Radicals from One-Electron Oxidation of 4-Aminoresorcinol: Models for the Active Site Radical Intermediate in Copper Amine Oxidases

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Received: January 24, 2000; In Final Form: March 24, 2000

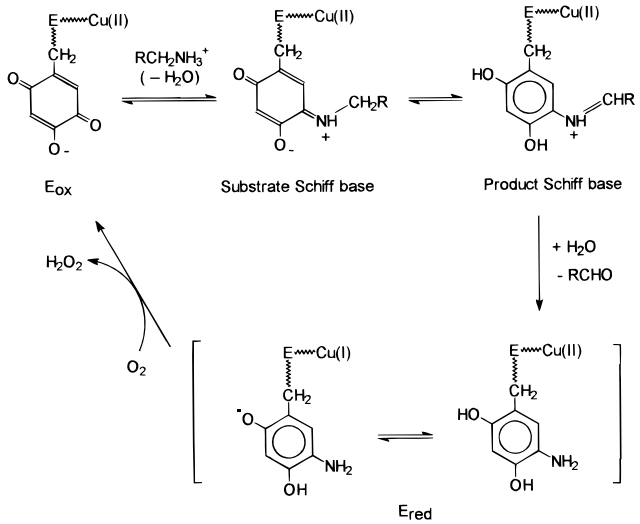
Pulse radiolysis, laser flash photolysis, and time-resolved resonance Raman spectroscopy have been used to study radicals derived by one-electron oxidation of 4-aminoresorcinol as models for the active site free-radical intermediate in the catalytic cycle of copper amine oxidases containing the trihydroxyphenylalanine (TOPA) quinone cofactor. The 4-aminoresorcinol radical at neutral pH has an absorption maximum at 450 nm, which is similar to that of the enzyme radical. At pH 5 the resonance Raman spectrum of the radical from one-electron oxidation of 4-aminoresorcinol resembles that in the enzyme. The radical protonates at lower pH values with a pK_a of 3.4 to give a species with a blue shifted absorption and different resonance Raman spectrum. Time-resolved resonance Raman spectroscopy shows that above pH 6.4 the radical from 4-aminoresorcinol deprotonates again to give a species that has a resonance Raman spectrum quite different from that of the enzyme radical. This second deprotonation is not immediately obvious from the transient absorption spectra obtained by pulse radiolysis. Interpretation of these spectra and comparison with related systems indicates that the enzyme intermediate is the singly deprotonated form of the radical. The results are discussed with respect to a recently proposed mechanism for the oxidative half reaction of copper amine oxidases (Su and Klinman, *Biochemistry* **1998**, *37*, 12513) in which the rate-limiting step is the oxidation by molecular oxygen of the fully reduced cofactor to the radical intermediate.

Introduction

Free radicals form an important aspect of the biogenesis and enzymatic mechanism of copper amine oxidase. This widely distributed enzyme is a homodimer with each monomer containing one TOPA (trihydroxyphenylalanine) quinone (TPQ) residue[1] and one cupric ion.[2] Because of the unusual TPQ residue and the involvement of free-radical species, copper amine oxidases have been intensively studied[3−5] with crystallographic structures available for the enzyme from several sources.[6−9] The TPQ residue arises from an original tyrosine residue in a conserved sequence[10] and is formed post-translationally through a sequence of free-radical reactions, dependent on the presence of the cupric ion and oxygen.[11−14]

The proposed catalytic cycle of copper amine oxidases is outlined in Scheme 1. Reaction between amine substrate and the TPQ residue of the enzyme (which is close, but not coordinated, to the cupric ion of the active site) in the oxidized state (E_{ox}) proceeds through Schiff base formation followed by hydrolysis to release aldehyde product and creating an enzyme-bound aminoquinol. This intermediate reduced state of the enzyme (E_{red}) containing aminoquinol and a cupric ion is in equilibrium with a form of the enzyme in which electron transfer leads to a cuprous ion and an iminosemiquinone recognized by its visible absorption spectrum (λ_{max} 464 nm)[15−17] and paramagnetic properties.[18−20] Resonance Raman spectroscopy has also been used to show that the free-radical intermediate contains the substrate-derived nitrogen atom.[21−23] In the pea seedling amine oxidase (PSAO) the iminosemiquinone is readily observed after amine substrate addition under anaerobic conditions, whereas in bovine serum amine oxidase (BSAO) it is more readily seen in the presence of cyanide.[15]

SCHEME 1: Proposed Catalytic Cycle of Copper Amine Oxidases



In the second (oxidative) half-reaction, the reduced enzyme is reoxidized by molecular oxygen to give hydrogen peroxide as the second product. It was proposed that the cuprous

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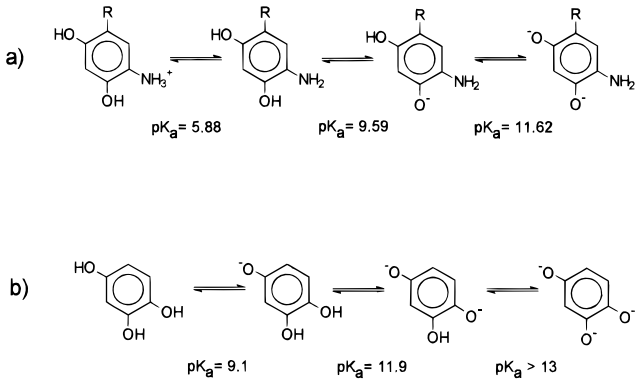
10.1021/jp000296v CCC: $19.00 © 2000 American Chemical Society
Published on Web 05/31/2000

SCHEME 2: (a) Deprotonation of 4-Aminoresorcinol (R = −H) and Associated pK_a Values for 6-Amino-4-ethylresorcinol (R = $-C_2H_5$) from Ref 28 and (b) Deprotonation of Benzene-1,2,4-triol and Associated pK_a Values from Ref 39



iminosemiquinone intermediate undergoes initial one-electron oxidation by molecular oxygen[3,15] in order to avoid two-electron reduction of oxygen which is impeded by spin restriction.[24] Recently it has been shown[25,26] that, although Cu^{2+}-depleted BSAO is inactive, catalytic activity is restored by reconstitution of the apoprotein with Co^{2+}, suggesting that the cuprous iminosemiquinone state is not an intermediate in the catalytic pathway. A study of isotope and viscosity effects on the oxidative half-reaction of BSAO has also led Su and Klinman[27] to rule out rapid one-electron reduction of oxygen to superoxide on reaction with cuprous ion as a key step. Instead, they propose that oxygen binds at a hydrophobic site identified in the crystal structure of *H. polymorpha* copper amine oxidase, placing the oxygen molecule adjacent to O-2 of the reduced TPQ residue. Here it is proposed the aminoquinol is oxidized in a one-electron step to give the iminosemiquinone, assisted by stabilization of the resulting superoxide ion by the nearby cupric ion. Subsequently, transfers of protons and a second electron attain the products, the proton transfer being assisted by suitably placed water molecules also identified from the X-ray structures.

