Characterization of the Topa Quinone Cofactor in Amine Oxidase from *Escherichia coli* by Resonance Raman Spectroscopy†

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ABSTRACT: The aromatic amine oxidase from *Escherichia coli* (ECAO) utilizes Cu(II) and 2,4,5-trihdroxyphenylalanine quinone (TPQ) as cofactors in enzymatic catalysis. The TPQ cofactor is clearly identified by a set of characteristic vibrational modes between 1200 and 1700 cm^{-1} in the resonance Raman (RR) spectrum of the native enzyme. This is the first report of a RR spectrum for an underivatized TPQ cofactor in an enzyme, showing that it is possible to study changes in the cofactor during the natural reaction cycle. The RR spectrum of ECAO closely matches that of a 2-hydroxy-1,4-benzoquinone model compound, particularly in the deprotonated state in aqueous solution. The principal in-phase C=O symmetric stretching mode of the quinone occurs at 1681 cm^{-1} in ECAO and at 1666 cm^{-1} in the model compound and, in both cases, undergoes a downshift of ~25 cm^{-1} upon substitution of *one* of the carbonyl oxygens with ^{18}O. The overall similarity of the ^{18}O and D shifts in their RR spectra shows that the TPQ cofactor and model compound have the same structure and reactivity, with oxygen exchange occurring at the carbonyl adjacent to the hydroxyl group. Substrate reduction of ECAO under anaerobic conditions leads to a stable semiquinone (λ_{max} at 442 and 468 nm) with a RR spectrum characteristic of an amine-substituted semiquinone. The intense ring mode at 1647 cm^{-1} undergoes a shift of −4 cm^{-1} in enzyme reacted with ^{15}N-containing substrate and a shift of −6 cm^{-1} upon addition of cyanide, indicating that the semiquinone cofactor is perturbed by formation of the Cu(I)CN complex. Thus, the Cu(I) cofactor appears to be sufficiently close to undergo electrostatic interactions with the semiquinone cofactor in this catalytic intermediate of amine oxidase.

The redox-active organic cofactor of copper amine oxidases has been identified as 2,4,5-trihydroxyphenylalanine (topa) in the reduced enzyme and topa quinone (TPQ) in the oxidized enzyme (Janes et al., 1990; Klinman & Mu, 1994). This quinone cofactor is derived by the posttranslational modification of a protein tyrosine residue that occurs as a copper-dependent, autocatalytic reaction (Mu et al., 1992; Cai & Klinman, 1994; Matsuzaki et al., 1994). The structure of TPQ was originally solved by performing NMR and mass spectroscopic analyses on the stable phenylhydrazine adduct of a pentapeptide from bovine serum amine oxidase (BSAO) (Janes et al., 1990). The cofactor appears to be a 2,5-quinone with nucleophilic substitutions occurring at the C5 carbonyl, adjacent to the hydroxyl group (Mure & Klinman, 1993). The phenylhydrazone adduct of TPQ has a distinctive resonance Raman (RR) spectrum, and this has led to the identification of a number of homologous TPQ-containing amine oxidases from animal, plant, and bacterial sources (Brown et al., 1991; Janes et al., 1992). These enzymes are dimeric, and each subunit has a molecular weight of ~85 000, 1.0 Cu, and 0.5−0.8 TPQ, which is dependent on enzyme-specific activity (Janes & Klinman, 1991).

Amine oxidases catalyze the conversion of an aliphatic amine to an aldehyde, followed by a two-electron reduction of O_2 to H_2O_2 to regenerate the oxidized enzyme. The following sequence of reactions has been proposed (Klinman & Mu, 1994; Turowski et al., 1993):

$$RCH_2NH_2 + Cu^{II}\text{-}topa_Q \rightarrow RCHO + Cu^{II}\text{-}topa_{NH_2} \quad (1)$$

$$Cu^{II}\text{-}topa_{NH_2} \rightleftharpoons Cu^{I}\text{-}topa_{NHSQ} \quad (2)$$

$$Cu^{I}\text{-}topa_{NHSQ} + O_2 + H_2O \rightarrow Cu^{II}\text{-}topa_Q + H_2O_2 + NH_3 \quad (3)$$

First the topa quinone ($topa_Q$) cofactor is reduced by substrate to an aminoresorcinol ($topa_{NH_2}$). Then the cofactor undergoes a one-electron oxidation by Cu(II) to form the semiquinone ($topa_{NHSQ}$). Finally, reaction with O_2 and water regenerates the quinone and Cu(II). Evidence for the formation of Cu(I)-$topa_{NHSQ}$ comes from EPR and pulsed EPR studies (McCracken et al., 1992; Warnke et al., 1994). They have shown that the free-radical signal of the enzyme reacted with substrate under anaerobic conditions is sensitive to the N-isotope composition of the substrate. We have now

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confirmed the formation of a nitrogen-containing semiquinone by RR spectroscopy.

