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Research Article

Chemical Pathways of Peptide Degradation. V. Ascorbic Acid Promotes Rather than Inhibits the Oxidation of Methionine to Methionine Sulfoxide in Small Model Peptides

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The effect of primary structure and external conditions on the oxidation of methionine to methionine sulfoxide by the ascorbate/Fe^{3+} system was studied in small model peptides. Degradation kinetics and yield of sulfoxide formation were dependent on the concentration of ascorbate and H^+, with a maximum rate observed at pH 6–7. Phosphate buffer significantly accelerated the peptide degradation compared to Tris, HEPES, and MOPS buffers; however, the formation of sulfoxide was low. The oxidation could not be inhibited by the addition of EDTA. Other side products besides sulfoxide were observed, indicating the existence of various other pathways. The influence of methionine location at the C terminus, at the N terminus, and in the middle of the sequence was investigated. The presence of histidine in the sequence markedly increased the degradation rate as well as the sulfoxide production. The histidine catalysis of methionine oxidation occurred intramolecularly with a maximum enhancement of the oxidation rate and sulfoxide production when one residue was placed between the histidine and the methionine residue.

KEY WORDS: methionine; methionine sulfoxide; free radical; ascorbate; EDTA; histidine; catalysis.

INTRODUCTION

The therapeutic use of proteins has resulted in an increased need for understanding of protein stability at the molecular level (1). The stability of methionine (Met) toward oxidation to Met sulfoxide has been a particular problem in the pharmaceutical industry. Sulfoxide formation can occur during isolation, synthesis, formulation, and storage of proteins. Oxidation of Met residues to their corresponding sulfoxides is associated with loss of biological activity and structure alteration for many peptide hormones, nonhormonal peptides, and proteins. Within a given protein, the reactivity of Met residues toward oxidation may differ depending upon their positions. For example, in human growth hormone (hGH), Met-170 was found to be completely resistant to oxidation by hydrogen peroxide (2). Solid-state studies on recombinant human growth hormone (rhGH) indicate that Met-14 is the most labile methionine (3). However, oxidation by hydrogen peroxide (2) results in preferred oxidation of Met-125. Consequently, hydrogen peroxide might not always be an appropriate oxidation test for stability studies.

In biological systems, oxidation of Met is observed during inflammation, conditions of oxidative stress and ageing processes (4,5). Several pathological conditions may involve the oxidation of Met in proteins and concurrent loss of biological activity, including emphysema, rheumatoid arthritis, and cataract. Under oxidative stress, biological systems are exposed to a variety of reactive oxygen species such as hydroxyl radicals (·OH), peroxyl radicals (ROO·), oxyl radicals (RO·), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (6–8). It is likely that pharmaceutical proteins under various different conditions of processing (synthesis, formulation, and isolation) undergo oxidation via pathways resembling protein oxidation *in vivo* under conditions of oxidative stress.

Thus, it is of great interest to understand the underlying mechanisms and the effects of external factors as well as the influence of neighboring groups on Met oxidation. The external factors which might influence the oxidation are the temperature, pH, nature of the buffer, nature and type of catalyst (e.g., traces of metal ions), and oxygen tension. In order to obtain basic information on Met oxidation, small model peptides of defined sequence were used in our study to elucidate the influence of the primary sequence as well as the influence of external factors on Met oxidation in peptides and proteins.

Ascorbate is a powerful electron donor and can act as both an efficient antioxidant and a prooxidant *in vivo* and *in vitro*. The general view is that ascorbate acts mainly as an antioxidant. It will become evident from this study that the prooxidant, and therefore the damaging role of ascorbate in the presence of iron, leads to effective oxidation of Met to Met sulfoxide in small model peptides. The evidence for the chemical oxidation of aromatic thioethers through the ascorbate/iron system has already been reported in the literature (9).

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MATERIALS AND METHODS

His-Met (HM), His-Gly-Met (HGM), His-Gly-Gly-Met (HGGM), His-Gly-Gly-Gly-Met (HGGGM), His-Gly-Gly-Gly-Gly-Met (HGGGGM), His-Pro-Met (HPM), His-Pro-Pro-Met (HPPM), Gly-Met (GM), Gly-Gly-Met (GGM), Gly-Gly-Gly-Met (GGGM), Pro-Met (PM), Gly-Met-Gly (GMG), Met-Gly-Gly (MGG), and Met-His (MH) were selected as model peptides for our studies.

HM, GM, GGM, GMG, PM, and MH were directly purchased from Bachem, Bioscience Inc., Philadelphia, PA. Other peptides were synthesized by standard techniques using Fmoc derivatives (10). The starting materials Fmoc-His(Trt), Fmoc-Gly, Fmoc-Pro, and Fmoc-Met were also purchased from Bachem and the products were purified by HPLC using a Keystone Hypersil ODS C_{18} reversed-phase column (250 × 10 mm).

All other chemicals, including ascorbic acid (from Aldrich Chemical Company, Inc., Milwaukee, WI), buffers, and ferric chloride (from Sigma Chemical Co., St. Louis, MO) were analytical grade and were used as received from the commercial suppliers. Trifluoroacetic acid (TFA; HPLC grade) was purchased from Pierce Chemicals (Rockford, IL). HPLC-grade acetonitrile was supplied by Fisher Chemical (Fair Lawn, NJ). The water used in all studies was from a Millipore MILLI-Q water system.

The standard Met sulfoxide derivatives of each peptide were synthesized by the following general procedure. A solution of the peptide (20 m*M*) in water was adjusted to pH 3.7 with concentrated HCl. Excess H_2O_2 (3%) was added and the solution was stored at room temperature for 1 day. The peptide sulfoxides were purified by HPLC using a Keystone Hypersil ODS C_{18} reversed-phase column (250 × 10 mm) and lyophilized. ^{1}H NMR (QE-300) and FAB mass spectrometry (VG ZAB-HS mass spectrometer interfaced with a 11/250 data system) were used for the characterization of the Met sulfoxide peptide. In general, Met sulfoxide-containing peptides were 16 U higher in mass than the Met-containing peptide. ^{1}H NMR analysis confirmed sulfoxide formation through the shift of the ε-methyl resonance at ca. δ = 2.2 ppm (s) for Met to δ = 2.7 ppm (s) for Met sulfoxide.