This mechanism proposed by Klinman and co-workers[27] in part rely on investigations performed on model compounds of the fully oxidized and reduced forms of TPQ. These show that the 2-hydroxyl group of TPQ is ionized in the enzyme with pK_a 3.0 compared with 4.13 in a model quinone (see Scheme 1).[28] Also in the enzyme the protonated amino group of reduced TPQ ionizes with a pK_a of 7.2 (compared with pK_a 5.9 in a model aminoquinol, see Scheme 2a) and is one of two ionizations which control the rate of the oxidative half reaction.[27] The protonation state of the iminosemiquinone radical, important in governing reactivity and redox potential, is more difficult to establish since model radical species are expected to be short-lived. In PSAO we have been able to detect only one form of the radical[22] by resonance Raman spectroscopy at pH values between 5 and 9. We now report studies of an iminosemiquinone model for the radical site in copper amine oxidases generated by pulse radiolysis and laser flash photolysis,[29] and characterized by transient absorption spectroscopy and time-resolved resonance Raman spectroscopy.[30]

Materials and Methods

4-Aminoresorinol (4-AR) was obtained from Aldrich and used as received. All other chemicals were of AnalaR grade or equivalent. Solutions were prepared in water purified in a Millipore Milli Q system or by triple distillation. In neutral or alkaline solutions 4-AR was found to readily oxidize in air. Stock solutions of 4-AR were prepared in dilute HCl (10^{-2} mol dm^{-3}) and all solutions degassed before mixing and adjustment to the desired pH value. Pulse radiolysis experiments made use of either the Febetron electron accelerator at the University of Salford or the Paterson Institute for Cancer Research (PICR) linear accelerator to deliver single pulses (ca. 20 ns) of between 2 and 15 Gy.

Time-resolved resonance Raman (TR^3) experiments were undertaken at the Lasers for Science Facility, Rutherford Appleton Laboratory. The pump laser pulse was provided by an XeCl excimer-pumped dye laser and the tunable probe pulse obtained using a Continuum Sunlight OPO system. Raman spectra were measured using a Spex triple monochromator and CCD detector as previously described.[31] For both lasers the energies were typically between 0.1 and 0.5 mJ, focused to ~150 μm at the sample. The sample cell was a 2 mm internal diameter quartz tube through which the sample flowed to waste. In the TR^3 experiments at pH > 6, oxidation of 4-AR was minimized by use of a flow system in which the deaerated solutions of 4-AR in HCl and buffer of the desired pH were mixed in the flow system immediately before exposure to the laser beams. Spectral subtractions and other manipulations were accomplished with Princeton CSMA software.

Results and Discussion

Oxidation of 4-AR by inorganic radicals was investigated by pulse radiolysis. The product aminoresorcinyl radicals were characterized by their transient absorption spectra, decay rates, and pK_a values. The same radicals were generated by laser flash photolysis using triplet duroquinone as the oxidant. Time-resolved resonance Raman spectra were obtained using the same laser as for flash photolysis in combination with a second pulsed laser, the probe, tuned to the maxima of the absorption spectra identified in the pulse radiolysis experiments.

1. Pulse Radiolysis Studies of 4-Aminoresorcinol. Both $Br_2^{\bullet-}$ and $N_3^{\bullet}$ radicals were used as one-electron oxidants. They were generated in N_2O-saturated aqueous solutions of the corresponding anion (Br^- or N_3^-).[29] At pH 6.9 the rate of decay of $Br_2^{\bullet-}$ was demonstrated to be first order in 4-AR concentration (data not shown). The second-order rate constant for reaction of $Br_2^{\bullet-}$ with 4-AR was determined from the pseudo-first-order decay of its transient absorption at 360−380 nm at various concentrations of 4-AR over the pH range of 2−8. The inset to Figure 1 shows that the second-order rate constant increases from 3.0×10^7 dm^3 mol^{-1} s^{-1} at pH 2.1 to 1.1×10^9 dm^3 mol^{-1} s^{-1} at pH 7.7. Such an increase in reactivity is typically associated with deprotonation of a reactive site in the solute and in this instance the curve in Figure 1 (inset) may be fitted to a single pK_a of 6.14 ± 0.10. This is the pK_a of the first deprotonation of 4-AR determined spectrophotometrically at 305 nm as 6.10 ± 0.1 (data not shown). This is very similar to the value of 5.88 determined for the deprotonation of the amino group in 6-amino-4-ethylresorcinol by Mure and Klinman[28] (Scheme 2a). Above pH 8 the experiments became increasingly irreproducible due to oxidation of 4-AR, despite exhaustive deaeration by bubbling with N_2O. This prevented measurements that may have otherwise observed a further increase in reactivity due to subsequent deprotonation of the phenolic groups, the pK_a values of which have been determined[28] for 6-amino-4-ethylresorcinol as 9.59 and 11.62. The second-order rate of reaction of the azidyl radical ($N_3^{\bullet}$) with 4-AR was determined from the rate of product radical formation at 460 nm (see below).

