SULFOXIDATION OF METHIONINE IN SMALL MODEL PEPTIDES: EVALUATION OF THE FERRIC CHLORIDE/MERCAPTOETHANOL OXIDIZING SYSTEM

by

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Submitted to the Department of Chemistry and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science

ABSTRACT

Conversion of methionine to methionine sulfoxide can take place during postranslational modification, formulation, storage or synthesis of proteins. Therefore, methionine sulfoxidation is a major concern to pharmaceutical industries. Small model peptides containing methionine were oxidized by mercaptoethanol/$FeCl_3$ system in phosphate buffer. These studies were carried out to investigate the factors affecting methionine oxidation and the mechanism involved in the sulfoxidation. The effect of pH, buffer concentration, Fe^{3+} concentration, type of thiol, mercaptoethanol concentration and EDTA concentration on the formation of methionine sulfoxide was examined. The model peptides studied included His-Met, Met-His, Gly-Met, Gly-Met-Gly, Gly-Gly-Met and Met-Gly-Gly. The initial rate of formation of methionine sulfoxide was observed to be dependant on the iron concentration and thiol concentration. However, the initial rate of formation of methionine sulfoxide was independent of the phosphate buffer concentration. The presence of EDTA not only suppressed the formation of sulfoxide but also caused an increase in peptide degradation. The influence of the location of methionine on the yield of sulfoxide was also investigated. Maximum sulfoxide formation was observed when methionine was in the carboxyl-terminal position in the

peptide, His-Met. The possible role of various reactive oxygen species was studied by using various trapping agents. The involvement of freely diffusible H_2O_2, $O_2^{-\cdot}$ and $OH^{\cdot}$ in this oxidation process was found to be negligible. It may be also possible that these reactive oxygen species may not be involved in the rate determining step. However, the presence of bound reactive oxygen species cannot be ruled out, as these may not be trapped by the trapping agents used in this study. These studies and future elucidation of the chemical mechanisms involved in methionine sulfoxidation will provide a better understanding of the susceptibility of various proteins and peptides to degradation.

ACKNOWLEDGEMENTS

The guidance and supervision by Dr. George Wilson aided me in the completion of this thesis. I would like to thank him for his understanding and patience. I would also like to thank Dr. Christian Schöneich for introducing me to both the theoretical and practical aspects of metal-catalyzed oxidation mechanisms.

I greatly appreciate the help and support offered by the members of the Wilson group. I would specifically like to thank Binodh, Chandrani and Tina for their help in figuring out problems with the HPLC. I also benefitted from numerous scientific and non-scientific discussions with Dongbo and Karina. The continual support of Department of Chemistry in the form of a teaching assistantship is sincerely acknowledged.

I would like to extend my sincere thanks to my husband, Ajay, who encouraged me at all times during my graduate study. His patience and moral support played a very important role in the completion of this thesis. I would also like to thank my parents, my husbands parents, my brother-in-law and his wife for their encouragement and support.

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SECTION I. INTRODUCTION

The commercial production of proteins as therapeutic agents has increased the need for the understanding of various factors involved in protein stability.

A) Mechanisms of Protein and Peptide Degradation

Proteins and peptides can undergo various physical and chemical degradation processes. Some of the mechanisms of the physical degradation process are:

1) Denaturation of Proteins

Denaturation causes the unfolding of proteins resulting in a loss of globular structure. When denatured, proteins lose their tertiary structure and also frequently their secondary structure (Havel et al., 1986). The denaturation of proteins can be caused by a variety of conditions such as change in temperature, extreme acidic or basic pHs, organic solvents, increase in salt concentration, reduction of disulfide bonds, or by shear forces.

2) Aggregation of Proteins

Aggregation of proteins involves the association of hydrophobic domains to form dimers, trimers, tetramers or even polymers. Between the native and unfolded state of a protein there may be several partially unfolded states (Yphantis and Arakawa, 1987; Brems et al., 1985). For instance, bovine growth hormone (Brems et al., 1988) and interferon-γ (Arakawa et al., 1987) form partially folded intermediates that can lead to their inactivation by the formation of aggregates.

3) Surface Adsorption

Adsorption of proteins to surfaces of containers can cause drug delivery-related problems as has been reported for insulin (Massey and Sheliga, 1988; Sato et al., 1983).

4) Precipitation

Macroscopic aggregation leads to the precipitation of proteins, for instance insulin precipitates upon storage (Massey and Sheliga, 1988).

The chemical degradation mechanisms of proteins and peptides include the following:

1) Hydrolysis of the Peptide Bond

Hydrolysis of the peptide bond can be effected by chemical or enzymatic means. This usually involves the attack of a water molecule at the peptide bond resulting in the formation of free amino and carboxyl groups.

2) Deamidation of Asparagine (Asn) or Glutamine (Gln)

Under neutral and alkaline conditions Asn and Gln residues undergo deamidation, where the side chain amide linkage is hydrolyzed to form a free carboxylic acid (Patel and Borchardt, 1990). This process may affect the function and activity of many proteins, e.g., triosephosphate isomerase (Yuan et al., 1981), hen egg white lysozyme (Ahern and Klibanov, 1985) and calmodulin (Johnson et al., 1989). Using synthetic peptides it has been shown that deamidation is increased by increased pH, temperature and ionic strength (Patel and Borchardt, 1990).

3) Oxidation of amino acids

Methionine, histidine, cysteine, tryptophan and tyrosine residues are potential oxidation sites in proteins and peptides. For example, oxidation of methionine to methionine sulfoxide occurs in proteins. Such oxidation can alter the function of some proteins and hormonal as well as nonhormonal peptides (Manning et al., 1989).

4) Disulfide Exchange Between Proteins

The interchange of disulfide bonds upon their reduction can lead to incorrect folded structures thus altering the tertiary structure of the proteins and possibly their activity. For instance, the inactivation of bovine pancreatic ribonuclease A at 90°C at pH 8.0 is mainly due to disulfide interchange (Zale and Klibanov, 1986).

5) β-Elimination

Cysteine, serine, threonine, phenylalanine and lysine can undergo elimination of the β-groups, a process that is usually influenced by pH, temperature and the presence of metal ions (Nashef et al., 1977 Sen et al., 1977).

6) Racemization of Amino Acids

Racemization of amino acids in proteins involves the abstraction of the alpha proton by base to generate D-amino acids or create peptide bonds inaccessible to proteolytic enzymes.

B) Role of Reactive Oxygen Species in Degradation of Proteins and Peptides

The interaction of reactive oxygen species with proteins and peptides constitutes one of the major pathways of degradation of proteins in biological media (Simic et al., 1988). Reactive oxygen species may cause changes in activity, formation or breakage of disulfide bonds, fragmentation, increased proteolytic susceptibility and aggregation of proteins (Simic et al., 1988; Davies et al., 1987).

In living systems, under conditions of oxidative stress, various reactive oxygen species such as hydroxyl radicals ($^{\cdot}OH$), peroxyl radicals ($ROO^{\cdot}$), superoxide radicals ($O_2^{-\cdot}$), oxyl radicals ($RO^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) may be generated (Simic et al., 1988; Stadtman, 1992). The reactive oxygen species are usually present in the

body in very low concentrations (10^{-5} M to 10^{-9} M). Several enzymes and electron transport proteins in various metabolic reactions generate free radicals *in vivo*. For instance, xanthine oxidase and aldehyde oxidase generate superoxide anion radical by adding a single electron to molecular oxygen. In aerobic eukaryotic cells mitochondria are the major source of endogenous free radical generation. Changes attributed to free radical reactions occurring in the body include: 1) oxidative alterations of proteins such as alpha-1-proteinase inhibitor and elastin (Harman, 1984) 2) oxidation of cysteine and methionine during senile cataract formation (Truscott et al., 1977) 3) breakdown of mucopolysaccharides by oxidative degradation and (Harman, 1984) 4) changes in membrane characteristics of mitochondria and lysosomes due to lipid peroxidation (Harman, 1984; Kukielka and Cederbaum, 1989). Therefore the oxidative modifications of several proteins are due to the reactive oxygen species formed under conditions of oxidative stress. The *in vitro* oxidation of proteins may occur during their synthesis, formulation, purification or storage. For instance, when recombinant human interleukin 2 is stored for a long periods in aqueous solutions, oxidation of methionine residues to methionine sulfoxide is observed (Sasaoki

et al., 1989). It has been shown that some proteins that are exposed to ·OH radicals can be recognized and degraded rapidly by intracellular proteolytic systems (Davies, 1987; Davies et al., 1987). The combination of ·OH, $O_2^{-\cdot}$ and molecular oxygen that may mimic biological exposure to O_2 radicals has been shown to produce extensive protein fragmentation (Davies, 1987). Thus, it may be possible that the *in vivo* reactive oxygen species-mediated oxidation reactions may be similar to those by which protein oxidation occurs *in vitro*.

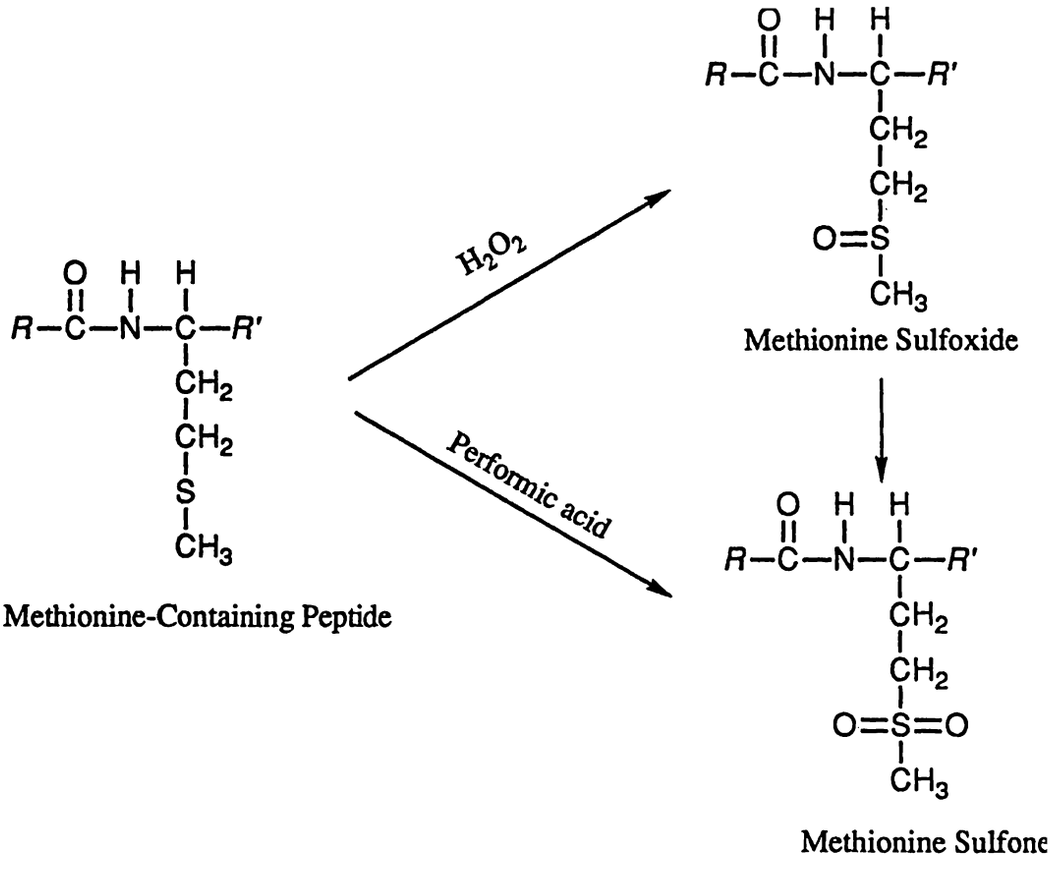
C) Oxidation of Sulfur in Proteins

1) Methionine Sulfoxidation

The amino acid, methionine, has a thioether group that is weakly nucleophilic. At low pH methionine is not protonated and thus can be easily oxidized. Therefore, the primary reaction of hydrogen peroxide with proteins under acidic conditions is the oxidation of methionine to methionine sulfoxide. However, at neutral or slightly alkaline conditions hydrogen peroxide can also oxidize imidazole, sulfhydryl, disulfide or phenol groups. Other reagents which have been used to oxidize methionine include

iodine, performic acid, $NaIO_4$, dimethyl sulfoxide, chloramine-T and N-chlorosuccinimide. Oxidizing agents such as hydrogen peroxide, $NaIO_4$, iodine and N-chlorosuccinimide oxidize methionine to methionine sulfoxide, whereas stronger oxidants such as performic acid can further oxidize methionine sulfoxide to methionine sulfone (Figure 1). In living systems under conditions of "oxidative stress" free radicals are produced which may lead to methionine sulfoxidation. This has been associated with the loss of biological activity of many peptide hormones such as corticotrophin (Dedman et al., 1961), α- and β-melanotropins (Dixon, 1956), parathyroid hormone (Tashjian et al., 1964), gastrin (Morley et al., 1965), calcitonin (Riniker et al., 1968), corticotropin releasing factor (Vale et al., 1981) as well as other proteins and non hormonal peptides. α-I-antiproteinase loses its activity upon exposure to reactive oxygen species (Travis and Salvesen, 1983). α-I-antiproteinase is an inhibitor of elastase, a digestive enzyme responsible for cleaving small neutral residues such as alanine, glycine etc. Inactivation of α-I-antiproteinase can lead to tissue damage in several disease states including rheumatoid arthritis and emphysema (Travis and Salvesen 1983; Carp and Janoff, 1980). In contrast, there are other proteins whose function is not affected by methionine oxidation such as pancreatic ribonuclease (Neumann et al., 1962) and α-

Figure 1: Oxidation of methionine to methionine sulfoxide and methionine sulfone.


H2O2
Performic acid
Methionine-Containing Peptide
Methionine Sulfoxide
Methionine Sulfone

chymotrypsin (Schachter et al., 1963). Also, when biosynthetic human growth hormone is chemically oxidized at methionine-14, it exhibits full biological activity and has similar immunoreactivity as authentic human growth hormone. In contrast, oxidation of methionine-64 and/or methionine-179 in human chorionic somatomamotropin decreases its affinity for lactogenic receptors and its *in vitro* biological potency (Teh et al., 1987).

The detection of methionine sulfoxide in proteins can be difficult because during conventional amino acid analysis by acid hydrolysis, methionine sulfoxide is unstable and can be converted back to methionine. However, other methods are available:

1. Exhaustive and selective alkylation of methionine residues is performed (methionine sulfoxide is resistant to alkylation) with the subsequent oxidation of methionine sulfoxide to methionine sulfone by performic acid. Methionine sulfone is stable to acid hydrolysis and can be analyzed by amino acid analysis under acidic conditions (Neumann, 1972).

2. Methionine sulfoxide is stable to base hydrolysis and thus after hydrolyzing the protein with 4N sodium hydroxide, the amount of methionine sulfoxide present

is determined by amino acid analysis (Patai et al., 1988).

3. Another method is the treatment of the protein with cyanogen bromide which specifically cleaves at the C-terminal end of methionine but does not cleave methionine sulfoxide. This results in the formation of methylthiocyanate and peptides containing homoserine lactone and methionine sulfoxide. The peptides are then subjected to acid hydrolysis in the presence of a sulfhydryl reagent which reduces the methionine sulfoxide to methionine. If methionine is formed after the hydrolysis, it indicates that methionine sulfoxide was initially present, whereas the amount of homoserine is an indication of the amount of methionine in the protein (Patai et al., 1988).

4. Alternatively, methionine and methionine sulfoxide containing peptides and proteins have been separated by high performance liquid chromatography (HPLC) (George-Nascimonto et al., 1988; Hugh et al., 1977) and affinity chromatography (Shaw and West, 1980).

2) Oxidation of Other Thiol Groups in Proteins

In addition to methionine, thiol groups in proteins can be oxidized to various products such as disulfides (RSSR), sulfenic acids (RSOH), sulfinic acids (RSO_2H) or sulfonic acids (RSO_3H). The nature of the products formed depends on the reaction conditions, such as, temperature, pH, buffer medium, type of trace metals used as catalysts, and oxygen tension (Jocelyn, 1972). In cases where the thiol groups are in spatial proximity then the formation of the disulfide is favored and the protein can covalently aggregate. Thiol groups can be oxidized in the presence of chemical oxidants such as iodine, hydrogen peroxide and ferricyanide. Thiol groups can be also oxidized by molecular oxygen in the presence of catalytic amounts of transition metal ions such as iron or copper. The rate of oxidation of the thiols depends on the neighboring groups of the thiols. It has been reported that the rate of oxidation of dithiols is increased when the distance between the two thiol groups is decreased. Electronegative neighboring groups such as carboxyl groups also increase the rate of oxidation of thiols by raising the pKa of the thiol group. Thus, a mercaptide ion can be oxidized more readily than a protonated thiol. Also

the oxidation rate usually increases with pH (Philipson, 1962).

D) Trace Metal Catalyzed Activation of Oxygen by Reductant

Metal ion catalyzed oxidation of proteins plays an important role in biological aging and this has been reviewed in detail by Stadtman (1990a, 1990b, 1992). These systems are partly responsible for the alterations of proteins *in vivo* and are implicated in the accumulation of altered forms of enzymes during aging. Transition metals act as catalysts in such auto-oxidation reactions. Thus, one route of formation of reactive oxygen species involves the transition metal catalyzed activation of oxygen by reductants. These reductants are referred to as prooxidants. Hence, in the presence of O_2, Fe^{3+} or Cu^{2+} and an appropriate electron donor, several enzymatic and non enzymatic oxygen free radical-generating systems are able to catalyze the oxidative modification of proteins. For instance, Levine (1981) reported the oxidative inactivation of glutamine synthetase by the ascorbate/Fe^{3+} system. It is believed that the Fe^{2+} binds to a metal binding site on the enzyme. The Fe^{2+}-enzyme complex may react with H_2O_2, produced during the autooxidation of ascorbate, to yield reactive

oxygen species at the metal binding site of the protein. This can result in the loss of catalytic activity of the enzyme and increase the susceptibility of the protein to proteolytic degradation. In the metal catalyzed inactivation of glutamine synthetase and other enzymes, free radical scavengers do not inhibit this inactivation (Levine et al., 1981; Fucci et al., 1983) and furthermore only a few amino acid residues of a given protein are modified. This has led to the proposition that metal catalyzed oxidative modification of proteins is a site specific process (Samuni et al., 1983). Furthermore, the interaction of NADPH with ferric complexes to catalyze microsomal generation of reactive oxygen species has been well studied (Rashba-Step and Cederbaum, 1993). However the catalytic ability of iron in aiding microsomal generation of reactive oxygen species is complex and depends upon the nature of the iron complex and the specific reaction under investigation. For instance complexes such as ferric-EDTA or ferric-diethylenetriaminepentaacetic acid (DTPA) are very effective catalysts for NADPH-dependent $^{\cdot}OH$ radical production in microsomes but are inhibitory towards lipid peroxidation. In contrast, complexes such as ferric ATP or ferric histidine show the opposite catalytic effectiveness (Kukielka and Cederbaum, 1989; Rashba-Step and Cederbaum, 1993).

