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Manganese(II) catalyzes the bicarbonate-dependent oxidation of amino acids by hydrogen peroxide and the amino acid-facilitated dismutation of hydrogen peroxide

(Fenton reactions/oxygen radical/leucine oxidation/α-methylalanine oxidation/radical-mediated cage reactions)

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ABSTRACT **In bicarbonate/CO_2 buffer, Mn(II) and Fe(II) catalyze the oxidation of amino acids by H_2O_2 and the dismutation of H_2O_2. As the Mn(II)/Fe(II) ratio is increased, the yield of carbonyl compounds per mole of leucine oxidized is essentially constant, but the ratio of α-ketoisocaproate to isovaleraldehyde formed increases, and the fraction of H_2O_2 converted to O_2 increases. In the absence of Fe(II), the rate of Mn(II)-catalyzed leucine oxidation is directly proportional to the H_2O_2, Mn(II), and amino acid concentrations and is proportional to the square of the HCO_3^- concentration. The rate of Mn(II)-catalyzed O_2 production in the presence of 50 mM alanine or leucine is about 4-fold the rate observed in the absence of amino acids and accounts for about half of the H_2O_2 consumed; the other half of the H_2O_2 is consumed in the oxidation of the amino acids. In contrast, O_2 production is increased nearly 18-fold by the presence of α-methylalanine and accounts for about 90% of the H_2O_2 consumed. The data are consistent with the view that H_2O_2 decomposition is an inner sphere (cage-like) process catalyzed by a Mn coordination complex of the composition Mn(II), amino acid, $(HCO_3^-)_2$. Oxidation of the amino acid in this complex most likely proceeds by a free radical mechanism involving hydrogen abstraction from the α-carbon as a critical step. The results demonstrate that at physiological concentrations of HCO_3^- and CO_2, Mn(II) is able to facilitate Fenton-type reactions.**

Whereas Mn is an active-site catalyst in some superoxide dismutases (1, 2) and catalases (3), the biological significance of nonprotein Mn complexes in catalysis of Fenton-type reactions was discounted until recently because of the unfavorable redox potentials of such complexes under physiological conditions. Nevertheless, Mn(II)–inorganic pyrophosphate complexes (4–6), Mn(II)–desferrioxamine derivatives (7–9), and Mn(II)–aminopolycarbonate complexes (10) catalyze the dismutation of superoxide anion (O_2^-), and Mn(II)–bicarbonate complexes catalyze the disproportionation of H_2O_2 (11–13) at physiological pH values. Such complexes provide protection of some cells against oxygen toxicity (5) and may explain the ability of Mn(II) to protect enzymes from Fe(III)-catalyzed inactivation by O_2 in the presence of enzymic (14–16) and nonenzymic (17) electron-donor systems. In the preceding paper (13), we showed that in bicarbonate buffer, Mn(II) catalyzes the disproportionation of H_2O_2.

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad [1]$$

We now report that Mn(II) alters the distribution of products formed in the oxidation of amino acids by Fenton's reagent [i.e., Fe(II) or Fe(III) + H_2O_2] in bicarbonate buffer and that, in bicarbonate buffer, Mn(II) by itself [in the absence of Fe(II)] catalyzes the oxidation of amino acids. Moreover, amino acids stimulate considerably the Mn(II)–bicarbonate-dependent disproportionation of H_2O_2.

In the next paper (18), we will present evidence for the generation of OH· and O_2^- radicals in H_2O_2/HCO_3^-/Mn(II) reaction mixtures and for the generation of an amino acid-derived radical species when amino acids are added to such mixtures (18).