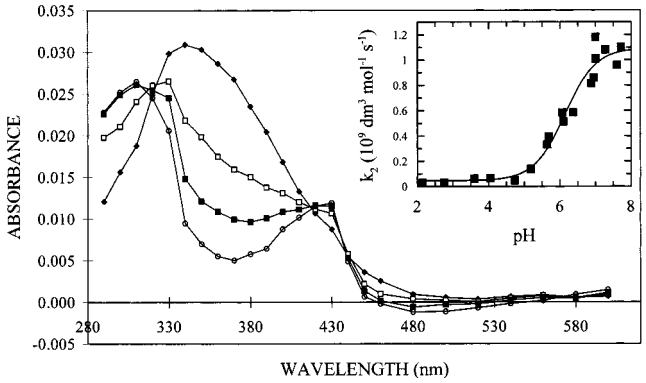


Figure 1. Transient absorption spectra from pulse radiolysis of N_2O-saturated solutions containing 4-aminoresorcinol (2 mmol dm^{-3}), KBr (0.1 mol dm^{-3}) EDTA (1 mmol dm^{-3}), and phosphate (50 mmol dm^{-3}) adjusted to pH 1.9 with HCl, measured 2.5 μs (◆), 5 μs (□), 15 μs (■) and 75 μs (○) after the pulse. Dose = 3 Gy pulse^{-1}. Inset: Effect of pH on the second-order rate constant (k_2) for reaction of $Br_2^{\bullet-}$ with 4-aminoresorcinol in solutions containing KBr (0.1 mol dm^{-3}) and buffered using phosphate (50 mmol dm^{-3}) and HCl.

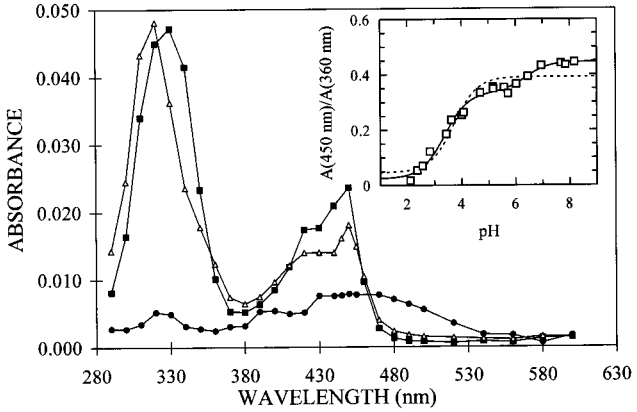


Figure 2. Transient absorption spectra from pulse radiolysis of N_2O-saturated solutions containing 4-aminoresorcinol (0.1 mmol dm^{-3}), sodium azide (0.1 mol dm^{-3}), phosphate buffer (50 mmol dm^{-3}), and EDTA (0.1 mmol dm^{-3}), at pH 6.04 measured 60 μs (△) and 8 ms (●) after the pulse, and at pH 7.74 measured 60 μs after the pulse (■). Dose = 3 Gy pulse^{-1}. Inset: Effect of pH on the intensity of the transient absorbance at 450 nm, relative to the initial aborbance at 360 nm, measured in solutions of 4-aminoresorcinol and KBr. The full curve shows the fit to two pK_a's and the dashed curve shows the fit to a single pK_a.

At pH 7.74 the second-order rate constant was (5.8 ± 0.2) × 10^9 dm^3 mol^{-1} s^{-1}. For $N_3^{\bullet}$ the rate constant is close to the diffusion-controlled limit while $Br_2^{\bullet-}$ is slightly less reactive, but is consistent with the more reactive nature of the azidyl radical toward both inorganic and organic solutes.[32]

Transient absorption spectra recorded during the reaction of $Br_2^{\bullet-}$ with 4-AR at pH 1.9 are shown in Figure 1. Almost immediately (2.5 μs) after the radiation pulse the spectrum is that of the $Br_2^{\bullet-}$ radical with λ_{max} 360 nm. As $Br_2^{\bullet-}$ reacts with 4-AR this spectrum disappears and is simultaneously replaced by the spectrum with λ_{max} 430 nm as shown in Figure 1 taken at 65 μs after the pulse. This transient spectrum is ascribed to the one-electron oxidation product of 4-aminoresorcinol. Assuming that reaction occurs only by electron transfer the 4-aminoresorcinyl radical has $\epsilon_{430\ nm} = 3.8 \times 10^3$ dm^3 mol^{-1} cm^{-1} by comparison with the intensity of the $Br_2^{\bullet-}$ spectrum ($\epsilon_{360\ nm} = 9.9 \times 10^3$ dm^3 mol^{-1} cm^{-1}).[33]

Spectra of the 4-aminoresorcinyl radicals at pH 6.04 and 7.74 formed by oxidation with $N_3^{\bullet}$ are shown in Figure 2. Similar spectra (not shown) were obtained using $Br_2^{\bullet-}$ as one-electron oxidant indicating that in each case the same product is formed by one-electron oxidation rather than by radical addition. At these higher pH values the peak of the long-wavelength absorption band in the transient spectrum is shifted to 450 nm and at pH > 7.5 has $\epsilon_{450\ nm} = 4.5 \times 10^3$ dm^3 mol^{-1} cm^{-1}. The decay of the transient absorption at 450 nm was found to be well fitted by nonlinear least-squares fitting to second-order kinetics leading to a much longer lived species with λ_{max} 440−470 nm as shown in Figure 2. This decay is most probably a disproportionation reaction forming the iminoquinone. The observed maximum at 440−470 nm in the spectrum measured at 8 ms after the pulse is consistent with that reported (λ_{max} 448−454 nm) reported for the iminoquinone by Mure and Klinman.[28,34]

The variation of the yield of the transient absorbance at 450 nm was determined as a function of pH using $Br_2^{\bullet-}$ as the oxidant. In the inset to Figure 2 the results are shown relative to the initial absorbance of $Br_2^{\bullet-}$ at 360 nm, thereby correcting for any variation in radiation chemical yield with pH. The full line shows the fit of the data to a double pK_a curve, yielding pK_a values of 3.37 ± 0.09 and 6.39 ± 0.25. The dashed line indicates an inferior fit to a single pK_a of 3.71 ± 0.13. This result suggests that within the pH range 1−8 there are three different protonated states of the radical. The resonance Raman studies (see below) support the existence of three different protonated forms of the radical and substantiate the interpretation of the transient absorbance data in terms of two pK_a values. Furthermore, analysis of the second-order decays of the 450 nm absorption of the radical gives $2k_2 = (1.4 \pm 0.3) \times 10^9$ dm^3 mol^{-1} s^{-1} at pH 6.04 and $2k_2 = (5.9 \pm 1.0) \times 10^8$ dm^3 mol^{-1} s^{-1} at pH 7.74. This indicates that differing species are involved below and above the pK_a of 6.4.

