Topaquinone-Dependent Amine Oxidases: Identification of Reaction Intermediates by Raman Spectroscopy†

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ABSTRACT: Resonance Raman (RR) spectroscopy has proven to be an excellent technique for providing structural information about the 2,4,5-trihydroxyphenylalaninequinone (TPQ) cofactor and for identifying the source of oxygen atoms during the posttranslational synthesis of the cofactor. Through specific labeling of the C2, C4, and C5 oxygens of TPQ in phenylethylamine oxidase (PEAO) from *Arthrobacter globiformis*, we have identified the C═O stretch of the C5 carbonyl at 1683 cm^{-1} (−27 in ^{18}O) and the C═O stretch of the C2 carbonyl at 1575 cm^{-1} (−21 in ^{18}O). These vibrational frequencies show that the C−O moiety at C5 has far greater double-bond character than at C2 or C4, thereby explaining the exclusive nucleophilic attack at the C5 position by substrates and substrate analogs. Bovine serum amine oxidase (BSAO) exhibits a similar ν(C═O) mode at 1678 cm^{-1} (−22 cm^{-1} in ^{18}O). Aniline reacts with the TPQ cofactor of PEAO to form a new derivative (λ_{max} at 450 nm) with properties similar to the proposed substrate−imine intermediate in the catalytic cycle. It retains the C2═O spectral features of the native enzyme and exhibits a new C5═N stretch at 1603 cm^{-1} (−29 in ^{15}N). In contrast, methylamine reacts with both PEAO and BSAO under anaerobic conditions to form a different stable adduct (λ_{max} at 385 nm) with properties closer to the proposed product-imine intermediate in the catalytic cycle. This species has a distinctive RR spectrum with a C═N stretch at 1617 cm^{-1} that corresponds to the atoms of the added methylamine (−58 cm^{-1} with CD_3NH_2, −19 cm^{-1} with $CH_3{}^{15}NH_2$). The lack of D_2O dependence of ν(C═N) shows that this is a deprotonated imine, which would be more stable toward hydrolysis than the postulated protonated imine in the enzymatic reaction. However, the BSAO product imine (from methylamine) does undergo hydrolysis and conversion to semiquinone upon addition of cyanide. It is possible that the inactive form of the product imine is stabilized by deprotonation and flipping of the TPQ ring [Cai, D., Dove, J., Nakamura, N., Sanders-Loehr, J., and Klinman, J. P. (1997) *Biochemistry 36*, 11472−11478].

The redox-active organic cofactor of copper amine oxidases has been identified as 2,4,5-trihydroxyphenylalanine (topa) in the reduced enzyme and topaquinone (TPQ)[1] in the oxidized enzyme (*1*, *2*). This quinone cofactor is derived by the posttranslational modification of a protein tyrosine residue that is accomplished in a copper-dependent, autocatalytic reaction (*3*−*6*). The X-ray crystal structures of the amine oxidases from *Escherichia coli* (ECAO), pea seedlings (PSAO), and *Arthrobacter globiformis* (PEAO) reveal that the TPQ is situated next to a copper ion and that a cavity on the other side of the cofactor is the substrate binding site (*7*, 8) (M. C. J. Wilce, D. M. Dooley, H. C. Freeman, J. M. Guss, H. Matsunami, W. S. McIntire, K. Tanizawa, and H. Yamaguchi, personal communication). Covalent adduct formation is known to occur at the C5 carbonyl of TPQ from the reaction of the enzyme and model compounds with substrate analogs such as hydrazines (*9*−*11*). Protein crystal structures show that these hydrazine adducts are located on the cavity side of the cofactor next to a conserved aspartate that serves as a catalytic base (*12*) (R. Li, L. Chen, D. Cai, J. P. Klinman, and F. S. Mathews, personal communication). However, in the resting state, which lacks a bulky substituent at C5, the TPQ ring can rotate, placing the C5 carbonyl adjacent to the copper (*7*, *8*). Although facile ring flipping is important in cofactor biosynthesis (*3*), the TPQ ring is expected to have the C5 side of the cofactor adjacent to the catalytic base during its reaction with substrate (Figure 1).

Amine oxidases catalyze the oxidative deamination 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 reaction has been proposed to proceed by a transamination mechanism (Figure 1). The covalent addition of the substrate to the cofactor produces the substrate Schiff base intermediate (substrate imine) that has been detected by

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[1] Abbreviations: TPQ, 2,4,5-trihydroxyphenylalaninequinone; BSAO, bovine serum amine oxidase; ECAO, *Escherichia coli* amine oxidase; HAO, histamine oxidase; PEAO, phenylethylamine oxidase; PSAO, pea seedling amine oxidase.

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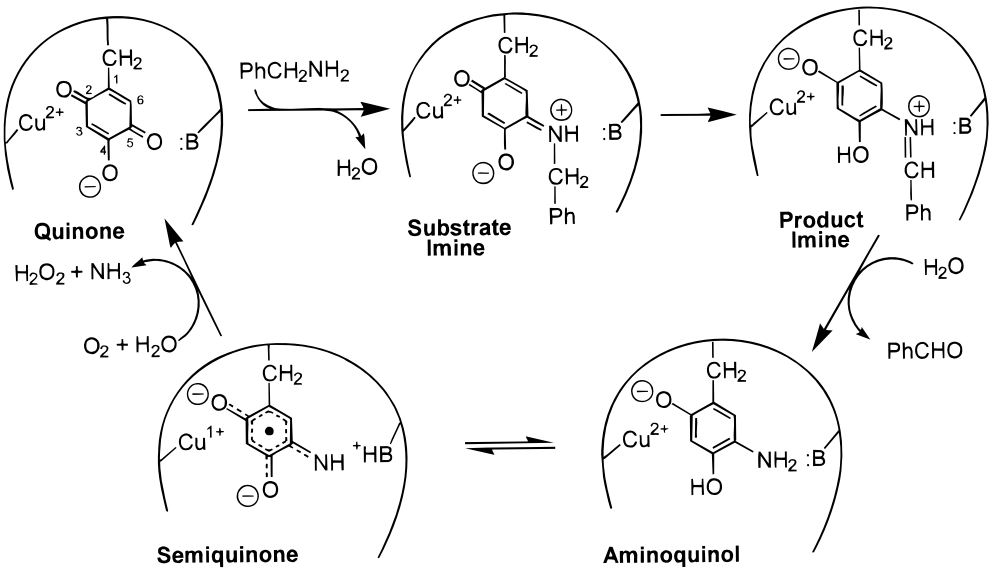

