One-Electron Photooxidation of *N*-Methionyl Peptides. Mechanism of Sulfoxide and Azasulfonium Diastereomer Formation through Reaction of Sulfide Radical Cation Complexes with Oxygen or Superoxide

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Abstract: We have characterized and quantified pathways by which sulfide radical cation complexes of *N*-methionyl peptides (Met-Met, Met-Met-Ala, and Met-Leu) transform into various products through the reaction with superoxide or oxygen. Sulfide radical cations were generated photolytically by the reaction of the peptides with either triplet carboxybenzophenone (^{3}CB) or hydroxyl radicals (HO$^\bullet$). Sulfide radical cations of Met-Met and Met-Met-Ala, generated through ^{3}CB, formed intramolecularly sulfur−sulfur three-electron-bonded radical cation complexes, $[R_2S\therefore SR_2]^+$, which efficiently reacted with superoxide to yield the respective disulfoxides Met(O)-Met(O) and Met(O)-Met(O)-Ala. Competitively, monomeric sulfide radical cations and $[R_2S\therefore SR_2]^+$ converted intramolecularly into sulfur−nitrogen three-electron-bonded complexes, $[R_2S\therefore N(R)H_2]^+$, which reacted with both superoxide and molecular oxygen to yield azasulfonium derivatives. Among these azasulfonium derivatives the C(S),S(R) diastereomers (AS II) were generally formed in about 1.5−3.8-fold excess over the C(S),S(S) diastereomers (AS I), indicating some diastereoselectivity in the reaction mechanism. Representative quantum mechanical calculations for the azasulfonium diastereomers of L-Met showed that the energy difference between both diastereomers was small, 1.9 kcal/mol (electronic energy) or 1.3 kcal/mol (gas-phase free energy). In complementary experiments, complex $[R_2S\therefore N(R)H_2]^+$ was generated through the reaction of the peptides with HO$^\bullet$. Here, the azasulfonium diastereomers were generated predominantly by the reaction of $[R_2S\therefore N(R)H_2]^+$ with molecular oxygen. The diastereomeric ratios [AS II]/[AS I] were generally higher when the azasulfonium products were formed via the reaction of $[R_2S\therefore N(R)H_2]^+$ with superoxide instead of with molecular oxygen. The reaction of superoxide with the sulfur radical cation complexes most likely proceeded via an inner-sphere mechanism, i.e. radical−radical combination where the addition of superoxide to $[R_2S\therefore SR_2]^+$ yielded an intermediary persulfoxide, $R_2S^{(+)}-O-O^{(-)}$, and the addition of superoxide to $[R_2S\therefore N(R)H_2]^+$ gave an intermediary hydroperoxysulfurane, $R(H)N-S(R_2)OOH$.

Introduction

Organic sulfides are highly susceptible toward oxidation, and several mechanisms have been established mostly by investigation of small organic model compounds. Such studies have provided important information on reactive intermediates as well as steric and electronic effects of substituents on the reactivity of such intermediates.[1−15] When an oxidation-sensitive sulfide is located in a macromolecule, e.g. a methionine (Met) residue in a peptide or protein, higher order structure may bring functional groups into close proximity of the sulfide which otherwise might be quite remote on the basis of primary sequence only. Many peptides are sufficiently flexible to allow contacts between otherwise remote functional groups on the nano- to picosecond time scale.[16] Therefore, mechanistic studies

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on the oxidation of the sulfide function of Met in peptides and proteins should always examine the possibility of effects of remote (on the basis of primary sequence) functional groups on reaction products and kinetics. As one- and two-electron oxidation mechanisms of small organic sulfides are significantly influenced by interaction of reaction intermediates with neighboring hydroxy, amino, carboxylate, and sulfide substituents,[6,9−11,13−15] similar effects may be expected for the oxidation of Met in peptides and proteins. Here, such functional groups can be present through the amino acids Thr, Ser, Lys, Asp, Glu, and a second Met.

The one-electron oxidation of Met to the Met sulfur radical cation, Met(>S$^{\bullet+}$), may play an important role for protein oxidation during conditions of oxidative stress and biological aging as certain conditions promote the conversion of sulfur radical cations into sulfoxide.[12,17] In addition, methionine oxidation presents a problem for the biotechnology industry in its attempts of processing and stabilizing formulations of recombinant protein pharmaceuticals.[18] Here, N-terminal Met residues are of particular interest as many recombinant proteins contain an N-terminal Met residue due to biotechnological processing (e.g., *N*-methionyl human growth hormone or brain-derived neurotrophic factor, BDNF). Thus, a mechanistic understanding of the processes which lead to the conversion of peptide- or protein-bound Met(>S$^{\bullet+}$) into Met(O) is important.

The present paper reports on the mechanisms of conversion of Met(>S$^{\bullet+}$) into Met(O) in small *N*-methionyl model peptides as a function of pH, oxygen concentration, peptide sequence, and the presence of superoxide. For this, we have subjected Met-containing peptides to one-electron photooxidation by triplet carboxybenzophenone (^{3}CB) in air-saturated aqueous solution. It will be shown that Met(>S$^{\bullet+}$) efficiently reacts with superoxide under formation of a zwitterionic intermediate like the one formed during the reaction of Met with singlet oxygen. Such a sulfur radical cation−superoxide reaction had originally been proposed for small organic sulfides[19−21] and recently quantified by us for dimethyl sulfide (DMS) in aqueous solution.[17] However, an alternative mechanism involves the conversion of Met(>S$^{\bullet+}$) into sulfur−nitrogen bonded intermediates which convert into azasulfonium (AS) salts with different diastereomeric excesses depending on the actual mechanism and the peptide sequence. These AS derivatives can subsequently hydrolyze to Met(O). By investigating the model peptide Met-Met, we were able to demonstrate competitive sulfoxide and AS derivative formation through the reaction of superoxide with sulfur−sulfur or sulfur−nitrogen bonded radical intermediates, respectively.

The following reactions have been quantified with dimethyl sulfide[17] and are of importance for the design of the experiments with the peptides presented in this paper. A type I photochemical oxidation of dimethyl sulfide by ^{3}CB yields the sulfur radical cation **1** (reaction 2), which exists as a three-electron sulfur−sulfur bonded complex **2** with a second molecule DMS (reaction 3). The product CB$^{\bullet-}$ exists in the protonation equilibrium 4 ($pK_{a,4} = 8.2$[22]). In the presence of oxygen, CBH$^\bullet$ and CB$^{\bullet-}$ generate superoxide via reaction 5.

$$CB + h\nu \rightarrow {}^3CB \quad (1)$$

$${}^3CB + S< \rightarrow CB^{\bullet-} + >S^{\bullet+}\ (\mathbf{1}) \quad (2)$$

$$>S^{\bullet+} + S< \rightleftharpoons [>S\therefore S<]^+\ (\mathbf{2}) \quad (3)$$

$$CB^{\bullet-} + H^+ \rightleftharpoons CBH^\bullet \quad (4)$$

$$CB^{\bullet-}/CBH^\bullet + O_2 \rightarrow CB + O_2^{\bullet-}/H^+, O_2^{\bullet-} \quad (5)$$

A highly efficient reaction of superoxide with **2** yields the persulfoxide **3**, which can hydrate[7] to the hydroperoxy sulfurane **4**.

$$\mathbf{2} + O_2^{\bullet-} \rightarrow >S + >S^{(+)}-O-O^{(-)}\ (\mathbf{3}) \quad (6)$$

$$\mathbf{3} + H_2O \rightarrow HO-(H_3C)_2S-OOH\ (\mathbf{4}) \quad (7)$$

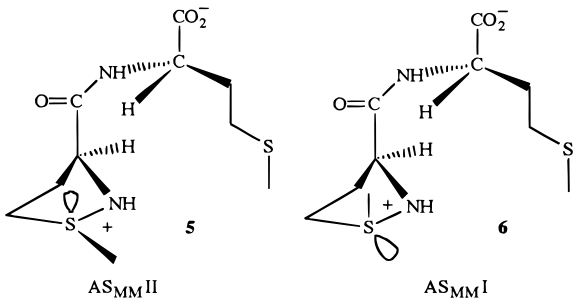
Overall, species **3** will yield 2 equiv of sulfoxide per persulfoxide either through reaction 8 or 9, respectively.

$$\mathbf{3} + S< \rightarrow 2DMSO \quad (8)$$

$$\mathbf{4} + S< \rightarrow DMSO + (H_3C)_2S(OH)_2 \rightarrow H_2O + 2DMSO \quad (9)$$

Results

A. Structural Assignment of the AS Diastereomers. 1. ^{1}H NMR Spectra of AS$_{MM}$I, AS$_{MM}$II, AS$_M$I, and AS$_M$II. Two major oxidation products of Met-Met by ^{3}CB are the azasulfonium (AS) diastereomers AS$_{MM}$I and AS$_{MM}$II (for all AS species the subscripts denote the respective peptide/amino acid reactant and the roman numbers the elution order during reversed-phase HPLC). AS$_{MM}$II and AS$_{MM}$I are tentatively assigned to the schematic structures **5** and **6**, respectively, where the sulfur is of *R* configuration in **5** and of *S* configuration in **6**. Their absolute configurations were confirmed by comparison of their ^{1}H NMR spectra with those of AS$_M$I and AS$_M$II obtained through the oxidation of L-Met. In the 2.4−4.6-ppm region, the cyclic AS systems of AS$_{MM}$I and AS$_{MM}$II show characteristic patterns which are slightly shifted with respect to each other (see Figure 1S; Supporting Information). For example, the ϵCH$_3$−S$^+$< methyl resonances appear at δ 2.85 ppm for AS$_{MM}$I and at δ 2.80 ppm for AS$_{MM}$II. More significantly, the resonances for γCH$_A$ and γCH$_B$ are well-separated multiplets for AS$_{MM}$II but merge for AS$_{MM}$I. The ϵCH$_3$−S$^+$< methyl



resonances appear at δ 2.74 ppm for AS$_M$I and δ 2.76 ppm for AS$_M$II, i.e. they show nearly identical chemical shifts for both