2. Laser Flash Photolysis and Time-Resolved Resonance Raman Spectroscopy. The radicals identified by the pulse radiolysis in section 1 were studied using time-resolved resonance Raman (TR^3) spectroscopy. Since TR^3 was not available to us in conjunction with pulse radiolysis, laser-induced one-electron oxidation of 4-AR was accomplished using triplet duroquinone (^{3}DQ) as a strongly oxidizing sensitizer.[33,37] In these experiments acetonitrile:water (50/50 v/v) was used as solvent, since DQ is insoluble in water alone. The second-order rate constant for reaction of ^{3}DQ with 4-AR in ethanol was (2.2 ± 0.1) × 10^9 dm^3 mol^{-1} s^{-1}, determined by measurement of the quenching kinetics for decay of the ^{3}DQ absorption at 500 nm. Transient absorption spectra obtained by laser flash photolysis revealed overlapping spectra of aminoresorcinol radicals and duroquinone radicals (neutral durosemiquinone radical λ_{max} 410 nm, durosemiquinone anion λ_{max} 440 nm, pK_a = 5.0).[36]

The TR^3 spectra of the 4-AR radical(s) were obtained with a nanosecond pump laser pulse between 340 and 355 nm to produce the reactive duroquinone triplet, which then reacted with 4-AR. Resonance Raman spectra of the triplet excited state and radical intermediates were produced using a second delayed nanosecond laser pulse at a wavelength tuned to the 4-aminoresorcinyl radical absorption bands between 430 and 460 nm. Figure 3 shows the TR^3 spectra obtained at various time delays between pump and probe laser pulses for reaction of ^{3}DQ with 4-AR at pH 5.2 and probed at 460 nm. At this wavelength, both ^{3}DQ ($\lambda_{max} \sim 500$ nm)[35] and the durosemiquinone radical anion are also in resonance. Almost immediately after excitation of the sample (20 ns delay, curve a) the spectrum consists of a dominant peak at 1550 cm^{-1} which can be ascribed to triplet duroquinone.[31] With increasing time delays between pump and

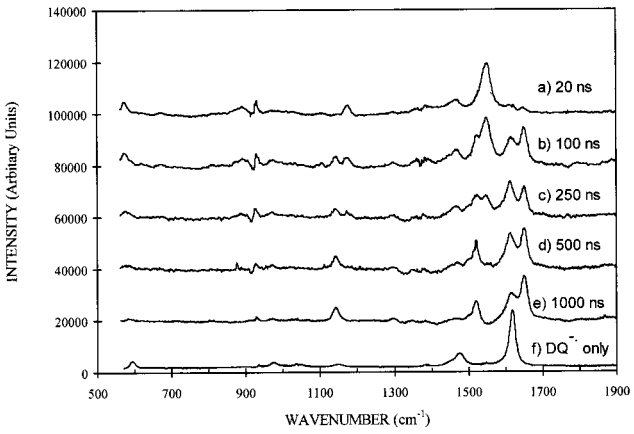


Figure 3. Time-resolved resonance Raman spectra obtained during reaction of triplet duroquinone with 4-aminoresorcinol. The solution (in 50/50 MeCN/H_2O v/v) contained DQ (5 mmol dm^{-3}), 4-AR (0.5 mmol dm^{-3}), and phosphate buffer (25 mmol dm^{-3} at pH 5.2). The time delays between pump laser (340 nm, 2 mJ/pulse) and probe laser (460 nm, 1 mJ/pulse) were (a) 20 ns, (b) 100 ns, (c) 250 ns, (d) 500 ns, and (e) 1000 ns. Also shown as curve f is the TR^3 spectrum of the durosemiquinone radical anion measured using the same wavelengths at a pump−probe delay of 1 μs in a solution containing DQ (5 mmol dm^{-3}) and ascorbate (1 mmol dm^{-3}) buffered to pH 7 with phosphate (25 mmol dm^{-3}). Probe-only spectra and sloping baselines have been subtracted.

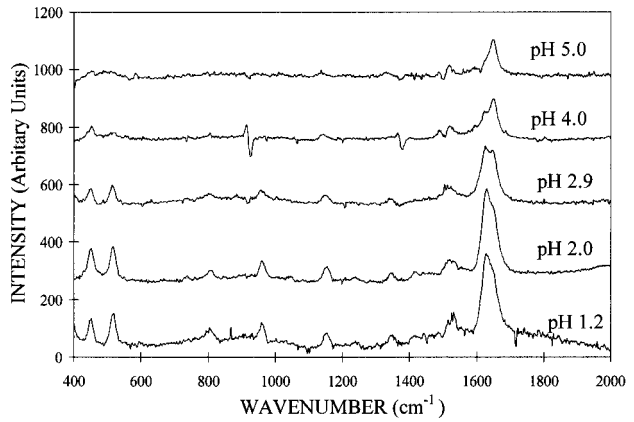


Figure 4. Time-resolved resonance Raman spectra of radicals formed by one-electron oxidation of 4-aminoresorcinol at the indicated pH values using a pump pulse at 355 nm and a probe pulse at 430 nm delayed by 1 μs. Solutions contained DQ (2.5 mmol dm^{-3}) and 4-AR (1 mmol dm^{-3}) buffered and the pH adjusted with HCl or acetate buffer (25 mmol dm^{-3}). Spectra of the durosemiquinone radicals determined at the same pH values have been subtracted (see text).