FIGURE 1: Proposed mechanism for the amine-oxidase catalyzed oxidation of benzylamine (*13, 14*). According to the crystal structure of the quinone form of yeast amine oxidase (R. Li, L. Chen, D. Cai, J. P. Klinman, and F. S. Mathews, personal communication), there is an intervening water molecule between the C5 carbonyl and the catalytic base, and the C2 carbonyl is H-bonded to a water ligand of the Cu cofactor.

rapid-scanning spectroscopy (*13*). Base-catalyzed abstraction of a methylene proton leads to the formation of a product Schiff base (product imine) that would be rapidly hydrolyzed to yield the aminoquinol form of the reduced cofactor. This reaction sequence is supported by extensive studies on model compounds (*10, 11, 15*−*17*). Under anaerobic conditions a further intermediate, the Cu(I) aminosemiquinone, has been detected by EPR spectroscopy (*14, 18*). In the present work we demonstrate that resonance Raman (RR) spectroscopy is an excellent technique both for detecting the different topa intermediates and for providing additional information about their structures.

Raman spectroscopy was initially performed on hydrazine adducts of amine oxidases because of their intense color and strong resonance enhancement, and these studies led to the definitive identification of the same TPQ cofactor in a number of different enzymes (*19, 20*). Subsequently, we were able to obtain RR spectra of the native quinone cofactor in amine oxidases from *E. coli* (ECAO), *A. globiformis* (PEAO and HAO), and bovine serum (BSAO) and showed that they also yield similar vibrational spectra, particularly the presence of a carbonyl stretching mode near 1680 cm^{-1} (*21, 22*). Specific labeling of the oxygen at the C2 or C5 position indicated that the 1680-cm^{-1} vibration was due only to the C5 carbonyl, thereby proving it has greater double-bond character than the C2 carbonyl (*22*). In addition, we identified a N═C stretch at 1618 cm^{-1} in a methylamine adduct, suggesting an analog of the product imine (*22*) and ^{15}N dependence in the semiquinone intermediate, confirming that the amino group is still present in this form of the enzyme (*21*). We have now been able to obtain RR spectra of amine oxidases specifically labeled with ^{18}O at the C4 position, and we have detected an aniline adduct that appears to be an analog of the substrate imine. The combined analysis of these isotopically substituted cofactors in the native as well as in the substrate imine, product imine, and semiquinone states has allowed us to make more definitive assignments of the structure of the cofactor throughout the catalytic reaction cycle.

EXPERIMENTAL PROCEDURES

PEAO and HAO. Recombinant phenylethylamine oxidase (PEAO) and histamine oxidase (HAO) from *A. globiformis* were overproduced in *E. coli* as apoproteins (deficient in copper and TPQ) and then activated by addition of $CuSO_4$ under aerobic conditions (*5, 23*). The C5 carbonyl of the TPQ cofactor was labeled by exchange with solvent $H_2{}^{18}O$, and the C2 carbonyl was labeled by activation of the apoprotein in the presence of $H_2{}^{18}O$ (*22*). The C4 carbonyl was labeled by growing the *E. coli* strain on ^{18}O-L-tyrosine (Stable Isotope Resource Program at Los Alamos, 94 atom % ^{18}O, ring-labeled) at a concentration of 40 mg/L in M9 minimal medium (*24*). All samples were in 50 mM HEPES (pH 6.8).

BSAO. Bovine serum amine oxidase was isolated and purified as described previously (*25*). An additional final step of gel filtration on Superdex 200 (HR 10/30) in an FPLC system (Pharmacia) was used to remove fluorescent impurities. The final purified enzyme had a specific activity of 0.47 units/mg of protein. Protein concentration was determined from 280 nm using $E^{1\%}_{1cm} = 20.8$ (*26*) and a molecular weight of 172 000 (*27*). The BSAO protein was concentrated to 0.75 mM (1.5 mM TPQ) in 20 mM potassium phosphate (pH 7.0) in a Microcon 30 (Amicon) ultrafiltration device for all subsequent spectroscopic experiments.

Exchange of the C5 carbonyl of TPQ was accomplished by diluting the protein 10-fold in $H_2{}^{18}O$ (97 atom %, ICON) containing 10 mM phosphate (pH 7.0) that was prepared by the addition of concentrated phosphate to yield a final concentration of 82 atom % ^{18}O. The protein was reconcentrated to 0.7 mM by centrifugation in a Microcon 30 ultrafiltration device (Amicon). For exchange of the C3-H of TPQ, the protein was diluted 20-fold in D_2O (99 atom %, Merck) containing 20 mM phosphate (pH 7.0) to yield a final concentration of 94 atom % D and was reconcentrated as above. The protein was incubated 2 days in each isotope prior to analysis.

Aniline Adduct of PEAO. A sample of 1.3 mM PEAO in 50 mM HEPES (pH 6.8) in a capillary tube was mixed with

60 mM aniline to yield final concentrations of 12 mM aniline and the 1.1 mM enzyme. Isotope labeling was performed with ^{15}N-aniline (98 atom %, Cambridge Isotope Laboratories).

Methylamine Adducts. A concentrated sample of BSAO (0.75 mM) in 50 mM phosphate (pH 7.0) was placed in a capillary tube sealed with a serum stopper and flushed with argon (*28*). An anaerobic solution of 50 mM methylamine substrate was added to the enzyme solution to yield 0.6 mM BSAO and 10 mM methylamine. The BSAO sample in D_2O, prepared as described above, contained a final concentration of 82% D_2O after reaction with methylamine. N-Isotope labeling of BSAO was performed with ^{15}N-methylamine (99 atom %, Cambridge Isotope Laboratories) generously provided by Dr. Victor Davidson. The methylamine adduct of PEAO was prepared as for BSAO but with 1.3 mM enzyme being mixed with 50 mM methylamine to yield 1.1 mM enzyme and 8 mM methylamine. D-Isotope labeling of the methylamine adduct was performed with methyl-d_3-amine (Aldrich, 98 atom % D).