HPLC Analysis

High-performance liquid chromatography (HPLC) was used with a system consisting of a Shimadzu SCL-10A system controller, a Shimadzu LC-10AS pump, an SPD-10A UV spectrophotometric detector, an SIL-10A autoinjector, a sample cooler, and a C-R4A chromatopac integrator. The analysis of the oxidation of Met-containing peptides in ascorbic acid was performed by HPLC using an Alltech Econosphere C_{18} reversed-phase column (250 × 4.6 mm) at ambient temperature (25°C), using an isocratic system at a flow rate of 1 mL/min. The solvent systems used in the analysis varied from 17 to 25% of acetonitrile/water and 0.02–0.033% trifluoroacetic acid, depending on the peptide. Detection of the Met-containing peptides was done at 214 nm. The Met-containing peptides and their corresponding sulfoxides were quantified by measuring peak areas and were standardized using purified Met-containing peptides and Met sulfoxide derivatives. The standard Met sulfoxide derivatives were compared chromatographically with the products that arose from the degradation of the peptides in ascorbic acid solutions. The retention time of the chemically synthesized sulfoxide was indistinguishable from that of the component, which arose at much slower rates from peptide oxidation by the ascorbic acid/$FeCl_3$ system. The initial rates of peptide degradation and Met sulfoxide formation were calculated by fitting the data according to zero-order kinetics. The peptide remaining and sulfoxide formation were measured after the reactions reached a plateau and were unchanged over 5 days.

RESULTS

Oxidation of GGM, GMG, and MGG

To demonstrate the role of ascorbate in Met oxidation, solutions with and without ascorbate were compared for their propensity to oxidize GGM, GMG, and MGG. The oxidation reaction was followed in solutions containing 0.2 m*M* peptide, 5 m*M* Tris · HCl, and 0.02 m*M* $FeCl_3$ in the absence and presence of 2 m*M* ascorbic acid, respectively, at pH 7.4. Figure 1a shows that sulfoxide formation and peptide degradation were pronounced in the solutions with ascorbate present, while no peptide degradation was observed in the solutions in the absence of ascorbate within the same time period. It is evident that ascorbate functions at least partially as a prooxidant in the process of Met oxidation. All reactions reached a plateau after ca. 4 to 5 days. Sulfoxide formation from GGM, GMG, and MGG amounted to 15.6, 13.0, and 19.7 μ*M*, respectively. The consumption of peptide was 39.8 μ*M* for GGM, 24.4 μ*M* for GMG, and 20.0 μ*M* for MGG. This product distribution indicates that the major oxidation product in ascorbate solution is indeed Met sulfoxide (see also Table I).

Effect of Histidine (His) on Met Oxidation

If HM is oxidized under the same conditions as GGM, GMG, and MGG, sulfoxide formation amounts to 63.2 μ*M*, while the consumption of HM is 102.4 μ*M* after the same period of time, giving an efficiency of sulfoxide formation of 61.7%. A comparison with the data for GGM, GMG, and MGG shows that both Met sulfoxide formation and peptide loss are much more significant in HM. Apparently, His in a neighboring position to Met catalyzes Met oxidation. The following experiments were done to examine the oxidation of HM in more detail.

Effect of pH. Solutions of 0.2 m*M* HM, 2 m*M* ascorbic acid, 5 m*M* Tris · HCl, and 0.02 m*M* $FeCl_3$ were prepared at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The pH values were measured using a POPE Model 1501 pH/ion meter. The solutions were kept at 25°C in autosampler vials and the progress of the oxidation reactions was monitored by HPLC. Figure 1b gives a typical time course for the oxidation of HM to HM(O) at pH 7.4. Although an excess amount of ascorbate (2 m*M* ascorbate over 0.2 m*M* peptide) was applied, HM did not completely oxidize in the course of the reaction. This is due simply to the two opposing functions of ascorbate in the solution. As an electron donor, ascorbate acts as a prooxidant to form reactive oxygen species through metal-catalyzed reaction with oxygen. The formation of superoxide radicals, hydroxyl radicals, and hydrogen peroxide