MATERIALS AND METHODS

Manometric Measurement of H_2O_2 Decomposition. The metal-ion-catalyzed oxidation of amino acids by H_2O_2 is almost entirely accounted for by reactions **2–4** (19).

$$2H_2O_2 + RC\overset{+}{H}NH_3COO^- \rightarrow RCOO^- + CO_2 + NH_4^+ + 2H_2O \quad [2]$$

$$H_2O_2 + RC\overset{+}{H}NH_3COO^- \rightarrow RC(O)COO^- + NH_4^+ + H_2O \quad [3]$$

$$H_2O_2 + RC\overset{+}{H}NH_3COO^- \rightarrow RCHO + NH_4^+ + HCO_3^- \quad [4]$$

In bicarbonate buffer, the Mn(II)-dependent oxidation of leucine proceeds mainly (80–90%) by reaction **2**. Therefore, the oxidation of amino acids in bicarbonate/CO_2 buffer can be monitored by manometric measurement of the change in pressure associated with the production of CO_2 (20). Because Mn(II) also catalyzes reaction **1** (13), the change in pressure observed during the oxidation of amino acids is due to both O_2 and CO_2. Therefore, two Warburg flasks containing identical reaction mixtures were incubated in parallel. An oxygen scavenger, Oxsorbant (Burrell, Pittsburgh), was present in the sidearm of just one of the two flasks throughout the incubation period. Thus, the pressure changes in the samples incubated in the presence and absence of Oxsorbant were due to CO_2 and $CO_2 + O_2$, respectively, and the difference in pressure changes between the two samples is a measure of O_2. Because Oxsorbant is rapidly decomposed when exposed to air, a Hamilton syringe was used to introduce 0.3 ml of Oxsorbant through the capillary stem of the vented stopper, but only after prior equilibration of the vessel and its contents with the CO_2/N_2 gas mixture, and before H_2O_2 in the other sidearm was mixed with reactants in the main compartment. To determine the effect of pH and HCO_3^- concentration on the rate of H_2O_2 utilization, the

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reaction mixtures containing various concentrations of $NaHCO_3$ were equilibrated with N_2 gas mixtures containing 5%, 10%, 15%, or 20% CO_2. The relationship between pH and HCO_3^- concentration and the partial pressure of CO_2 in the gas phase was determined as described by Umbriet *et al.* (20).

Solutions of H_2O_2 were prepared by dilution of a 30% stock solution from Fisher and were standardized by absorbance measurements at 240 nm (ε = 44.5 $M^{-1}\cdot cm^{-1}$). The content of H_2O_2 in reaction mixtures was measured by the method of Thurman *et al.* (21). Amino acids were determined by spectrophotometric measurement of the *o*-phthaldialdehyde derivative as described by Church *et al.* (22), following removal of NH_3 by microdiffusion (23). Ammonia was determined by reaction with Nessler's reagent (24) after its separation by microdiffusion into 0.25 M H_2SO_4 (23).

Isovaleric and α-ketoisocaproic acids produced in the oxidation of leucine were identified and quantified by HPLC. Reaction mixtures were adjusted to pH 2.25 with phosphoric acid, and 100–200 μl of the acidified mixture was injected onto an IBM C_{18} column equilibrated with an aqueous solution of phosphoric acid (pH 2.25). Reaction products were eluted with a 0–50% (vol/vol) methanol gradient in aqueous phosphoric acid (pH 2.25). The elution profile was monitored at 210 nm, using absorbance at 550 nm as a reference. With a flow rate of 2.0 ml/min, the retention times for isovaleric acid, α-ketoisocaproic acid, and leucine were 10.3, 9.2, and 2.5 min, respectively. Significant quantities of other compounds were not detected.

