

Manganese(II)–bicarbonate-mediated catalytic activity for hydrogen peroxide dismutation and amino acid oxidation: Detection of free radical intermediates

(oxygen radicals/electron paramagnetic resonance/spin trapping)

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ABSTRACT To examine the structural identities of reactive free radicals and the mechanism of the oxidative modification of proteins, we used EPR and spin-trapping methods to investigate the oxidation of amino acids by H_2O_2 as well as the decomposition of H_2O_2 itself catalyzed by Mn(II) ions. Superoxide and hydroxyl radicals ($O_2^{\cdot-}$ and $OH\cdot$) were trapped by a spin trap, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), in a reaction mixture containing Mn(II) and H_2O_2 in bicarbonate/ CO_2 buffer. When Hepes was used in place of bicarbonate buffer, superoxide radical was not observed, indicating the importance of bicarbonate buffer. With addition of L-leucine to a similar reaction mixture, a leucine-derived radical that replaced the DMPO–superoxide adduct was detected in the absence and presence of DMPO. Using various isotope-enriched L-leucines, we successfully identified this radical as a hydroxynitroxide, $^-\text{OOC(R)CHNHO}\cdot$. The data are consistent with the formation of a transient “caged” $OH\cdot$ in the inner coordination sphere of Mn(II). This caged $OH\cdot$ is likely to undergo an intramolecular hydrogen-atom abstraction from the Mn-bound H_2O_2 or amino acid. Two reaction schemes are proposed to account for the experimental results shown here and in the preceding papers.

Mn ions are known to participate in certain superoxide dismutases (1, 2) and catalases (3, 4). Low molecular weight complexes of Mn have also been observed to protect cells from oxygen damage (5, 6). This capability has been attributed to the fact that Mn(II) when complexed with certain anionic ligands is capable of catalyzing the dismutation of superoxide anion, $O_2^{\cdot-}$. Catalase-like activity has also been observed for nonprotein Mn(II) complexes (7, 8). In addition, transition metal ions such as Fe and Cu are known to catalyze oxygen radical-mediated oxidative modification of proteins, a process implicated in protein turnover, aging, and oxygen toxicity (9, 10).

In the preceding papers (8, 11), we showed that Mn(II)–bicarbonate complexes are capable of catalyzing the disproportionation of H_2O_2 to form O_2 and the oxidation of amino acid by H_2O_2 to form the carbonyl derivatives of the amino acid, NH_4^+ , and CO_2 . To investigate the mechanisms of these catalytic reactions, we have used an EPR spectrometer together with a spin trap to detect short-lived free radical intermediates for these reactions. Various isotope-enriched leucines were also used for identifying the leucine-derived radical. Based on the results of this study and the kinetic and product analyses presented in the preceding papers (8, 11), two mechanisms are proposed, one for the catalase-like activity and the other for the oxidation of amino acids.

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MATERIALS AND METHODS

The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), purchased from Aldrich, was further purified with activated charcoal under N_2 gas in the dark (12). L-[^{13}C]Leucine (99 atom %), L-[^{15}N]leucine (95%), L-leucine- d_{10} [(CD_3) $_2$ CDCD $_2$ -CD(NH_2)COOH, where D is deuterium] (98%), and D_2O (99.9%) were purchased from Cambridge Isotope Laboratories (Woburn, MA). Sodium [^{13}C]bicarbonate (99%) and L-[^{13}C]leucine (99%) were obtained from MSD Isotopes.

EPR spectra were recorded at 100-kHz frequency modulation and 9.87-GHz microwave irradiation with a Bruker ESP 300 spectrometer operated in the TM_{110} mode. An aqueous flat cell ($\approx 150 \mu l$) was used and the spectrometer was tuned with the cell filled with water. The reaction mixture was transferred to the flat cell through stainless steel needle tubing by applying pressure to the connected closed reaction vessel (13). A Bruker ESP 1600 data system was used for spectral acquisition, which began 40 sec after initiation of the reaction by an injection of H_2O_2 . The conditions for the acquisition of spectral data were as follows: temperature, 25°C; microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 10.24 msec; time constant, 82 msec; sweep time, 21 sec; sweep width, 100 G with 2048-point resolution.

