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Communication

Characterization of the Native Lysine Tyrosylquinone Cofactor in Lysyl Oxidase by Raman Spectroscopy*

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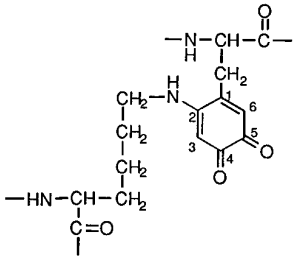
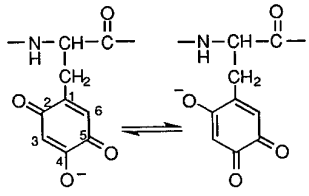
Lysine tyrosylquinone (LTQ) recently has been identified as the active site cofactor in lysyl oxidase by isolation and characterization of a derivatized active site peptide. Reported in this study is the first characterization of the underivatized cofactor in native lysyl oxidase by resonance Raman (RR) spectrometry. The spectrum is characterized by a unique set of vibrational modes in the 1200 to 1700 cm^{-1} region. We show that the RR spectrum of lysyl oxidase closely matches that of a synthetic LTQ model compound, 4-*n*-butylamino-5-ethyl-1,2-benzoquinone, in aqueous solutions but differs significantly from those of other topa quinone-containing amine oxidases under similar conditions. Furthermore, we have observed the same ^{18}O shift of the C=O stretch in both the lysyl oxidase enzyme and the LTQ cofactor model compound. The RR spectra of different model compounds and their D shifts give additional evidence for the protonation state of LTQ cofactor in the enzyme. The overall similarity of these spectra and their shifts shows that the lysyl oxidase cofactor and the model LTQ compound have the same structure and properties. These data provide strong and independent support for the new cofactor structure, unambiguously ruling out the possibility that the structure originally reported had been derived from a spurious side reaction during the derivatization of the protein and isolation of the active site peptide.

Quinoproteins are a class of enzymes that utilize quinones as their active site redox cofactor (1). Among the well-known quinocofactors, topa quinone (TPQ,[1] Structure 2) is ubiquitous, existing in both prokaryotic and eukaryotic enzymes that include a divergent class of copper-containing amine oxidases (CAOs); tryptophan tryptophylquinone and pyrroloquinoline quinone have thus far been found only in prokaryotic systems.

One of the mammalian CAOs, lysyl oxidase, plays an important role in the development of connective tissues through its catalysis of the post-translational oxidative modification of the ε-amino group of the lysine side chains to form inter- and intrachain protein cross-links (2). Recently, the active site cofactor in lysyl oxidase was identified as lysine tyrosylquinone (LTQ, Structure 1), derived from the cross-linking of a modified tyrosine residue to the ε-group of a lysyl side chain (3). The identification of this cofactor involved the isolation of an active site cofactor-containing peptide from phenylhydrazine-derivatized lysyl oxidase and the subsequent structural characterization of this peptide by Edman sequencing, mass spectrometric, and resonance Raman spectrometric studies (3).



STRUCTURE. 1. **LTQ**.



STRUCTURE 2. **TPQ**.

As described (3), [^{14}C]phenylhydrazine was used to inactivate and stabilize the enzyme, as well as to provide a radioactive label that could be monitored during the peptide purification. A model system containing the phenylhydrazine adduct of 4-ethyl-1,2-benzoquinone was found to be unreactive toward propylamine (3). Although this result made it unlikely that a lysyl residue-cross-linked cofactor would be generated by fortuitous side reactions during the course of phenylhydrazine inhibition and active-site peptide isolation, the final proof of the cofactor structure has awaited a structural analysis of the underivatized cofactor in native lysyl oxidase. In this study, we show that the resonance Raman spectra of underivatized lysyl oxidase are in excellent agreement with those of the synthetic LTQ model compound used in the initial elucidation of the cofactor structure. This finding provides strong evidence for an amino substituent at C-2 of the quinone ring in native lysyl oxidase. Furthermore, the resonance Raman study on different LTQ compounds supports the view that LTQ is a neutral *ortho*-quinone structure under physiologic conditions.[2]

EXPERIMENTAL PROCEDURES

Lysyl oxidase was purified from calf aorta by a modification of previously described methods (5, 6). The precipitated enzyme was obtained after dialyzing the purified enzyme (eluted from gel filtration column in 6 M urea buffer) against 16 mM phosphate buffer, pH 7.8. Yeast amine

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[1] The abbreviations used are: TPQ, 2,4,5-trihydroxyphenylalanine (topa) quinone; CAO, copper amine oxidases; LTQ, lysine tyrosylquinone; RR: resonance Raman.