Semiquinones. The semiquinone form of BSAO was prepared as described above for the methylamine adduct but with the addition of 40 mM KCN to the anaerobic methylamine solution to yield a final concentration of 7 mM KCN. The PEAO semiquinone was similarly produced but with phenylethylamine as the reductant and no added KCN, yielding 1.1 mM enzyme and 8 mM phenethylamine.

Spectroscopy. Absorption spectra were recorded on a Perkin-Elmer Lambda 9 spectrophotometer. Raman spectra were obtained (unless otherwise stated) 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 4-cm^{-1} spectral resolution and 10 min of data accumulation. Excitation sources were Coherent Innova 90-6 Ar and Innova 302 Kr lasers. BSAO samples in glass capillaries were inserted into a cold finger immersed in a water-ice mixture (*28*), whereas PEAO samples were measured at room temperature. 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}. Absorption spectra, taken on the same sample capillaries that were used for Raman experiments, showed that none of the samples were affected by laser irradiation. Difference RR spectra for PEAO were obtained by subtracting the spectrum of the apoprotein as described previously (*22*).

RESULTS AND DISCUSSION

RR Spectra of Native Amine Oxidases. Excitation of native PEAO within its 475-nm absorption band produces a RR spectrum that is characteristic of the TPQ chromophore (Figure 2A). The large number of vibrational modes between 1200 and 1700 cm^{-1} are due to a combination of C═C, C−C, and C═O stretching and C−H bending motions. A similar set of frequencies and intensities are observed in the RR spectra of HAO, BSAO, and ECAO as well as a TPQ hydantoin model compound (*21, 22*). Assignment of the vibrational spectrum is dependent on the ability to perform heavy-atom substitution and observe frequency shifts to lower energy. The PEAO system has proven to be ideal for such studies because a copper-deficient apoenzyme can be isolated with no tyrosine modification (*4, 23*). This has permitted us to obtain high-quality RR spectra of the TPQ moiety by subtracting out the vibrational contributions of the apoprotein and to specifically label the C2 oxygen with ^{18}O during cofactor generation in the presence of Cu and $H_2{}^{18}O$ (*22*). In addition, the C5 oxygen can be specifically labeled when the TPQ-containing holoenzyme is exposed to $H_2{}^{18}O$, and specific labeling of the C4 oxygen has now been achieved by growing the bacteria on ^{18}O-tyrosine.

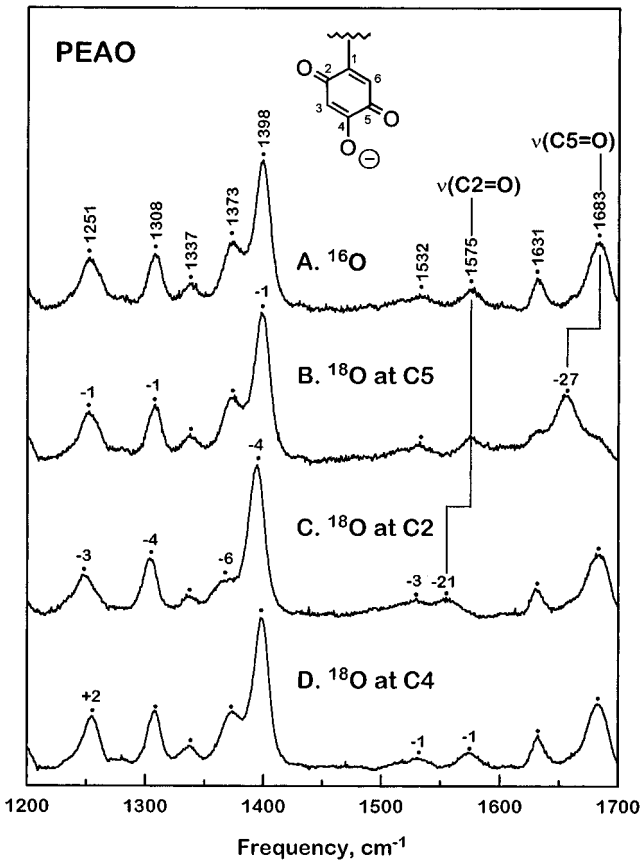

FIGURE 2: Raman spectra of native PEAO with ^{18}O-substituted TPQ. Apoprotein was activated (A) in $H_2{}^{16}O$, (B) in $H_2{}^{16}O$ followed by incubation in $H_2{}^{18}O$, and (C) in $H_2{}^{18}O$ followed by incubation in $H_2{}^{16}O$. (D) Bacteria were grown on ^{18}O-tyrosine and the resulting apoprotein was activated in $H_2{}^{16}O$. Spectra were obtained from 1.3 mM protein using 514.5-nm (30 mW) excitation and corrected by subtraction of the apoprotein spectrum. Frequency shifts relative to ^{16}O in spectrum A are listed above each peak. Peaks with frequencies identical to spectrum A are unlabeled.

