Identification by Electron Spin Resonance of Free Radicals Formed during the Oxidation of 4-Hydroxyanisole Catalyzed by Tyrosinase*

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We have observed the formation of free radicals during the oxidation of the melanocytotoxic agent 4-hydroxyanisole with the enzyme tyrosinase as a catalyst. The first free radical to form is identified as the 4-methoxy-1,2-benzoquinone radical anion. The peak concentration of this radical increases with tyrosinase concentration; a minimum concentration of 50 µg/ml of tyrosinase was needed to observe this radical. The peak concentration of this radical is independent of 4-hydroxyanisole concentration. This radical is produced by reverse dismutation of the primary product, 4-methoxy-1,2-benzoquinone and 4-methoxycatechol produced indirectly.

The depigmenting agent 4-hydroxyanisole (4-methoxyphenol, 1) was studied by Riley (1) and found to be oxidized by the enzyme tyrosinase to cytotoxic products (1–3). There is interest in the possible therapeutic application of melanocytotoxic chemicals for the treatment of malignant melanoma. 4-Hydroxyanisole has been found to cause regression of Harding-Passey melanoma tumors transplanted into mice (4). In preliminary clinical trials (5), 4-OHA administered intra-arterially caused regression of localized malignant melanomas.

The cytotoxic products of the oxidation of 4-OHA in the presence of tyrosinase were not isolated by Riley, but he (2) postulated and presented evidence that there is a toxic intermediate which is a free radical. He observed that several free radical scavengers such as ascorbate (2, 3) reduced 4-OHAn-mediated damage to melanocytes in cell cultures. He observed an increase in a melanin-like ESR signal from skin biopsies of guinea pigs that had had topical application of 4-OHA. More recent work with Harding-Passey melanoma cell lines has shown that the greatest toxicity occurs when cells are placed in culture media that had been incubated in tyrosinase and 4-OHA for 12 min and that for longer incubation times the cell killing is minimal (6, p. 94). The relationship between the reactivity (and hence observability) and toxicity of free radicals is complex. Very stable radicals are more readily observed but the low reactivity implied by their stability makes it less likely that they are toxic. Very reactive free radicals have the potential to damage many cell components but their reactivity limits the distance they will diffuse and therefore their toxicity is affected by the site of their production. The determination of the biological significance of free radicals therefore can best be achieved by understanding the detailed reactions involved, including the reactivity of the various components. With such knowledge we may be able to maximize the selective toxicity of reactions that produce free radicals and other reactive species. The aim of the present study is to elucidate some of the free radical chemistry involved in the metabolism of a compound known to lead to selective biological damage which may occur via reactions of a free radical intermediate. This paper describes experiments which determine if and under what conditions free radical intermediates will form during the oxidation of 4-OHA catalyzed by tyrosinase.

EXPERIMENTAL PROCEDURES

4-Hydroxyanisole (4-methoxyphenol, 1, Aldrich) was recrystallized from xylene and stored in a vacuum desiccator prior to use. The unpurified 4-OHA appears to contain p-hydroquinone as an impurity, as we were sometimes able to detect its semiquinone ESR signal at high pH. Mushroom tyrosinase (monophenol monoxygenase, EC 1.14.18.1, 2000 units/mg) and horseradish peroxidase (d-norehydrogen peroxidase oxido-reductase, EC 1.11.1.7, 105 units/mg) were obtained from Sigma. 4-Methoxy-1,2-benzoquinone, 2, was synthesized according to the method of Teuber and Staiger (7) starting with 4-OHA and potassium nitrosodisulfonate (Fremy's salt). Quinone 2 was recrystallized (bright red crystals) from CHCl3 and stored in a vacuum desiccator prior to use. Because quinone 2 readily decomposes, it was used within a week after synthesis. Solutions of tyrosinase and 4-OHA were made in 0.1 M phosphate buffer solutions of appropriate pH and were used within 12 h. Solutions of 4-methoxy-1,2-benzoquinone were prepared in nitrogen-saturated phosphate buffer and used within 1 h. When reactions were studied in the presence of Zn(SO4), tris(hydroxymethyl)aminomethane buffer solutions were used instead of phosphate solutions due to the low solubility of zinc phosphate.

ESR measurements were carried out on a Bruker ER 200D spectrometer using 100-kHz modulation for signal detection. The magnetic field was calibrated with an NMR Gaussmeter. We determined...
g-factors using Fremy's salt as a standard ($g = 2.0057(8)$) and determined spin concentrations by comparison with a solution of Fremy's salt of known concentration.