[2] S. X. Wang, M. Mure, and J. P. Klinman, manuscript in preparation.

oxidase was expressed and purified as described (7), and pea seedling amine oxidase was provided by D. M. Dooley and D. E. Brown. The LTQ model compounds used in this study were prepared as described by Wang *et al.*[2] For mass spectrometric analyses, 2-hydroxy-5-ethyl-1,4-benzoquinone and 4-butylamino-5-ethyl-1,2-benzoquinone were employed as the model compounds for TPQ and LTQ, respectively. 3 mg of each described compound was first dissolved in 20 μl of acetonitrile and then diluted with 100 μl of $H_2{}^{18}O$ (97.1 atom %, Isotec Inc.). The quinone solution was incubated overnight (approximately 15 h) at 25 °C before it was lyophilized and submitted for mass spectrometric analysis. High-resolution mass spectra were obtained by electron impact ionization (70 eV) on a JEOL JMX-DX 300 instrument.

Raman spectra were obtained on a McPherson 2061 spectrograph (0.67 m, 1800-groove grating) using Kaiser Optical holographic supernotch filters and a Princeton Instruments liquid N_2-cooled (LN-1100PB) CCD detector with 514.5 nm and 488.0 nm excitations from a Coherent Innova 90-6 Ar^+ laser. The samples were placed in glass capillaries and measured at room temperature in a 90° scattering geometry for model compounds and a back scattering geometry for precipitated enzyme. Peak frequencies were calibrated relative to an indene standard and are accurate to ± 1 cm^{-1}. Spectra of isotopically substituted samples were obtained under identical instrumental conditions such that frequency shifts are accurate to ± 0.5 cm^{-1} (8).

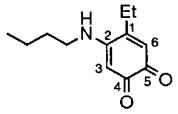
For ^{18}O-isotope exchange, the precipitated lysyl oxidase was centrifuged and supernatant was removed. The enzyme was then suspended in 4 volumes of 16 mM potassium phosphate (pH 7.8) that was prepared by the addition of concentrated potassium phosphate to $H_2{}^{18}O$ (97 atom %, Icon). This cycle was repeated two more times to yield 16 mM potassium phosphate (pH 7.8) and 89 atom % ^{18}O.

RESULTS AND DISCUSSION

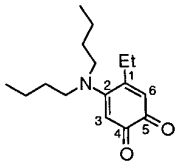
RR Spectra of Amine Oxidases—The UV-Vis spectrum of the LTQ cofactor in native lysyl oxidase shows a characteristic absorption maximum at approximately 510 nm, which is 20 to 30 nm red-shifted relative to other TPQ-containing CAOs (3). The synthetic LTQ model compound (NHR-LTQ, Structure 3) displays a λ_{max} of 504 nm which is an excellent match to that of the native enzyme (3). Using excitation within this absorption band, resonance Raman spectra can be obtained whose peak frequencies represent the vibrational modes of the LTQ chromophore itself. As shown in Fig. 1, the RR spectrum of the LTQ cofactor in the resting state of lysyl oxidase is significantly different from the RR spectrum of the TPQ cofactor in other amine oxidases. The lysyl oxidase spectrum is dominated by peaks at 1386 cm^{-1} and 1529 cm^{-1}, whereas the TPQ-containing amine oxidases have their most intense feature 10–15 cm^{-1} higher than 1386 cm^{-1} and no feature corresponding to the peak at 1529 cm^{-1}. The difference in state between lysyl oxidase (precipitate) and the amine oxidases (solution) does not appear to be a factor, as no changes in RR vibrational frequencies are observed for amine oxidase in crystals or in ammonium sulfate precipitates.

The spectra of the amine oxidase samples are more similar to one another in both frequency and intensities and appear to be relatively unaffected by the orientation of the TPQ cofactor in the active site. In the crystal structure of pea seedling amine oxidase, the TPQ ring has a flipped conformation in the resting enzyme with the C-5 carbonyl hydrogen-bonded by the side chains of Tyr^{286} and Asn^{386} (9). In contrast, the TPQ cofactor in yeast amine oxidase is in the active conformation with the C-5 carbonyl pointing toward the substrate binding pocket.[3] Since the different TPQ environments in amine oxidases are not affecting the electronic structure of the cofactor (as shown in Fig. 1), the distinctly altered frequencies and intensities in the RR spectrum of lysyl oxidase are indicative of a change in the chemical structure of the LTQ cofactor rather than a change of environment.

