, and the bacteria were further cultivated at 30°C for 2–3 h until the cell density reached A_{600} > 1.0. The harvested cells were suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM *N*,*N*-diethyldithiocarbamate (buffer A), supplemented with 0.1 mg/ml each of phenylmethylsulfonyl fluoride and *N*-tosyl-L-phenylalanine chloromethylketone, and then disrupted at 4°C by ultrasonic disintegration. The resulting lysate was centrifuged at 30,000 × *g* for 30 min, and the supernatant solution was dialyzed overnight against 2 liters of buffer A. The enzyme solution was applied to a DEAE-Toyopearl column (70 ml) equipped on a Pharmacia FPLC system and equilibrated with buffer A. The column was washed thoroughly with buffer A, and the bound protein was eluted with a 120-min linear gradient of 0–0.3 M KCl in the same buffer at a flow rate of 5 ml/min. The active fractions were pooled and concentrated by ultrafiltration through a UK-10 membrane (Advantec). The enzyme solution was then added with solid ammonium sulfate to 20% saturation and applied to a Phenyl-Toyopearl column (25 ml) precquilibrated with 20% saturated ammonium sulfate in buffer A. The bound protein was eluted with a 100-min linear gradient of 20–0% saturation of ammonium sulfate at a flow rate of 1 ml/min. The enzyme eluted at about 10% saturation of ammonium sulfate was pooled, concentrated by ultrafiltration, and dialyzed against buffer A. Finally, the enzyme was purified by repeating twice the anion-exchange chromatography with a Resource Q (Pharmacia) column (6 ml), first eluted by a

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60-min linear gradient of 0–0.3 M KCl in buffer A, and second eluted by a 60-min linear gradient of 0–0.3 M KCl in 20 mM sodium acetate buffer (pH 5.1) containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM *N,N*-diethyldithiocarbamate. The enzyme thus purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis was dialyzed thoroughly against 50 mM HEPES (pH 6.8) and stored at −20°C until use. A typical yield of the enzyme from 20 g of wet *E. coli* cells was about 60 mg (total activity, 1440 units, assayed after incubation with Cu^{2+} as described below). The Y382F mutant enzyme overproduced in *E. coli* cells carrying pPEAO2-Y382F [11] was purified to homogeneity by the same procedure as described above for the wild-type enzyme, based on their identical chromatographic behaviors. Since the mutant enzyme had no activity even after incubation with Cu^{2+}, the purification steps were monitored by SDS-polyacrylamide gel electrophoresis, and its identity as phenylethylamine oxidase was confirmed by N-terminal amino acid sequencing of the purified protein.

2.2. Enzyme assay and protein and copper content determinations

In all cases unless otherwise stated, the enzyme was preincubated at 30°C for 30 min with its ten times concentration of $CuSO_4$ in 50 mM HEPES (pH 6.8), before assaying the activity. The assay for phenylethylamine oxidase was carried out at 30°C in 100 mM potassium phosphate buffer (pH 7.0) with 0.1 mM phenylethylamine sulfate as substrate, by monitoring H_2O_2 production coupled to the oxidation of 4-aminoantipyrine ($\Delta\varepsilon_{505} = 4.6\ mM^{-1}\cdot cm^{-1}$) using phenol and horseradish peroxidase [12]. Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficients of 12.3 and 13.2 for 1% solutions of the precursor enzyme and the active, copper/quinone-containing enzyme, respectively, determined in 100 mM potassium phosphate (pH 7.0) by a refractometric method reported previously [13]. For calculation of the concentration of enzyme subunit, a molecular weight of 70,600 [11] was employed. The copper content of the enzyme was determined by atomic absorption analysis using a Shimadzu AA-670G atomic absorption spectrophotometer with a GFA-4A graphite furnace atomizer.