A flow system was used to observe transient free radicals. Reactants were fed with syringes for stopped flow and with a gravity feed for continuous flow. The reactants were mixed with a home-built plexiglass mixer and then directed into a quartz flat cell in a Varian E-238 X-band TM10 cavity. A Wilmad WG-804 combination mixing chamber and flat cell was used to search for radicals with very short lifetimes.

Experiments were performed at room temperature (20 °C). A modulation amplitude between 0.125 and 0.2 G and a nominal microwave power of 0.8 milliwatt were used; these instrument settings were found to give the best compromise for sensitivity versus modulation broadening and power saturation for the radicals which we observed in solution.

RESULTS

A weak symmetric ESR spectrum (Fig. 1A) with a minimum of 17 lines at $g = 2.0045$ is observed when an oxygenated solution of the enzyme tyrosinase (0.5 mg/ml) is mixed with an equal volume of a solution of 4-OHA at pH 7.7. There is a lag time between 30 s and 15 min before the ESR signal is observed. The ESR signal rapidly reaches a maximum after its appearance and then decays to an undetectable level within the next 15 min (see Fig. 2). The ESR spectrum becomes slightly asymmetric as it decays, indicating the formation of a second radical. The spectrum of a fairly stable radical is then seen. This spectrum consists of a single symmetric line at $g = 2.0044$ with a width of 0.2 G.

No ESR signal is seen when L-tyrosine is used as a substrate under similar reaction conditions. No ESR-detectable autooxidation of 4-OHA is observed, even under strong alkaline conditions. No free radical is seen when tyrosinase and 4-OHA solutions are saturated with nitrogen before mixing. The peak intensity of the ESR signal increases when the concentration of oxygen present initially in the reaction solutions is increased, although the rate of decay of the signal is slower for lower initial concentrations of oxygen.

The final product of the reaction of 4-OHA with tyrosinase is a black polymeric material. This material was collected, washed, and dialyzed at pH 7 to remove any unreacted material. The purified material gives an ESR signal at $g = 2.0034$ with a peak-to-peak line width of 4.5 G. This ESR signal increases as the pH is increased, in the presence of Zn(SO$_4$)$_2$, and upon irradiation with light. The signal is stable when the material is heated to 90 °C and decreases in the presence of paramagnetic ions such as dysprosium. These results are similar to those observed for natural and synthetic melanin (9). Thus, the final product for the reaction of 4-OHA and tyrosinase should be an analog of tyrosine-based melanin.

We assign the 17-line spectrum to a radical with three equivalent protons which have a hyperfine splitting of 1.15 G and 3 additional nonequivalent protons which have splittings of 4.19, 1.30, and 0.61 G. A computer simulation$^2$ using these values is shown in Fig. 1C. This spectrum consists of 32 nondegenerate transitions, but only 17 peaks are resolved when the radical is formed from 4-OHA since some of the nearly degenerate transitions overlap. No radical has been reported in the literature with these hyperfine splittings. The splitting of 1.15 G for the 3 equivalent protons is characteristic of methoxy-substituted semiquinones (10) and the most likely assignment for this radical would be the 4-methoxy-1,2-benzoquinone radical anion, $^3$.

The splitting constants for this radical have been reported by Dixon et al. (11). They report a methoxy proton splitting of 1.1 G and splittings for

$^2$ Computer program written by M. J. N. Program simulates isotropic spectra with input for four different nuclei with any spin and any number of equivalent nuclei.

$^3$
protons at positions 3, 5, and 6 to be $a_3 = 0.75$ G, $a_5 = 3.7$ G, and $a_6 = 0.0$ G, respectively, but they do not show a spectrum. While our hyperfine splittings are close to those of Dixon et al., the differences are too large to be attributable to errors in measurement. For four-substituted $o$-benzosemiquinones, one would expect a splitting larger than 3.6 G for the proton at position 5 and this splitting should increase with the electron-donating ability of the substituent at position 4 (11). For 4-methyl-1,2-benzosemiquinone and 4-hydroxy-1,2-benzosemiquinone, this splitting is 3.80 G and 5.98 G, respectively (11). One would expect $a_5$ for semiquinone 3 to have a value between these and our observed value of 4.19 G seems more in line with this expectation.