RR Spectra of Lysyl Oxidase and LTQ Models—In contrast to the data for the TPQ enzymes (Fig. 1), the RR spectrum of the lysyl oxidase enzyme shows striking similarities to the NHR-LTQ (Structure 3) model compound (Fig. 2). Although the spectra of Structure 3 were obtained with 488 nm excitation, identical RR spectra were also observed with 514 nm excitation (data not shown). The spectra of the LTQ model indicate intense peaks at 1676, 1536, and 1392 cm^{-1} and weaker peaks at 1635, 1590, 1340, 1286, and 1255 cm^{-1} (Fig. 2*A*). In $H_2{}^{18}O$, the peak at 1676 cm^{-1} undergoes a -23 cm^{-1} shift whereas the peak at 1635 cm^{-1} loses its intensity (Fig. 2*B*). The frequency of 1676 cm^{-1} is within the 1640 to 1695 cm^{-1} range observed for the C=O stretch in a large number of substituted benzoquinones (11) and is attributed to the quinone carbonyl group. Based on a simple two-body system, the observed shift of -23 cm^{-1} for this peak in $H_2{}^{18}O$ suggests the existence of a singly exchanged carbonyl group in the LTQ model compound (11). This assignment is supported by the mass spectrum of NHR-



STRUCTURE 3. **NHR-LTQ**. R = *n*-butyl.



STRUCTURE 4. **NR_2-LTQ**. R = *n*-butyl. Model compounds (Structures 3 and 4) are numbered to agree with the LTQ structure (Structure 1), rather than by the conventional nomenclature for organic compounds.

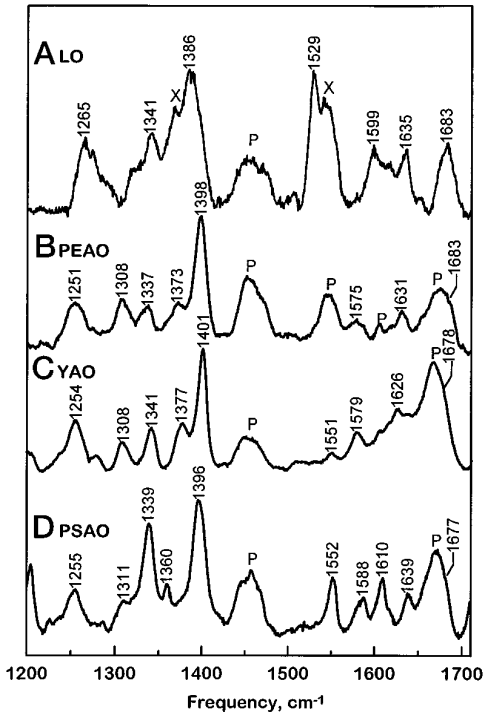


FIG. 1. **Resonance Raman spectra of lysyl oxidase and amine oxidases.** *A*, lysyl oxidase, precipitated from 16 mM phosphate (pH 7.8). *B*, phenylethylamine oxidase (1.3 mM) from *Arthrobacter globiformis* in 50 mM HEPES (pH 6.8). Data from Ref. 13. *C*, yeast amine oxidase (1.3 mM) in 50 mM phosphate (pH 6.8). *D*, pea seedling amine oxidase (1.4 mM) in 100 mM phosphate (pH 7.2). *Spectra B–D* were obtained with 514.5 nm (30–40 milliwatts) excitation, 4 cm^{-1} spectral resolution, and 10 min of data accumulation, whereas *spectrum A* required 60 milliwatts of laser power and 40 min of accumulation. *P* denotes protein vibrational mode (10), *X* denotes instrumental artifact.

[3] R. Li, J. P. Klinman, and F. S. Mathews, manuscript in preparation.

