The Oxidation of N-Substituted Aromatic Amines by Horseradish Peroxidase*

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The mechanism of N-dealkylation by peroxidases of the Ca²⁺ indicator quin2 and analogs was investigated and compared with the mechanism of N-dealkylation of some N-methyl-substituted aromatic amines. Nitrogen-centered cation radicals were detected by ESR spectroscopy for all the compounds studied. Further oxidation of the nitrogen-centered cation radicals, however, was dependent upon the structure of the radical formed. In the case of quin2 and analogs, a carbon-centered radical could be detected using the spin trap 5,5-dimethyl-1-pyrroline N-oxide. By using the spin trap 2-methyl-2-nitrosopropane (tert-nitrosobutane), it was determined that the carbon-centered radical was formed due to loss of a carboxylic acid group. This indicated that bond cleavage most likely occurred through a rearrangement reaction. Furthermore, extensive oxygen consumption was detected, which was in agreement with the formation of carbon-centered radicals, as they avidly react with molecular oxygen. Thus, reaction of the carbon-centered radical with oxygen most likely led to the formation of a peroxyl radical. The peroxyl radical decomposed into superoxide that was spin trapped by 5,5-dimethyl-1-pyrroline N-oxide and an unstable iminium cation. The iminium cation would subsequently hydrolyze to the monomethyl amine and formaldehyde. In the case of N-methyl-substituted aromatic amines, carbon-centered radicals were not detected during the peroxidase-catalyzed oxidation of these compounds. Thus, rearrangement of the nitrogen-centered radical did not occur. Furthermore, little or no oxygen consumption was detected, whereas formaldehyde was formed in all cases. These results indicated that the N-methyl-substituted amines were oxidized by a mechanism different from the mechanism found for quin2 and analogs.

In a previous paper, we have shown that the fluorescent Ca²⁺ chelators quin2 and its analogs are susceptible to peroxidase-mediated oxidation (1). Quin2 and its analogs served as reducing cofactors for the hydroperoxidase activity of prostataglandin H synthase, undergoing oxidation in the process. At the same time, archidonic acid metabolism was stimulated. Oxidation of these compounds resulted in a decrease in quin2 fluorescence as well as a loss of its ability to bind calcium. These results indicated that one or more of the -N-CH₂-COOH groups, responsible for the binding of calcium, were oxidized by the peroxidase.

Little is known about the mechanism by which these N-substituted aromatic amines are oxidized by peroxidases. The oxidation of N-methyl-substituted aromatic amines, on the other hand, has been well documented. However, several mechanisms of N-demethylation have been proposed, and there is still some controversy about the exact mechanism. Galliani et al. (2) reported that horseradish peroxidase, the enzyme most commonly used in these studies, catalyzed the H₂O₂-dependent N-demethylation of a number of N,N'-di-methyl- and N,N'-dibutylaniline derivatives. They suggested a mechanism involving one-electron oxidation of the nitrogen followed by deprotonation (-H⁺) to form a neutral carbon-centered radical (Scheme 1A). This carbon-centered radical can either be oxidized to the iminium cation, which is subsequently hydrolyzed to the monomethyl amine and formaldehyde (Scheme 1, pathway A) or, it can react with oxygen to form a peroxyl radical, which then decomposes to the monomethyl amine and formaldehyde (Scheme 1, pathway B).

Griffin et al. (3–5) showed that oxidation of N-methyl-substituted aromatic amines by horseradish peroxidase led to the formation of nitrogen-centered cation radicals and, in some cases, to a series of more complex reactions including dimerization to give a substituted benzidine. It was suggested that one-electron oxidation results in the formation of the nitrogen-centered cation radical, which loses a hydrogen atom to form an iminium cation. This leads to the formation of formaldehyde (Scheme 1, pathway C) or to radical dimerization (not shown), depending upon radical stability of the parent compound and, most likely, also on enzyme, H₂O₂, and substrate concentration.

Another possible mechanism has been proposed by Eling et al. (6, 7) for the N-demethylation of aminopyrine. Aminopyrine was oxidized to a nitrogen-centered radical cation, which then disproportionates to the iminium cation and aminopyrine. The iminium cation is hydrolyzed to the monomethyl amine and formaldehyde (Scheme 1, pathway D).

