Chemical Pathways of Peptide Degradation. VIII. Oxidation of Methionine in Small Model Peptides by Prooxidant/Transition Metal Ion Systems: Influence of Selective Scavengers for Reactive Oxygen Intermediates

Shihong Li,[1] Christian Schöneich,[1] and Ronald T. Borchardt[1,2]

Received June 9, 1994; accepted October 1, 1994

In the presence of oxygen, Fe(III), and an appropriate electron donor (e.g. ascorbic acid, dithiothreitol), the oxidation of methionine residues to methionine sulfoxides in small model peptides can be induced. It is shown in this study that these oxidations can be retarded by catalase in a pH-dependent manner, by some hydroxyl radical scavengers, and by azide. In contrast, superoxide dismutase has only a minimal effect, indicating that the superoxide radical does not contribute significantly to the oxidation of the methionine residue. The experimental results can be interpreted by invoking hydrogen peroxide as the major oxidizing species at pH $\leq$ 7, whereas the involvement of free hydroxyl radicals seems to be negligible. Other reactive oxygen intermediates such as iron-bound hydroperoxy, or site-specifically generated reactive oxygen species may be actively involved in the oxidation of methionine residues at pH > 7.

KEY WORDS: methionine; methionine sulfoxide; free radicals; ascorbate; dithiothreitol; catalase; superoxide dismutase.

INTRODUCTION

The oxidation of methionine (Met) residues is one of the major pathways of covalent post-translational modification of proteins (1), which may result in the loss of their biological activities. This chemical degradation pathway of Met residues in proteins has been associated with several pathological conditions including emphysema, rheumatoid arthritis, and cataracts, as well as aging (2,3). In addition, oxidation of Met residues in therapeutic peptides and proteins presents a major challenge to pharmaceutical scientists as they attempt to develop effective and safe formulations of these drugs for human and animal uses (4).

The rates and yields of Met sulfoxide formation in peptides and proteins have been shown to be dependent on internal (e.g., primary sequence) and external (e.g., pH, metal ions, prooxidants) factors (5,6). The oxidation of Met residues and organic sulfides is generally initiated through the generation of reactive oxygen species (7). Mechanistic studies concerning specifically the superoxide radical ($O_2\cdot^-$) (8), hydroxyl radical (·OH) (6,8), peroxyl radical (ROO·) (9), hydrogen peroxide (H_2O_2) (10), and singlet oxygen ($^1O_2$) (11) have shown that their individual propensities for sulfoxide formation vary considerably.

Recently, studies in our laboratory have shown that prooxidant/transition metal systems such as ascorbic acid (AsA)/Fe(III), and dithiothreitol (DTT)/Fe(III) can effectively promote the oxidation of Met residues in model peptides through the activation of oxygen (5,6). In the present study, we have attempted to identify the reactive oxygen intermediates which are generated by the AsA/Fe(III) and DTT/Fe(III) systems and are ultimately responsible for the oxidative degradation.

METHODS

Materials. His-Met (HM) and Gly-Gly-Gly-Met-Gly-Gly-Gly (GGGMGGG) were selected as model peptides. HM was purchased from Bachem Bioscience, Inc. (Philadelphia, PA). GGGMGGG was synthesized by the Biochemical Service Laboratory at the University of Kansas. Catalase (CAT) (bovine liver, thymol-free, 199,000 U/mg), superoxide dismutase (SOD) (bovine erythrocyte, 5,1000 U/mg), DTT, D-mannitol, and sodium azide were purchased from Sigma Chemical Company (St. Louis, MO). Sodium formate, thiourea, and acetonitrile (HPLC grade) were supplied by Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA, HPLC grade) was purchased from Pierce Chemical Company (Rockford, IL). The water used in all studies was from a Millipore MILLI-Q™ Water System. All the reagents were obtained commercially as the analytical grade.

Reactions. The typical reaction mixtures contained 0.2 mM peptide, 2 mM AsA/0.02 mM $FeCl_3$ or 2 mM DTT/0.02 mM $FeCl_3$ in 10 mM phosphate buffer. The possible involvement of various reactive oxygen intermediates in the oxidation process was examined by the addition of CAT, SOD, ·OH scavengers (such as methanol, formate, mannitol, and thiourea), or a $^1O_2$ scavenger (azide) to the reaction mixtures.

HPLC Analysis. HPLC analysis was performed on a system consisting of a Shimadzu SCL-10A system controller, a Shimadzu LC-10AS pump, a SPD-10A UV spectrophotometric detector, a SIL-10A autoinjector, a sample cooler and a C-R4A chromatopac integrator. The analysis of Met-containing peptides was performed by HPLC using a Keystone Hypersil ODS C_{18} reversed-phase column (4.6 × 250 mm) with a Brownlee RP-18 Spheri-5 cartridge (4.6 × 30 mm) at ambient temperature (25°C), using an isocratic system at a flow rate of 1 ml/min. The mobile phase was a mixture of acetonitrile/water (10:90, v/v) containing 0.1% TFA for HM, and 0.025% TFA for GGGMGGG. Detection of the Met-containing peptides was achieved at 214 nm. The Met-containing peptides and their corresponding sulfoxides were quantified by measuring peak areas, and were standardized by using purified Met-containing peptides and the Met sulfoxide derivatives synthesized in our laboratory by reaction with H_2O_2 (5,6).

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66045.

[2] To whom correspondence should be addressed at Department of Pharmaceutical Chemistry, 3006 Malott Hall, School of Pharmacy, The University of Kansas, Lawrence, Kansas 66045.

ABBREVIATIONS: AsA-ascorbic acid; DTT-dithiothreitol; CAT-catalase; SOD-superoxide dismutase; TFA-trifluoroacetic acid; D-electron donor; L_x-Ligand.