I) Reactivity of Oxygen with Biomolecules

Ground state dioxygen is a triplet molecule and its reaction with most biomolecules is spin forbidden. Although dioxygen cannot directly react with biomolecules at a significant rate, transition metals act as catalysts of redox reactions and their reactions with dioxygen are less spin restricted. They act as catalysts because coordination of transition metals to most biomolecules usually involves the d-orbitals of the transition metal. Furthermore, dioxygen can also bind to transition metals through their d-orbitals (Valentine, 1973). Thus the transition metals act as a bridge between the biomolecules and dioxygen as it can simultaneously bind to the biomolecules and dioxygen. It has been reported that ascorbate, iron and dioxygen can form such complexes (Khan et al., 1967).

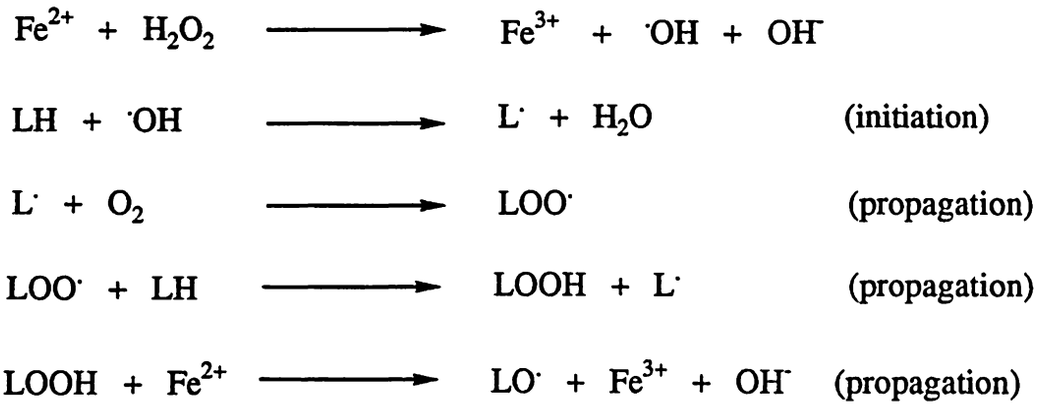
The various biomolecules reacting with oxygen and transition metals include ascorbate, cysteine, dopamine, epinephrine, glutathione, cysteamine and dithiothreitol. It has been shown that ascorbate and DTT act as both antioxidant and prooxidants (Miller et al., 1990; Li et al., 1993; Schöneich et al., 1993; Kim et al., 1985). It has also been shown that the antioxidant activity of many of these compounds is metal dependent. For instance, the prooxidant and antioxidant activity of ascorbate in iron

catalyzed lipid peroxidation (Miller and Aust, 1989) has been related to the degree of iron reduction. Similarly, lipid peroxidation initiated by thiols such as cysteine, glutathione, or DTT has been related to the reduction of iron (Tien et al., 1982).

2) Chemistry of Iron

Iron is a known catalyst of lipid peroxidation, and for DNA and protein oxidation. The most widely accepted theory of iron-catalyzed lipid peroxidation is that iron catalyzes $^{\cdot}OH$ formation from H_2O_2 in an acidic medium, which in turn reacts with polyunsaturated fatty acids, initiating a chain reaction (Figure 2). However, due to the low solubility of iron (Fe^{3+}) at physiological pH (10^{-18} M) (Miller et al., 1990), biological systems must exploit a chelation chemistry so that the metals can be solubilized and can be transported to and from storage proteins. Similarly, in vitro, when ferric chloride is used as a catalyst in phosphate buffer solutions (pH 6-10), the concentration of free hydrated ferric iron is low as predicted from the solubility products of $Fe(OH)_3$(Ksp $Fe(OH)_3 = 10^{-38.8}$) and $FePO_4$ (Ksp $FePO_4 = 10^{-26.4}$) (Kotrly and Sucha, 1985). Ferric ion can be retained in solution by the chelation capability of phosphate buffer due to the formation of complexes of ferric with $H_2PO_4^-$ and HPO_4^-

Figure 2: Mechanism of lipid peroxidation.


$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$
$LH + {}^{\cdot}OH \longrightarrow L^{\cdot} + H_2O$ (initiation)
$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$ (propagation)
$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$ (propagation)
$LOOH + Fe^{2+} \longrightarrow LO^{\cdot} + Fe^{3+} + OH^-$ (propagation)

$[Fe_{aq}.H_2PO_4]^{+2}$ and $[Fe_{aq}.HPO_4]^{+}$ (Lanford and Kiehl, 1942; Kotrly and Sucha, 1985). Using hydroxylation of salicylates as a model, it has been shown that with ferric chloride as catalyst in phosphate buffer, free hydroxyl radicals are formed from superoxide and hydrogen peroxide (Flitter et al., 1983). Ferric ion can also be solubilized by chelation to amino acids, peptides or disulfides. For instance,

$$\log \beta 1 = 10.0 \text{ for } Fe^{+3} + Gly = [Fe_{aq}.Gly]^{n+},$$

$$\log \beta 1 = 9.10 \text{ for } Fe^{+3} + Gly\text{-}Gly = [Fe_{aq}.Gly\text{-}Gly]^{n+},$$

and in the presence of hydroxide ion,

$$\log \beta = 30.7 \text{ when } Fe^{+3} + 2,3,\text{-dimercaptopropanol} = [Fe(OH)(2,3,\text{-dimercaptopropanol}],$$

where β is the stability constant of the complex.

These reactions can be influenced by the presence of chelating agents. Chelators, in which oxygen atoms ligate transition metals, generally prefer the oxidized forms of the transition metals, thereby decreasing the redox potential of these metals. Chelators in which nitrogen atoms ligate transition metals prefer their reduced forms and tend to increase the redox potential of these metals (Miller et al., 1990).

E) Description of the Research Problem

Proteins such as human growth hormone (Teh et al., 1987) and interleukin-2 (Sasaoki et al., 1989) undergo a variety of degradation processes, one of which is the sulfoxidation of the sulfur containing amino acid methionine. Conversion of methionine to methionine sulfoxide is of major concern to the pharmaceutical industry as this conversion can occur during posttranslational modification of recombinant proteins, isolation of proteins and peptides from biological materials, formulation, storage or synthesis. In certain cases, oxidation of methionine residues to their corresponding sulfoxides can lead to the loss of biological activity. For instance, oxidation of two methionyl residues on human α1-proteinase inhibitor inactivates this inhibitor towards elastolytic proteinases (Johnson and Travis, 1979). Oxidation of methionine residue β(55)D6 at the α1-β1 interface in haemoglobin completely destabilizes the T state of the hemoglobin molecule and thus the functional behavior is drastically changed which is characterized by high oxygen affinity, absence of cooperativity and lack of Bohr effect (Amiconi et al., 1989). Conformation changes can also be induced by the oxidation of methionine residues such as in the parathyroid hormone (Zull et al., 1990). In living systems methionine sulfoxide can be formed under

conditions of oxidative stress, aging and inflammation (Patai and Rappoport, 1988). Several disease states, for example cataracts and rheumatoid arthritis, are associated with the loss of biological activity of the proteins which may be related to the conversion of methionine to methionine sulfoxide. Thus the stability of proteins is extremely important in controlling and monitoring biotechnological processes. It is also important in understanding aging and inflammation under conditions where protein degradation catalyzed by trace metals and oxygen is taking place.

Oxidation of methionine is known to be influenced by the environment around methionine in the protein. For example, in recombinant human growth hormone, of the three methionines present, methionine 125 is prone to oxidation when the protein is exposed to chemical oxidants such as hydrogen peroxide, while solid state stability tests show that methionine 14 is mainly oxidized (Becker et al., 1988). The mechanism of the oxidation of methionine is not known and thus it is of interest to study the formation of methionine sulfoxide in small model peptides and to investigate the influence of adjacent amino acids on the formation of methionine sulfoxide. This study will aid in predicting the methionine stability towards oxidation depending on the type of adjacent amino acids. Recent

studies have suggested the potential reactivity of various reactive oxygen species including hydrogen peroxide, $O_2^{-\cdot}$, $ROO^{\cdot}$, $HO^{\cdot}$ in this process of sulfoxidation (Schöneich et al., 1991a; Li et al., 1993). The reactive oxygen species can be generated during the oxidation of methionine containing peptides in the presence of transition metals and reductant. In the present study various monothiols and in particular, mercaptoethanol (MSH) in combination with $FeCl_3$ was chosen as the prooxidant/transition metal system. Some of the potential pathways of formation of reactive oxygen species by thiol/iron systems (Tien et al., 1982; Cavallini et al., 1968; Searle and Tomasi, 1982; Seaz et al., 1982)' are as follows

$$2MSH + 2\ Fe^{3+} \rightarrow MSSM + 2Fe^{2+} + 2H^{+} \quad (1)$$

$$2H^{+} + 2Fe^{2+} + O_2 \rightarrow 2Fe^{3+} + H_2O_2 \quad (2)$$

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

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

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + {}^{\cdot}OH \quad (5)$$

$$RH + {}^{\cdot}OH + O_2 \rightarrow ROO^{\cdot} + H_2O \quad (6)$$

Specific Aims:

The aim of the present study was to investigate:

1) Sulfoxidation of methionine in small model peptides will be studied to obtain information about the effect of the environment around the methionine. For example, the influence of adjacent amino acids such as histidine will be studied because histidine is known to bind to transition metals and to form reactive oxygen species. The information obtained from the use of peptides will help in investigating methionine sulfoxidation in the complete protein.

2) The various factors which may influence the sulfoxidation of methionine include pH, nature of buffer, trace metal ions (such as iron, copper), peptide and mercaptoethanol (reducing agent) concentration. The effect of these factors will be studied.

3) The role of different reactive oxygen species involved in this oxidation process will be investigated to help in elucidation of the mechanism of methionine sulfoxide formation.

4) The effect of the primary sequence of the proteins or peptides on methionine sulfoxidation will also be studied. This will help in predicting the sulfoxidation of methionine in any given protein.

These studies will provide a basis for the further investigation of methionine oxidation in proteins. The understanding of the mechanism of oxidation will enable the pharmaceutical industry to design oxidation resistant proteins in different formulations or to find ways of preventing oxidation from occurring.

SECTION II. MATERIALS AND METHODS

Materials:

The peptides His-Met (HM), Met-His (MH), Gly-Met (GM), Gly-Gly-Met (GGM), Gly-Met-Gly (GMG) and Met-Gly-Gly (MGG) were obtained from Bachem, Bioscience Inc. (Philadelphia, PA). Dithiothreitol (DTT), $FeCl_3$, trifluoroacetic acid, catalase (bovine liver, EC 1.11.1.6), superoxide dismutase (bovine erythrocyte, EC 1.15.1.1), cysteine and glutathione were purchased from Sigma Chemical Company (St. Louis, MO). Monobasic sodium phosphate, dibasic sodium phosphate and methanol were obtained from Fisher Scientific (St. Louis, MO). HPLC-grade acetonitrile was supplied by Fisher Chemical (Fair Lawn, NJ). 2-Hydroxyethyl disulfide $(OHCH_2CH_2)_2S_2$, sodium disulfide and ethylenediaminetetraacetic acid (EDTA) were obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). The water used in all reaction solutions was of Millipore Q quality (18 MΩ resistance).

Synthesis of Methionine Sulfoxide in Methionine-Containing Peptides:

The sulfoxides of each of HM, MH, GM, GGM, GMG and MGG were synthesized by reacting the peptide solution with an excess of hydrogen peroxide. Briefly, a 25 mM peptide solution in water (pH 3.0) was mixed with an excess of hydrogen peroxide (ca 35 mM) in a final volume of 1 mL. The formation of the corresponding methionine sulfoxide was monitored by HPLC. Upon completion of the reaction, the methionine sulfoxide formed was purified by preparative reverse phase HPLC (C18 column; 250 x 10 mm; 10 μm particle size; Alltech Associates, Deerfield, IL). The mobile phase used for the separations varied from 2-20% (v/v) acetonitrile, 0.01-0.02% (v/v), trifluoroacetic acid and 80-98% (v/v) water depending on the peptide. The flow rate was 1 mL/minute. The eluate from the column was monitored using a UV detector at 214 nm. The fractions containing the sulfoxides were collected and pooled. The pooled fractions were frozen in a dry-ice-acetone bath and lyophilized. The solid residue obtained after lyophilization was characterized by ^{1}H NMR (300 MHz) and positive FAB-MS (VG ZAB-HS mass spectrometer interfaced with a 11/250 data system). The peptide converted to methionine sulfoxide was used as a standard. The mass spectra of HM and HM(SO) are shown in Figures 3 and 4 and the ^{1}H NMR spectrum of HM(SO)

is shown in Figure 5. The molecular ion peak of HM is (MH^+ 287) and that of HMS(0) is 16 mass units higher than HM (molecular ion peak is MH^+ (303). This was confirmed by ^1H NMR which shows a downfield shift of the thioether sulfur upon formation of the sulfoxide (from σ = 2.2 ppm to σ = 2.7 ppm).

Reaction Mixture for Studying Methionine Sulfoxidation:

Methionine sulfoxidation was typically studied in a 0.5 ml reaction mixture containing 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 1 mM peptide and 240 μM $FeCl_3$. The concentration of $FeCl_3$ (240 μM) indicates the amount of $FeCl_3$ added and not the true of concentration of iron in the reaction solution. The effect of changing the following reaction conditions was also determined:

1) The effect of different thiols was studied by substituting MSH with cysteine, glutathione or DTT.
2) The effect of MSH concentration was studied at 0 mM, 1 mM, 3 mM, 4 mM, 5 mM and 10 mM final concentrations.
3) The effect of sodium phosphate buffer concentration was studied at 10 mM, 20 mM and 50 mM.
4) The effect of final pH of the reaction mixture was studied at various pH's ranging from 6.0 to 9.0.

Figure 3: Positive FAB-MS of His-Met (HM).

The commercially obtained HM was subjected to positive FAB-MS in thioglycerol matrix. This was done as a control to confirm the fragmentation pattern of HM(SO). The molecular ion peak of HM was found to be 287 amu.

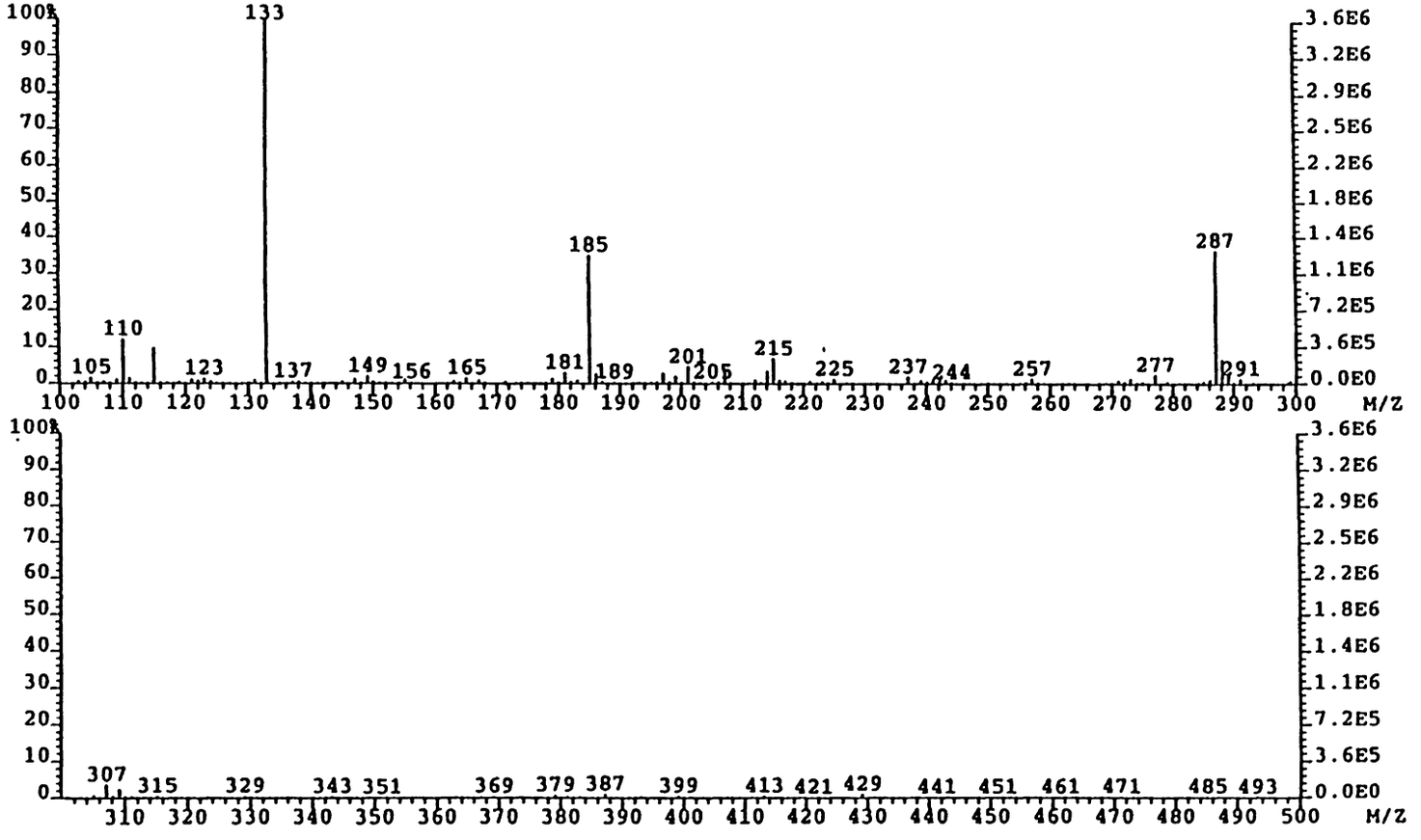

100%
90
80
70
60
50
40
30
20
10
0
133
185
287
110
105
123
137
149
156
165
181
189
201
205
215
225
237
244
257
277
291
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300
M/Z
3.6E6
3.2E6
2.9E6
2.5E6
2.2E6
1.8E6
1.4E6
1.1E6
7.2E5
3.6E5
0.0E0
307
315
329
343
351
369
379
387
399
413
421
429
441
451
461
471
485
493
310
320
330
340
350
360
370
380
390
400
410
420
430
440
450
460
470
480
490
500

Figure 4: Positive FAB-MS of His-Met sulfoxide {(HM(SO)}.