In contrast, Hellenga and co-workers (8–12) suggested that N-demethylation of N,N'-dimethylaniline by horseradish peroxidase involved hydrogen atom abstraction (-H·) from the methyl group, leading directly to the formation of a neutral carbon-centered radical rather than to the formation of a nitrogen-centered radical cation (Scheme 1, pathway E). One-electron oxidation would subsequently lead to the formation of the iminium cation and formaldehyde.

The mechanism of N-demethylation is obviously not clearly defined and might furthermore depend upon the compound studied. The mechanisms outlined in Scheme 1 provide various possibilities for the mechanism of the oxidation of the

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more complex N-substituted aromatic amines like quin2 and its analogs. We therefore decided to study the formation of the various free radical intermediates in the oxidation of these compounds by horseradish peroxidase and \( \text{H}_2\text{O}_2 \). We have used the technique of electron spin resonance spectroscopy, which enabled us to detect moderately stable radicals directly (nitrogen-centered cation radicals) and to detect reactive radicals by spin trapping (carbon-centered radicals) in order to try to elucidate the mechanism of oxidation.

**MATERIALS AND METHODS**

5,5'-(BAPTA), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, 5,5'-dimethyl BAPTA, \(^3\)C-labeled 5,5'-dimethyl BAPTA, and 5,5'-difluoro BAPTA were obtained from Molecular Probes Inc. Quin2, horseradish peroxidase (type VI), \( \text{H}_2\text{O}_2 \) (30%), DMPO, and \( \text{t-NB} \) were purchased from Sigma. DMPO was distilled prior to use. \( N,N' \)-Dimethyl-p-toluidine and \( N,N' \)-dimethylaniline were obtained from Aldrich, \( N,N' \)-Dimethyl-p-toluidine, \( H_2\text{O}_2 \), or horseradish peroxidase were obtained from Aldrich, \( N,N' \)-Dimethyl-p-toluidine, \( H_2\text{O}_2 \), or horseradish peroxidase were precipitated using \( \text{H}_2\text{O}_2 \) and \( \text{Ba(OH)}_2 \), and the samples were centrifuged. Subsequently, 1 ml of the supernatant was incubated with 1 ml of the Nash reagent (consisting of 15 g of \( \text{NH}_4\text{Ac} \), 0.2 ml of acetylacetone, and 0.3 ml of glacial acetic acid in 100 ml of \( \text{H}_2\text{O} \)) for 5 min at 58 °C. To correct for color formation that occurred during some reactions, a 1-ml sample was also incubated with the Nash reagent without acetylacetone. Absorption was measured at 412 nm.

Oxygen consumption was determined using a Clark-type electrode. All these experiments were performed at room temperature, and incubation conditions are described in the figure legends.

**RESULTS**

The Oxidation of N-Methyl-substituted Aromatic Amines: Formation of Nitrogen-Centered Cation Radicals—Incubation of \( N,N' \)-dimethyl-p-toluidine with horseradish peroxidase and \( \text{H}_2\text{O}_2 \) in 0.2 M sodium acetate buffer, pH 4.0, yielded the nitrogen-centered radical cation, as was shown previously by Griffin et al. (5). The spectrum is shown in Fig. 1A. When \( N,N' \)-dimethyl-p-toluidine, \( \text{H}_2\text{O}_2 \), or horseradish peroxidase was omitted from the incubation mixture, no ESR signals could be observed (results not shown). The spectrum was analyzed by using a computer correlation technique (15-17). Hyperfine splitting constants were found for all nuclei with spin in the radical cation, and they are listed in Table I. The simulation is shown in Fig. 1B. The Fourier transform of the experimental spectrum and the simulation are shown in Fig. 1, C and D, respectively. The nitrogen-centered cation from \( N,N' \)-dimethyl-p-toluidine has been described before, and the splitting constants are in agreement with those reported by Griffin et al. (5) (in buffer, pH 5.0) and by Latta and Taft (in acetoni trie) (18). When the experiment was performed in \( \text{H}_2\text{O} \) buffer, pH 4.0, the same spectrum was obtained (data not shown).

