ESR Spin-Trapping Studies of Free Radicals Generated by Hydrogen Peroxide Activation of Metmyoglobin

Yuan Xu,† Ali Asghar,‡ J. Ian Gray,‡ Albert M. Pearson,‡ Alfred Haug,§ and Eric A. Grulke*,†

Department of Chemical Engineering, Department of Food Science and Human Nutrition, and Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824

The reaction of metmyoglobin (MetMb) with hydrogen peroxide (H_2O_2) generated a free radical that was detectable by ESR at room temperature only in the presence of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxyl (DMPO). The formation of the free radical was favored at pH ≥7 and was depressed at low pH. The free radical spin-trapped by DMPO may be a ferryl radical or an amino acid free radical originating from the myoglobin moiety.

Studies on lipid peroxidation in meats have recently attracted a great deal of attention (Gray and Pearson, 1987; Kanner et al., 1987). A number of free radicals (e.g., superoxide radical, hydroxyl radical, perferryl radical, and ferryl radical) have been proposed as initiators of lipid peroxidation in biological systems. This aspect has been discussed in detail by several researchers (Aust and Svingen, 1985; Halliwell and Gutteridge, 1986; Asghar et al., 1988). However, some recent studies (Kanner and Harel, 1985a,b) have suggested that membranal lipid peroxidation is initiated by hydrogen peroxide activated metmyoglobin (MetMb) produced endogenously in the muscle system. On the basis of theoretical considerations, the activated MetMb was believed to be a porphyrin cation (ferryl) radical ($P^+Fe^{IV}=O$). In contrast, some researchers also consider that the reaction between MetMb and H_2O_2 produces hydroxyl and ferryl radicals (George and Irvine, 1955). Others believe that an oxidized product of the globin molecule of the reaction represents the radical (Gibson et al., 1958).

Electron spin resonance (ESR) techniques are used for direct detection of free radicals in solution. However, ESR methodology is restricted to observing stable free radicals that accumulate to measurable quantities or unstable radicals that reach a high steady-state concentration. The free radical generated during MetMb oxidation has been detected only at subfreezing (−50 to −120 °C) temperatures (Gibson et al., 1958; King and Winfield, 1963). Above these temperatures, the free radical decays so rapidly that its concentration is less than the minimum required for the ESR detection limit of 10^{-6} M. In a frozen sample, however, the free radical is no longer in a fluid environment and anisotropic effects from freezing may interfere with identification of the radical. Moreover, this procedure is limited by the concentration of the free radical prior to freezing and the length of time required to freeze the sample, which is about 5–10 ms (Gibson et al., 1958).

Some researchers have successfully detected free radicals at room temperature using an ESR spectrometer equipped with a continuous-flow apparatus (Shiga and Imaizumi, 1975; Harada and Yamazaki, 1987). Alternatively, the spin-trapping technique overcomes some of the difficulties associated with the detection of unstable free radicals in aqueous solutions. The spin trap stabilizes the unstable free radical by reacting with it covalently, thus forming a spin trap adduct. The adduct exists in a "long-lived form" and can be detected at room temperature with conventional ESR instrumentation. The stable spin adduct usually accumulates because of its low rate of degeneration. Spin trapping is an integrative method of measuring free radicals and is more sensitive than just measuring the instantaneous or steady-state level of free radicals.

In this paper, we report the results of an investigation of the free radical formed during the reaction of MetMb with H_2O_2 by the spin-trapping method. The goal of the present work was to study the MetMb/H_2O_2 lipid peroxidation mechanism under experimental conditions similar to those under which membranal lipids are oxidized. It is known that peroxidation of fresh uncooked meat occurs even at low temperatures between +4 and −20 °C (Asghar et al., 1988). The study presented here deals with the effect of temperature, pH, and buffer system on free-radical formation from the reaction between MetMb and H_2O_2.