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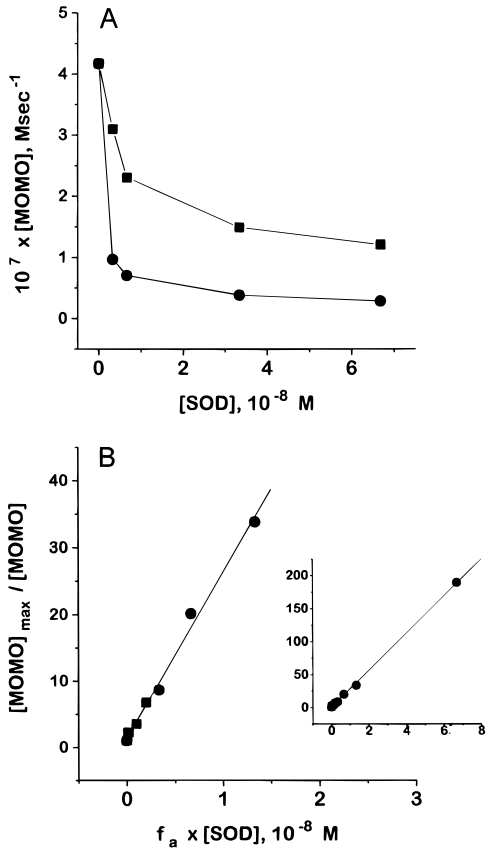

Figure 1. (A) Inhibition of Met(O)-Met(O) (denoted as MOMO) by (●) native and (■) dematallated SOD. Experimental conditions: photolysis (four 350-nm lamps) of air-saturated aqueous solutions containing 2.0×10^{-3} M Met-Met, 2.0×10^{-4} M CB, and 1.0×10^{-2} M sodium phosphate, pH 8.0. (B) Plot of $[MOMO]_{max}/[MOMO]$ vs f_a[SOD], where f_a represents the fraction of active SOD, i.e. $f_a = 1.0$ for native SOD (●) and $f_a = 0.03$ for dematallated SOD (■).

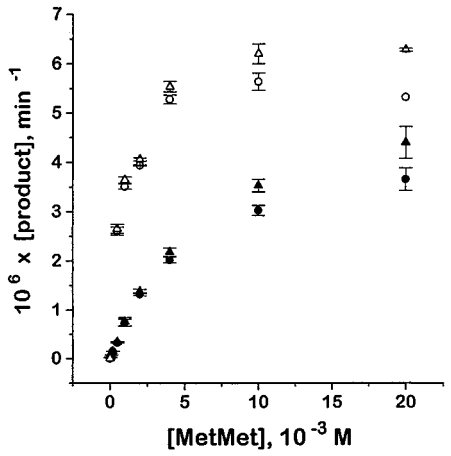

Figure 2. Photolytic formation of sulfoxide products as a function of [Met-Met] in air-saturated aqueous (H_2O, D_2O) solution containing 9.0×10^{-5} M rose bengal and 5.0×10^{-4} M sodium phosphate, pL 7.4 (L = H, D). Closed symbols, H_2O: ●, Met(O)-Met(O); ▲, Met(O)-Met(O) + 0.5 [Met(O)-Met + Met-Met(O)]. Open symbols, D_2O: ○, Met(O)-Met(O); △, Met(O)-Met(O) + 0.5 [Met(O)-Met + Met-Met(O)]

diastereomers (see Figure 2S; Supporting Information). When the ^{1}H NMR spectra were recorded at pD 2.2,[23] both $\epsilon CH_3-S^+<$ methyl resonances were slightly shifted downfield with δ 2.79 ppm for AS_MII and δ 2.80 ppm for AS_MI, respectively (data, see the Experimental Section). Thus, partial protonation of the carboxylate group (for AS_M, $pK_{a,CO_2H} = 2.52$[24]) has a small effect on the chemical shift of the $\epsilon CH_3-S^+<$ methyl resonances with the larger pD-induced shift ($\Delta = 0.06$) observed for AS_MI (with a protonated carboxylate group, the AS_M derivatives should be better comparable to the AS_{MM} derivatives in which the AS system does not contain a carboxylate substituent but a neutraly charged amide).

For the C_γ protons of the AS ring systems significant differences are observed depending on whether they are located in AS_MI or AS_MII. For example, a clear separation of the multiplets for γCH_A and γCH_B is observed for AS_MII whereas both multiplets merge for AS_MI (Figure 2S). The same tendency was subsequently not only observed for $AS_{MM}I$ and $AS_{MM}II$ (Figure 1S) but for all the characterized AS diastereomers, i.e. separated multiplets for $AS_{ML}II$ and $AS_{MMA}II$ and merging multiplets for $AS_{ML}I$ and $AS_{MMA}I$ (see data in the Experimental Section). This appears to be a general characteristic by which AS I and AS II diastereomers of all investigated peptides may be differentiated and suggests (i) that the ring conformations of the AS derivatives are identical for all AS I and identical for all AS II diastereomers and (ii) that the retention order during reversed-phase HPLC of the AS diastereomers is a function of ring conformation.

2. Polarimetry. Values for the specific rotation, of both AS diastereomers of L-Met as well as for the diastereomeric Met sulfoxides resulting from the hydrolysis of the respective AS diastereomers are known.[25−27] By comparison of our measured values for AS_MI (+17 ± 1°) and AS_MII (+86 ± 3°) we assigned the αC(S),S(S) conformation to AS_MI and the αC(S),S(R) conformation to AS_MII. The hydrolysis of AS_MI and AS_MII by 1 N HCl yielded the expected sulfoxide diastereomers, i.e. the αC(S),S(R) configured L-Met(O) (−55 ± 4°) from AS_MI and the αC(S),S(S) configured L-Met(O) (+132 ± 4°) from AS_MII. That the hydrolysis of the AS_M diastereomers by 1 N HCl gave optically pure sulfoxide products attests to the fact that acid-catalyzed racemization of the sulfoxide was not occurring over the time period of the measurements.

On the basis of our NMR data (see above), we have concluded that AS I derivatives of all investigated peptide substrates are of the same configuration. Thus, we conclude that the sulfur is of *S* configuration in all AS I diastereomers and, consequently, that the sulfur is of *R* configuration in all AS II diastereomers of the investigated peptides. This conclusion is in accord with the assignment of $AS_{MM}II$ to structure **5** and $AS_{MM}I$ to structure **6**.

B. Oxidation of Met-Met by ^{3}CB. 1. Product Formation as a Function of pH and Presence of Superoxide Dismutase. Table 1 displays the loss of Met-Met and the formation of products for the photolysis of air-saturated solutions containing 2.0×10^{-4} M CB, 2.0×10^{-3} M Met-Met, and 1.0×10^{-2} M sodium phosphate at pH 6.5, 8.0, and 9.0, respectively. Several features shall be noted. (i) Irradiation times were between 0 and 48 s, and the loss of Met-Met as well as the formation of products was linearly dependent on the irradiation time. Even at the maximum irradiation time of 48 s (eight lamps), the

(23) Samples were acidified with DCl. At pD 2.2, azasulfonium derivatives undergo hydrolysis to the respective sulfoxides. However, NMR spectra of the AS derivatives can be recorded within 5 min after acidification to pD 2.2.

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Table 1. Yields of Products and Loss of Substrate as a Function of pH and the Presence of SOD[a]

	yield, 10^{-7} M s^{-1}					
	pH 6.5		pH 8.0		pH 9.0	
compd	−SOD	+SOD	−SOD	+SOD	−SOD	+SOD
$AS_{MM}II$	5.6 ± 0.2	7.2 ± 0.4	5.1 ± 0.3	10.8 ± 0.8	4.8 ± 0.2	11.9 ± 0.2
$AS_{MM}I$	1.9 ± 0.2	3.0 ± 0.2	1.4 ± 0.1	4.3 ± 0.4	1.6 ± 0.1	4.8 ± 0.1
Met(O)-Met(O)	6.8 ± 0.3	<0.2	11.9 ± 0.1	0.9 ± 0.1	10.8 ± 1.0	0.5 ± 0.1
Met(O)-Met	1.8 ± 0.1	0.7 ± 0.1	2.0 ± 0.1	1.0 ± 0.1	2.3 ± 0.2	1.3 ± 0.2
Met-Met(O)	2.2 ± 0.1	0.4 ± 0.1	1.2 ± 0.1	0.6 ± 0.1	1.6 ± 0.1	1.0 ± 0.1
thiols	3.0 ± 0.1	0.2 ± 0.1[b]	1.4 ± 0.1	0.2 ± 0.1[b]	2.3 ± 0.6	0.2 ± 0.1[b]
formaldehyde	3.5 ± 0.2	3.3 ± 0.3	2.0 ± 0.1	2.1 ± 0.1	2.3 ± 0.2	nd
−[Met-Met]	27.0 ± 5.0	27.0 ± 5.0	26.6 ± 5.0	26.6 ± 5.0	22.0 ± 3.0	21.0 ± 4.0

[a] Conditions: photolysis (eight 350-nm lamps) of air-saturated aqueous solutions containing 2.0×10^{-3} M Met-Met, 2.0×10^{-4} M CB, and 1.0×10^{-2} M sodium phosphate in the absence (−SOD) or presence (+SOD) of 7.8×10^{-6} M SOD dimer (nd = not determined).