In the case of \( N,N' \)-dimethylaniline, the nitrogen-centered cation radical could not be detected under the conditions used for \( N,N' \)-dimethyl-p-toluidine due to the instability of this radical. However, by using the ESR fast-flow technique, we were able to detect the nitrogen-centered cation radical of \( N,N' \)-dimethylaniline (Fig. 2A). No signal could be detected when \( N,N' \)-dimethylaniline, \( \text{H}_2\text{O}_2 \), or the enzyme was omitted from the reaction mixture (results not shown). The assignment of the hyperfine splitting constants was accomplished

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1 The abbreviations used are: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; \( \text{t-NB} \), 2-methyl-2-nitrosopropane (tert-nitrosobutane); half-dimethyl BAPTA, \( N,N' \)-dimethyl-carboxyethyl)-4-methylaniline; \( W \), watts; \( \alpha \), hyperfine splitting constant.
by computer simulation (Table I), and the simulation is shown in Fig. 2B. The Fourier transform of the experimental spectrum and the simulation are shown in Fig. 2, C and D, respectively.

The Oxidation of N-CH2-COOH-substituted Aromatic Amines: Formation of Nitrogen-centered Cation Radicals—In this study, half-dimethyl BAPTA and 5,5'-dimethyl BAPTA were used as model compounds for the Ca2+ chelator quin2 and its analogs. Half-dimethyl BAPTA has a structure similar to that of N,N'-dimethyl-p-toluidine, with the two -CH2-COOH groups replacing the -CH3 groups. Incubation of half-dimethyl BAPTA with horseradish peroxidase and H2O2 at pH 4.0 resulted in the detection of a nitrogen-centered radical cation, as shown in Fig. 3A. When half-dimethyl BAPTA, horseradish peroxidase, or H2O2 was omitted from the reaction mixture, no ESR signal could be detected (results not shown).

The radical detected for half-dimethyl BAPTA was rather unstable and decayed during the scan. To improve the signal-to-noise ratio, several scans were accumulated. Although half-dimethyl BAPTA has a structure similar to that of N,N'-dimethyl-p-toluidine, it was not possible to simulate the observed spectrum using the hyperfine splitting constants found for N,N'-dimethyl-p-toluidine with four protons instead of six. We therefore decided to perform the experiment in D2O buffer, pH 4.0, to see whether one or more protons attached to the carboxylic acid groups could be detected under our experimental conditions. As can be seen in Fig. 4A, the spectrum changed considerably when D2O buffer was used, indicating that under these conditions, protons attached to the carboxylic acid groups could be detected. Using the computer correlation technique, we were able to obtain an additional hyperfine splitting constant of approximately 1.6 G for a deuterium ion, which implies a hyperfine splitting constant of approximately 10 G for the corresponding proton (Table I). By using the hyperfine splitting constants found for N,N'-dimethyl-p-toluidine and an additional hyperfine splitting constant of 10.0 G for two protons attached to the carboxylic acid groups, we were able to simulate the spectrum of half-dimethyl BAPTA (Fig. 3B). Fig. 4B shows the simulation for the spectrum detected in D2O buffer. The splitting constants are listed in Table I. The Fourier transform from the spectrum obtained in regular buffer is shown in Fig. 3C (experimental) and 3D (simulation) and from the spectrum in D2O buffer in Fig. 4C (experimental) and 4D (simulation).

Incubation of 5,5'-dimethyl BAPTA with H2O2 and horseradish peroxidase at pH 4.0 resulted in the formation of a nitrogen-centered radical cation as shown in Fig. 3E. No ESR spectrum was detected when 5,5'-dimethyl BAPTA, horseradish peroxidase, or H2O2 was omitted from the reaction mixture (results not shown). Although the spectrum was similar to that found for half-dimethyl BAPTA, we were unable to analyze this spectrum. Comparison of the hyperfine splitting constants found for the protons on the phenyl ring shows that for all compounds studied, these hyperfine splitting constants are very similar (Table I). Variation occurs in the hyperfine splitting constants obtained for the nitrogen and for the protons from the -CH2/-CH3 groups attached to the nitrogen. However, by using these hyperfine splitting constants, we were still unable to simulate the spectrum obtained for the nitrogen-centered radical from 5,5'-dimethyl BAPTA (Fig. 3E). The presence of the -O-CH2-R group attached to the phenyl ring most likely causes significant changes in the hyperfine splitting constants.