2.3. Isolation and sequencing of topa quinone-containig peptide

The purified enzyme (about 20 mg) in 5 ml of 50 mM HEPES (pH 6.8) was first incubated with 0.5 mM $CuSO_4$ at 30°C for 60 min (to fully generate the topa quinone, see below) and then labeled at 30°C by the addition of a slight excess of 1 mM *p*-nitrophenylhydrazine in several portions over a 30-min period. The reaction mixture was repeatedly concentrated by a Centricon-30 (Amicon) ultrafiltration cartridge and diluted with 50 mM HEPES (pH 6.8) to remove unreacted *p*-nitrophenylhydrazine. The derivatized enzyme solution was added with solid urea to 8 M, incubated at 40°C for 10 min, and diluted with 0.1 M Tris-HCl (pH 8.0) to a final urea concentration of 2 M. Digestion with thermolysin was carried out at 40°C for 12 h at a protease-to-substrate ratio of 1:50 (w/w) followed by further digestion for 6 h after the second addition of thermolysin. The peptide mixture was separated on a Tosoh HPLC system equipped with a semipreparative reverse phase column (Cosmosil 5C18-300, 0.8 × 25 cm) using a solvent system of 0.3% (v/v) triethylamine acetate, pH 7.0 (A) and 0.3% triethylamine acetate containing 90% acetonitrile (B), as reported previously [5]. A 60-min linear gradient from 0 to 60% B was used to elute peptides at a flow rate of 0.5 ml/min with continuous monitoring of the absorbance at 215 nm (peptide absorbance) and at 350 nm (cofactor-*p*-nitrophenylhydrazone absorbance). The *p*-nitrophenylhydrazone peptide was further purified using an analytical column (Vydac C18) with a shallower gradient of acetonitrile concentration. The sequences of the peptide fragments purified were determined with an Applied Biosystems Model 477A protein sequencer linked with an Applied Biosystems Model 120A PTH analyzer.

3. Results and discussion

The *E. coli* BL21(DE3) cells carrying pPEAO2, an overexpression plasmid for phenylethylamine oxidase of *Arthrobacter globiformis* [11], were cultivated in the Cu^{2+}-depleted medium, and the inactive enzyme overproduced was purified to homogeneity in the presence of a strong Cu^{2+}-chelating agent, *N,N*-diethyldithiocarbamate. The Cu^{2+}-deficient enzyme thus purified showed very low specific activity, less than 0.5 unit/mg unless incubated with Cu^{2+}. It contained only a trace amount of Cu^{2+} (0.043 mol atom of Cu^{2+}/mol of enzyme subunit) when analyzed by atomic absorption and exhibited no absorption peak in the visible wavelength region. Remarkably however, when the purified enzyme (10–50 μM subunit) was incubated with excess Cu^{2+} (0.1–0.5 mM $CuSO_4$) at 30°C for about 30 min, the specific activity increased to a level of 20–24 units/mg