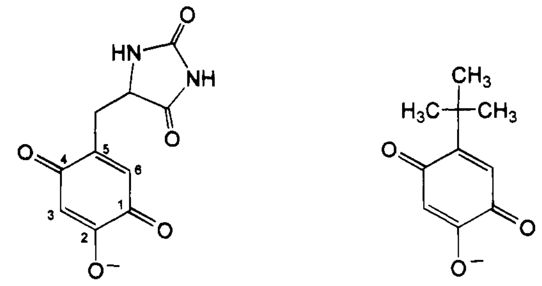
All of the RR spectra previously reported for amine oxidases were obtained on phenylhydrazine or *p*-nitrophenylhydrazine derivatives of TPQ which have a molar absorptivity 10-fold greater (ϵ ~30 000 M^{-1} cm^{-1}) than the underivatized cofactor (Brown et al., 1991). Previous attempts to observe the RR spectrum of underivatized TPQ in amine oxidases have been hampered by protein fluorescence (Dooley et al., 1990). Our studies of a topa-hydantoin quinone model compound showed that it is possible to obtain RR spectra of the underivatized cofactor (Sanders-Loehr et al., 1991). Through the use of a highly purified enzyme, we have now been able to obtain RR spectra of the native TPQ cofactor in *Escherichia coli* amine oxidase (ECAO) from strain W3350. This amine oxidase was originally isolated from *E. coli* strain K-12 and shown to contain the same TPQ cofactor as in BSAO (Cooper et al., 1992). We find that the RR spectrum of native ECAO is closely similar to the RR spectrum of a 2-hydroxy-1,4-benzoquinone model compound, thus definitively identifying the ECAO cofactor as TPQ. In addition, we have found that ECAO, despite its preference for bulky substrates such as 2-phenylethylamine, will utilize methylamine as an alternative, slow substrate. This has enabled us to characterize the N-isotope dependence of the RR spectrum of the semiquinone intermediate. We find that the substrate amine group is still bound to the cofactor in the semiquinone state and that formation of a Cu(I) cyanide complex perturbs both the absorption and RR spectra of the $topa_{NHSQ}$.

EXPERIMENTAL PROCEDURES

ECAO. Overexpression of *E. coli* amine oxidase was achieved by inserting the *maoA* gene for aromatic amine oxidase from strain W3350, substrain PPA207 into a pUC18 plasmid containing the lac promoter.[1] The resultant pBCP467 plasmid was incorporated into *E. coli* strain TG2. Highly purified ECAO was achieved in three column steps using DEAE-Sepharose fast-flow, phenyl-Sepharose, and MonoQ (Pharmacia) and yielded a specific activity of 15.4 units/mg with 2-phenylethylamine as substrate.[1] The enzyme in 50 mM potassium phosphate (pH 7.0) was concentrated using a Microcon 30 (Amicon) ultrafiltration device. Protein concentration was determined from E_{280} (0.1%) = 1.67. Samples contained 1.0−1.15 phenylhydrazine-titratable TPQ cofactors per enzyme dimer.

ECAO Semiquinone. A concentrated enzyme sample (0.4 mM ECAO) in 50 mM phosphate (pH 7) was placed in a capillary tube sealed with a serum stopper (Loehr & Sanders-Loehr, 1993) and flushed with argon. Reduction was performed by the addition of an anaerobic solution of 50 mM methylamine substrate with or without the subsequent addition of an anaerobic solution of 40 mM KCN to yield final concentrations of 10 mM methylamine, 8 mM KCN, and ~0.3 mM ECAO. Isotopic labeling was performed with methylamine containing 99 atom % ^{15}N (Cambridge Isotope Laboratories), generously provided by Dr. Victor Davidson. For copper analyses, the Cu in ECAO-SQ was reduced by boiling for 5 min in 0.05% SDS, 1 mM ascorbate, and bicinchonic acid (Pierce). The copper content was determined using an ϵ_{562} of 7280 M^{-1} cm^{-1} for Cu(I) bicinchonate.

[1] V. Steinebach, J. Benen, P. Postma, P. Loreti, S. de Vries, and J. A. Duine, manuscript in preparation.



TPQ-Hydantoin **tBuQO⁻**

FIGURE 1: 2-Hydroxy-1,4-benzoquinone models for TPQ in amine oxidases. The hydantoinmethyl substituent at C5 in TPQ-hydantoin is analogous to the $(CH)_\alpha(CH_2)_\beta$-polypeptide substituent at C1 in TPQ-ECAO.

Synthesis of Model Compounds. The sodium salt of 2-hydroxy-5-(5′-hydantionmethyl)-1,4-benzoquinone (TPQ-hydantoin) was prepared as described previously (Mure & Klinman, 1993). The 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (tBuQOH) and its deprotonated analog ($tBuQO^-$) were prepared as described elsewhere (Mure & Klinman, 1995).

Isotope Exchange. ECAO was diluted 20-fold in 20 mM phosphate buffer (pH reading 7.0, prepared by the addition of concentrated phosphate) in D_2O (99 atom %, Merck) to yield a a final concentration of 94 atom % D. The protein was reconcentrated to 0.4 mM by centrifugation in a Microcon 30 ultrafiltration device (Amicon). ECAO was diluted in $H_2{}^{18}O$ (97 atom %, ICON) to yield 10 mM phosphate (pH 7.0) and 82 atom % ^{18}O and was reconcentrated as above. The *tert*-butylammonium salt of the $tBuQO^-$ model was dissolved directly in pure water (same D and ^{18}O isotopes as above) to yield a final concentration of ~2 mM and a pH reading of 9.6. Isotope exchange was monitored by mass spectrometry (VG Analytical 7070E) using FAB in the negative ion mode in diethanolamine. Isotope exchange was rapid (complete in 2 min or less for ECAO in $H_2{}^{18}O$) and the extent of exchange did not increase with time.