probe pulses (curves a−e) the ^{3}DQ peak decays and major new peaks at 1520, 1614, and 1652 cm^{-1} are formed. Curve f in Figure 3 shows the TR^3 spectrum of the durosemiquinone radical anion formed in solutions of duroquinone and ascorbate at pH 7, and identifies the peaks at 1612 and 1470 cm^{-1} as belonging to $DQ^{\bullet-}$ as previously observed.[31] Resonance Raman spectra attributable to 4-AR radical(s) were obtained by subtraction of the durosemiquinone radical ($DQH^{\bullet}$ and/or $DQ^{\bullet-}$) spectra (such as (f)) measured at the same pH value. Typical resultant spectra are shown in Figure 4 for a range of pH values when probed at 430 nm in order to be in resonance with both forms of the radical observed in the pulse radiolysis experiments. The spectra change substantially on increasing the pH of the solutions from pH 1.2 to pH 5.0. This is most clearly seen in the region of the C−C stretching vibration where the peak shifts from 1629 cm^{-1} at pH 1.2 to 1648 cm^{-1} in the spectrum at pH 5.0. At intermediate pH values the spectra appear to be superimpositions of these

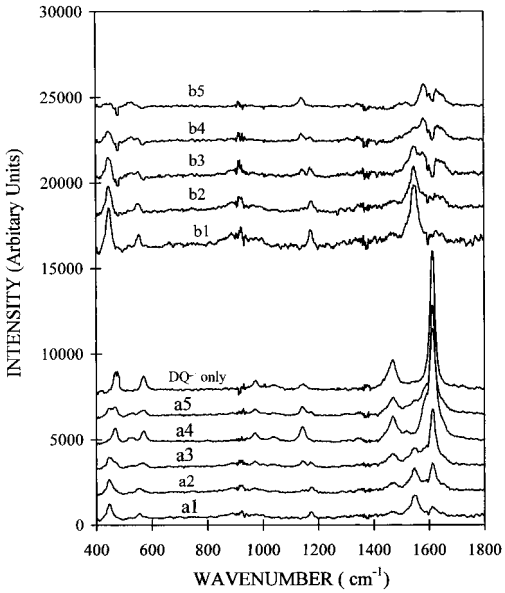


Figure 5. Time-resolved resonance Raman spectra measured from a solution of DQ (1 mmol dm^{-3}), 4-AR (0.2 mmol dm^{-3}), and EDTA (50 μmol dm^{-3}) buffered to pH ∼ 8 with phosphate (50 mmol dm^{-3}) using pump and probe wavelengths of 355 and 450 nm, respectively. The set of spectra marked (a) are as measured, while the set marked (b) are shown after subtraction of the durosemiquinone ($DQ^{\bullet-}$) radical anion spectrum. The time delays between pump and probe pulse were 20 ns (1), 100 ns (2), 250 ns (3), 500 ns (4), and 2 μs (5).

two extreme spectra and are entirely consistent with the first deprotonation of the radical with a pK_a of 3.4 determined from the pulse radiolysis experiments.

The TR^3 spectra a1−a5 in Figure 5 obtained at pH 8 with a 450 nm probe laser pulse, appropriate to the longer wavelength radical absorption maximum at pH > 6, indicate reaction of triplet duroquinone (peak at 1550 cm^{-1} in the 20 ns spectrum a1) to yield product radicals, predominantly $DQ^{\bullet-}$ with peaks at 1612 and 1470 cm^{-1}. However, comparison of the spectra of the products at 2 μs (spectrum a5) with that of $DQ^{\bullet-}$ indicates contributions from a second species as revealed when the spectrum of $DQ^{\bullet-}$ is subtracted, as shown in spectra b1−b5. The TR^3 spectrum of this new radical obtained by one-electron oxidation of 4-AR at pH 8 has major peaks at ca. 1581 and 1629 cm^{-1} and is clearly different from those shown in Figure 4. Using a 460 nm probe wavelength, TR^3 spectra were obtained between pH 4.5 and pH 8.3 and are illustrated in Figure 6 after subtraction of the durosemiquinone radical contributions. Although pH 4.5 is close to the lower pK_a value of the 4-aminoresorcinyl radical identified by pulse radiolysis, the resonance Raman spectrum obtained with a 460 nm probe at this pH appears to select only the singly deprotonated form of the radical. As the pH is increased between pH 7.3 and pH 8.3, the spectral change clearly shows the formation of a new radical. This seems to indicate a somewhat higher pK_a for the second deprotonation of the 4-aminoresorcinyl radical than the value of 6.4 obtained from the spectrophotometric titration shown in Figure 2. However, it must be noted that the aqueous solvent for the TR^3 experiments contains 50% acetonitrile rather than water alone as used for the pulse radiolysis and it may be that the radical pK_a is changed slightly by the presence of the organic solvent in these experiments. Recent experiments with other phenols[37] suggest that the pK_a value may be about one unit higher in acetonitrile/water than in water alone. The results in Figure 6 confirm the second deprotonation reaction indicated by the changes in extinction of the transient absorption spectrum

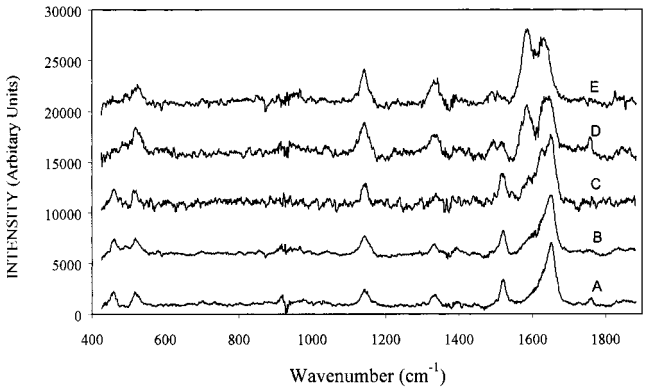


Figure 6. Time-resolved resonance Raman spectra of radicals from one-electron oxidation of 4-AR measured using a probe wavelength of 460 nm (pump wavelength 340 nm). After mixing in a flow system, the final solutions contained DQ (5 mmol dm^{-3}), 4-AR (2.5 mmol dm^{-3}), EDTA (50 μmol dm^{-3}), and phosphate buffer (50 mmol dm^{-3}). All spectra were recorded with a delay of 2 μs between pump and probe pulses. The solutions were at pH 4.5 (A), pH 6.7 (B), pH 7.3 (C), pH 8.0 (D), and pH 8.3 (E).

