Oxidation of Threonylmethionine by Peroxynitrite. Quantification of the One-Electron Transfer Pathway by Comparison to One-Electron Photooxidation

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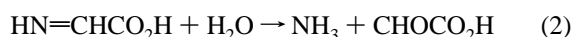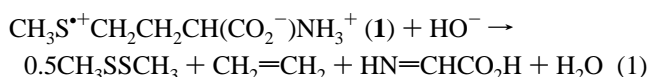
Abstract: Peroxynitrite can modify methionine by one- and two-electron oxidation pathways. Here, we have quantified the extent of one-electron oxidation of threonylmethionine (Thr-Met) by peroxynitrite using a characteristic reaction according to which Thr-Met sulfur radical cations decompose via fragmentation of the Thr side chain, yielding acetaldehyde. The efficiencies, $f_{acet,photo}$, for the formation of acetaldehyde from Thr-Met sulfur radical cations were obtained by means of one-electron photooxidation using triplet 4-carboxybenzophenone. Exact quantum yields for the formation of Thr-Met sulfur radical cations by triplet 4-carboxybenzophenone were obtained by laser flash photolysis and time-resolved UV spectroscopy. Acetaldehyde yields were measured for the reaction of peroxynitrite with Thr-Met, and division of these acetaldehyde yields by $f_{acet,photo}$ yielded the extents to which peroxynitrite reacted with Thr-Met via the one-electron transfer pathway. There was little one-electron oxidation of Thr-Met by peroxynitrite at pH 7.4, i.e., 1.5%, 1.8%, and 5.3% based on the total chemical conversion of Thr-Met for Thr-Met concentrations of 1×10^{-3}, 5×10^{-4}, and 1.75×10^{-4} M, respectively. In all cases the major reaction product was the two-electron oxidation product threonylmethionine sulfoxide. However, at pH 6.0, one-electron oxidation of Thr-Met showed a significantly higher efficiency of 14% for [Thr-Met] = 1.75×10^{-4} M. Under all experimental conditions the extent of one-electron oxidation increased with decreasing peptide concentration in agreement with a recently established mechanism according to which the one-electron oxidation of Met by peroxynitrite requires a unimolecular transformation of peroxynitrous acid to an excited species which is the ultimate one-electron oxidant.
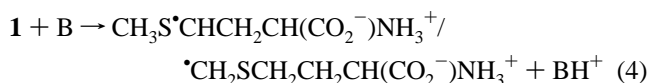
Introduction

Peroxynitrite, $ONOO^-$, is a biologically important oxidant,[1] generated through the reaction of nitric oxide, NO, with superoxide, $O_2^{\bullet-}$,[2,3] in tissues exposed to oxidative stress[4] where peroxynitrite can modify tyrosine,[5] tryptophan,[6] cysteine,[7] and methionine (Met)[8,9] residues. Through Met oxidation, peroxynitrite inhibits the α-1-proteinase inhibitor[8] and calmodulin stimulation of neuronal NO synthase.[10] Recently, Pryor et al. reported that peroxynitrite can modify Met via a one-electron (1e) and a two-electron (2e) pathway, yielding ethylene and methionine sulfoxide, respectively.[9] It was proposed that the

1e pathway generated an intermediary methionine sulfide radical cation, $MetS^{\bullet+}$ (**1**), decomposing via reactions 1 and 2.

$$CH_3S^{\bullet+}CH_2CH_2CH(CO_2{}^-)NH_3{}^+ \ (\textbf{1}) + HO^- \rightarrow$$
$$0.5CH_3SSCH_3 + CH_2{=}CH_2 + HN{=}CHCO_2H + H_2O \quad (1)$$

$$HN{=}CHCO_2H + H_2O \rightarrow NH_3 + CHOCO_2H \quad (2)$$

However, on the basis of the established chemistry for **1**, we do not expect reaction 1 to be very efficient. For example, when **1** was generated at neutral pH by pulse radiolysis, 51% of **1** underwent direct decarboxylation (reaction 3), yielding 1-amino-3-(methylthio)prop-1-yl radicals **2**.[11] In complementary experiments **1** was generated by 1e photooxidation of Met by triplet 4-carboxybenzophenone, $^3CB^*$, where 60% of **1** underwent direct decarboxylation.[12] These respective efficiencies of 50−60% decarboxylation do not necessarily mean that the residual fractions of **1** directly yield ethylene since other competing pathways such as deprotonation in the α-position to the sulfur (reaction 4) must also be considered.

$$\textbf{1} \rightarrow CO_2 + H^+ + CH_3SCH_2CH_2C^{\bullet}HNH_2 \ (\textbf{2}) \quad (3)$$

$$\textbf{1} + B \rightarrow CH_3S^{\bullet}CHCH_2CH(CO_2{}^-)NH_3{}^+/$$
$$^{\bullet}CH_2SCH_2CH_2CH(CO_2{}^-)NH_3{}^+ + BH^+ \quad (4)$$

Thus, any quantification of the 1e pathway between peroxynitrite and Met must take into account all of these competing

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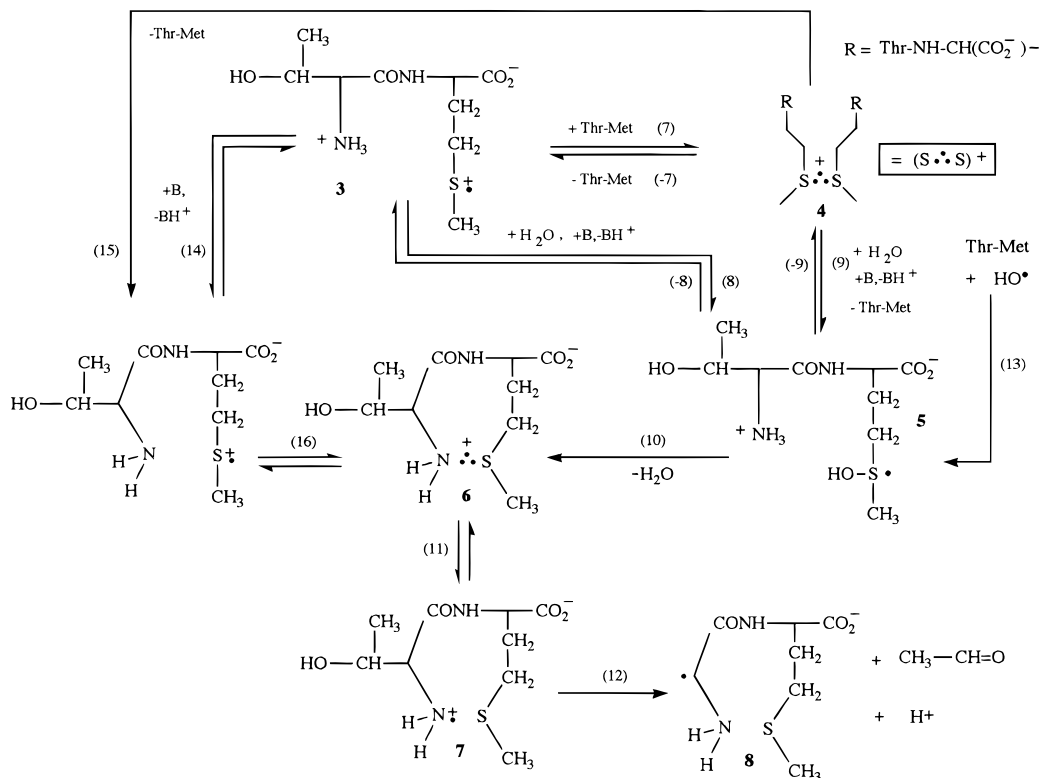(8) Moreno, J. J.; Pryor, W. A. *Chem. Res. Toxicol.* **1992,** *5,* 425.
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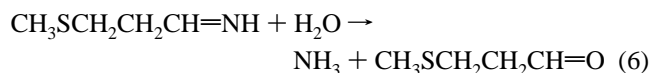(11) Hiller, K.-O.; Asmus, K.-D. *Int. J. Radiat. Biol.* **1981,** *40,* 583.
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Scheme 1