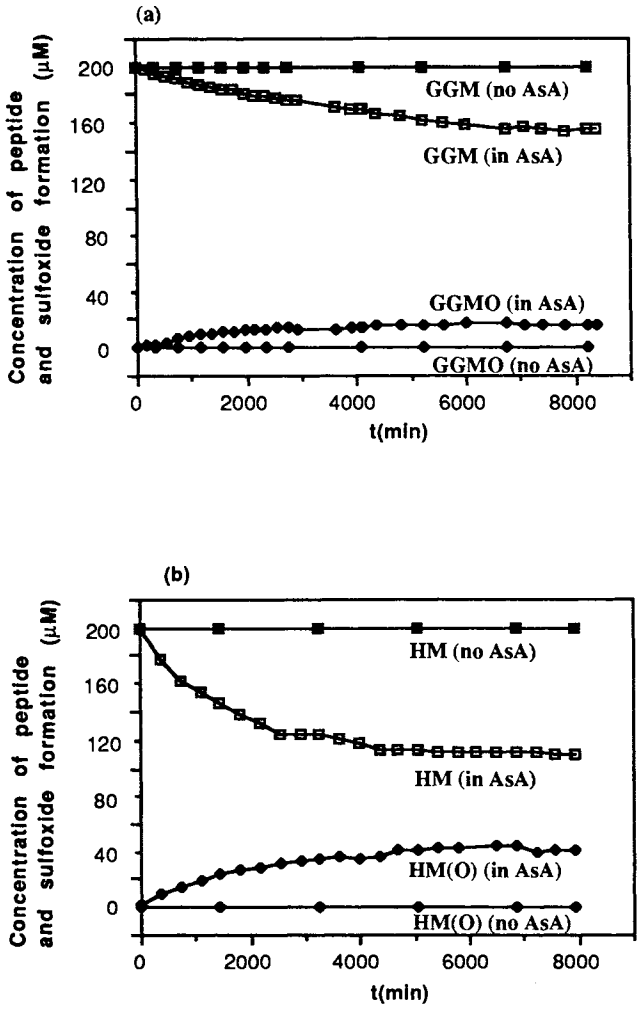


Fig. 1. Typical time course of degradation of GGM (0.2 m*M*) (a) and HM (0.2 m*M*) (b) with and without ascorbic acid (AsA) (2 m*M*) in $FeCl_3$ (0.02 m*M*) and Tris · HCl (5 m*M*) buffer solution at pH 7.4, 25°C.

in the metal-catalyzed oxidation of ascorbic acid was reported previously (11–15). However, in its well-known function as an antioxidant, ascorbate would also be able to scavenge the generated reactive oxygen species (see Ref. 16). Thus, only a certain fraction of the generated reactive oxygen species can oxidize Met. The extent of Met oxidation then depends on the individual rate constants of the oxidizing species with both ascorbate and Met. A quantitative evaluation of those rate constants would, however, require the characterization of the oxidizing species; this was not attempted in this study.

The initial rate of HM oxidation can be derived from zero-order kinetics of both HM degradation and HM(O) formation. The effect of pH on the observed rate constants is shown in Fig. 2a. The maximum degradation rate, k_d, was observed around pH 6.5. In Fig. 2b the formation of sulfoxide and HM degradation are plotted as a function of pH. Maximum sulfoxide formation and peptide degradation were seen between pH 7.0 and pH 8.0.

Nature of the Buffers. Oxidation of HM was performed in various buffers including Tris · HCl, phosphate, MOPS, and HEPES (all at 5 m*M* concentration) at pH 7.4. The solutions contained 0.2 m*M* HM, 2 m*M* ascorbic acid, and 0.02 m*M* $FeCl_3$. Kinetic data obtained as function of the buffer are presented in Table II. It evolves that the oxidation rate decreases in the order phosphate> Tris · HCl> MOPS> HEPES. Phosphate buffer significantly increased both the absolute yield and the rate of degradation of HM. However, the phosphate system yields the lowest sulfoxide formation.

Effect of Temperature. Solutions containing 0.2 m*M* HM, 2 m*M* ascorbic acid, 5 m*M* Tris · HCl and 0.02 m*M* $FeCl_3$ at pH 7.4 were kept at four temperatures (4, 25, 37, and 50°C). The Arrhenius plot of the HM degradation rate in ascorbic acid/$FeCl_3$ solution (data not shown) suggests that the complex oxidation process has an apparent overall activation energy (E_a) of 23.94 ± 1.99 kJ/mol. It remains to be investigated, after characterization of the oxidizing species, if this activation energy corresponds to the formation of the oxidizing species through metal-catalyzed ascorbate oxidation, the actual process of Met oxidation, or both.

Effect of Ascorbic Acid Concentration. The effect of ascorbic acid concentration was studied in solutions containing 0.2 m*M* HM, 5 m*M* Tris · HCl, and 0.02 m*M* $FeCl_3$ at pH 7.4 containing 0.5, 0.75, 1.0, 2.0, and 5.0 m*M* ascorbic acid, respectively. The rate of HM degradation increased with increasing ascorbate concentration, whereas the apparent rate of sulfoxide formation remained about constant (see Fig. 3). The consumption of HM also increases with increasing ascorbate concentration. The yield of sulfoxide amounts to 20, 43, 53, 60, and 30 μ*M* in 0.5, 0.75, 1.0, 2.0, and 5.0 m*M*

Table I. Effect of Met Location and Neighboring Amino Acid on the Oxidation Rate and Product Distribution in Small Model Peptides (0.2 m*M*), Ascorbic Acid (2 m*M*), $FeCl_3$ (0.02 m*M*), and Tris Buffer (5 m*M*) Solutions at pH 7.4, 25°C[a]

Peptide	$k_d \times 10^8$ (*M*/min)[b]	$k_{so} \times 10^8$ (*M*/min)[c]	Peptide consumption (μ*M*)	Sulfoxide formation (μ*M*)	Efficiency of sulfoxide formation (%)[d]
HM	2.86 ± 0.38	0.92 ± 0.01	102.4 ± 4.2	63.2 ± 0.1	61.7
MH	1.98 ± 0.51	1.11 ± 0.33	47.6 ± 9.6	30.8 ± 0.3	64.7
MGG	0.96 ± 0.29	0.70 ± 0.08	20.0 ± 0.2	19.7 ± 0.85	98.5
GMG	0.74 ± 0.21	0.27 ± 0.48	24.4 ± 0.4	13.0 ± 0.40	53.3
GGM	0.98 ± 0.10	0.57 ± 0.16	39.8 ± 7.2	15.6 ± 0.44	39.2

[a] Values are means ± SE based on data from two experiments.
[b] k_d, initial rate constant for peptide degradation.
[c] k_{so}, initial rate constant for sulfoxide formation.
[d] Related to consumed peptide.