To establish the correct identity of the radical formed from 4-OHA and tyrosinase, we generated semiquinone 3 by flowing a deaerated solution of quinone 2 against an oxygenated pH 10 buffer solution. The radical produced by dilute alkali should be the simple semiquinone and not a secondary radical which is seen under conditions of strong alkali or when most of the quinone has been consumed (12–14). The ESR spectrum of the radical produced from quinone 2 is shown in Fig. 1B and is identical with that formed from 4-OHA and tyrosinase. Thus, we assign both of these spectra to semiquinone 3 with $a_3 = 1.30$ G, $a_4(OCH_3) = 1.15$ G, $a_5 = 4.19$ G, and $a_6 = 0.61$ G. When quinone 2 is flowed against an oxygenated pH 14 buffer, the line width of semiquinone 3 is smaller than that at pH 10, resulting in a better resolved spectrum (Fig. 3). Only 29 of the 32 lines are seen since several transitions near the center of the spectrum still overlap. The extra peaks near the center part of the spectrum (indicated by arrows) are due to (a) secondary radical(s). When the flow of quinone 2 against a pH 10 buffer is stopped, the ESR signal of semiquinone 3 decays and the signal of the secondary radical increases and then also decays. The secondary radical ESR signal is always obscured by the presence of the signal of semiquinone 3 but is shown more clearly in Fig. 4 when this interference is at a minimum. The appearance of a secondary radical is due to the conversion of the quinone of the primary radical to a new hydroquinone and the resultant secondary radical from this

![Fig. 4. X-band (9.5 GHz) spectrum of a secondary radical produced during the oxidation of 4-methoxy-1,2-benzoquinone. Upper spectrum was recorded 1 min after stopping the flow of a solution of 4-methoxy-1,2-benzoquinone against a pH 10 buffer solution at 20 °C. Lower spectrum is a computer simulation for a system consisting of two inequivalent protons with hyperfine splittings of 0.55 G and 1.0 G and three equivalent protons with a hyperfine splitting of 1.15 G. A Lorentzian line shape with a peak-to-peak line width of 0.085 G.](image)

![Fig. 3. X-band (9.5 GHz) ESR spectrum of the 4-methoxy-1,2-benzoquinone radical anion produced by flowing a solution of 4-methoxy-1,2-benzoquinone against a pH 14 buffer solution at 20 °C. The arrows point to features due to a secondary radical.](image)
new hydroquinone dominates only when the concentrations of the quinone and hydroquinone of the primary radical have greatly diminished. This second radical, which has a \( g = 2.0045 \), can be assigned to a species with 3 equivalent protons having splittings of 1.15 G and having two other nonequivalent protons having splittings of 1.0 G and 0.55 G. The computer simulated spectrum using these parameters is shown in Fig. 4. This radical could be assigned to the 2-hydroxy-5-methoxy-1,4-benzosemiquinone radical anion, 4, based on analogy with other four-substituted o-benzoquinones (13). One could also assign the splittings to a dimeric radical in which a phenolic group is coupled to semiquinone 3 at the 5-position, semiquinone 5 (12, 14). The marked asymmetry in the experimental spectrum indicates the presence of semiquinone 3 or another secondary radical.

When quinone 2 is oxidized in the presence of Zn\(^{2+}\) ions, a spectrum which is different and much stronger than that of semiquinone 3 is observed as shown in Fig. 5. This spectrum can be assigned to the Zn\(^{2+}\)-complexed 4-methoxy-1,2-benzoquinone radical anion with the following hyperfine splittings: \( a_o = 0.97 \) G, \( a_o (OCH_3) = 1.35 \) G, \( a_5 = 4.51 \) G, and \( a_5 = 0.75 \) G. The increases in proton splittings for positions 4 and 5 and the decreases for positions 3 and 6 (proton splitting at position 6 is predicted to be negative (11)) are similar to changes observed with other Zn\(^{2+}\)-complexed four-substituted o-benzoquinones (15). The observation of this Zn\(^{2+}\)-complexed radical lends further support to our assignment of semiquinone 3. This Zn\(^{2+}\)-complexed radical is also seen instead of semiquinone 3 when 4-OHA is reacted with tyrosinase in the presence of Zn(SO\(_4\)).

No ESR signal could be observed corresponding to that (11, 16) of the 4-methoxyphenoxo radical anion, even at very short times after mixing of tyrosinase and 4-OHA. This phenoxy radical is readily observed (Fig. 6) when horseradish peroxidase in the presence of H\(_2\)O\(_2\) is used instead of tyrosinase. Unlike the oxidation of 4-OHA with tyrosinase, the final product of this reaction is a yellow-green water-insoluble material. The radical signal appears immediately and decays to an undetectable level within 20 s (pH 7.7), as expected for a direct 1-electron oxidation. The hyperfine splitting for the three methoxy protons is 5.00 G and for the two ortho protons it is 2.05 G; the hyperfine splitting for the two para protons is too small to observe; the \( g \)-factor is 2.0046.