pathways. Another complication arises from the fact that **2** reduces O_2[13] $[E°(O_2/O_2^{•-}) = -0.16$ V[14]], ultimately yielding 3-(methylthio)propionaldehyde (methional) via reactions 5 and 6. In fact, methional was detected as a major product subsequent to the 1e oxidation of Met by $^3CB*$.[15]

$$2 + O_2 \rightarrow O_2^{•-} + H^+ + CH_3SCH_2CH_2CH=NH \quad (5)$$

$$CH_3SCH_2CH_2CH=NH + H_2O \rightarrow$$
$$NH_3 + CH_3SCH_2CH_2CH=O \quad (6)$$

Methional itself has been shown to yield ethylene as a result of oxidation by a variety of oxidants.[16] Thus, one possible route to ethylene in the peroxynitrite/Met system would be the conversion of Met to methional, followed by a conversion of methional to ethylene, i.e., a multistep mechanism.

In this paper we report a chemical system which permits the direct quantification of 1e oxidation of a Met residue in a model peptide by peroxynitrite, based on a previously established mechanism by which sulfide radical cations from Thr-Met, TM-$(S^{•+})$ (**3**), specifically generated by reaction of Thr-Met with $SO_4^{•-}$, intramolecularly form acetaldehyde via Thr side chain cleavage, displayed in Scheme 1 (reactions 7−16).[17]

The underlying mechanism, displayed in Scheme 1, is primarily based on the reaction of water and a base with TM-$(S^{•+})$ (**3**) or its three-electron-bonded dimeric sulfide radical cation $(S\therefore S)^+$ (**4**). In one potential pathway the addition of water to **3** or **4** yields the hydrated sulfur radical cations ($>S^{•+}\cdots OH_2$ from **3** or $[(S\therefore S)OH_2]^+$ from **4**) which subsequently transfer a proton to a base, generating the hydroxysul-

furanyl radical TM(S•OH) (**5**) (reactions 8 and 9). Species **5** undergoes a coupled proton/electron transfer with the protonated N-terminal amino group of Thr-Met (reaction 10; $k_{10} > 2.2 \times 10^6$ s^{-1} [17]) yielding the three-electron $S\therefore N$-bonded intermediate **6**. This pathway has been characterized by pulse radiolysis coupled to time-resolved UV spectroscopy where **5** was generated specifically by the reaction of Thr-Met with hydroxyl radicals (reaction 13).[17] Species **6** exists in equilibrium 11 with the N-centered radical cation **7** which suffers heterolytic cleavage of the C_α−C_β bond of the Thr residue (reaction 12), yielding protonated acetaldehyde and the capto-dative stabilized radical **8**. The latter has been detected by time-resolved EPR spectroscopy.[17] Mechanistically, it is important to note that the radical cation **3** itself does not directly react with the protonated N-terminal amino group (p$K \approx 8.2$[18]). An alternative pathway for the formation of **6** would require proton transfer to a base from the protonated N-terminal amino group of **3** (reaction 14) or **4** (reaction 15) to allow bond formation between the radical cationic sulfur center and the electron lone pair of the deprotonated N-terminal amino group (reaction 16). The base required for the proton transfer reactions in both pathways will essentially be water and, for more alkaline pH values, hydroxide ion.

In this paper we have measured the efficiency of acetaldehyde formation from **3** by means of 1e photooxidation experiments with $^3CB*$ and used this efficiency to quantify the extent of the 1e pathway for the reaction of peroxynitrite with the thioether moiety of Thr-Met. All necessary information about quantum yields for the formation of **3** during the reaction of $^3CB*$ with Thr-Met was obtained by complementary time-resolved laser photolysis experiments. These experiments with Thr-Met do not only provide an alternative method of quantitation of the 1e oxidation but also an example for 1e oxidation of a Met residue embedded in a peptide for comparison to that of the free amino acid Met. A quantitative understanding of the 1e

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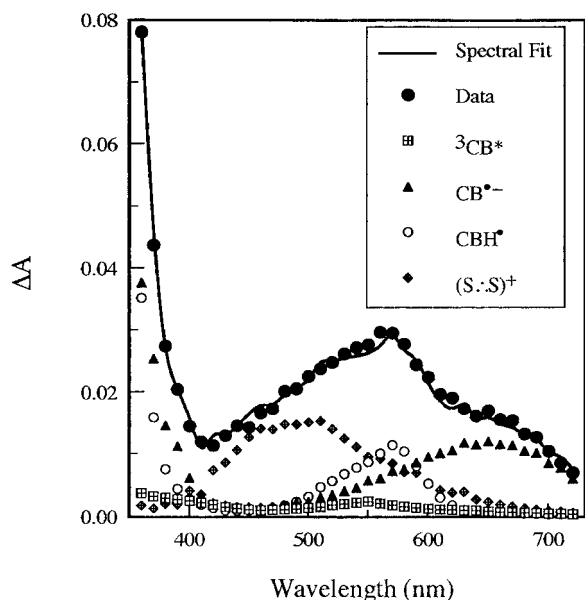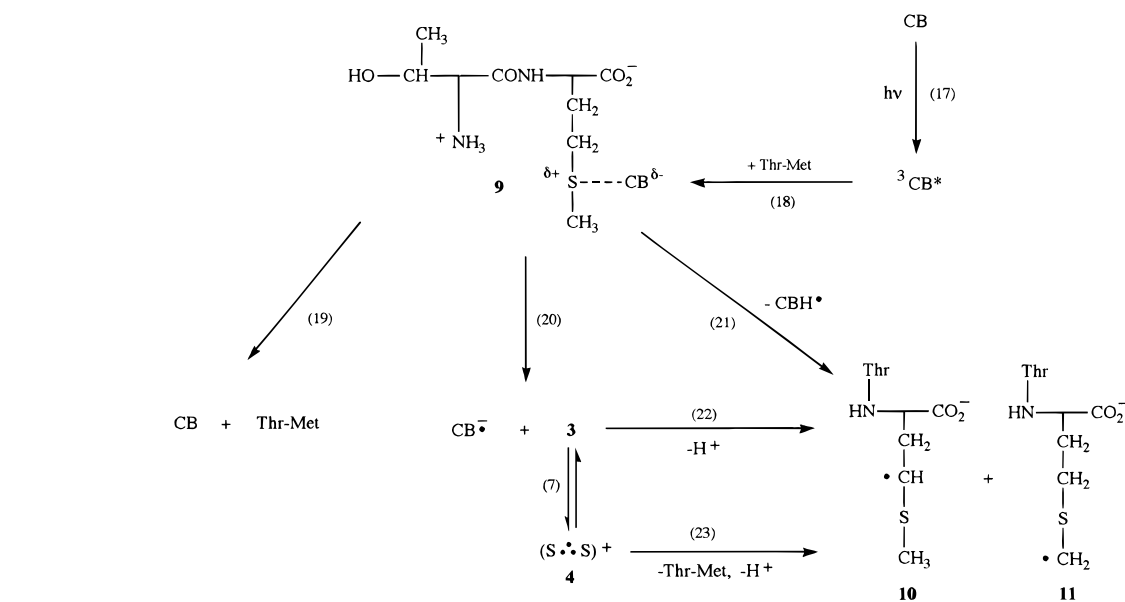
Scheme 2