RESULTS

Formation of Superoxide Radical. The first-derivative EPR absorption spectra shown in Fig. 1 were obtained with a solution containing 0.1 mM $MnCl_2$, 100 mM DMPO, and 30 mM H_2O_2 in 23.5 mM $NaHCO_3/CO_2$ buffer at pH 7.5. The six hyperfine splitting lines observed in an 800-G sweep width (spectrum A) are attributed to the interaction of a Mn(II) ion ($I = 5/2$) with average hfc constant of 95 G. In addition, a number of sharp lines from DMPO–radical adducts appear at the center of the spectrum. This part of the spectrum has been expanded and the sloping baseline due to the Mn(II) absorption signal has been corrected as shown in spectra B and C. Two sets of resonance lines can be distinguished. One set is attributable to a DMPO–OH adduct on the basis of the characteristic spectral pattern of the intensity ratio 1:2:2:1 with hfc constants of 14.9 G from nitrogen and hydrogen nuclei. The relative amplitude of the two sets of resonance lines varies with time. By subtracting spectrum B from C, a second set of absorption signals, spectrum D, is obtained. The simulated spectrum, E, obtained with use of hfc constants of 14.33 G (A^N), 11.43 G (A^H), and 1.30 G (A^H) and 1.40 G of peak-to-peak linewidth corresponds well with spectrum D, indicating that it originates from DMPO–OOH (14).

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; hfc, hyperfine coupling.

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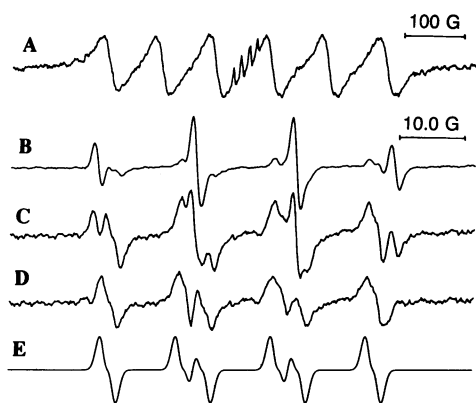


FIG. 1. First-derivative EPR spectra of Mn(II) ion complex and DMPO-radical adducts. The sample contained 0.1 mM MnCl₂, 100 mM DMPO, and 30 mM H₂O₂ in NaHCO₃ buffer bubbled with a 5% CO₂/95% N₂ gas mixture (pH 7.5). Other experimental conditions and spectrometer settings were as described in *Materials and Methods*. Spectrum A was obtained in 800-G sweep width, showing six hyperfine coupling (hfc) resonance lines of the Mn(II) nucleus. In spectrum B, the sharper lines at the center of spectrum A are expanded and the sloping base lines are subtracted. The spectrum was recorded at 6 min, 41 sec. Spectrum C was obtained at 30 min, 31 sec. Spectrum D was obtained by subtracting B from C after normalization. Spectrum E is a simulation of spectrum D obtained with use of hfc constants of 14.3 G (A^N), 11.4 G (A^H), and 1.30 G (A^J) and 1.40 G of peak-to-peak linewidth.

A similar experiment performed in the presence of ¹³C-enriched bicarbonate buffer exhibits a spectrum identical to that obtained in natural-abundance bicarbonate, and no CO₂⁻ adduct ($A^N = 15.6$, $A^H = 18.7$) (15, 16) was observed throughout EPR measurements in this investigation. It has been suggested that DMPO-OOH can decompose or disproportionate partially into DMPO-OH or 5,5-dimethyl-2-ketopyrrolidino-1-oxyl (DMPOX) (17, 18) and partially into a nonradical species, such as hydroxylamine (17-20). However, no absorption lines from DMPOX ($A^N = 7.1$ G, $A^H = 4.2$ G) or nitroxide from oxidized DMPO ($A^N = 15.3$ G, $A^H = 22.0$ G) (18) were observed in this investigation. Thus, OH· and O₂⁻/HO₂⁻ are the only oxidation products trapped by DMPO.