Carbonyl Compounds. The concentrations of α-keto acids and of aldehydes were determined by HPLC of the 2,4-dinitrophenylhydrazone derivatives. Aliquots (0.1 ml) of the reaction mixtures were added to 0.3 ml of reagent solution (0.1% 2,4-dinitrophenylhydrazine in 2 M HCl). After 15 min at room temperature, 1.6 ml of absolute methanol was added and 100 μl of the mixture was injected onto an IBM C_{18} column equilibrated with dilute phosphoric acid solution (pH 2.25). The hydrazones were eluted with an 80–94% methanol gradient obtained by mixing appropriate amounts of absolute methanol with aqueous phosphoric acid (pH 2.25). The elution profile was monitored at 360 nm, using absorbance at 575 nm as the reference, with a flow rate of 1 ml/min. The cis and trans isomers of α-ketoisocaproic hydrazone were eluted at 3 and 4 min, respectively; the isovaleraldehyde derivative was eluted at 4.5 min. The amount of each derivative was calculated from the areas under the respective peaks by using solutions of authentic hydrazone derivative, treated in an identical manner, as reference standards. Significant amounts of hydrazones other than those of α-ketoisocaproic acid and isovaleraldehyde were not detected; however, the sum of the α-ketoisocaproic and isovaleraldehyde determined in this manner was about 75% of the amount of total carbonyl compound as determined by direct assay of the reaction mixtures by the method of Friedeman and Haugen (25).

Materials. Reagents were obtained from commercial sources as follows: Nessler's reagent from Mallinckrodt; 2,4-dinitrophenylhydrazine, isovaleraldehyde, and Hepes from Fluka; α-ketoisocaproic acid, *o*-phthaldialdehyde, ADP, and leucine from Sigma; 2-mercaptoethanol from Aldrich; sodium dodecyl sulfate, from Bio-Rad; alanine from Calbiochem. Sources of other reagents were as previously reported (13). All buffers and amino acid solutions were treated with Chelex 100 (Bio-Rad) prior to use.

RESULTS

Leucine Oxidation in the Presence of Fe(II) and Mn(II). The oxidation of leucine by Fenton's reagent [H_2O_2/Fe(II)] plus ADP in bicarbonate/CO_2 buffer (pH 7.6) leads to the production of NH_4^+, isovaleraldehyde, α-ketoisocaproate, and isovalerate (19). Addition of Mn(II) to Fe(II)-containing reaction mixtures decreases the rate of leucine oxidation as measured by the amounts of NH_4^+ and carbonyl compounds formed (Fig. 1*A*). As the concentration of Mn(II) was increased from 0.1 to 1.0 mM, the total yield of carbonyl compounds (isovaleraldehyde and α-ketoisocaproate) per mole of NH_4^+ produced remained essentially constant; however, there was a progressive increase in the yield of α-ketoisocaproate and a progressive decrease in the yield of isovaleraldehyde per mole of leucine oxidized, i.e., per mole of NH_4^+ produced (Fig. 1*B*). At all concentrations of Mn(II), little or no H_2O_2 remained after 10 min. By taking into account the amounts of carbonyl compounds and NH_4^+ produced, it can be calculated (see legends to Table 1 and Fig. 1) that in reaction mixtures containing 5 μM Fe(II), the fraction of H_2O_2 which was decomposed to O_2 (reaction **1**) increased from 11% to 40% as the level of Mn(II) was varied from 0 to 1 mM (Fig. 1*A*).

Mn(II)-Dependent Oxidation of Amino Acids in the Absence of Fe(II). In bicarbonate buffer, Mn(II) by itself [no Fe(II)] catalyzes the dismutation of H_2O_2 and the oxidation of amino acids (Fig. 2, Table 1). Negligible amounts of gas are produced in the absence of added divalent cation. For reaction mixtures containing amino acids, the production of carbonyl compounds, O_2, and CO_2 by reactions **1–4** account for about 90% of the H_2O_2 consumed (Table 1). In the absence of amino acid, O_2 is the only gas formed. About 0.5 mole of O_2 is formed per mole of H_2O_2 decomposed, as predicted by reaction **1** (Table 1).