Raman Spectroscopy. Spectra were obtained either on a computerized Jarrell-Ash spectrophotometer using a cooled Hamamatsu R943−02 photomultiplier and an Ortec model 9302 amplifier-discriminator or on a McPherson 2061 spectrograph (0.6 m) using a Princeton Instruments liquid-N_2-cooled (LN-1100PB) CCD detector. Excitation sources were Spectra-Physics 165-04 Ar and 2025-11 Kr lasers. Samples in glass capillaries were inserted into a cold finger immersed in a water−ice mixture (Loehr & Sanders-Loehr, 1993), and spectra were collected using a 90° scattering geometry. 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}. The same sample capillaries were used to obtain absorption spectra before and after RR experiments. None of the samples were affected by laser irradiation.

RESULTS AND DISCUSSION

TPQ Absorption Spectra. The TPQ cofactor in native amine oxidase from *E. coli* strain W3350 has a characteristic absorption band at 474 nm (Figure 1A) with an ϵ of 3100

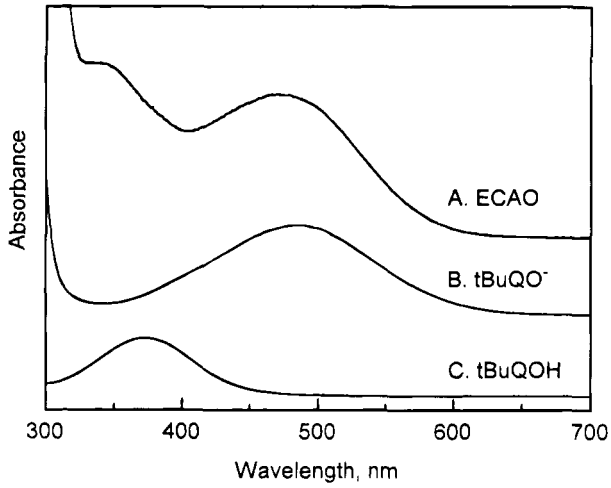


FIGURE 2: Absorption spectra of the TPQ cofactor in *E. coli* amine oxidase and model compounds. (A) ECAO at pH 7.0. (B) $tBuQO^-$ model at pH 9.6. (C) tBuQOH model in CH_3CN.

M^{-1} cm^{-1} per TPQ, and it contains 0.50−0.58 TPQ per monomer based on a phenylhydrazine titration.[1] This TPQ content appears to be lower than the value of ~1 TPQ per monomer reported previously for AO from *E. coli* strain K-12, which was based on an ϵ_{480} value of 2200 M^{-1} cm^{-1} per TPQ for *Arthrobacter P1* AO (Cooper et al., 1992). The absorption spectra of the amine oxidases have been found to closely match that of a TPQ hydantoin model compound (Figure 1) in which the 4-hydroxyl group is deprotonated (Mure & Klinman, 1993). The corresponding pK_a values for BSAO and TPQ hydantoin are 3.0 and 4.1, respectively. The 5-*tert*-butyl derivative of 2-hydroxy-1,4-benzoquinone ($tBuQO^-$, Figure 1) has proven to be more stable than TPQ hydantoin during isolation and, therefore, may be more suitable for extended spectroscopic studies. The deprotonated species, $tBuQO^-$, is red with an aborption maximum at 484 nm and an ϵ of 1800 M^{-1} cm^{-1} in H_2O (Figure 2B), which resembles the behavior of ECAO (Figure 2A). In contrast the protonated species, tBuQOH, is yellow with its absorption maximum shifted to 372 nm in H_2O (pH 2) or CH_3CN with an ϵ of 700 M^{-1} cm^{-1} in CH_3CN (Figure 2C).

TPQ Resonance Raman Spectra. Raman spectroscopy is a light scattering technique that detects molecular vibrations (Carey, 1982). It works particularly well in systems with conjugated double bonds such as quinones. In addition, a resonance phenomenon leads to enhanced intensities for vibrational modes that are part of a chromophore. This allows contributions of a quinone cofactor to be magnified many-fold relative to the normal vibrational modes of the protein (Dooley & Brown, 1992). Previously, RR spectroscopy has been used to characterize the tryptophan tryptophylquinone (TTQ) cofactor in native methylamine dehydrogenase (Backes et al., 1991). However, in the case of the amine oxidase, the RR spectrum of the native enzyme was overshadowed by sample fluorescence (Dooley et al., 1990), and only the more chromophoric, TPQ−phenylhydrazone inhibitor complex was detectable by this technique (Brown et al., 1991).

Using an overexpressed ECAO which could be highly purified, we have now been able to obtain a RR spectrum of the native enzyme by excitation within the 476-nm absorption band (Figure 3B). The majority of the peaks between 1100 and 1700 cm^{-1} are resonance-enhanced and,