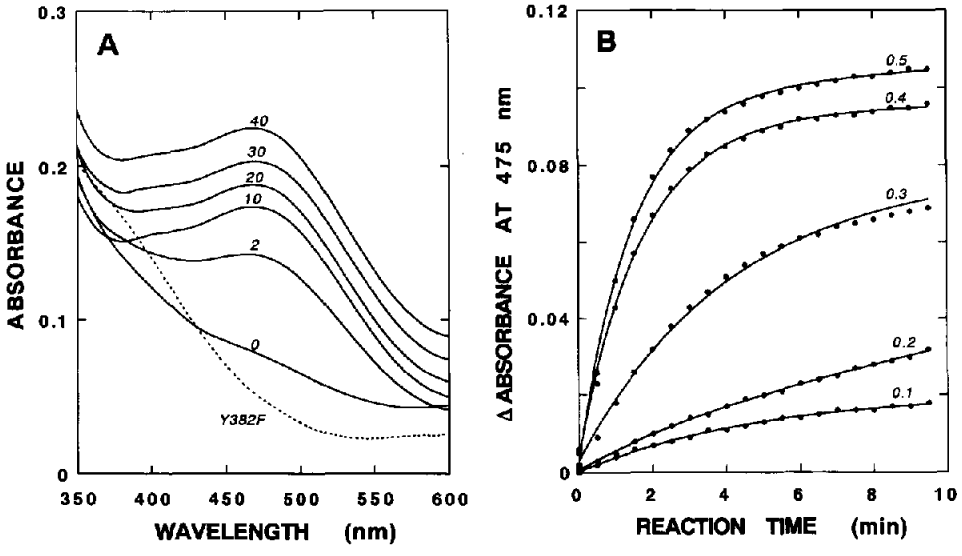

Fig. 1. Cu^{2+}-dependent formation of a quinone compound absorbing at 470–480 nm. (A) Spectral change of the purified Cu^{2+}-deficient enzyme (0.1 mM subunit) upon addition of 0.5 mM $CuSO_4$ in 50 mM HEPES (pH 6.8). Absorption spectra were recorded at indicated times (min) with a Hewlett Packard 8452A diode array spectrophotometer in a 1.0-cm path-length cell placed in a thermostated cell holder at 30 ± 0.2°C. The 0-min spectrum was taken in an evacuated cell. The spectrum of the Y382F mutant enzyme (0.1 mM subunit) was also measured after aerobic incubation with 0.5 mM $CuSO_4$ for 30 min (*dotted line*). (B) Time course of the increase in 475-nm absorption. The Cu^{2+}-deficient enzyme (0.1 mM subunit) was incubated with indicated concentrations of $CuSO_4$ (mM), and the absorbance change at 475 nm was monitored. The curves were drawn by the least-squares best fit of the data to two parallel first-order kinetics (see text).

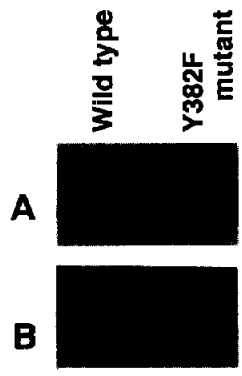

Fig. 2. In situ quinone formation by incubation with Cu^{2+} of the membrane-immobilized enzyme. The purified Cu^{2+}-deficient wild-type and Y382F mutant enzymes (about 5 μg each) were electrophoresed in a polyacrylamide gel containing no SDS and electrotransferred onto a polyvinylidene difluoride membrane. Pieces of the membrane were cut off and stained for proteins (A) with 0.1% (w/v) Ponceau S and for quinone compounds (B), after incubation with 1 mM $CuSO_4$ for 30 min, with the alkaline nitroblue tetrazolium/glycinate reagent [16].

protein, which is much higher than those of monoamine oxidases purified from other sources [5,6]. After removal of unbound Cu^{2+} by dialysis against 1 mM EDTA in 50 mM HEPES (pH 6.8), the copper content in the fully activated enzyme was determined to be 0.9–1.0 mol atom/mol of subunit. Although incubation of the purified inactive enzyme with Fe^{2+} (0.5 mM $FeCl_2$) resulted in about 10% activation as compared with the specific activity of Cu^{2+}-activated enzyme, other divalent metal ions including Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Be^{2+}, Mo^{2+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, and Zn^{2+} were all inert.

Concomitant with the Cu^{2+}-dependent appearance of enzymatic activity, the enzyme solution gradually colored brownish pink with an absorption maximum at 470–480 nm (Fig. 1A), which is ascribable to the formation of a quinone compound, most likely the topa quinone [5]. In contrast, when the enzyme was anaerobically mixed with excess Cu^{2+} in an evacuated cell, there was no change in the absorption spectrum, indicating that the formation of the chromophore is strictly dependent on the presence of the dissolved oxygen. In an air-saturated solution with the dissolved oxygen concentration being about 0.2 mM at 1 atom and containing various concentrations of Cu^{2+}, the increases in 475-nm absorption could be best fitted to two parallel first-order reactions: $f(t) = L_a(1 - e^{-k_a t}) + L_b(1 - e^{-k_b t}) + C$, where k_a and k_b are first-order rate constants with the corresponding limits L_a and L_b and C is a constant (Fig. 1B). The calculated k_a values were found to be dependent on the Cu^{2+} concentration, while the k_b values were much smaller than the k_a values and independent on the Cu^{2+} concentration. In the case of addition of 4 equivalents of Cu^{2+} (0.4 mM) per enzyme subunit (0.1 mM), the overall reaction proceeded with rate constants of 0.59 and 0.008 min^{-1} with about 70% of the reaction following the faster of the two reactions ($L_a/L_{a+b} = 0.67$). The molar absorption coefficient (ε_M) of the chromophore produced in the fully activated enzyme (after incubation with excess Cu^{2+} for 12 h and dialysis against 1 mM EDTA) was calculated to be about 1,400 $M^{-1} \cdot cm^{-1}$ in 50 mM HEPES at pH 6.8, assuming that each polypeptide (M_r 70,600) contains 1 mol of the chromophore as does the bovine serum enzyme [14]. These results altogether indicate that the Cu^{2+}- deficient inactive enzyme is the precursor form devoid of the quinone cofactor.