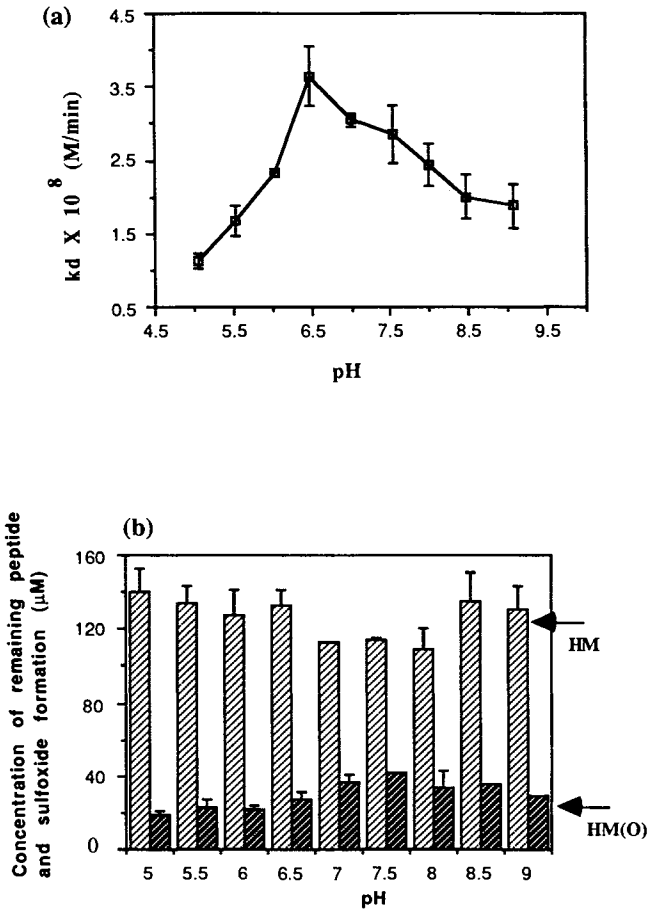


Fig. 2. Effect of pH on the degradation rate k_d (a) and product distribution (b) of HM (0.2 m*M*) oxidation in an ascorbic acid (2 m*M*)/$FeCl_3$ (0.02 m*M*) and Tris · HCl buffer (5 m*M*) system at 25°C. Mean ± SE based on data from two experiments.

ascorbate solutions, respectively. The efficiency of sulfoxide formation was calculated to be 62.5% for 0.5 m*M*, 62.3% for 0.75 μ*M*, 61.6% for 1 m*M*, 60% for 2 m*M*, and 21.4% for 5 m*M* initial ascorbate concentrations. This change in efficiency indicates that other oxidation pathways and/or subsequent degradation of the sulfoxides become increasingly important with the increase of ascorbate concentrations. In a separate experiment (data not shown), it was demonstrated that, indeed, further degradation of sulfoxide occurred at high ascorbate concentrations.

Effect of Concentration of $FeCl_3$. Figure 4a shows the concentration vs time profiles for systems containing 0.2 m*M* HM and 2 m*M* ascorbic acid in 5 m*M* Tris · HCl at pH 7.4 in the absence of added $FeCl_3$ and the presence of added 0.02 m*M* $FeCl_3$. It can be seen that the kinetics in the system containing 0.02 m*M* $FeCl_3$ are faster than in the absence of the added $FeCl_3$. However, the fact that the oxidation still occurred in the absence of added iron reflects the potential contamination of the buffer as well as the peptide with trace amounts of iron or other transition metals which are sufficient to drive the oxidation process.

Effect of EDTA. Figure 4b shows that the addition of the chelator EDTA (0.02 m*M*) to the solution containing 0.2 m*M* HM, 2 m*M* ascorbic acid, 5 m*M* Tris · HCl, and 0.02 m*M* $FeCl_3$ at pH 7.4 accelerated rather than inhibited the degradation of the Met-containing peptide. However, Met sulfoxide formation was suppressed.

Detection of Oxygen Uptake. In order to elucidate the relationship between sulfoxide formation and ascorbate oxidation, oxygen uptake was measured in the reaction mixtures. The uptake of oxygen was measured with a polarographic oxygen sensor and an LC-4B amperometric detector controlled with an applied potential of −0.6 V. The oxygen sensor was immersed in 10 mL of a reaction mixture containing 0.2 m*M* HM, 2 m*M* ascorbic acid, 5 m*M* Tris · HCl, 0.02 m*M* $FeCl_3$, and 10 m*M* KCl at pH 7.4 in a closed vial. Oxygen uptake as well as Met oxidation was observed only in the presence of ascorbic acid (see Fig. 5). This result shows that the oxidation of HM is initiated by the metal-catalyzed oxidation of ascorbate. Taking an initial oxygen content of 0.258 μmol/mL O_2 in air-saturated aqueous solution, the current dropped to a plateau value of ca. 260 nA, i.e., by ca. 32.5% with respect to the initial value of 385 nA. This corresponds to an uptake of 0.084 μ*M*/mL O_2 at 25°C within about 100 min of reaction time in the present system.

Effect of Location of His and Met in the Primary Sequence on the Rate of Met Oxidation

In order to elucidate the catalytic effect of His on Met oxidation as a function of primary sequence, some spacing amino acid residues were placed between His and Met. As shown in Table III, the presence of His in the Met-containing peptide catalyzes the sulfoxide formation in every His- and

Table II. Effect of Buffer Species on the Rate and Product Distribution of HM Oxidation in an Ascorbic Acid/$FeCl_3$ Buffer Solution at pH 7.4, 25°C[a]

Buffer	$k_d \times 10^8$ (*M*/min)[b]	$k_{so} \times 10^8$ (*M*/min)[c]	HM consumption (μ*M*)	HM(O) formation (μ*M*)	Efficiency of sulfoxide formation (%)[d]
Tris-HCl	2.86 ± 0.38	0.917 ± 0.012	102.4 ± 4.2	63.2 ± 0.2	61.7
Phosphate	3.08 ± 0.08	0.611 ± 0.021	164.4 ± 4.0	17.3 ± 2.2	10.5
MOPS	2.26 ± 0.07	0.767 ± 0.081	121.6 ± 7.8	54.8 ± 4.6	45.1
HEPES	1.79 ± 0.53	0.707 ± 0.092	89.2 ± 4.4	38.4 ± 1.2	43.0

[a] Conditions are the same as for Table I. Values are means ± SE based on data from two experiments.
[b] k_d, initial rate constant for HM degradation.
[c] k_{so}, initial rate constant for HM(O) formation.
[d] Related to consumed peptide.