In the presence of higher concentrations of 5,5'-dimethyl BAPTA, H2O2, and enzyme, another spectrum could be detected (results not shown). Although we were unable to simulate this spectrum, we think it might be the result of a dimerization reaction, as was observed previously for N-

### Table I

| Hyperfine splitting constants for the nitrogen-centered cation radicals in Gauss | Parent compound |
|---|---|
| $a^0$ | $a^0$ (N-CH3) | $a^0$ (N-CH2) | $a^0$(orth) | $a^0$(meta) | $a^0$(para) | $a^0$(CH3) | $a^0$(COOH) | $a^0$(COOH) |
| N,N'-Dimethyl-p-toluidine | 11.0 | 11.90 | 5.16 | 1.30 | 10.10 |
| N,N'-Dimethyl-p-toluidine (5) | 10.55 | 11.35 | 5.20 | 1.20 | 9.70 |
| N,N'-Dimethyl-p-toluidine (18) | 11.17 | 12.22 | 5.20 | 1.36 | 9.97 |
| N,N'-Dimethylaniline | 11.70 | 13.9 | 5.50 | 1.66 | 7.76 |
| Half-dimethyl BAPTA | 11.0 | 11.86 | 5.20 | 1.24 | 10.16 | 10.0 |
| Half-dimethyl BAPTA (H2O) | 11.0 | 12.0 | 5.20 | 1.20 | 10.12 | 1.66 |
prior to entering the flat cell at a total flow rate of 40 ml/min. In 0.2 M sodium acetate buffer, pH 4.0. Equal volumes of N,N'-dimethylaniline/peroxide and peroxidase were mixed milliseconds prior to entering the flat cell at a total flow rate of 40 ml/min. Instrumental conditions were: microwave power, 20.9 mW; modulation amplitude, 0.26 G; time constant, 0.66 s; scan range, 140 G; scan time, 60 min. B, computer simulation of A. Hyperfine splitting constants are listed in Table I. Line width, 0.8 G. C, Fourier transform of the computer simulation.

The Formation of Carbon-centered Radicals: Detection with the Spin Trap DMPO—Addition of the spin trap DMPO to a mixture of horseradish peroxidase, H_2O_2, and 5,5'-dimethyl BAPTA at pH 4.0 resulted in the detection of a six-line spectrum, as shown in Fig. 5A. Similar results were obtained when 5,5'-dimethyl BAPTA was replaced by half-dimethyl BAPTA, BAPTA, 5,5'-difluoro BAPTA, or quin2 (results not shown). Due to the greater sensitivity of the spin-trapping technique as compared with direct ESR, we were able to detect the carbon-centered radical at lower substrate and enzyme concentrations. Furthermore, under the conditions used in our experiments, the addition of H_2O_2 was not required to produce the radical (Fig. 5D), although addition of H_2O_2 did enhance the intensity of the carbon-centered radical adduct spectrum. This signal increased in time (results not shown). No ESR signal was found when horseradish peroxidase, 5,5'-dimethyl BAPTA, or DMPO was omitted from the incubation mixture (Fig. 5, C, E, and F). The six-line spectrum detected under these conditions is characteristic of a carbon-centered radical adduct with coupling constants a_N = 15.7 G and a_C = 22.9 G. A similar radical adduct was detected when quin2 and its analogs were incubated with prostaglandin H synthase and arachidonic acid at pH 7.4 (1). More structural information about the trapped carbon-centered radical could be obtained by isotope labeling of the compounds at specific positions. 5,5'-Dimethyl BAPTA was ^13C-labeled at the CH_2 moiety in the acetate side group, and it was shown that the radical was formed at this ^13C-labeled position (1). Incubation of ^13C-labeled 5,5'-dimethyl BAPTA with horseradish peroxidase and H_2O_2 at pH 4.0 gave similar results (Fig. 5B). The radical is trapped at the same position, and the presence of the [^13C]carbon with spin ½ causes doubling of all the peaks in the observed spectrum. The hyperfine splitting constants are a_N = 15.7 G, a_C = 22.9 G, and a_N = 7.6 G, which are similar to those obtained previously at pH 7.4.

