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TIME-RESOLVED RESONANCE RAMAN STUDIES OF RADICALS FROM 4-AMINORESORCINOL AS MODELS FOR THE ACTIVE SITE RADICAL INTERMEDIATE IN COPPER AMINE OXIDASES

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Radicals formed by one-electron oxidation of 4-aminoresorcinol have been studied as models for the active site free radical intermediate which forms as a stable product during anaerobic incubation of amine oxidases with amine substrates. Pulse radiolysis shows that the radical undergoes ionisations with pK_a's of 3.4 and 6.4. Although the two deprotonated forms are difficult to distinguish by absorption spectroscopy, they have clearly different resonance Raman spectra. Comparison with the resonance Raman spectrum of the enzyme intermediate shows it to be the singly deprotonated form of the radical.

Keywords: Resonance Raman; enzyme; amine oxidase; free radical; aminoresorcinol; spectroscopy

The trihydroxyphenylalanine (TOPA) quinone residue at the catalytic site of copper amine oxidases is a recently discovered enzyme cofactor formed from post-translational modification of a tyrosine residue [1, 2]. The copper amine oxidases belong to the group of protein–radical

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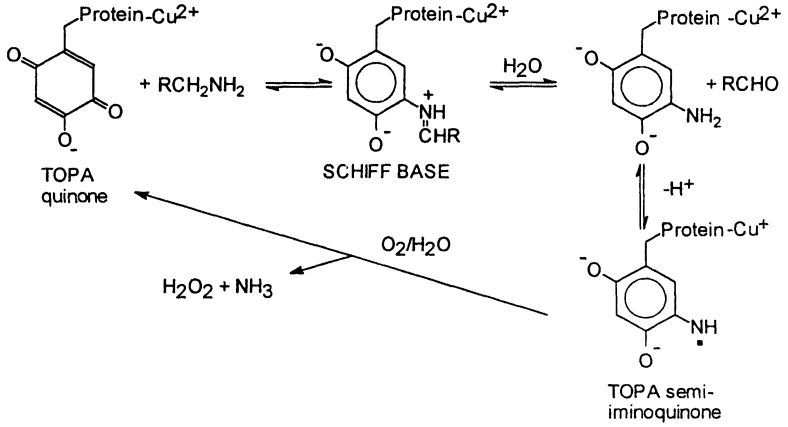
enzymes in which the catalytic mechanisms each involve a free radical site in the protein [3]. In the case of copper amine oxidases the TOPA quinone residue is reduced by substrate to a free radical, believed to be a TOPA-derived semi-iminoquinone [4, 5] whilst a cupric ion, serving as a second redox site, is simultaneously reduced to Cu(I) [6] as shown in Scheme 1 (adapted from Ref. [10]). In the X-ray crystal structures



SCHEME 1

obtained recently for E. coli [7] and pea seedling [8] copper amine oxidases the TOPA quinone site is within about 6 Å, but is not a ligand, of the copper atom.

Several previous studies [6, 9, 10] have reported the formation of a stable absorption with λ_{max} ca. 464 nm upon anaerobic incubation of copper amine oxidases with amine substrate. This rapidly disappears upon admission of oxygen with a second order constant [11] of $2.5 \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. The resonance Raman spectrum of this intermediate has been measured using both ^{14}N- and ^{15}N-containing substrates [5, 12]. Small changes in the frequencies of some modes indicate incorporation of the nitrogen atom from substrate into the intermediate and identify it as a semi-iminoquinone. The formation of the enzyme radical and cuprous atom appears to be a mechanism to activate reduction of dioxygen [13]. The TOPA-semi-iminoquinone/Cu(I) intermediate is in equilibrium with the fully reduced aminoquinol/Cu(II) form [6].

4-Aminoresorcinol (4-AR) therefore serves as a model for the two-electron reduced form of the TOPA quinone in the enzyme. Ground state pK_a's of 6-amino-4-ethylresorcinol have been determined [14], showing the 4-hydroxyl group of TOPA quinone to be ionised in active copper amine oxidase.

Free radicals which might serve as models for the protein–radical site in copper amine oxidases have been generated by one-electron oxidation of 4-AR in both pulse radiolysis and time-resolved resonance Raman (TR^3) experiments. In the pulse radiolysis experiments either $Br_2^{\cdot-}$ or $N_3^{\cdot}$ radicals were used as one-electron oxidants. Transient absorption spectra of 4-AR radicals obtained between pH 1.9 and pH 7.8 are shown in Figure 1. At pH 1.9 the long wavelength absorption maximum is at 430 nm, whereas at the two