In the earlier period of the reaction, the resonance amplitude of DMPO-OH is much larger than that of DMPO-OOH (spectrum B). However, the rate constant of trapping OH· by DMPO, $2.1-5.7 \times 10^9$ M⁻¹·sec⁻¹ (16, 21, 22), is also much greater than those of O₂⁻ (10 M⁻¹·sec⁻¹), and HOO· (6.6×10^3 M⁻¹·sec⁻¹) (22). In view of these data, the major product of the reaction is O₂⁻/HO₂⁻, at least 10⁵-fold more concentrated than OH· radical. Spectra B and C illustrate that the relative amplitude of the resonance lines of DMPO-OOH and DMPO-OH increases with time. This observation is particularly unexpected when we consider previous reports (17, 18, 23) showing that the O₂⁻/HO₂⁻ adduct is unstable in aqueous solution. However, a recent report (24) showed that DMPO-OH adducts are unstable in the presence of excess O₂⁻. Experiments carried out in the presence of superoxide dismutase showed that the resonance signals due to DMPO-OOH disappeared completely, but the signal amplitudes of DMPO-OH adducts increased (data not shown). Thus, a similar reaction may occur in this system. When a reaction was carried out in 50 mM Hepes buffer at pH 7.5, no resonance lines due to DMPO-OOH adduct were detected, indicating the importance of bicarbonate/CO₂ buffer or the radical-scavenging effect of organic buffer.

Formation and Identification of L-Leucine-Derived Hydroxynitroxide Radical. Addition of L-leucine followed by an injection of H₂O₂ into a similar reaction mixture containing Mn(II) and DMPO in bicarbonate/CO₂ buffer generates a

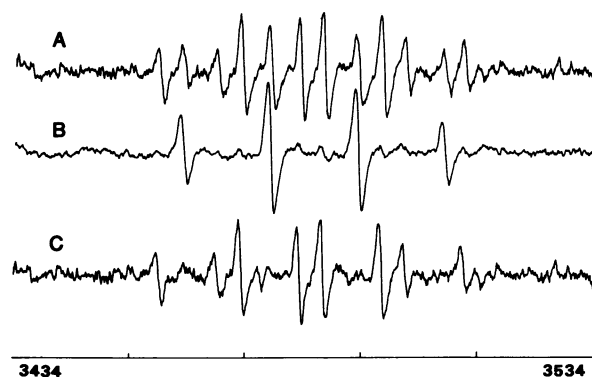


FIG. 2. First-derivative EPR spectra (3434-3534 G) of DMPO-radical adducts formed in a reaction mixture containing 50 mM L-leucine, 0.1 mM MnCl₂, 100 mM DMPO, and 30 mM H₂O₂ in NaHCO₃/CO₂ buffer (pH 7.5). Spectrum A was recorded at 1 min, 55 sec. Spectrum B was taken at 2 hr, 30 min. Spectrum C resulted by subtraction of B from A.

new set of absorption lines (Fig. 2, spectra A and B) which replace those of the DMPO-OOH adduct. Spectrum C in Fig. 2 (and upper spectrum A in Fig. 3) is the resultant spectrum obtained by subtraction of B from A. The resonance signal pattern represents hyperfine interaction with $A^N = 14.04$, $A^H = 10.07$, and $A^H = 14.04$ G as shown in the simulated spectrum (lower spectrum A in Fig. 3). The hfc constants, 14.04 G (1 N) and 10.07 G (1 H), are similar to those of DMPO-peroxy radical adducts (17, 25-27). However, an identical spectrum can also be obtained in the absence of DMPO.

For the assignment of hfc constants and the structural identification of this radical, similar experiments were performed in various isotope-enriched reaction mixtures. Some of these spectra, with subtraction of resonance signals originating from the DMPO-OH adduct, corresponding simulated spectra, and their EPR parameters are shown in Figs. 3 and 4 and Table 1. The upper spectrum B in Fig. 3, obtained from the reaction mixture prepared with ¹⁵N-enriched L-leucine, exhibits a different hyperfine splitting pattern that can be analyzed with hfc constants of $A^{15N} = 19.75$, $A^H = 10.07$, and $A^H = 14.04$ G. The ratio of hfc constants, $A^{15N}/A^{14N} = 1.40$, is identical to that of nuclear *g* factors, clearly indicating that the resonance signals observed involve the amino group of leucine.