overall loss of Met-Met (e.g., at pH 6.5) of = 1.3×10^{-4} M (=48 s × 2.7×10^{-6} M s^{-1}) is lower than 7% of the starting material so that secondary reactions between ^{3}CB and oxidation products can be excluded. (ii) The products Met-Met(O), Met-(O)-Met, Met(O)-Met(O), $AS_{MM}I$, and $AS_{MM}II$ mainly originate from initial one-electron oxidation of Met-Met (see Discussion) and account for a fraction of 0.67−0.96 (depending on pH) of the chemical quenching of ^{3}CB by Met-Met. Thiols and formaldehyde originate from the same precursor, an α-(alkylthio)alkyl radical, which is mainly the product of initial hydrogen transfer between Met-Met and ^{3}CB (see Discussion). Considering that hydrogen transfer should occur approximately equally well from the ϵCH_3 and the γCH_2 group of Met, and considering that formaldehyde only forms after hydrogen transfer from the ϵCH_3 or $\epsilon C'H_3$ group from Met-Met, each equivalent of formaldehyde is representative for approximately 2 equiv of hydrogen transfer. Thus, representatively for pH 6.5 in the absence of SOD, we calculate the fraction of hydrogen transfer contributing to chemical quenching of ^{3}CB by Met-Met as ca. ($2 \times 3.5 \times 10^{-7}$ M s^{-1}/27×10^{-7} M s^{-1}) = 0.26 (we did not rely on the yields of thiols in these calculations due to their instability in an oxidative environment). On the basis of the combined fractions of electron and hydrogen transfer, 0.67 + 0.26 = 0.93, it is evident that the reaction products well account for the loss of starting material through chemical quenching. It must be noted that in particular the quantification of the loss of starting material carries quite large error limits as we kept the total conversion of substrate <7% to minimize secondary reactions. None of the observed reaction products were obtained in the absence of oxygen (i.e., N_2-saturated solutions; data not shown), indicating that the presence of molecular oxygen was an important requirement for product formation.

(iii) When the oxidation of Met-Met was carried out in the presence of 7.8×10^{-6} M superoxide dismutase (SOD) dimer,[28] there was a ca. 13−34-fold pH-dependent reduction of the yields of Met(O)-Met(O) and a ca. 2−3-fold decline of the yields of Met(O)-Met and Met-Met(O). The remaining Met(O)-Met(O) yields in the presence of SOD will be from here on referred to as *SOD-independent* yields, whereas subtraction of the *SOD-independent* Met(O)-Met(O) yields from the total Met(O)-Met-(O) yields will be referred to as *SOD-dependent* Met(O)-Met(O) yields. The yields of $AS_{MM}I$ and $AS_{MM}II$ increased ca. 1.3−3-fold in the presence of SOD. In general, the ratios of [AS_{MM}-II]:[$AS_{MM}I$] were higher in the absence of SOD (2.9−3.6) than in the presence of SOD (ca. 2.5).

Sulfide radical cations such as $R_2S^{\bullet+}$ are very strong one-electron oxidants.[29] Therefore, we had to confirm that the inhibition of Met(O)-Met(O) formation by SOD was not merely caused by the competitive reaction of $R_2S^{\bullet+}$ with easily oxidizable amino acids X of the SOD protein skeleton (reaction 10), where X in bovine erythrocyte SOD could be Tyr or Met.[30,31]

$$R_2S^{\bullet+} + \text{SOD-(X)} \rightarrow R_2S + \text{SOD-(X}^{\bullet+}\text{)} \qquad (10)$$

For this purpose, a demetalated des-Cu variant (apo-SOD) of SOD was prepared by partial unfolding of the enzyme, extraction of Cu^{2+} with EDTA, and refolding (see the Experimental Section). Our preparation of apo-SOD showed ca. 3% of the residual activity as compared to the native enzyme, most probably caused by an incomplete removal of Cu^{2+}. When air-saturated solutions containing 1.0×10^{-2} M sodium phosphate, pH 8.0, 2.0×10^{-4} M CB, and 2.0×10^{-3} M Met-Met were photolyzed in the presence of various concentrations of either native SOD or apoenzyme, the apoenzyme was much less effective in inhibiting the formation of Met(O)-Met(O) than was native SOD, as shown in Figure 1a. These data suggest that it is the dismutation of superoxide by SOD which causes the observed inhibition of sulfoxide formation and not an unspecific oxidation of amino acid residues of SOD by $R_2S^{\bullet+}$ according to reaction 10.

The fact that the apoenzyme still inhibits sulfoxide formation, though to a lesser extent, can be related to its residual SOD activity. This becomes evident from Figure 1b, which shows a plot of $[\text{Met(O)-Met(O)}]_{max}/[\text{Met(O)-Met(O)}]$ vs f_a[SOD], where $[\text{Met(O)-Met(O)}]_{max}$ and [Met(O)-Met(O)] represent the yields of Met(O)-Met(O) in the absence and presence of SOD, respectively, f_a represents the fraction of active SOD, and [SOD] represents the concentration of the SOD dimer. In this plot, all the points fall on a line regardless of whether obtained with native SOD or apoenzyme.

Thus, we have strong evidence that in the CB-photosensitized system the majority (ca. 95−97%, depending on pH) of Met-(O)-Met(O) is formed through the reaction of superoxide with a Met-Met sulfide radical cation, [Met-Met]$^{\bullet+}$, according to the general reaction 11.

$$\text{Met-Met}^{\bullet+} + O_2^{\bullet-} \rightarrow \text{Met(O)-Met(O)} \qquad (11)$$

(28) Earlier experiments with DMS had shown that such concentration of SOD was sufficient for a complete inhibition of all superoxide-dependent processes.[17]

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Table 2. Efficiencies, f = [product]/[loss of Met-Met], of Product Formation from Met-Met at pH 9.0 as a Function of Light Intensity[a]

	efficiency, f			
	−SOD		+SOD	
product	four lamps	eight lamps	four lamps	eight lamps
$AS_{MM}II$	0.343	0.216	0.584	0.540
$AS_{MM}I$	0.117	0.073	0.220	0.216
Met(O)-Met(O)	0.348	0.490	0.020	0.024
Met-Met(O)	0.090	0.074	0.054	0.046
Met(O)-Met	0.077	0.098	0.053	0.059

[a] Conditions: 2.0×10^{-3} M Met-Met, 2.0×10^{-4} M CB, and 1.0×10^{-2} M sodium phosphate, pH 9.0, irradiated with either four lamps (corresponding to an output of 1.6×10^{-5} M s^{-1} photons) or eight lamps (3.2×10^{-5} M s^{-1} photons). The loss of Met-Met was 1.17×10^{-6} M s^{-1} for four lamps and 2.2×10^{-6} M s^{-1} for eight lamps, respectively.

However, only a smaller fraction of ca. 30−50% of the monosulfoxides Met(O)-Met and Met-Met(O) appears to be formed by the reaction of superoxide with an intermediate, most likely also $[Met\text{-}Met]^{\bullet+}$. A direct oxidation of Met-Met by superoxide (reaction 12) can be excluded on the basis of competition experiments with formate. When air-saturated solutions, pH 10, containing 8×10^{-3} M phosphate, 2×10^{-4} M CB, and 2×10^{-3} M Met-Met were photolyzed in the additional presence of 1.0 M sodium formate, there was a 70% reduction of the Met(O)-Met(O) yields as compared to formate-free systems. This result will be rationalized in more detail in the Discussion.

$$\text{Met-Met} + O_2^{\bullet-} \rightarrow \text{Met(O)-Met(O)} + \text{products} \quad (12)$$

2. Influence of Radical Concentration. Table 2 displays the efficiencies f for product formation at pH 9.0 in the absence and presence of SOD as a function of light intensity which controls the formation rate of radical species. The efficiencies f were calculated by dividing the rate of product formation by the rate of substrate loss. The reaction mixtures were exposed to either four or eight lamps, corresponding to 1.6×10^{-5} and 3.2×10^{-5} M s^{-1} photons, respectively. Under these conditions the loss of Met-Met was $(1.17 \pm 0.07) \times 10^{-6}$ M s^{-1} for four lamps and $(2.2 \pm 0.3) \times 10^{-6}$ M s^{-1} for eight lamps, i.e. showed the expected 2-fold increase for a 2-fold higher light intensity.

However, significant differences were observed for the efficiencies of product formation in the absence of SOD. The major product during the photolysis with eight lamps was Met(O)-Met(O), whereas its efficiency of formation significantly dropped at the expense of the formation of $AS_{MM}I$ and $AS_{MM}II$ in the four-lamp setup. In contrast, there were no differences between the experimental systems when the photolysis was carried out in the presence of SOD. As expected, higher steady-state concentrations of $[Met\text{-}Met]^{\bullet+}$ and superoxide favor the formation of Met(O)-Met(O). However, as they disfavor the production of the AS diastereomers, it may be concluded that at least a fraction of the latter requires the competitive conversion of an initially formed $[Met\text{-}Met]^{\bullet+}$ into a second intermediate which constitutes a precursor for $AS_{MM}I$ and $AS_{MM}II$. In the presence of SOD, the radical−radical reaction 11 is inhibited and comparable product efficiencies are observed for both light intensities.