The second small radical adduct signal that was detected is the DMPO-superoxide radical adduct (Fig. 6A, marked by arrows). Addition of superoxide dismutase (130 µg/ml) inhibited the formation of the DMPO-superoxide radical adduct.
the trapping of a peroxyl radical, which decomposes to super-ylaniline with horseradish peroxidase and H$_2$O$_2$ in the presence of DMPO under identical conditions did not result in a carbon-centered radical. The very weak nitrogen coupling constant observed is most likely a decomposition product of the DMPO-superoxide radical adduct (19) since the addition of superoxide dismutase also inhibited the formation of this radical adduct. The very weak superoxide dismutase-insensitive component may be due to the trapping of a peroxy radical, which decomposes to superoxide (20). The complete simulation is shown in Fig. 4A. D, Fourier transform of the experimental spectrum shown in Fig. 4A.

The spectrum shown in Fig. 7A is the result of two different radical species. The first species is due to the trapped carbon-centered radical of 5,5'-dimethyl BAPTA, and its structure is shown in Fig. 7A. The large nitrogen-coupling constant arises from the nitroxide, and this hyperfine splitting constant of 16.1 G is typical for a spin adduct of a carbon-centered radical.

Identification of the Carbon-Centered Radical Using the Spin Trap t-NB—A major disadvantage of using a nitrene spin trap like DMPO is the lack of structural information obtained about the trapped radical. Although the site at which the carbon-centered radical is formed can be determined by using specifically labeled compounds, additional information about the structure of the trapped radical is required. Further information can be obtained using the nitroso spin trap t-NB. Nitroso compounds have a distinct advantage over nitrones because the reactive free radical attaches directly to the nitrogen atom of the spin trap, which will give rise to additional hyperfine splitting constants (21).

Incubation of 5,5'-dimethyl BAPTA with horseradish peroxidase and H$_2$O$_2$ in the presence of t-NB at pH 7.4 resulted in the spectrum shown in Fig. 7A. These experiments were performed at pH 7.4, because t-NB was more soluble at higher pH. When H$_2$O$_2$ was omitted from the reaction mixture, a signal was detected, when 5,5'-dimethyl BAPTA, horseradish peroxidase, or t-NB was omitted from the reaction mixture, no ESR signal could be detected (results not shown).

The spectrum shown in Fig. 7A is the result of two different radical species. The first species is due to the trapped carbon-centered radical of 5,5'-dimethyl BAPTA, and its structure is shown in Fig. 7A. The large nitrogen-coupling constant arises from the nitroxide, and this hyperfine splitting constant of 16.1 G is typical for a spin adduct of a carbon-centered radical. Furthermore, a hyperfine splitting constant of 2.2 G for the nitrogen of 5,5'-dimethyl BAPTA is observed. The hyperfine splitting constants for the two protons ($a_1^N = 10.3$ G and $a_2^N = 7.8$ G) are inequivalent, due to the conformational inequivalence of the two methylene protons. The simulation for this spin adduct is shown in Fig. 7C. The second spin adduct comes from t-butyl hydronitroxide, a species that can be formed due to the reduction of t-NB, with splitting constants $a_1^N = 14.3$ G and $a_2^N = 14.9$ G and is shown in Fig. 7D.
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FIG. 6. ESR spectrum of the DMPO-superoxide radical adduct. A, the incubation conditions were as in Fig. 5A. Instrumental conditions were: microwave power, 20.9 mW; modulation amplitude, 0.5 G; time constant, 0.66 s; scan range, 80 G; scan time, 3 min. B, computer simulation of A. Hyperfine splitting constants are, for species 1: \( a^N(t-NB) = 16.1 \) G, \( u^N(BAPTA) = 2.2 \) G, \( u^p = 10.3 \) G, and \( a^p = 14.3 \) G; for species 2: \( a^N = 15.0 \) G and \( a^p = 15.0 \) G. C, as A, but with superoxide dismutase (130 \( \mu \)g/ml).

