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Copper-Promoted Overall Transformation of 4-*tert*-Butylphenol to its *para*-Hydroxyquinonic Derivative, 2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone. Biomimetic Studies on the Generation of Topaquinone in Copper Amine Oxidases

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Abstract—Topaquinone (TPQ) is a cofactor present at the active site of copper amine oxidases, derived from a Tyr residue inserted in the polypeptide chain through a copper-dependent but otherwise largely unknown mechanism. A simple model system was developed that permits to obtain the overall transformation of 4-*tert*-butylphenol, chosen as a model for Tyr, into a TPQ-like, *para*-hydroxyquinonic structure in the presence of Cu(II)-imidazole mononuclear complexes. © 2000 Elsevier Science Ltd. All rights reserved.

Copper amine oxidases (CuAOs, EC 1.4.3.6) are ubiquitous enzymes that catalyze the oxidative deamination of primary amines to produce aldehydes, hydrogen peroxide, and ammonia.[1] These enzymes contain an active-site redox cofactor, identified by Klinman and coworkers as 2,4,5-trihydroxyphenylalanine quinone **1** (topaquinone, TPQ; Fig. 1).[2] Recent studies have shown that TPQ is produced by the post-translational modification of a strictly conserved active-site tyrosine residue by enzyme-bound copper and oxygen,[1b,3] in which appears to be a unique process among known enzymatic systems. In addition to the several fascinating mechanistic implications of the reaction, the full elucidation of TPQ biogenesis is also stimulated by the increasing medical interest in amine oxidases.[4] Indeed, among the CuAOs, the physiologically relevant lysyl oxidase has been recently shown to contain a modified form of TPQ, designated lysine tyrosylquinone (LTQ),[5] that is expected to be generated in a manner similar to that of TPQ. In patients with Menkes disease, which is characterized by a state of copper deficiency, the activity of lysyl oxidase is strongly impaired, giving rise to several clinical manifestations.[6] Very recent data indicate that copper deficiency does not influence lysyl oxidase expression, but results in an enzyme with reduced levels of its active site cofactor LTQ.[7]

Although the detailed mechanism of TPQ and LTQ biogenesis has yet to be elucidated, several plausible pathways leading from Tyr to TPQ (or LTQ) have been proposed that invoke a certain number of intermediates. In his simplest form (Fig. 2), the overall reaction is postulated to involve the initial hydroxylation of Tyr to 3,4-dihydroxyphenylalanine (dopa), followed by oxidation of dopa to dopaquinone. Nucleophilic attack by water (or hydroxide ion) on the dopaquinone ring leads to topa. Further oxidation of topa would finally give TPQ. Alternatively, the reaction could produce directly dopaquinone without passing through the compulsory intermediacy of the catechol, as suggested by Sayre and Nadkarni.[8]

We have previously addressed the issue of TPQ generation by means of biomimetic studies that utilized non-enzymatic model compounds for TPQ and putative biogenetic intermediates.[1b,9] In particular, the steps following the formation of dopa, have been investigated. More

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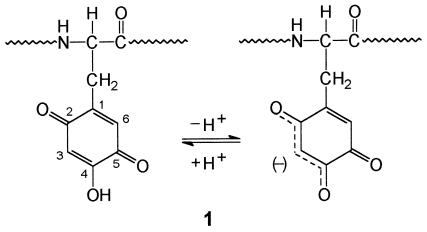

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Figure 1. Structure of TPQ and of its depronated form. The anionic form shows a characteristic absorbtion band in the visible region, centered around 485–490 nm.

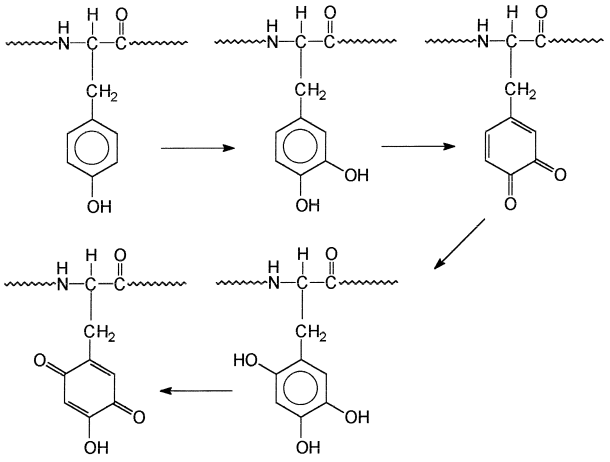

Figure 2. Postulated pathway for the biogenisis of TPQ cofactor from a tyrosine residue in CuAOs. LTQ cofactor in lysyl oxidase is thought to derive from Tyr through a similar mechanism.

recently, we started to focus on the first step of the TPQ generation, i.e., the oxidative conversion of tyrosine to dopa, which is by far the least understood portion of the biogenetic reaction. We herewith report some data showing that Cu(II)–imidazole mononuclear complexes are able, under experimental conditions, to induce the overall transformation of 4-*tert*-butylphenol, chosen as a model for Tyr, into a TPQ-like structure, namely 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (H*t*BQ). This model system might therefore offer a frame of reference for interpretation of studies on TPQ biogenesis in CuAOs.