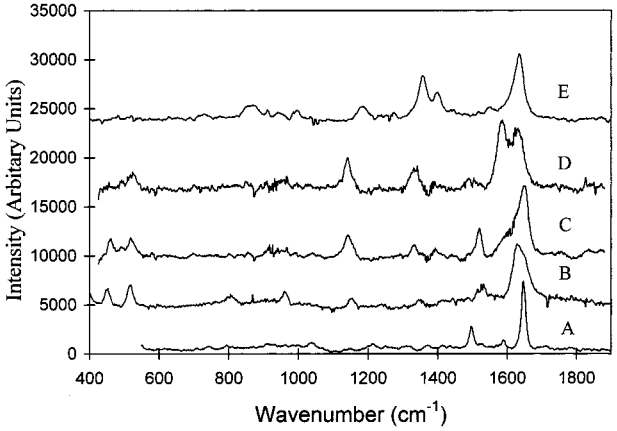


Figure 7. Comparison of resonance Raman spectra for the active site radical in pea seedling amine oxidase (A, from ref 22, probe wavelength 457.9 nm); the 4-AR radical at pH 1.2 (B), at pH 6.7 (C), and at pH 8.3 (D). For spectra B−D, conditions are as described for Figure 6. Also shown is the steady-state resonance Raman spectrum of the long-lived Schiff base intermediate formed on reaction of pea seedling amine oxidase (1.0 mg/mL) with 4-(dimethylamino)benzylamine (3 mmol dm^{-3}) at pH 8.0 using a probe wavelength of 406 nm.

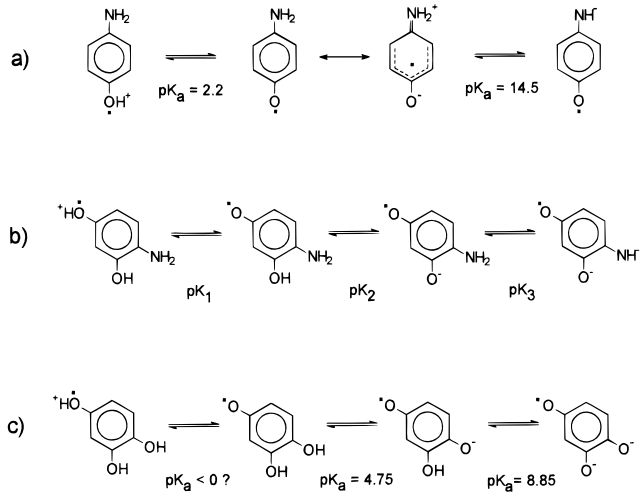
and the second-order decay rate of the radical in the pulse radiolysis experiments.

3. Comparison of Resonance Raman Spectra of Radicals from 4-AR and Amine Oxidase. The TR^3 spectra of 4-aminoresorcinyl radicals may be compared with the resonance Raman spectrum of the active site free-radical intermediate of copper amine oxidases. On the basis of spectra from PSAO after reaction with ^{14}[N]- and ^{15}[N]-containing substrates, we previously supported the conclusion[18,21] that the radical contains the nitrogen atom of the substrate and is a semi-iminoquinone.[22] TR^3 spectra of each protonation state of the iminosemiquinone radical obtained from oxidation of 4-aminoresorcinol are compared with the pea seedling amine oxidase (PSAO) radical spectrum and with a long-lived Schiff base intermediate in Figure 7. It is immediately obvious that the singly deprotonated 4-aminoresorcinyl radical spectrum (spectrum C at pH 6.7) most closely resembles the PSAO radical spectrum. It has a dominating band at 1650 cm^{-1}, compared with 1647 cm^{-1} in the enzyme, ascribed to the ring C−C stretching vibration (Wilson ν_{8a}) and another moderately strong band at 1520 cm^{-1}, observed at 1496 cm^{-1} in PSAO which we previously ascribed to a mixed C−O/C−N stretch (Wilson ν_{7a}). It is reasonable to expect that this latter frequency might be altered in the enzyme due to environmental effects, including hydrogen-bonding interactions with the nitrogen or oxygen atoms, leading to changes in the strengths and mixing of the vibrational modes. The spectra in Figure 7 measured at lower pH (pH 1.2) and higher pH (pH 8.3) are rather unlike the PSAO radical spectrum with bands in the C−C region between 1583 and 1632 cm^{-1}.

Stopped-flow studies have established that earlier intermediates prior to radical formation in the catalytic cycle of copper amine oxidase are the Schiff base adducts.[38] It is therefore useful to show at this stage that the resonance Raman spectrum of the PSAO radical intermediate may also be readily distinguished from that of a Schiff base. The resonance Raman spectrum shown as Figure 7E was measured for the long-lived Schiff base intermediate formed on reaction of 4-(dimethylamino)benzylamine (DMAB) with PSAO[39] and measured with a probe wavelength of 406 nm in resonance with the long wavelength absorption band of this species. Although the spectrum of this adduct again contains a dominant band at 1634 cm^{-1} in a similar region to the C−C stretching mode of the radicals described above, it also contains additional bands at 1357 and 1399 cm^{-1} which appear to be characteristic of the aromatic C−N stretching vibration in the adduct. The resonance Raman spectrum of the DMAB adduct with PSAO appears somewhat different from the adducts of BSAO and phenethylamine oxidase with aniline and methylamine reported previously.[23]