In reaction mixtures containing leucine or alanine, about half of the H_2O_2 is due to reaction **2**; the other half is attributable to the disproportionation of H_2O_2 (reaction **1**). However, in the presence of α-methylalanine, the disproportionation of H_2O_2 accounts for 90% of the H_2O_2 consumed (Table 1). Very little oxidation of α-methylalanine occurs, as evidenced by the low yields of CO_2, NH_4^+, and carbonyl compounds. These results are therefore consistent with the view (26) that metal ion-catalyzed oxidation of α-amino acids

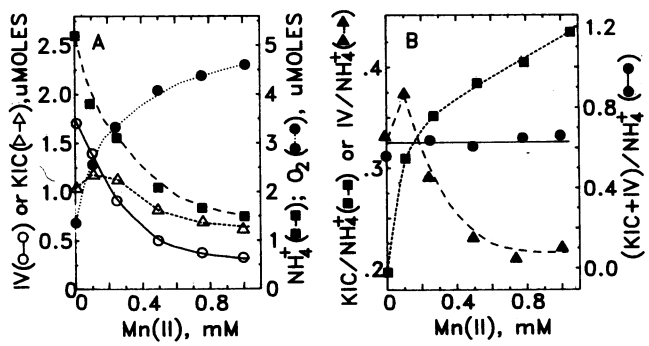


FIG. 1. Effect of Mn(II) on Fe(II)-catalyzed oxidation of leucine and H_2O_2 decomposition. Reaction mixtures (0.37 ml) in 12 × 75 mm test tubes contained initially 23.5 mM $NaHCO_3$, 125 μM ADP, 50 mM leucine, and 0–1 mM Mn(II) as indicated. By means of a 20-gauge hypodermic needle inserted along the side of the loosely stoppered test tube, each sample was deaerated for 30 sec by passing a vigorous stream of gas (5% CO_2/95% N_2) over the surface of the solution. Then 0.01 ml of 2 mM $FeSO_4$ was added. After further gassing for 30 sec, 0.02 ml of 600 mM H_2O_2 was added. Gassing was continued for 30 sec and then the needle was withdrawn and simultaneously the stopper was tightly sealed. After incubation at 37°C for 10 min, the concentrations of isovaleraldehyde (IV), α-ketoisocaproic acid (KIV), NH_4^+, H_2O_2, and leucine were measured as described in *Materials and Methods*. The amount of O_2 produced was calculated as 0.5[H_2O_2 consumed − (NH_4^+ produced + isovalerate produced)]. This assumes that the oxidation of leucine and the formation of O_2 are almost entirely attributable to reactions **1–4** (see Table 1 for verification).

Table 1. Mn(II)-dependent oxidation of amino acids and H_2O_2 decomposition

	Amount, μmol			
	− Leu	+ Leu	+ Ala	+ α-MeAla
CO_2 evolved	—	11.5	11.6	1.9
O_2 formed	8	12.1	16.5	24.1
Carbonyl compounds formed	—	3.2	0.74	0.56
NH_4^+ formed	—	12.4	12.9	3.7
Leucine consumed	—	11.8	—	—
H_2O_2 consumed				
Observed	17	53	60	60
Calculated	16	50	57	53

Reactions were carried out at 37°C in double-sidearm Warburg flasks. The reaction mixture (2.0 ml) contained initially 23.5 mM $NaHCO_3$, 30 mM H_2O_2, 50 μM Mn(II), and, where indicated, 50 mM amino acid. After 60 min, the amounts of H_2O_2, leucine, NH_4^+, and carbonyl compounds in the reaction mixtures were measured. In the last two lines of the table, the amount of H_2O_2 consumed during the reaction as determined by direct measurement (observed) is compared with the amount expected (calculated) on the assumption that 2 moles of H_2O_2 are consumed for each mole of CO_2 (reaction **2**) and each mole of O_2 (reaction **1**) produced and that only 1 mole of H_2O_2 is consumed for each mole of carbonyl compound formed (reactions **3** and **4**).

involves radical-mediated abstraction of the hydrogen atom from the α-carbon (see *Discussion*).