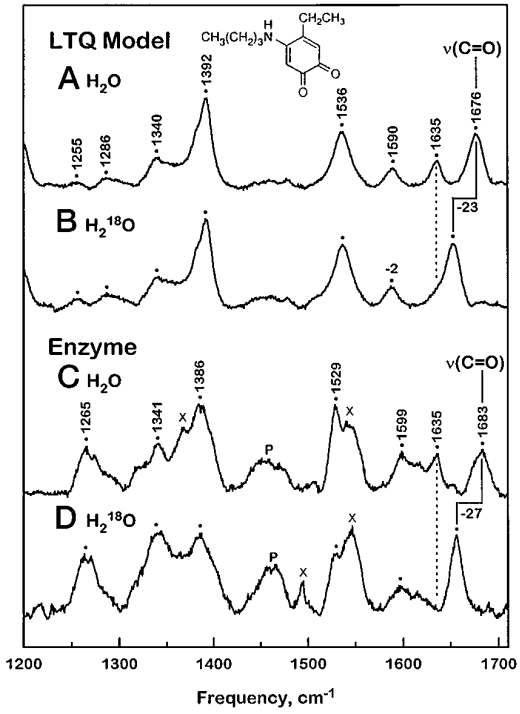


FIG. 2. **Raman spectra of N-*n*-butyl-LTQ model compound and lysyl oxidase.** The LTQ model (Structure 3, NHR-LTQ) was dissolved in $H_2{}^{16}O$ (*A*) and $H_2{}^{18}O$ (*B*) and examined with 488.0 nm (10 milliwatts) excitation, 5 cm^{-1} spectral resolution, and 5 min of data accumulation. The lysyl oxidase precipitate was suspended in $H_2{}^{16}O$ (*C*) and $H_2{}^{18}O$ (*D*) buffer (pH 7.8) and examined as in Fig. 1*A*. Frequency shifts in ^{18}O relative to ^{16}O are listed above each peak, with unchanged frequencies being unlabeled.

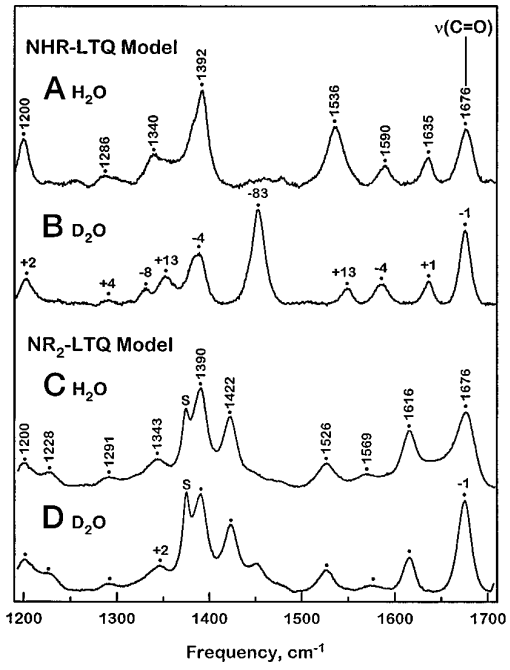


FIG. 3. **Raman spectra of LTQ models with R = *n*-butyl.** The mono-*n*-butyl model (**3**, NHR-LTQ) was dissolved in H_2O (*A*) and D_2O (*B*) and analyzed as in Fig. 2*A*. The stock solution (1.6 mM, 20% acetonitrile/water) of the di-*n*-butyl model (**4**, NR_2-LTQ) was diluted 10-fold by H_2O (*C*) and D_2O (*D*) and examined with 514.5 nm (12 milliwatts) excitation, 4 cm^{-1} spectral resolution, and 5 min of data accumulation. *S* denotes solvent (acetonitrile) mode. Frequency shifts in D relative to H are listed above each peak, with unchanged frequencies being unlabeled.

LTQ which shows only a 2 mass unit increase in $H_2{}^{18}O$, indicating that only one of the two carbonyl groups has reacted. Studies have shown that the C-5 carbonyl (C=O) of this model compound is susceptible to nucleophilic attack to form stable phenylhydrazine adducts (3)[2] and thus is potentially capable of exchange with solvent oxygen. The intense peak at 1676 cm^{-1} is therefore assigned to the C=O stretch of the C-5 carbonyl. There are no other changes of the spectrum in $H_2{}^{18}O$ except that the C=O bend at 502 cm^{-1} shifts by −3 cm^{-1} (data not shown).