3. Comparison of Met-Met with Met-Leu and Met-Met-Ala. An important observation is that ^{3}CB oxidation of the peptides shows some diastereoselectivity for the AS diastereomers whereas that is not the case for the oxidation by KI_3 (which generally yielded 1:1 ratios of AS II and AS I for all investigated compounds). Quantum mechanical calculations (see the Supporting Information) revealed a small difference in stability (ca. 1.9 kcal/mol electronic energy or 1.3 kcal/mol gas-phase free energy) between the AS diastereomers of L-Met. However, more likely it is the nature of the radical intermediates and their interactions with superoxide and molecular oxygen (see Discussion) which may be responsible for the diastereoselectivity. To investigate this possibility, we have compared the diastereomeric ratios [AS II]:[AS I] for Met-Met, Met-Leu, and Met-Met-Ala as a function of peptide concentration (Table 3) and as a function of pH (Table 4). These sequences were not selected randomly but on the basis of the following considerations. An important characteristic of Met-Met is that one-electron oxidation by ^{3}CB leads to sulfur radical cations **7**-N and **7**-C (where the suffix N and C denote the N-terminal and the C-terminal Met residue) which can form an intramolecular sulfur−sulfur three-electron bond as shown in structure **8**.[32] Alternatively, an oxygen−sulfur bond[33] (a sulfuranyl type radical) could form via interaction between the C-terminal carboxylate group and a radical cation on the C-terminal Met residue (structure **9**), though pulse radiolytic studies with Met-Met have shown that such sulfur−oxygen bonds may at best exist transiently.[34] However, even as short-lived transients, such intermediates may affect the overall product formation, and their potential influence on the mechanism has to be tested. Sulfuranyl radicals **9** cannot form when the carboxylate group and the Met residue are separated by a peptide bond,[35] i.e. in Met-Met-Ala. Therefore, any difference in the product pattern between Met-Met and Met-Met-Ala may indicate that the sulfuranyl structure **9** plays a role in product formation. Met-Leu can neither form a sulfuranyl intermediate **9** nor form an intramolecular sulfur−sulfur three-electron bond, and a com-

Table 3. Diastereomeric Ratios [AS II]/[AS I] as a Function of Peptide Sequence, Peptide Concentration, and the Presence of SOD[a]

	[AS II]/[AS I] (±10%)					
	Met-Leu		Met-Met		Met-Met-Ala	
[peptide], M	−SOD	+SOD	−SOD	+SOD	−SOD	+SOD
0.5×10^{-3}	1.50	2.4	3.7	2.6	3.7	2.8
1.0×10^{-3}	1.6	2.4	3.5	2.6	3.8	2.7
2.0×10^{-3}	1.6	2.3	3.6	2.5	3.8	2.8

[a] Conditions: air-saturated aqueous solution containing 2.0×10^{-4} M CB, 8×10^{-3} M sodium phosphate, pH 8.0, various concentrations of peptide, and no or 7.8×10^{-6} M SOD dimer.

Table 4. Diastereomeric Ratios [AS II]/[AS I] as a Function of Peptide Sequence, pH, and the Presence of SOD[a]

	[AS II]/AS I (±10%)					
	Met-Leu		Met-Met		Met-Met-Ala	
pH	−SOD	+SOD	−SOD	+SOD	−SOD	+SOD
6.5	2.1	1.9	2.9	2.4	3.2	2.8
8.0	1.6	2.3	3.6	2.5	3.8	2.8
9.0	1.7	2.6	3.0	2.5	3.7	2.9

[a] Conditions: air-saturated aqueous solution containing 2.0×10^{-3} M peptide, 2.0×10^{-4} M CB, 8×10^{-3} M sodium phosphate, various concentrations of peptide, and no or 7.8×10^{-6} M SOD dimer.

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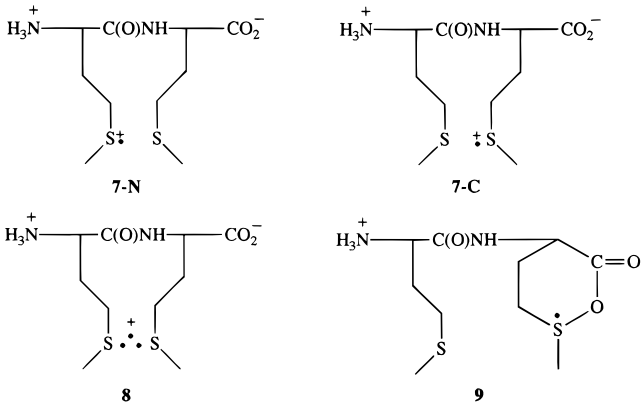
parison of Met-Leu and Met-Met may demonstrate the importance of structure **8** for product formation.



For reactions in the absence of SOD, there was a significant difference between the ratios [AS II]:[AS I] of Met-Leu and Met-Met but not between Met-Met and Met-Met-Ala. However, all three peptides showed comparable diastereomer ratios in the presence of SOD at pH ≥ 8.0. Thus, it appears that in particular the potential to form an intramolecular sulfur−sulfur three-electron bond has some influence on the reaction of peptide sulfur radical cations with superoxide. On the other hand, the inhibition of potential oxygen−sulfur bond formation (as in Met-Met-Ala) showed little effect on the diastereomer ratio.

C. Oxidation of Met-Met by Singlet Oxygen. 1. Product Formation and Rate Constant. Alternatively to the type I photooxidation of organic sulfides (i.e., reaction 2), ^{3}CB may be quenched by oxygen to yield singlet oxygen (reaction 13).[36−38]

$$^3\mathrm{CB} + {}^3\mathrm{O_2} \rightarrow \mathrm{CB} + {}^1\mathrm{O_2}\ (^1\Delta_g,\ ^1\Sigma^+_g) \qquad (13)$$

We subjected Met-Met to oxidation by singlet oxygen to assess whether the reaction products derived from the ^{3}CB system were caused by a type I or a type II (via singlet oxygen) photoprocess. The illumination of rose bengal (RB) with visible light produces singlet oxygen according to the general reactions 14 and 15.[1,39]

$$\mathrm{RB} + h\nu \rightarrow \mathrm{RB}^* \qquad (14)$$

When air-saturated aqueous (H_2O and D_2O) solutions, containing 5.0×10^{-4} M phosphate buffer and 9.0×10^{-5} M RB were exposed to visible light in the presence of $(0.1-1.0) \times 10^{-3}$ M Met-Met, there was almost exclusive formation of Met(O)-Met(O), as displayed in Table 5.

$$\mathrm{RB}^* + {}^3\mathrm{O_2} \rightarrow \mathrm{RB} + {}^1\mathrm{O_2} \qquad (15)$$

All other products accounted for <10% of the product yields. Moreover, the presence of additional 7.8×10^{-6} M SOD dimer did not affect the yields of Met(O)-Met(O), indicating that Met(O)-Met(O) was not the product of a type I photoprocess followed by a reaction of superoxide with $[\text{Met-Met}]^{\bullet+}$. Thus, reactions 16 and 17, followed by reaction 11 were not responsible for Met(O)-Met(O) formation in the RB system. If Met(O)-Met(O) would have been the result of such a type I photoprocess, we would have expected not only an effect of SOD but also significant yields of AS_{MM}II and AS_{MM}I, as in the ^{3}CB system.

Table 5. Yields of Products and Consumption of Met-Met by Singlet Oxygen[a]

	yields, 10^{-7} M s^{-1}		
compd	1×10^{-4} M Met-Met, 1:1 (v/v) H_2O/D_2O, pL 8.0	1×10^{-3} M Met-Met, H_2O, pH 7.0	1×10^{-3} M Met-Met, D_2O, pD 7.0
−[Met-Met]	1.63 ± 0.24	nd[b,c]	nd[b,c]
Met(O)-Met(O)	1.62 ± 0.04	7.5 ± 0.4	36 ± 2
Met-Met(O)	0.02 ± 0.004	0.26 ± 0.07	0.55 ± 0.16
Met(O)-Met	0.02 ± 0.04	0.23 ± 0.07	0.47 ± 0.26
AS_{MM}II	0.0	0.09 ± 0.02	0.41 ± 0.05
AS_{MM}I	0.0	<0.08	<0.4

[a] Conditions: air-saturated aqueous (L_2O, L = H, D) solutions containing 5.0×10^{-4} M phosphate buffer, 9.0×10^{-5} M rose bengal, and various concentrations of Met-Met. [b] nd = not determined. [c] Too low conversion for satisfactory determination of loss of Met-Met.

$$\mathrm{RB}^* + \text{Met-Met} \rightarrow \mathrm{RB}^{\bullet-} + [\text{Met-Met}]^{\bullet+} \qquad (16)$$

$$\mathrm{RB}^{\bullet-} + \mathrm{O_2} \rightarrow \mathrm{RB} + \mathrm{O_2}^{\bullet-} \qquad (17)$$

Further confirmation that singlet oxygen was the actual oxidant in the RB system but not in the ^{3}CB system was derived from solvent isotope effects. Inspection of the third and fourth column in Table 5 reveals that ca. 5-fold higher yields of Met(O)-Met(O) were formed in the RB system when the experiments were carried out in D_2O as compared to H_2O. This finding is rationalized on the basis of the 10−16-fold longer lifetime of $^1O_2$ in D_2O,[40,41] promoting Met-Met oxidation at the expense of quenching by the solvent (reaction 18; L = H, D).

$$^1\mathrm{O_2} + \mathrm{L_2O} \rightarrow {}^3\mathrm{O_2} + \mathrm{L_2O} \qquad (18)$$

In contrast, the *SOD-dependent* Met(O)-Met(O) formation via one-electron oxidation in the ^{3}CB system (type I photoprocess) showed only a 1.2-fold increase upon changing the solvent from H_2O to D_2O (see below).