Kinetics of the Mn(II)-Dependent Reactions. Semilogarithmic plots of H_2O_2 concentration versus time, at various concentrations of either HCO_3^-, Mn(II), leucine, or alanine, at different partial pressures of CO_2, and at various pH values, are all linear for at least 20 min (Fig. 3*A*). In contrast, semilogarithmic plots of data obtained with α-methylalanine are biphasic (Fig. 3*B*). The slope of the log[H_2O_2]-versus-time curve during the first 20 min is only about half that ultimately obtained. It is therefore apparent that in the presence of α-methylalanine, the catalytic efficiency of the Mn complex increases with time, suggesting that there is a time-dependent change in the composition and/or structure of the Mn coordination complex.

The rate of H_2O_2 decomposition is directly proportional to the concentrations of Mn(II) and leucine (Fig. 4).

Decomposition of H_2O_2 in the Presence of Amino Acids Is Second-Order with Respect to HCO_3^- Concentration. The oxidation of leucine and α-methylalanine was investigated at various pH values ranging from 7.4 to 8.0 and at HCO_3^- concentrations varying from 15 to 60 mM. To maintain the pH at a given value, the samples were equilibrated with N_2/CO_2 gas mixtures containing 5%, 10%, or 20% CO_2 (13). The apparent rate constant, k_{app}, for H_2O_2 decomposition in the presence of either leucine or α-methylalanine increases linearly with respect to the square of the HCO_3^- concentration (Fig. 5).

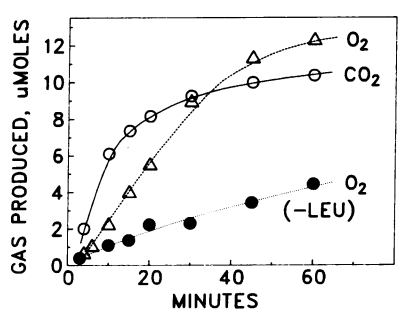


FIG. 2. Production of CO_2 and O_2 during the Mn(II)-dependent decomposition of H_2O_2 in the presence or absence of leucine. Reactions were carried out in a Warburg apparatus as described in the legend to Table 1. The mixtures (2.0 ml) contained initially 50 μM Mn(II), 23.5 mM $NaHCO_3$, 30 mM H_2O_2, and either 50 mM (open symbols) or 0 mM (closed symbols) leucine.

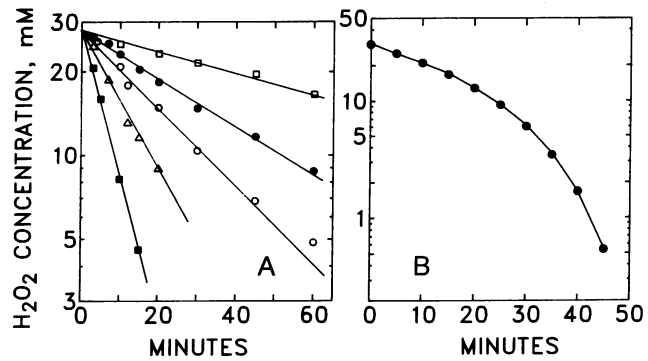


FIG. 3. Semilogarithmic plots of H_2O_2 concentration versus time under various experimental conditions. All reactions were carried out in a Warburg apparatus. The amount of gas produced under each condition was monitored manometrically. The concentration of H_2O_2 at various times was calculated on the assumption that 2 moles of H_2O_2 are consumed for each mole of gas formed (reactions **1** and **2**). This calculation does not take into account the relatively small amount of H_2O_2 consumed in the production of carbonyl compounds (*cf.* Table 1). (*A*) Initially, all reaction mixtures contained 27 mM H_2O_2 and were equilibrated with a CO_2/N_2 gas mixture containing 5% or 20% CO_2. Otherwise, mixtures were varied as follows: □, 23.5 mM $NaHCO_3$, 100 μM Mn(II), 5% CO_2, pH 7.6; ●, 50 μM Mn(II), 50 mM leucine, 18.5 mM $NaHCO_3$, 5% CO_2, pH 7.5; ○, 50 μM Mn(II), 50 mM leucine, 23.5 mM $NaHCO_3$, 5% CO_2, pH 7.6; △, 50 μM Mn(II), 50 mM leucine, 66 mM $NaHCO_3$, 20% CO_2, pH 7.45; ■, 50 μM Mn(II), 50 mM leucine, 94 mM $NaHCO_3$, 20% CO_2, pH 7.6. (*B*) Initially, the reaction mixture contained 50 mM α-methylalanine, 30 mM H_2O_2, 50 μM Mn(II), and 23.5 mM $NaHCO_3$, and were equilibrated with 5% CO_2/95% N_2 (pH 7.6).