MATERIALS AND METHODS

MetMb from horse heart, the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxyl (DMPO), and the metal chelator diethylenetriaminepentaacetic acid (DETAPAC) were purchased from Sigma Chemical Co. (St. Louis, MO). H_2O_2 was obtained from J. C. Baker (Phillipburg, NJ), while Chelex-100 resin was purchased from Bio-Rad (Rockville Center, NY). The DMPO contained impurities as evidenced by pronounced ESR single absorption peaks. To remove the impurities, 0.5 g of activated charcoal was added to 1 g of DMPO in 15 mL of double-distilled water and the resultant mixture then stirred for 20 min in the dark at room temperature (Thornalley and Bannister, 1985). The charcoal containing the absorbed impurities was filtered off, and the process was repeated two more times. The filtrate was monitored by ESR at high gain (10^{-5}) to ensure that the radical impurities were removed. The final solution was assayed for DMPO concentration spectrophotometrically with a molar extinction coefficient of $\epsilon = 7700\ M^{-1}\ cm^{-1}$ at 234 nm in ethanol (Thornalley and Bannister, 1985). The final H_2O_2 concentration was standardized (Halliwell and Gutteridge, 1986) at 240 nm with a molar extinction coefficient of $\epsilon = 43.6\ M^{-1}\ cm^{-1}$ (Abraham and Bleaney, 1970).

All solutions were prepared with double-distilled water passed through a Chelex-100 column to remove trace metals. The phosphate buffers were also passed through the column. Finally, the metal chelating agent, DETAPAC (0.1 mM), was added

† Department of Chemical Engineering.
‡ Department of Food Science and Human Nutrition.
§ Pesticide Research Center.

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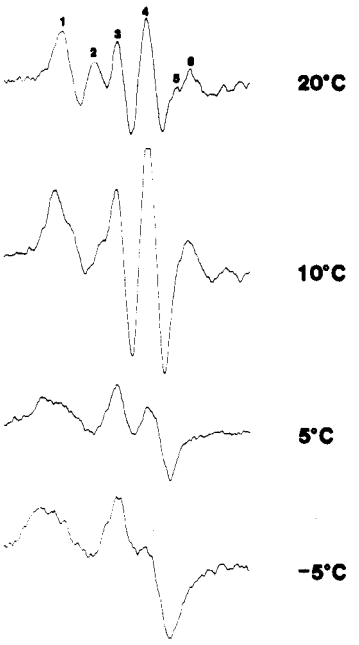

Figure 1. Effect of temperature on the spectra of the spin trap adduct produced on reacting MetMb with H_2O_2 in the presence of DMPO (pH 7.0, buffer 0.1 M phosphate containing DETAPAC): peak 1, 3197 G; 2, 3206 G; 3, 3212.5 G; 4, 3220.5 G; 5, 3229.5 G; 6, 3233 G.

to the buffers to ensure that trace metals in H_2O_2 and MetMb were removed before the reaction was initiated. A Varian E-Line Century Series Model 112 spectrometer was used to record ESR spectra under the following conditions: gain, 5×10^4; microwave power, 20 mW; modulation intensity, 1 G; time constant, 0.5 s; scan time, 2 min. The magnetic field was set at 3220 G, and the spectra were recorded over a 100-Ga sweep range (3220 ± 50). In a typical experiment, the reaction medium contained 0.01 M DMPO, 0.4 mM MetMb, 0.4 mM H_2O_2, and sufficient buffer to reach a final volume of 0.5 mL.

RESULTS AND DISCUSSION

A defined sequence of adding chemicals was chosen to permit reaction of the chelating agent with metal ions in DMPO, MetMb, and H_2O_2. The order of addition was as follows: buffer, DMPO, MetMb, and H_2O_2. These reagents were also tested in different combinations such as buffer, DMPO, and MetMb; buffer, DMPO, and H_2O_2; and buffer, MetMb, and H_2O_2 to examine for the presence of free radicals. The first two control experiments demonstrated that MetMb and H_2O_2 did not produce any free radical in the absence of each other. The third control experiment demonstrated that the reaction between MetMb and H_2O_2 was too rapid to be detected by ESR at room temperature in the absence of a spin trap.