As mentioned above and shown in Fig. 1, there is a lag time before semiquinone 3 is formed from 4-OHA and tyrosinase. This lag time decreases and the peak concentration of free radical increases when the concentration of enzyme or the pH is increased. The lag time increases as the 4-OHA concentration is decreased from 40 mM to 10 mM and then decreases as the concentration is lowered from 10 mM to 1 mM. Near concentrations of 1 mM, the lag time becomes irreproducible and sometimes the radical never formed. The radical never formed below 1 mM concentration of 4-OHA. Providing the radical forms, its peak concentration is independent of 4-OHA concentration. For a tyrosinase concentration of 0.25 mg/ml (5 \( \times 10^{11} \) \( \mu \)M units) and a pH of 7.7, the peak steady state concentration of semiquinone 3 is about 0.5 \( \mu \)M. The ESR signal of the radical is too weak to see at enzyme concentrations less than 0.05 mg/ml (pH 7.7).

When quinone 2 is oxidized, there is no lag time before semiquinone 3 is observed and the formation of semiquinone 3 could be observed as low as pH 7.7. When quinone 2 is oxidized at pH 7.7 and tyrosinase is added to the aerated buffer solution, the semiquinone signal decreases and is undetectable at tyrosinase concentrations of 0.5 mg/ml.

A lag time has been observed for the reaction of tyrosine with tyrosinase (17, 18). This lag time decreases with increasing enzyme concentration or when an ortho-diphenol is added. The same observations are noted for the lag time in the formation of semiquinone 3. However, the lag time for the oxidation of tyrosine increases with increasing concentrations of tyrosine, whereas the lag time for the formation of semiquinone 3 is not a monotonic function of 4-OHA. We have performed preliminary experiments with 4-OHA in parallel reactions one of which was monitored by ESR and the other by observation of the first optical product at 264 nm (6). The first optical product is seen before the appearance of the semiquinone ESR signal and the appearance of this signal roughly corresponds to a deviation from the normal sigmoid kinetic curve seen for the oxidation of phenols using tyrosinase (17). The deviation of formation of a plateau is associated with the inactivation of the enzyme (6, 19). This observation,
the inability to observe the radical at 4-OHA concentrations below 1 mM, and inhibition of formation of semiquinone 3 directly from quinone 2 when tyrosinase is present leads to the hypothesis that free radicals are produced in a secondary reaction and are detectable only when an initial reaction involving tyrosinase is saturated or a free radical scavenger is consumed.

**DISCUSSION**

The enzyme tyrosinase is generally believed to have two inter-related functions, the ortho-hydroxylation of phenols (cresolase) and the oxidation of ortho-diphenols to their corresponding quinones (catecholase). These processes are believed to be 2-electron transfers, with no direct formation of free radicals, in contrast to peroxidases (19, 20). Although the reaction of catechol with tyrosinase produces free radicals (21), this is believed to be due to reverse dismutation of catechol and its quinone rather than the direct production of radicals via 1-electron oxidation. In the case where tyrosine is the initial substrate, the oxidation of tyrosine is coupled stoichiometrically to the oxidation of dopa to dopaquinone (19). Thus, dopaquinone is the first product of tyrosine oxidation and there is no direct formation of dopa. Reverse dismutation of dopa and dopaquinone to give the semiquinone would not occur after the initial oxidation of tyrosine. In the case of 4-OHA, one would expect the first intermediate product to be quinone 2 (Equation 1) with no direct formation of free radicals.

$$\begin{align*}
\text{OCH}_3 & + \text{O}_2 \xrightarrow{\text{tyrosinase}} \text{OCH}_3 & \quad \text{OCH}_3
\end{align*}$$

Four-substituted o-benzoquinones should readily undergo nucleophilic attack by OH~ at the 5-position. The methoxy group of quinone 2 would lead to resonance stabilization of the expected intermediate for conjugate addition (Equation 2). This reaction would produce 2,4,5-trihydroxyanisole, 6, which can act as a reducing agent. Triol 6 will equilibrate with quinone 2 to give 2-hydroxy-5-methoxy-1,4-benzoquinone, 7, and 4-methoxycatechol, 8, (Equation 3). This equilibrium will be shifted far to the right because the reduction potential for the hydroxyquinone 7 is expected to be smaller than that for the simple quinone 2 (e.g. \(E_v = +0.09\) V for topa versus \(E_v = +0.30\) V for dopa (22)). Quinone 7 should be the first stable product formed and is believed (6) to be the first optical product, which is a yellow chromophore. The steady state concentration of quinone 2, which is a red chromophore, is too small to be detected optically.