HM(SO) was synthesized by hydrogen peroxide-mediated oxidation of HM as described in the methods. The purified compound was subjected to positive FAB-MS in thioglycerol matrix. The molecular ion of HM(SO) was found to be 303 amu. The remaining fragmentation pattern of this compound is essentially similar to that observed for the parent compound, HM.

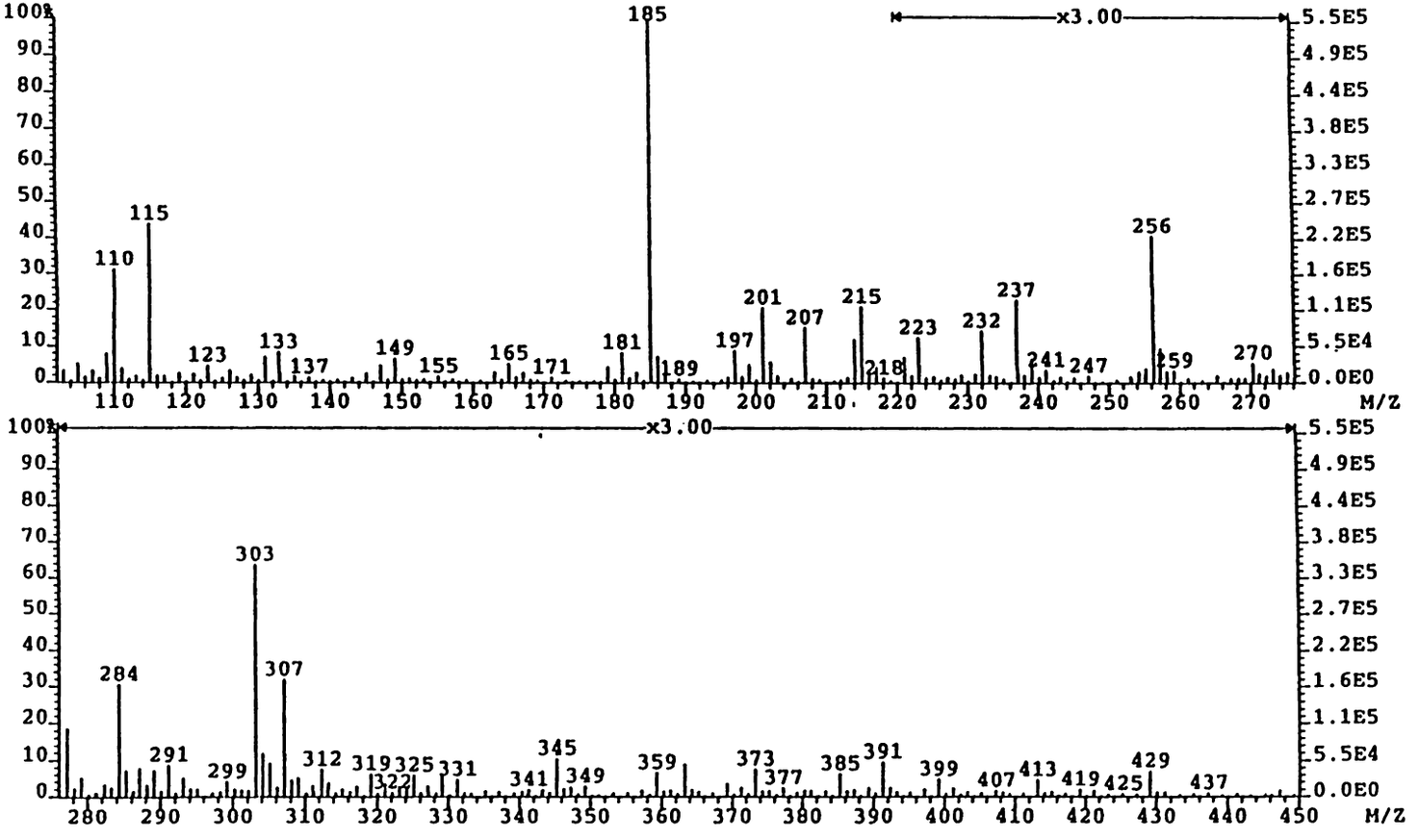
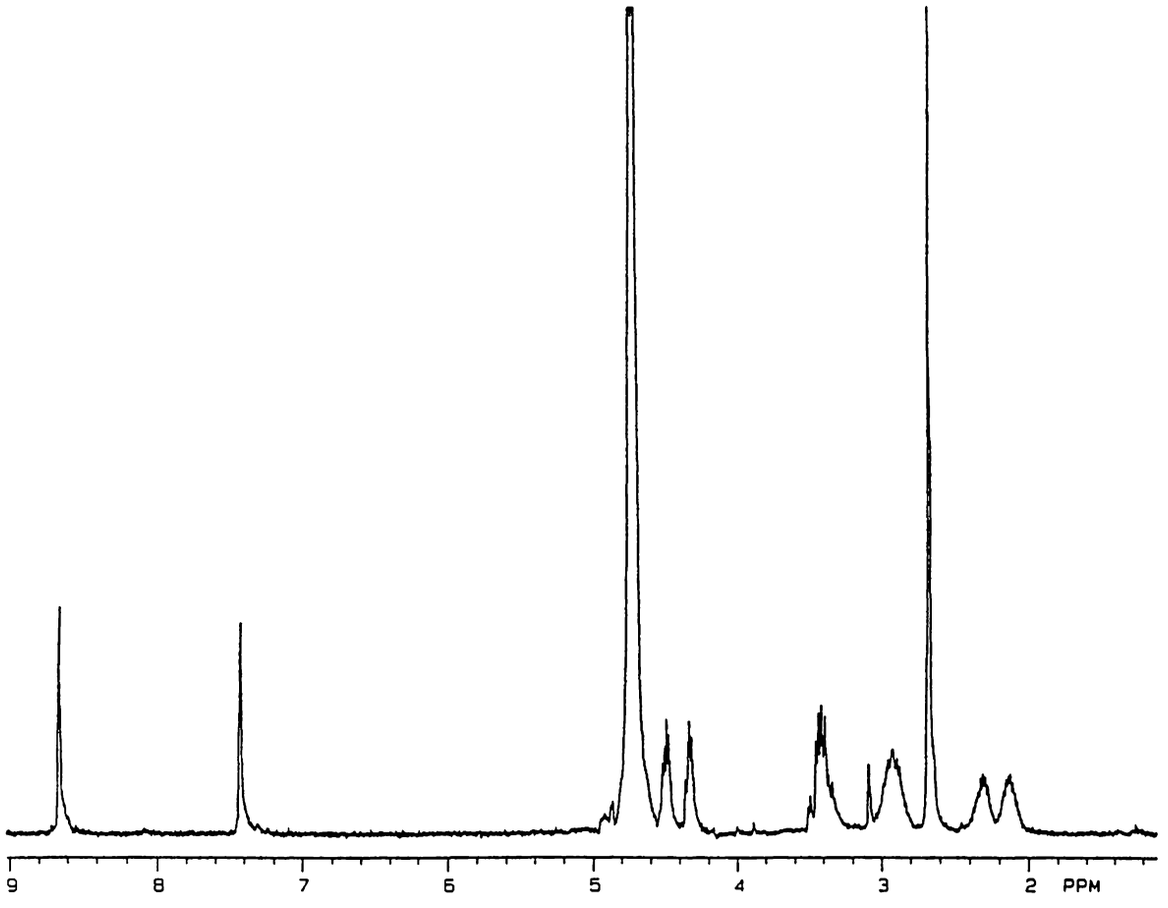

x3.00
185
115
110
256
201
215
207
237
223
232
123
133
137
149
155
165
171
181
189
197
218
241
247
259
270
M/Z
x3.00
303
284
307
291
299
312
319
322
325
331
345
341
349
359
373
377
385
391
399
407
413
419
425
429
437
M/Z

Figure 5: ^{1}H NMR of His-Met sulfoxide {HMS(0)} in D_2O.

HM(SO) was synthesized by hydrogen peroxide-mediated oxidation of HM as described in the Methods section. The purified compound was dissolved in 0.6 ml D_2O and subjected to ^{1}H NMR (300 MHz; QE-300). The chemical shift of S-methyl protons was found to be 2.7 ppm.


9
8
7
6
5
4
3
2
PPM

5) The effect of $FeCl_3$ concentration was studied at 0, 20, 200, and 2000 μM.

6) Various blank reactions were studied. Reactions were conducted in the absence of $FeCl_3$, MSH, peptide or phosphate buffer.

7) Various peptides were studied and these included HM, MH, GM, GGM, GMG and MGG. The effect of changing the above conditions (1-6) was studied using HM and GMG as representatives of the other peptides.

Stock solutions of various thiols including MSH, DTT, cysteine, glutathione and $FeCl_3$ were made fresh on the day of the experiment. After all the ingredients were added to the reaction mixture, the pH was adjusted as specified and the volume of the reaction mixture was made up to 0.5 mL.

Determination of Methionine Sulfoxidation by HPLC:

HPLC analysis was performed on a Shimadzu SIL-6B instrument consisting of a Shimadzu system controller, auto injector (SIL-6B), Shimadzu pump (LC-6A), UV detector (SPD 6AV) and Chromatopac integrator (C-R4A). The column used for the analytical separation of the peptide and its corresponding sulfoxide was: Alltech (250 mm x 4.6 mm) 5 μm particle size, C18 reverse phase column. The mobile phase was pumped isocratically at 1 ml/min and the detection

carried out at 214 nm. The HPLC analysis was carried out at ambient temperature (25°C). The remaining conditions, e.g. mobile phases, retention times etc. varied between peptides and these are summarized in Table 1. HPLC retention times of the sulfoxides produced in the reaction mixtures were compared with the retention times of the sulfoxide standards and in some cases the sulfoxide peak was collected and analyzed by positive FAB-MS. The observed initial rate constants for the reactions were calculated by fitting the data to a zero order reaction (Figure 6). The final yields of sulfoxide formed and consumed peptide were determined after the reaction was completed. All reactions were studied in duplicate.

Effect of Ethylenediaminetetraacetic acid (EDTA) on Methionine Sulfoxidation:

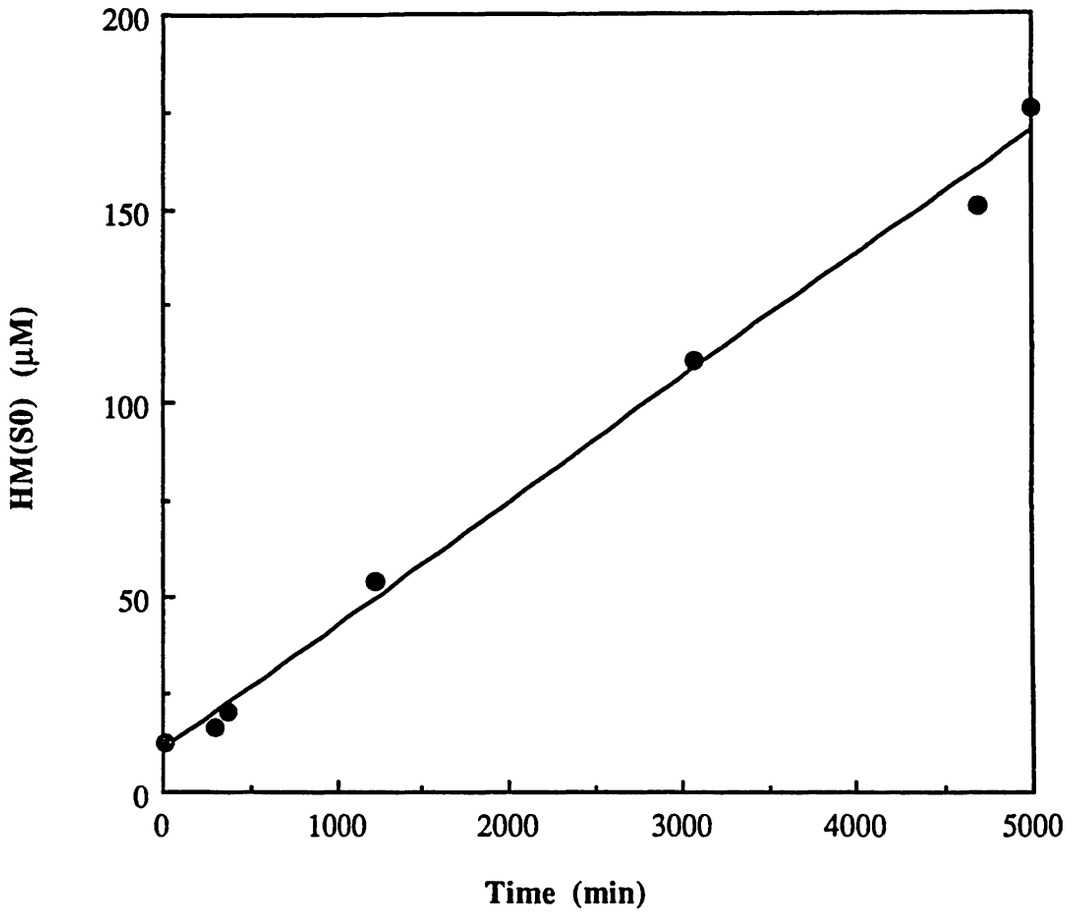
EDTA is a chelator of metal ions. The effect of EDTA on methionine sulfoxidation was studied in a 0.5 ml reaction mixture containing 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 1 mM peptide, 240 μM $FeCl_3$ and 0, 120, 240 or 480 μM EDTA. The amount of His-Met sulfoxide [HM(SO)] and HM consumed was determined by the HPLC method described above.

Table 1. Summary of the HPLC conditions for the various peptides and their corresponding sulfoxides.

Peptide	Product studied	Mobile Phase Composition			Retention Time (min)	
		ACN %(v/v)	Water %(v/v)	TFA %(v/v)	Peptide Sulfoxide	Peptide
HM	HM(SO)	15	85	0.02	11.3	19
GMG	GM(SO)G	10	90	0.02	5.2	12.8
MGG	M(SO)GG	5	95	0.02	6.7	13
MH	M(SO)H	15	85	0.02	9.8	18.5
GGM	GGM(SO)	7	93	0.01	6.6	15.8
GM	GM(SO)	5	95	0.01	7.3	16

Figure 6: Initial rate of formation of His-Met sulfoxide [HMS(O)].

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ and 5 mM MSH. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


200
150
100
50
0
HM(S0) (μM)
0
1000
2000
3000
4000
5000
Time (min)

Effect of Catalase on Methionine Sulfoxidation:

Catalase acts as a scavenger of free H_2O_2. The influence of catalase on methionine sulfoxidation was studied in 0.5-mL reaction solutions containing 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 1 mM peptide and 240 μM ferric chloride and in the presence or absence of 150 U/mL, 300 U/mL, 600 U/mL or 1200 U/mL catalase. The amount of HM(SO) formed was monitored by HPLC as described above. In addition, reaction mixtures were prepared consisting of 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 240 μM $FeCl_3$ and equimolar amounts of cytochrome c or catalase. The activity of catalase was examined using solutions that consisted of 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 240 μM $FeCl_3$ in the presence or absence of 1200 U/mL catalase. After the reaction plateaued, excess H_2O_2 (18 mM) was added to the solutions and the HPLC analysis was carried out 60 minutes after the addition of the H_2O_2 to determine the amount of HM(SO) formed.

Effect of Superoxide Dismutase on Methionine Sulfoxidation:

Superoxide dismutase is an enzyme that scavenges $O_2^{\cdot -}$ radicals. The effect of superoxide dismutase on methionine sulfoxidation was studied in reaction solutions using 1 mM

HM, 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 240 μM $FeCl_3$ and 0, 10, 25, 52.5 or 105 U/mL of superoxide dismutase. The amount of HM(SO) formed was determined by HPLC as described above.

Effect of Methanol on Methionine Sulfoxidation:

The formation of ·OH radicals was investigated by using a ·OH radical scavenger, methanol. Reaction solution containing 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 240 μM $FeCl_3$ were prepared in the absence or presence of 1 M methanol to observe the effect of methanol on methionine sulfoxidation. The amount of HM(SO) formed was determined by HPLC as described above.

Effect of RS- on Methionine Sulfoxidation:

The effect of RS^- species on the sulfoxidation process was studied using HM and GMG as the model peptides. HS^- (5 mM) was generated *in situ* in the reaction solutions by adding Na_2S to the solutions containing 1 mM peptide (HM or GMG), 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$. The effect of different concentrations of HS^- was also studied. The amount of HM(SO) or GMG(SO) was determined by the HPLC method described in the method section.

SECTION III. RESULTS AND DISCUSSION

Sulfoxidation of methionine in small peptides was also shown to occur in systems containing ascorbate/$FeCl_3$ (Li et al., 1993) and in systems containing DTT/$FeCl_3$ (Schöneich et al., 1993). The present study was carried out to investigate the characteristics and mechanisms of methionine oxidation in small model peptides in the presence of a monothiol (MSH) and $FeCl_3$. These studies were designed such that a comparison between methionine sulfoxidation by a monothiol (MSH) and a dithiol system (Schöneich et al., 1993) can be made. The peptides chosen for the study contained glycine and methionine or histidine and methionine, at various positions to observe the effect of neighboring amino acids on the oxidation of methionine.

The catalytic effect of histidine on methionine sulfoxidation has been shown by Li et al. (1993) and Schöneich et al. (1993) in ascorbate/FeCl3 and DTT/$FeCl_3$ systems, respectively. Therefore in this study, HM was compared to GMG, MGG, GGM to decide if there is a catalytic effect of histidine on methionine sulfoxidation in systems containing MSH/$FeCl_3$.