0724-8741/95/0300-0348$07.50/0

CAT Activity Assay. CAT activity was measured using a Shimadzu UV-2100 spectrophotometer coupled to a microcomputer. The assay procedure was based on the UV method outlined by Aebi (12), in which CAT activity was followed by the decomposition of H_2O_2 at 240 nm. H_2O_2 was diluted into 0.05 M phosphate buffer at pH 7.0 to yield a final concentration of 0.01 M. Fresh solutions containing CAT alone and CAT with AsA (5 mM)/$FeCl_3$ (0.02 mM) or DTT (5 mM)/$FeCl_3$ (0.02 mM) were prepared in phosphate buffers (pH 7.0) to yield a final concentration of 2000 U/ml CAT. In a quartz cuvette 3 ml of a diluted H_2O_2 solution was incubated in the spectrophotometer at 25°C. An aliquot (10 μl) was withdrawn from the CAT solution or from its reaction mixtures and rapidly mixed into the cuvette containing H_2O_2 in phosphate buffer. The initial absorbance was between 0.55 and 0.60. The decreases in absorbance at 240 nm were recorded for 2–3 min. ΔA_{240}/min was calculated from the initial (40 s) linear portion of the curve.

RESULTS

Oxidation of HM and GGGMGGG by AsA/Fe(III) and DTT/Fe(III) Systems

Two peptides (HM and GGGMGGG) of different size and sequence were chosen for the present study in order to confirm that the active oxygen species generated in the system was independent of primary sequence. Both peptides were degraded under similar reaction conditions employing 0.2 mM peptide (HM or GGGMGGG), and 2 mM AsA/0.02 mM $FeCl_3$ or 2 mM DTT/0.02 mM $FeCl_3$ in 10 mM phosphate buffer, pH 7.0. The degradation rate of Met peptide and the yield of Met sulfoxide were found to be dependent on the primary sequence (e.g., on the presence of His) and the pH. It was also shown that the kinetics of peptide degradation were dependent on the nature of the prooxidant. Zero order kinetics could be used to fit the HM degradation rate in the AsA/Fe(III) system, whereas the degradation in the DTT/Fe(III) system followed approximately first-order kinetics (see Fig. 1). Higher yields of Met sulfoxide were generally observed in the DTT/Fe(III) system. A mixture of other oxidation products was not characterized in this study.

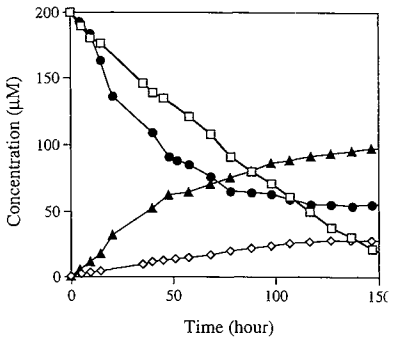


Fig. 1. The oxidation profile of HM to HMSO initiated by the AsA/Fe(III) or the DTT/Fe(III) systems. The reaction mixture contained 0.2 mM HM, 2 mM AsA/0.02 mM $FeCl_3$ or 2 mM DTT/0.02 mM $FeCl_3$ in 10 mM phosphate buffer, pH 7.0. □, HM, AsA/Fe(III): ●, HM, DTT/Fe(III); ◇, HMSO, AsA/Fe(III); ▲, HMSO, DTT/Fe(III).

Determination of the Minimum Concentration of CAT Needed to Inhibit the H_2O_2-Initiated Oxidation of HM

CAT can be used to examine the potential involvement of H_2O_2 in the oxidation of Met residues in peptides and proteins since it catalyzes the decomposition of H_2O_2 to H_2O and O_2 ($k > 10^7\ M^{-1}s^{-1}$) (13). In preliminary studies, the ability of CAT to inhibit the H_2O_2-initiated oxidation of HM was examined at various concentrations of the enzyme. The reaction mixture contained 0.2 mM HM, 2 mM H_2O_2, and different concentrations at CAT ranging from 2 to 2000 U/ml in 10 mM phosphate buffer, pH 7.0. In the absence of CAT, HM was almost completely oxidized to HM sulfoxide (HMSO) within 24 hours. However, 2.39 μM of HMSO were produced in the presence of 2 U/ml of CAT, and 0.81 μM of HMSO in 10 U/ml of CAT. It appeared that a minimum of 20 U/ml of CAT was necessary to completely inhibit the formation of HMSO under the experimental conditions. Theoretically, 2 mM H_2O_2 represents the maximum concentration of H_2O_2 expected from the oxidation of either 2 mM AsA or 2 mM DTT since two reduction equivalents are necessary for the reduction of O_2 to O_2^{2-}. With this in mind, we further investigated the role of H_2O_2 in the oxidation of Met peptides initiated by the AsA/Fe(III) and the DTT/Fe(III) systems.

Effect of CAT on the AsA/Fe(III) or DTT/Fe(III) Initiated Oxidation of HM and GGGMGGG at pH 7.0

The potential role of H_2O_2 in the oxidation of the Met residues in HM and GGGMGGG was investigated by the addition of CAT to the AsA/Fe(III) and DTT/Fe(III) reaction mixtures. Fresh solutions of CAT were prepared and added to the reaction mixture prior to the addition of AsA or DTT. The pH of the reaction mixtures was adjusted to 7.0. As the concentrations of CAT were varied from 200 to 2000 U/ml, there were decreases in the rate of peptide degradation and Met sulfoxide formation. In the AsA system, the addition of CAT produced relatively low inhibition of HM oxidation (Table 1, Fig. 2), whereas the enhancement of HM stability and the inhibition of HMSO formation were far more significant in the DTT/Fe(III) system. For example, 61.1 μM of HMSO was produced in the DTT/Fe(III) solution in the absence of CAT within 48 hours. The formation of HMSO was decreased to 43.3 μM, 27.7 μM and 13.3 μM with the addition of 200 U/ml, 1000 U/ml and 2000 U/ml of CAT, respectively. Similar effects of CAT were also observed for the oxidation of GGGMGGG (Table II). In contrast, heat-inactivated CAT had no effect on the oxidation of HM or GGGMGGG by the AsA/Fe(III) or DTT/Fe(III) systems (Tables I and II). It needs to be mentioned that the yield of Met sulfoxide formation of HM was low. This is simply due to the fact that the His residue can also be oxidized under the same condition. However, the degradation products of His was not characterized in this study.