The spectra of the native lysyl oxidase in solution are difficult to acquire due to sample fluorescence caused by the high concentration of urea required to solubilize the enzyme. Removal of urea produces a hydrated enzyme precipitate from which we have succeeded in obtaining the RR spectra of native lysyl oxidase in both $H_2{}^{16}O$ and $H_2{}^{18}O$ (Fig. 2, *C* and *D*). Overall, the spectral frequencies, intensities, and the ^{18}O shifts are remarkably similar to those of the LTQ model (summarized in Table I). This indicates that the cofactor in lysyl oxidase enzyme has a similar quinone structure to model compound (Structure 3) and that the *n*-butylamino group at the C-2 position and ethyl group at the C-1 position accurately model the respective vibrational contributions of the ϵ-NH_2 side chain of lysine and the β-CH_2 group of tyrosine residue in the cross-linked cofactor. Analogous to Structure 3, the 1683 cm^{-1} peak undergoes a −27 cm^{-1} shift in $H_2{}^{18}O$ whereas the peak at 1635 cm^{-1} loses its intensity. The 1683 cm^{-1} peak is similarly assigned to the C=O stretch of the C-5 carbonyl group in the cofactor (Structure 1). As will be discussed later, the RR spectra of other TPQ-containing CAOs also show an exchangeable carbonyl group at similar frequency (12, 13).

During the identification and the characterization of the LTQ cofactor, we synthesized several other organic model compounds,[2] including 4-(di-*n*-butylamino)-5-ethyl-1,2-benzoquinone (NR_2-LTQ, Structure 4). The λ_{max} of this compound is 20 to 30 nm red-shifted compared with that of the monosubstituted LTQ model compound (Structure 3) or to that of lysyl oxidase itself.[2] Based on the pK_a analysis of these quinone model compounds and their UV-Vis properties, we have proposed that, at physiological pH, the LTQ cofactor exists as the neutral NH-containing *ortho*-quinone structure (as shown in Structures 1 and 3) rather than the protonated quaternary amine or deprotonated imine.[2] In support of the pK_a assignment,[2] we have found that the RR spectrum of NHR-LTQ is altered only at pH values <2 (data not shown).

The resonance Raman spectra of the mono- and disubstituted LTQ model compounds are compared in Fig. 3. Overall, the Raman spectrum of NR_2-LTQ, Structure 4, has a pattern similar to that of NHR-LTQ, Structure 3, with intense peaks at 1676 and 1390 cm^{-1} (Fig. 3*A*). The 1676 cm^{-1} peak of Structure 4 can be assigned to the C=O stretch of the C-5 carbonyl group by analogy to Structure 3. This C=O stretch is an isolated vibrational mode whose frequency is unaffected by substitution

TABLE I
Resonance Raman frequencies and isotope shifts for LTQ in lysyl oxidase protein and the model compound (NHR-LTQ)

Boldface indicates the most intense peaks. ↓ means decrease in intensity.

LTQ model		Lysyl oxidase enzyme	
Frequency	$\Delta\nu$ ($H_2{}^{18}O$)	Frequency	$\Delta\nu$ ($H_2{}^{18}O$)
1676	−23	**1683**	−27
1635	↓	1635	↓
1590		1599	
1536		**1529**	
1392		**1386**	
1340		1341	

at the C-2 position. While the peaks below 1400 cm^{-1} show close resemblance in Structures 3 and 4, the peaks between 1400 and 1650 cm^{-1} are very different (Fig. 3, *A* and *C*), which could reflect the nature of the substituent at the C-2 position.

The 1400 to 1600 cm^{-1} region also shows the greatest sensitivity to deuterium exchange in NHR-LTQ (Fig. 3*B*). Almost every peak in the spectrum shifts, and the shifts are complete within the few minutes that are needed for dissolution in D_2O and data collection. Most noticeably, the peak at 1536 cm^{-1} disappears and is replaced by a new peak at 1453 cm^{-1}. It is likely that the 1536 cm^{-1} peak in H_2O has actually been replaced by a Fermi resonance doublet at −83 and +13 cm^{-1} in D_2O; similarly the 1340 cm^{-1} peak in H_2O appears to produce a Fermi doublet at +13 and −4 cm^{-1} in D_2O. In contrast, the addition of D_2O to NR_2-LTQ has essentially no effect on its RR spectrum (Fig. 3*D*). Based on these facts, we conclude that the D shifts observed in Fig. 3*B* are due to the D-exchange of the hydrogen on the C-2 NH and not the C-3 hydrogen. This is supported by the ^1H NMR spectroscopy of NHR-LTQ which shows no incorporation of D at the C-3 position even after 3–7 days of incubation in D_2O.[2] Incubation of the lysyl oxidase enzyme for 1 h in D_2O causes a similar loss of intensity at 1529 cm^{-1} (data not shown), indicative of NH exchange in the C-2 substituent. Thus, the D-isotope effects observed in the RR spectrum provide direct evidence for the proposed cofactor structure and the existence of the N-H hydrogen in that structure.