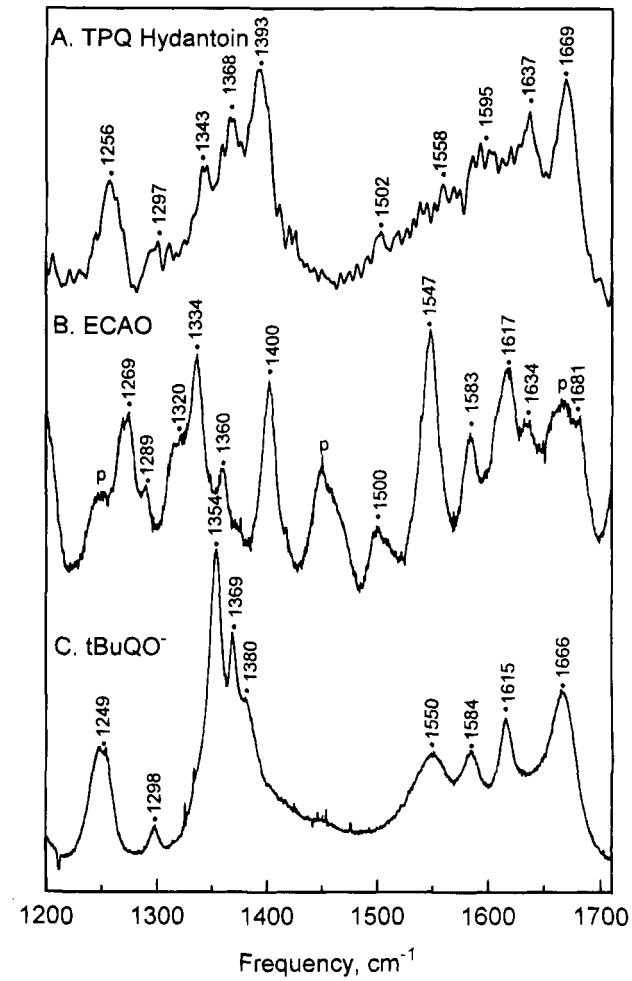


FIGURE 3: Resonance Raman spectra of the TPQ cofactor in *E. coli* amine oxidase and model compounds. (A) TPQ hydantoin model. Solid sample (sodium salt) sealed in a capillary under vacuum and inserted into a cold finger immersed in liquid nitrogen (sample at 90 K). The spectrum was obtained on the Jarrell-Ash instrument with 488.0-nm excitation (50 mW) in a 150° backscattering geometry with a scan rate of 1 cm^{-1}/s, spectral resolution of 7 cm^{-1}, and accumulation of 14 scans. (B) Native *E. coli* amine oxidase (0.4 mM) in 50 mM phosphate (pH 7.0) at 278 K. The spectrum was obtained on a McPherson spectrograph (1800-groove grating) with a Kaiser Optical holographic super-notch filter using 514.5-nm excitation (10 mW), an exposure time of 50 s, and an accumulation of 16 scans. P indicates protein modes (Lord & Yu, 1970). (C) $tBuQO^-$ model compound (~2 mM) in aqueous solution (pH 9.6) obtained as in panel B with an exposure time of 200 s and an accumulation of 4 scans.

thus, due to the quinone cofactor. Raman spectra obtained using a series of excitation wavelengths reveal that the resonance-enhanced modes of ECAO (Table 1) have maximum Raman intensity at ~500 nm, slightly to the red of the 476-nm absorption maximum. Residual protein vibrational modes (labeled P in Figure 3B) are observed at 1002 cm^{-1} (phenylalanine ring), 1247 cm^{-1} (amide III), 1454 cm^{-1} (CH_2 deformation), and 1665 cm^{-1} (amide I). The nonresonance character of these Raman modes is affirmed by their becoming the dominant spectral features with 647.1-nm excitation. We have recently obtained a RR spectrum from bovine serum amine oxidase (manuscript in preparation) similar to that of ECAO (Figure 3B), but with poorer signal quality due to fluorescence from the less highly purified material.

Table 1: Raman Frequencies of Quinones and Semiquinones in ECAO and Model Compounds[a]

quinones[b]								semiquinones[c]			
ECAO-TPQ			tBuQO⁻			TPQ-hyd	*p*-benzoquinone		ECAO-SQ		*p*-aminophenoxyl	
ν	[Δ^{18}O]	(ΔD)	ν	[Δ^{18}O]	(ΔD)	ν	ν	[Δ^{18}O]	ν	{Δ^{15}N}	ν	(ΔD)
1269			**1249**			**1256**	1234	[−1]	1269			
1289		(−9)	1298	[−2]	(−25)	1297			1316			
1334			**1354**		(−10)	1343	1357	[−7]	1335			
1360			**1369**			1368						
1400			1380			**1393**	1394	[−1]				
1500		(−6)							1454	{−3}	1434	(+13)
1547		(−4)	1550		(−10)	1558	1540	[−8]	1485			
1583			1584			1595			1548		1518	(−6)
1617		(−2)	**1615**	[−5]			1616	[−21]	1612	{+4}		
1634						1637			**1647**	{−4}	**1636**	(+6)
1681	[−26]	(−1)	**1666**	[−23]		**1669**	**1665**	[−11]			1656	(+7)
						1684		[−14]				

[a] Frequencies in cm^{-1}. Boldface denotes most intense peaks. [b] RR spectra of ECAO-TPQ, tBuQO$^-$, and TPQ-hydantoin from Figures 3, 4, and 5. Raman spectrum of *p*-benzoquinone, [^{16}O,^{16}O], and [^{18}O,^{18}O], from Becker (1991). [c] RR spectrum of ECAO-SQ (topa$_{NHSQ}$) obtained as in Figure 4. RR spectrum of *p*-aminophenoxyl radical, ($NH_2C_6H_4O^{\bullet}$) and ($ND_2C_6H_4O^{\bullet}$), from Tripathi and Schuler (1984).