The key processes for the reaction of $^1O_2$ with Met-Met in aqueous solution are displayed in Scheme 1, based on analogous reactions of $^1O_2$ with organic sulfides in protic solvents.[1,2,7] It should be noted that in structures **10** and **11** the persulfoxide or the hydroperoxysulfurane may be formed at either the N- or the C-terminal Met residue. It is also important to understand that persulfoxide **10** represents only one possible structure initially formed through the addition of $^1O_2$ to a sulfide. An alternative structure such as a cyclic thiadioxirane has been suggested in particular in view of the presence of both a nucleophilic (likely the persulfoxide) and an electrophilic intermediate (likely the thiadioxirane) during the reaction of $^1O_2$ with sulfides in aprotic solvents.[2]

Stoichiometrically, every chemical reaction of $^1O_2$ with Met-Met yields 1 equiv of Met(O)-Met(O) or 2 equiv of the monosulfoxides. The total amount of $^1O_2$ reacting with Met-Met is equal to Σ = [Met(O)-Met(O)] + 0.5 ([Met-Met(O)] + [Met(O)-Met]). Figure 2 displays the yields of Met(O)-Met(O) and Σ obtained as a function of [Met-Met] in L_2O (L = H,

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Scheme 1

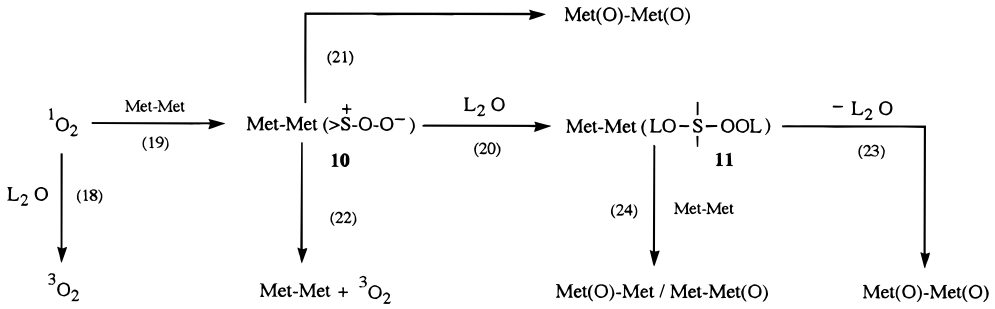


Table 6. Solvent Isotope Effects on the Efficiency, f = [Product]/[Loss of Met-Met], during the Oxidation of Met-Met by the ^{3}CB System in the Absence or Presence of SOD at PL 9.0 (L = H, D)

	−SOD		+SOD	
	H_2O	D_2O	H_2O	D_2O
$AS_{MM}II$	0.216	0.207	0.540	0.483
$AS_{MM}I$	0.073	0.059	0.216	0.188
Met(O)-Met(O)	0.490	0.580	0.024	0.141
Met-Met(O)	0.074	0.079	0.046	0.082
Met(O)-Met	0.098	0.082	0.059	0.067

D) during the photolysis of air-saturated aqueous solutions, pL = 7.4, containing 5×10^{-4} M sodium phosphate and 9.0×10^{-5} M RB.

In D_2O, Σ reaches a plateau, Σ_{max}, for [Met-Met] = 10^{-2} M, whereas in H_2O, we observe increasing values of Σ for [Met-Met] > 10^{-2} M. At Σ_{max}, $^1O_2$ reacts exclusively with Met-Met (reaction 19) at the expense of solvent quenching (reaction 18), and the absolute value of Σ_{max} is determined by the branching ratios among reactions 20−22. For sufficiently low concentrations of Met-Met, the branching ratios of reactions 20−22 should remain constant (i.e., we do not have to include a direct reaction of **10** with a second molecule of Met-Met as the hydration reaction 20 is expected to be very fast). Thus, the absolute values of Σ and Σ_{max} in H_2O can be used to compute the rate constant for reaction 19 in H_2O according to eqs I and II, assuming that Σ_{max} is similar in H_2O and D_2O. A plot of $d(\Sigma_{max}/\Sigma_{max} - \Sigma)/d$[Met-Met] gave a straight line with a slope of 126 ± 14 (intercept 1.0). Taking $k_{18} = 5 \times 10^5$ s^{-1} in H_2O,[40] we obtain $k_{19} = (6.3 \pm 0.7) \times 10^7$ M^{-1} s^{-1}.

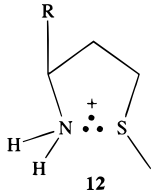
$$\frac{\Sigma_{max}}{\Sigma_{max} - \Sigma} = 1 + \frac{k_{19}[\text{Met-Met}]}{k_{18}} \quad \text{(I)}$$

$$\frac{\partial(\Sigma_{max}/\Sigma_{max} - \Sigma)}{\partial[\text{Met-Met}]} = \frac{k_{19}}{k_{18}} \quad \text{(II)}$$

2. Potential Contribution of Type II Photooxidation in the ^{3}CB System. We can now estimate any potential contribution of type II photooxidation to the product yields observed in the ^{3}CB system. Table 6 diplays product yields obtained with 2×10^{-3} M Met-Met in L_2O, pL 9.0 (L = H, D), in the absence and presence of SOD.

In H_2O, pH 9.0, the addition of SOD reduces the yields of Met(O)-Met(O) by ca. 95%, indicating that the majority of Met(O)-Met(O) derives from the reaction of Met-Met$^{\bullet+}$ with superoxide. When, in the presence of SOD, the solvent is changed to D_2O, there is a ca. 5.9-fold increase in the SOD-*independent* yields of Met(O)-Met(O), suggesting that they derive from the reaction of singlet oxygen, formed via reaction 13, with Met-Met. We note that this 5.9-fold increase in the efficiency of Met(O)-Met(O) formation is somewhat higher than the ca. 3-fold increase in absolute Met(O)-Met(O) yields observed for a change in solvent from H_2O to D_2O in the RB system for 2×10^{-3} M Met-Met (see Figure 2). However, as we do not know whether a change from H_2O to D_2O has an effect on the energy transfer reaction 13, we prefer to compare these absolute values qualitatively rather than quantitatively. In the absence of SOD, the total yields of Met(O)-Met(O) are ca. 18% higher in D_2O as compared to H_2O. We know that one-electron oxidation of sulfides to sulfide radical cations by ^{3}CB can be less efficient in D_2O as compared to H_2O (though the magnitude of such a solvent isotope effect depends on various factors such as the structure of the charge-transfer complex, subsequent transition states/intermediates, and the substituents on the sulfide).[17,42] Thus, the 18% increase of the total Met(O)-Met(O) yields is likely the result of an overall lower efficiency of the SOD-*dependent* formation offset by a significantly higher efficiency of the SOD-*independent* formation. In the presence of SOD, a change from H_2O to D_2O has only a small effect on the formation of $AS_{MM}I$ and $AS_{MM}II$. This is not unexpected as we have shown that both AS diastereomers are not formed through the interaction of singlet oxygen with Met-Met. The slight isotope effect likely results from a solvent isotope effect on the formation pathways of the AS derivatives.

D. Oxidation of *N*-Methionyl Peptides by Hydroxyl Radicals. During the photooxidation of Met-Met by ^{3}CB, significantly higher yields of AS diastereomers were formed in the presence of SOD, though with different diastereomeric ratios as compared to the absence of SOD. These results suggest that the AS diastereomers may form via at least two different pathways, a superoxide-*dependent* and a superoxide-*independent* mechanism. As will be shown in the discussion, one key intermediate for AS formation is an intramolecularly sulfur−nitrogen three-electron-bonded radical cation, $[>H_2N\therefore S<]^+$, displayed in structure 12. Such sulfur−nitrogen bonded radical cations can be conveniently generated and monitored by pulse radiolysis coupled to time-resolved UV spectroscopy,[43] and their structures have been confirmed by ESR[44] and theoretical calculations.[44,45]



12

a : R = C(O)-Met

b : R = C(O)-Met-Ala

c : R = C(O)-Leu

The reaction of *N*-methionyl peptides with hydroxyl radicals, HO$^{\bullet}$, provides a convenient means for the generation of

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Table 7. Yields of Products and Loss of Substrate and during the Reaction of Peptides with Photolytically Produced Hydroxyl Radicals ($HO^{\bullet}$)[a]

		yield, 10^{-8} M s^{-1}	
peptide	compd	pH 6.5	pH 8.0
Met-Met	−[Met-Met]	14.3 ± 3.6	12.0 ± 1.0
	$AS_{MM}II$	6.1 ± 0.2	4.4 ± 0.5
	$AS_{MM}I$	2.4 ± 0.1	1.6 ± 0.2
	Met(O)-Met(O)	< 0.2	< 0.2
	Met(O)-Met	< 0.2	< 0.2
	Met-Met(O)	< 0.2	< 0.2
Met-Met-Ala	−[Met-Met-Ala]	17.0 ± 6.0	14.4 ± 3.5
	$AS_{MMA}II$	6.6 ± 0.4	5.7 ± 0.4
	$AS_{MMA}I$	2.8 ± 0.2	2.2 ± 0.2
	Met(O)-Met(O)-Ala	< 0.2	< 0.2
	Met(O)-Met-Ala	< 0.2	< 0.2
	Met-Met(O)-Ala	< 0.2	< 0.2
Met-Leu	−[Met-Leu]	15.2 ± 1.6	12.1 ± 1.0
	$AS_{ML}II$	4.5 ± 0.4	4.5 ± 0.2
	$AS_{ML}I$	2.2 ± 0.3	1.8 ± 0.1
	Met(O)-Leu	0.14 ± 0.02	0.1 ± 0.02

		ratio	
peptide	ratios	pH 6.5	pH 8.0
Met-Met	$[AS_{MM}II]/[AS_{MM}I]$	2.5	2.8
Met-Met-Ala	$[AS_{MMA}II]/[AS_{MMA}I]$	2.4	2.6
Met-Leu	$[AS_{ML}II]/[AS_{ML}I]$	2.0	2.5

[a] Conditions: photolysis (253.7 nm) of air-saturated aqueous solutions containing 1.0×10^{-4} M H_2O_2, 1.0×10^{-4} M peptide, and 5.0×10^{-5} M sodium phosphate.