Effect of pH on the Inhibition of Met Oxidation in HM and GGGMGGG by CAT

Previous results with a variety of model peptides indicated that the oxidation rate of Met and the yield of Met-sulfoxide were dependent on pH, particularly in the pH

Table I. Effects of CAT and SOD on the Oxidation of HM by AsA/Fe(III) and DTT/Fe(III) Systems*

Inhibitor added	Hm (μM) consumption	HMSO (μM) formation	% Inhibition of HMSO
AsA/Fe(III)			
None	62.4	14.0	0
CAT[a] (200 U/mL)	56.9	12.3	2
(1000 U/mL)	50.0	8.24	41
(2000 U/mL)	55.0	6.98	50
SOD[a] (100 U/mL)	43.2	11.5	18
SOD (100 U/mL) + CAT (2000 U/mL)	55.9	6.18	56
DTT/Fe(III)			
None	116	61.6	0
CAT (Boiled)	107	59.7	3
CAT (200 U/mL)	84.8	43.3	30
(1000 U/mL)	45.2	27.7	55
(2000 U/mL)	37.4	13.3	78
SOD (100 U/mL)	118	75.5	0
SOD (100 U/mL) + CAT (2000 U/mL)	39.5	10.0	84

[a] Units of CAT are μmol H_2O_2 destroyed/min and units of SOD activity are defined by the cytochrome C assay.

* Reaction mixtures contained HM (0.2 mM), and AsA (2 mM)/$FeCl_3$ (0.02 mM) or DTT (2 mM)/$FeCl_3$ (0.02 mM) in phosphate buffers (10 mM) at pH 7.0. The incubations were controlled at 25°C for 48 h. All the data are the averages of duplicates. The standard error is less than 5%.

range of 6–8 (5,6). In order to investigate the possibility that different reactive oxygen intermediates were involved in the oxidation process at different pH values, we examined the oxidation of HM initiated by the DTT/Fe(III) system in the presence of CAT (1000 U/ml) and the absence of CAT at pH 6.5, 7.0, 7.5 and 8.0, respectively. As shown in Fig. 3, the addition of CAT produced almost a complete inhibition of HM oxidation and HMSO formation at pH 6.5 and 7.0 over a time period of 1400 min. (Fig. 3a, b). However, only partial inhibition of the oxidation of Met was observed at pH 7.5 (Fig. 3c) and 8.0 (Fig. 3d). Furthermore, the addition of 5 mM H_2O_2 to these reaction mixtures after 400 and 1200 minutes of reaction resulted in a rapid oxidation of HM to HMSO in the solutions lacking CAT, while no additional HMSO formation was observed in solutions containing CAT (Fig. 4). These results indicated that the activity of CAT was still sufficient to scavenge all the H_2O_2 generated in the reaction during this time period. When the same reaction conditions were applied to GGGMGGG, we observed the same trend of pH dependency, i.e., total inhibition by CAT at pH ≤ 7 and partial inhibition at pH > 7 (data not shown).

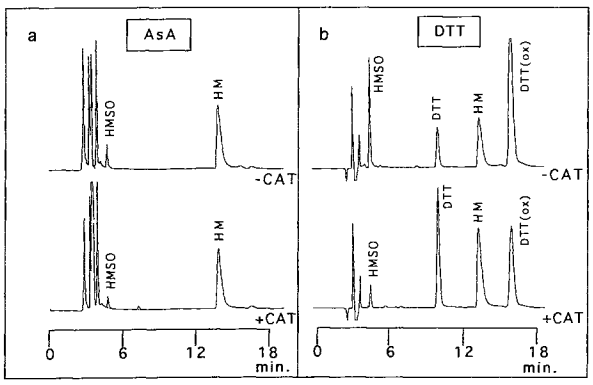


Fig. 2. Chromatograms of the oxidation of HM to HMSO recorded at 48 h in the absence of CAT (top) and the presence of CAT (bottom). (a) The AsA/Fe(III) system contained HM (0.2 mM), AsA (2 mM)/$FeCl_3$ (0.02 mM) and the absence or the presence of CAT (2000 U/ml) in phosphate buffer (10 mM) at pH 7.0, 25°C (b) The DTT system contained HM (0.2 mM), DTT (2 mM)/$FeCl_3$ (0.02 mM) and the absence or the presence of CAT (2000 U/ml) in phosphate buffer (10 mM) at pH 7.0, 25°C.

CAT Activity Assay

Since a considerably higher concentration of CAT (>200 U/ml) was needed for the inhibition of Met oxidation in the AsA/Fe(III) system or DTT/Fe(III) system than for the H_2O_2-induced oxidation, it was possible that CAT was unstable under the reaction conditions employed. The propensity of AsA to inactivate CAT has been reported (14). Under our experimental conditions it was observed that there was a continuous loss of CAT activity when CAT was incubated with either AsA or DTT in the presence of Fe(III) (Fig. 5). In contrast, CAT itself was fairly stable in the absence of AsA/Fe(III) and DTT/Fe(III) and no loss of activity was observed during the same time period. However, based on the results shown in Fig. 4, there must be sufficient CAT activity even after 400 or 1200 min. of incubation to scavenge exogeneously added H_2O_2. Thus the enzyme could not have been totally inactivated within this time period under the experimental conditions.