Figure 1. Absorption spectra recorded 200 ns after laser flash photolysis of an Ar-saturated aqueous solution containing 2×10^{-3} M CB, 1×10^{-2} M Thr-Met, and 2×10^{-2} M sodium phosphate, pH 7.43. The closed circles represent the experimental spectrum. The experimental spectrum was deconvoluted into the individual spectra of its components, ^{3}CB*, CB$^{•-}$, CBH$^•$, and $(S\therefore S)^+$, and a computer fit of the composite spectrum (solid line) obtained by summation of the absorbances of the individual components at their respective concentrations.

oxidation by peroxynitrite is important in view of its biological relevance: in tissue protein methionine sulfoxide residues can be enzymatically reduced to Met by methionine sulfoxide reductase[19] whereas 1e oxidation often leads to an irreversible decomposition of Met residues.

Results

Photooxidation of Thr-Met. Laser Photolysis. Figure 1, closed circles, shows the optical spectrum recorded at 200 ns after the laser flash photolysis of an Ar-saturated aqueous solution containing 2×10^{-2} M phosphate buffer, pH 7.43, 2×10^{-3} M CB, and 1×10^{-2} M Thr-Met. It is the composite

spectrum of all transients in solution at this specific time, generated via reactions 17–21, displayed in Scheme 2.

The initial product immediately after (and during) the laser pulse is ^{3}CB* (reaction 17) which rapidly reacts with Thr-Met by formation of a charge transfer complex **9** with the sulfide moiety of Thr-Met (reaction 18; $k_{18} \approx 2 \times 10^9$ M^{-1} s^{-1} [20]). The initial yields of ^{3}CB* after the laser flash were independently determined in aqueous solution, pH 11.5, as 1.98×10^{-5} M, taking $\lambda_{max}(^3CB^*) = 535$ nm and $\epsilon_{535} = 6250$ M^{-1} cm^{-1} [21–23]. For 1×10^{-2} M Thr-Met, reaction 18 is nearly complete at 200 ns after the pulse (see below) so that there are only minor residual amounts of ^{3}CB* in the solution at this time which will not contribute significantly to the composite spectrum. Generally, radical cation **3** exists in equilibrium 7 with $(S\therefore S)^+$ (**4**) where equilibrium 7 will be located virtually completely on the side of the dimer at peptide concentrations of 10^{-2} M.[24] For Thr-Met $(S\therefore S)^+$ is characterized by an optical absorption with $\lambda_{max} = 480$ nm.[17] The original value of $\epsilon_{480} = 6540$ M^{-1} cm^{-1} [17] has recently been corrected to $\epsilon = 8690$ M^{-1} cm^{-1} [25] which was taken as a reference value in this paper. The spectral characteristics of the other reaction products in solution are well established; i.e., for the ketyl radical anion (CB$^{•-}$) $\lambda_{max} = 660$ nm and $\epsilon_{660} = 7660$ M^{-1} cm^{-1},[21,22] for the ketyl radical (CBH$^•$) $\lambda_{max} = 570$ nm and $\epsilon_{570} = 5200$ M^{-1} cm^{-1},[21,22] and for species **10** and **11** $\lambda_{max} \approx 280$ nm and $\epsilon_{280} \approx (3000 \pm 600)$ M^{-1} cm^{-1}.[26] Thus, at 200 ns after the laser flash the composite spectrum in the range between 360 and 720 nm essentially contains contributions from CB$^{•-}$, CBH$^•$, and $(S\therefore S)^+$. On the basis of their known individual spectra and extinction coefficients, the composite spectrum can be resolved into the spectra of the individual components by a linear regression technique[27] of the form of eq I where $\Delta A(\lambda_j)$ is the observed absorbance

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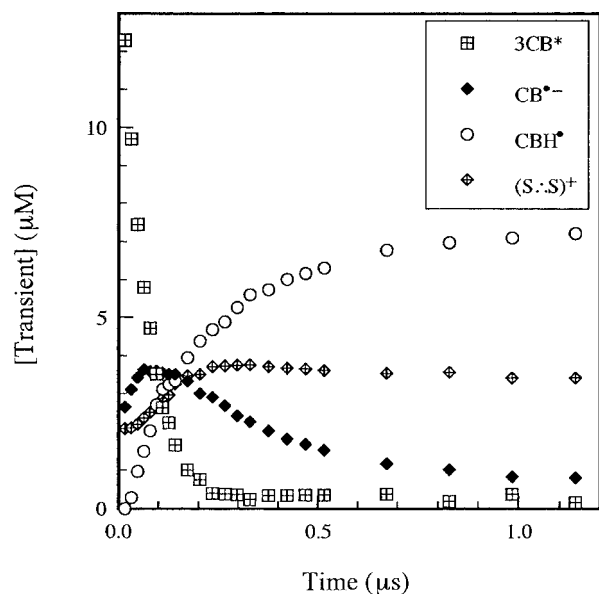
Figure 2. Concentration vs time profiles for intermediates obtained after laser flash photolysis of an Ar-saturated aqueous solution, containing 2×10^{-3} M CB, 1×10^{-2} M Thr-Met, and 2×10^{-2} M sodium phosphate, pH 7.43.

change of the composite spectrum, and $\epsilon_i(\lambda_j)$ is the molar extinction coefficient of the ith species at the jth wavelength of observation.

$$\Delta A(\lambda_j) = \sum_{i=1}^{n} \epsilon_i(\lambda_j) a_i \qquad (I)$$

The linear regression coefficients correspond to $c_i l$ where c_i is the concentration of the ith transient and l is the optical path length of the monitoring light. Further details of this method have been described elsewhere.[28] Figure 1 displays a quantitative calculation of the spectra of the individual components contributing to the composite spectrum (closed circles), and the solid line represents a simulated composite spectrum based on the calculated concentrations of the individual components. By application of this linear regression technique to composite spectra recorded at various times after the laser flash, the concentration vs time profiles of the individual components can be monitored, as displayed in Figure 2 for an Ar-saturated aqueous solution containing 2×10^{-2} M phosphate buffer, pH 7.43, 2×10^{-3} M CB, and 1×10^{-2} M Thr-Met.