structures **12** without the parallel formation of superoxide anion[34] to assess whether the reaction of **12a−c** with O_2 produces AS diastereomers. Hydroxyl radicals were generated by photolytic cleavage of hydrogen peroxide at 254 nm according to reaction 25.[46]

$$H_2O_2 + h\nu \rightarrow 2HO^{\bullet} \qquad (25)$$

In a standard experiment, air-saturated solutions contained 1×10^{-4} M H_2O_2, 1×10^{-4} M peptide, and 5×10^{-5} M sodium phosphate of desired pH. There was no pH shift during the experiment. Photolysis conditions were adjusted such as to convert less than 20% of the substrate in order to minimize reactions of hydroxyl radicals with primary oxidation products. All product yields are reported in Table 7. Control experiments confirmed that there was only negligible direct oxidation of the peptides (to sulfoxides) by the added hydrogen peroxide during the time of photolysis. Photolysis in the absence of H_2O_2 showed only trace amounts of oxidation products. Two characteristic features are apparent. (i) At both pH values, the yields of the AS diastereomers are significantly higher than those of the sulfoxides. Such a product pattern is similar to that found for the ^{3}CB system in the presence of SOD. (ii) The diastereomeric ratios [AS II]:[AS I] for all three peptides are close to the ratios of the ^{3}CB system in the presence of SOD. Thus, it appears that product formation in the hydroxyl radical system and in the ^{3}CB/SOD system involves similar pathways. When the photolysis was carried out in N_2-saturated solutions, peptide consumption was identical to that in air-saturated solutions but none of the oxidation products was formed, indicating that molecular oxygen is required for their formation. Representatively for Met-Leu at pH 6.5 (1×10^{-4} M Met-Leu, 1×10^{-4} M H_2O_2, 5×10^{-5} M sodium phosphate), we determined that hydrogen peroxide was consumed at a rate of $(7.24 \pm 0.7) \times 10^{-8}$ M s^{-1} (this particular experiment was carried out in N_2-saturated aqueous solution in order to avoid any hydrogen peroxide formation through secondary processes). As every photolytic conversion of H_2O_2 into free hydroxyl radicals yields 2 equiv of $HO^{\bullet}$, we expect a consumption of Met-Leu at a rate of 1.45×10^{-7} M s^{-1}, in good agreement with our experimental value of $(1.52 \pm 0.16) \times 10^{-7}$ M s^{-1}. Any potential reaction of $HO^{\bullet}$ with H_2O_2 (reaction 26) can be excluded for our experimental systems based on $k_{26} = 2.7 \times 10^7$ M^{-1} s^{-1} [47] as compared to $k_{27} = 1.2 \times 10^{10}$ M^{-1} s^{-1}.[34]

$$HO^{\bullet} + H_2O_2 \rightarrow H_2O + H^+ + O_2^{\bullet -} \qquad (26)$$

$$HO^{\bullet} + \text{Met-Met} \rightarrow \text{products} \qquad (27)$$

In control experiments we confirmed that the additional presence of 1×10^{-4} M H_2O_2 had no influence on the conversion of reactive intermediate(s) into $AS_{MM}I$ and $AS_{MM}II$, respectively. For this, 2×10^{-3} M Met-Met was oxidized by ^{3}CB (four 350-nm lamps, air-saturated solution, 2×10^{-4} M CB, 1×10^{-2} M sodium phosphate, pH 7.4) in the presence of no and $(0.1-1.0) \times 10^{-3}$ M H_2O_2. There was no change in the absolute yields of $AS_{MM}I$ and $AS_{MM}II$ as well as the diastereomeric ratio for $[H_2O_2] < 1 \times 10^{-3}$ M.

The oxidation products listed in Table 7 account for ca. 50−60% of lost substrate for Met-Met and Met-Met-Ala and ca. 50% of lost substrate for Met-Leu. Mechanistically, the formation of the AS diastereomers requires an initial one-electron oxidation of the peptides. However, it has been shown that at least 20% of hydroxyl radical attack at Met[48] and Met-containing peptides[34] proceeds via hydrogen transfer directly yielding α-(alkylthio)alkyl radicals so that only ca. 80% of the available hydroxyl radicals actually form one-electron oxidation products. Considering this additional pathway, the AS diastereomers account for 63−75% of the products derived from one-electron oxidation of Met-Met and Met-Met-Ala. Any reaction of $HO^{\bullet}$ with the Ala residue in Met-Met-Ala may be neglected based on the low rate constant for the reaction of $HO^{\bullet}$ with Ala ($k = 7.7 \times 10^7$ M^{-1} s^{-1} [47]) as compared to Met-Met ($k = 1.2 \times 10^{10}$ M^{-1} s^{-1} [34]). However, on the basis of the rate constants for the reaction of $HO^{\bullet}$ with Leu ($k = 1.7 \times 10^9$ M^{-1} s^{-1} [47]) and Met ($k = 8.3 \times 10^9$ M^{-1} s^{-1} [47]), we expect that in the case of Met-Leu 17% of the hydroxyl radicals directly react with the Leu residue. Of the remaining 83% reacting with the Met residue, a fraction of 0.2 will react via hydrogen transfer (see above). Thus, overall only 66% of the total amount of hydroxyl radicals will react with Met-Leu under formation of sulfide radical cations so that $AS_{ML}I$ and $AS_{ML}II$ represent 75% of the one-electron oxidation products of Met-Leu.

Discussion

Formation of the Intermediates. The primary steps of the reaction of ^{3}CB with organic sulfides[49] and Met-containing

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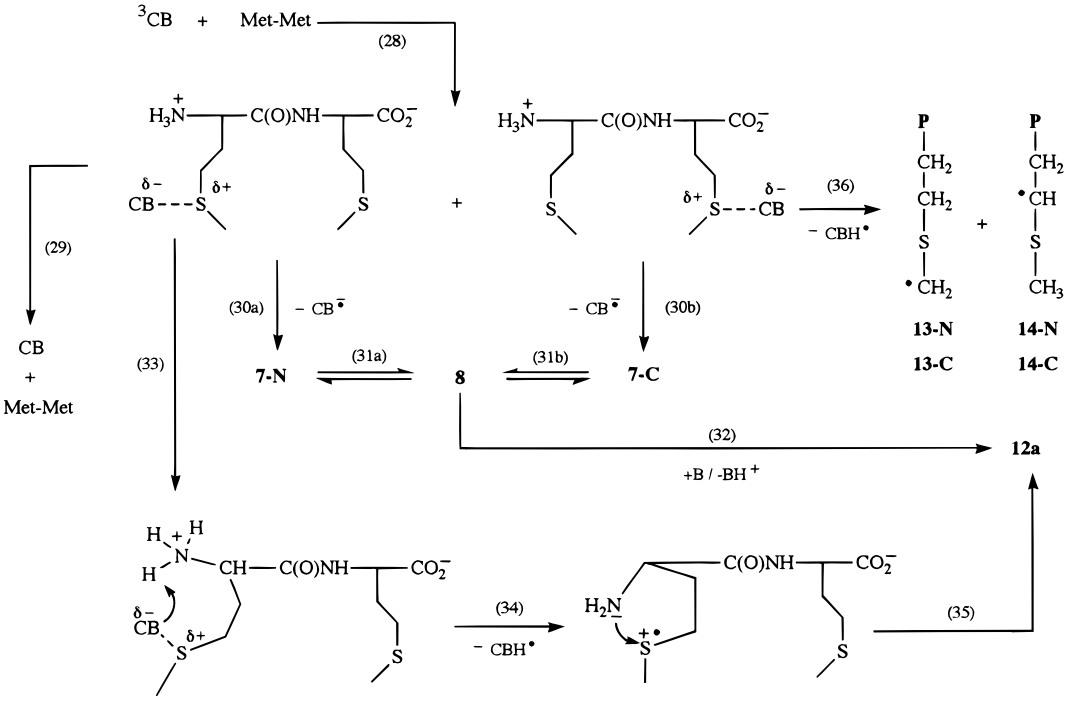
Scheme 2



peptides[32,50] have been established and are, representatively for Met-Met, displayed in Scheme 2. Initially, a charge-transfer complex forms (reaction 28) which decomposes either into ground-state CB and Met-Met (reaction 29; physical quenching) or via chemical quenching into various products (reactions 29−36). Electron-transfer yields $CB^{\bullet-}$ and the monomeric radical cations **7**-N and **7**-C which can subsequently form the sulfur−sulfur bonded complex **8**. Recently, it has been established with γ-hydroxyalkyl sulfides[42] (and a similar mechanism appears to operate with *N*-methionyl peptides[50]) that intramolecular proton transfer to the developing $CB^{\bullet-}$ from the charge-transfer complex can promote the one-electron transfer pathway as it directly leads to the neutral product $CBH^{\bullet}$ (a species less prone to back electron transfer within the charge-transfer complex or solvated ion pair as compared to $CB^{\bullet-}$). For Met-Met, a potential pathway is shown in reactions 33−35. An important feature of this reaction sequence is that it simultaneously produces a sulfur radical cation and a deprotonated N-terminal amino group which can rapidly cyclize to form the sulfur−nitrogen bonded intermediate **12a**. We note that reactions 34 and 35 do not necessarily have to occur stepwise but **12a** may form in a concerted process as well. Cyclization is not possible for structures **7**-N, **7**-C, or **8** unless base-catalyzed deprotonation of the N-terminus permits ring closure (reaction 32). In fact, on a longer time scale, reaction 32 is likely responsible for a conversion of **8** into **12a**. Finally, a fraction of the initial charge-transfer complex will also decompose via hydrogen transfer (reaction 36), yielding α-(alkylthio)alkyl radicals **13**-N/C and **14**-N/C, where the suffixes N and C symbolize that the radical center is located on the N- or C-terminal Met residue and P denotes the skeleton of the residual peptide molecule. It is important to note that in phosphate-buffered solution any proton transfer between $CB^{\bullet-}$ and **7**-N or **7**-C to yield **13**-N/C or **14**-N/C is precluded due to the rapid pH-dependent protonation of $CB^{\bullet-}$ by the buffer.[50,51] Usually, in oxygenated solutions, α-(alkylthio)alkyl radicals convert into peroxyl radicals and ultimately into thiols and aldehydes (S-dealkylation pathway). These intermediary peroxyl radicals may contribute to a small (SOD-*independent*) part of the observed monosulfoxides as peroxyl radicals are known to oxidize sulfides to sulfoxides.[52] However, for kinetic and mechanistic reasons (i.e., the effect of SOD), alkylperoxyl radicals are not involved in the formation of the AS diastereomers, Met(O)-Met(O), and the majority of the monosulfoxides (see also below). The fact that the yields of both thiols and formaldehyde are relatively low in the ^{3}CB system (cf. Table 2) indicates that the hydrogen transfer pathway plays a less important role than the electron-transfer pathway in Scheme 2. In general, chemical quenching of ^{3}CB by Met and Met-containing peptides (i.e., reactions 30, 33, and 36) occurs with quantum yields of $\Phi \approx 0.3-0.7$[32,50,51] depending on the structure with the residual fraction of ^{3}CB reacting via physical quenching (reaction 29). In summary, the electron-transfer pathway leads to the formation of sulfur−sulfur and sulfur−nitrogen bonded intermediates which are precursors for the sulfoxides and AS diastereomers, and the hydrogen transfer pathway leads to α-(alkylthio)alkyl radicals which mainly convert into thiols and aldehyde.