Initial Rate of MSH-Dependent Formation of HM(SO):

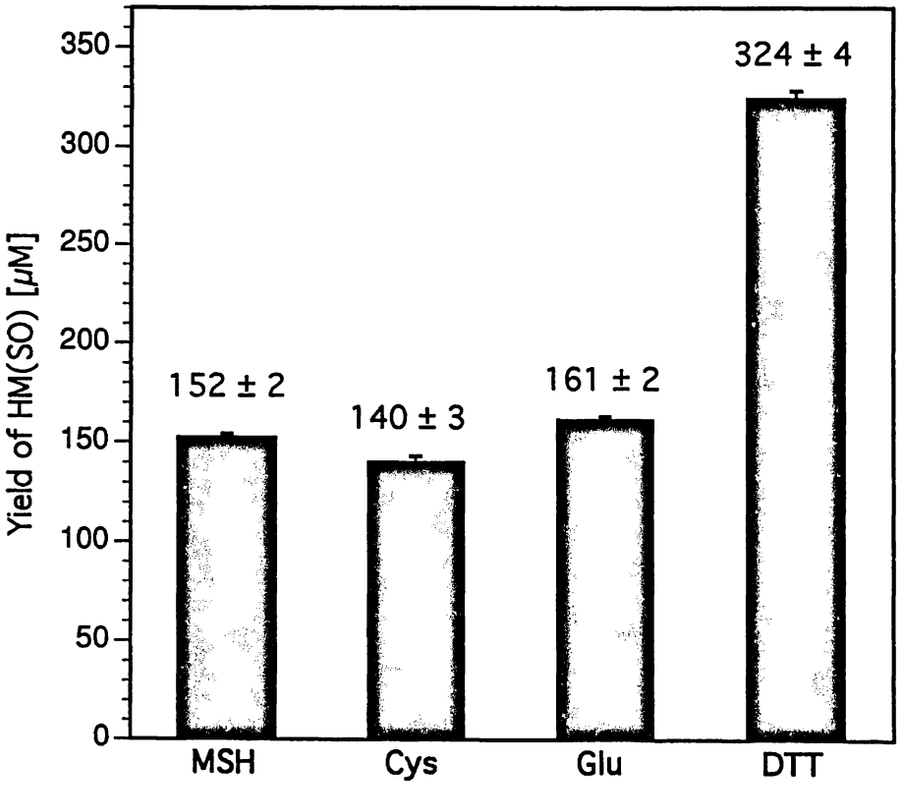
Incubation systems containing sodium phosphate buffer, peptide and $FeCl_3$ were studied with or without MSH. The sulfoxidation of HM or GMG was observed only when MSH was present in the reaction mixture. The initial rate of methionine sulfoxidation was measured. The increase in the concentration of the sulfoxide was linear with time at the start of the reaction. The formation of sulfoxide finally plateaus giving the final yields of the sulfoxide. Figure 6 shows a typical plot of the change in concentration of HM(SO) with time (kobs = 0.16 nmole/min). The initial zero order rate constant was calculated by a linear regression of the data.

Effect of Different Thiols on Methionine Sulfoxidation

In order to compare the influence of various monothiols and dithiol on the formation of methionine sulfoxide, MSH, DTT, cysteine and glutathione was studied using HM as the model peptide. Solutions containing 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $Fecl_3$ and 5 mM thiol were studied. The presence of MSH, cysteine or glutathione resulted in the formation of similar amounts of HM(SO) (Figure 7). When DTT was used as the reductant, the

Figure 7: Effect of different thiols on methionine sulfoxidation.

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ and 5 mM thiol [MSH, DTT, cysteine (Cys) or glutathione (Glu)]. The amount of HM(SO) formed was determined by HPLC as described in the methods section. The percentage yield of HM(SO) was calculated with respect to amount of initial HM present in the reaction solution. The data represent averages of duplicate observations.


Yield of HM(SO) [μM]
350
300
250
200
150
100
50
0
152 ± 2
140 ± 3
161 ± 2
324 ± 4
MSH
Cys
Glu
DTT

methionine sulfoxide yield was approximately 2-fold higher when compared to the monothiols (Figure 7). Thus DTT appears more effective in generating reactive oxygen species. Also, the rate of formation of methionine sulfoxide by DTT (k_{obs} = 0.90 ± 0.10 nmol/min) was faster than with the monothiols, MSH (k_{obs} = 0.18 ± 0.05 nmol/min), glutathione (k_{obs} - 0.15 ± 0.10 nmol/min), cysteine (k_{obs} = 0.10 ± 0.05 nmol/min). This rate difference may be based on the structural characteristics of DTT as compared to other thiols. DTT has two thiol groups which upon oxidation can form intramolecular disulfide bond, a kinetically favored process. In contrast, MSH, cysteine and glutathione have a single thiol group, therefore two molecules are required to make a intermolecular disulfide bond. The ease of formation of an intramolecular bond in DTT as compared to an intermolecular disulfide bond in MSH, cysteine and glutathione may dictate the extent of methionine sulfoxidation.

Alternatively, since 5 mM DTT contains twice the number of free thiols as compared to 5 mM MSH, the higher concentration of free thiol groups in DTT-containing reaction mixtures may be responsible for the higher yield of HMSO. In order to test this possibility, methionine sulfoxidation was studied at various concentrations of a

monothiol. MSH was chosen as the monothiol for further studies with the model peptides.

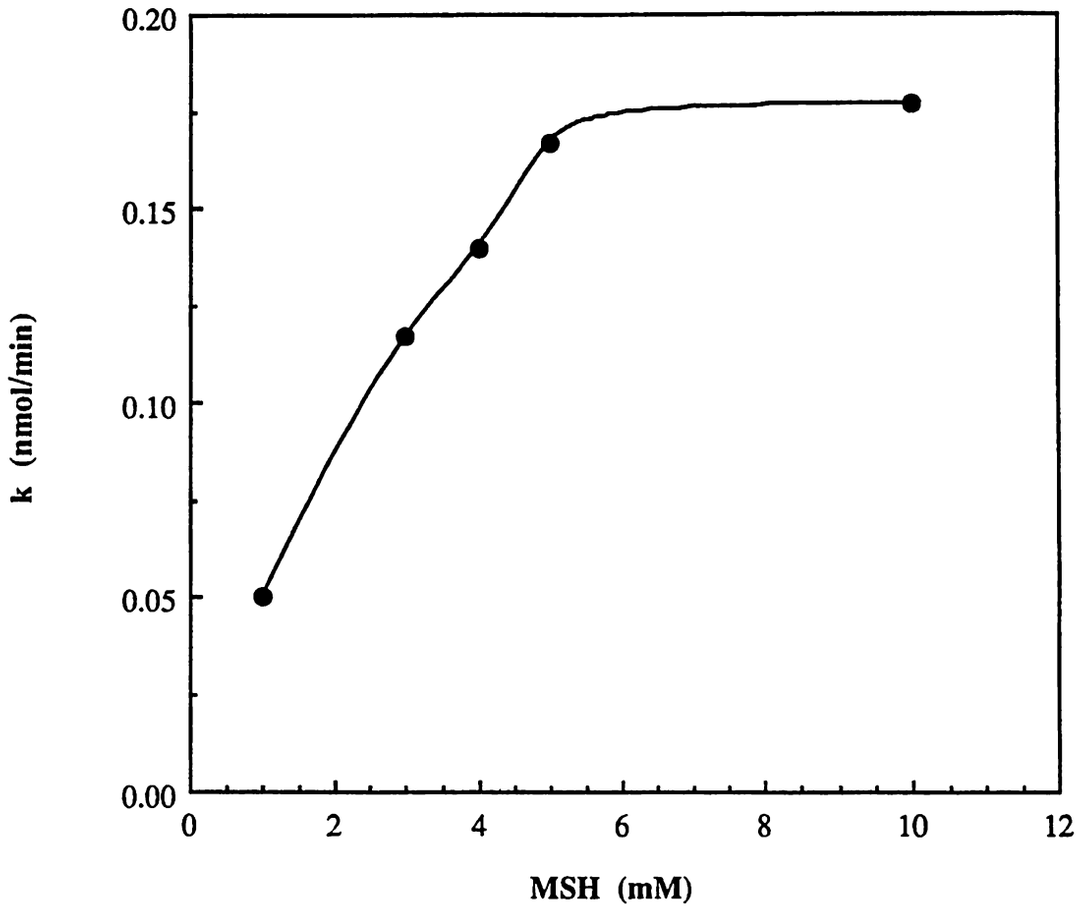
Effect of MSH Concentration on Sulfoxide Formation:

In order to observe the effect of the increase in the concentration of MSH on the oxidation of methionine, reaction solutions containing 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ were prepared at various concentrations of MSH. The MSH concentration was varied from 1 mM to 10 mM. The initial rate of sulfoxide formation increased and plateaued at 5 mM MSH (Figure 8). Similar results were obtained when the yields of HM(SO) were compared at 5 mM (220 ± 20 μM) and 10 mM MSH (240 ± 25 μM). These data suggest that at 5 mM concentrations of MSH, the free thiols are not the limiting factor for the formation of HM(SO). Therefore, a higher concentration of free thiol groups was not responsible for the observed 2-fold increase in yield with the dithiol, DTT (Figure 7).

Reductants, such as MSH, act as a prooxidant and as a scavenger in transition metal catalyzed reactions. As the reaction proceeds a fraction of the reactive oxygen species react with HM and the remaining fraction oxidizes MSH. Therefore, the efficiency of the methionine oxidation

Figure 8: Initial rate constant (nmol/min) for formation of HM(SO) at different MSH concentrations.

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0) 240 μM $FeCl_3$ and 1 mM, 3 mM, 4 mM, 5 mM or 10 mM MSH. The amount of HM(SO) formed was determined by HPLC as described in the methods section. The data represent averages of duplicate observations.


0.20
0.15
0.10
0.05
0.00
k (nmol/min)
0
2
4
6
8
10
12
MSH (mM)

should depend on the individual rate constants of the reactive oxygen species with respect to methionine containing peptides. Further, the value of these rate constants largely depends on the nature of the oxidizing species involved in the reaction. These rate constants may change as the reaction proceeds. Since the nature and quantity of the reactive oxygen species was not known, the rate constants of their formation could not be studied. Therefore, even though the initial rate constant for the formation of MSSM (k_{obs} = 0.21 ± .02 nmol/min) is similar to the initial rate of formation of HM(SO) (k_{obs} = 0.18 ± 0.05 nmol/min), these data does not allow us to predict the reaction mechanism involved. Such predictions would result in an oversimplification of a complex series of reactions.

For the remaining studies, MSH was used at 5 mM concentration because methionine sulfoxidation was maximum at that concentration.

In addition to the oxidation described above, MSH undergoes air oxidation as the reaction proceeds. Blank solutions containing 5 mM MSH and 20 mM phosphate buffer were monitored to account for the air oxidation of MSH. The loss of MSH and the concomitant formation of its disulfide form (MSSM) was monitored (Figure 9). The rates of MSSM

formed in all reactions were corrected for the air oxidation of MSH.

Effect of Phosphate Buffer Concentration on Sulfoxidation of Methionine:

Phosphate buffer strongly binds to Fe^{3+} and according to Equation 1, MSH reduces Fe^{3+} to Fe^{2+} and subsequent reactions (Equations 1-6; see Introduction) lead to the generation of reactive oxygen species. Therefore the influence of phosphate buffer on the formation of reactive oxygen species, and the sulfoxidation of methionine was studied using HM as the model peptide. The phosphate buffer concentration was varied from 10 mM to 50 mM in solutions containing HM, sodium phosphate buffer (pH 7.0), MSH and $Fecl_3$. The initial rates of formation of sulfoxide were found to be largely independent of the concentration of phosphate buffer (Figure 10), although there is a slight rate increase with increasing phosphate concentration. Hence, the concentration of phosphate buffer does not appear to influence the rate limiting step in methionine sulfoxidation. Reactions were also carried out in the absence of phosphate buffer. However, the pH in these reactions could not be carefully controlled and hence the data were found to be inconclusive.

Figure 9: Change in concentration of MSH and its disulfide form (MSSM) with time (minutes).

Blank solutions consisted of 20 mM sodium phosphate buffer (pH 7.0) and 5 mM MSH. The amount of MSH and MSSM were determined by HPLC as described in the Methods section. The data represent averages of duplicate observations. Dissolved $[O_2]$ in aqueous buffer is known to be approximately 200 μM. The conversion of MSH to MSSM was observed to be stoichiometric.

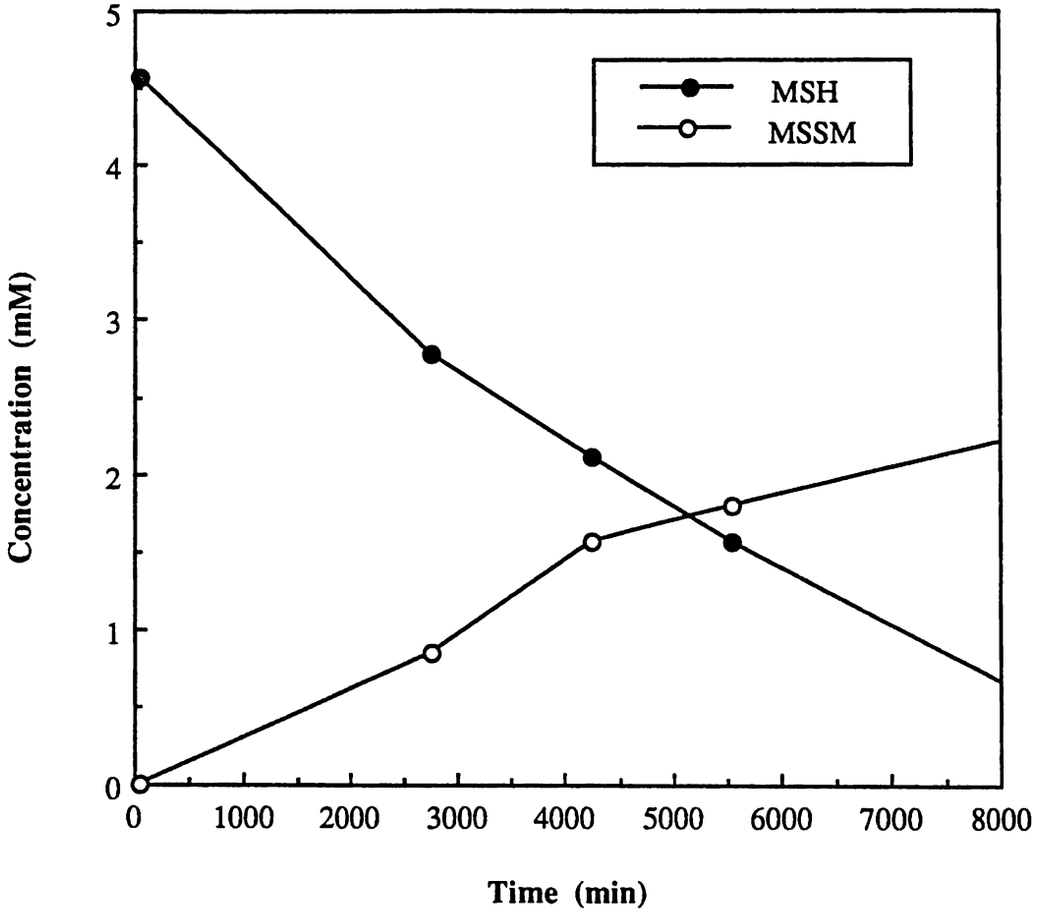
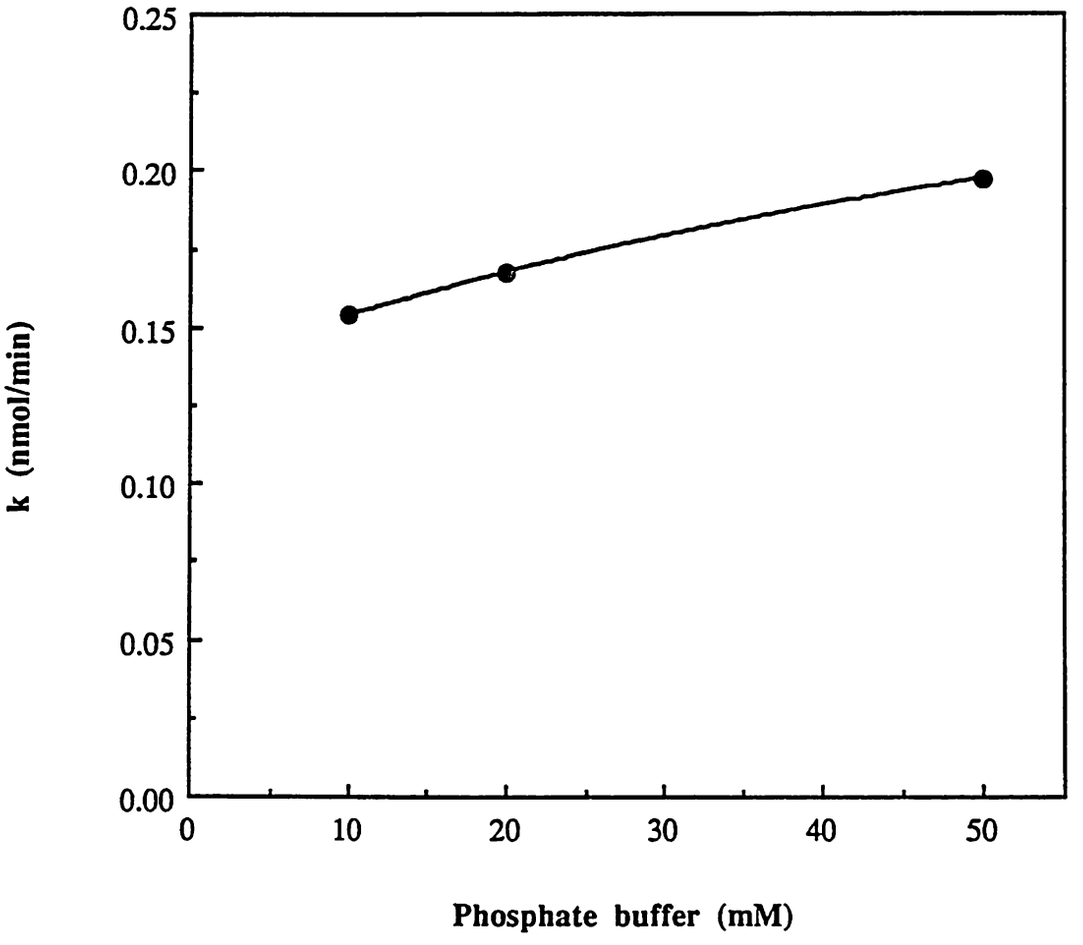

MSH
MSSM
Concentration (mM)
Time (min)
0
1
2
3
4
5
0
1000
2000
3000
4000
5000
6000
7000
8000

Figure 10: Initial rate constant (nmol/minute) for formation of HM(SO) at different phosphate buffer concentrations.

Reaction solutions consisted of 1 mM HM, 240 μM $FeCl_3$, 5 mM MSH and 10 mM, 20 mM, or 50 mM sodium phosphate buffer (pH 7.0). The amount of HM(SO) formed was determined by HPLC as described in the methods section. The data represent averages of duplicate observations.