Within 300 ns after the laser flash there is a complete decay of ^{3}CB* paralleled by the formation of $(S\therefore S)^+$, $CB^{\bullet-}$, and $CBH^{\bullet}$. The dimeric sulfide radical cation $(S\therefore S)^+$ remains rather stable over a time period of 1 μs. However, the formation of $CB^{\bullet-}$ is superimposed by a subsequent decay, paralleled by the formation of additional yields of $CBH^{\bullet}$. This process reflects the protonation equilibrium 24 ($pK_{24} = 8.2^{21}$) which is nearly completely established at ca. 1.1 μs after the laser flash.

$$CBH^{\bullet} \rightleftharpoons CB^{\bullet-} + H^+ \qquad (24)$$

On the basis of $pK_{24} = 8.2$, we expect that at pH 7.43 ($[CB^{\bullet-}]/[CBH^{\bullet}])_{\text{theor}} = 0.17$, in reasonable agreement with our experimental value of $([CB^{\bullet-}]/[CBH^{\bullet}])_{\text{exp}} = 0.14$ at 1.1 μs after the laser flash. The combined yields of $CB^{\bullet-}$ and $CBH^{\bullet}$ at 1.1 μs after the laser flash are representative of the fraction of ^{3}CB* which reacts via chemical quenching (reactions 20 and 21) with

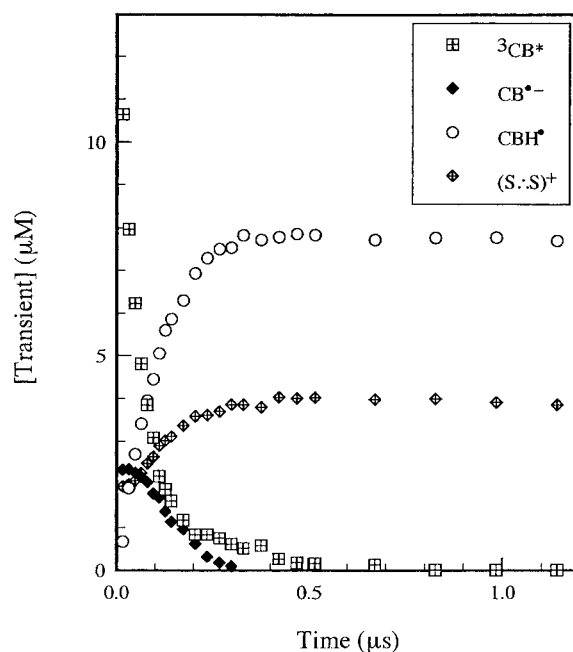(28) Marciniak, B.; Bobrowski, K.; Hug, G. L. *J. Phys. Chem.* **1993**, *97*, 11937.

Figure 3. Concentration vs time profiles for intermediates obtained after laser flash photolysis of an Ar-saturated aqueous solution, containing 2×10^{-3} M CB, 1×10^{-2} M Thr-Met, and 2×10^{-2} M sodium phosphate, pH 5.96.

Thr-Met. Photochemical quantum yields can be calculated as $\Phi(\text{product}) = [\text{product}]/[^3\text{CB*}]_{\text{initial}}$ where $[^3\text{CB*}]_{\text{initial}} = 1.98 \times 10^{-5}$ M (see above). We derive $\Phi(CB^{\bullet-} + CBH^{\bullet}) = 0.4$, indicating that 40% of the reaction of ^{3}CB* with Thr-Met proceeds via reactions 20 and 21, and 60% afford physical quenching (reaction 19), yielding ground state CB. An inspection of the first 200 ns of the concentration vs time profile reveals a 1:1 stoichiometry of $CB^{\bullet-}$ and $(S\therefore S)^+$, reflecting the 1e oxidation of Thr-Met according to reaction 20. The quantum yield for $(S\therefore S)^+$ amounts to $\Phi[(S\therefore S)^+] = 0.20$, indicating that 50% of the chemical quenching is accounted for by electron transfer (reaction 20). The quantum yield for the hydrogen transfer reaction 21 is then calculated as $\Phi(CBH^{\bullet}) = \Phi(CB^{\bullet-} + CBH^{\bullet}) - \Phi[(S\therefore S)^+] = 0.20$. Figure 3 displays a concentration vs time profile for all transients at pH 5.96. At this pH the protonation of $CB^{\bullet-}$ is faster and equilibrium 24 is nearly completely located on the left hand side. The photochemical quantum yields at pH 5.96 are $\Phi(CB^{\bullet-} + CBH^{\bullet}) = 0.40$, $\Phi(CBH^{\bullet}) = 0.20$, and $\Phi[(S\therefore S)^+] = 0.20$.

An important detail of Figures 2 and 3 is that the protonation of $CB^{\bullet-}$ does not afford deprotonation of $(S\therefore S)^+$ (cf. reactions 22 and 23; Scheme 2) but rather involves proton transfer from the solvent or the buffer.

Steady-State Photolysis. Steady-state photolysis experiments in N_2-saturated aqueous solutions containing 2.5×10^{-4} M CB, 2×10^{-2} M phosphate buffer, and various concentrations of Thr-Met were carried out to determine the efficiency of photochemical acetaldehyde formation, $f_{\text{acet,photo}}$, per equivalent of initial radical cation **3**. The laser photolysis experiments had shown that chemical quenching of ^{3}CB* by Thr-Met in phosphate buffer proceeds via hydrogen transfer (50%) and electron transfer (50%). Under N_2 each electron and hydrogen transfer process is associated with the formation of 1 equiv of $CB^{\bullet-}/CBH^{\bullet}$ which, at pH < 8, reacts bimolecularly to yield 1,2-dihydroxy-1,2-bis(4′-carboxyphenyl)-1,2-diphenylethane. Of this loss of CB (ΔCB), 50% is due to the electron transfer reaction 20. The yields of acetaldehyde and ΔCB were measured for various concentrations of Thr-Met at pH 6.0 and

Table 1. Product Yields from the Oxidation of Thr-Met by ^{3}CB* and Peroxynitrite[a]

	conditions			^{3}CB* + Thr-Met	peroxynitrite (ONOO$^-$) + Thr-Met[c]					
entry	[Thr-Met], 10^{-3} M	pH	atmosphere	$f_{acet,photo}$[b]	[ONOO$^-$], 10^{-4} M	$f_{acet,PN}$	$f_{Thr-Met(O),PN}$	$f_{NH3,PN}$	$\Sigma_{prod,PN}$[d]	$f_{1e,PN}$[e]
1	1.0	7.4	N$_2$	0.78	5.0	0.012	0.96	0.028	1.0	0.015
2	0.5	7.4	N$_2$	0.80	2.55	0.014	0.72	0.033	0.77	0.018
3	0.175	7.4	N$_2$	0.58	0.875	0.031	0.44	0.023	0.50	0.053
4	1.0	7.4	air	f	5.0	0.014	0.73	0.06	0.81	0.018
5	0.5	7.4	air	f	2.5	0.028	0.51	0.07	0.61	0.035
6	0.175	7.4	air	f	0.875	0.035	0.35	0.11	0.50	0.060
7	1.0	7.4	2.5 × 10^{-2} M HCO$_3^-$, air	f	5.0	0.050	0.12	0.02	0.19	0.064
8	1.0	6.0	N$_2$	0.18	g	g	g	g	g	g
9	0.5	6.0	N$_2$	0.17	g	g	g	g	g	g
10	0.175	6.0	N$_2$	0.17	g	g	g	g	g	g
11	1.0	6.0	air	f	5.0	0.008	0.76	g	g	0.044
12	0.5	6.0	air	f	2.5	0.015	0.68	g	g	0.088
13	0.175	6.0	air	f	0.875	0.024	0.50	g	g	0.140

[a] All yields are reported with an error limit of ±20%. [b] $f_{acet,photo}$ = [acetaldehyde]/[TM(S$^{\bullet+}$)] = [acetaldehyde]/0.5ΔCB. [c] $f_{product/PN}$ = [product]/[loss of TM]. [d] $\Sigma_{prod,PN} = f_{acet,PN} + f_{Thr-Met(O),PN} + f_{NH3,PN}$. [e] $f_{1e,PN} = f_{acet,PN}/f_{acet,photo}$. [f] Not determined because of competitive reaction ^{3}CB* + O$_2 \rightarrow$ CB + ^{1}O$_2$. [g] Not determined.