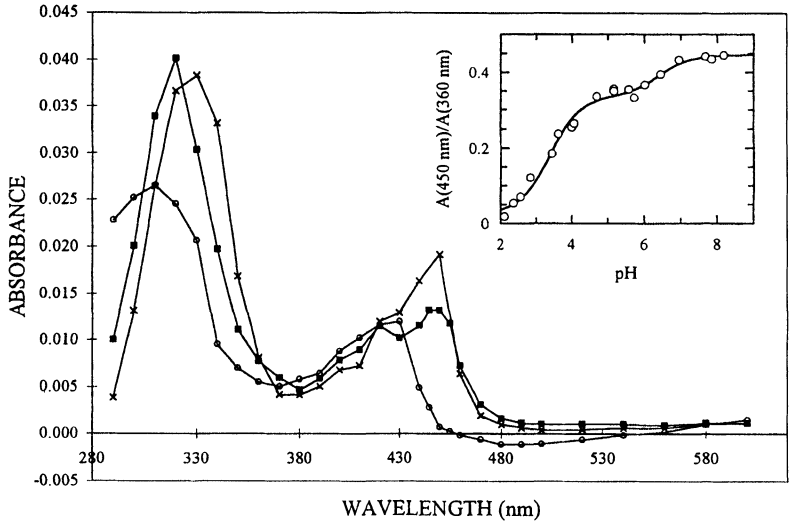


FIGURE 1 Transient spectra generated from one-electron oxidation of 4-aminoresorcinol (4-AR) by pulse radiolysis. Spectra were obtained in N_2O-saturated aqueous solutions at (a) pH 1.9 in solutions containing 4-AR (2 mM) and KBr (0.1 M), measured 65 μs after the pulse (O—O); (b) pH 6.0 in solutions containing 4-AR (0.25 mM) and NaN_3 (0.1 M), measured 20 μs after the pulse (■—■); and (c) pH 7.7 in solutions containing 4-AR (0.1 mM) and NaN_3 (0.1 M), measured 20 μs after the pulse (X—X). N_2O was used to convert the hydrated electron to hydroxyl radical, which was then scavenged by the Br^- or N_3^-. INSET:– effect of pH on the transient absorbance of 4-AR radicals at 450 nm, formed by oxidation with $Br_2^{\cdot-}$, normalised to the $Br_2^{\cdot-}$ radical absorbance at 360 nm immediately after the pulse.

higher pH values this shifts to 450 nm. At pH 6.0 and 7.8 the spectra are somewhat similar, although the extinction coefficient is slightly increased at the higher pH with a value of approximately $5 \times 10^3 M^{-1} cm^{-1}$. A plot of transient absorbance at 450 nm as a function of pH is shown as the inset to Figure 1. The data are best fit to a double pK curve with pK_a values of 3.37 ± 0.09 and 6.39 ± 0.25, suggesting that within the pH range 2–8 the radical exists in three differently protonated forms. Both of the two deprotonated forms have absorption spectra which are similar, but not identical, to that of the amine oxidase free radical. All three aminoresorcinol radicals are found to be unstable in the pulse radiolysis experiment and decay on a millisecond timescale.

In the TR^3 experiments, radicals from oxidation of 4-AR were generated using triplet duroquinone (^3DQ) which we have previously found useful in the one-electron oxidation of phenols [15, 16]. Using pump and probe wavelengths of 355 and 450 nm respectively, the TR^3 spectra shown in Figure 2A were measured at pH 8.0. At this probe wavelength the deprotonated 4-AR radicals, the durosemiquinone ($DQ^{-\cdot}$) radical anion and ^3DQ are all in resonance. With the shortest time delay between pump and probe pulses (20 ns) the spectrum is predominantly that of ^3DQ with bands at 1546, 1173 and 447 cm^{-1} [15]. With increasing time delays between pump and probe pulses, this species decays by reaction with 4-AR and the spectrum becomes dominated by the 1612 cm^{-1} band (C=C stretch, ν_{8a}) of the durosemiquinone radical anion [15]. However, it is seen that this band appears to overlay additional features at both the high and low frequency edges. Spectral subtraction of the component due to the durosemiquinone radical anion, obtained by reduction ^3DQ by ascorbate and also shown in Figure 2A, reveals the growing spectrum of the 4-aminoresorcinol radical anion with increasing pump-probe delay as illustrated in Figure 2B. This doubly deprotonated radical anion present at pH > 6.4 has a resonance Raman spectrum with dominant bands at 1581 and 1628 cm^{-1}. Following previous assignments [17] these are likely to be C—O/C—N (ν_{7a}) and C=C (ν_{8a}) stretching modes respectively. An additional feature is observed at 1140 cm^{-1} which may be assigned to a C—H bend (ν_{9a}). The low frequency mode at 529 cm^{-1} is assigned to the — CCC —bend (ν_{6a}).