Effects of SOD and Mixtures of SOD and CAT on the Oxidation of Met Peptides at pH 7.0

SOD catalyzes the dismutation of $O_2\cdot^-$ to H_2O_2. The available evidence indicates that $O_2\cdot^-$ itself exhibits fairly low reactivity toward organic sulfides and Met. However, it may serve as a source for secondary reactive oxygen species such as H_2O_2. Both SOD and CAT solutions were freshly prepared and added to the same reaction mixtures prior to the addition of electron donors (i.e. AsA or DTT). As shown in Tables I and II, SOD alone had only a slight effect on the oxidation of HM and GGGMGGG in the DTT/Fe(III) system. Even though a slight inhibitory effect of SOD was observed for the HM oxidation in the AsA/Fe(III) system, the role of $O_2\cdot^-$ can be considered negligible. A combination of CAT and SOD would ensure that all the resultant H_2O_2 from the SOD-catalyzed dismutation of $O_2\cdot^-$ be removed from the reaction mixture (15). However, the protective effect of a combination of SOD and CAT in our experiments is not statistically more significant than that when using CAT alone. For example, without the presence of either CAT or SOD, the formation of HMSO amounts to 61.6 μM in the DTT/Fe(III) system. Whereas 10.0 μM of HMSO was observed in the combination of CAT and SOD compared to 13.3 μM of HMSO formation in the presence of CAT.

Effect of ·OH Scavengers on the Oxidation of Met Peptides at pH 7.0

Formate, mannitol, thiourea, and methanol are known

Table II. Effects of CAT and SOD on the Oxidation of GGGMGGG by AsA/Fe(III) and DTT/Fe(III) Systems*

Inhibitor added	GGGMGGG consumption (μM)	GGGM(O)GGG[a] formation (μM)	% inhibition of GGGM(O)GGG
AsA/Fe(III)			
None	39.5	12.1	0
CAT (200 U/mL)	45.4	11.0	9
SOD (100 U/mL)	31.1	11.7	3
SOD (100 U/mL) + CAT (200 U/mL)	42.8	10.9	10
DTT/Fe(III)			
None	64.0	43.2	0
CAT (200 U/mL)	35.7	31.6	27
CAT (Boiled)	69.0	47.3	0
SOD (100 U/mL)	71.7	37.7	13
SOD (100 U/mL) + CAT (200 U/mL)	33.1	26.7	38

[a] GGGM(O)GGG = GGGMGGG sulfoxide.

* Reaction mixtures contained GGGMGGG (0.2 mM), and AsA (2 mM)/$FeCl_3$ (0.02 mM) or DTT (2 mM)/$FeCl_3$ (0.02 mM) in phosphate buffers (10 mM) at pH 7.0. The incubations were controlled at 25°C for 72 h. All the data are the averages of duplicates. The standard error is less than 10%.

to be scavengers of ·OH (16). An inspection of their respective rate constants reveals that these scavengers react with ·OH at quite similar rates (17) (shown in the second column of Table III). Addition of these scavengers (2 mM) to the AsA/Fe(III) or the DTT/Fe(III) system, however, resulted in different inhibition of Met oxidation (Table III). Thiourea is most effective in retarding the oxidation of Met. In the AsA/Fe(III) system, thiourea reduces the HM consumption from 62.4 to 42.0 μM and it inhibits HMSO formation by ca. 5.2 μM. The effect of thiourea in the DTT system is more significant; the HM consumption decreases from 116 μM to 38 μM and the formation of HMSO was decreased by 39.3 μM. In contrast to thiourea, formate produced no observable effects. Similar results were also observed for the oxidation of GGGMGGG. Shown in Table III, the inhibitory effect of mannitol at 2 mM was not very pronounced. A more significant protection was observed as the concentration of mannitol was increased to 20 mM (Fig. 6). Minimum protective effect of methanol, however, could only be observed if the concentration of methanol was increased to 0.2 M (Table IV). From these inconsistent results, it seems that a free diffusible ·OH is not of major importance for Met oxidation and sulfoxide formation by both the AsA/Fe(III) and DTT/Fe(III) systems.

Effect of a $^1O_2$ Scavenger on the Oxidation of Met Peptides

Azide is a known scavenger of $^1O_2$, which is a powerful oxidant for Met (18). In the prooxidant/transition metal systems, $^1O_2$ can be generated through the bimolecular reaction of $O_2\cdot^-$ or other peroxyl radicals (19). If the Met oxidation is

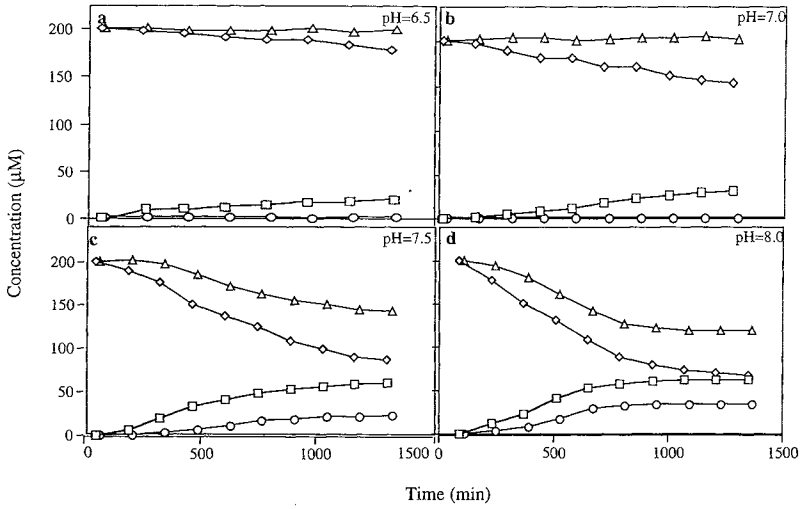


Fig. 3. The effect of pH on the inhibition of Met oxidation by CAT. The reactions contained 0.2 mM HM, 2 mM DTT/0.05 mM $FeCl_3$ in 10 mM phosphate buffer, in the presence of CAT (1000 U/ml) or the absence of CAT. (a) pH = 6.5; (b) pH = 7.0; (c) pH = 7.5; (d) pH = 8.0. △, HM(+CAT); ◇, HM(−CAT); ○, HMSO(+CAT); □, HMSO(−CAT).