When the C5 oxygen is substituted with ^{18}O, the peak at 1683 cm^{-1} undergoes a 27-cm^{-1} shift to lower energy (Figure 2B), identifying this as the C═O stretching mode of the C5 carbonyl. This ν(C═O) is a fairly pure vibration with little coupling to other modes since none of the other peaks in the high-frequency region have shifts greater than 1 cm^{-1}. In contrast, substitution of the C2 oxygen with ^{18}O has no effect on the 1683-cm^{-1} mode but causes the 1575-cm^{-1} mode to undergo a 21-cm^{-1} shift to lower energy (Figure 2C). The 1575-cm^{-1} frequency is low for a pure C═O stretch, suggesting that it has some single-bond character arising from the delocalization of electron density from the phenyl ring. This interpretation is supported by the additional ^{18}O shifts of -3 to -6 cm^{-1} in the features at 1251, 1308, 1373, 1398, and 1532 cm^{-1}, which arise from coupling

Table 1: RR Frequency Shifts upon Substitution of ^{18}O at C2, C4, or C5 of TPQ in PEAO[a]

	$\Delta^{18}O$							
ν	C2	C4	C5	C2 + C4	C2 + C5	C4 + C5	C2 + C4 + C5	assignment[b]
411	−7	−1	−3	−9	−9	−4	−11	δ(C═O) + δ(C−O)
489	−2	−2	−1	−4	−3	−3	−5	
507	−3	−1	−1	−4	−4	−2	−5	
522	−2	0	0	−2	−2	0	−2	
655	0	0	0	0	0	0	0	
724	−8	0	0	−8	−8	0	−8	
1189	0	0	0	0	0	0	0	
1251	−3	+2	−1	0	−3	0	0	ν(C═C) + ν(C2═O)
1308	−4	0	−1	−4	−4	0	−5	
1337	0	0	0	0	0	0	0	
1373	−6	0	0	−7	−6	0	−7	
1398	−4	0	−1	−5	−4	−1	−5	
1532	−3	−1	0	−4	−3	−2	−4	
1575	−21	−1	0	−22	−21	−1	−22	ν(C2═O)
1631	0	0	0	0	0	0	0	
1683	0	0	−27	0	−28	−27	−27	ν(C5═O)

[a] Frequencies in reciprocal centimeters. $\Delta^{18}O$ denotes shift upon substitution of ^{18}O. Data for C2 (generation of TPQ in $H_2{}^{18}O$), C4 (growth on ^{18}O-tyrosine), and C5 (incubation in $H_2{}^{18}O$) are from Figure 2. Data for mixtures were obtained by preparing enzyme under combined conditions (e.g., C2 + C4 obtained by growth on ^{18}O-tyrosine, TPQ generation in $H_2{}^{18}O$, and incubation in $H_2{}^{16}O$). [b] δ denotes bending mode, and ν denotes stretching mode.

of the C2−O stretch with lower energy vibrational motions. Finally, substitution of ^{18}O at C4 has considerably less effect on the RR spectrum (Figure 2D), indicating that the C4−O has mainly single-bond character and does not contribute significantly to the $\pi \rightarrow \pi^*$ transitions of the TPQ chromophore. These results demonstrate that the C−O moiety at C5 has the greatest double-bond character, thereby explaining the selective reactivity of the C5 carbonyl toward nucleophiles in both the enzyme and model compounds (*1*, *10*). The partial single-bond character of the C2 and C4 carbonyl groups detected by RR is in agreement with previous evidence for resonance delocalization of electrons from the C4 oxyanion to C2 (*11*), but RR spectroscopy indicates considerably more C═O at C2 than C4.

The oxygen-isotope shifts in PEAO were further verified by obtaining RR spectra on samples with ^{18}O-substitution at two or three positions of the TPQ cofactor (Table 1). In all cases, the shifts in the multiply-substituted samples correspond to the addition of the shifts from the individual substitutions. For example, the peak at 1398 cm^{-1} decreases by −4 cm^{-1} when both C2 and C4 are substituted with ^{18}O, which is the sum of −3 cm^{-1} for C2 alone and −1 cm^{-1} for C4 alone. This same ^{18}O additivity is observed in the 400−500-cm^{-1} region (Table 1), where deformations of the carbonyl groups and the aromatic ring are known to occur (*22*). Furthermore, a closely similar set of vibrational frequencies and isotope shifts was observed for HAO prepared in a similar manner with ^{18}O at the C2, C4, or C5 positions (data not shown). Thus, we can be quite confident of the C═O assignments listed in Table 1.

The RR spectra of native BSAO and ECAO are very similar to PEAO, with almost a one-to-one correspondence in peak frequencies and intensities (Table 2). In all three proteins, the ∼1400-cm^{-1} feature is one of the strongest peaks in the spectrum (indicated by boldface type). All three proteins also exhibit a similar C5═O stretching mode at ∼1680 cm^{-1} with a similar downshift of 22−27 cm^{-1} in $H_2{}^{18}O$ (Table 2) and a similar decrease in intensity of the adjacent peak at ∼1630 cm^{-1} (Figure 2B) (*21*). Furthermore, the frequency of the C5═O stretch varies by only 5 cm^{-1} between the three proteins. Similar constancy of vibrational

Table 2: Comparison of RR Frequencies and Isotope Shifts in Different Amine Oxidases[a]

BSAO[b]			ECAO[c]			PEAO[d]		
ν	[$\Delta^{18}O$]	(ΔD)	ν	[$\Delta^{18}O$]	(ΔD)	ν	[$\Delta^{18}O$]	(ΔD)
1263			1269			1251	[−1]	(−30)
1310		(−23)	1289		(−9)	1308	[−1]	(−16)
1335			**1334**			1337		
1373			1360			1373		
1398			**1400**			**1398**	[−1]	
1499			1500		(−6)			
1556		(−5)	**1547**		(−4)	1532		
1587			1583			1575		(−2)
1616		(−3)	1617		(−2)			
1633			1634			1631		
1678	[−22]		1681	[−26]	(−1)	1683	[−27]	(−3)

[a] Frequencies in reciprocal centimeters. Boldface type denotes the most intense peaks. $\Delta^{18}O$ denotes shift upon substitution of ^{18}O at the C5 position of TPQ. ΔD denotes shift in D_2O. [b] Spectrum from Nakamura et al. (*22*); isotope data from this work. [c] Spectra and isotope data from Moënne-Loccoz et al. (*21*). [d] Spectra obtained as in Figure 2.

frequency is observed for the peaks at ∼1335, 1400, 1500, 1615, and 1635 cm^{-1}. In contrast, the peaks at ∼1260, 1310, and 1550 cm^{-1} vary by ∼20 cm^{-1}. These three peaks also show the greatest deuterium isotope sensitivity (Table 2), a property that has been shown to be due to exchange of the hydrogen at the C3 position (*21*). It is likely that the greater range of energies in these three modes is due to variability in the extent of their C3−H bending character. In addition, the frequency of the C2═O stretch at ∼1580 cm^{-1} varies by 12 cm^{-1}, showing that it is more sensitive to the protein environment than the C5═O stretch.