The resonance-enhanced Raman modes of ECAO are quite similar to those reported previously for the solid sodium salt of TPQ hydantoin (Sanders-Loehr et al., 1991), particularly in the 1200−1400-cm^{-1} region (Figure 3A). This region is dominated by C−C and C−O stretching and C−H bending modes and appears to be sensitive to the nature of the C5 substitutent in the model. The C5 substituent in TPQ hydantoin (Figure 1) is similar to the amino acid backbone of TPQ in ECAO, and there is a good match between the spectral features at 1269, 1334, 1360, and 1400 cm^{-1} in ECAO and 1256, 1343, 1368, and 1393 cm^{-1} in TPQ hydantoin. The higher frequency region between 1500 and 1700 cm^{-1}, which is dominated by C=C and C=O stretching modes, appears to be more sensitive to the environment and the ionization state of the cofactor. In this region, the RR frequencies and intensities of ECAO are closer to those of the deprotonated model, tBuQO$^-$, in aqueous solution (Figure 3C and Table 1) with the peaks at 1547, 1583, 1617, and 1681 cm^{-1} in ECAO being comparable to those at 1550, 1584, 1615, and 1666 cm^{-1} in tBuQO$^-$. The similarity of vibrational frequencies between ECAO, TPQ hydantoin, and tBuQO$^-$ indicates that ECAO does contain a TPQ cofactor, as had already been proposed from the RR spectrum of its phenylhydrazone (Cooper, 1992). The similarity of the absorption spectra for these three species (Figure 2; Mure & Klinman, 1993) indicates that the hydroxyl group of the quinone is deprotonated in ECAO. The better match between ECAO and tBuQO$^-$ in the high frequency region may be due to the fact that the tBuQO$^-$ sample is in an aqueous environment whereas the TPQ hydantion sample is in the solid state.

Isotope Shifts in ^{18}O and D. Quinone carbonyl groups are susceptible to nucleophilic attack and, thus, are potentially capable of exchange with solvent oxygen. When the tBuQO$^-$ model compound is equilibrated in $H_2{}^{18}O$, the peak at 1666 cm^{-1} immediately shifts by −23 cm^{-1} whereas the peak at 1615 cm^{-1} shifts by −5 cm^{-1} and loses intensity (Figure 4C). These are the only spectral changes that occur in $H_2{}^{18}O$, even after 3 days of incubation. The frequency of 1666 cm^{-1} is within the 1640−1695-cm^{-1} range observed for the C=O stretch in a large number of substituted benzoquinones (Berger & Rieker, 1974). The ν(C=O) assignment is confirmed by the ^{18}O dependence of this vibrational mode.

Based on a simple two-body system, the predicted isotope shift for ν(C=O) is −40 cm^{-1} for a fully-substituted [^{18}O,^{18}O] quinone and −20 cm^{-1} for a half-substituted [^{16}O,^{18}O] quinone. The observed shift of −23 cm^{-1} for tBuQO$^-$ in $H_2{}^{18}O$ is consistent with a singly-exchanged carbonyl group. This was verified by mass spectrometry of tBuQO$^-$ where the major ion in $H_2{}^{18}O$ was at *m/z* 181 compared to *m/z* 179 in $H_2{}^{16}O$. Both masses are as expected for the deprotonated hydroxyquinone. Studies of the reaction of the TPQ hydantoin model compound with (4-nitrophenyl)-hydrazone have shown that only the carbonyl carbon adjacent to the hydroxyl group (C1 in Figure 1) is susceptible to nucleophilic attack (Mure & Klinman, 1993). Presumably, this is the carbon that also undergoes nucleophilic attack by solvent, resulting in oxygen isotope exchange.

Although the vibrational assignments for unsubstituted *p*-benzoquinone have been studied extensively (Becker, 1991 and references therein), this simple molecule gives surprisingly complex Raman and infrared spectra, apparently due to extensive Fermi resonance coupling of ν(C=O) and ν(C=C) with other vibrational modes. Thus, almost every Raman spectral feature of *p*-benzoquinone shows substantial ^{18}O dependence, and the sum of the shifts is large because it is fully substituted with ^{18}O (Table 1). In contrast, with the tBuQO$^-$ model compound, the majority of the ^{18}O dependence occurs in a single mode at 1666 cm^{-1}, implying that the in-phase stretch of the two carbonyl groups (C=^{16}O and C=^{18}O) is a fairly pure vibrational mode. Similarly, the TTQ cofactor in MADH reacts with $H_2{}^{18}O$ to give a half-substituted product whose principle ν(C=O) mode near 1625 cm^{-1} downshifts by −16 to −27 cm^{-1} (Backes et al., 1991). This same type of behavior is exhibited by ECAO upon equilibration with $H_2{}^{18}O$ for 1 h to 3 days. The only observed ^{18}O dependence is that the peak at 1681 cm^{-1} in $H_2{}^{16}O$ (Figure 5A) downshifts by −26 cm^{-1} in $H_2{}^{18}O$ (Figure 5C). This points to the TPQ cofactor in ECAO having the same *p*-quinone structure as in the tBuQO$^-$ model compound, with solvent oxygen exchange occuring only at C5 (the carbonyl adjacent to the hydroxyl group).