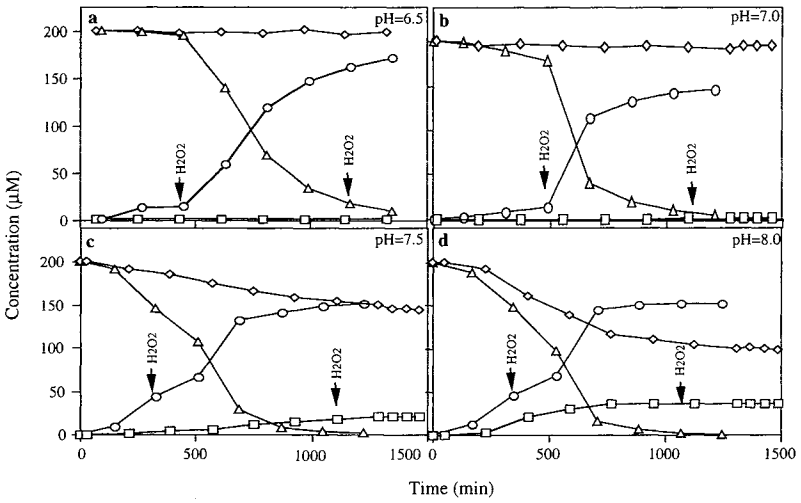


Fig. 4. Influence of H_2O_2 on the oxidation of HM in the absence or the presence of CAT (1000 U/ml) at different pH values. The reaction conditions were the same as described in Fig. 3. 5 mM H_2O_2 was added to the reaction mixtures at approximately 400 and 1200 min, respectively. (a) pH = 6.5; (b) pH = 7.0; (c) pH = 7.5; (d) pH = 8.0. ◇, HM(+CAT); △, HM(−CAT); □, HMSO(+CAT); ○, HMSO(−CAT).

not affected by the addition of azide, the participation of $^1O_2$ can be excluded. However, even if it does inhibit, this still does not provide unambiguous proof of the role of $^1O_2$ in the oxidation process since azide can also react with ·OH (with an even greater rate constant) (17). Upon the addition of azide, slight effects on the oxidation of HM and GGGMGGG were observed in the AsA/Fe(III) system (Table III). In contrast, significant inhibitory effects of azide were observed for the DTT/Fe(III) system. For example, the consumption of HM was reduced from 116.5 to 54.8 μM and the HMSO formation was inhibited by ca. 25.6 μM. Nevertheless, the participation of $^1O_2$ in the reaction cannot be definitely concluded due to the high reactivity of azide with ·OH radicals, as well as its tendency to complex Fe(III) (20).

DISCUSSION

The $O_2\cdot^-$ radical is reported to be a weak oxidizing radical in aqueous solution, and it does not react with free Met at a measurable rate (the rate constant is less than 1 $M^{-1}s^{-1}$) (21). The insignificant effects of SOD on the oxidation of Met peptides and sulfoxide formation in our experiments are consistent with the low reactivity of $O_2\cdot^-$. Consequently, the direct damaging role of $O_2\cdot^-$ towards Met residues in these model peptides does not appear to be significant.

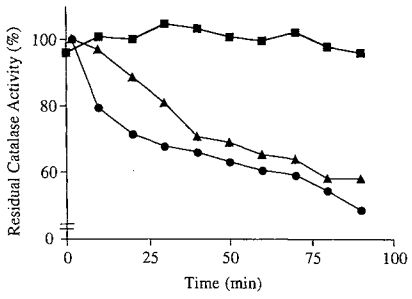


Fig. 5. Inhibition of CAT by AsA/Fe(III) and DTT/Fe(III) systems. CAT activity was measured in terms of the rate of decrease of H_2O_2 absorbance at 240 nm. The incubation mixtures included the addition of 10 μl of CAT solution (2000 U/ml) to 0.01 M H_2O_2 in 0.05 M phosphate buffer at pH 7.0 (■, CAT only); 2 mM AsA with 0.02 mM $FeCl_3$ in phosphate buffer at pH 7.0 (●, CAT in AsA/Fe(III)); and 2 mM DTT with 0.02 mM $FeCl_3$ in phosphate buffer at pH 7.0 (▲, CAT in DTT/Fe(III)). Incubations were controlled at 25°C. The estimated S. D. is ± 2.9%.

However, $O_2\cdot^-$ undergoes dismutation to H_2O_2. In a separate experiment (data not shown), it was confirmed that H_2O_2 stoichiometrically oxidizes the Met-containing peptides to Met sulfoxide without the formation of other degradation products. In the H_2O_2-induced oxidation reaction containing only 0.2 mM HM and 2 mM H_2O_2 in 10 mM phosphate buffer, a concentration as low as 20 U/ml of CAT was shown to be sufficient for complete suppression of the formation of HMSO. Based on these results, we assume that any free H_2O_2 which was generated in the prooxidant/Fe(III) reactions would be consumed by higher concentrations of CAT (200–2000 U/ml) added to the reaction mixture, thus protecting the peptides from the oxidation. At pH ≤ 7.0, almost total inhibition of Met oxidation was observed in the presence of CAT (Fig. 3a, b). This result may indicate that at pH ≤ 7.0 free H_2O_2 is the major oxidizing species responsible for Met peptide oxidation and sulfoxide formation. In contrast, only a partial inhibition of Met oxidation by CAT was observed at higher pH (7.5 and 8.0) (Fig. 3c, d). Most importantly, it was shown that H_2O_2 could not be the only oxidizing species responsible for Met oxidation at pH > 7. Consequently, other reactive oxygen intermediates must be involved in this oxidation process. Such reactive oxygen intermediates might include: (i) site-specifically formed H_2O_2 (which is not accessible to CAT), or (ii) other reactive oxygen species promoted through the binding of the transition metal ions to the peptide, such as reactive iron-oxy or iron-peroxy species.