The C5═O stretch at ∼1680-cm^{-1}exhibits a small deuterium dependence of −1 to −3 cm^{-1} in ECAO and PEAO, most likely due to hydrogen bonding of the C5-carbonyl oxygen with exchangeable protons in the protein. An important question is whether the RR spectrum of the TPQ is sensitive to the orientation of the cofactor in the protein. In the X-ray crystal structure of pea seedling amine oxidase (PSAO) (*8*), the C5 carbonyl of the TPQ ring is flipped away from the substrate binding pocket and H-bonded to the side chains of Tyr286 and Asn386. In contrast, in the crystal

structure of yeast amine oxidase (YAO), the C5 carbonyl of the TPQ cofactor is flipped into the substrate binding pocket and is hydrogen-bonded to a water molecule (R. Li, L. Chen, D. Cai, J. P. Klinman, and F. S. Mathews, personal communication). We have observed an identical frequency of 1677 cm^{-1} for the C5═O stretch in PSAO and YAO (N. Nakamura, J. P. Klinman, D. M. Dooley, and J. Sanders-Loehr, unpublished results), suggesting that the C5 carbonyl group forms equally strong H-bonds in the two cofactor orientations. In contrast, the frequency of the C2═O stretch is more variable, occurring at 1580 cm^{-1} in YAO and at 1588 cm^{-1} in PSAO. The 8-cm^{-1} lower energy in YAO could reflect the flipping of the C2═O toward the Cu and its strong H-bonding to a water ligand of the Cu (seen in the crystal structure).

Aniline Adduct as a Substrate Imine. Aniline behaves as a competitive inhibitor of BSAO (*29*), but the nature of this reaction has not been well studied. We have found that PEAO at pH 6.8 forms a complex with aniline, causing its 475-nm absorption band to blue-shift to 450 nm. This reaction appears to be similar to that of BSAO with ammonia at pH 9.1, whose product has an absorption maximum at 440 nm (*10*). These values are close to the 454-nm absorption maximum of 2-hydroxy-5-*tert*-butyl-*N*-cyclohexyl-1,4-iminoquinone (compound **8)** (*11*), suggesting that the aniline and ammonia adducts of PEAO and BSAO are stable iminoquinones and, thus, models for the substrate imine intermediate (Figure 1).

The RR spectrum of anilino-PEAO is remarkably similar to that of native PEAO, with peaks at 1257, 1313, 1375, and 1405 cm^{-1} being close in energy as well as intensity (Figure 3A,D). Further evidence for related structures comes from the reaction of ^{18}O-labeled PEAO (C2 position) with aniline (Figure 3B). These same four peaks show ^{18}O shifts of −3 to −6 cm^{-1}, similar to the behavior of ^{18}O-labeled PEAO (C2 position) in its native state (Figure 2C). Furthermore, the 1553-cm^{-1} peak has undergone a shift of −26 cm^{-1}, thereby identifying it as the C═O stretch of the C2 carbonyl. This proves that the aniline adduct has maintained a quinone-like structure. The major RR spectral differences occur at higher energy, where anilino-PEAO is missing the 1683-cm^{-1} C═O stretch from the C5 carbonyl and instead has a new intense mode at 1591 cm^{-1}. Reaction of PEAO with ^{15}N-aniline leads to a frequency shift of −5 cm^{-1} for the peaks at 1553 and 1591 cm^{-1} (Figure 3C), showing that the nitrogen from aniline has been incorporated into the TPQ cofactor. In addition, the peak at 1603 cm^{-1} has disappeared, with new intensity appearing as a 1574-cm^{-1} shoulder on the 1586-cm^{-1} peak. This ^{15}N-shift of −29 cm^{-1} is ascribed to the C═N stretch of the aniline adduct at the C5 position, lending further support to the iminoquinone formulation.

The aniline adduct of PEAO appears to be a deprotonated imine, based on its 450-nm absorption maximum. This is corroborated by the RR spectrum in D_2O, where the ν(C═N) mode at 1603 cm^{-1} sharpens and upshifts by 2 cm^{-1}, behavior that is indicative of H-bonding rather than protonation of the imine nitrogen. In the compound **8** model, the 454-nm absorption maximum of the deprotonated imine shifts to 352 nm upon protonation of the imine nitrogen (*11*). The 340-nm intermediate of BSAO observed during enzymatic turnover has consequently been ascribed to a protonated substrate imine (Hartmann et al., 1993). The observation of a deprotonated aniline adduct of PEAO at pH 6.8 is surprising given the pK_a of 9.2 for the reaction of BSAO with ammonia (*10*). The more facile loss of a proton in the reaction with aniline is presumably due to the inductive effective of the phenyl ring.