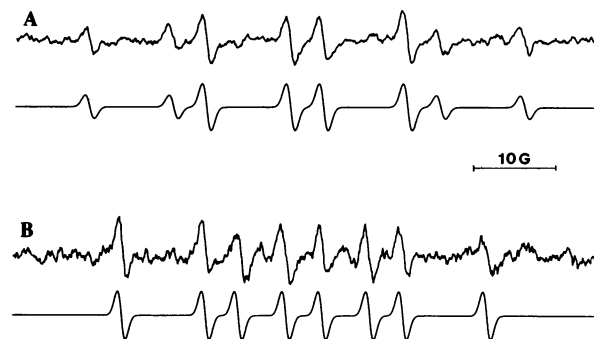


FIG. 3. First-derivative EPR spectra of leucine-derived radicals (not DMPO adducts) produced in a reaction mixture containing 0.1 mM MnCl₂, 100 mM DMPO, 30 mM H₂O₂, and isotope-enriched 50 mM L-leucine in NaHCO₃/CO₂ buffer in H₂O (pH 7.5). The sloping baseline and the absorption lines due to DMPO-OH adduct were subtracted. Spectra A, natural-abundance L-leucine in H₂O (upper spectrum) and its simulated spectrum (lower spectrum). Spectra B, L-[¹⁵N]leucine in H₂O and its simulation. The hfc constants are listed in Table 1.

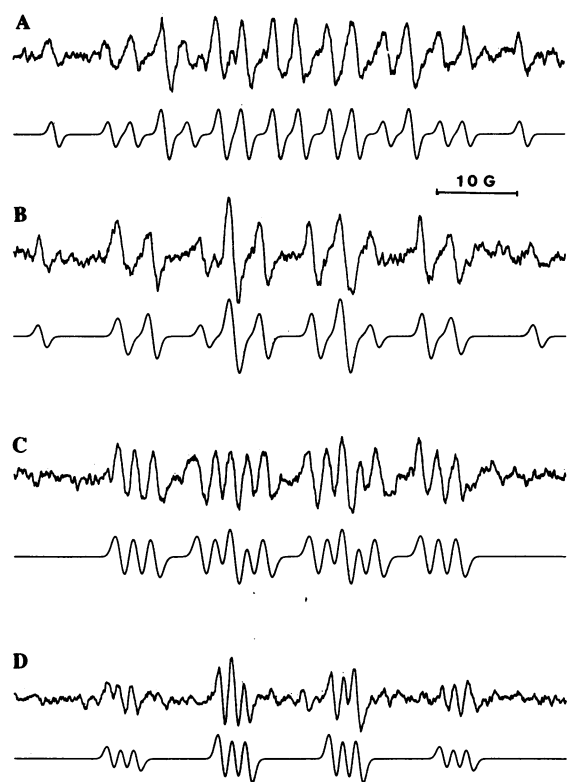


FIG. 4. First-derivative EPR spectra and simulated spectra (below each spectrum) of leucine-derived radicals produced in a reaction mixture containing 0.1 mM MnCl₂, 100 mM DMPO, 30 mM H₂O₂, and NaHCO₃/CO₂ buffer (pH 7.5). The sloping baselines and the absorption peaks due to DMPO-OH adduct were subtracted. Spectra: A, L-[2-¹³C]leucine in H₂O; B, L-[1-¹³C]leucine in H₂O; C, natural-abundance L-leucine in D₂O; D, L-leucine-*d*₁₀ in H₂O. The hfc constants are listed in Table 1.

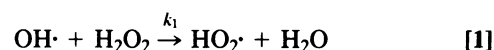
Spectra A and B in Fig. 4 were obtained, respectively, with the reaction mixtures in the presence of L-[2-¹³C]leucine and L-[1-¹³C]leucine. They exhibit extra hyperfine interactions due to 2-¹³C with $A^{13C} = 7.25$ G and 1-¹³C with $A^{13C} = 10.58$ G. To probe the location of two hydrogen atoms with hfc constants of 10.07 and 14.04 G, we have carried out experiments with the reaction mixture prepared in D₂O in the presence of natural-abundance L-leucine (Fig. 4, spectrum C) and in H₂O solution in the presence of L-leucine-*d*₁₀ (Fig. 4, spectrum D). With L-leucine-*d*₁₀ in H₂O, the spectrum shows one deuterium hyperfine interaction with $A^D = 1.65$ G that replaces $A^H = 10.07$ G. From the reaction mixture of natural-abundance L-leucine in D₂O solution, we observed one deuterium hyperfine interaction of $A^D = 2.16$ G in place of $A^H = 14.04$ G, indicating one exchangeable and one nonexchangeable hydrogen atom.

These data demonstrate clearly that the radical produced from L-leucine contains the nitrogen atom of the amino group, α -carbon, carboxyl carbon, one exchangeable hydrogen atom, and one nonexchangeable hydrogen atom. The A^N and exchangeable A^H values of the leucine-derived radical observed here are of the magnitudes expected for an aliphatic alkyl hydronitroxide (28–31) listed in Table 1. Since we observed only one exchangeable hydrogen (the one in the amino group), it is likely that the side-chain residue of the amino acid remains in the radical. We therefore assigned this radical as $\text{OOC(R)CHNH}\cdot$, a hydronitroxide of leucine.