Since 50% and 90% of the H_2O_2 consumed in the presence of leucine and α-methylalanine, respectively, is due to reaction **1**, it is evident from the data in Fig. 5 that the dismutation of H_2O_2 is increased nearly 4-fold by the presence of leucine and about 18-fold by the presence of α-methylalanine.

DISCUSSION

In the absence of amino acids, the rate of Mn-catalyzed disproportionation of H_2O_2 in bicarbonate buffer is directly proportional to the third power of the HCO_3^- concentration (13), suggesting that three equivalents of HCO_3^- react with

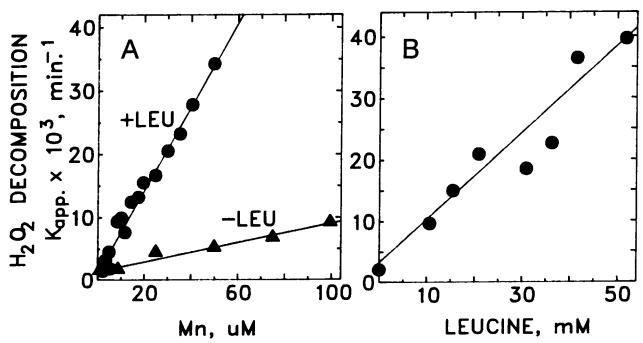


FIG. 4. Effect of leucine and Mn(II) concentration on the apparent rate constant (k_{app}) for H_2O_2 decomposition. All reaction mixtures were equilibrated with 5% CO_2/95% N_2 (pH 7.6) and contained 23.5 mM $NaHCO_3$, and 27 mM H_2O_2. (*A*) Mixtures contained 0 or 50 mM leucine and various amounts of Mn(II) as indicated. (*B*) Mixtures contained 50 μM Mn(II) and leucine as indicated. The half times, $t_{0.5}$, for H_2O_2 decomposition used in calculations of the apparent first-order rate constant ($k_{app} = 0.693/t_{0.5}$) were estimated from linear semilogarithmic plots of H_2O_2 concentration versus time, based on the assumptions described in the legend to Fig. 3.