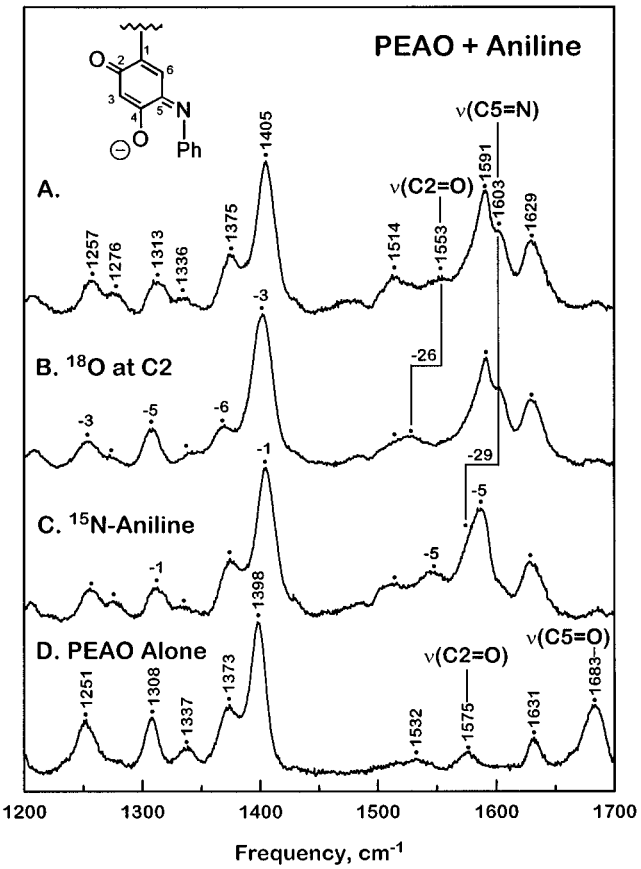

FIGURE 3: Raman spectra of aniline adduct of PEAO. Samples were prepared with (A) ^{14}N-aniline, (B) ^{14}N-aniline and PEAO activated in $H_2{}^{18}O$ as in Figure 2C, and (C) ^{15}N-aniline. These spectra were obtained on 1.1 mM protein using 514.5-nm excitation (30 mW). (D) The spectrum of native PEAO with no added aniline was collected as in Figure 2A.

The RR spectrum of anilino-PEAO is dominated by vibrations of the quinone ring of the TPQ cofactor. The characteristic vibrations of the aniline ring, such as peaks at 1030 and 1280 cm^{-1} (*30*), are simply not observed. This behavior is in striking contrast to the *p*-nitrophenylhydrazine adduct (a product imine analog) where the RR spectrum is dominated by vibrations of the azo and *p*-nitrophenyl groups and is unaffected by isotopic substitution of the reduced quinone ring (*22*). The lack of contribution of the aniline ring to the RR spectrum is most likely due to its being twisted and not coplanar with the TPQ ring. Such a rotation of the substituent ring has been observed in the crystal structure ECAO reacted with 2-hydrazinopyridine, which also serves as a model for the substrate imine intermediate (*12*).

Methylamine Adduct as a Product Imine. The preferred substrates of BSAO are amines with bulky side chains that are either aromatic (benzylamine) or aliphatic (spermine), with smaller molecules such as ethylamine showing no measurable activity in an assay based on O_2 consumption (*31*). Similarly, the preferred substrate for PEAO and ECAO is 2-phenylethylamine. In our earlier work we found that ECAO does react slowly with methylamine, yielding an aminosemiquinone intermediate (Figure 1) under anaerobic conditions (*21*). We further found that PEAO and BSAO

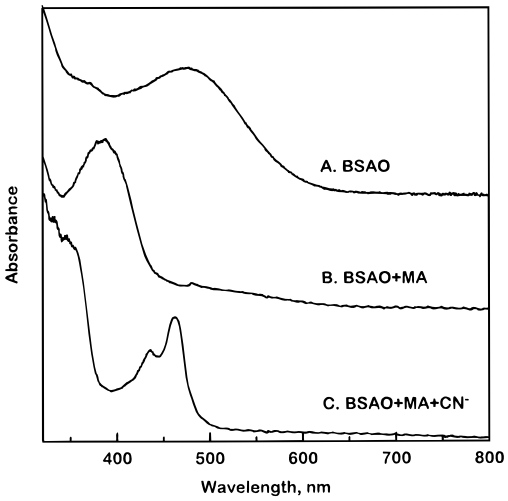

FIGURE 4: Absorption spectra of oxidized BSAO (A), methylamine adduct of BSAO (B), and semiquinone produced by anaerobic reaction of BSAO with methylamine and cyanide (C) in 20 mM sodium phosphate (pH 7.0).

react differently with methylamine, forming an imine rather than a semiquinone (*22*). Thus, anaerobic incubation of 0.6 mM BSAO with 10 mM methylamine results in an immediate loss within seconds of visible absorption, followed by the appearance within a few minutes of a stable colored species absorbing at 385 nm (Figure 4A,B). The same 385-nm absorption is observed for the methylamine adduct of PEAO (*22*). This absorption maximum is close to the 368-nm value for a deprotonated analog of the product imine (Figure 1), prepared by reacting benzaldehyde with 4-amino-6-*tert*-butylresorcinol (compound **11**) (*11*). Protonation of the imine nitrogen in compound **11** causes its absorption maximum to shift to 406 nm.

The RR spectrum of the methylamine adduct of either BSAO or PEAO (Figure 5A,D) is totally different from that of the aniline adduct (Figure 4A), with no spectral features corresponding to the quinonoid form of the cofactor. Instead of the ν(C2═O) and ν(C5═N) modes at 1553 and 1603 cm^{-1}, respectively, the methylamine adduct has its most intense RR mode at ∼1617 cm^{-1}. This feature downshifts by 19 cm^{-1} with ^{15}N-methylamine in BSAO (Figure 5C) and by 58 cm^{-1} with deuterated methylamine (CD_3NH_2) in PEAO (Figure 5F). It is assigned to the C═N stretch of an imine derived from the atoms of methylamine and, thus, identifies the structure as a product imine (Figure 1). Further evidence for this structure comes from the magnitude of the D dependence. On the basis of a simple two-body calculation, ν(N═CD_2) in a product imine has a predicted isotope shift of −51 cm^{-1}, which agrees well with the observed value of −58 cm^{-1}. In contrast, the D shift predicted for ν(C═NCD_3) in a substrate imine is only −22 cm^{-1}. The other peaks at ∼1450, 1500, and 1575 cm^{-1}, which shift significantly with $^{15}NH_2$- or CD_3-methylamine (Figure 5C,F), are probably due to coupling of ν(C═N) with ν(C═C) and δ(C−H) motions of the TPQ ring and pendant methine group.