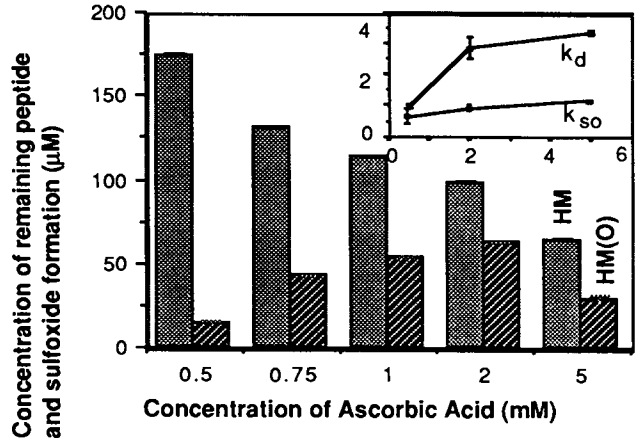
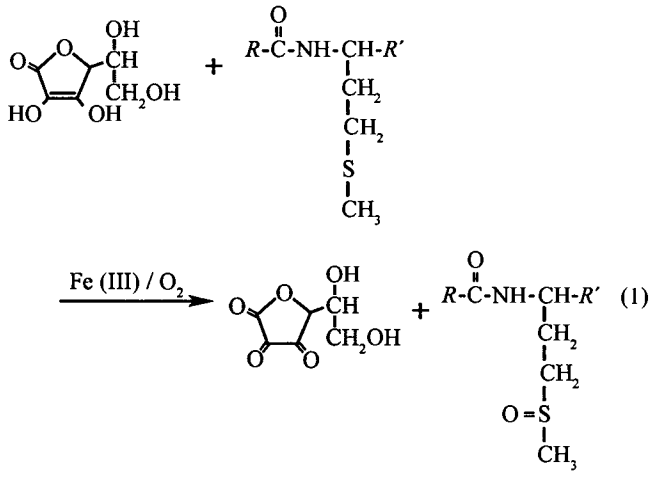


Fig. 3. Effect of ascorbic acid (AsA) concentration on the product distribution and degradation rate k_d, sulfoxide formation rate k_{so} [inset: x axis is AsA concentration (mM), y axis is $k \times 10^8$ (M/min)] in the reaction of HM (0.2 mM) oxidation in ascorbic acid/$FeCl_3$ (0.02 mM) and Tris · HCl buffer (5 mM) solution at pH 7.4, 25°C. Mean ± SE based on data from two experiments for the concentration of 0.5, 2.0, and 5.0 mM AsA solutions. The 0.75 and 1.0 mM AsA solutions are single experiments.

Met-containing peptide. Catalysis occurs even if His and Met are separated by up to four Gly residues or by two Pro residues. Both the peptide consumption and the sulfoxide formation are much higher with His in the sequence. No catalytic effect was observed for the oxidation of GGM in the presence of added His or imidazole under the same experimental conditions (see Table IV), indicating that catalysis by His may be intramolecular. The maximum oxidation rate is observed for HGM and HPM. Obviously, the chemical susceptibility of the residue between His and Met can also influence the kinetics and product distribution. Other side products besides sulfoxide were observed for Pro-containing peptides, which have not yet been characterized in our systems. Several reports in the literature, however, indicate the likelihood of Pro undergoing metal-catalyzed oxidation (17).



The effect of Met being located at the N terminus, at the C terminus, or in the middle of the sequence on the kinetics and product distribution was small (see Table I). However, C-terminal Met is slightly more labile than Met at the other locations. Similar findings were observed for the oxidation of Met by a comparable oxidizing system constituting of DTT_{red} (reduced dithiothreitol)/$FeCl_3$ (18). If HM is compared with MH, it appears that Met in the sequence MH (N terminal) is more stable than HM (C terminal).

DISCUSSION

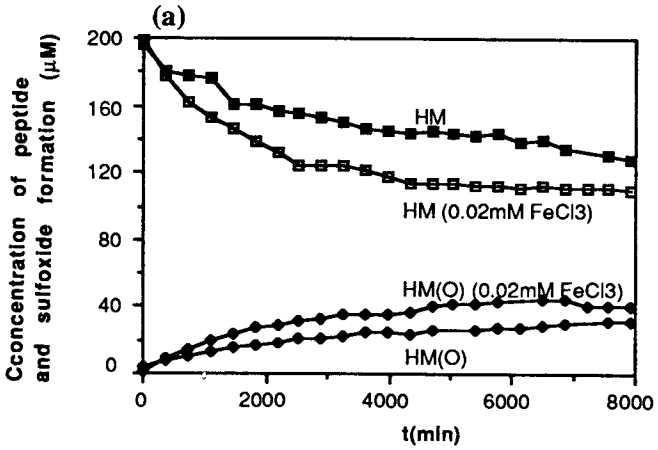
The results indicate that the major degradation pathway for the oxidation of Met-containing peptides through the ascorbic acid/$FeCl_3$ system is the formation of Met sulfoxide as described in Eq. (1).