The interaction of H_2O_2 and MetMb rapidly produced highly reactive free-radical species that never reached the concentration necessary for detection at room temperature. This radical was only observable by ESR after it had been spin-trapped. The spectra of the spin trap adduct were strongly dependent on temperature and the pH of the solution, whereas buffer concentration in the range 0–0.1 M had little effect. We further studied the conditions influencing spin trap adduct stability and the conditions affecting radical formation.

Temperature-Dependent Formation of Free Radicals. Figure 1 shows a typical temperature dependence for the formation of free radicals. The recording of the spectra was started 2 min after the initiation of the reaction and continued over a period of time in each case. However, for the sake of brevity, only selective spectra are presented herein. The feature of the spectra was that, at high temperature, more free radicals were spin-trapped than at low temperatures. The control experiments showed that the reaction of MetMb with H_2O_2 was too fast to be observed by ESR in the absence of DMPO.

Figure 1 demonstrates that, at 20 °C, the spin trap adduct was less stable and decayed faster than at 10 °C. Since the rate of decay of the free radical was greater than its rate of formation at the higher temperatures, the amplitude of the spectrum at 20 °C was smaller than that at 10 °C. As the temperature was decreased, the formation of the free radical also decreased; thus, only a relatively small amount of free radical was trapped, as is apparent in Figure 1. One of the most important characteristics of the ESR spectra is that as the temperature changed, the spectra also varied. The spectrum at 20 °C originally had peaks located respectively at G = 3197, 3206, 3212.5, 3220.5, 3229.5, and 3233. In the spectrum obtained at 10 °C, however, the peaks at G = 3206 and 3229.5 became shoulders and nearly vanished. At 5 °C, more peaks vanished while the remaining peaks broadened. The peaks that persisted were at G = 3197, 3212.5, and 3220.5. When the temperature reached −5 °C, only two peaks at G = 3197 and 3212.5 remained. The peak at G = 3220.5 became a shoulder.

A possible interpretation of these observations is that as the temperature was lowered, the rate of conformational changes decreased and certain lines in the spectrum became broader (Abraham and Bleaney, 1970; Wertz and Bolton, 1972). Those lines that broadened correspond to transitions for which the conformational interconversion leads to a change in spin state.

A further lowering of temperature caused an increase in the length of time during which every molecule attained a specific conformation. Eventually, the spectrum approaches that of a blocked or frozen conformation (Abraham and Bleaney, 1970; Wertz and Bolton, 1972). We expect that if the temperature was further lowered, the spectrum would finally be reduced to that obtained by King and co-workers (King and Winfield, 1963; King et al., 1964). The most pronounced peaks in the spectrum were obtained at 10 °C (Figure 1); therefore, this temperature was used for subsequent experiments.

Effect of pH on the Formation of Free Radicals. Figure 2 shows the pH-dependent formation of free radicals. The spectra were recorded 2 min after the initiation of the reaction. Only a small amount of free radicals was spin-trapped at pH 5 and 6. However, a strong spectrum appeared at pH 7, and even stronger spectra were obtained at pH >7. This suggests that, under acidic conditions, the reaction rate was low since only small amounts of free radicals were spin-trapped. On the other hand, under neutral and basic conditions, high concentrations of free radicals were formed.

Decay of Spin Adducts. *Effect of pH on the Decay of Spin Adduct at 10 °C.* Figure 2 shows that basic or neutral conditions were better than acidic conditions for the generation of free radicals from the MetMb and H_2O_2 reaction. Under basic conditions, however, the spin trap adduct decayed more rapidly than under neutral conditions. The change in the spin trap adduct concentration with time was difficult to analyze because the spectrum was dependent on both the reaction temperature and the pH of the solution. Under acidic conditions (pH 5 and 6), the spin adduct concentration did not reach its maximum until approximately 10 min after the initiation of the reaction. Although there was