Comparison of LTQ and TPQ in Amine Oxidases—Although lysyl oxidase is considered a member of the CAO family and catalyzes similar redox reactions, it differs greatly from other TPQ-containing amine oxidases. Besides the lack of the TPQ consensus sequence and its reduced size, the most fundamental difference lies in its unique cofactor structure. From the studies herein, we conclude that the LTQ cofactor (Structure 1) has a neutral *ortho*-benzoquinone structure, whereas the active TPQ cofactor (Structure 2) exists as an *ortho-/para*-quinone resonance hybrid with significant charge localized on the C-4 oxygen. However, the exchangeable C=O stretch assigned to the C-5 carbonyl of LTQ cofactor at 1683 cm^{-1} has essentially the same energy as the analogous carbonyl vibrations at 1677–1683 cm^{-1} in the TPQ cofactors (Fig. 1). The fact that the reactive carbonyl group has the same bond strength in the *ortho*- and *para*-quinone forms of the cofactor is consistent with the similar chemical reactivities of this position in LTQ and TPQ toward nucleophiles. On the other hand, the C=O stretch of the C-2 carbonyl in TPQ has been identified at 1575 cm^{-1} in phenylethylamine oxidase from its −21 cm^{-1} shift with ^{18}O at the C-2 position (13). An analogous peak is present at 1579–1588 cm^{-1} in other TPQ-containing CAOs, including yeast amine oxidase and pea seedling amine oxidase (Fig. 1), as well as *E. coli* amine oxidase (12), histamine oxidase (13), and bovine serum amine oxidase (4), but is missing from the RR spectrum of lysyl oxidase (Fig. 1). A possible assignment for the C=O stretch of the C-4 carbonyl in lysyl oxidase is the peak at 1599 cm^{-1}. Further evidence for the chemical difference of the two carbonyl groups in each cofactor comes from the observation of only one exchangeable oxygen in the mass spectrum of both NHR-LTQ and TPQ (12) model compounds.

A unique feature in the RR spectrum of lysyl oxidase is the intense peak at 1529 cm^{-1}, which corresponds to the peak at 1536 cm^{-1} in the model compound (Fig. 2). Its strong deuterium dependence in both lysyl oxidase and the LTQ model suggests that it is due to the lysyl side chain (the NH group) at the C-2 position. The high frequency of this band implies double bond character. Thus, it could involve C=C stretching combined with C-N stretching or C-N-H bending at C-2. The other intense peak at 1386 cm^{-1} in lysyl oxidase is at lower energy than the intense peak at 1396–1401 cm^{-1} in other amine oxidases (Fig. 1). In phenylethylamine oxidase, the 1396 cm^{-1} peak has some contribution from C_2=O stretching (13). The sharp drop in the intensity of the ~1390 cm^{-1} peak in both lysyl oxidase (data not shown) and the LTQ model (Fig. 3*B*) in D_2O indicates that the amino group at C-2 is a contributor to the 1386 cm^{-1} mode in lysyl oxidase.

A significant difference between LTQ and TPQ is that the C-3 hydrogen of TPQ readily exchanges with D_2O in both *E. coli* amine oxidase enzyme and a TPQ model compound (12), whereas no such exchange has been observed for NHR-LTQ. The C-3 hydrogen exchange in TPQ is promoted by the anionic character of the cofactor and provides evidence for a resonance delocalization between the C-2 and C-4 oxygens as shown in Structure 2. The lack of exchange in LTQ is indicative of a more localized orthoquinone structure.

In summary, the resonance Raman spectrum of the underivatized lysyl oxidase closely matches that of a synthetic analog of the cofactor (NHR-LTQ) and shows the same ^{18}O shift as the latter. The C=O stretch at 1683 cm^{-1} (1676 cm^{-1} in the model) is identified as that of the C-5 carbonyl in the cofactor; this is the position shown to undergo derivatization with exogenous nucleophiles.[2] The intense peak at 1536 cm^{-1} and its shift in D_2O provide direct evidence for the existence of the N-H hydrogen in the lysyl side chain, indicating that under physiologic pH conditions LTQ exists as a neutral *ortho*-quinone structure. This contrasts with TPQ that exists as a resonance-stabilized hybrid with a net (−1) negative charge. The properties of LTQ may confer unique chemical reactivity to this cofactor[2] and explain the evolution of a quinocofactor structure that is distinct from the other copper amine oxidases.

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