The mechanisms of oxidation of ascorbate (A) to dehydroascorbate (DHA) may involve the generation of reactive oxygen species according to reactions (2)–(6) (6,12–14).

$$A + 2\ Fe^{3+} \rightarrow DHA + 2\ Fe^{2+} \quad (2)$$

$$Fe^{2+} + O_2 \rightarrow O_2\cdot^- + Fe^{3+} \quad (3)$$

$$2\ O_2\cdot^- + 2\ H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$



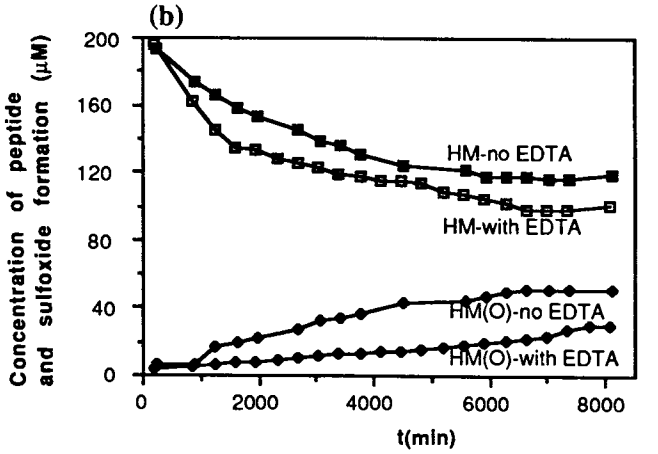


Fig. 4. Effect of $FeCl_3$ concentration (a) and EDTA (b) on HM (0.2 mM) oxidation in an ascorbic acid (2 mM)/Tris · HCl buffer (5 mM) solution at pH 7.4, 25°C.

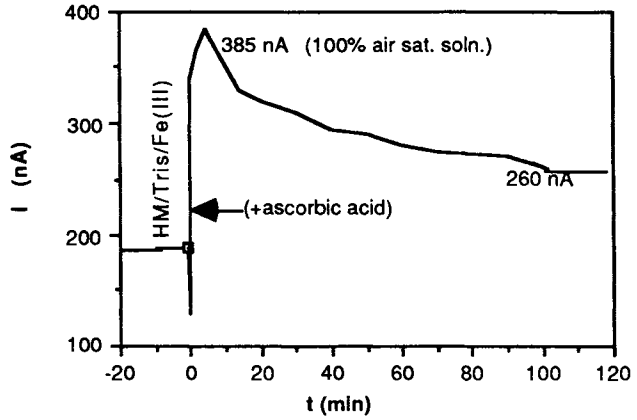


Fig. 5. Oxygen uptake by the HM (0.2 m*M*)/Tris (5 m*M*)/$FeCl_3$ (0.02 m*M*) system with the addition of ascorbic acid (2 m*M*).

$$Fe^{2+} + H_2O_2 \xrightarrow[O_2]{} Fe^{3+} + HO^- + HO\cdot \quad (5)$$

$$HO\cdot + RH \longrightarrow H_2O + ROO\cdot \quad (6)$$

The reactivity of these different active oxygen species toward Met differs with regard to the kinetics and the efficiency of sulfoxide formation (18). For example, hydrogen peroxide oxidizes Met to Met sulfoxide with 100% stoichiometric conversion without the formation of other side products. A peroxide species is therefore a likely candidate for the oxidant leading to sulfoxide formation. Hydroxyl radicals can also induce the formation of Met sulfoxide. However, in the reaction of hydroxyl radicals with Met peptides, only about 20% of Met sulfoxides were observed and various other products were more dominant (18). Nevertheless, their reactions with Met might still be considered for the peptide degradation. The reaction of superoxide radicals with organic sulfides has not been proven to yield sulfoxide with appreciable yields (C. Schöneich, unpublished results), whereas peroxyl radicals on reaction with organic sulfides do quite efficiently lead to formation of sulfoxide (19). Thus, the likely candidates for Met sulfoxide formation in our system appear to be hydrogen peroxide or the peroxyl radical. The hydroxyl radical, if formed, would not result in high yield of sulfoxide formation even though it rapidly reacts with Met.

The effect of pH on HM oxidation (Fig. 2) by ascorbate can be rationalized by simply invoking ionization of ascorbic acid. Deprotonation of ascorbic acid will facilitate electron donation to Fe(III) and, therefore, promote a faster rate of peptide consumption. However, ascorbate also increasingly functions as an antioxidant at higher pH and in turn scavenges the generated oxidizing species. On this basis, the maximum rate at pH 6.5 and maximum oxidation yields between pH 7.0 and pH 8.0 might be explained. It should be mentioned that Levine observed a similar maximum rate upon oxidative inactivation of glutamine synthetase by the ascorbate/Fe(III) system around pH 7.0, which does not differ remarkably from our results (20). It is important to note that the pH of the maximum initial rate for peptide consumption (Fig. 2a) does not correspond to the maximum yield of sulfoxide formation (Fig. 2b). The initial rate is only a measure for the overall degradation process. The deviation of the maxima in Figs. 2a and b is most likely caused by the fact that the stoichiometry of the conversion from Met to Met sulfoxide is not 1:1 in this reaction.

The nature of the buffer plays an important role in both the kinetics and the efficiency of sulfoxide formation. The observed differences with various buffers are probably caused by their different affinity to metal ions and different ability to scavenge free radicals. Phosphate buffer strongly binds Fe(III) (21), while the binding of Tris or HEPES with metal ions is relatively weak (22,23). According to reaction (2), ascorbic acid reduces Fe(III) to Fe(II). The subsequent reduction of molecular oxygen by Fe(II) leads to the generation of the reactive oxygen species. Since it is known that phosphate buffer promotes the electron transfer of Fe(II) to oxygen (21), the overall radical initiation process is enhanced as the activation of oxygen is faster in phosphate buffer. This might be part of the reason that we observed the maximum degradation rate of peptide in phosphate buffer. It also appears that the efficiency of sulfoxide formation (the ratio of the sulfoxide formation vs the peptide degradation) is

Table III. Effect of Met Location in the Primary Sequence on Peptide Degradation by the Ascorbic Acid/$FeCl_3$ System at pH 7.4, 25°C[a]