The hypothesis of the site specific formation of H_2O_2 or

Table III. Effects of ·OH Scavengers and $^1O_2$ Scavenger on the Oxidation of HM and GGGMGGG by the AsA/Fe(III) and DTT/Fe(III) Systems*

Inhibitor added	$k \times 10^{-9}$[a] ($M^{-1}s^{-1}$)	HM (μM) consumption	HMSO (μM) formation	% Inhibition of HMSO	GGGMGGG consumption (μM)	GGGM(O)GGG formation (μM)	% Inhibition of GGGM(O)GGG
AsA/Fe(III)							
None		62.4	14.0	0	39.5	12.1	0
Formate	3.5	68.0	13.6	3	36.8	12.1	0
Mannitol	1.7	67.3	13.4	4	32.9	11.8	2
Thiourea	3.9	42.0	8.84	37	24.5	7.10	41
Sodium azide	12	49.9	13.2	6	25.7	12.2	0
DTT/Fe(III)							
None		116	61.6	0	64.0	43.2	0
Formate	3.5	85.9	57.9	6	63.8	45.2	0
Mannitol	1.7	76.3	50.5	18	50.9	38.1	12
Thiourea	3.9	38.0	22.3	64	24.6	18.4	57
Sodium azide	12	54.8	36.0	42	39.4	34.6	20

[a] Rate constant of ·OH with inhibitor (ref. 17).

* Reaction mixtures contained HM (0.2 mM), and AsA (2 mM)/$FeCl_3$ (0.02 mM) or DTT (2 mM)/$FeCl_3$ (0.02 mM) in phosphate buffers (10 mM) at 25°C, pH 7.0. HM reactions were incubated for 48 h; and GGGMGGG reactions were incubated for 72 h. The concentrations of the inhibitors were all at 2 mM. All the data are the averages of duplicates. The standard error is less than 10%.

other metal-bound reactive oxygen species takes into account the fact that metal ion binding with peptides through the N-terminal amino group, amide group and side chain (e.g., imidazole of His) are favored by an increase in pH (22) (at the pH range of 6–8, the C-terminal anionic carboxylic group would always be available for complexation with metal ions). H_2O_2 or other reactive oxygen species formed at specific sites of the peptides might immediately react with Met before they are able to diffuse into the solution. Findings consistent with the site-specific formation of reactive oxygen species were reported for the oxidation of several proteins and discussed in terms of a "caged" process (23). Despite all the evidence for this hypothesis, the nature of the oxidizing species as well as the detailed reaction mechanism still remains to be characterized.

The effect of CAT at higher pH values in our study may help to identify the underlying mechanisms. It has been documented that CAT reacts not only with H_2O_2 but also other hydroperoxides, although with lower efficiency (e.g., with methylperoxide $K = 8.5 \times 10^5\ M^{-1}s^{-1}$, and ethylperoxide $k = 2 \times 10^4\ M^{-1}s^{-1}$) (13). The partial inhibition seen in the presence of CAT at pH > 7 might, in fact, occur via the decomposition of some other yet unidentified hydroperoxide species. One hydroperoxide possibly generated from the prooxidant/transition metal system is a reactive iron-hydroperoxy species. The chemistry of such species formed by the addition of H_2O_2 to transition metal ions has been extensively studied by Sawyer and coworkers (24). Since free H_2O_2 does not appear to be of importance at higher pH, we infer that an iron-bound hydroperoxy species (eq. 1; species 1) may be formed by the addition of O_2 to Fe(II). The subsequent formation of other plausible iron-bound hydroperoxy species (species 2 and 3) are illustrated in equations 1–2. This type of iron-bound hydroperoxide species has also been discussed in the connection with the iron-EDTA/H_2O_2 catalyzed oxidation of several organic substrates (25).

$$L_xFe(II) + O_2 + H^+ \rightarrow \underset{1}{L_xFe(III)\text{-}O\text{-}O\text{-}H} \xrightarrow{+e^-} \underset{2}{L_xFe(II)\text{-}O\text{-}O\text{-}H} \xrightarrow{+e^-} \underset{3}{L_x^-Fe(II)\text{-}O\text{-}O\text{-}H} \quad (1)$$

$$L_xFe(II) + [H_2O_2]_{bound} \rightarrow \mathbf{3} \quad (2)$$

The ·OH is one highly reactive radical which might be formed in the prooxidant/transition metal system. It is re-