Another difference between the substrate imine (aniline adduct) and product imine (methylamine adduct) is in the influence of the C2 substituent on the RR spectrum. Whereas the aniline adduct of PEAO with ^{18}O at C2 undergoes shifts of ∼3 cm^{-1} in at least five vibrational modes

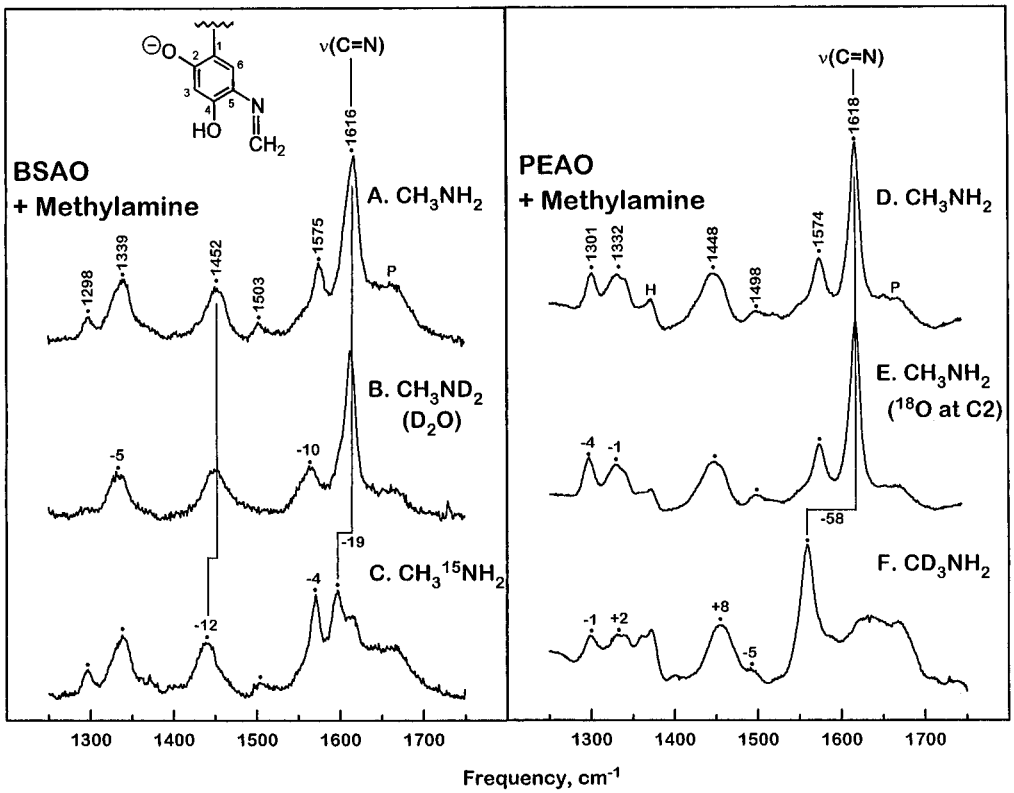

FIGURE 5: Raman spectra of methylamine adduct of BSAO and PEAO. Samples of BSAO (0.6 mM) were prepared by anaerobic addition of (A) CH_3NH_2, (B) CH_3ND_2 in D_2O, and (C) $CH_3{}^{15}NH_2$. Spectra were obtained on a Jarrell-Ash instrument with RCA C31034 photomultiplier, 406.7-nm excitation (50 mW), scan rate of 1 cm^{-1}/s, spectral resolution of 8 cm^{-1}, and accumulation of 8 scans. Samples of PEAO (1.1 mM) were prepared by anaerobic addition of (D) CH_3NH_2, (E) CH_3NH_2 to PEAO activated in $H_2{}^{18}O$ as in Figure 2C, and (F) CD_3NH_2 in H_2O. Spectra were obtained on the CCD instrument with 413.1-nm excitation (20 mW), 6-cm^{-1} resolution, and 5-min accumulation. P denotes protein mode (*32*); H denotes ν_4 mode of heme contaminant.

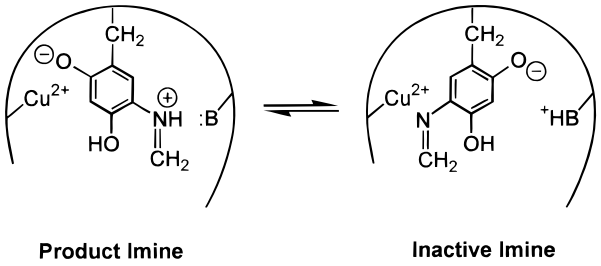

FIGURE 6: Flipping of TPQ ring in methylamine adducts of BSAO and PEAO, based on the mechanism of Cai et al. (*33*). The product imine is expected to yield catalytic turnover as in Figure 1.

(Figure 3B), the methylamine adduct of this same material only exhibits a significant shift of -4 cm^{-1} for the peak at 1301 cm^{-1} (Figure 5E). The low frequency of this ν(C2−O)-containing mode provides further evidence of its single-bond character, compared to the 1575-cm^{-1} value for ν(C2═O) in native PEAO (Figure 2).

Finally, the D isotope dependence of BSAO reacted with ND_2-methylamine in D_2O was investigated. Substantial D isotope downshifts of -5 at 1339 cm^{-1} and -10 at 1575 cm^{-1} were observed (Figure 5B). Shifts of this magnitude are most likely due to the exchange of the C3−H in the TPQ cofactor since they are similar to the shifts observed for native amine oxidases in D_2O (Table 1). Since the protein was prepared in D_2O, it would have undergone H exchange at the C3 position prior to the addition of methylamine. Significantly, the principal ν(C═N) mode at 1616 cm^{-1} has no detectable frequency change in D_2O. This result proves that the imino group of the methylamine adduct is not protonated, in agreement with its absorption maximum being similar to that of the deprotonated imine model **11** (*11*). Furthermore, since no changes in the optical or RR spectrum are observed upon lowering the pH 5.6, the deprotonated state of the PEAO-imine must be maintained even at this low pH.

The lack of protonation of the imine would explain the unusual stability of the methylamine adduct in BSAO and PEAO. In the proposed enzymatic reaction (Figure 1), nucleophilic attack on the product imine by water is facilitated by protonation of the imine nitrogen. It is possible that the deprotonated state of the imine nitrogen is favored by a flipping of the TPQ ring and interaction with the copper cofactor (Figure 6). This would be similar to the flipping observed for underivatized cofactors (*7, 8*) and presumably still feasible with small substituents on the TPQ ring. This pathway was originally proposed for the E406N mutant of yeast amine oxidase on the basis of the observation that enzymatic turnover was inhibited by methylamine but not larger substrates (*33*).