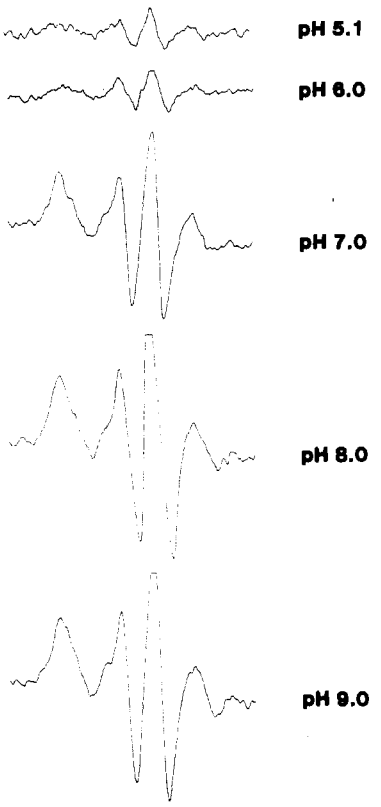

Figure 2. Effect of pH on the spectra of the spin trap adduct produced on reacting MetMb with H_2O_2 in the presence of DMPO at 10 °C.

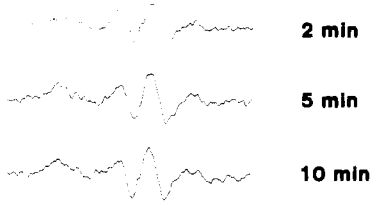

Figure 3. Changes with time in the spectra of the spin trap adduct produced by MetMb on reacting with H_2O_2 in the presence of DMPO at pH 6.0 and 10 °C.

only a small amount of free radical spin-trapped under acidic conditions, the spin adduct spectrum was very similar for 5–20 min after the reaction. Figure 3 shows the time dependence for the formation of free radicals at pH 6. It is evident that the amplitude of the signals is small relative to that produced at higher pH at corresponding times.

At pH 7.0, the spin trap adduct concentration reached a maximum within 2 min of initiation of the reaction. However, the spin adduct also decayed quickly. Approximately 10 min after the initiation of reaction, the spin adduct concentration was less than half of its maximum (Figure 4). At pH 8.0, similar results were obtained as at pH 7.0. At pH 9.0, the spin adduct concentration was nearly the same as at pH 7.0 but it decayed much faster. These spectra are not included in the text. These data suggested that the most suitable pH condition for the generation and observation of free radicals was between 7 and 8.

Effect of Temperature on the Decay of the Spin Adduct. At 20 °C and pH 7, the spin adduct was unstable (Figure 5). It started decaying soon after the free radicals were spin-trapped. The spectral amplitudes in Figure 5 were smaller than those in Figure 4, which were obtained under exactly the same experimental conditions except for

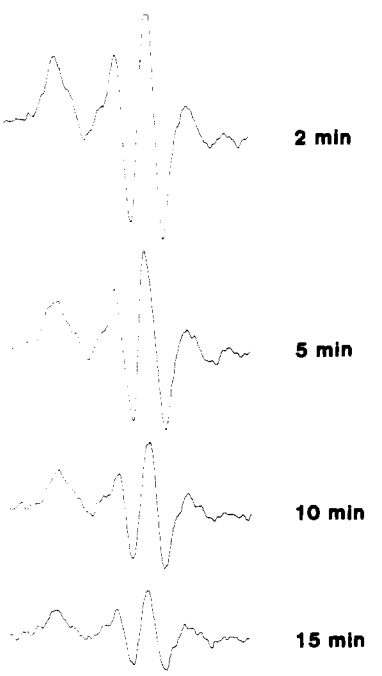

Figure 4. Changes with time in the spectra of the spin trap adduct produced on reacting MetMb with H_2O_2 in the presence of DMPO at pH 7.0 and 10 °C.

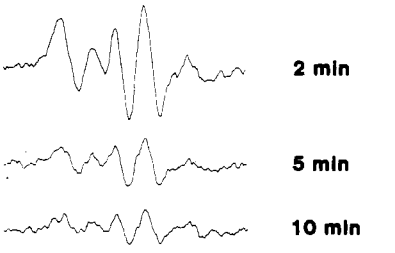

Figure 5. Changes with time in the spectra of the spin trap adduct produced on reacting MetMb with H_2O_2 in the presence of DMPO at pH 7.0 and 20 °C.