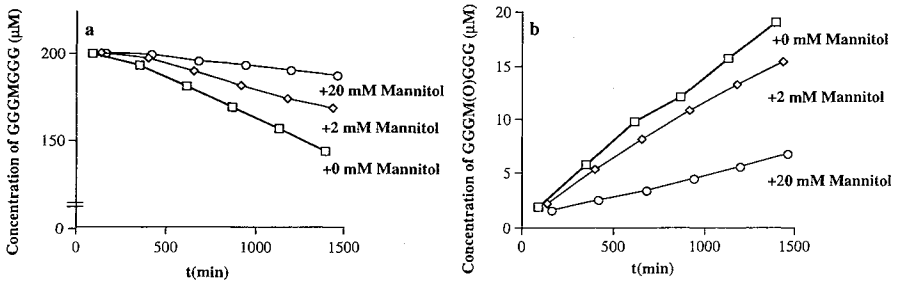


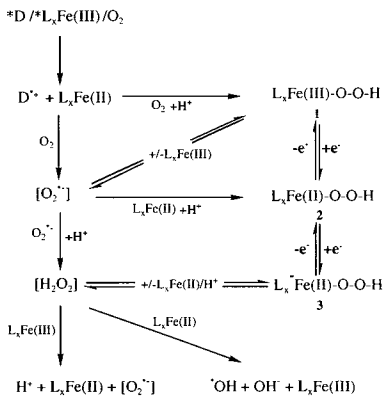
Fig. 6. Effect of mannitol concentration on the oxidation of GGGMGGG at pH 7.0. The reaction mixtures contained 0.2 mM GGGMGGG, 2 mM DTT/0.05 mM $FeCl_3$ in 10 mM phosphate buffer plus 0, 2, 20 mM mannitol. (a) The remaining concentration of GGGMGGG. (b) The formation of GGGM(O)GGG.

Table IV. Effects of Methanol (MeOH) on the Oxidation of HM by DTT/Fe(III) System*

Inhibitor added	t = 24 h		t = 48 h	
	HM (μM) consumption	HMSO (μM) formation	HM (μM) consumption	HMSO (μM) formation
MeOH[a] (0 M)	65	48	102	64
(0.2 M)	55	42	91	55
(1 M)	44	34	60	45

[a] Rate constant of ·OH with MeOH is 1.0×10^9 $M^{-1}s^{-1}$ (ref. 17).
* Reaction mixtures contained HM (0.2 mM), DTT (2 mM) and $FeCl_3$ (0.02 mM) in phosphate buffers (10 mM) at pH 7.0. The incubations were controlled at 25°C. All the data are the average of duplicates. The standard error is less than 10%.

ported to be reactive toward Met-peptides with the formation of various degradation products, other than considerable yields of Met sulfoxides (6,8,26). The inhibitory effects of the ·OH scavengers on Met oxidation in our experiments do not completely comply with the reported rate constants for their reactions with ·OH (Table III). Formate, which exhibits a similar rate constant (3.5×10^9 $M^{-1}s^{-1}$) for the reaction with ·OH as thiourea (3.9×10^9 $M^{-1}s^{-1}$) (17), does not show any protection on Met oxidation. The low inhibitory effect of formate might be explained by secondary generation of $O_2\cdot^-$, and the subsequent formation of H_2O_2 according to equations 3–4. However, if free ·OH were indeed involved in the process, we would be able to observe a significant effect from methanol even at a concentration of 2 mM. In spite of the fact that mannitol (1.7×10^9 $M^{-1}s^{-1}$) and methanol (1.0×10^9 $M^{-1}s^{-1}$) exhibit similar rate constants for their reaction with ·OH, they also showed very different effects on the oxidation of Met. Although mannitol inhibited the oxidation of Met-peptides to a certain extent, the concentration needed was much more than expected on the basis of simple competition kinetics. The inhibitory effect of mannitol was further complicated by the fact that mannitol was found to interact with iron in some manner which affected the conversion of Fe(II) to Fe(III) (27). Obviously, the direct involvement of ·OH in the oxidation under such conditions could not be concluded. The detailed mechanisms of the mannitol effect on the metal-catalyzed oxidation of peptides and proteins are currently under investigation in our laboratory.



*D = Electron Donor
*L$_x$ = Ligand (e.g. Buffer, Prooxidant, and Peptide)

Scheme 1.

$$\cdot OH + HCO_2^- \rightarrow H_2O + \cdot CO_2^- \quad (3)$$

$$\cdot CO_2^- + O_2 \rightarrow CO_2 + O_2\cdot^- \quad (4)$$

Although the common belief is that Fenton reagents (i.e., $L_xFe(II)/H_2O_2$) lead to the formation of free ·OH (included in Scheme 1) the evidence provided in recent studies (28,29) suggests that free ·OH is not the dominant reactant in Fenton reactions, and that the nucleophilic addition of H_2O_2 to $L_xFe(II)$ yields the major reactive intermediate L_x-Fe(II)-O-O-H (species 3) which can react directly with substrates. As summarized in Scheme 1, such reactive intermediates might be produced through various pathways in the prooxidant/transition metal ion system and become a major oxidizing species for Met peptides. Based on this theory and our experimental results, we have to conclude that free ·OH radicals are not a major species responsible for the formation of Met sulfoxide in the prooxidant/Fe(III) systems.

Analogous mechanisms as obtained for the Met oxidation in small peptides might also be expected in proteins. The understanding of Met oxidation in small model peptides may then help to shed some light on the complicated mechanisms of the oxidative modification of Met and other oxidation labile residues in proteins.

ACKNOWLEDGMENTS

S. Li would like to acknowledge the financial support from PMA Foundation and a Syntex Predoctoral Fellowship. The financial support for C. Schöneich by Hoffmann-La Roche and the Deutsche Forschungsgemeinschaft (DFG) (postdoctoral fellowship) is also gratefully acknowledged.