4. Identification of 4-AR Radical Protonation States. So far, the different radicals formed on one-electron oxidation of 4-aminoresorcinol have been described as "singly" and "doubly" deprotonated relative to the state in the experiments under the most acidic conditions investigated (pH $\approx$ 1). However, it is of importance to identify the absolute protonation state of the 4-aminoresorcinyl radicals observed here and this may be accomplished by comparison with various related compounds. Possible sites of oxidation of 4-AR are the two hydroxyl groups and the amino group. As is clear from the work on radicals from benzene-1,2,4-triol[40] (see Schemes 2b and 3c), the delocalization of the unpaired electron involves mainly the groups para to one another and the effect of the 2-hydroxyl substituent is minor until it undergoes ionization allowing complete electron delocalization between all three oxygen atoms. Therefore, the behavior of 4-aminoresorcinyl radicals may be approached by considering them as substituted 4-aminophenoxyl radicals. Above pH 9.59, the first pK_a involving one of the phenolic −OH groups (see Scheme 2a), the obvious site of oxidation is the ionized phenolate oxygen atom. Similarly, at pH values below the pK_a of 5.9 relating to protonation of the amino group, the phenolic groups are also the most likely oxidation sites. However, at intermediate pH values near neutrality both amino and hydroxyl groups are possible one-electron donors, having similar one-electron oxidation potentials. For 4-aminophenol in both acidic and neutral solutions, it is well established from both ESR[41,42] and resonance Raman[43−45] spectroscopy that oxidation of the hydroxyl group is favored over that of the amino group. The one-electron oxidation product is essentially a phenoxyl radical with semiquinone-like features, or the corresponding radical cation. The pK_a values of phenoxyl radical cations are generally in the region of −5 to −1,[42,46] but for 4-aminophenoxyl it has been firmly established that in acidic solutions the radical is protonated at the phenoxyl oxygen, and not at the amino group, with a pK_a of 2.2.[45] The neutral radical has substantial zwitterionic character as indicated in Scheme

SCHEME 3: Deprotonation of Radical Species and Their Associated pK_a Values: (a) 4-Aminophenoxyl Radicals, Values from Ref 44; (b) 4-Aminoresorcinyl Radicals; and (c) Benzene-1,2,4-triol Radicals, Values from Ref 39



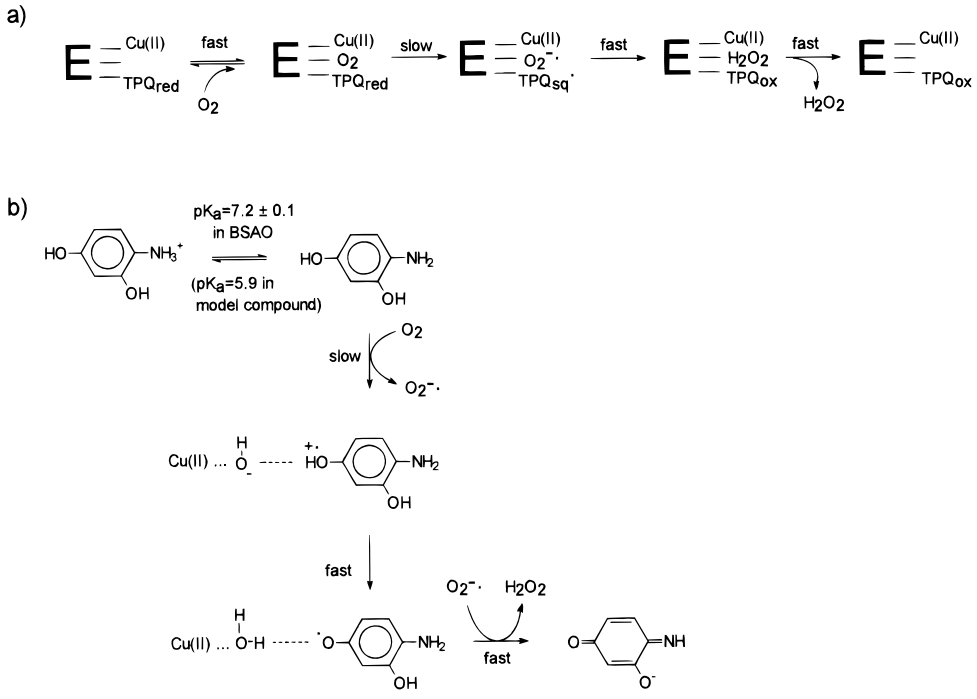
3a, and, as a result, protonation of the phenoxyl oxygen atom occurs with a relatively high pK_a. Conversely, the zwitterionic character of the neutral radical also facilitates deprotonation of the amino ($-NH_2$) group with an abnormally low pK_a of 14.5. This is comparable with pK_a values of 13.2 for the deprotonation of the isoelectronic *p*-phenylenediamine radical cation[47] and 11.1 for the *N*-acetyl-4-aminophenoxyl radical,[48] but much lower than the pK_a of ~35 for deprotonation of the neutral ammonia molecule.[49]

The lower pK_a of 3.4 for the radical from 4-AR observed in the pulse radiolysis experiments is therefore identified with pK_1 in Scheme 3b and represents deprotonation of the radical cation at the phenoxyl oxygen atom, leading to the overall neutral radical with zwitterionic character. The next experimentally identified ionization with a pK_a of 6.4 cannot be that of the amino group since this would be expected to occur at pH ~ 13−14. Instead, it is the deprotonation step shown with pK_2 in Scheme 3b and leads to the radical monoanion. It is expected from the above discussion that pK_3 in Scheme 3b would be ~14. We have not attempted to observe this deprotonation step due to experimental difficulties associated with autoxidation of 4-AR at pH > 8.5. The enzyme radical is identified by these results as the neutral (zwitterionic) iminosemiquinone radical. This supports our previous analysis of the resonance Raman spectrum of the PSAO radical site[22] in that the 5-hydroxy group in the cofactor iminosemiquinone free radical is not deprotonated in the stable form of the enzyme radical that is observed after substrate addition to anaerobic solutions. This conclusion conflicts somewhat with evidence from electron spin-echo envelope modulation spectroscopy of the enzyme radical. This indicates only a single proton bound to the substrate-derived nitrogen atom and that the two oxygen atoms are deprotonated,[20] implying that the enzyme radical is the dianion (i.e., the final radical species shown after pK_3 in Scheme 3b). In view of the discussion above, this appears unlikely. The presence of cyanide in these experiments, which substantially influences the aminoquinol−iminosemiquinone equilibrium, may perturb the pK_a values in the environment of the amine oxidase active site, but we are not able to comment further.