DISCUSSION

It was shown in the preceding papers (8, 11) that Mn(II) ions catalyze the dismutation of H₂O₂ to O₂, and the rate of dismutation follows first-order kinetics with respect to H₂O₂ and Mn(II) and third-order kinetics with respect to HCO₃⁻. The EPR results presented here indicate that OH \cdot and O₂⁻ are intermediates in this dismutation, and that the O₂⁻ radicals are the major product trapped by DMPO. When a similar reaction was carried out in the presence of excess (1 M) ethanol, CH₃CHOH radicals in addition to OH \cdot and O₂⁻ radicals were trapped (data not shown).

The “free” OH \cdot in the bulk solution will react with H₂O₂ and ethanol,



with rate constants $k_1 = 2 \times 10^7$ and $k_2 = 1.8 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$, respectively (32). Since the trapping rates of CH₃CHOH and OH \cdot radicals by DMPO are much larger than that of O₂⁻, it is inconceivable that sufficient amounts of O₂⁻ can be produced by “free” OH \cdot in the medium to provide a detectable level of DMP-OOH adduct under our experimental conditions where DMPO, ethanol, and H₂O₂ were present at 100, 1000, and 30 mM, respectively. Furthermore, when a reaction was carried out under identical conditions except with leucine, a better ligand to metal ions, substituted for ethanol, no resonance lines due to DMPO-OOH adducts were detected. It is therefore very likely that formation of O₂⁻ takes place in the inner coordination sphere of the metal ions where the OH \cdot radicals are generated. Thus, we propose Scheme I as a tentative pathway for the dismutation of H₂O₂ catalyzed by the Mn(II) ion. The Mn(II) ion in the initial complex, 1, is coordinated by three bicarbonate ions (L), two molecules of H₂O₂, and a ligand (X) which is most likely a solvent molecule. Transfer of an electron from Mn(II) to the H₂O₂ at the axial site results in homolytic cleavage of the H₂O₂ and the formation of a transient “caged” OH \cdot radical in 2. Some of the caged OH \cdot radicals will be released to the bulk solution

Table 1. hfc constants of alkyl hydronitroxides (R-NH-O \cdot)

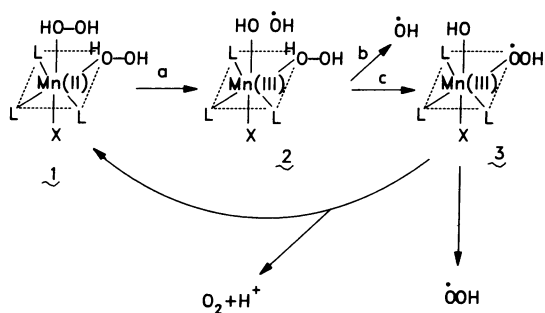
R-	hfc, G				Ref(s).
	A^N	A^{N-H}	$A^{C\alpha-H}$	Others	
H-	12.8	12.8 (2)*			28, 29
CH ₃ -	14.8	14.8	14.8 (3)*		29, 30
CH ₃ CH ₂ -	14.15	14.15	14.15 (2)*		29, 30
(CH ₃) ₂ CH-	14.25	14.25	13.35 (1)*		29, 30
(CH ₃) ₃ C-	14.4	13.9			30, 31
OOC(R)CH-	14.04 [†]	14.04 [‡]	10.07 [§] (1)*	7.25 (2- ¹³ C) 10.58 (1- ¹³ C)	This work

*Number of nuclei.

[†] $A^{15N} = 19.75$ G.

[‡] $A^D = 2.16$ G.

[§] $A^D = 1.60$ G.