In our model system,[10] Cu(II)–imidazole mononuclear complexes were used in order to account for the active-site copper ion, ligated by three histidines. 4-*tert*-Butylphenol was chosen as a model for Tyr, since the two compounds have a similar value of pKa for the phenol moiety, and the hydroxylated and quinonoid derivatives of 4-*tert*-butylphenol have been already studied.[9] The higher yield of H*t*BQ was obtained when 4-*tert*-butylphenol was incubated in the presence of $CuIm_4(ClO_4)_2$, in 20% aqueous MeOH at pH 11. Tables 1 and 2 list the results obtained varying the pH value from 8 to 12, and in the presence of other Cu(II)–imidazole complexes or uncomplexed copper. Attempts to lower (10%) or to increase (50 and 80%) the proportion of MeOH in the reaction solution, or to substitute it for another organic solvent (acetonitrile, dioxane, acetone) did always lead to a decrease in the yield of H*t*BQ (data not shown). In some cases, the formation of a brown precipitate, possibly due to the production of polymeric products, was

Table 1. Dependence on pH value of the production of H*t*BQ from 4-*tert*-butylphenol in the presence of $CuIm_4(ClO_4)_2$[a]

pH	H*t*BQ [mM]	Efficiency relative to that at pH 11 (100)
8	0.01	2.23
10	0.16	35.56
11	0.45	100
12	0.20	44.45

[a]Experimental conditions as reported in the Notes.[10] H*t*BQ yield estimated from absorbance at 485 nm ($\varepsilon = 1800\ M^{-1}\ cm^{-1}$).

Table 2. Relative efficiency in the production of H*t*BQ from 4-*tert*-butylphenol in the presence of free or complexed Cu(II)[a]

Cu(II) compound	H*t*BQ [mM]	Efficiency relative to that with $CuIm_4(ClO_4)_2$ (100)
$CuIm_4(ClO_4)_2$	0.45	100
$CuIm_4Cl_2$	0.43	95.56
$CuIm_2Cl_2$	0.36	80.00
free $Cu(ClO_4)_2$	0.27	60.00
free $CuCl_2$	0.26	57.78

[a]All experiments were performed at pH 11. Other experimental conditions as reported in the Notes.[10] H*t*BQ yield estimated from absorbance at 485 nm ($\varepsilon = 1800\ M^{-1}\ cm^{-1}$).

noticed. The achieved yield of conversion of 4-*tert*-butylphenol into H*t*BQ was low (approx. 2.25%), and most of the starting material remained unreacted (data not shown). However, this is not surprising, given the difficulty to get the bulk production of *para*-hydroxyquinonic compounds under alkaline conditions even when starting from catechols.[11a]

The identity of the final product of 4-*tert*-butylphenol hydroxylation and oxidation in our model system, H*t*BQ, is proven by its absorption spectrum (Fig. 3), showing a broad peak centered around 485 nm, which closely resembles that of an authentic sample of H*t*BQ,[9b] of other TPQ-like models,[11] and of native CuAOs. Moreover, in both cases, a very similar spectroscopic behaviour can be observed when the model compound is reacted with a 10-fold molar amount of the carbonyl reagent phenylhydrazine, with the progressive bleaching of the peak at 485 nm and the rapid increase at shorter wavelength (~430 nm) (Fig. 3). TPQ in native CuAOs is also well known to give stable hydrazine adducts when reacted with phenylhydrazine and 4-nitrophenylhydrazine, with comparable effects on the absorption spectrum.