The methoxycatechol 8 produced as shown in Equation 3 can also undergo autooxidation to give its quinone, 2, with the possible formation of the semiquinone, 3. Methoxycatechol 8 can also undergo reverse dismutation (Equation 4) with quinone 2 which is continually being produced by the enzyme (Equation 1) to give semiquinone 3. Since the equilibrium for Equation 4 lies significantly to the left at pH 7.7 (for example, the semiquinone formation constant for 2-methoxy-1,4-benzoquinone is calculated to be \(5.8 \times 10^{-7}\) using the parameters and Equation 11 in reference (23)), both methoxycatechol 8 and quinone 2 must build up to significant concentrations in order to see the ESR signal of the semi-
none. The steady state concentration of semiquinone 3 will be proportional to the square root of the product of the concentration of catechol 8 and quinone 2 (equilibrium constant for Equation 4 is equal to [3]²/[8]·[2]) and the decay of the signal as shown in Fig. 2 is due to the disappearance of catechol 8 and/or quinone 2.

The above reaction scheme explains how a semiquinone could be produced with an enzyme that carries out 2-electron oxidations. A similar scheme, involving conjugate addition to a quinone, is used (12) to explain the apparent reduction of quinones to semiquinones under oxidizing conditions. Indeed, the formation of semiquinone 3 starting directly from quinone 2 is an example of this. The formation of semiquinone 3 appears to occur fairly easily since the ESR signal of semiquinone 3 can be observed at pH values as low as 7 and the Zn²⁺-stabilized semiquinone signal can be observed at pH values as low as 5 (the stability of the semquinone and the equilibrium constant for Equation 4 decreases with pH).

While the above mechanism accounts for most of our observations, it explains neither the lag period which occurs even though quinone 7 has already formed nor the abrupt cessation (rather than a gradual decrease) of radical formation when the concentration of 4-OHA is lowered even though quinone 7 is still formed. These phenomena could be explained if quinone 7 could be formed without the formation of its hydroquinone, 4-methoxycatechol, or if the methoxycatechol is rapidly consumed. A plausible mechanism for the rapid consumption of methoxycatechol is the enzymatic oxidation of its quinone, 2. Inactivation of the enzyme would then lead to the observed phenomena. Reasoning by analogy from the observation that 4-substituted phenols cause irreversible inactivation of tyrosinase (19, 24), we looked for such irreversible inactivation by 4-OHA but found that after a 90-min incubation of the enzyme (0.1 mg/ml) at pH 7.7 in 2.5 mM 4-OHA there is no measurable inactivation of the enzyme. We did find, however, that a depletion of oxygen does provide a satisfactory means to explain the inactivation of the enzyme reversibly. We found, using a Lazar DO-166 electrode in a 25-ml reaction vessel containing 20 mM 4-OHA and 0.25 mg/ml of tyrosinase (as shown in Fig. 2), that the oxygen decreased from 20 ppm to less than 0.2 ppm within 4 min after mixing. Thus, the appearance of semiquinone 3 is associated with the complete or nearly complete consumption of oxygen. Since the initial concentration of oxygen, 20 ppm, corresponds to 0.7 mM and the stoichiometry of Equation 2 requires 1 mol of O₂ to be consumed per mol of 4-OHA, the lack of formation of semiquinone 3 below 4-OHA concentrations of 1 mM can be explained as follows. Only when there is an excess of 4-OHA versus O₂ will semiquinone 3 form. One might also argue that the lack of formation of semiquinone 3 before the depletion of O₂ might arise from rapid reaction of the semiquinone 3 with O₂. Our observation that semiquinone 3 could be produced from quinone 2 in the presence of O₂ but not in the presence of O₂ and tyrosinase eliminates this possibility.

Activity was measured with 4-OHA as a substrate and absorbance was monitored at 264 nm. One-tenth ml of enzyme solution was added to a cuvette containing 1 ml of 1 mM 4-OHA, 1 ml of pH 6.5 buffer, and 0.9 ml of H₂O.

CONCLUSIONS

We have elucidated some of the steps involved in the reaction of 4-OHA with the enzyme tyrosinase, including the formation of the 4-methoxy-1,2-benzo-semiquinone radical. We are presently working to collect better quantitative kinetic data and working on the development of methods to control and monitor oxygen concentrations inside the ESR flat cell in order to better understand the conditions necessary to form this radical. As mentioned in the introduction, the observation of a free radical does not imply that it is the toxic species. We have not shown that it is produced under true physiological conditions and have not shown that it will cause cell damage. Determination of the biological significance of the methoxysemiquinone radical will require biological data on its effects in normal and melanin-containing cells.

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J. Biol. Chem. 1984, 259:2446-2451.

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