Peptide	$k_d \times 10^8$ (*M*/min)[b]	$k_{so} \times 10^8$ (*M*/min)[c]	Peptide consumption (μ*M*)	Sulfoxide formation (μ*M*)	Efficiency of sulfoxide formation (%)[d]
HM	2.86 ± 0.38	0.92 ± 0.01	102.4 ± 4.2	63.2 ± 0.2	61.7
HGM	9.63 ± 0.68	5.59 ± 0.58	107.4 ± 9.8	63.4 ± 4.8	59.0
HGGM	5.28 ± 0.44	2.24 ± 0.02	110.6 ± 5.6	67.4 ± 4.6	60.9
HGGGM	2.50 ± 0.23	1.38 ± 0.18	90.4 ± 5.6	53.6 ± 3.4	59.3
HGGGGM	5.74 ± 0.34	2.51 ± 0.48	119.4 ± 15.6	51.4 ± 1.4	43.0
GM	1.01 ± 0.26	0.47 ± 0.03	—	—	—
GGM	0.98 ± 0.13	0.57 ± 0.16	39.8 ± 7.2	15.6 ± 0.8	39.2
GGGM	0.97 ± 0.14	0.92 ± 0.18	40.4 ± 11.4	16.6 ± 3.8	41.1
PM	2.01 ± 0.93	0.18 ± 0.02	31.4 ± 1.4	8.6 ± 1.8	27.4
HPM	12.2 ± 0.07	8.15 ± 0.96	151.0 ± 1.6	70.4 ± 0.4	46.6
HPPM	4.88 ± 1.44	1.27 ± 0.15	97.4 ± 2.6	21.0 ± 1.2	21.6

[a] Conditions are the same as for Table I. Values are means ± SE based on data from two experiments.
[b] k_d, initial rate constant for HM degradation.
[c] k_{so}, initial rate constant for HM(O) formation.
[d] Related to consumed peptide.

Table IV. Effect of His on the Oxidation Rate and Product Distribution of Met-Containing Peptides by the Ascorbic Acid/$FeCl_3$ System[a]

Peptide	$k_d \times 10^8$ (M/min)[b]	$k_{so} \times 10^8$ (M/min)[c]	Peptide consumption (μM)	Sulfoxide formation (μM)	Efficiency of sulfoxide formation (%)[d]
HGGM	5.28 ± 0.44	2.24 ± 0.02	110.6 ± 5.6	67.4 ± 8.6	60.9
GGM	0.98 ± 0.10	0.57 ± 0.16	39.8 ± 7.2	15.6 ± 0.8	39.2
GGM(+His)	1.42 ± 0.10	0.39 ± 0.13	37.8 ± 3.4	9.48 ± 1.00	25.1
GGM(+Imd)[e]	1.24 ± 0.05	0.42 ± 0.14	35.2 ± 7.5	14.2 ± 1.4	40.3

[a] Conditions are the same as for Table I. Values are means ± SE based on data from two experiments.
[b] k_d, initial rate constant for HM degradation.
[c] k_{so}, initial rate constant for HM(O) formation.
[d] Related to consumed peptide.
[e] Imidazole.

very low in phosphate buffer. Thus it is likely that the hydroxyl radical rather than peroxyl radical or hydrogen peroxide might be the dominant active species formed in the phosphate buffer solution. On the other hand, Tris and HEPES are efficient scavengers of hydroxyl radicals (24). By this function they might intercept hydroxyl radical induced peptide degradation. Therefore, a much lower hydroxyl radical-induced consumption of HM would be observed in Tris and HEPES buffers. Since neither Tris nor HEPES readily interacts with the other reactive oxygen species (e.g., peroxyl radical and hydrogen peroxide), the sulfoxide formation constitutes the major pathway.

EDTA does not simply inhibit the oxidation process by acting as a chelating agent. In the presence of transition metal ions such as iron, HO· radicals can be generated via a metal-catalyzed Haber–Weiss reaction, which is depicted for Fe-EDTA in reactions (7)–(9) (25).

$$Fe^{3+} - EDTA + O_2\cdot^- \rightarrow Fe^{2+} - EDTA + O_2 \quad (7)$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow Fe^{3+} - EDTA + HO\cdot + HO^- \quad (8)$$

$$\text{Sum: } O_2\cdot^- + H_2O_2 \xrightarrow{\text{Fe(III)} - \text{EDTA}} O_2 + HO\cdot + HO^- \quad (9)$$

On the other hand, hydroxyl radicals react (diffusion-controlled) with Met-containing peptides (26), while, on the other hand, they do not efficiently yield sulfoxide (18). The involvement of reactions (7)–(9) might explain the observed peptide degradation with lack of sulfoxide formation in the presence of EDTA.

The catalytic effect of His on Met oxidation is obvious by comparing the overall product distribution and efficiency of sulfoxide formation for GGM (39.2%) and HM (61.7%) or HGM (59.0%) (Table III). In addition, a number of other small peaks have been observed in the HPLC analysis during the reaction, which might be due to the degradation of His (27,28). The characterization of these minor products is currently under study. The catalysis by His may be due to the strong binding of transition metal ions by the imidazole ring (29). The complexation of iron with the peptide (including also C-terminal and N-terminal binding) and the formation of an oxidizing species at the metal ion would eventually bring the oxidizing species close to the substrate Met, thus promoting the oxidation process (30). The optimum spatial arrangement is apparently created when one residue (Gly or Pro) is located between His and Met, yielding the highest oxidation rate.

CONCLUSIONS

The ascorbate/Fe(III) system in the presence of oxygen induces the oxidation of Met to Met sulfoxide in small model peptides. Thus, although ascorbate is an efficient antioxidant, the data presented here demonstrate the potential of ascorbate for promoting the generation of reactive oxygen species and subsequent degradation of the peptides.