There are some precedents for the *ortho*-hydroxylation of phenols by mononuclear copper systems to obtain catechols without cleavage of the aromatic system.[12] More recently, Urano and co-workers reported the selective *ortho*-hydroxylation of *para*-substituted phenols by a Cu^{2+}–ascorbic acid-O_2 system.[13] When *para*-substituted phenols bearing electron-donating groups (as *para*-cresol) were used as substrates, only small amounts of hydroxylated products were obtained (3%),[13] comparable to the yield obtained in this study, confirming that electron-donating groups probably retard the initial hydroxylation reaction of the phenol to the corresponding catechol. Other catalytic systems lead to oxidative

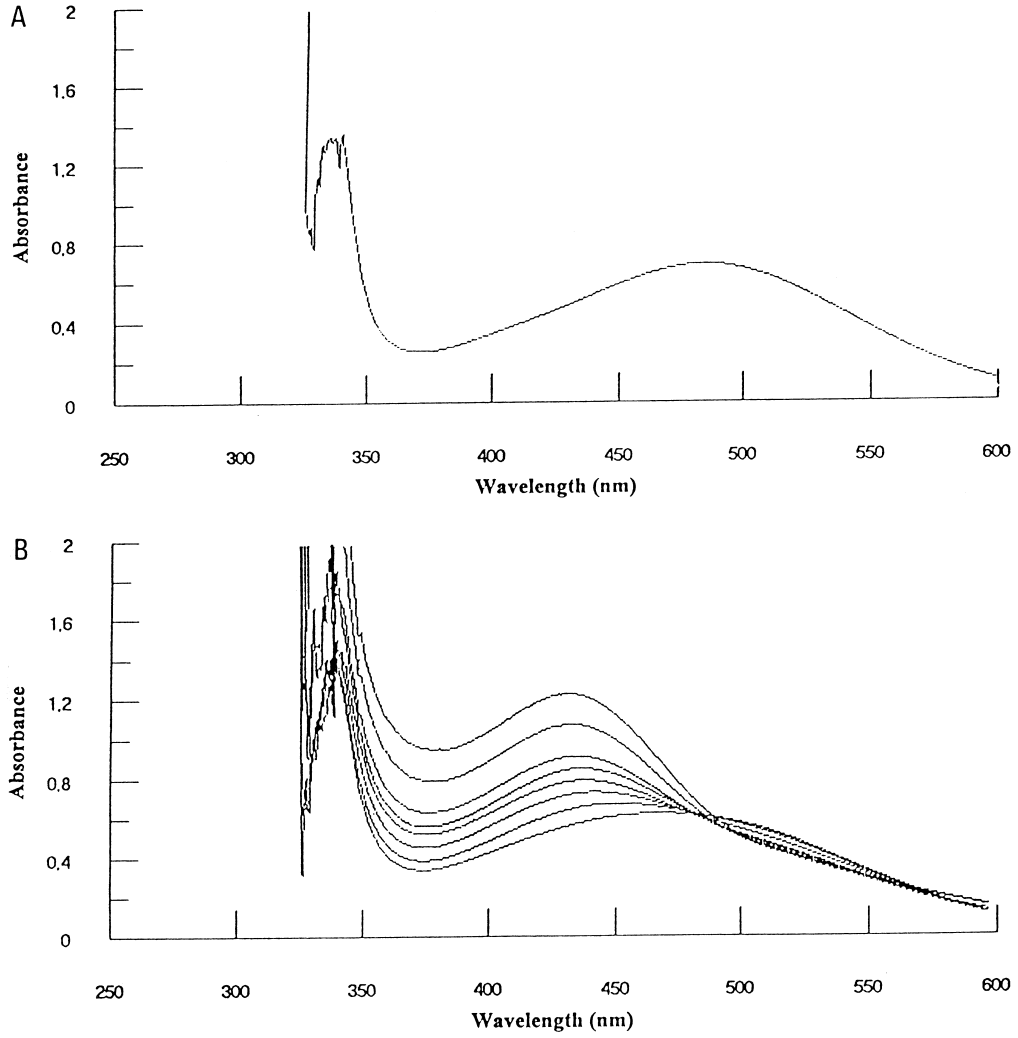

Figure 3. Absorbtion spectrum of H*t*BQ (0.39 mM, estimated from $\varepsilon_{485\ nm}$), in aqueous solution at pH 11, obtained from 4-*tert*-butylphenol.[10] (B) Time of reaction of H*t*BQ, as derived from 4-*tert*-butylphenol, with phenylhydrazine. H*t*BQ, 0.39 mM in aqueous solution, pH 11, was reacted with a 10-fold molar amount of phenylhydrazine. Spectra have been recorded at several times after addition of phenylhydrazine: 5, 10, 15, 20, 25, 30, 45, and 60 min. A similar spectroscopic behaviour was observed when an authentic sample of H*t*BQ[9b] was reacted with phenylhydrazine under similar experimental conditions (data not shown).

aromatic ring cleavage or to the formation of coupling products.[14] Also the direct conversion of phenols to *ortho*-quinones by Cu(I) in the presence of O_2, and the copper catalyzed oxidation of phenol to hydroquinone, have been demonstrated.[8,15]