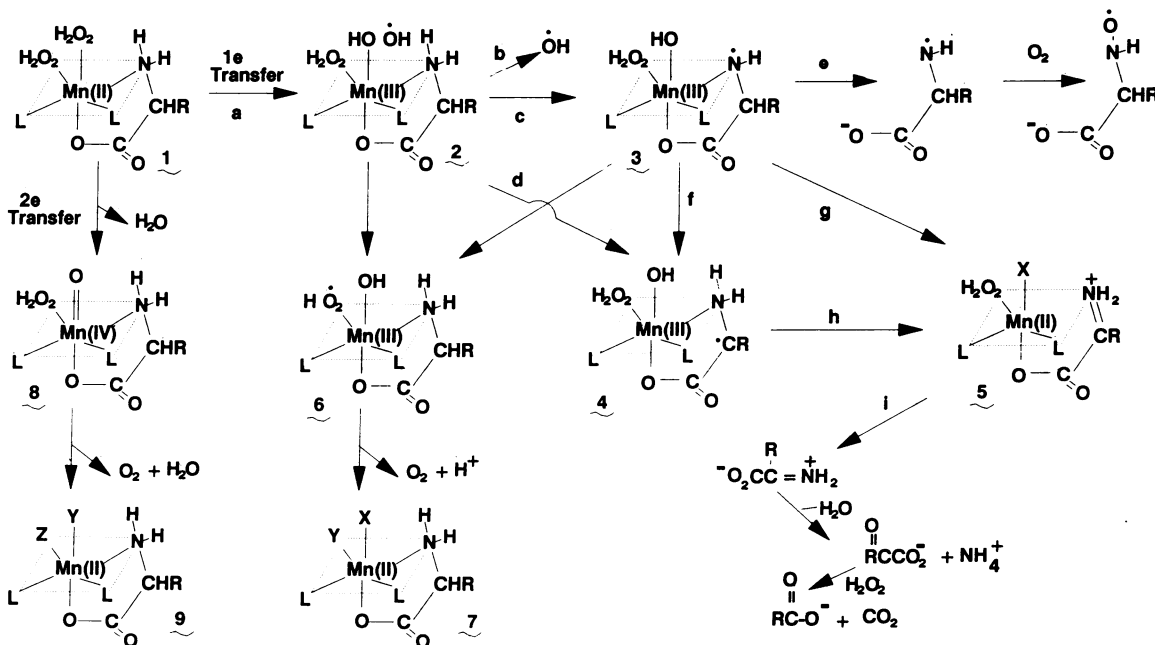
Scheme I.

as free OH· that can be trapped by DMPO or produce CH₃CHOH radical in the presence of ethanol. However, the majority of the "caged" OH· radicals in 2 will undergo intramolecular abstraction of the hydrogen atom from the H₂O₂ bound at an equatorial position to form HO₂· radical. Since the consumption of H₂O₂ is directly proportional to H₂O₂ concentration (8), the homolytic cleavage of H₂O₂ at the axial site is most likely the rate-limiting step.

In the presence of leucine, a leucine-derived hydronitroxide radical is observed. To our knowledge, this is the first report on the observation of an amino acid-derived hydronitroxide radical in the oxidation reaction of amino acids, although stable secondary nitroxides generated from proline and hydroxyproline (33–35) and a hydronitroxide produced from *N*-hydroxyglycine (34) were reported among many amino acids being investigated. In most cases, carbon-centered radicals are the only products in the oxidation of amino acids by H₂O₂ catalyzed by metal ions detected directly by continuous-flow experiments (36, 37) or by the spin-trapping method (37, 38). Thus, the observation of the hydronitroxide of leucine may imply an alternative route in the oxidation of amino acids by the H₂O₂/metal ion system. It is certain that an α -carbon radical is not the precursor for the observed radical, because we did not observe α -hydrogen or α -deuterium exchange in the reaction mixture of leucine-*d*₁₀ in H₂O or natural-abundance leucine in D₂O.

In the Mn(II)/HCO₃⁻ system, leucine enhances the rate of O₂ formation by a factor of 4, and it also alters the HCO₃⁻ dependence to second-order (11). In addition to O₂ generation,