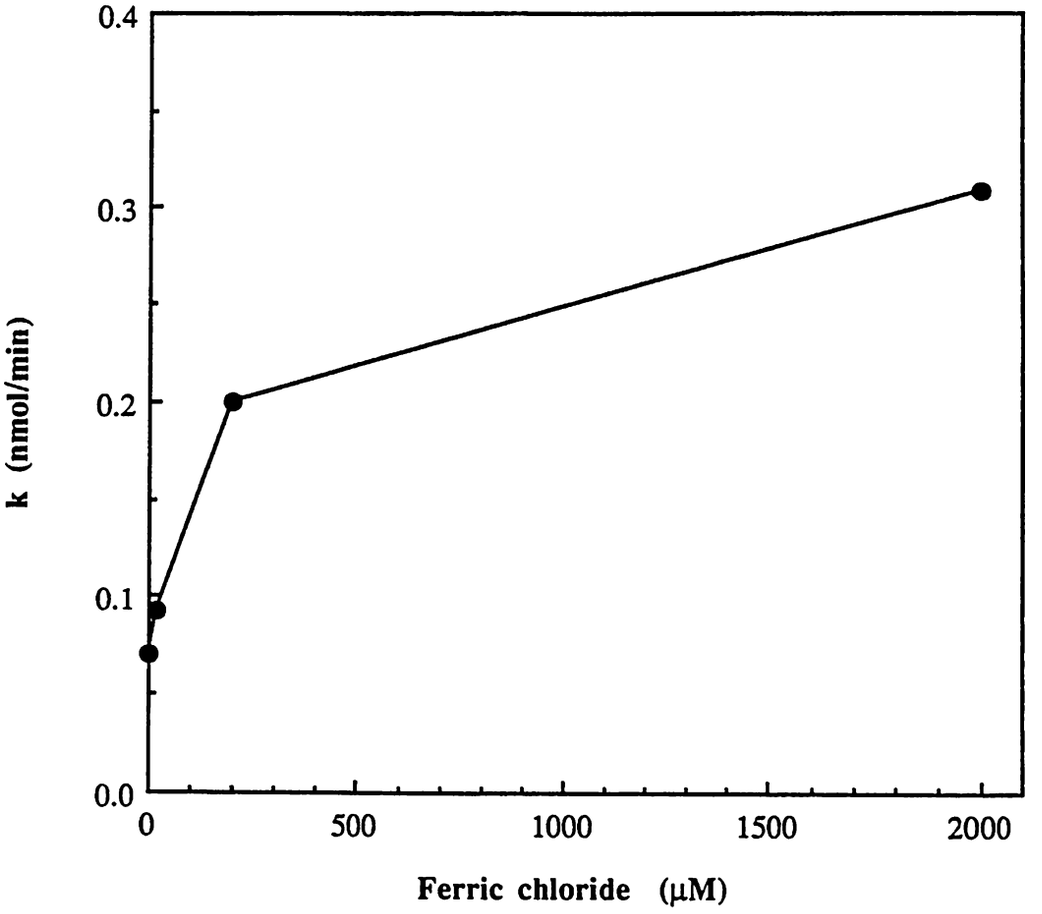

0.25
0.20
0.15
0.10
0.05
0.00
k (nmol/min)
0
10
20
30
40
50
Phosphate buffer (mM)

Effect of $FeCl_3$ Concentration:

The importance of iron in the reaction solutions was studied in reaction mixtures containing 1 mM HM, 5 mM MSH and 20 mM phosphate at pH 7.0, with 0, 20, 200 or 2000 μM concentrations of $FeCl_3$, respectively. The concentration of the added $FeCl_3$ is more than the solubility of free hydrated (Fe^{3+}) ion (approximately 10^{-18} M) (Miller et al., 1990) at pH 7.4. An increase in the initial rates of sulfoxide formation was observed as the $FeCl_3$ concentration was increased (Figure 11). This indicates that the catalytically active form is a complex of iron and not free hydrated Fe^{3+}. Also, significant sulfoxide formation was observed in solutions that did not contain $FeCl_3$. This may have been due to the trace amounts of iron present in commercially available buffer salts. The quantity of iron initially present in the amount of buffer used was calculated to be 0.6 μM. These calculations were based on the quality-control data provided by the supplier of monobasic sodium phosphate and dibasic sodium phosphate. This indicates that small quantities of iron can drive the oxidation process.

Figure 11: Effect of ferric chloride concentration on HM(SO) formation.

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer, 5 mM MSH and 0 μM, 20 μM, 200 μM or 2000 μM $FeCl_3$. The amount of HM(SO) formed was determined by HPLC as described in the methods section. The data represent averages of duplicate observations.


0.4
0.3
0.2
0.1
0.0
k (nmol/min)
0
500
1000
1500
2000
Ferric chloride (μM)

Effect of EDTA on the Oxidation of HM and GMG

Since the presence of iron was important in the formation of methionine sulfoxide, the influence of a chelator on the oxidation process was investigated. Solutions were prepared that consisted of peptide, sodium phosphate buffer (pH 7.0), MSH and $FeCl_3$ with 0, 120 μM, 240 μM or 480 μM EDTA. The sulfoxide formation was reduced when EDTA was present (Figure 12). For instance, when EDTA (100 μM) {Figure 12} is added the % yield of methionine sulfoxide with respect to consumed peptide drops from 53% to 15% suggesting that EDTA inhibits the formation of methionine sulfoxide. Higher concentrations of EDTA (960 μM and 1000 μM) were also used, however, at these concentrations, methionine sulfoxide could not be accurately determined because of interfering peaks. These peaks appeared apparently as a result of an increased degradation of HM (Figure 13). An attempt to alter the HPLC conditions did not improve the resolution of the peaks of interest.

Thus the chelation of iron by EDTA prevents the formation of the methionine sulfoxide. However, complete inhibition of sulfoxide formation was not observed at the highest EDTA concentrations studied (Figure 12). The increase in peptide degradation with the increase in EDTA

Figure 12: Effect of EDTA concentration on the formation of HM(SO).

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$, 5 mM MSH with 0 μM, 120 μM, 240 μM or 480 μM EDTA. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

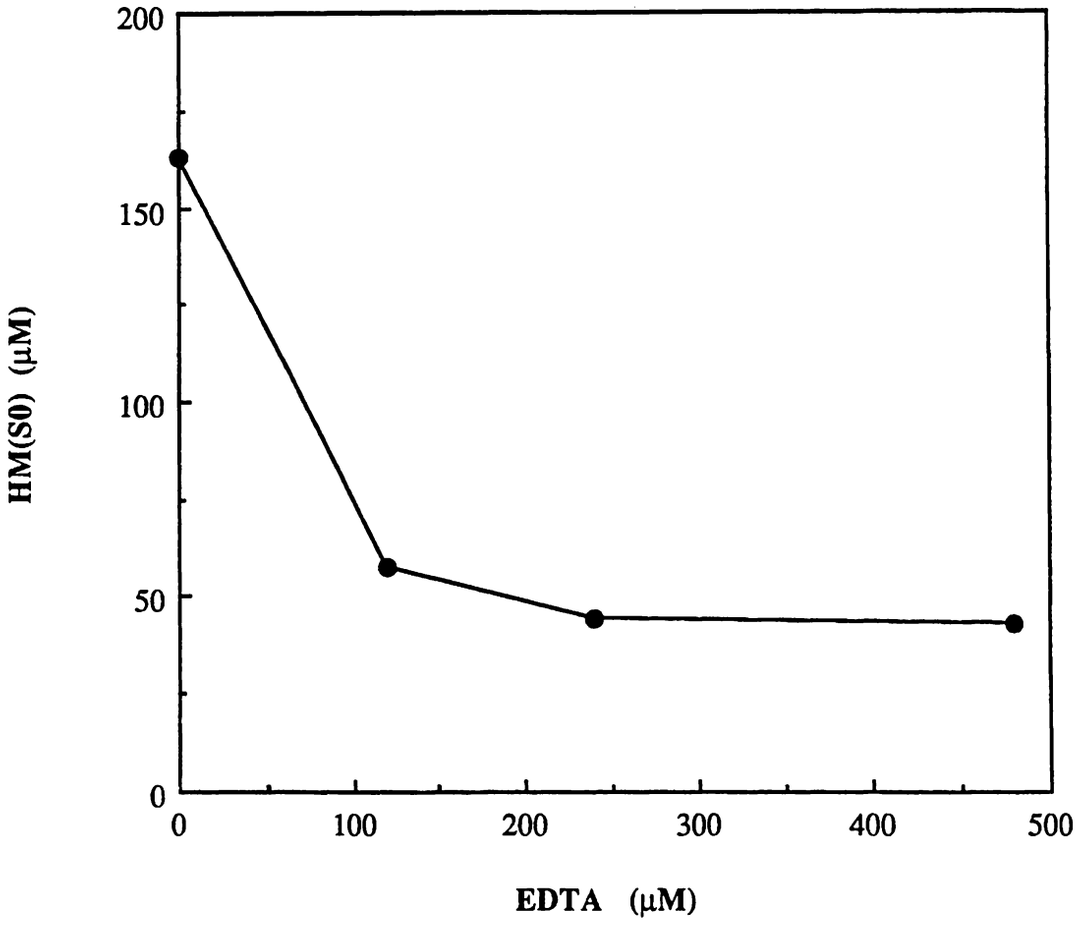
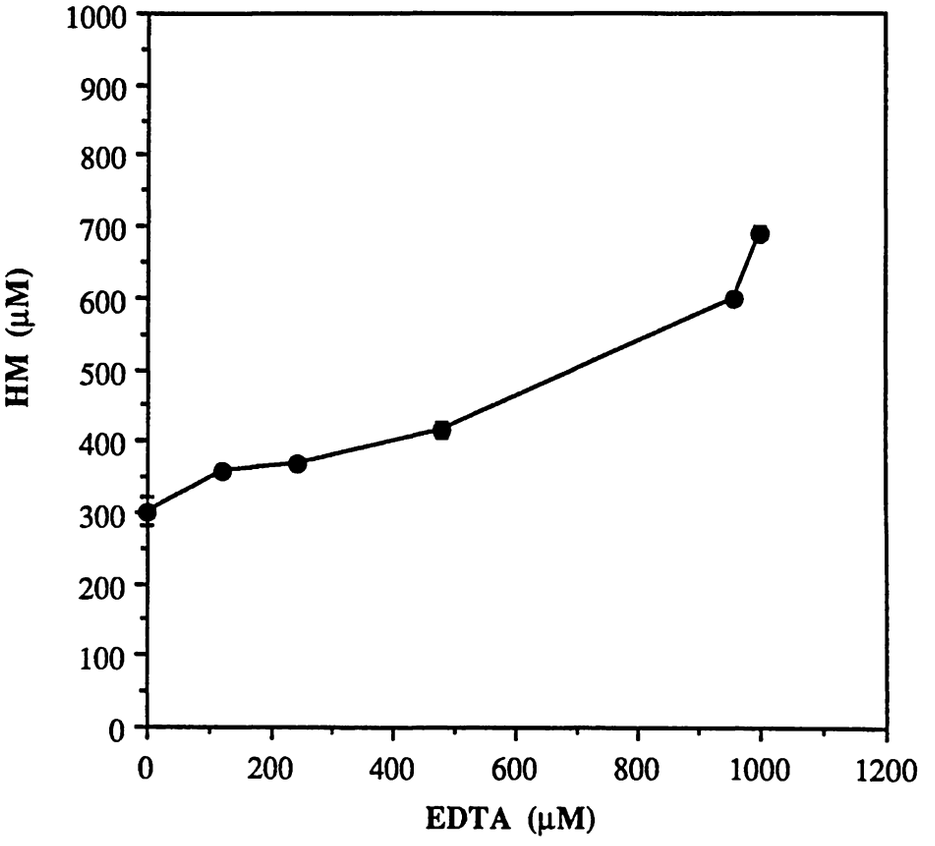

200
150
100
50
0
HM(S0) (μM)
0
100
200
300
400
500
EDTA (μM)

Figure 13: The effect of EDTA concentration on the consumption of HM.

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$, 5 mM MSH with 0 μM, 120 μM, 240 μM or 480 μM EDTA. The amount of HM remaining was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


1000
900
800
700
600
500
400
300
200
100
0
HM (μM)
0
200
400
600
800
1000
1200
EDTA (μM)

concentration may be due to the formation of $OH^{\cdot}$ radicals. These radicals may be generated by the metal catalyzed Haber-Weiss reaction (Greenwald, 1985), which can be modified for an iron-EDTA complex as shown below:

$$Fe^{3+}\ (EDTA) + O_2^{-\cdot} \quad \text{-----> } Fe^{2+}\ (EDTA) + O_2 \qquad (9)$$

$$Fe^{2+}\ (EDTA) + H_2O_2 \quad \text{-----> } Fe^{3+}\ (EDTA) + OH^- + {\cdot}OH \qquad (10)$$

Thus the overall reaction gives O_2, OH^- and $\cdot OH$. It has been reported by Schöneich et al. (1993) that $\cdot OH$ radicals react with methionine containing peptides (GGM, GMG, HM, MGG, HM etc.), however, this does not result in efficient formation of methionine sulfoxide. Thus equations 9 and 10 may explain the decreased sulfoxide formation with EDTA and increased peptide degradation with the increase in EDTA concentration. However, apparently the formation of $\cdot OH$ does not occur under neutral conditions (Rush and Koppel, 1986). In neutral and biological media, other oxidizing species have been postulated (Greenwald, 1985). One of the species postulated is the ferryl radical, which is considered to be as reactive as the hydroxyl radical (Walling, 1975). Upon reaction of the Fe^{2+}(EDTA) with H_2O_2, an intermediate, $[FeOH]^{3+}$, is formed, which can be viewed as a $\cdot OH$ bound to the Fe^{3+}(EDTA). Ionization of the intermediate yields $[FeO]^{2+}$, the ferryl radical ion. If this species is being

formed, it may be behaving similarly to the ·OH radicals thus causing the degradation of HM.

Effect of pH on Sulfoxidation of MGG and GMG:

The influence of H^+ concentration on the formation of reactive oxygen species and thereby the formation of methionine sulfoxide was investigated. Solutions containing peptide, phosphate buffer, MSH and $FeCl_3$ were prepared at pH 6, 7, 8, or 9. The sulfoxide yield and the peptide consumed for GMG and MGG as a function of pH are shown in Figures 14 and 15, respectively. The sulfoxide yields and the peptide consumed in all reactions decreased as the pH was increased. The deprotonation of MSH is facilitated at a higher pH. This is supported by the observation that the rate of loss of MSH is increased as the pH increases (Figure 16). It can only be speculated that deprotonation of MSH can result in the formation of reactive oxygen species by electron transfer to ferric ion ($2MSH + 2Fe^{3+} \rightarrow MSSM + 2Fe^{2+} + 2H^+$). It is also possible that the deprotonated form of MSH binds to the ferric ion thereby facilitating the formation of iron-complexed species, which in turn may be involved methionine sulfoxidation. For instance, Schöneich et al. (1993) have postulated that iron-bound peroxyl species can be formed by the ligation of

Figure 14: Effect of pH on the consumption of Gly-Met-Gly (GMG) and the formation of GMG sulfoxide {GMG(SO)}.

Reaction solutions consisted of 1 mM GMG, 20 mM sodium phosphate buffer, 5 mM MSH and 240 μM $FeCl_3$ at pH 6.0, 7.0, 8.0 or 9.0. The amount of GMG consumed and GMG(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

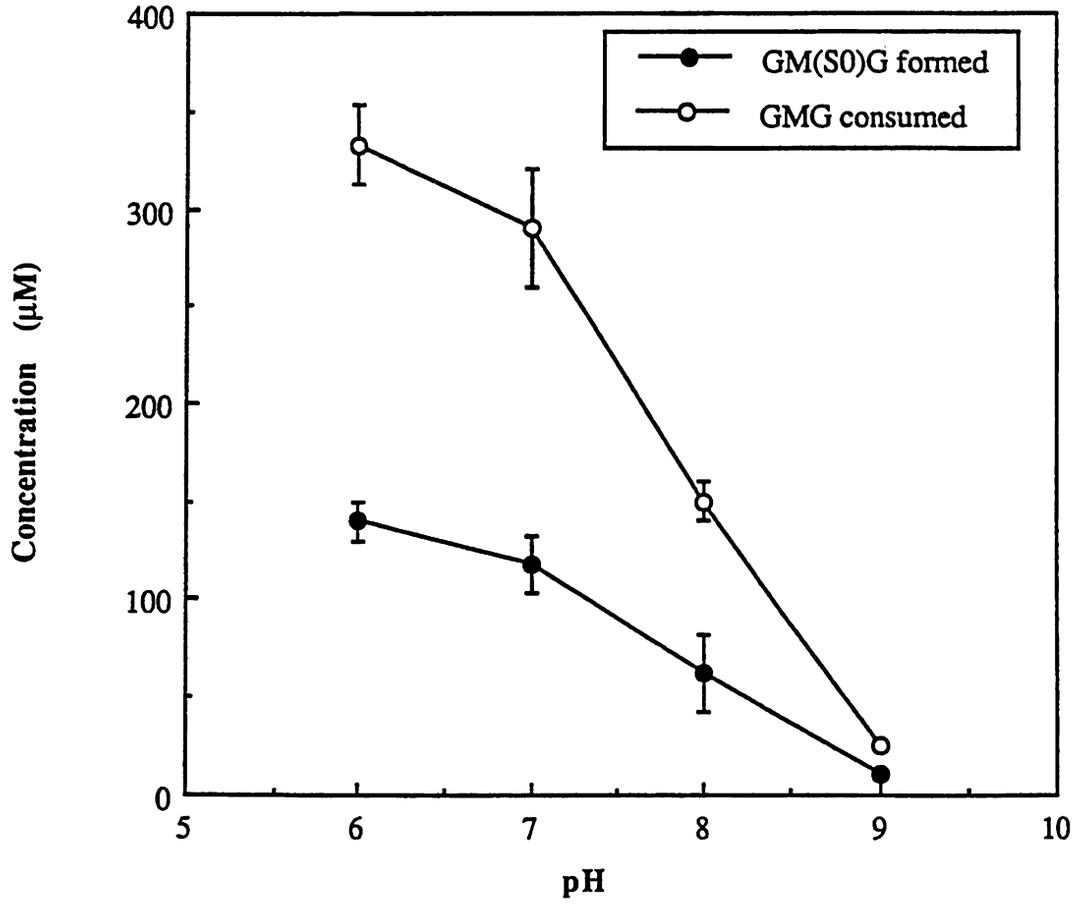

GM(S0)G formed
GMG consumed
Concentration (μM)
400
300
200
100
0
5
6
7
8
9
10
pH

Figure 15: Effect of pH on the consumption of Met-Gly-Gly (MGG) and the formation of MGG sulfoxide {M(SO)GG}.