Effect of Cyanide on the Methylamine Adduct. Reaction of PEAO or BSAO with their normal substrates, phenylethylamine and benzylamine, respectively, under anaerobic conditions leads to the formation of aminoquinol (Figure 1). The Cu(II) can then remove an electron from the aminoquinol to generate a Cu(I) semiquinone, with the equilibrium distribution between these two species varying for different proteins (*14, 34*). The Cu(I) semiquinone concentration can be enhanced by the addition of cyanide, which binds and stabilizes Cu(I) (*14*). In the case of BSAO, addition of cyanide to the methylamine adduct (product imine) actually drives the reaction to completion, resulting in the appearance

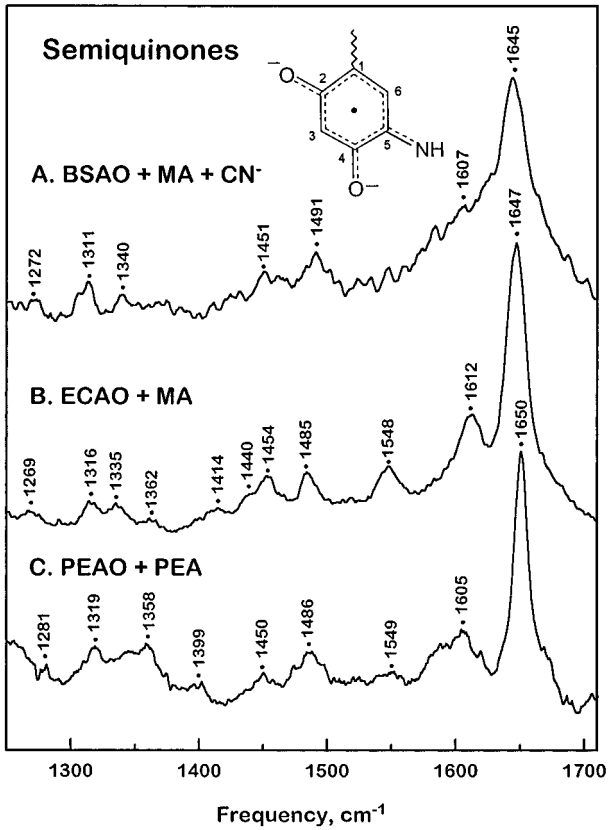

FIGURE 7: Raman spectra of semiquinones prepared by anaerobic reduction of BSAO, ECAO, and PEAO. (A) BSAO reduced with methylamine in the presence of KCN. Spectrum was obtained on a Jarrell-Ash instrument with 457.9-nm excitation (50 mW), scan rate of 1 cm^{-1}/s, spectral resolution of 6 cm^{-1}, and accumulation of 16 scans. (B) ECAO reduced with methylamine. Spectrum was obtained on a Jarrell-Ash instrument with 476.5-nm excitation (50 mW), spectral resolution of 8 cm^{-1}, and accumulation of 4 scans. (C) PEAO reduced with 2-phenylethylamine. Spectrum was obtained on the CCD instrument with 457.9-nm excitation (20 mW), 6-cm^{-1} resolution, and 5-min accumulation.

of absorption bands at 438 and 462 nm that are characteristic of the semiquinone (Figure 4C). This spectrum is identical to those reported previously for the semiquinone forms of pea seedling and *Arthrobacter* P1 amine oxidases prepared under anaerobic conditions using a variety of substrates (*34, 35*). The lack of substrate dependence is due to the fact that only the amino group of the substrate remains in the semiquinone intermediate (*18*).

The RR spectrum of the semiquinone form of BSAO, produced with methylamine and cyanide, is essentially identical to the RR spectra of the semiquinones produced by the addition of methylamine to ECAO or phenylethylamine to PEAO (Figure 7). All of the spectra are dominated by an intense feature at ∼1650 cm^{-1}, as has also been observed for the semiquinone produced by the addition of benzylamine to PSAO (*36*). Our earlier work on ECAO proved that the peaks at ∼1450 and 1650 have partial ν-(C═N) character from their shifts of -3 and -4 cm^{-1}, respectively, with ^{15}N-labeled substrate (*21*). The 1650-cm^{-1} feature was assigned as primarily a benzene ring stretching mode by comparison with the *p*-aminophenoxy and 1,2,4-benzenetriol radicals, whose RR spectra are dominated by similar intense peaks at 1636 and 1625 cm^{-1}, respectively (*37, 38*). Based on the extent of the ^{15}N shifts, the C5−N of the semiquinone has less double-bond character than the

C5−N of the substrate imine (Figure 3). Thus, the semiquinone is likely to have its unpaired electron delocalized over the C2 and C4 oxygens, as well as the C5 nitrogen, in agreement with the conclusions from ENDOR studies (*18*).

The above results on the reaction of BSAO with methylamine and cyanide (Figure 7A) show that the methylamine adduct has been hydrolyzed and that the resulting aminoquinol cofactor has undergone oxidation to yield the same semiquinone intermediate as in ECAO and PEAO. Although this reaction does not occur at a noticeable rate in BSAO with methylamine alone, it is apparently pushed toward completion by the formation of a copper−cyanide complex. This supports our proposal that the Cu(II) in BSAO actually helps to stabilize the methylamine adduct by favoring the flipped conformation of the inactive imine (Figure 6). Coordination of cyanide to the copper could disrupt this interaction and thereby promote the return to the normal reaction pathway. The substantial differences among amine oxidases in the formation of methylamine adducts and in the stabilization of the semiquinone intermediate may reflect variations in their copper−quinone interactions. Copper has been shown to play an important role in the generation of the TPQ cofactor (*3*, *4*) and in the reoxidation of the aminoquinol (*14*). Our results suggest that the copper complex may also influence the turnover of product imine in the reductive half of the reaction cycle.

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