leucine is oxidized to yield α -keto acid, isovaleric acid, NH₄⁺, and CO₂ (11). To account for all of the experimental observations we propose Scheme II for the oxidation of leucine. Complexes 1 and 2 and steps a–d are similar to those in Scheme I, except for a bidentate coordination of an amino acid in place of a bicarbonate and a solvent molecule. The caged OH· generated in complex 2 can be released to the bulk medium or undergo intramolecular hydrogen-atom abstraction from (i) the amino group of the metal-bound leucine to produce an acid amine radical complex 3, (ii) the α -carbon group to produce aminoalkyl radical complex 4, or (iii) the metal-bound H₂O₂ to form a metal-bound O₂⁻ as shown in complex 6. Some of the metal-bound acid amine radicals may also be released into the bulk medium and proceed to form leucine-derived hydronitroxides by reacting with O₂ or OH· (39). However, the majority of complex 3 will undergo rearrangement to form aminoalkyl radical 4, followed by one-electron transfer from the radical to reduce metal ion and the release of alkylimine as indicated by the reactions j and i. Alternatively, complex 3 can also proceed via reactions h and i to form alkylimine. The latter will be hydrolyzed to produce α -keto acid, isovaleric acid, NH₄⁺, and CO₂. Although processes of the rearrangement and further oxidation may occur after detachments of free radicals from the metal ion, so far we have been unable to detect or trap an acid amine radical or an aminoalkyl radical. It has been reported that alkylamine radicals are very unstable and can be detected only with a flow system in conjunction with spin traps (30). Aminoalkyl radicals, however, have been observed during photolysis of the amino acid/H₂O₂ system (37). Therefore, we tentatively favor a mechanism in which the rearrangement and oxidation processes occur within the inner coordination sphere. The formation of a metal-coordinated amine radical is an important step and the ability to form an alkylimine intermediate is essential for the oxidation of amino acid. Our preliminary results in the catalytic system of α -methylalanine show that while the hydronitroxide radical of this amino acid is formed and the dismutation of H₂O₂ occurs in this reaction mixture, the oxidation products of the amino acid are negligible. The most probable reason for this phenomenon is (i) the difficulty of rearranging its acid amine radical to form the corresponding alkylimine, either directly or via the aminoalkyl radical, or (ii) the difficulty of forming the aminoalkyl radical



Scheme II.

through step d. A full description of these results will be published later.

The formation of CO₂ can be accounted for by the reaction pathways involving steps c–i. However, to explain the observed O₂ generation it is necessary to include two alternative pathways in Scheme II. One is a one-electron transfer and the other is a two-electron transfer mechanism. The first mechanism calls for a homolytic cleavage of H₂O₂ and oxidation of Mn(II) to Mn(III). The caged OH· could extract a hydrogen atom intramolecularly from either the amino group or H₂O₂, to form a metal-bound O₂^{•-} (complex 6) which then reduces Mn(III) to Mn(II) and generates O₂. The second mechanism involves a two-electron transfer from Mn(II) to H₂O₂ followed by heterolytic cleavage of the O—O bond. This process invokes the generation of a Mn(IV)=O complex (complex 8) that will then react with another H₂O₂ molecule to form O₂ and H₂O. The involvement of higher-oxidation-state Mn-oxo complexes, Mn(IV)=O and Mn(V)=O, in the catalytic transfer of oxygen atoms is well documented in the Mn(III)/porphyrin system in nonaqueous solution (40–43). Mansuy and coworkers (42) proposed a Mn(V)=O complex as the intermediate oxidant for the dismutation of H₂O₂ and hydrocarbon oxidation catalyzed by Mn(III)/porphyrin system. The Mn(III) ⇌ Mn(V) redox cycle is a two-electron process that does not involve free radical intermediates. In our system, this corresponds to the Mn(II) ⇌ Mn(IV) redox cycles.

While our investigation is continuing for the detection of acid amine and aminoalkyl radicals as well as metal ion complexes, the proposed Schemes I and II essentially account for the combined results of this EPR study and the preceding papers on kinetic and product analysis (8, 11). Our reaction schemes are similar to the mechanism proposed by Anderson and Norman (30) for the oxidation of *N*-alkylhydroxylamines by Ti(III) ions. Our EPR study showing that OH· and O₂^{•-} are intermediates of the H₂O₂ dismutation reaction catalyzed by Mn(II)/HCO₃⁻ is in accord with the work of Sychev *et al.* (44). They concluded that OH· and O₂^{•-} are intermediates of the reaction based on the finding that OH· and O₂^{•-} scavengers, such as *N,N*-dimethyl-*p*-nitrosoaniline and tetranitromethane, respectively, inhibit the Mn(II)/HCO₃⁻-catalyzed dismutation of H₂O₂. In addition, our data indicate that formation of OH·, O₂^{•-}, and leucine-derived hydronitroxide radicals occurs mainly in the inner coordination sphere of Mn. Inner- and outer-coordination sphere mechanisms have been proposed by Sychev *et al.* (45, 46) for the peroxidation of organic dyes catalyzed by Mn/HCO₃⁻ systems. However, their mechanistic schemes (47) and kinetics of HCO₃⁻ dependency are different from those determined in our studies.

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