Laser flash experiments have confirmed and quantified the formation of **8** during the reaction of ^{3}CB with Met-Met at pH 5.9.[32] The published spectrum also suggests the formation of **12a** at pH 5.9, but this species was not quantified. As the formation of **12a** is likely paralleled by the formation of $CBH^{\bullet}$ [50] (reactions 33−35), an exact quantification of the electron-transfer and the hydrogen-transfer pathway based on the formation of $CB^{\bullet-}$ and $CBH^{\bullet}$, respectively, is not possible. That the direct formation of **12a** is possible was demonstrated by analogous laser flash experiments with Met-Gly and Met-Gly-Gly in the absence of buffer at pH 6.0 and 6.2, respectively, where the sulfur−nitrogen bonded intermediate $[>H_2N\therefore S<]^+$

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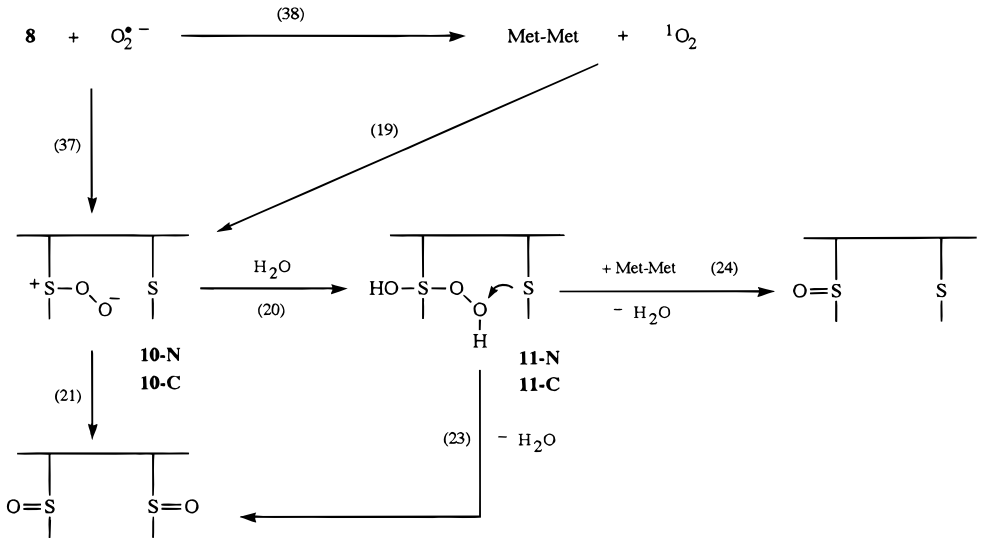
Scheme 3



was the predominant one-electron oxidation product paralleled by the formation of CBH$^{\bullet}$.[50]

The Mechanism of Met(O)-Met(O) Formation. The majority of Met(O)-Met(O) is formed through the reaction of **8** with superoxide. The underlying reactions are displayed in Scheme 3.

Essentially, **10**-N and **10**-C (where the suffices N and C represent a transformation at the N- or C-terminal Met, respectively) can be generated by a direct addition of $O_2^{\bullet-}$ to **8** (reaction 37) or through electron transfer (reaction 38), followed by addition of the product $^1O_2$ to Met-Met (reaction 19). Subsequently, Met(O)-Met(O) is formed through reactions 20, 21, and 23. The SOD-*dependent* yields of the monosulfoxides will form via reactions 37 and 24 whereas the SOD-*independent* yields are the result of peroxyl radical reactions (see above) and reactions 38, 19, 20, and 24. On the basis of the yields of Met(O)-Met(O) in H_2O and D_2O (see Table 7), we may conclude that reaction 38 is of minor importance as compared to the radical addition pathway (reaction 37). By comparison with the rose bengal system, we would have expected the yields of Met(O)-Met(O) to increase ca. 3-fold upon changing the solvent from H_2O to D_2O if singlet oxygen were the major oxidant leading to Met(O)-Met(O) even in the SOD-*dependent* reaction pathway. In reality, we only observe a ca. 18% increase of the Met(O)-Met(O) yields and most of it appears to originate from a higher efficiency of the SOD-*independent* pathway, i.e. from the reaction of singlet oxygen which had been generated by the reaction of ^{3}CB with oxygen.

Exclusion of a Direct Oxidation of Met-Met by Superoxide. A direct oxidation of Met-Met by superoxide (reaction 12) can be excluded on the basis of the experiments performed in the presence of formate. Formate scavenges ^{3}CB[17] to yield CBH$^{\bullet}$ and $^{\bullet}CO_2^-$ (reaction 39), both of which generate $O_2^{\bullet-}$ (reactions 5, 40, and 41; $k_{41} = 4 \times 10^9$ M^{-1} s^{-1} [53]).

$$^3\mathrm{CB} + \mathrm{HCO_2^-} \rightarrow \mathrm{CBH^{\bullet}} + {}^{\bullet}\mathrm{CO_2^-} \qquad (39)$$

$$\mathrm{CB^{\bullet-}/CBH^{\bullet}} + \mathrm{O_2} \rightarrow \mathrm{CB} + \mathrm{O_2^{\bullet-}/H^+},\ \mathrm{O_2^{\bullet-}} \qquad (5)$$

$${}^{\bullet}\mathrm{CO_2^-} + \mathrm{CB} + \mathrm{H^+} \rightarrow \mathrm{CBH^{\bullet}} + \mathrm{CO_2} \qquad (40)$$

$${}^{\bullet}\mathrm{CO_2^-} + \mathrm{O_2} \rightarrow \mathrm{CO_2} + \mathrm{O_2^{\bullet-}} \qquad (41)$$

On the basis of $k_{39} = 3.5 \times 10^7$ M^{-1} s^{-1} [17] and $k_{28} = 2.9 \times 10^9$ M^{-1} s^{-1},[32] we expect that the photoconversion of 2×10^{-3} M Met-Met by ^{3}CB should decrease by ca. 86% when additional 1.0 M formate is present. Experimentally, we find a decrease of 70%. However, every reaction of ^{3}CB with formate in oxygenated solution produces 2 equiv of superoxide per ^{3}CB whereas every chemical reaction of ^{3}CB with Met-Met generates only 1 equiv of superoxide per ^{3}CB (reactions 28−36, followed by reaction 5). Thus, in a first approximation, the formation rate of superoxide in the formate-containing solution (1.0 M formate, 2×10^{-3} M Met-Met, pH 10) should be at least 1.86 times higher as compared to the formate-deficient solution. The experimental fact that increased formation rates of superoxide are paralleled by a decreased formation rate of Met(O)-Met(O) clearly argues against a direct oxidation of Met-Met to Met-(O)-Met(O) by superoxide.

Formation of AS$_{MM}$I and AS$_{MM}$II. Several features are important for the formation of AS$_{MM}$I and AS$_{MM}$II. (i) An efficient reaction of superoxide with **8** at higher radical formation rates favors the formation of Met(O)-Met(O) at the expense of the AS diastereomers. However, at lower radical formation rates, higher yields of the AS diastereomers are formed at the expense of Met(O)-Met(O). (ii) In the presence of SOD, increased yields of the AS diastereomers are formed at the expense of Met(O)-Met(O). (iii) The AS products do not form through reaction of Met-Met with singlet oxygen, but they do form when the sulfur−nitrogen bonded intermediates **12** are generated in the presence of O_2, as shown in the hydroxyl radical system. The diastereomeric excess [AS$_{MM}$II]:[AS$_{MM}$I] is similar for the hydroxyl radical-system and the ^{3}CB system in the presence of SOD. However, it is different for the ^{3}CB system in the absence of SOD, suggesting the operation of a superoxide-*dependent* and a superoxide-*independent* pathway of AS diastereomer formation. All of these facts are accounted for by the general reactions displayed representatively for Met-Met in Scheme 4. The one-electron oxidation of Met-Met by ^{3}CB yields **8** and **12a**. The sulfur−sulfur bonded species **8** reacts with superoxide to form a precursor for the generation of Met(O)-Met(O), Met-Met(O), and Met(O)-Met (see Scheme 3). However, **8** can convert into **12a** after deprotonation of the N-terminal amino group (reaction 32; Scheme 2). Both pathways are competitive, and at a given pH and buffer