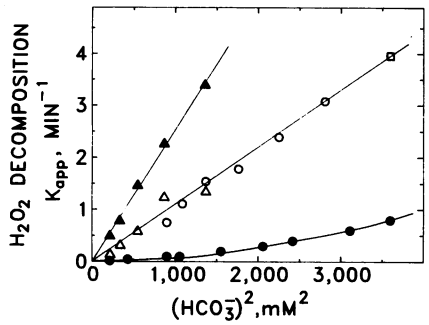


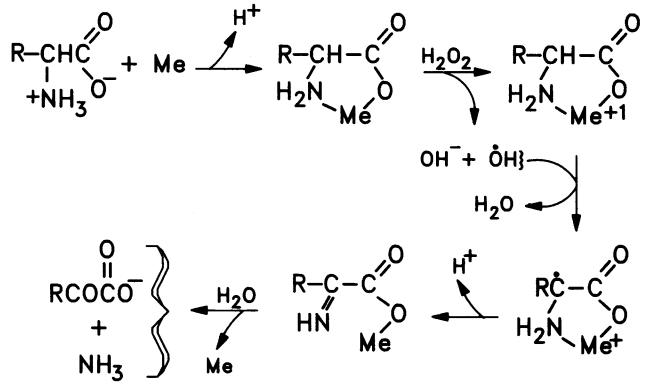
FIG. 5. Decomposition of H_2O_2 in the presence of leucine or α-methylalanine is apparently second-order with respect to HCO_3^- concentration. Reaction mixtures (37°C) contained 2–50 μM Mn(II), 30 mM H_2O_2, $NaHCO_3$ as indicated, and either no amino acid (●), 50 mM α-methylalanine (▲), or 50 mM leucine (open symbols). Mixtures containing no amino acid or α-methylalanine were equilibrated with 5% CO_2/95% N_2. Samples containing leucine (pH 7.6) were equilibrated with gas mixtures containing N_2 and 5% (△), 10% (○), or 20% (□) CO_2. k_{app} is the observed first-order rate constant normalized with respect to the Mn(II) concentration; i.e., $k_{app} = 0.693/t_{0.5}[Mn(II)]$.

Mn(II) to form the catalytically active complex, $Mn^{II}(HCO_3^-)_3$. In contrast, the rate of H_2O_2 decomposition in the presence of amino acids is proportional to the square of HCO_3^- concentration (Fig. 5). Among other possibilities this suggests that an amino acid (Xaa) can displace one equivalent of HCO_3^- in the $Mn^{II}(HCO_3^-)_3$ complex to form a complex, $Mn^{II}(HCO_3^-)_2Xaa$, having an even greater catalytic efficiency, presumably because Mn in the latter complex has a more favorable redox potential.

In addition to simple coordination complexes of Mn(II) with a single amino acid moiety and two equivalents of HCO_3^-, complexes of Mn(II) with carbamino acids and HCO_3^- should also be considered. Carbamino acids are formed by reaction of HCO_3^- with the amino group of amino acids and are known to form stable salts with divalent metal cations (27). Further reaction of the Mn salts of carbamino acids with HCO_3^- could yield complexes containing 2 or 3 equivalents of HCO_3^-. Interconversion of such complexes may be responsible for the time-dependent acceleration in the rate of H_2O_2 decomposition that occurs in the presence of α-methylalanine (Fig. 5). The possibility that such a shift may reflect conversion of the catalytic complex from a Mn(II) ⇌ Mn(III) redox system to a Mn(III) ⇌ Mn(IV) system deserves consideration in light of the implication of cycles of the latter type in the catalytic activity of catalase (3) and nonenzymic systems (28–31). This possibility is discussed further in the following paper (18).

It is noteworthy that unstable, readily oxidizable ternary complexes of amino acids, Fe(II), and ferrozine have been shown to occur as transient intermediates in the formation of the stable, oxidation-resistant Fe^{III}(ferrozine)$_3$ complex (ref. 19 and unpublished data).

It is generally assumed (26) that the oxidation of amino acids by OH· radicals involves abstraction of a hydrogen atom from the α-carbon atom to form a carbon-centered radical; this undergoes further oxidation to the imine, which spontaneously hydrolyzes to yield NH_3 and the α-keto acid, or CO_2 and the aldehyde containing one less carbon atom. Since the metal-ion-catalyzed oxidation of amino acids is not significantly affected by the presence of radical scavengers (unpublished data), the oxidation probably involves a cage-type process in which radical generation and its dissipation occur within the metal (Me)–Xaa–HCO_3^- complex (Scheme I). In this mechanism, Mn(II) is presumed to react with the amino acid (or the carbamino acid) to form a Mn(II)–Xaa complex, with liberation of a proton. Mn(II) in the Mn(II)–



Scheme I.

Xaa complex is then oxidized by H_2O_2 to form OH· plus OH^- and the Mn(III)–Xaa complex. Because the OH· is formed in close proximity to the amino acid moiety of the complex, it will preferentially abstract a hydrogen atom from the α-carbon of the amino acid to form a carbon-centered amino acid radical. This radical can then react with Mn(III) in the complex to regenerate Mn(II) and form the imino acid derivative, which upon spontaneous hydrolysis will yield the α-keto acid and NH_3.

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