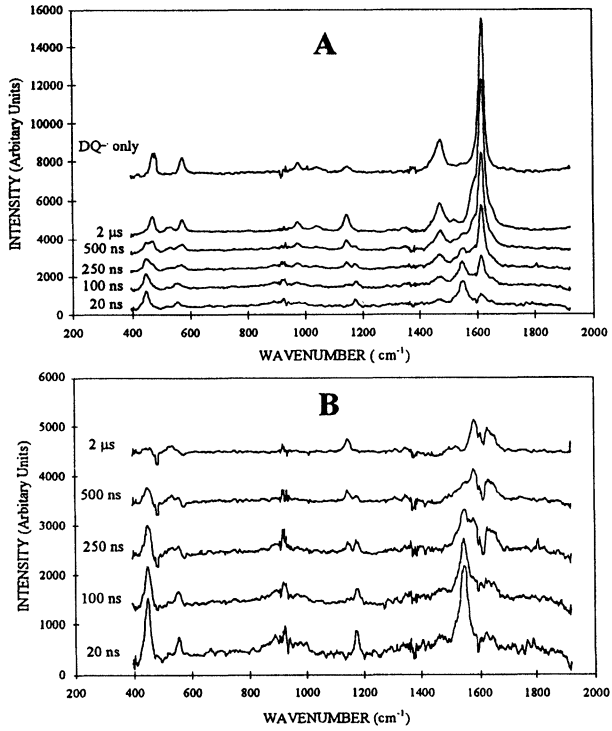


FIGURE 2 Time-resolved resonance Raman spectra obtained from solutions (H_2O/acetonitrile 50/50 v/v) of 4-AR (125 μM) and duroquinone (1 mM) buffered to pH 8.0 with phosphate (50 mM). Spectra were obtained at the indicated intervals between pump pulse (355 nm) and probe pulse (450 nm). Panel A shows spectra obtained after subtraction of the probe-only spectra. The spectrum marked "$DQ^{-\cdot}$ only" was measured in a solution containing duroquinone (1 mM) and ascorbate (1 mM) also at pH 8.0. Panel B shows the same spectra after subtraction of the durosemiquinone radical anion ($DQ^{-\cdot}$) component using the spectrum shown in panel A.

In a similar manner TR[3] spectra were obtained for 4-AR radicals at pH 1.2 and pH 5. The 4-AR radical spectra (after subtraction of appropriate durosemiquinone spectra) at all three pH values are shown in Figure 3, together with the resonance Raman spectrum of the pea seedling amine oxidase free radical, obtained by anaerobic incubation of the enzyme with benzylamine. At pH 1.2 the resonance Raman spectrum of the 4-AR radical has a dominant peak in the C=C stretching region at 1629 cm^{-1} which was shown by band

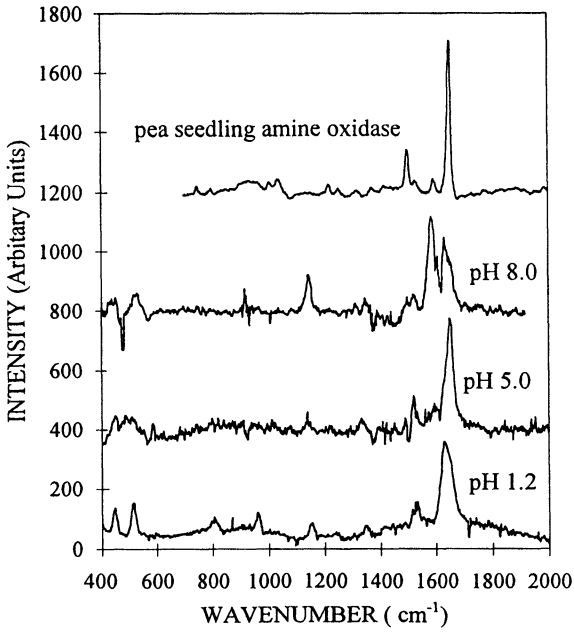


FIGURE 3 Resonance Raman spectra of 4-AR radicals measured at pH 1.2, pH 5.0 and pH 8.0, compared with the spectrum obtained from the radical site in pea seedling amine oxidase after substrate addition under anaerobic conditions.

analysis to contain components at 1627 and 1645 cm^{-1} in an intensity ratio of approximately 1 : 4. At pH 5 the peak in the C=C stretching region shifts to 1648 cm^{-1}, which is very close to our previous determination of 1647 cm^{-1} for the C=C stretching mode in the copper amine oxidase radical [12].

The pH dependent changes in transient absorption spectra and resonance Raman spectra observed here for the 4-AR radicals are somewhat similar to those observed in radicals formed by one-electron oxidation of 1,2,4-benzenetriol [18] for which the singly and doubly deprotonated forms were virtually indistinguishable by absorption spectroscopy, but which were clearly discriminated between by resonance Raman spectroscopy.

Resonance Raman spectroscopy therefore confirms that there are three differently protonated forms of the 4-AR radical in the range pH 1–8, supporting the identification of two pK_a's in the titration

curve obtained in the pulse radiolysis experiments. The resonance Raman spectra obtained for each protonated form of the 4-AR radicals show that the singly deprotonated form is that which occurs as an intermediate in the catalytic cycle of copper amine oxidases.

Acknowledgements

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