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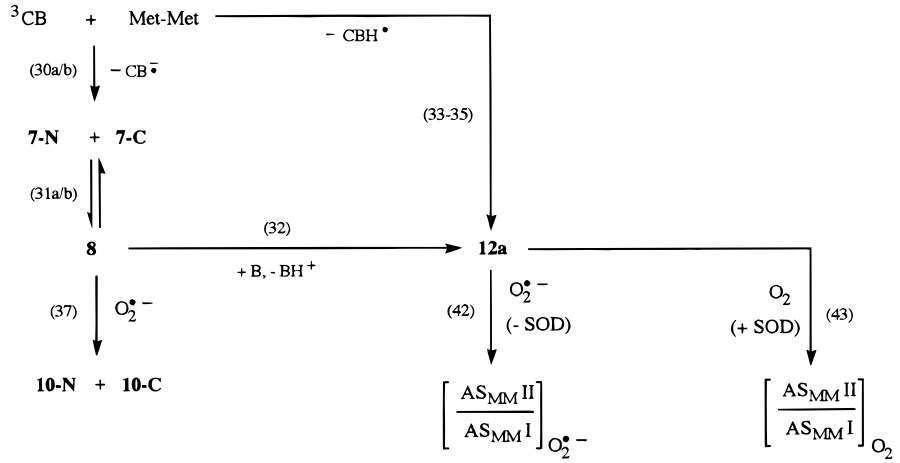
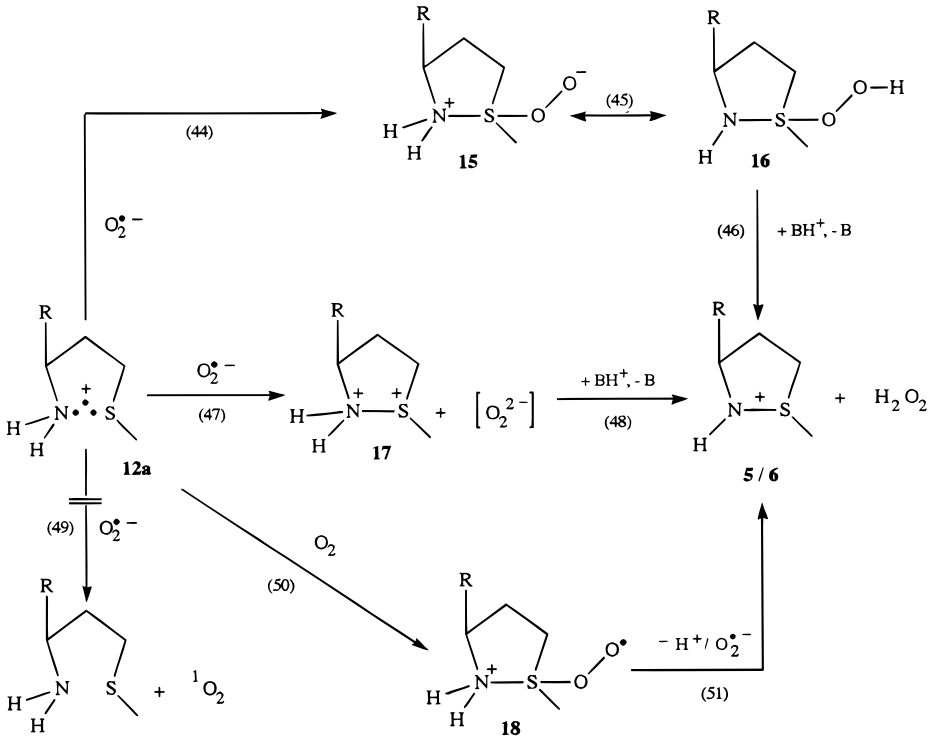
Scheme 4



Scheme 5



concentration, an increased concentration of primary photoproducts, i.e. **8** and $O_2^{\bullet-}$, would favor reaction 37 at the expense of reaction 32, rationalizing the higher efficiency of Met(O)-Met(O) formation at higher light intensities (higher production rates of radical intermediates). On the other hand, the elimination of superoxide by SOD inhibits reaction 37 which leads to a significant increase in the formation of products originating from species **12a**. Structure **12a** represents a precursor for the formation of $AS_{MM}I$ and $AS_{MM}II$ by reaction with $O_2^{\bullet-}$ or O_2, respectively (reactions 42 and 43). Preliminary pulse radiolysis experiments indicate that $k_{42} \approx 5 \times 10^9$ M^{-1} s^{-1} [54] and that k_{43} is on the order of 10^7 M^{-1} s^{-1}.[55]

There are several possibilities for a reaction of superoxide with **12a**, summarized in Scheme 5.

The addition of superoxide to **12a** would lead to the sulfurane intermediates **15** and **16** (reactions 44 and 45) which could eliminate hydrogen peroxide (reaction 46). In particular, structure **16** resembles a cyclic sulfurane intermediate which has been proposed for the oxidation of Met by I_2.[56] Alternatively, **12a** may transfer an electron to superoxide to generate the intermediary dication **17** (reaction 47), which subsequently deprotonates (reaction 48). We can exclude a possible reduction of **12a** by superoxide (reaction 49) as such reaction is expected to produce singlet oxygen which only yields negligible amounts of $AS_{MM}I$ and $AS_{MM}II$ in its reaction with Met-Met (cf. Table 5).

We propose that the reaction of **12a** with O_2 proceeds via addition, followed by elimination of superoxide (reactions 50 and 51; Scheme 5). The lifetime of a peroxyl radical **18** cannot be specified, i.e. it is unclear whether it would represent a relatively stable intermediate or only a transition state. However, earlier we had found kinetic evidence for a relatively stable analogous sulfur-based structure **19**,[57] suggesting that **18** might exist as an intermediate. We note that reaction 51 generates

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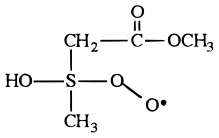

19

superoxide which may subsequently contribute to the formation of AS derivatives via reactions 44−48. However, as superoxide formation requires the initial reaction of **12a** with oxygen, this would be in support of reaction sequence 50−51, which is further supported by the fact that high yields of AS diastereomers are formed in the ^{3}CB/SOD system, i.e. in the absence of any superoxide available for reaction with **12a**.

It is interesting to note that the pH has only a negligible effect over a range which covers the pK_a of the N-terminal amino group of Met-Met (pK_a = 7.4[58]). This fact indicates that the radical cation complex **8** must live long enough to react with superoxide even in the presence of the free N-terminal amino group available for cyclization to **12a**. Supporting this hypothesis, significant yields of **8** were observed during laser photolysis of a CB/Met-Met system at pH 10.[32] Alternatively, it may be possible that initially higher yields of **15** or **16** are formed at higher pH but that a fraction of those species is converted to **11**-N by reaction with hydroxide (reaction 52).

$$\mathbf{15}/\mathbf{16} + HO^- \rightarrow \mathbf{11}\text{-N} \qquad (52)$$

It must be noted that, even under conditions where the N-terminal amino group is fully deprotonated, ^{3}CB reacts predominantly with the sulfide functions of Met-Met. For example, the rate constant for the reaction of ^{3}CB with a free amino group (of Ala) was determined as $k \approx 2 \times 10^8$ M^{-1} s^{-1} [59] whereas $k_{28} = 2.9 \times 10^9$ M^{-1} s^{-1}.[32]

The reaction of $^1O_2$ with Met-Met yielded almost exclusively Met(O)-Met(O), which is in interesting contrast to the reaction of $^1O_2$ with Met which, at pH 7−11, produced significant yields of AS_M.[60] For Met, an initially formed persulfoxide intermediate was proposed to undergo cyclization to AS_M with parallel elimination of hydrogen peroxide.[60] In Met-Met, such cyclization has to compete with intramolecular oxygen transfer to the second unoxidized Met residue, and the nearly exclusive formation of Met(O)-Met(O) suggests that the oxygen transfer process is significantly faster than the cyclization.

For a rationalization of the diastereoselectivity in the formation of the AS diastereomers, several facts need to be considered. An inherent higher stability of AS_{MM}II over AS_{MM}I may be discarded as a rationale: The oxidation of all *N*-methionyl peptides and L-Met by I_3^- generates both diastereomers in a 1:1 ratio. Therefore, it appears more likely that not the stability per se but the mechanism of formation, i.e. one-electron oxidation of Met-Met followed by the reaction with molecular oxygen or superoxide, has some influence on the extent of diastereoselectivity.

A comparison of the diastereomeric ratios [AS II]:[AS I] of Met-Met, Met-Met-Ala, and Met-Leu shows a significantly higher excess of AS II over AS I for the peptides containing the Met-Met sequence in the absence but not the presence of SOD. Thus, the propensity to form intramolecular sulfur−sulfur bonded complexes may play an important role for product formation and diastereoselectivity particularly when the radical cationic intermediates react with superoxide. At present we cannot specify the exact nature of the parameters contributing to the observed diastereoselectivity. However, it is important to realize that intramolecular complex formation of radical intermediates in peptides can determine the nature and configuration of the ultimate molecular products. The efficient formation of Met(O)-Met(O) through the reaction of sulfur−sulfur bonded radical cation complexes with superoxide is particularly interesting with regard to our observations with the protein calmodulin. Calmodulin isolated from the brains of aged rats contains high levels of Met sulfoxide.[61] Considering the spatial proximity of some of the Met residues in calmodulin, mechanisms such as that characterized in this paper may contribute to the accumulation of age-related modifications of Met in vivo, particulary as superoxide levels are elevated in aged tissue.[62]

Experimental Section

See Supporting Information.

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Supporting Information Available: Experimental section, quantum mechanical calculations, and Figures 1S and 2S with legends (17 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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