REFERENCES

1. N. Brot and H. Weissbach. Biochemistry and physiological role of methionine sulfoxide residues in proteins. *Arch. Biochem. Biophys.* 223:271–81 (1983).
2. E. R. Stadtman. Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Rad. Biol. & Med.* 9:315–25 (1990).
3. E. R. Stadtman. Protein oxidation and aging. *Science.* 257 1220–24 (1992).
4. M. C. Manning, K. Patel, and R. T. Borchardt. Stability of protein pharmaceuticals. *Pharm. Res.* 6:903–18 (1989).
5. S. Li, C. Schöneich, G. S. Wilson, and R. T. Borchardt. Chemical pathways of peptide degradation. V. Ascorbic acid promotes rather than inhibits the oxidation of methionine to methionine sulfoxide in small model peptides. *Pharm. Res.* 10:1572–79 (1993).
6. C. Schöneich, F. Zhao, G. S. Wilson, and R. T. Borchardt. Iron-thiolate-induced oxidation of methionine to methionine

sulfoxide in small model peptides. Catalysis by histidine. *Biochim. Biophys. Acta.* **1158**:307–22 (1993).
7. K. L. Maier, E. Matejkova, H. Hinze, L. Leuschel, H. Weber, and I. Beck-Speier. Different selectivities of oxidants during oxidation of methionine residues in the α-1-proteinase inhibitor. *FEBS Lett.* **250**:221–26 (1989).
8. C. Schöneich, A. Aced, and K-D Asmus. Mechanism of oxidation of aliphatic thioethers to sulfoxides by hydroxyl radicals. The importance of molecular oxygen. *J. Am. Chem. Soc.* **115**:11375–83 (1993).
9. C. Schöneich, A. Aced, and K-D Asmus. Halogenated peroxyl radicals as two-electron transfer agents. Oxidation of organic sulfides to sulfoxides. *J. Am. Chem. Soc.* **113**:375–76 (1991).
10. M. A. P. Dankleff, R. Curci, J. O. Edwards, and H-Y Pyun. The influence of solvent on the oxidation of thioxane by hydrogen peroxide and by t-butyl hydroperoxide. *J. Am. Chem. Soc.* **89**:3209–18 (1967).
11. P. K. Sysak, C. S. Foote, and T-Y Ching. Chemistry of singlet oxygen-XXV. Photooxygenation of methionine. *Photochem. Photobiol.* **26**:19–27 (1977).
12. Aebi, H. Catalase. In H. U. Bergmeyer (ed.), *Methods of Enzymatic Analysis, Vol. 2,* Academic Press, New York, 1974, pp. 673–84.
13. G. R. Schonbaum, and B. Chance. Catalase. in P. D. Boyer (ed.), *The Enzymes, Vol. XIII,* Academic Press, New York, 1976, pp. 363–408.
14. A. J. Davison, A. J. Kettle, and D. J. Fatur. Mechanism of the inhibition of catalase by ascorbate. *J. Biol. Chem.* **261**:1193–200 (1986).
15. G. D. Mao, P. D. Thomas, G. D. Lopaschuk, M. J. and Poznansky. Superoxide dismutase (SOD)-catalase conjugates. *J. Biol. Chem.* **268**:416–20 (1993).
16. D. A. Rowley, and B. Halliwell. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: a physiologically significant reaction? *Arch. Biochem. Biophys.* **225**:279–84 (1983).
17. G. V. Buxton, C. L. Greenstock, W. P. Helman, and A. B. Ross. Critical review of rate constants for reactions of hydrated electrons. Hydrogen atoms and hydroxyl radicals (·OH/·O$^-$) in aqueous solution. *J. Phys. Chem. Ref. Data.* **17**:513–886 (1988).
18. I. B. C. Matheson, and J. Lee. Chemical reaction rates of amino acids with singlet oxygen. *Photochem. Photobiol.* **29**:879–81 (1979).
19. E. J. Corey, M. M. Mehvotra, and A. U. Khan. Water induced dismutation of superoxide anion generates singlet molecular oxygen. *Biochem. Biophys. Res. Commun.* **145**:842–46 (1987).
20. G. V. Buxton, and I. Janovsky. Mechanism of the oxidation of iron (II) by the azide radical. *J. C. S. Faraday I.* **72**:1884–86 (1976).
21. B. Halliwell, and J. M. C. Gutteridge. *Free Radicals in Biology and Medicine (2nd Ed.),* Clarendon Press, Oxford, 1989, pp. 76–77.
22. D. L. Rabenstein, S. A. Daignault, A. A. Isab, A. P. Arnold, and M. M. Shoukry. Nuclear magnetic resonance studies of the solution chemistry of metal complexes. 21. The complexation of zinc by glycylhistidine and alanylhistidine peptides. *J. Am. Chem. Soc.* **107**:6435–39 (1985).
23. E. R. Stadtman. Oxidation of free amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **62**:797–821 (1993).
24. H. C. Tung, C. Kang, and D. T. Sawyer. Nature of the reactive intermediates from the iron-induced activation of hydrogen peroxide. *J. Am. Chem. Soc.* **114**:3345–455 (1992).
25. C. Walling, M. Kurz, and H. J. Schugar. The iron-ethylenediamine-tetraacetic acid-peroxide system. *Inorg. Chem.* **9**:931–37 (1969).
26. C. Schöneich, K. Bobrowski, J. Holcman, and K-D Asmus. Oxidation mechanisms of methionine containing peptides by hydroxyl and peroxyl radicals. In K. J. A. Davies (ed.), *Oxidative Damage and Repair.* Plenum Press, New York, 1991, pp. 380–85.
27. J. M. Braughler, L. A. Duncan, and R. L. Chase. The involvement of iron in lipid peroxidation. *J. Biol. Chem.* **261**:10282–89 (1986).
28. D. T. Sawyer, C. Kang, A. Llobet, and C. Redman. Fenton reagents (1:1 $Fe^{II}L_x$/HOOH) react via [$L_xFe^{II}OOH(BH^+)$] (1) as hydrolases (RH → ROH), not as generators of free hydroxyl radicals (HO·). *J. Am. Chem. Soc.* **115**:5817–18 (1993).
29. I. Yamazaki, and L. H. Piette. EPR spin-trapping study on the oxidizing species formed in the reaction of the ferrous ion with hydrogen peroxide. *J. Am. Chem. Soc.* **113**:7588–93 (1991).