7.4, respectively, and $f_{acet,photo}$ calculated according to eq II. The results for $f_{acet,photo}$ are displayed in Table 1 (column 5).

$$f_{acet,photo} = \frac{[\text{acetaldehyde}]}{0.5[\Delta\text{CB}]} \qquad (\text{II})$$

As expected, $f_{acet,photo}$ is higher at pH 7.4 due to a more efficient formation of **6** at higher pH. In control experiments we photooxidized mixtures of 5 × 10^{-4} M Thr-Leu and 5 × 10^{-4} M Gly-Met under conditions similar to those of Thr-Met. They showed no significant yields of acetaldehyde at both pH 6.0 and 7.4, respectively. The design of these control experiments should be rationalized briefly. Generally, ^{3}CB* reacts ca. 10 times faster ($k \approx 2 \times 10^9$ M^{-1} s^{-1} [20]) with the sulfide moiety of Met as compared to a deprotonated N-terminal amino group (e.g., of Ala at alkaline pH, $k \approx 2 \times 10^8$ M^{-1} s^{-1} [29]). One apparently obvious control experiment for the importance of Met oxidation in Thr-Met for intramolecular acetaldehyde formation would, therefore, be the photooxidation of Thr-Leu. However, at pH 7.44 ca. 15% of Thr-Leu (p$K_a \approx 8.2$[18]) will exist in the N-terminal deprotonated form. In the absence of any sulfide function, electron transfer between ^{3}CB* and the fraction of the N-terminal deprotonated amino group would become a possibility, directly yielding the N-centered radical cation **7** (see Scheme 1), an efficient precursor for acetaldehyde. In a 1:1 mixture of Thr-Leu and Gly-Met at pH 6.0 or 7.4, ^{3}CB* will predominantly react with the sulfide moiety of Gly-Met with ca. 60% of this chemical quenching, yielding Gly-Met(S$^{\bullet+}$).[20] The fact that no acetaldehyde was formed in such systems shows (i) that intermolecular electron transfer between Gly-Met(S$^{\bullet+}$) and Thr-Leu does not occur, suggesting that intermolecular electron transfer between Thr-Met(S$^{\bullet+}$) and the N-terminal amino group of a second Thr-Met molecule is unlikely, (ii) that acetaldehyde formation from X-Met(S$^{\bullet+}$) requires X = Thr, and (iii) that a direct reaction of ^{3}CB* with the fraction of the deprotonated N-terminus of Thr-Met at both pH 6.0 and 7.4 can be considered negligible.

Oxidation of Thr-Met by Peroxynitrite. Stopped-Flow Experiments. By stopped-flow rapid scan UV spectroscopy we measured the decomposition kinetics of the peroxynitrite anion (ONOO$^-$; λ_{max} = 302 nm[30]) at pH 7.4 (25 °C) in the absence and presence of various concentrations of Thr-Met and, for comparison, Gly-Met. Some details are important for the

derivation of the mathematical expressions for k_{obs}. In alkaline aqueous solution, peroxynitrite exists predominantly as *cis*-ONOO$^-$, as concluded from Raman spectroscopy[31] and ^{15}N-NMR.[32] Peroxynitrite anion is quite stable toward decomposition into nitrate with $k_{obs} \approx 0.01$ s^{-1} for pH 8.6 at 25 °C[1] (via protonation of peroxynitrite to peroxynitrous acid, ONOOH). Peroxynitrous acid converts rapidly into nitrate with $k = 1.3$ s^{-1} at pH < 6.0 and 25 °C.[1] Theoretical calculations[32] predict an equilibrium between *cis*-ONOO$^-$ and the *cis,cis* isomer of ONOOH (reaction 25) with pK_{25} = 6.8, and the pH−rate profile for the unimolecular decomposition of peroxynitrite showed a sigmoidal behavior with pK_a at 6.8,[1,9b] indicating that ONOOH is an important intermediate in the overall conversion of *cis*-ONOO$^-$ to NO$_3^-$ (reactions 25 and 26).

$$cis,cis\text{-ONOOH} \rightleftharpoons cis\text{-ONOO}^- + \text{H}^+ \qquad (25)$$

$$cis,cis\text{-ONOOH} \longrightarrow \text{NO}_3^- + \text{H}^+ \qquad (26)$$

We note that, though pK_a = 6.8 appears to be characteristic for the protonation of *cis*-ONOO$^-$ to *cis,cis*-ONOOH[32] (reaction 25), it is difficult to experimentally measure the preferred solution conformation of ONOOH. In fact, additional conformers of ONOOH might form in the course of the isomerization to nitrate (reaction 26) such as *trans-perp*-ONOOH[32] and/or several possible excited states (see the Discussion).

In general, ONOOH is a much better oxidant as compared to peroxynitrite anion,[1] and earlier results with Met[9] suggest that any oxidation of Met by ONOO$^-$ may be neglected at pH ≤ 7.4 due to the fast equilibrium 25 (i.e., for ONOO$^-$ + Met, $k = 0.2$ M^{-1} s^{-1}, and for ONOOH + Met, $k = 2060$ M^{-1} s^{-1} [33]). Thus, we expect that the oxidation of Thr-Met and Gly-Met at pH 7.4 exclusively involves ONOOH. As will be discussed in detail below, ONOOH can directly react with the X-Met peptides (reaction 27; X = Thr or Gly), yielding the respective

$$\text{ONOOH} + \text{X-Met} \rightarrow \text{X-Met(O)} + \text{H}^+ + \text{NO}_2^- \qquad (27)$$

sulfoxides, X-Met(O), or unimolecularly convert into an excited

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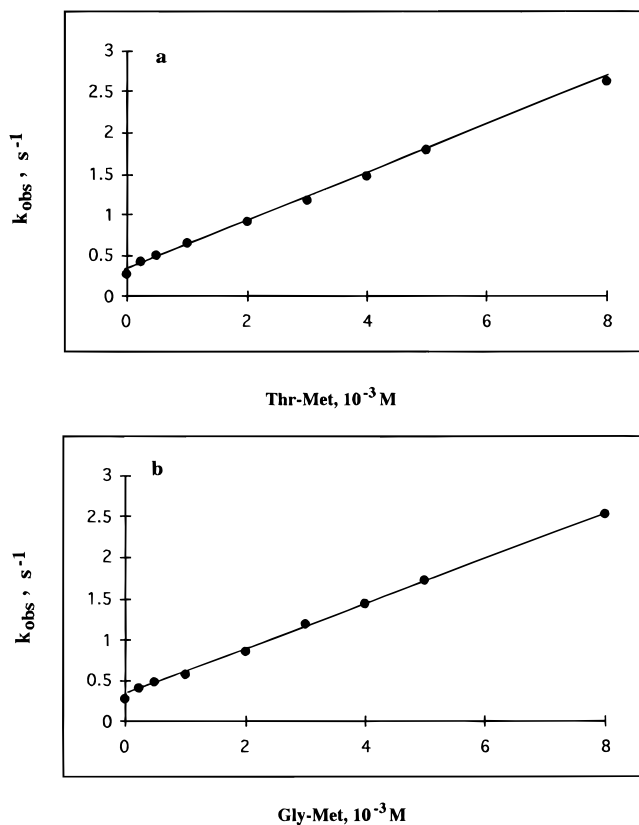(30) Hughes, M. N.; Nicklin, H. G. *J. Chem. Soc. A* **1968**, 450.