FIG. 7. Spin adduct (R) trapped with \( t-NB \). A, the incubation mixture contained 4 mM 5,5'-dimethyl BAPTA, 30 \( \mu \)g/ml horseradish peroxidase, 0.14 mM \( H_2O_2 \), and 1 mg/ml \( t-NB \) in 0.1 M sodium phosphate buffer, pH 7.4. Instrumental conditions were: microwave power, 20.9 mW; modulation amplitude, 0.8 G; time constant, 0.98 s; scan range, 80 G; scan time, 300 s. Five scans were accumulated. B, composite computer simulation of A. Hyperfine splitting constants are, for species 1: \( a^N(t-NB) = 16.1 \) G, \( a^N(BAPTA) = 2.2 \) G, \( a^p = 10.3 \) G, and \( a^p = 7.8 \) G; for species 2: \( a^N = 14.3 \) G and \( a^p = 14.9 \) G. C, computer simulation of species 1. D, computer simulation of species 2.

FIG. 8. Formation of formaldehyde and oxygen consumption during the oxidation of 5,5'-dimethyl BAPTA as a function of horseradish peroxidase concentration. The incubation mixtures contained 5,5'-dimethyl BAPTA (1 mM) and horseradish peroxidase (HRP) (concentration was varied from 0 to 2.5 \( \mu \)g/ml) in 0.2 M sodium acetate buffer, pH 4.0. Samples were incubated for 5 min at room temperature. Formaldehyde formation and oxygen consumption were determined as described under "Materials and Methods." O, nmol of formaldehyde formed/ml of sample; ●, nmol of oxygen consumed/ml of sample; ■, nmol of oxygen consumed/min.

Formaldehyde Formation and Oxygen Consumption—From the experiments with the spin traps DMPO and \( t-NB \), it was clear that incubation of 5,5'-dimethyl BAPTA with horseradish peroxidase and \( H_2O_2 \) resulted in the oxidative cleavage of the -N-CH\(_2\)-COOH group, releasing the carboxylic acid group. In order to investigate the fate of the carbon-centered radical, we decided to look for the formation of formaldehyde. Previously we showed that incubation of quin2 and its analogs with prostaglandin H synthase and arachidonic acid resulted in the formation of formaldehyde and that this formaldehyde formation could be used as an indication of the oxidation of the -N-CH\(_2\)-COOH group (1). Formaldehyde can be measured using a colorimetric method developed by Nash (22). The Nash reagent contains acetylacetone, which reacts with formaldehyde in the presence of excess amounts of ammonium salts to form a yellow product, diacetyl-dihydrolutidine, that can be measured quantitatively at 412 nm. Incubation of 5,5'-dimethyl BAPTA with horseradish peroxidase (concentration varied from 0 to 2.5 \( \mu \)g/ml) resulted in the formation of formaldehyde, as illustrated in Fig. 8. The addition of \( H_2O_2 \) was not necessary for formaldehyde formation to occur. The amount of formaldehyde formed increased with enzyme concentration until a maximum was reached at approximately 1.5 \( \mu \)g/ml of horseradish peroxidase.

Oxygen consumption was measured under identical conditions, and the results are shown in Fig. 8. Oxygen consumption increased with enzyme concentrations, at approximately 1 \( \mu \)g/ml horseradish peroxidase, oxygen consumption was complete.
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Fig. 9. Formation of formaldehyde and oxygen consumption during the oxidation of the N-methyl-substituted amines by horseradish peroxidase as a function of \( \text{H}_2\text{O}_2 \) concentration. A, the incubation mixtures contained \( N,N'\)-dimethylaniline (1 mM), horseradish peroxidase (0.5, 1.0, or 5.0 \( \mu \text{g/ml} \)), and \( \text{H}_2\text{O}_2 \) (concentration was varied from 0 to 1 mM) in 0.2 M sodium acetate buffer, pH 4.0. Samples were incubated for 5 min at room temperature. Formaldehyde formation was measured as described under "Materials and Methods." C, 0.5 \( \mu \text{g/ml} \) horseradish peroxidase; D, 1 \( \mu \text{g/ml} \) horseradish peroxidase; E, 5 \( \mu \text{g/ml} \) horseradish peroxidase. B, as A. Oxygen consumption was measured as described under "Materials and Methods." C, as A, but with \( N,N'\)-dimethyl-p-toluidine. D, as B, but with \( N,N'\)-dimethyl-p-toluidine. E, as A, but with aminopyrine. F, as B, but with aminopyrine.