Reaction solutions consisted of 1 mM MGG, 20 mM sodium phosphate buffer, 5 mM MSH and 240 μM $FeCl_3$ at pH 6.0, 7.0, 8.0 or 9.0. The amount of MGG consumed and MGG(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

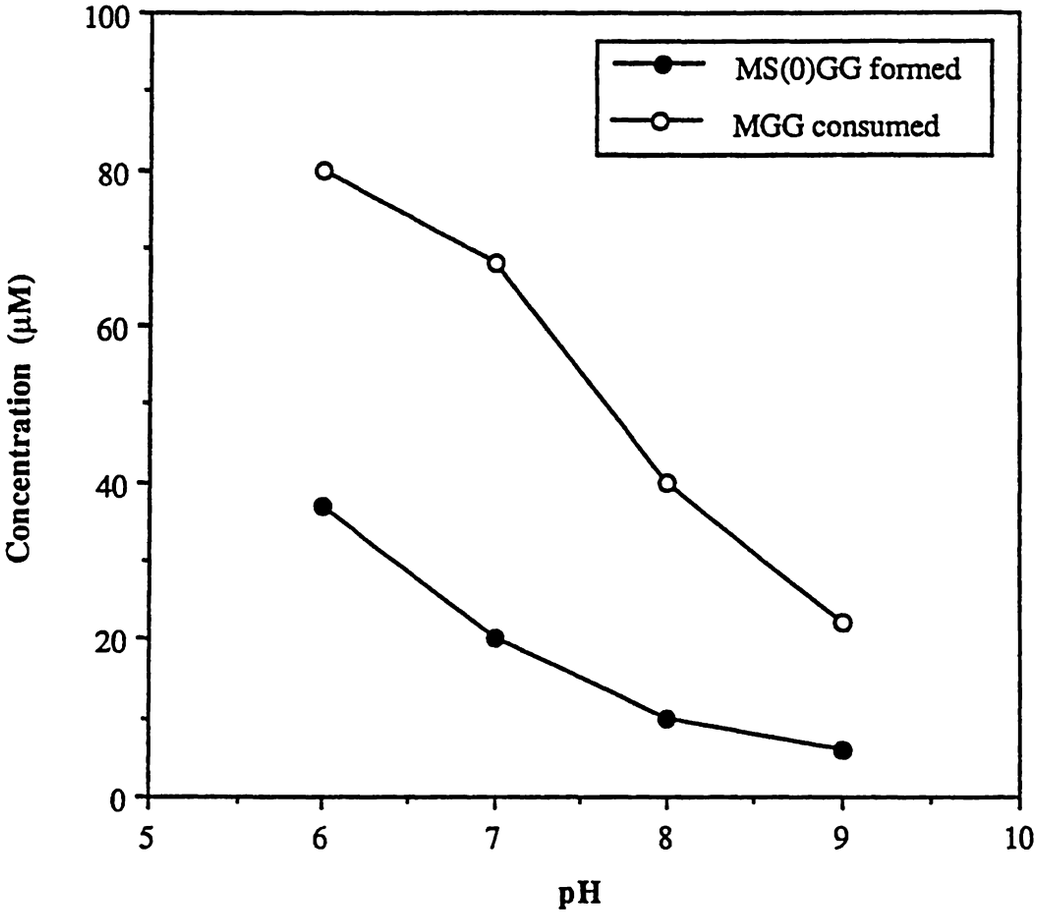
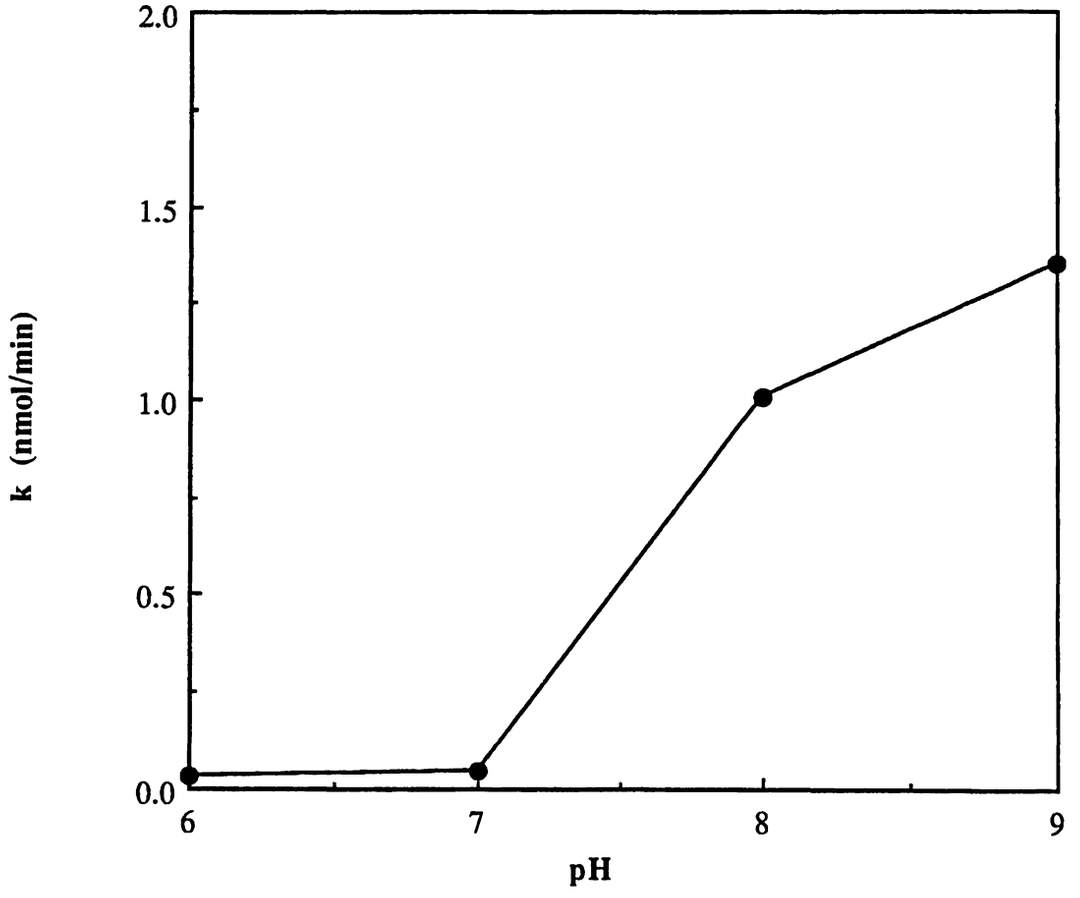

MS(0)GG formed
MGG consumed
Concentration (μM)
100
80
60
40
20
0
5
6
7
8
9
10
pH

Figure 16: Initial rate constant (nmol/min) for the loss of MSH with pH.

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer, 240 μM $FeCl_3$ and 5 mM MSH at pH 6.0, 7.0, 8.0 and 9.0. The amount of MSH consumed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


2.0
1.5
1.0
0.5
0.0
k (nmol/min)
6
7
8
9
pH

oxygen to a DTT-iron complex [(S-(DTT)-S)Fe-OO·]. This species may oxidize methionine-containing peptides by a mechanism that is similar to the oxidation caused by peroxyl radicals. The reaction of peroxyl radicals with organic sulfides have been shown to efficiently form the corresponding sulfoxide (Schöneich et al., 1991b). Similar intermediates have been shown for reactions between porphyrins and non heme iron complexes and enzymes with oxygen (Menage et al., 1992; Kurtz, 1990).

At a lower pH (Figure 14 and Figure 15) the oxidizing species were more efficient in forming the sulfoxide. This is evident from the higher yields of sulfoxide at the end of the reaction. However, the yield of sulfoxide and the total amount of peptide consumed do not follow a 1:1 stoichiometry at lower pH (Figure 14 and Figure 15). This suggested that products other than sulfoxide are also formed under these conditions. It is interesting that the sulfoxide yield and the peptide consumed approach a 1:1 stoichiometry as the pH is increased from 6 to 9. Therefore, it appears that at higher pH the reactive oxygen species are not only less efficient in oxidizing methionine but also less efficient in converting the peptide to other products.

The possibility also exists that the reactive oxygen species with a greater capability of sulfoxide formation may be converted into another species at higher pH which has lower efficiency for forming the methionine sulfoxide.

Effect of pH on Oxidation of HM and MH:

In the case of HM and MH the sulfoxide yields go through a maximum at pH 7.0 (Figure 17 and Figure 18). The pKa for the deprotonation of the imidazole nitrogen in histidine is at 6.9 and thus the deprotonated imidazole group could act as a metal binding site and could thus bring the oxidizing species close to the methionine thereby facilitating the sulfoxidation. Comparison of the reactions of the different peptides at pH 7.0 (Table 2 on page 75) shows that both sulfoxide formation and peptide consumption were highest for HM. The pH profile for MH was similar to HM but the sulfoxide yield was significantly lower than HM at all pH values (Figure 18).

Influence of Catalase:

Experiments were conducted to determine the role of various reactive oxygen species shown in Equations 1-6 (see Introduction) in the sulfoxidation of methionine. H_2O_2 was used for the synthesis of the sulfoxides of the peptides

Figure 17: Effect of pH on the consumption of HM and the formation of HM(SO).

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer, 5 mM MSH and 240 μM $FeCl_3$ at pH 6.0, 6.5, 7.0, 8.0 or 9.0. The amount of HM consumed and HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

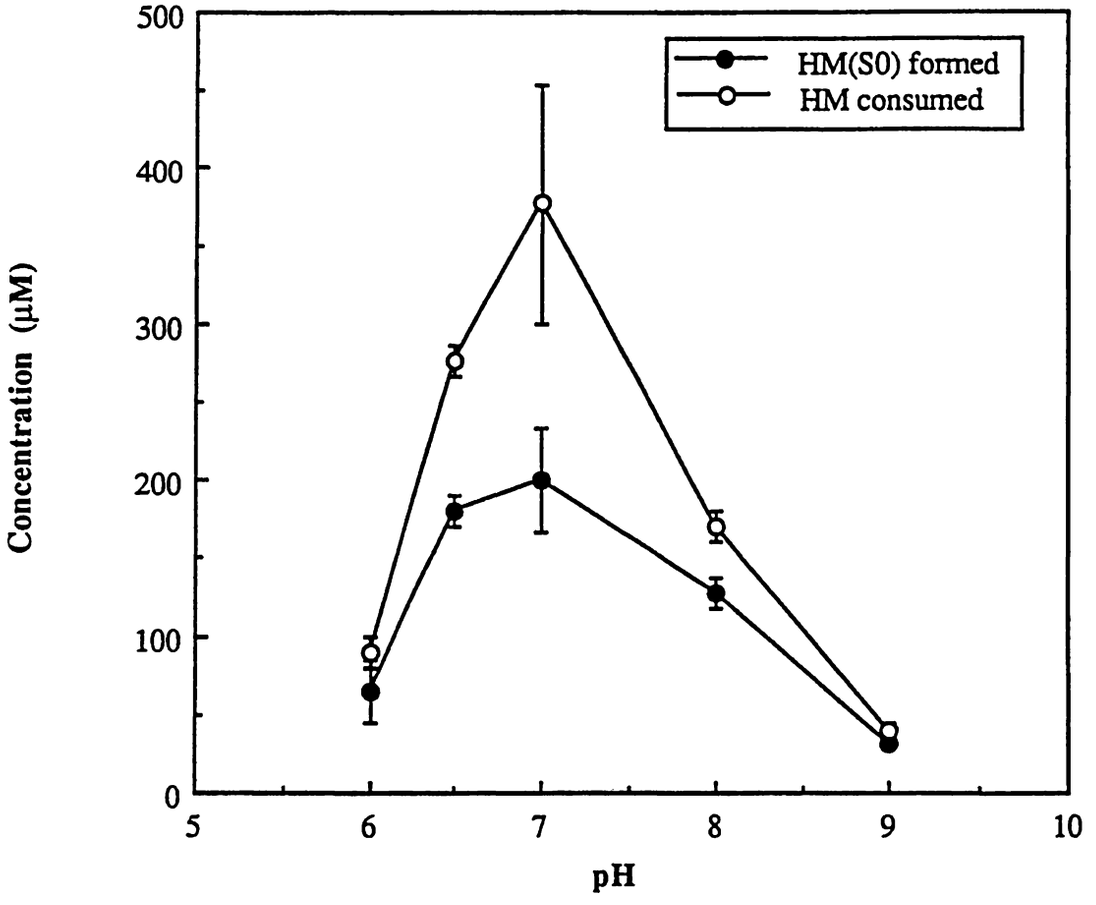
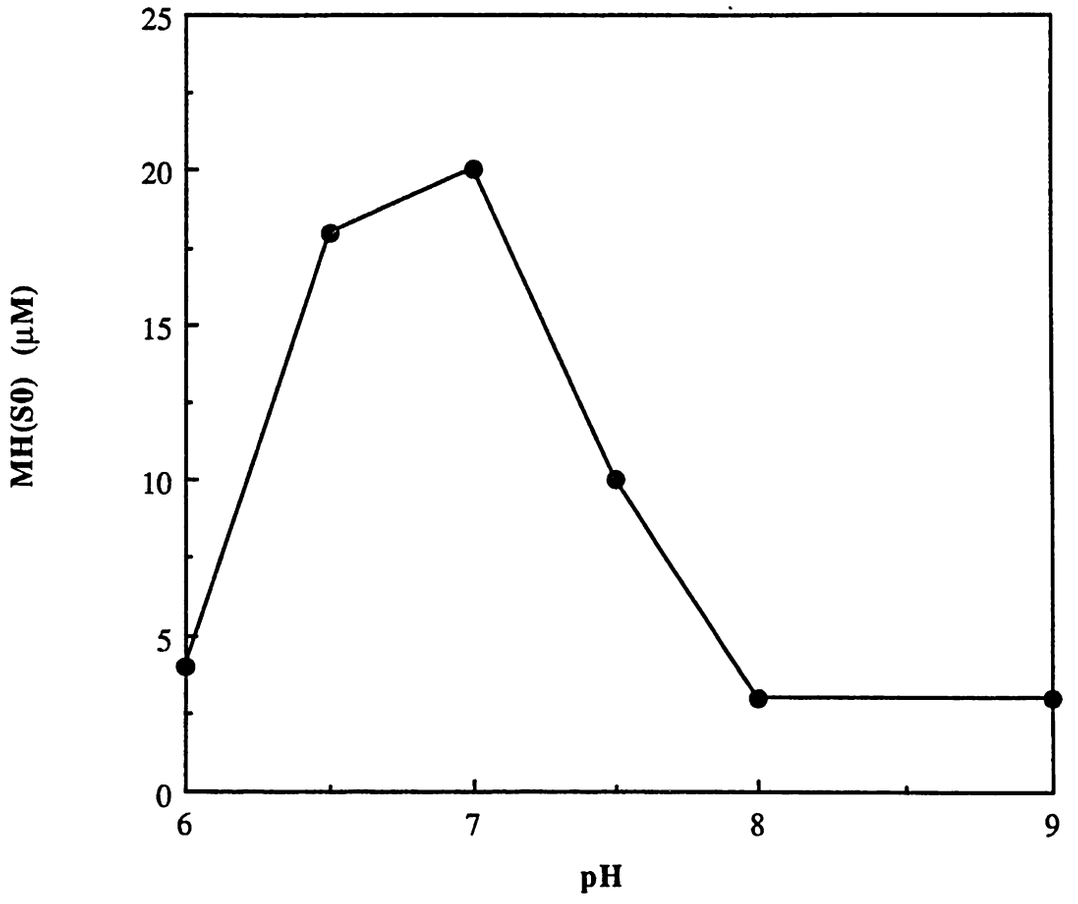

HM(S0) formed
HM consumed
Concentration (μM)
pH
0
100
200
300
400
500
5
6
7
8
9
10

Figure 18: The yield of {MH(SO)} as a function of pH.

Reaction solutions consisted of 1 mM GMG, 20 mM sodium phosphate buffer, 5 mM MSH and 240 μM $FeCl_3$ at pH 6.0, 6.5, 7.0, 7.5, 8 or 9.0. The amount of MH(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


25
20
15
10
5
0
MH(S0) (μM)
6
7
8
9
pH

used in the study (see methods) and complete conversion of the peptide to its corresponding sulfoxide was observed without the formation of side products. Complete conversion of the peptides to their corresponding sulfoxides was not observed in the systems used. However, for further confirmation regarding the role of freely diffusible H_2O_2, studies were carried out to determine if H_2O_2 is the reactive oxygen species involved in the reaction. The solutions containing 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 5 mM MSH, 240 μM $FeCl_3$ were prepared in the presence or absence of 150 U/mL, 300 U/mL, 600 U/mL or 1200 U/mL catalase. A very slight decrease in sulfoxide yield was observed in presence of higher concentrations of catalase (600 U/mL and 1200 U/mL) (Figure 19). There can be several explanations for this phenomenon:

a) In the reaction solutions, catalase may be rendered inactive.
b) catalase at high activity can compete with the peptide for the reactive oxygen species and thus decrease the sulfoxide formation.

In order to test the first possibility, the activity of catalase was checked by adding excess of H_2O_2 to both systems, i.e., with or without catalase. This was carried out only after the methionine sulfoxidation had plateaued.

It was observed that the sulfoxide formation was increased significantly in the absence of catalase whereas there was no increase in the sulfoxide formation in solutions containing 1200 U/mL catalase (Figure 20). This showed that the activity of catalase was enough to suppress the reaction of HM with even high concentrations of free H_2O_2. However bound H_2O_2 could be present as catalase scavenges only freely diffusible H_2O_2.

To test the second possibility that catalase was competing with the peptide for reactive oxygen species, the following study was conducted. Solutions were prepared which consisted of 1 mM HM, 20 mM phosphate, 5 mM MSH, 240 μM $FeCl_3$, pH 7.0 and equimolar amounts of cytochrome c or catalase. Cytochrome c was chosen as a control because it has two methionine residues which is similar to catalase possessing three methionine residues. Figure 21 shows that the initial rate of sulfoxide formation was decreased in the presence of both catalase and cytochrome c, suggesting that the catalase may be competing with the peptide.

Furthermore, experiments were carried out where catalase was heat inactivated by heating the enzyme (1200 U/mL) at 100°C for 5 minutes at pH 7.0. The sulfoxide yield of solution containing 1200 U/mL active catalase (105 ±5 uM) was similar to that obtained from reaction solutions

Figure 19: The yield of HM(SO) at different catalase concentrations.