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Figure 4. k_{obs} vs concentration of (a) Thr-Met and (b) Gly-Met for the reaction of peroxynitrite with both peptides in aqueous solution containing 0.1 M sodium phosphate buffer, pH 7.4. The rate constants were obtained by following the disappearence of the 302 nm absorbance of peroxynitrite anion using stopped-flow rapid scan UV spectroscopy.

species which subsequently reacts with the peptides via 1e oxidation. At pH 7.4, we observed very little 1e oxidation of the peptides for [Thr-Met] > 1.75×10^{-4} M (see below), indicating that reactions 25−27 are sufficient for a kinetic analysis of our stopped-flow experiments.

The kinetics of peroxynitrite decomposition were first-order in the absence of peptides with $k_{obs} = 0.27 \pm 0.01$ s^{-1}, in good agreement with earlier measurements at pH 7.4 which yielded $k_{obs} = 0.25 \pm 0.04$ s^{-1} [1] and 0.26 ± 0.01 s^{-1},[9b] respectively. In general, the concentration vs time profiles of peroxynitrite anion could be well fitted with a single exponential function. Only at very early time points a biexponential fit was slightly better. This can be rationalized by the fact that mixing of the alkaline (pH 12) stock solution of peroxynitrite with the phosphate-buffered stock solution of the peptide (pH 7.4) causes a rapid equilibration of peroxynitrite with peroxynitrous acid according to reaction 25. However, the fact that equilibrium 25 is fast as compared to the subsequent processes (i.e., reactions 25 and 26) is evident from the result that, except for very early time points, the concentration vs time profile for peroxynitrite anion can be well fitted with a single exponential function. A similar kinetic scheme was derived for the radiative and nonradiative decay of protonated and nonprotonated triplet benzophenone in very acidic solution.[34] In the presence of peptides the kinetics changed to pseudo-first-order where k_{obs} (=(ln 2)/$t_{1/2}$) increased with increasing peptide concentrations. Plots of k_{obs} vs peptide concentration were linear, as displayed in Figure 4.

Equations III−V represent mathematical expressions for $k_{obs,pH\ 7.4}$, derived on the basis that the kinetics were measured

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under pseudo-first-order conditions ([ONOO$^-$] ≪ [X-Met]), that

$$-\frac{d[\text{ONOO}^-]}{dt} = k_{obs,pH\ 7.4}[\text{ONOO}^-] \qquad \text{(III)}$$

$$k_{obs,pH\ 7.4} = k_{26,pH\ 7.4} + k_{27,pH\ 7.4}[\text{peptide}] \qquad \text{(IV)}$$

$$k_{obs,pH\ 74} = (k_{26} + k_{27}[\text{peptide}])\left(\frac{[\text{H}^+]}{[\text{H}^+] + K_{25}}\right) \qquad \text{(V)}$$

ONOOH is the key intermediate for unimolecular decomposition of peroxynitrite into nitrate[1] and for oxidation of X-Met,[9] that reaction 27 represents the predominant pathway of X-Met oxidation at pH 7.4, and that equilibrium 25 is rapid compared to reactions 26 and 27.[1,9]

The slopes of the straight lines yield $k_{27,pH\ 7.4} = 283 \pm 3$ M^{-1} s^{-1} for Thr-Met and $k_{27,pH\ 7.4} = 280 \pm 2$ M^{-1} s^{-1} for Gly-Met. These values are comparable to the rate constants for the bimolecular reaction of peroxynitrite with 4-methylthiobutanoic acid ($k_{pH\ 7.4} = 283$ M^{-1} s^{-1}) and Met [$k_{pH\ 7.4} = 181$[9a]-416[33] M^{-1} s^{-1}]. On the basis of $K_{25} = 1.58 \times 10^{-7}$ M^{-1} [1,9b] and, at pH 7.4, [H$^+$] = 4×10^{-8} M, we derive that for Thr-Met $k_{27} = 1400 \pm 15$ M^{-1} s^{-1} and for Gly-Met $k_{27} = 1386 \pm 10$ M^{-1} s^{-1}. The value for the unimolecular decomposition of peroxynitrite in the absence of peptide corresponds to $k_{26,pH\ 7.4} = 0.27 \pm 0.01$ s^{-1} (see above).

Product Yields. When 5×10^{-4} M peroxynitrite reacted with 1.0×10^{-3} M Thr-Met in N$_2$-saturated aqueous phosphate buffer (2×10^{-2} M, pH 7.4), (2.5 ± 0.5) × 10^{-4} M Thr-Met was lost, but only very small yields of acetaldehyde [(3.0 ± 0.3) × 10^{-6} M] were formed together with small yields of ammonia, NH$_3$ [(7 ± 1) × 10^{-6} M], and major yields of threonylmethionine sulfoxide, Thr-Met(O) [(2.4 ± 0.5) × 10^{-4} M]. In a first approximation, a loss of ca. 2.5×10^{-4} M Thr-Met under these experimental conditions is not unexpected on the basis of our experimentally derived rate constants for the reaction of peroxynitrite with Thr-Met at pH 7.4. Taking $k_{26,pH\ 7.4} = 0.27$ s^{-1} and $k_{27,pH\ 7.4} = 283$ M^{-1} s^{-1} for Thr-Met, competition kinetics predict that in the presence of 1×10^{-3} M Thr-Met ca. 51% (corresponding to 2.55×10^{-4} M) of the added peroxynitrite should react with Thr-Met and the residual 49% of peroxynitrite should suffer unimolecular decomposition.