There is considerable agreement in the literature that copper plays an essential role in the initial conversion of Tyr to dopa during TPQ biogenesis in CuAOs, although it is not yet clear whether copper is required for activating the phenol ring of tyrosine, dioxygen, or both. Mechanisms proposed so far involve an oxidation of the precursor tyrosine to a tyrosyl radical, concomitant with the conversion of active site Cu(II) to Cu(I), with a copper-dependent activation of both the phenol ring and dioxygen,[16] or the formation of a charge-transfer complex of Cu(II) and a tyrosinate ion,[17] with dioxygen reacting directly with the activated phenol ring, without the necessity to generate Cu(I) and tyrosyl radical as discrete precursor species. A recent study on a native CuAO from *Arthrobacter globiformis* has ascertained that the oxidation of tyrosine to TPQ consumes two mol of dioxygen and produces one mol of H_2O_2 for every mol of TPQ generated.[18]

Our results indicate that, under experimental conditions, it is possible to obtain a TPQ-like compound starting from a phenol, and that this reaction is promoted by Cu(II) reagents. In particular, complexation of copper by imidazoles enhances the yield of H*t*BQ with respect to uncomplexed Cu(II) species, possibly favoring the initial hydroxylation of the phenol ring to a catechol. Site-directed mutagenesis studies have shown that the copper-ligating histidines in native CuAOs are required for TPQ formation, confirming the key role played by coordinated copper in the biogenesis reaction. Moreover, data reported in Table 1 suggest that proton abstraction from the phenol moiety to give a phenolate ion, obtained by raising the pH of the reaction solution (pK_α value of the hydroxyl group of 4-*tert*-butylphenol being $\cong$10.0), it is a prerequisite for the effective *ortho*-hydroxylation of the aromatic system. This is consistent

with the expectation that the phenolate ring would be more susceptible to direct, or indirect (through copper), attack by dioxygen.

Obviously, the results obtained from model studies must be in any case carefully evaluated and interpreted before exporting to the relevant enzymatic system, and is therefore not possible to translate in a straightforward manner our results to the mechanism of TPQ biogenesis in the protein active site. However, present data tend to support the hypothesis that a tyrosinate ion may be involved in the first step of cofactor generation in native CuAOs.[17]

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10. Prior to use, 4-*tert*-butylphenol was analyzed by means of HPLC, to ensure it was free from contaminating 4-*tert*-butylcatechol. Solutions were made using deionized, distilled water but not purified further, so contained usual levels of trace metals. Cu(II)–Imidazole complexes $CuIm_2Cl_2$, $CuIm_4Cl_2$, and $CuIm_4(ClO_4)_2$ were prepared according to the methods reported in Goodgame, D. M. L.; Goodgame, M.; Rayner-Canham, G. W. *Inorg. Chim. Acta* **1969**, *3*, 406. and in Krushna, C.; Mohapatra, C.; Dash, K. C. *J. Inorg. Nucl. Chem*. **1977**, *39*, 1253. Analytical results (C, H, and N) for all the compounds were good. Characterization of the complexes involved measurement of infrared spectra, reflectance spectra, and electric conductance (data not shown). Hydroxylation of 4-*tert*-butylphenol was attempted as follows. In a typical experiment, a mixture of 20 mM 4-*tert*-butylphenol and 2 mM Cu(II)–imidazole complex (or uncomplexed copper) in 10 mL of 20% aqueous MeOH was adjusted to pH 11 with NaOH. The pH value was corrected for the mixed solvent system, and remained at the initial set value over the course of the reaction, or fell slightly. The solution was magnetically stirred vigorously at 40 °C in the dark. Several experiments at different pH values (in the range 8–12), or with organic solvents other than MeOH (dioxane, acetonitrile, acetone), were also performed. In another set of experiments, the MeOH concentration in the reaction solution was varied progressively from 10 to 80%. In all cases, after 48 h, the reaction mixture was acidified with HCl and then extracted with dichloromethane for three times, and the combined organic layer was dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure. The concentrate was then resuspended in 2 mL of 0.1 M NaOH, to ensure a rapid autoxidation of all the species present, and the absorbance spectra recorded at 10 and 60 min after addition of NaOH. Authentic samples of H*t*BQ and its deprotonated analogue were prepared from 4-*tert*-butylcatechol,[9b] and the concentration estimated using an $\epsilon_{484\ nm}$ of 1800 $M^{-1}\ cm^{-1}$ in H_2O, as reported by Moënne-Loccoz, P.; Nakamura, N.; Steinebach, V.; Duine, J. A.; Mure, M.; Klinman, J. P.; Sanders-Loehr, J. *Biochemistry* **1995**, *34*, 7020. The reaction of H*t*BQ, as derived from 4-*tert*-butylphenol, with phenylhydrazine is described in the caption of Figure 3.
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