Isotope dependence in D_2O is expected if there are exchangeable cofactor hydrogens or polar groups on the cofactor which are capable hydrogen bonding with other molecules in the environment. Both of these conditions exist for the TPQ cofactor. In their study of NMR-detectable

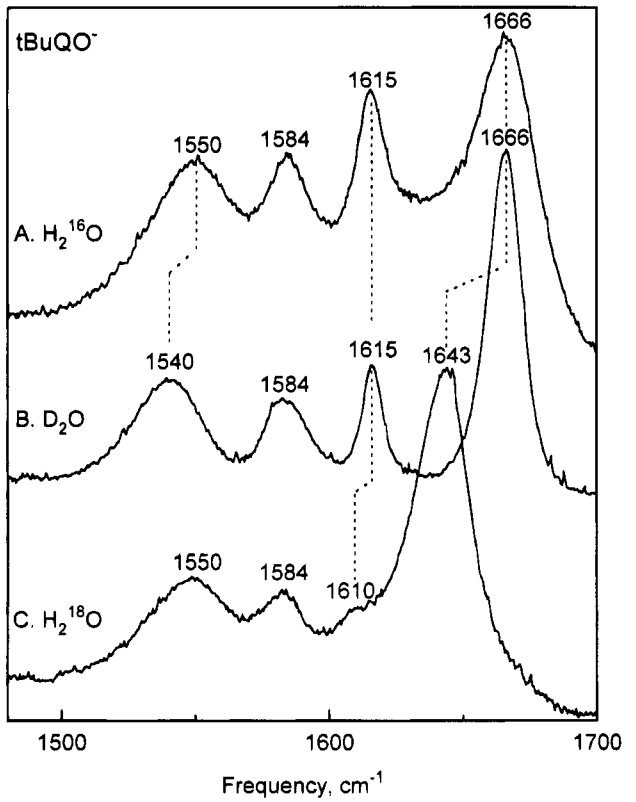


FIGURE 4: RR spectra of tBuQO⁻ model compound (~2 mM) dissolved in (A) $H_2{}^{16}O$, (B) D_2O, and (C) $H_2{}^{18}O$, all at a pH reading of 9.6. Spectra obtained as in Figure 3C.

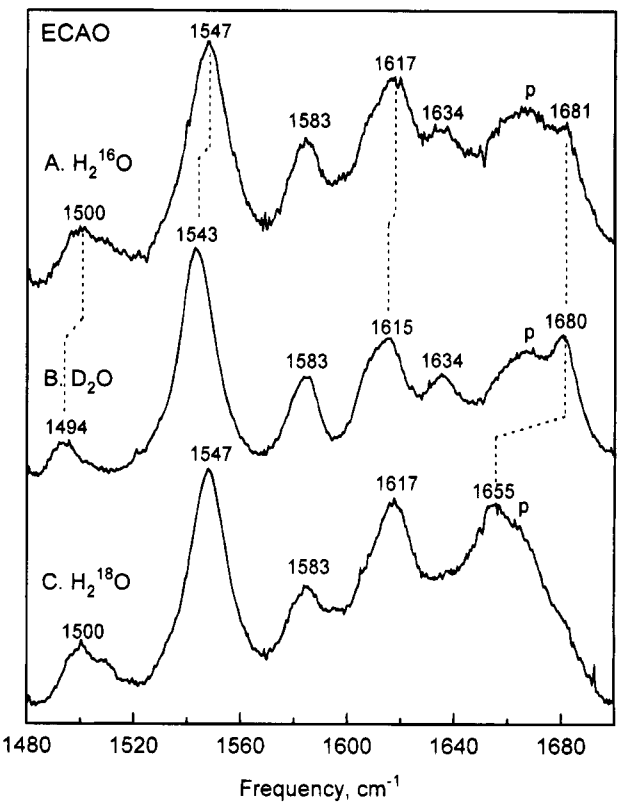


FIGURE 5: RR spectra of ECAO in (A) $H_2{}^{16}O$, (B) D_2O, and (C) $H_2{}^{18}O$. Protein (~0.4 mM) dissolved in 20 mM phosphate (pH reading 7.0). Spectra obtained as in Figure 3B.

protons, Janes et al. (1990) concluded that the C3 proton in the TPQ hydantoin (Figure 1) exchanges readily with D_2O. In addition, the isotope dependence of the ENDOR spectrum of the semiquinone form of ECAO reveals hydrogen bonds to the three polar (O and N) ring substituents (Warncke et al., 1994). The tBuQO⁻ model exhibits substantial D-isotope downshifts of -25 at 1298 cm^{-1}, -10 at 1354 cm^{-1}, and -10 at 1550 cm^{-1} (Table 1 and Figure 4B). Shifts of this magnitude are most likely due to the exchange of a ring H, presumably at the C3 position. Mass spectrometry of $tBuQO^-$ reveals the major ion at *m/z* 179 in H_2O and *m/z* 180 in D_2O, as expected for a deprotonated hydroxyquinone with a ring deuterium. ECAO exhibits similar deuterium-isotope shifts of -9 at 1289 cm^{-1}, -6 at 1500 cm^{-1}, and -4 at 1547 cm^{-1} (Table 1 and Figure 5B) that can also be ascribed to an exchangeable C3 hydrogen.

The principle ν(C=O) mode at 1666 cm^{-1} in the model and 1681 cm^{-1} in the protein shows very little frequency change in D_2O. However, in both cases, there is a marked increase in peak intensity in D_2O (Figures 4B and 5B). Vibrational intensity changes provide another indicator (in addition to frequency shifts) of the occurrence of hydrogen bonds in organic compounds (Hadzi & Bratos, 1976; Joesten & Schaad, 1974). Our observed intensity changes show that one or both of the carbonyl groups of the TPQ cofactor are hydrogen-bonded in the protein and in the isolated compound in water. The striking similarity in the ^{18}O and D isotope dependences of the enzyme and model compound again points to TPQ in the enzyme having the same structure and reactivity as 2-hydroxy-1,4-benzoquinones in aqueous solution.