in 5 min. Furthermore, the amount of oxygen consumed/min increased with enzyme concentrations. The total amount of oxygen consumed was always less than the total amount of formaldehyde formed, but both reached a maximum at an enzyme concentration of about 1.5 \( \mu \text{g/ml} \). When oxygen consumption was complete, a red color appeared in the incubation mixtures. When DMPO was added to the incubation mixtures, formaldehyde formation was inhibited by approximately 80%, and oxygen consumption was completely inhibited (results not shown).

The experiments were repeated for \( N,N'\)-dimethylaniline, \( N,N'\)-dimethyl-p-toluidine, and aminopyrine; the results are shown in Fig. 9, A–F. Incubation of these compounds with horseradish peroxidase without the addition of \( \text{H}_2\text{O}_2 \) did not result in the formation of formaldehyde, as was observed in the case of 5,5'-dimethyl BAPTA. Incubation of \( N,N'\)-dimethylaniline with horseradish peroxidase and \( \text{H}_2\text{O}_2 \) resulted in the formation of formaldehyde, and this formation was most affected by an increase in the \( \text{H}_2\text{O}_2 \) concentration (Fig. 9A). However, no oxygen consumption could be detected when \( N,N'\)-dimethylaniline was incubated with horseradish peroxidase and \( \text{H}_2\text{O}_2 \) under these conditions (Fig. 9B). When \( N,N'\)-dimethyl-p-toluidine was incubated with horseradish peroxidase and \( \text{H}_2\text{O}_2 \), formaldehyde could again be detected in the incubation mixtures (Fig. 9C). Formaldehyde formation was not affected significantly by an increase in enzyme concentration, but increasing amounts of \( \text{H}_2\text{O}_2 \) produced an increase in the formaldehyde formation. Oxygen consumption was determined under the same experimental conditions and amounted to a maximum of 30–40 nmol of oxygen/ml of sample, which was about 15% of the total amount of formaldehyde formed (Fig. 9D). Oxygen consumption was hardly affected by an increase in enzyme concentration, but an increase in \( \text{H}_2\text{O}_2 \) concentration caused a small increase in oxygen consumption. In the case of aminopyrine, a smaller amount of formaldehyde was formed, but no oxygen consumption was detected under these conditions (Fig. 9, E and F). For all these compounds, the addition of DMPO did not significantly affect formaldehyde formation (data not shown).