The efficiency of peroxynitrite-mediated product formation can be calculated as $f_{prod,PN} = $ [product]/[lost Thr-Met]. Thus, the efficiencies of product formation are $f_{acet,PN} = 0.012$ for acetaldehyde, $f_{NH3,PN} = 0.028$ for ammonia, and $f_{Thr-Met(O),PN} = 0.96$ for threonylmethionine sulfoxide, as displayed in Table 1, entry 1. All three products accounted for 100% of the converted Thr-Met, i.e., $\sum_{prod,PN} = f_{acet,PN} + f_{NH3,PN} + f_{Thr-Met(O),PN} = 1.0$ ($\sum_{prod,PN} < 1.0$ for lower concentrations of Thr-Met; see below). As expected,[17] the oxidation of Ala-Met or Thr-Leu by peroxynitrite did not yield acetaldehyde. The absence or presence of O$_2$ had little influence on the yields of acetaldehyde but apparently some small influence on the yields of Thr-Met-(O) (compare entries 1−3 with entries 4−6). However, it remains to be shown whether this effect may be caused by different levels of CO$_2$ in the oxygenated and the N$_2$-saturated solutions, respectively (see also below). There was no influence of added 5.0×10^{-4} M [Fe^{III}(CN)$_6$]$^{3-}$ on the yields of acetaldehyde and Thr-Met(O), indicating that the additional presence of a potent electron acceptor did not affect the product yields. This result is important with regard to the fact that peroxynitrite itself (in particular ONOOH) is a potent electron acceptor with $E^{o\prime,red,pH\ 7.4}(\text{ONOO}^-, 2\text{ H}^+/\text{NO}_{2,aq}) = 1.4$ V.[1] With increasing concentrations of Thr-Met at constant ratios of [Thr-

Met]:[peroxynitrite] = 2:1, $f_{acet,PN}$ decreased whereas $f_{Thr-Met(O),PN}$ increased. This feature of $f_{acet,PN}$ is different from the efficiency of photochemical acetaldehyde formation, $f_{acet,photo}$, where increasing concentrations of Thr-Met promoted no (at pH 6.0) or even a slight increase (at pH 7.4) of $f_{acet,photo}$ (see Table 1, column 5), and bears important mechanistic information (see the Discussion). We note that at concentrations of [Thr-Met] $< 1.0 \times 10^{-3}$ M, $\Sigma_{prod,PN} < 1.0$. This suggests that at lower concentrations of Thr-Met peroxynitrite may react via additional channels with Thr-Met, possibly via hydrogen abstraction. In fact, MALDI-TOF mass spectrometric analysis of our reactions indicated products with molecular masses corresponding to either MW(TM) + MW(NO) or MW(TM) + MW(O_2) − 2MW-(H) and MW(TM) + MW(NO_3) (TM = Thr-Met) which remain to be characterized. However, this detail does not affect our calculations of the efficiency of 1e oxidation of Thr-Met by peroxynitrite, determined by measurement of acetaldehyde, since we have provided a reference value for every concentration of Thr-Met by measurement of the photochemical efficiency of acetaldehyde formation, $f_{acet,photo}$ (see also the Discussion). In control experiments we confirmed that acetaldehyde was not a substrate for peroxynitrite under our reaction conditions. In a typical experiment the reaction of 8.75×10^{-5} M peroxynitrite with 1.75×10^{-4} M Thr-Met yielded 1.4×10^{-6} M acetaldehyde at pH 7.4. When a solution containing 1.75×10^{-4} M Thr-Met and 9×10^{-6} M acetaldehyde was reacted with 8.75×10^{-5} M peroxynitrite, the final concentration of acetaldehyde after completion of the reaction was 1.04×10^{-5} M, i.e., an amount corresponding to the sum of initially added acetaldehyde and the yield of acetaldehyde expected on the basis of an exclusive reaction of peroxynitrite with Thr-Met even in the presence of acetaldehyde.

When 1.75×10^{-4} M Thr-Met was oxidized by 8.75×10^{-5} M peroxynitrite in the presence of 3.5×10^{-3} or 1.75×10^{-2} M methanol, there was only a 10% or 20% decrease of $f_{acet,PN}$, respectively. This result indicates that acetaldehyde is not the product of a reaction of free hydroxyl radicals (HO•) with Thr-Met in the peroxynitrite system. Initially, it had been suggested that hydroxyl radicals may be generated by homolytic cleavage of peroxynitrite (reaction 28),[35] but both theoretical[1] and

$$ONOOH \rightarrow HO^{\bullet} + {}^{\bullet}NO_2 \qquad (28)$$
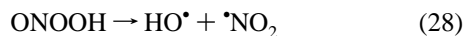
experimental[36,37] evidence against such a reaction has now been presented. If free hydroxyl radicals would have been responsible for acetaldehyde formation from Thr-Met in our systems, methanol would have reduced $f_{acet,PN}$ by 66% (3.5×10^{-3} M methanol) and 91% (1.75×10^{-2} M methanol), respectively, on the basis of $k(HO^{\bullet}+Thr\text{-}Met) \approx 9.8 \times 10^9$ M^{-1} s^{-1} [38] and $k(HO^{\bullet}+CH_3OH) = 9.7 \times 10^8$ M^{-1} s^{-1}.[39]

In the presence of a physiological concentration of 2.5×10^{-2} M HCO_3^-, the product yields were significantly changed (Table 1, entry 7), as we observed significantly higher values for $f_{acet,PN}$ but significantly lower values for $f_{Thr-Met(O),PN}$. Mechanistically, these features can be rationalized by the rapid

formation of an adduct between $ONOO^-$ and CO_2[40] (present through the equilibrium $HCO_3^- + H^+ \rightleftharpoons H_2O + CO_2$) in competition with the reaction of peroxynitrite with Thr-Met. Potential structures of such adducts between peroxynitrite and CO_2 have been proposed[40,41] but not experimentally confirmed.

Formation of Methional from Met and Thr-Met. As displayed in reactions 3, 5, and 6, methional is a stable molecular product originating from the 1e oxidation of Met. One potential mechanistic problem associated with the formation of ethylene from Met is the fact that ethylene may result not only from a direct decomposition of a Met-derived intermediate but also from further oxidation of methional, a product of the decomposition of **1**. Here, we have included experimental evidence that methional is, in fact, a product from the reaction of peroxynitrite with Met. We reacted different concentrations of Met (1×10^{-3}, 5×10^{-4}, 1.75×10^{-4} M) with peroxynitrite at ratios of [Met]:[peroxynitrite] = 2:1 in 2×10^{-2} M phosphate buffer, pH 7.4, similarly to the experimental system described for Thr-Met (see above). The exposure of 1×10^{-3} M Met to 5×10^{-4} M peroxynitrite resulted in the loss of 3.3×10^{-4} M Met, accompanied by the formation of 2.0×10^{-5} M methional. Thus, the efficiency for methional formation by peroxynitrite, $f_{methional,PN} = $ [methional]/[loss of Met] corresponds to $f_{methional,PN} = 0.062$. With decreasing concentrations of Met, $f_{methional,PN}$ increased to $f_{methional,PN} = 0.15$ for 5×10^{-4} M Met and $f_{methional,PN} = 0.31$ for 1.75×10^{-4} M Met. Thus, in particular at lower Met concentrations methional accounts for a significant fraction (up to 31%) of the products formed during the oxidation of Met by peroxynitrite. When we exposed Thr-Met to peroxynitrite, there was no formation of methional at all concentrations of Thr-Met, 1.75×10^{-4}, 5×10^{-4}, and 1×10^{-3} M.