The present results will have important implications for the further investigation of Met oxidation in proteins and the creation of oxidation-resistant formulations of protein pharmaceuticals. The simple addition of an antioxidant does not necessarily result in a complete inhibition of oxidation but might promote this process.

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REFERENCES

1. M. C. Manning, K. Patel, and R. T. Borchardt. Stability of protein pharmaceuticals. *Pharm. Res.* 6:903–918 (1989).
2. L. C. Teh, L. J. Murphy, N. L. Huq, A. S. Surus, H. G. Friesen, L. Lazarus, and G. E. Chapman. Methionine oxidation in human growth hormone and human chorionic somatomammotropin. *J. Biol. Chem.* 262:6472–6477 (1987).
3. G. W. Becker, P. M. Tackitt, W. W. Bromer, D. S. Lefeber, and R. M. Riggin. Isolation and characterization of a sulfoxide and a desamido derivative of biosynthetic human growth hormone. *Biotechnol. Appl. Biochem.* 10:326–337 (1988).
4. N. Brot and H. Weissbach. Biochemistry and physiological role of methionine sulfoxide residues in proteins. *Arch. Biochem. Biophys.* 223:271–281 (1983).
5. N. Brot and H. Weissbach. The biochemistry of methionine sulfoxide residues in proteins. *Trends Biochem. Sci.* 7:137–139 (1982).
6. D. Garland, J. S. Zigler, Jr., and J. Kinoshita. Structural changes in bovine lens crystallins induced by ascorbate, metal, and oxygen. *Arch. Biochem. Biophys.* 251:771–776 (1986).
7. C. Schöneich, K. Bobrowski, J. Holcman, and K.-D. Asmus.

Oxidation mechanisms of methionine containing peptides by hydroxyl and peroxyl radicals. In K. E. Davies (ed.), *Oxidative Damage and Repair,* Plenum Press, New York, 1991, pp. 380–385.
8. E. R. Stadtman. Oxidation of proteins by mixed-function oxidation systems: Implications in protein turnover, ageing and neutrophil function. *Trends Biochem. Sci.* **11**:11–12 (1986).
9. T. Numata, Y. Watanabe, and S. Oae. Oxygenation of alkyl sulfides with ferrous perchlorate/ascorbic acid/oxygen system. *Tetrahedron Lett.* **16**:1411–1414 (1979).
10. G. B. Fields and R. L. Noble. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res.* **35**:161–241 (1990).
11. Y. Ogata, Y. Kosugi, and T. Morimoto. Kinetics of the cupric salt-catalyzed autoxidation of L-ascorbic acid in aqueous solutions. *Tetrahedron* **24**:4057–4066 (1968).
12. M. M. T. Khan and A. E. Martell. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation. *J. Am. Chem. Soc.* **89**:4176–4185 (1966).
13. M. M. T. Khan and A. E. Martell. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric ion catalyzed oxidation. *J. Am. Chem. Soc.* **89**:7104–7111 (1967).
14. M. M. T. Khan and A. E. Martell. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. III. Vanadyl ion catalyzed oxidation. *J. Am. Chem. Soc.* **90**:6011–6017 (1968).
15. K. Yamamoto, M. Takahashi, and E. Niki. Role of iron and ascorbic acid in the oxidation of methyl linoleate micelles. *Chem. Lett.* **186**:1149–1152 (1987).
16. S. A. Levine and P. M. Kidd. *Antioxidant Adaptation: Its Role in Free Radical Pathology,* Biocurrents Division, San Leandro, CA, 1985, pp. 52–54.
17. Y. Kato, K. Uchida, and S. Kawakishi. Oxidative fragmentation of collagen and prolyl peptide by Cu(II)/H_2O_2. *J. Biol. Chem.* **267**:23646–23651 (1992).
18. 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.* (in press).
19. 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**:375–376 (1991).
20. R. L. Levine. Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *J. Biol. Chem.* **258**:11828–11833 (1983).
21. M. Cher and N. Davidson. The kinetics of the oxygenation of ferrous iron in phosphoric acid solution. *J. Am. Chem. Soc.* **77**:793–798 (1955).
22. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. Singh. Hydrogen ion buffers for biological research. *Biochemistry* **5**:467–477 (1966).
23. D. E. Gueffroy (ed.). *Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems,* Calibiochem Corp., 1990.
24. M. Hicksand and J. M. Gebicki. Rate constants for reaction of hydroxyl radicals with Tris, Tricine and Hepes buffers. *FEBS Lett.* **199**:92–94 (1986).
25. R. A. Greenwald (ed.). *Handbook of Methods for Oxygen Radical Research,* CRC Press, Boca Raton, FL, 1985.
26. K. Bobrowski and J. Holcman. Formation and stability of intramolecular three electron S.∴N, S.∴S, and S.∴O bonds in one-electron oxidized simple methionine peptides. Pulse radiolysis study. *J. Phys. Chem.* **93**:6381–6387 (1989).
27. K. Uchida and S. Kawakishi. Selective oxidation of tryptophan and histidine residues in protein through the copper-catalyzed autoxidation of L-ascorbic acid. *Agr. Biol. Chem.* **52**:1529–1535 (1988).
28. K. Uchida and S. Kawakishi. Selective oxidation of imidazole ring in histidine residues by the ascorbic acid-copper ion system. *Biochem. Biophys. Res. Commun.* **138**:659–665 (1986).
29. R. J. Sundberg and R. B. Martin. Interactions of histidine and other imidazole derivatives with transition metal ions in chemical and biological systems. *Chem. Rev.* **74**:471–512 (1974).
30. K. A. Williams, J. T. Doi, and W. K. Musker. Neighboring-group participation in organic redox reactions. 10. The kinetic and mechanistic effects of imidazole and benzimidazole nitrogen on thioether oxidations. *J. Org. Chem.* **50**:4–10 (1985).