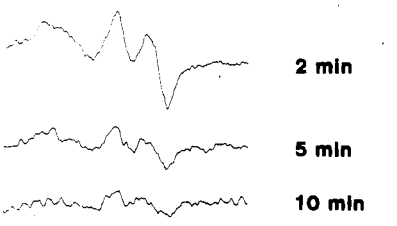

Figure 6. Changes with time in the spectra of the spin trap adduct produced on reacting MetMb with H_2O_2 in the presence of DMPO at 0 °C and pH 7.0.

temperature. The plot in Figure 5 shows that, at 20 °C, the spin adduct in the solution was almost undetectable at approximately 10 min after the initiation of the reaction. Figure 4 shows the spin adduct decay rates at 10 °C. Obviously, the spin adduct has a longer life at lower temperatures. At 10 °C, the spin adduct did not completely vanish until 1 h after the start of the reactions. The decay of the spin adduct at 0 °C is shown in Figure 6. When the temperature was further lowered to −5 °C, the spin adduct concentration did not reach its maximum until 5 min after the initiation of the reaction. About 10 min later, there was still a significant amount of spin adduct in the solution as can be seen from Figure 7.

The data obtained in these studies provide an explanation for the observations of Kanner and Harel (1985b) that neither MetMb or H_2O_2 alone could initiate membranal lipid oxidation in the model system. Our

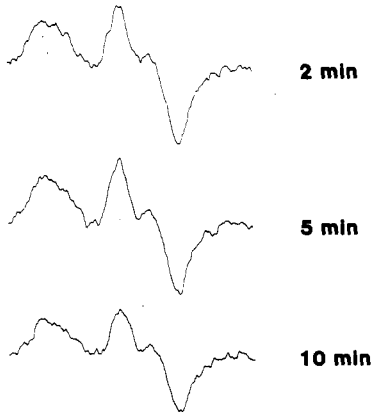

Figure 7. Changes with time in the spectra of the spin trap adduct produced on reacting MetMb with H_2O_2 in the presence of DMPO at -5 °C and pH 7.0.

results show that, in general, the spin adduct decreased during the first 5 min, and within approximately 10 min the spin adduct was reduced to about half of the maximum adduct concentration. However, the spin adduct did not completely disappear until at least 30 min after beginning the reaction. When the pH of the reaction system was below 6, the generation of free radicals was almost completely inhibited (Figure 2). This explains why Kanner and Harel (1985b) found less lipid peroxidation at pH 5.0 than at pH 6.5. However, this seems contrary to reports indicating higher oxidation rates in meat at low pH compared to higher pH (Owen and Lawrie, 1975; Judge and Aberle, 1980).

In our experiments, we found no evidence of any spin adduct of hydroxyl radicals. On the basis comparisons with spectra of known spin adducts (Bergmeyer et al., 1970), the trapped free radical is not a hydroxyl or a superoxide radical over the wide pH range of 5–9. During the preparation of this paper, we noted a recent study by Hong and Piette (1989) in which it was observed that the MetMb-H_2O_2 system in the presence of DMPO produced an asymmetric ESR spectrum consisting of five broad lines, which differed from that of the hydroxyl radical adduct. Similarly, Morehouse et al. (1989) did not observe the signal for a porphyrin cation radical during reaction of peroxidase with hematoporphyrin IX in the presence of hydrogen peroxide.

Because of the complexity of MetMb oxidation under various temperatures and pH values, it is difficult to assign a specific name for the free radical formed during MetMb oxidation. King et al. (1964) tentatively suggested the free radical to be a phenylalanine radical or tyrosine free radical, while Maples et al. (1988) suggested the formation of a protein-derived thiyl free radical during the reaction of hemoglobin with phenylhydrazine. We demonstrated in this study that the reaction of MetMb and H_2O_2 does produce a free radical that can be trapped by DMPO and detected at room temperature by ESR spectroscopy. This radical may be responsible for initiating membranal lipid peroxidation in muscle foods. Further work on the nature of the free radical is currently in progress.

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