Semiquinone Absorption Spectra. The semiquinone (SQ) intermediate, Cu(I)-topa$_{NHSQ}$, is generated during the anaerobic reduction of amine oxidases by substrate (eqs 1 and 2). This SQ has been observed with amine oxidases from bacterial, plant, and animal sources and is identified by the appearance of characteristic absorption bands near 430 and 465 nm and an ^{15}N-dependent free-radical EPR signal (Bellelli et al., 1985; Dooley et al., 1990, 1991). The reversible nature of reaction 2 has been demonstrated. Cu(II)-topa$_{NH_2}$ is favored at temperatures below 260 K or by the addition of chloride, and Cu(I)-topa$_{NHSQ}$ is favored by the addition of cyanide. Anaerobic reaction of ECAO with methylamine leads to the appearance of SQ absorption bands at 442 and 468 nm (Figure 6), which are identical to those obtained with 2-phenylethylamine. This indicates that methylamine can also serve as a substrate, presumably generating formaldehyde and the identical topa$_{NHSQ}$ product. However, the reaction of ECAO with methylamine is markedly slower than with its preferred aromatic amine substrates.

The tendency of ECAO to favor the SQ as the predominant species after anaerobic addition of substrate is similar to the behavior of *Arthrobacter* P1 amine oxidase (APAO) (Dooley et al., 1990). In contrast, substrate-reduced BSAO must be reacted with cyanide before it will form significant amounts of SQ (Dooley et al., 1991). Addition of cyanide to the ECAO-SQ causes the absorption bands at 442 and 468 nm to blue-shift to 438 and 462 nm, respectively (Figure 6), and makes the SQ resistant to reoxidation by O_2. A similar blue-shift in the SQ absorption spectrum is observed upon addition of cyanide to APAO (Dooley et al., 1990) and BSAO which has values of 434 and 464 nm (unpublished results). Copper analyses of ECAO after reaction with 2-phenylethylamine and cyanide (5 mM) indicate that ~90% of the Cu is still bound to the protein. The blue-shift in the absorption spectrum in going from Cu(I)-topa$_{NHSQ}$ to Cu(I)CN-topa$_{NHSQ}$

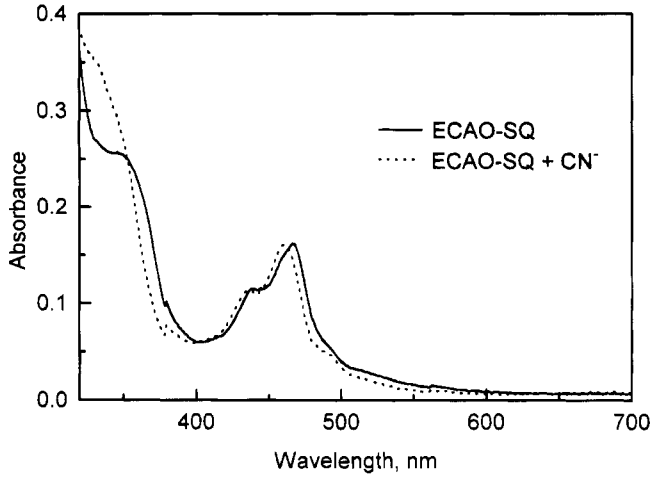


FIGURE 6: Absorption spectra of semiquionone prepared by anaerobic reduction of ECAO by methylamine (—) or methylamine plus KCN (- - -).

suggests that there is a significant chemical interaction between the copper and the SQ cofactor which is perturbed by the addition of cyanide.

Semiquinone Resonance Raman Spectra. The RR spectrum of ECAO-SQ is dominated by a peak at 1647 cm^{-1} with a series of weaker features between 1269 and 1612 cm^{-1} (Figure 7A and Table 1). The 1647-cm^{-1} peak can be assigned as a benzene ring stretching mode by comparison with the dominant spectral feature at 1636 cm^{-1} in the RR spectrum of the *p*-aminophenoxy radical (Table 1) and at 1625 cm^{-1} in the RR spectrum of the 1,2,4-benzenetriol radical (Qin et al., 1987). Reaction of ECAO with ^{15}N methylamine leads to frequency shifts of −3, +4, and −4 cm^{-1}, respectively, for the peaks at 1454, 1612, and 1647 cm^{-1} (Figure 7B). The N-isotope dependence shows that the nitrogen from substrate has been incorporated into the TPQ cofactor and remains in the semiquinone state. Given the reactivity of the carbonyl adjacent to the hydroxyl group in model compounds (Mure & Klinman, 1993), it is likely that the substrate amine group has replaced the oxygen at the C5 position of TPQ in amine oxidases.

The small value of the N-isotope shifts in ECAO means that there is no pure C−N stretching mode, which would have a ^{15}N downshift of 23−26 cm^{-1}. Rather, the C−N stretch appears to be coupled with other vibrational motions such as the C−C and C−O stretches and NH bends. Thus, N-isotope dependence is observed for both the ring mode at 1647 cm^{-1} (−4 cm^{-1}) and the C−O/C−N stretch at at 1454 cm^{-1} (−3 cm^{-1}). The analogous C−O/C−N stretch is assigned at 1434 cm^{-1} in the *p*-aminophenoxy radical (Tripathi & Schuler, 1984). This compound exhibits similar, but somewhat larger, shifts to lower and higher frequency upon deuterium substitution of the amino group (Table 1). The larger values in this case are due to the greater mass change (from two hydrogens) and the greater influence of D than N on modes with NH bending character. Both the C−O and C−N bonds of the *p*-aminophenoxy radical have bond orders of ~1.5 (Tripathi, 1989), indicating substantial imine character in the C−N bond.