Although the Cu^{2+}-deficient enzyme purified was homogeneous by SDS-polyacrylamide gel electrophoresis, the possibility that a trace of contaminating enzyme(s) is involved in the quinone formation in the recombinant *A. globiformis* oxidase should be considered; *E. coli* may have an enzymatic system to generate the topa quinone in its inherent monoamine oxidase [6]. However, the quinone was similarly formed by the in situ incubation with Cu^{2+} of the precursor enzyme immobilized on a polyvinylidene difluoride membrane by electroblotting (Fig. 2). It is therefore evident that the quinone formation requires no external enzymatic systems. Furthermore, it should be noted that the in situ quinone formation is possible only with the enzyme that was electrophoresed in the absence of SDS. Thus the binding of Cu^{2+} with the *native* enzyme is prerequisite to the formation of the quinone cofactor.

To identify the quinone compound, the Cu^{2+}-activated enzyme was first treated with *p*-nitrophenylhydrazine, followed by complete digestion with thermolysin, as has been employed as the general strategy for isolation of the topa quinone-containing peptide [2–5]. Like in the previous studies [2,4,5], a major yellow-colored peptide was eluted on the reversed phase liquid chromatography of the thermolysin digest (Fig. 3). The *p*-nitrophenylhydrazone-containing peptide further purified by repeating the reversed phase liquid chromatography as well as the derivatized native enzyme showed an absorption maximum at 455–470 nm at neutral pH and at 580–585 nm at alkaline pH (in 1 M KOH) (Fig. 4); the red shift in absorption of about 120 nm has been suggested to be unique to the topa quinone [5]. Automated Edman degradation of the *p*-nitrophenylhydrazone-containing peptide revealed its sequence as Ile-Gly-Asn-X-Asp-Tyr-Gly, where X is an unidentifiable residue. This sequence corresponds to that from Ile^{379} to Gly^{385}, except for position 382, in the primary structure of phenylethylamine oxidase from *A. globiformis*, deduced from the nucleotide sequence [11]. The unidentifiable residue X is located at the position corresponding to Tyr^{382} in the deduced amino acid sequence, and the sequence surrounding X coincides with the consensus Asn-Tyr(topa quinone)-Asp/Glu-Tyr sequence,

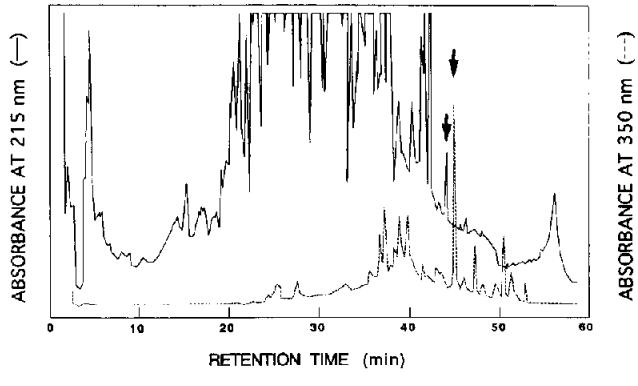

Fig. 3. Isolation of quinone-containing peptide from a thermolytic digest of the *p*-nitrophenylhydrazine-derivatized Cu^{2+}-activated enzyme. The chromatographic conditions are described in the text. The absorbances at 215 nm (peptide absorbance, —) and at 350 nm (*p*-nitrophenylhydrazone absorbance, --------) were continuously monitored. The peak of the quinone-containing peptide is indicated with arrows.

highly conserved in copper/topa quinone-containing monoamine oxidases [5]. Thus the quinone compound generated by aerobic incubation with Cu^{2+} of the Cu^{2+}-deficient inactive enzyme is most likely the topa quinone, derived from Tyr^{382}.

It has been established that resonance Raman spectroscopy is the most reliable method to identify the topa quinone in Cu^{2+}-containing amine oxidases [3–6]. Accordingly, we measured resonance Raman spectra of the *p*-nitrophenylhydrazine derivatives of the Cu^{2+}-activated enzyme, the quinone-containing peptide obtained by thermolysin digestion, and the topa quinone hydantoin model compound. All Raman spectra of these *p*-nitrophenylhydrazones were virtually identical (Fig. 5), leading to the conclusion that the Cu^{2+}-generated quinone compound is the topa quinone. Finally, the Y382F mutant enzyme, in which the precursor Tyr^{382} to the topa quinone is replaced by Phe [11], has also been purified to homogeneity. The Y382F mutant enzyme had almost no activity (< 0.05 unit/mg protein) and showed no absorption peak at 480 nm characteristic of the topa quinone (Fig. 1A), even after incubation with excess Cu^{2+}. No quinone compound was also produced by the in situ incubation with Cu^{2+} of the membrane-blotted mutant enzyme (Fig. 2). These results show that the precursor to the topa quinone must be a tyrosyl residue but not a phenylalanyl residue.