**DISCUSSION**

As stated in the Introduction, various mechanisms have been proposed for the oxidation of \( N \)-substituted aromatic amines by peroxidases (Scheme 1, A–E). Comparison of the pathways outlined in Scheme 1 shows that the major difference between pathways A–E is that the initial radical formed in the pathways A–D is nitrogen centered, whereas the initial radical formed in pathway E is carbon centered. The results in this paper clearly demonstrate that nitrogen-centered cation radicals are detected from both the \(-\text{CH}_2\)- and \(-\text{CH}_2\)-COOH-substituted aromatic amines during peroxidase-catalyzed N-dealkylation (Table 1). A carbon-centered radical, on the other hand, was detected only in the case of 5,5'-dimethyl BAPTA and its analogs and not for \( N,N'\)-dimethyl-p-toluidine or \( N,N'\)-dimethylaniline (Figs. 5–7). From the experiments with 5,5'-dimethyl BAPTA, it was clear that if carbon-centered radicals were formed during the oxidation of \( N,N'\)-dimethyl-p-toluidine or \( N,N'\)-dimethylaniline, they should be detected with the spin-trapping technique. Extensive oxygen consumption was measured during the horseradish peroxidase-catalyzed dealkylation of 5,5'-dimethyl BAPTA and analogs, whereas in the case of \( N,N'\)-dimethyl-p-toluidine, a small amount of oxygen consumption was observed (Figs. 8 and 9). No oxygen was consumed during the oxidation of \( N,N'\)-dimethylaniline or aminopyrine. Thus, there is general agreement between the ESR and the oxygen consumption data, as should be expected since carbon-centered free radicals react with molecular oxygen at near diffusion-limited rates (23). Formaldehyde formation, on the other hand, was detected for all the compounds, which indicates that \( N,N'\)-dimethyl-p-toluidine, \( N,N'\)-dimethylaniline, and aminopyrine are \( N \)-demethylated by horseradish peroxidase and \( \text{H}_2\text{O}_2 \) (Fig. 9). If the oxygen consumption had been stoichiometric with formaldehyde formation, it would have been easily detectable (Fig. 9). However, neither the ESR data (qualitatively) nor the oxygen consumption data (quantitatively) indicate that carbon-centered radicals are formed during the oxidation of \( N \)-methyl-substituted compounds by horseradish peroxidase and \( \text{H}_2\text{O}_2 \), with the possible exception of \( N,N'\)-dimethyl-p-toluidine when detectable but less than stoichiometric oxygen consumption was found. This clearly demonstrates that different mechanisms of N-dealkylation occur for \(-\text{CH}_2\)- and \(-\text{CH}_2\)-COOH-substituted aromatic amines. Any proposed carbon-centered radical intermediate would have to decompose very rapidly since its reaction with oxygen is known to be nearly diffusion controlled (23).

Hollenberg and co-workers (8–12) have studied extensively the oxidation of \( N \)-methyl-substituted aromatic amines by peroxidases. They measured formaldehyde formation as a result of the oxidation of \( N,N'\)-dimethylaniline and determined the effect of various spin traps on the formaldehyde formation (8). Although all the spin traps caused some inhi-
N-Dealkylation of Aromatic Amines

The iminium cation, which is formed upon decomposition of the peroxyl radical, is also unstable and will hydrolyze to the mono-substituted amine and formaldehyde. The amount of formaldehyde formed during the horseradish peroxidase-catalyzed oxidation of 5,5'-dimethyl BAPTA was always slightly higher than the total amount of oxygen consumed (Fig. 8), which indicates that formaldehyde formation might occur through more than one pathway. These results suggest that the major pathway for dealkylation requires oxygen (Scheme 2) but that some formaldehyde might be formed through a mechanism that is oxygen independent (pathway 1E).

In the case of our N-methyl-substituted amines, rearrangement of the nitrogen-centered radical cation to a carbon-centered radical is unlikely to occur since there is no good leaving group available. Thus, there are several possible pathways for the further oxidation of the nitrogen-centered radical, two of which are outlined in Scheme 1, C and D. Furthermore, studies by Slaughter and O’Brien (25) on the oxidation of N,N'-dimethyl-p-toluidine by horseradish peroxidase and H2O2 indicated that the oxidation might proceed along three separate pathways: N-demethylation to N-methylamine and formaldehyde, dimerization to N,N,N'-tetramethyl-p,p'-benzidine, and another dimerization to an unidentified water-soluble product, possibly an N-oxide. This suggests that further studies on the products, formed together with formaldehyde, might make it possible to elucidate the mechanism by which these N-methyl-substituted aromatic amines are oxidized.

In conclusion, the results in this paper clearly demonstrate that N-substituted aromatic amines are good electron donors and thus are easily oxidized in a system containing horseradish peroxidase and H2O2. One-electron oxidation leads to the formation of nitrogen-centered cation radicals. The subsequent mechanism of N-dealkylation, however, is dependent upon the structure of the nitrogen-centered radical formed. When the N-alkyl side group contains a good leaving group, like the carboxylic acid group in 5,5'-dimethyl BAPTA and analogs, bond breakage occurs through a rearrangement reaction, with the formation of a carbon-centered radical (Scheme 2). When a good leaving group is not available, the mechanism for the oxidation of the nitrogen-centered cation radical is still unknown, and further studies are required to elucidate this mechanism fully.

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