Reaction solutions consisted of 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ and 5 mM MSH in the presence and absence of 150 U/mL, 300 U/mL, 600 U/mL or 1200 U/mL catalase. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

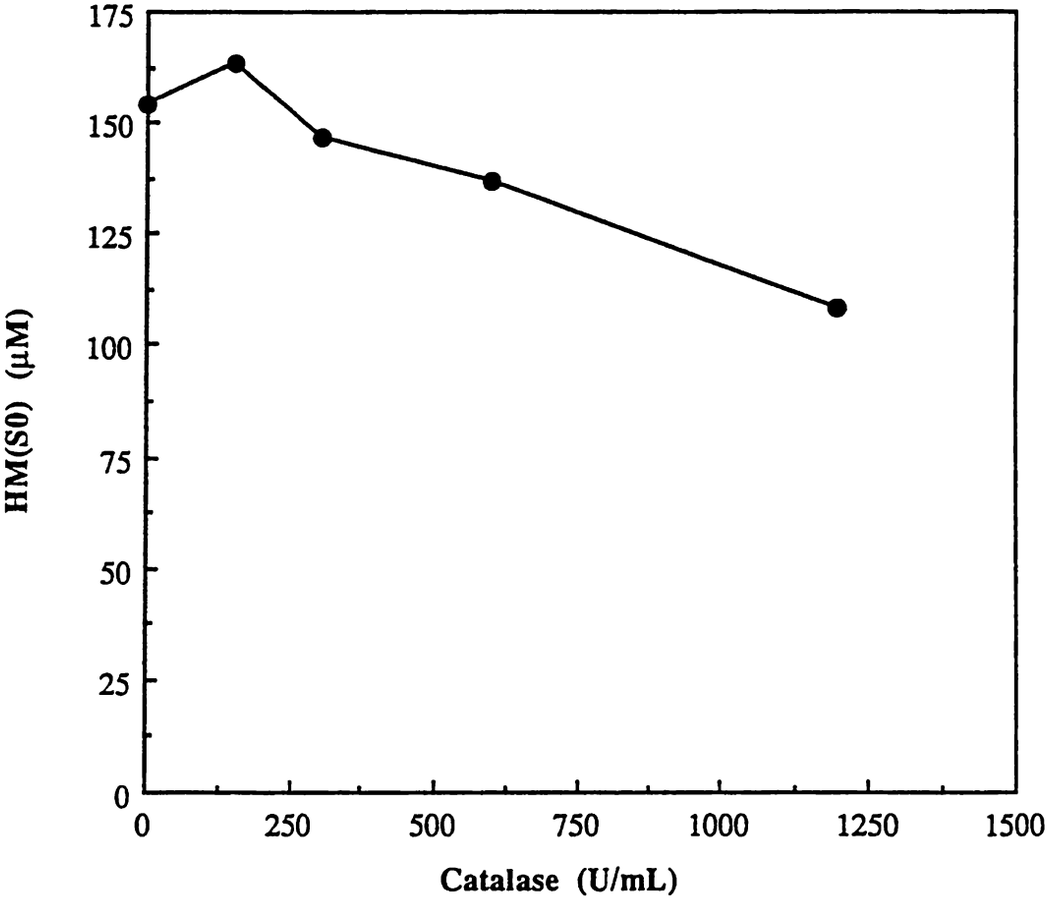

175
150
125
100
75
50
25
0
HM(S0) (μM)
0
250
500
750
1000
1250
1500
Catalase (U/mL)

Figure 20: The yield of HM(SO) in the absence (0 U/mL) and presence (1200 U/mL) of catalase with and without the external addition of H_2O_2.

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$, and 5 mM MSH. After the reaction was completed H_2O_2 (18 mM) was added to the reaction mixture. The amount of HM(SO) formed was determined 60 minutes after the addition of H_2O_2 by HPLC as described in the Methods section. The data represent averages of duplicate observations.

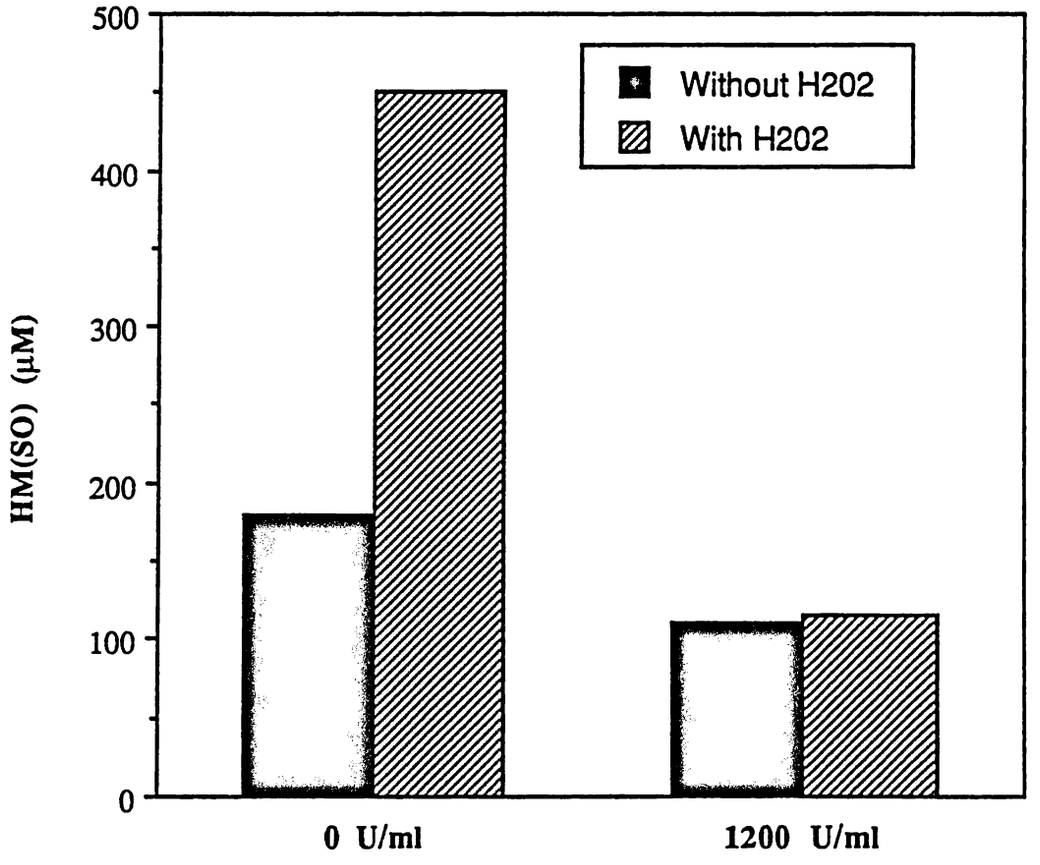
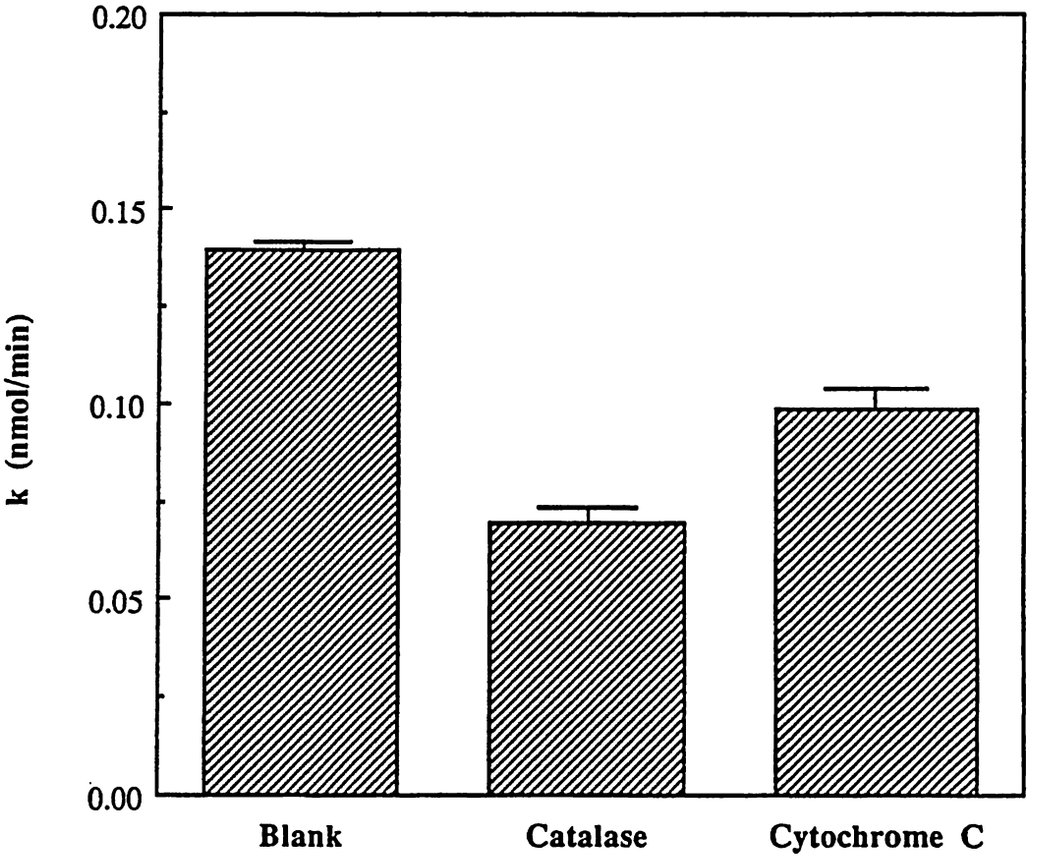

500
400
300
200
100
0
HM(SO) (μM)
Without H202
With H202
0 U/ml
1200 U/ml

Figure 21: The initial rate constant (nmol/min) for the formation of HM(S0) in the absence (blank) or the presence of catalase or cytochrome c.

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$, 5 mM MSH and equimolar amounts of catalase or cytochrome c. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


0.20
0.15
0.10
0.05
0.00
k (nmol/min)
Blank
Catalase
Cytochrome C

containing heat-inactivated catalase (125 ± 5 μM). Also, catalase is contaminated with aminopeptidase which can destroy the peptide and thus produce artifacts at higher concentration. Thus it appears that the yield of methionine sulfoxide was lower at high concentrations of catalase, due to the possibility of catalase competing with the peptide, for the reactive oxygen species. It is also apparent that freely diffusible H_2O_2 is not involved in the oxidation process. However, the effect of catalase on the yield of methionine sulfoxide may be masked because the formation of H_2O_2 may not be the rate determining step.

Effect of Superoxide Dismutase:

Superoxide dismutase is an efficient scavenger of the superoxide radical. Reaction solutions were prepared using HM, sodium phosphate buffer (pH 7.0), MSH, $FeCl_3$ and 0, 10, 25, 52.5 and 105 U/mL of superoxide dismutase respectively. The sulfoxide yield was not altered in the presence of superoxide dismutase (Figure 22). Similar experiments were performed using GMG and GM (Figure 23). Superoxide dismutase had no effect on the sulfoxidation of either GMG or GM. Therefore it appears that freely diffusible superoxide radical is also not involved in the sulfoxidation of methionine. However, the effect of

Figure 22: The yield of HM(SO) at different concentrations of superoxide dismutase (SOD).

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$, 5 mM MSH and 0 U/mL, 10 U/mL, 25 U/mL, 52.5 U/mL or 105 U/ml of SOD. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

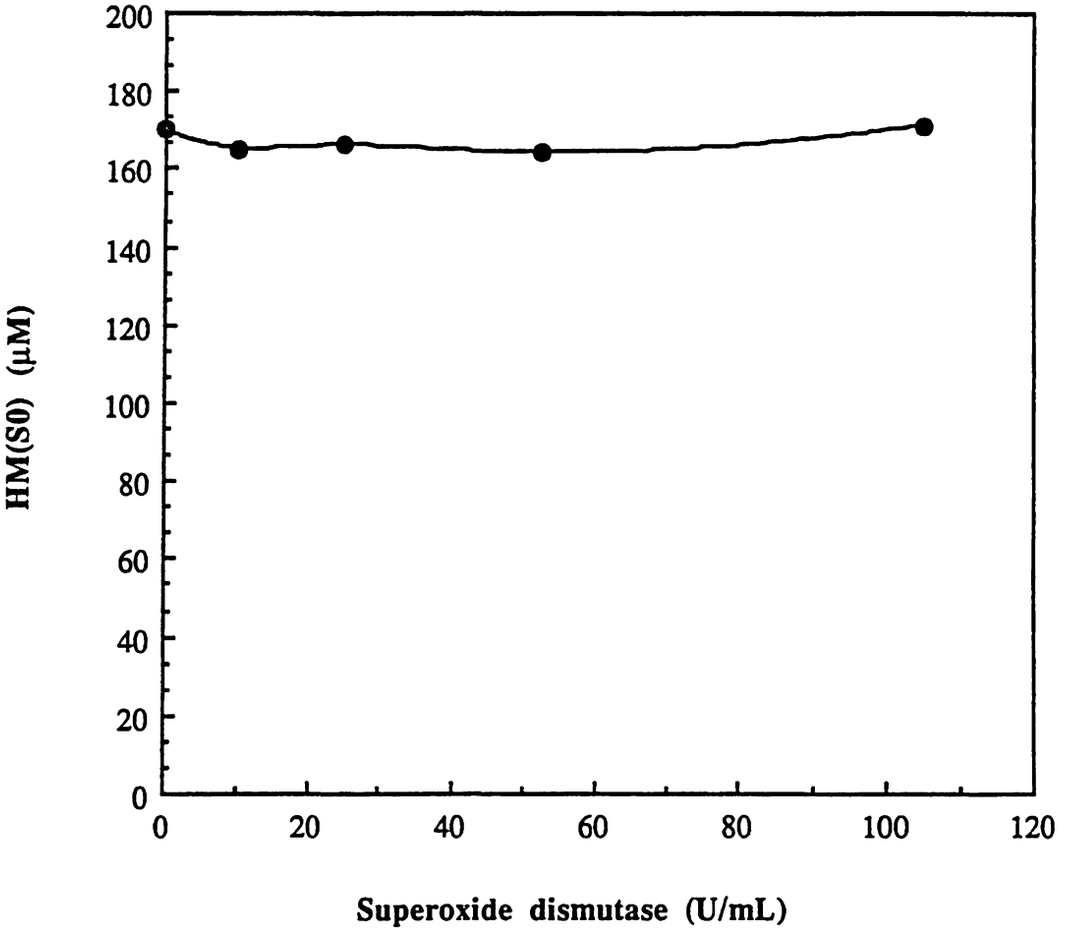
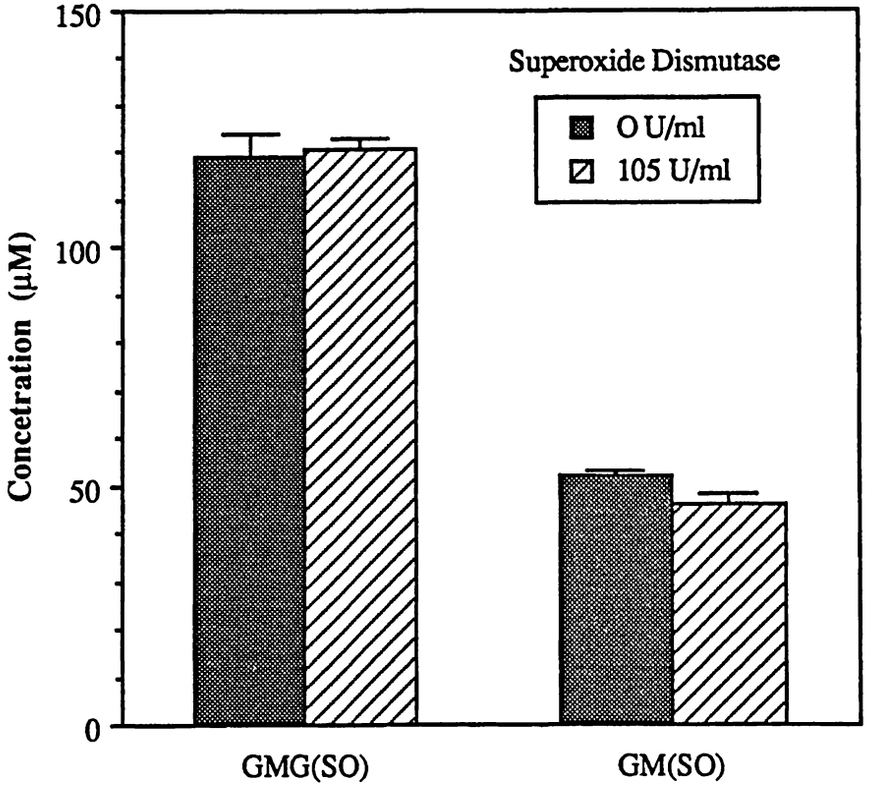

200
180
160
140
120
100
80
60
40
20
0
HM(S0) (μM)
0
20
40
60
80
100
120
Superoxide dismutase (U/mL)

Figure 23: The yield of GM(S0) and GM(S0)G in the absence and presence of 105 U/mL superoxide dismutase (SOD).

Reaction solutions were prepared containing 1 mM GM or GMG, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ and 5 mM MSH in the absence and presence of 105 U/ml of SOD. The amount of GM(S0) and GM(S0)G formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


150
100
50
0
Concetration (μM)
Superoxide Dismutase
0 U/ml
105 U/ml
GMG(SO)
GM(SO)

superoxide dismutase on the yield of methionine sulfoxide may be masked because the formation of superoxide radical may not the rate determining step.