Previous EPR and ESEEM studies of BSAO-SQ indicated that a substrate-derived nitrogen was part of the free radical species (McCracken et al., 1992). Thus, for amine oxidases in general, the amino group of the substrate remains attached

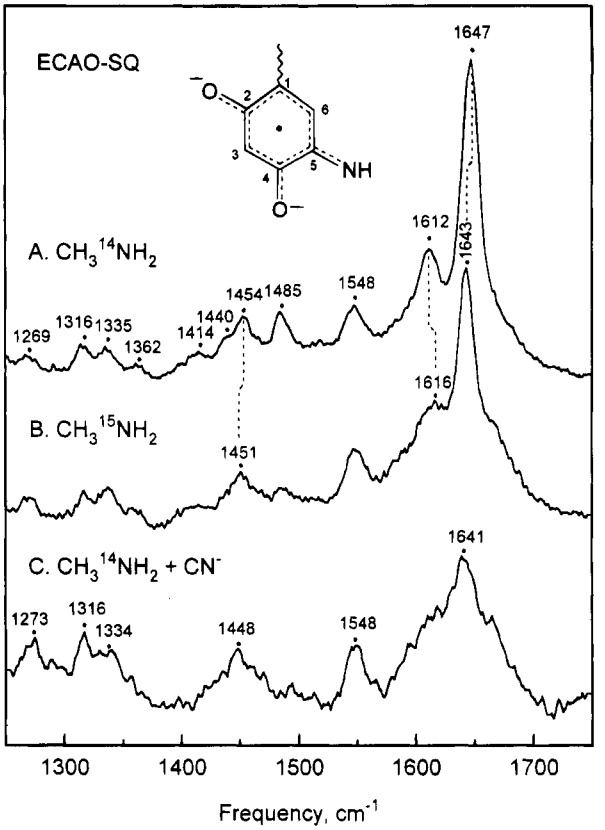


FIGURE 7: RR spectra of semiquionone prepared by anaerobic reduction of ECAO by methylamine. (A) ^{14}N-methylamine. (B) ^{15}N-methylamine. (C) ^{14}N-methylamine plus KCN. The spectra were obtained on a Jarrell-Ash instrument with 476.5-nm (50 mW) excitation, scan rate of 1 cm^{-1}/s, spectral resolution of 8 cm^{-1}, and accumulation of 4 scans.

to the TPQ cofactor during generation of the Cu(I)-topa$_{NHSQ}$ intermediate (eq 2). A similar conclusion regarding the retention of the substrate amino group in the semiquinone intermediate has been reached for the tryptophan-derived quinone cofactor in methylamine dehydrogenase (Warncke et al., 1993). The ENDOR studies have also suggested that the SQ in BSAO is more *imine-like* than in the *p*-aminophenoxy radical in that the nitrogen in the BSAO-SQ has only a single covalently bonded hydrogen. Our RR studies also indicate greater imine character for the ECAO-SQ based on ν(C−O/C−N) at 1454 cm^{-1} and ν(ring) at 1647 cm^{-1} which are 20 and 11 cm^{-1} higher, respectively, than the corresponding vibrations in the *p*-aminophenoxy radical (Table 1). The greater imine character of the semiquinone, which appears to be imposed by the enzyme, could play a role in facilitating oxidation of the TPQ cofactor to the subsequent iminoquinone intermediate (Warnke et al., 1994).

The addition of cyanide to ECAO-SQ perturbs its RR spectrum (Figure 7C), as well as its absorption spectrum (Figure 6). In particular, the peaks at 1454 and 1647 cm^{-1} each shift 6 cm^{-1} to lower energy to 1448 and 1641 cm^{-1}. The cyanide complex of BSAO-SQ exhibits a similar RR spectrum with its dominant intensity at 1645 cm^{-1} (manuscript in preparation). The cyanide complex of ECAO-SQ still retains ~90% of the copper content of the oxidized enzyme, indicating that the cyanide is primarily involved in Cu binding, as opposed to Cu removal. The sensitivity of the RR spectrum to cyanide binding at the Cu(I) site indicates that there is an electrostatic interaction between the Cu and

the TPQ cofactor in the SQ form of the enzyme. The 6-cm^{-1} downshifts of the ν(C−O/C−N) and ν(ring) modes at 1448 and 1641 cm^{-1}, respectively, suggests that cyanide binding to Cu alters its interaction with the SQ cofactor. We have observed contrasting behavior for the oxidized form of ECAO in that formation of the Cu(II)−azide complex (upon addition of 200 mM azide) has no effect on the RR spectrum of the $topa_Q$ cofactor (data not shown). Azide is known to mimic cyanide in its ability to bind to Cu and inhibit the reoxidation of reduced AO with O_2 (Barker et al., 1979; Dooley & Coté, 1985). Whereas reduced ECAO appears to support an electrostatic interaction between Cu(I) and $topa_{NHSQ}$, no such electrostatic interaction between Cu(II) and $topa_Q$ is apparent in oxidized ECAO.

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