In conclusion, the results described so far demonstrate that the aerobic incubation with Cu^{2+} of the Cu^{2+}-deficient precursor form of phenylethylamine oxidase leads to the generation of its topa quinone redox cofactor. The multi-step autooxidation of a specific tyrosyl residue, as postulated for a possible pathway of the topa quinone biogenesis [4], is certainly mediated by the protein-bound copper ions. Thus, the enzyme protein has ability to generate its own coenzyme without the need of external enzymatic systems. Recently, Åberg et al. [15] have also provided evidence for the autocatalytic generation of the dopa quinone in the F208Y mutant enzyme of *E. coli* ribonucleotide reductase by its intrinsic Fe^{2+}, although the dopa generated in the mutant enzyme has no coenzymatic function. The biogenesis of the topa quinone cofactor might represent a novel type of posttranslational modification to generate a covalently bound functional group involved in oxidoreduction. For the future it will be necessary to elucidate the mechanism how the enzyme-bound Cu^{2+} converts a specific tyrosyl residue into the topa quinone cofactor and to examine whether the same cofactor in copper amine oxidases from eukaryotic organisms is also produced by Cu^{2+}-dependent autooxidation.

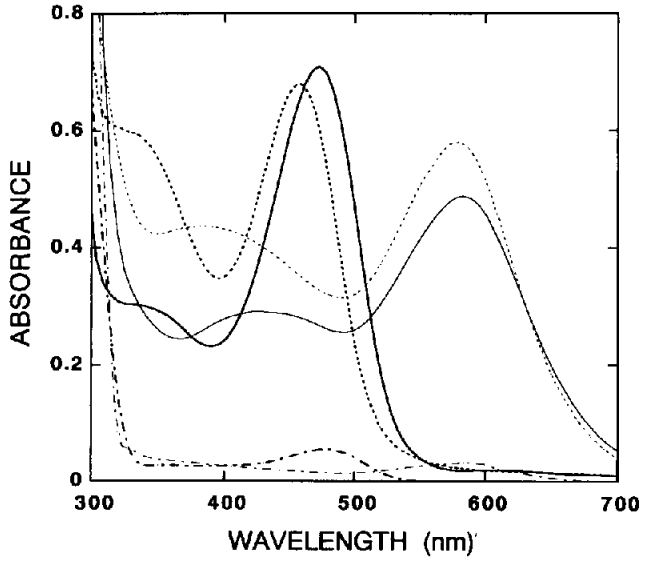

Fig. 4. Visible absorption spectra of *p*-nitrophenylhydrazone adducts. Spectra of the *p*-nitrophenylhydrazone derivatives of the Cu^{2+}-activated enzyme (26 μM subunit) (—), the quinone-containing peptide obtained from thermolytic digests (arbitrary concentration) (--------), and the Cu^{2+}-deficient precursor enzyme (26 μM subunit) (—·—·—) were measured in 50 mM HEPES (pH 6.8) (*heavy lines*) or in 1 M KOH (*thin lines*).