Effect of Methanol on the Oxidation of HM:

The involvement of hydroxy radicals in the oxidation of methionine was also studied. It has been reported (Schöneich, et al., 1993) that in the reactions of hydroxyl radicals with methionine containing peptides only 20% of sulfoxide is formed and various other products are formed in larger quantities. For instance, for the peptide MH, the major product formed is Met-Asp (Schöneich et al., 1993). The reaction of organic sulfides and methionine with hydroxyl radicals has been well studied (Hiller et al., 1981). The main products are the α-thioether radicals and subsequent oxidation of the α-thioether radicals to peroxyl radical. Methanol is an efficient scavenger of hydroxyl free radical. It reacts with free hydroxyl radicals generating hydroxymethyl radicals and the subsequent reactions finally yield hydroxymethylperoxyl radicals and hydrogen peroxide (Schöneich et al., 1993). These oxidizing species have long lifetimes for reaction to take place with methionine containing peptides. H_2O_2 was used as the oxidant for the synthesis of sulfoxides of the peptides used in

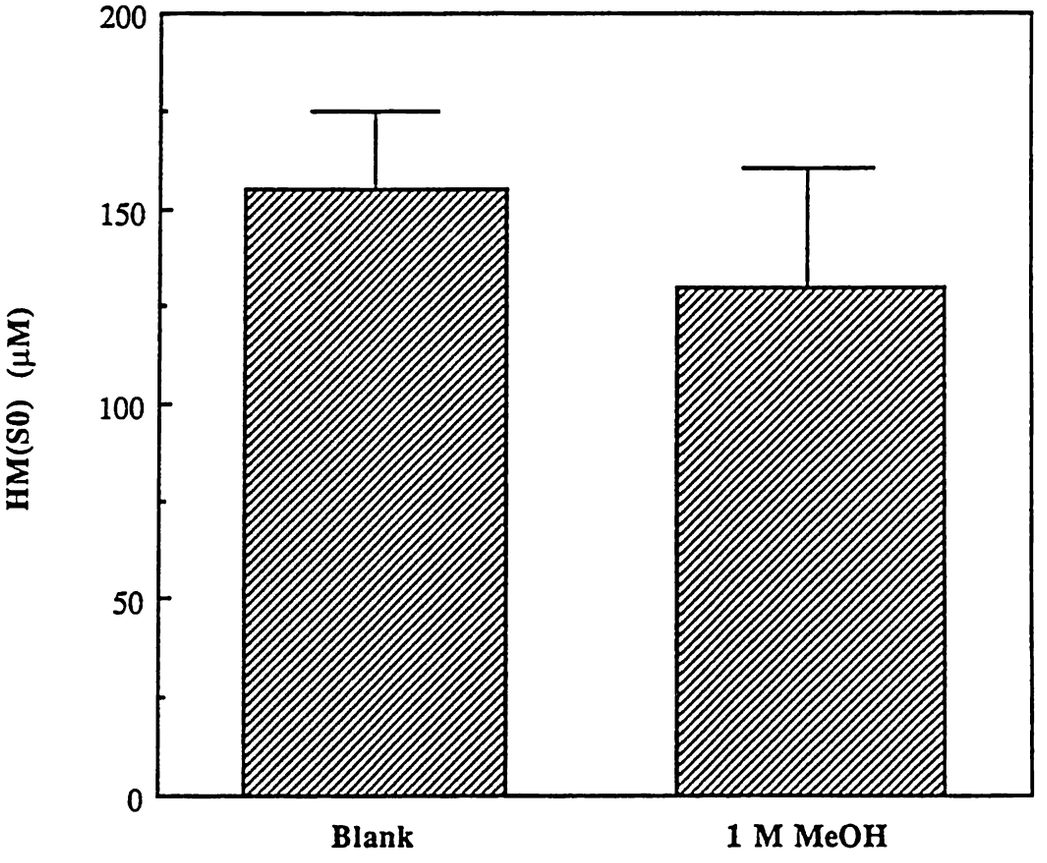
this study (see Methods). Complete conversion of the peptides to their corresponding sulfoxides was observed without the formation of side products. Therefore, if hydroxymethylperoxyl radicals and H_2O_2 were formed in the MSH/$FeCl_3$ system, then the formation of the sulfoxide and consumed peptide should be stoichiometric. Experiments were carried out in solutions containing HM, phosphate buffer (pH 7.0), MSH, and $FeCl_3$ in the absence and presence of 1 M methanol. The sulfoxide yields were not altered in the presence of methanol (Figure 24). Stoichiometric conversion of peptide to sulfoxide was not observed. Thus it appears that free hydroxyl radicals also do not account for the sulfoxide formation. However, the effect of methanol on the yield of methionine sulfoxide may be masked because the formation of hydroxyl radicals may not be the rate limiting step. These data suggest that hydroxyl radicals are not involved in the rate determining step of methionine sulfoxidation in MSH/$FeCl_3$ systems.

Effect of HS- Species on Sulfoxidation of Methionine in HM and GMG:

HS^- species can be formed in trace amounts through disulfide degradation of protein and may catalyze further chemical degradation. For instance in proteins containing

Figure 24: The yield of HM(SO) in the absence (blank) and presence of 1 M methanol (MeOH).

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$, 5 mM MSH, in the absence or presence of 1 M MeOH. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


200
150
100
50
0
HM(S0) (μM)
Blank
1 M MeOH

cysteine, HS^- can be formed which can cause degradation of proteins. In order to see the effect of HS^- on methionine oxidation, reactions were carried out where HS^- was generated *in situ* by the addition of Na_2S and adjusting the pH to 7.0 based on the following chemistry:

$$Na_2S + 2HCl \text{ ---> } 2\ NaCl + H_2S \qquad (7)$$

$$H_2S \text{ ----> } H_30^+ + HS^-,\ Ka = 1\ X\ 10^{-7} \qquad (8)$$

Solutions were prepared containing 1 mM peptide, 20 mM sodium phosphate buffer (pH 7.0), 5 mM Na_2S and 240 μM $FeCl_3$. Blank solutions had no externally added $FeCl_3$. Figure 25 shows that the sulfoxide yield and consumed peptide was higher in solutions containing externally added $FeCl_3$. Furthermore, the sulfoxide yield and consumed peptide yields were similar to those observed in solutions containing 5 mM MSH. Further experiments were carried out where the concentration of HS^- was varied in the reaction solutions. Solutions were prepared consisting of 1 mM peptide, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ with 100 μM, 500 μM, 1 mM, 5 mM or 10 mM Na_2S. The sulfoxide yield and consumed peptide increased with the increase in HS^- concentration and plateaued around 5 mM HS^- (Figure 26). The apparent initial rates of sulfoxide formation were similar to those observed in solutions containing 5 mM MSH (k = 0.1590 nmole/min for 5 mM MSH; k

Figure 25: Effect of HS^- on HM(SO) formation.

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ and 5 mM Na_2S. Blank solutions contained no added $FeCl_3$. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.

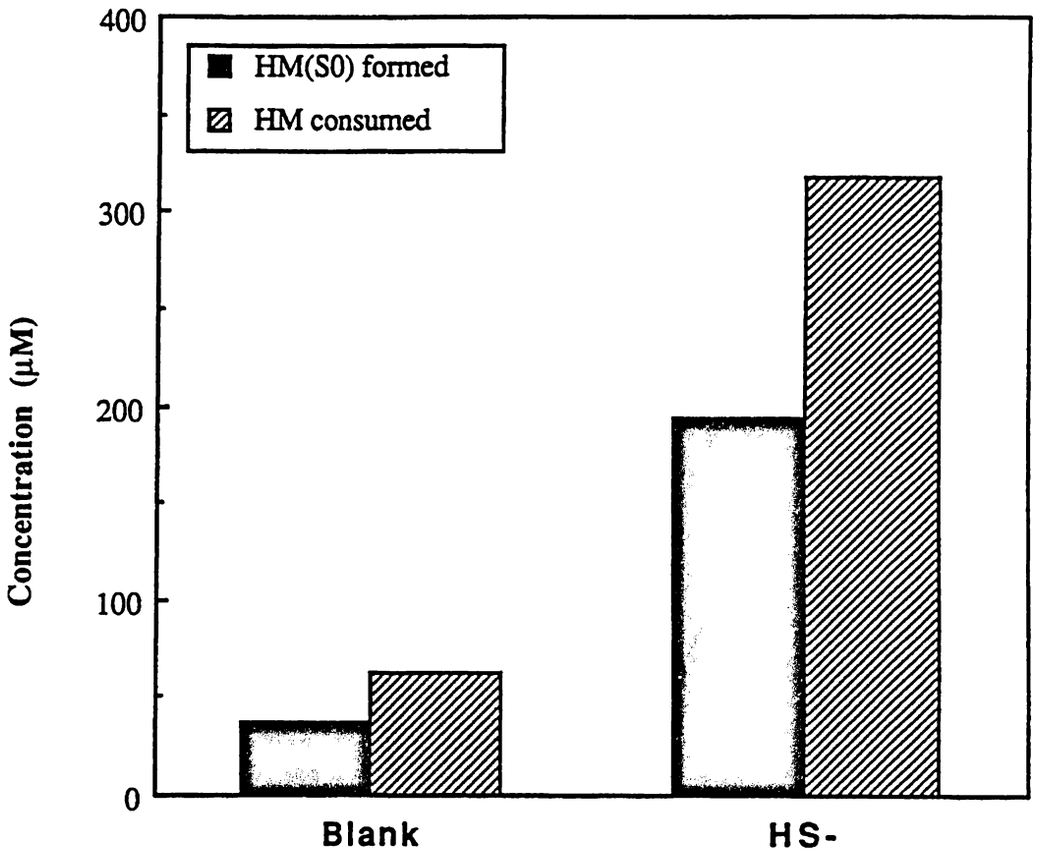
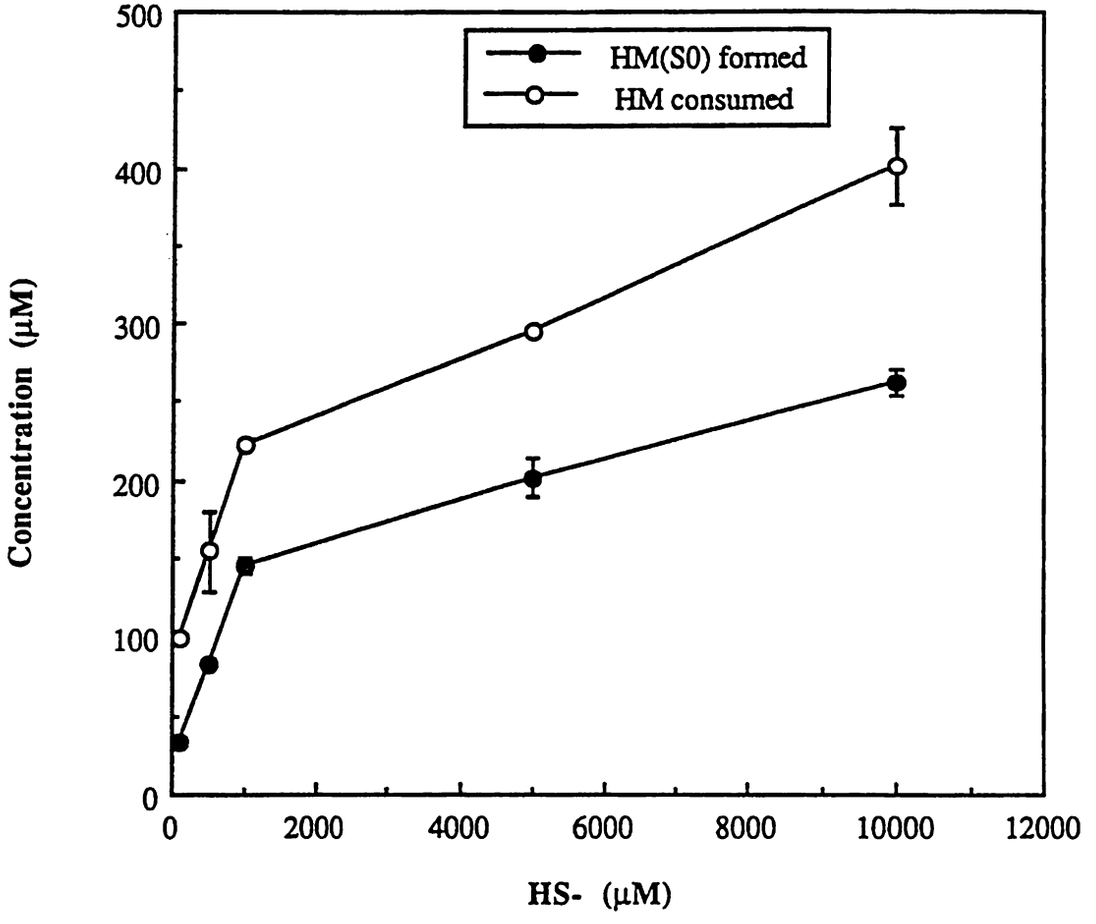

400
300
200
100
0
Concentration (μM)
HM(S0) formed
HM consumed
Blank
HS-

Figure 26: Effect of concentration of HS^- on the consumption of HM and the formation of HM(SO).

Reaction solutions contained 1 mM HM, 20 mM sodium phosphate buffer (pH 7.0), 240 μM $FeCl_3$ and 100 μM, 1 mM, 2 mM, 5 mM or 10 mM. The amount of HM(SO) formed was determined by HPLC as described in the Methods section. The data represent averages of duplicate observations.


HM(S0) formed
HM consumed
Concentration (μM)
HS- (μM)
0
100
200
300
400
500
0
2000
4000
6000
8000
10000
12000

= 0.1480 nmole/min for 5 mM HS^-). Therefore it appears that MS^- and HS^- are oxidizing methionine by similar mechanism.

Effect of the Location of Methionine on Sulfoxide Formation:

Table 2 shows that peptides containing methionine at their carboxyl-terminal give higher sulfoxide yields in comparison to the methionine present at the amino-terminal position. Another functional group which can undergo deprotonation is the N-terminal amino group (approx pka = 8.2) in the pH ranges investigated in this study. Thus the species responsible for the formation of the methionine sulfoxide may have to competitively attack either the thioether group or the amino group. Furthermore, the decreased sulfoxide yield in N-terminal methionine containing peptides could be due to strong binding of ferric ion to the carboxyl groups rather than the deprotonated N-terminal groups. Similar observations have been reported for methionine oxidation for DTT/$FeCl_3$ (Schöneich, et al., 1993; Li et al., 1993).

Although, histidine appears to catalyze the formation of methionine sulfoxide, the location of the methionine is also important in determining sulfoxide formation. This is

Table 2: Summary of the yields of methionine sulfoxide formed and peptide consumed at pH 7.0

Peptide	Sulfoxide formation (nanomoles)	Peptide consumed (nanomoles)
GMG	53 ± 10 (35.3 %)[a]	150 ± 30
MGG	10 ± 3 (25.0 %)	40 ± 5
HM	105 ± 33 (55.9 %)	188 ± 30
GGM	32 ± 2 (32.0 %)	100 ± 6
MH	11 ± 2 (24.4 %)	45 ± 8
GM	15 ± 2 (26.8 %)	56 ± 5

[a] Values in parenthesis represent the % yield of methionine sulfoxide with respect to amount of consumed peptide.

clear from the observation (Figure 17 and Figure 18) that when sulfoxidation of HM is compared to that of MH, the N-terminal methionine (MH) is more resistant to sulfoxidation than the C-terminal methionine in HM.

SECTION IV. CONCLUSION

The MSH/-iron system used in this study was shown to drive the oxidation of methionine in small peptides, and the major degradation product was methionine sulfoxide. Thus, although MSH is usually used as a reductant, the information obtained from this study shows that MSH in presence of iron and phosphate buffer promotes the oxidation of methionine. The monothiols when compared to the dithiol (DTT) are less efficient in the formation of the sulfoxide.

The involvement of freely diffusible H_2O_2, $O_2^{-\cdot}$ and $^{\cdot}OH$ in this oxidation process was negligible. The secondary reactive oxygen species formed by subsequent reactions of H_2O_2, $O_2^{-\cdot}$ and $^{\cdot}OH$ may or may not be involved in methionine sulfoxidation. However, the presence of bound reactive oxygen species cannot be ruled out, as these may not be trapped by the trapping agents used in this study. Also, the effect of the trapping agents on methionine sulfoxidation may be masked because the formation of H_2O_2, $O_2^{-\cdot}$and $^{\cdot}OH$ may not be involved in the rate determining step. Schöneich et al. (1993) have further shown that α-thioether radicals do not efficiently form methionine sulfoxide in small peptides such as HM, GMG, MGG etc. RS^- appears to be one of the species involved in the oxidation of methionine.

Eventually the rate determining step may be the complexation of Fe^{3+} with RS^-/RSH.

The formation of the sulfoxide depends on the presence of histidine, pH and the position of methionine in the peptide. The sulfoxide yield was more for HM when compared to GMG, GGM and MGG. Similar observations have been reported by Li et al. (1993) and Schöneich et al. (1993) for ascorbate/$FeCl_3$ and DTT/$FeCl_3$ systems respectively. The histidine molecule has three possible coordination sites in aqueous solution. The carboxyl group (pKa = 1.9), the imidazole nitrogen (pka = 6.9) and the amino nitrogen (pKa = 9.1) become available for complexation as the pH increases (Sundberg and Martin, 1974). The classical examples of biological histidine-metal interactions are haemoglobin and myoglobin. Catalysis by histidine may be due to a deprotonated imidazole group providing a binding site for iron and thus this can bring the iron bound reactive oxygen species closer to the methionine. The effect of pH is rather complex and depends on the peptide under investigation. The formation of methionine sulfoxide was found to be higher when methionine was present at the C-terminal of the peptide, e.g., GGM > MGG and HM > MH.

Schöneich et al. (1993) have speculated that iron bridged species may be involved in the sulfoxidation of

methionine in systems containing DTT/$FeCl_3$. Similarly, this study also provides indirect evidence for the formation of iron complexes that act as oxidizing species in methionine sulfoxidation. In order to be catalytically active it is not necessary for iron to be in solution as free ferric ion (Fe^{3+}_{aq}). For instance it has been shown that a complex of tetramethyl reductic acid and chloride and iron catalyzes the hydroxylation of saturated hydrocarbons by molecular oxygen (Feng et al., 1990).

Chelation of iron by EDTA decreases the sulfoxide formation as the EDTA may compete with the methionine containing peptide for the iron bound reactive oxygen species. Schöneich et al. (1993) have postulated that species such as (His-N)-Fe^{2+}-$00^{\cdot}$ or [(S-(DTT)-S)Fe-$00^{\cdot}$] can be reduced to the general form R-Fe^{2+}-00H or R-Fe^{2+}-00^{-}. These species can oxidize organic thioethers (Walling et al., 1970) and are expected to produce methionine sulfoxide. Furthermore two electron reduction of (His-N)-Fe^{2+}-$00^{\cdot}$ or [(S-(DTT)-S)Fe-$00^{\cdot}$] will produce the iron-oxo species (R-Fe^{2+}-$0^{\cdot}$). These two electrons can be provided by the conversion of DTT to its disulfide form, which is accelerated at higher pH. Bound (R-Fe^{2+}-$0^{\cdot}$) species appear to react similarly to hydroxyl radicals (Schöneich et al., 1993). In this study instead of DTT, MSH was used and the same analogy can be drawn, except that two molecules of MSH

must be converted to MSSM to provide two electrons for the reduction of (His-N)-Fe^{2+}-$00^{\cdot}$ or [(S-(DTT)-S)Fe-$00^{\cdot}$] to R-Fe^{2+}-$0^{\cdot}$. Thus at lower pH (His-N)-Fe^{2+}-$00^{\cdot}$ or [(S-(DTT)-S)Fe-$00^{\cdot}$] may be present which can efficiently form the sulfoxide and as the pH is increased the deprotonation of MSH is accelerated (Figure 16). Thus the formation of iron-oxo species can take place which act similarly to hydroxyl radicals giving lower yields of sulfoxide at higher pH (Figures 14 and 15). Several oxygen activating enzymes require the complexation of iron by histidine to form species having the general formula of (His-N)-Fe^{2+}-$00^{\cdot}$ (Menage et al., 1992; Kurtz, 1990). This may account for the increased sulfoxide yield for HM around pH 7.0 (Figure 17).

These studies have provided evidence that freely diffusible H_2O_2, $O_2^{-\cdot}$ and $^{\cdot}OH$ species may not be involved in the rate determining step in the formation of methionine sulfoxide. The importance of the location of methionine on the peptide is also crucial to the efficiency of oxidation of methionine. These studies and future elucidation of the chemical mechanisms involved in methionine sulfoxidation will provide a better understanding of the susceptibility of various proteins and peptides to degradation. Future studies along similar lines will help to predict the fate of pharmaceutically important proteins and peptides during

their preparation and storage. Also, methionine sulfoxidation is thought to be related to several clinical conditions such as aging, inflammation, cataracts, rheumatoid arthritis and emphysema. The understanding of the mechanisms of these reactions may help in the prognosis and treatment of such ailments.

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