Amine oxidases have optimal activity at slightly alkaline pH due to a requirement for ionization of Asp-383 in BSAO[3] with an abnormally high pK_a of 8.0. However, our previous steady state experiments with PSAO revealed the spectrum shown in Figure 7A as the only radical intermediate observable between pH 5 and pH 9, although the TR^3 results show that the model aminoresorcinyl radical undergoes further deprotonation with a pK_a of 6.4. This might suggest either that the pK_a values in model radical and the enzyme radical are substantially different and that the disproportionation reaction between Cu^{2+} and the fully reduced TOPA quinone is unfavorable when leading to the monoanion form of the radical, or that the monoanionic form of the radical is unstable in the enzyme environment. If the Cu^{+}−iminosemiquinone form of the reduced enzyme lies on the enzymic pathway, then the radical concentration is unlikely to be rate-limiting since its formation from aminoquinol is fast.[50] Additionally, the oxidation of the reduced enzyme, with a second-order rate constant[51] of 2×10^7 dm^3 mol^{-1} s^{-1}, has a pseudo-first-order rate of 4×10^3 s^{-1} at atmospheric oxygen concentration that is much greater than the turnover number.

5. Protonation States of the 4-AR Radical and the Mechanism of Copper Amine Oxidases. The alternative mechanism proposed recently for copper amine oxidases by Su and Klinman,[27] and supported by the results of experiments in which Cu^{2+} is replaced by Co^{2+},[25,26] outlines a scheme in which the Cu^{+}−iminosemiquinone state is not an intermediate in the catalytic pathway. The first role of the cupric ion is suggested to be in stabilizing intermediate formation of a superoxide radical anion generated from one-electron oxidation of the fully reduced aminoquinol. Additionally, the cupric ion supports a network of hydrogen-bonded water molecules whose function is to mediate proton transfers associated with the electron-transfer reactions. However, this alternative mechanism still provides a role for the iminosemiquinone radical as an intermediate. An important conclusion arising from their experimental results is that the rate-limiting step of the oxidative half-reaction is electron transfer to oxygen-forming superoxide as shown in Scheme 4a (adapted from ref 27) based on superoxide remaining at its original hydrophobic binding site and not moving to Cu(II). We propose a more detailed view of this mechanism as presented in Scheme 4b based on our description above of the ionization state of the iminosemiquinone radical in the enzyme. The results of our pulse radiolysis experiments with $Br_2^{\bullet-}$ as oxidant show that proton loss increases the oxidizability of the aminoquinol and are in accord with the assignment of one of the controlling ionizations (that with a pK_a of 7.2) to the deprotonation of the cationic amino group of the fully reduced cofactor. One-electron oxidation of the neutral aminoquinol will lead immediately to the radical cation and superoxide. Deprotonation of this species (with a pK_a of 3.4 in the model radical from 4-AR) would be fast, especially if facilitated by an adjacent base group. Su and Klinman[27] have suggested that the second group controlling the pH dependence of the oxidative half reaction with a pK_a of 6.2 might be a hydroxide bound to the cupric ion. One such water molecule, identified in the structure of the copper amine oxidase from *Hansenula polymorpha*,[9] appears adjacent to O-2 of the reduced TPQ and would be well suited to act in this fashion. Similarly, the crystal structure of the fully reduced from of *E. coli* copper amine oxidase shows a water ligand to the cupric ion which is also hydrogen bonded to O-2 of the aminoquinol.[52] Loss of the proton from the radical cation would generate the neutral iminosemiquinone radical, which is the protonation state identified by the TR^3 studies. The penultimate step of this part of the oxidative half-reaction is oxidation of the iminosemiquinone radical by superoxide. It is immediately clear from the mech-

SCHEME 4: (a) Outline of the Pathway for the Oxidative Half-Reaction of Copper Amine Oxidases Adapted from Ref 27 and (b) a More Detailed Scheme Based on Protonation States of the Cofactor Radical Derived from the Present Work



anism proposed in Scheme 4b that both protons could be provided by the neutral iminosemiquinone radical, and that there would be no requirement for additional proton transfer from water molecules at this stage. The other product is the iminoquinone in which O-4 is envisaged as being deprotonated at pH 7 by analogy with TPQ.[28]

The rate of reaction of the 4-AR radicals with oxygen will increase progressively at each deprotonation step, with the radical anion more reactive than the neutral zwitterionic radical. Oxidation of the radical anion might be expected to be close to the diffusion-controlled limit (ca. 10^9-10^{10} dm^3 mol^{-1} s^{-1}), so that the measured second-order rate constant for the second step of the enzyme mechanism (ca. 10^7 dm^3 mol^{-1} s^{-1}) easily accommodates the lower reactivity of the neutral radical. We have so far not been able to measure the reaction rate of oxygen with the radical species by pulse radiolysis. Measurements in which the neutral radical is generated by oxidation of 4-AR are made difficult by the rapid autoxidation of 4-AR in other than quite reasonably acidic aerobic solutions (i.e., pH < 3). The alternative approach using one-electron reduction of the iminoquinone is also likely to be unsuccessful due to the rapid hydrolysis of the iminquinone in aqueous solution.

Conclusions

Pulse radiolysis and TR^3 studies support the identification of pK_a values of 3.4 and 6.4 for the radical formed by one-electron oxidation of 4-AR. The upper pK_a value is reflected by a relatively small change in the absorption spectra determined from pulse radiolysis, but resonance Raman spectroscopy clearly identifies two different radical species at pH values immediately below and above this pK_a value. Taking the 4-aminoresorcinyl radicals as possible models for the TOPA-derived free radical intermediate in the enzyme cycle of copper amine oxidases, the enzyme radical is identified as being in the singly deprotonated state. Accordingly, the oxidative half-reaction of copper amine oxidases appears simplified in terms of requiring transfer of fewer protons.

Acknowledgment. The studentship to S.A.J. and access to The Lasers for Science Facility (Rutherford Appleton Laboratory) were provided by the Biological and Biotechnology Research Council (BBSRC). Pulse radiolysis experiments were performed at the Paterson Institute for Cancer Research Free Radical Research Facility, Manchester, UK, with the support of the European Commission through the Access to Large-Scale Facilities activity of the TMR program.

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