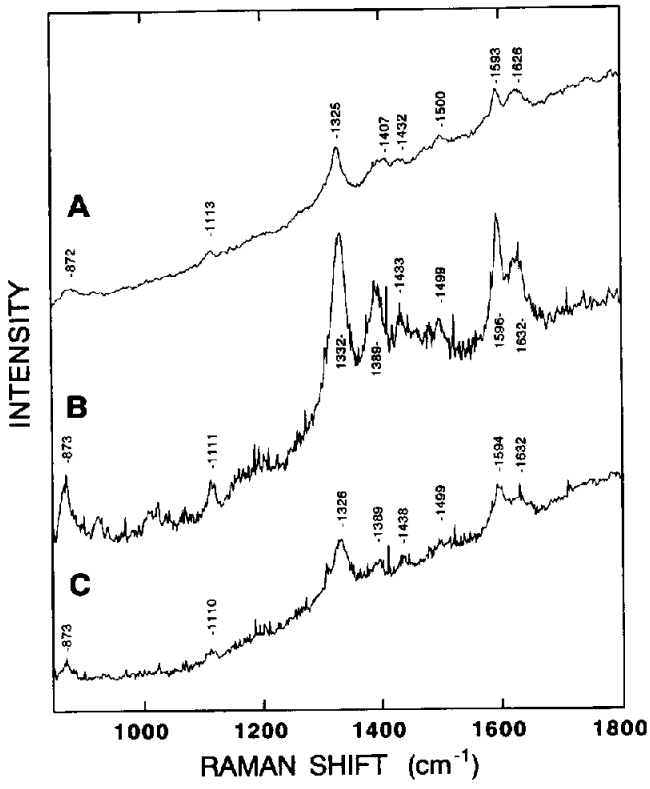

Fig. 5. Resonance Raman spectra for the *p*-nitrophenylhydrazone of the Cu^{2+}-activated enzyme (A), the quinone-containing peptide (B), and the topa quinone hydantoin model compound (C). Spectra were obtained with an apparatus consisting of a triple polychromator (Spex 1877c), an intensified photodiode array detector (PAR 1455R-HQ) operated at −20°C, and a personal computer (NEC 9801) for data collection. The 457.9-nm line from an Ar laser (Spectra Physics 2016–05) was used for excitation. The laser power at the sample position was about 15 mW, and a 5 cm^{-1} spectral slit width was employed. Frequency calibration was based on the Raman spectra of acetone, indene, and ethyl acetate, and estimated frequency errors were ± 2 cm^{-1} for well-resolved bands. The *p*-nitrophenylhydrazone of the topa quinone hydantoin model compound [2] was synthesized and purified according to the previously published procedure [5].

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References

[1] McIntire, W.S. and Hartmann, C. (1993) in: Principles and Applications of Quinoproteins (Davidson, V.L. Ed.) pp. 97–171, Marcel Dekker.

[2] 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.
[3] Brown, D.E., McGuirl, M.A., Dooley, D.M., Janes, S.M., Mu, D. and Klinman, J.P. (1991) J. Biol. Chem. 266, 4049–4051.
[4] Mu, D., Janes, S.M., Smith, A.J., Brown, D.E., Dooley, D.M. and Klinman, J.P. (1992) J. Biol. Chem. 267, 7979–7982.
[5] Janes, S.M., Palcic, M.M., Scaman, C.H., Smith, A.J., Brown, D.E., Dooley, D.M., Murc, M. and Klinman, J.P. (1992) Biochemistry 31, 12147–12154.
[6] Cooper, R.A., Knowles, P.F., Brown, D.E., McGuirl, M.A. and Dooley, D.M. (1992) Biochem. J. 288, 337–340.
[7] Bruinenberg, P.G., Evers, M., Waterham, H.R., Kuipers, J., Arnberg, A.C. and Geert, A.B. (1989) Biochim. Biophys. Acta 1008, 157–167.
[8] Zhang, X., Fuller, J.H. and McIntire, W.S. (1993) J. Bacteriol. 175, 5617–5627.
[9] Novotny, W.F., Chassande, O., Baker, M., Lazdunski, M. and Barbry, P. (1994) J. Biol. Chem. 269, 9921–9925.
[10] Mu, D., Medzihradszky, K.F., Adams, G.W., Mayer, P., Hines, W.M., Burlingame, A.L., Smith, A.J., Cai, D. and Klinman, J.P. (1994) J. Biol. Chem. 269, 9926–9932.
[11] Tanizawa, K., Matsuzaki, R., Shimizu, E., Yorifuji, T. and Fukui, T. (1994) Biochem. Biophys. Res. Commun. 199, 1096–1102.
[12] Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Clin. Chem. 20, 470–475.
[13] Shimizu, E., Kiguchi, S. and Yorifuji, T. (1989) Agric. Biol. Chem. 53, 2801–2803.
[14] Janes, S.M. and Klinman, J.P. (1991) Biochemistry 30, 4599–4605.
[15] Åberg, A., Ormö, M., Nordlund, P. and Sjöberg, B.-M. (1993) Biochemistry 32, 9845–9850.
[16] Paz, M.A., Flückiger, R., Boak, A., Kagan, H.M. and Gallop, P.M. (1991) J. Biol. Chem. 266, 689–692.