Discussion

Quantification of the 1e Oxidation of Thr-Met by Peroxynitrite. Scheme 1 displays two potential pathways according to which sulfide radical cations from Thr-Met produce acetaldehyde, (i) the formation of **5** (reactions 8 and 9) with subsequent conversion to **6** (reaction 10) or (ii) the deprotonation of the N-terminal amino group of **3** or **4** (reactions 14 and 15) to allow direct formation of **6** (reaction 16). We note, however, that acetaldehyde formation is only one possible pathway of the decomposition of threonylmethionine sulfide radical cations. Competing pathways include deprotonation in the α-position to the sulfur such as shown in reactions 22 and 23 (Scheme 2). In general, these deprotonation pathways are more efficient at higher pH and lower sulfide concentrations.[42] This fact may rationalize our result that photochemical acetaldehyde formation at pH 7.4 was less efficient for 1.75×10^{-4} M Thr-Met as compared to 5×10^{-4} and 1×10^{-3} M Thr-Met. However, the pathways leading to acetaldehyde also benefit from higher pH, rationalizing why acetaldehyde formation was generally more efficient at pH 7.4 as compared to pH 6.0. A quantitative prediction of acetaldehyde formation as a function of pH and Thr-Met concentration is currently not possible as not all rate constants for the individual (competing) processes are known. However, a semiempirical approach allows this prediction. We have measured the primary yields of sulfide radical cations,

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(42) Mönig, J.; Goslich, R.; Asmus, K.-D. *Ber. Bunsen-Ges. Phys. Chem.* **1986**, *90*, 115.

represented by $(S \therefore S)^+$ (**4**), during the oxidation of Thr-Met by $^3CB^*$ using laser photolysis. The only other primary reaction products formed during laser photolysis are the α-(alkylthio)-alkyl radicals **10** and **11**, but these do not cause acetaldehyde formation.[17] Sulfide radical cation dimers can be conveniently observed within 1.1 μs after the laser flash but earlier pulse radiolytic measurements have shown that such species decay with $t_{1/2} \leq 1 \times 10^{-5}$ s at peptide concentrations of $\leq 1 \times 10^{-3}$ M,[43] i.e., conditions representative for our steady-state photolysis and peroxynitrite experiments. For $(S \therefore S)^+$ (**4**), this subsequent decay yields acetaldehyde, **8**, **10**, and **11**. (Some additonal decarboxylation has been observed for hydroxyl radical-initiated oxidations of X-Met peptides[24] but was negligible for $^3CB^*$-initiated oxidations,[20] suggesting that branching leading to decarboxylation may not necessarily involve dimeric sulfide radical cations.) By measurement of the efficiency of photochemical acetaldehyde formation per threonylmethionine sulfide radical cation, $f_{acet,photo}$, for each concentration of Thr-Met at pH 6.0 and 7.4, we obtained reference values which can be used for the exact calculation of the 1e oxidation efficiency of peroxynitrite by measurement of the yields of acetaldehyde in the peroxynitrite/Thr-Met systems. For example, the efficiency of acetaldehyde formation during the anaerobic oxidation of 1 $\times 10^{-3}$ M Thr-Met by 5×10^{-4} M peroxynitrite was $f_{acet,PN} = 0.012$. Since under such conditions the decomposition of threonylmethionine sulfide radical cations yields 0.78 mol of acetaldehyde/mol of sulfide radical cation, determined photochemically (i.e., $f_{acet,photo} = 0.78$), the application of eq VI reveals that a fraction of $f_{1e,PN} = 0.015$ (i.e., 1.5%) of the peroxynitrite-induced oxidation of Thr-Met must have proceeded via 1e oxdiation.

$$f_{1e,PN} = f_{acet,PN}/f_{acet,photo} \qquad (VI)$$

In a similar way the efficiencies for 1e oxidation of Thr-Met by peroxynitrite were calculated for all experimental conditions, as displayed in the last column of the Table 1. In principle, the value of $f_{acet,photo}$ is not expected to be influenced by the absence or presence of oxygen. However, the photochemical measurement of $f_{acet,photo}$ in oxygenated solutions is difficult as oxygen reacts rapidly with $^3CB^*$, $CB^{\bullet-}$, and $CBH^{\bullet}$, producing singlet oxygen and superoxide, respectively, which may competitively react with Thr-Met or $(S \therefore S)^+$.[44] Therefore, the values $f_{acet,photo}$ obtained photochemically in N_2-saturated solutions were used for the calculation of $f_{1e,PN}$ for the peroxynitrite-mediated processes in oxygenated solutions.

One additonal feature should be discussed briefly. We had shown that protonation of any $CB^{\bullet-}$, initially formed during the photochemical 1e oxidation of Thr-Met (reaction 20), does not affect the lifetime of $(S \therefore S)^+$. However, for an exact quantification of $f_{acet,photo}$ we have to exclude that, at a later stage, $CBH^{\bullet}$ does not react significantly with $(S \therefore S)^+$ under the conditions of steady-state photolysis (i.e., via reaction 29), thereby reducing the yields of $(S \therefore S)^+$, effectively available for acetaldehyde formation.

$$CBH^{\bullet} + (S \therefore S)^+ \, (\mathbf{4}) \rightarrow CB + H^+ + 2Thr\text{-}Met \qquad (29)$$

Equations VII−XI allow the calculation of an upper limit of the steady-state concentration of $CBH^{\bullet}$, $[CBH^{\bullet}]_s$, assuming that $CBH^{\bullet}$ decays solely according to reaction 30 with $2k_{30} = 1.8 \times 10^9$ M^{-1} s^{-1}.[21] In eq VII, $\Phi(CB^{\bullet-}+CBH^{\bullet})$ corresponds to

(43) By analogy to Gly-Met: Bobrowski, K.; Holcman, J. *Int. J. Radiat. Biol.* **1987**, *52*, 139.

(44) Miller, B. L.; Williams, T. D.; Schöneich, Ch. *J. Am. Chem. Soc.* **1996**, *118*, 11014.

$$2CBH^{\bullet} \rightarrow (^-O_2CPh)(Ph)C(OH)C(OH)(Ph)(PhCO_2^-) \qquad (30)$$

$$\frac{d[CBH^{\bullet}]}{dt} = \frac{d[^3CB^*]}{dt}\Phi(CB^{\bullet-} + CBH^{\bullet}) - k_{30}[CBH^{\bullet}]^2 \qquad (VII)$$

the quantum yield for chemical quenching of $^3CB^*$ according to reactions 20 and 21 (Scheme 2), measured immediately after the laser flash (i.e., compare Figures 2 and 3). Subsequently, on a longer time scale, $CB^{\bullet-}$ accepts a proton to yield $CBH^{\bullet}$.

$$d[CBH^{\bullet}]/dt = 0 \qquad (VIII)$$

$$[CBH^{\bullet}]_s = \left[\frac{(d[^3CB^*]/dt)\Phi(CB^{\bullet-}+CBH^{\bullet})}{k_{30}}\right]^{1/2} \qquad (IX)$$

In our steady-state photolysis experiments we measured ΔCB as a function of time which is related to the chemical quenching of $^3CB^*$ through eq X. Combining eqs X and IX yields eq XI.

$$\frac{\Delta CB}{dt} = \frac{d[^3CB^*]}{dt}\Phi(CB^{\bullet-}+CBH^{\bullet}) \qquad (X)$$

$$[CBH^{\bullet}]_s = [(\Delta CB/dt)/k_{30}]^{1/2} \qquad (XI)$$

For our steady-state photolysis conditions we determined that $\Delta CB/dt = 7.4 \times 10^{-7}$ M s^{-1} so that $[CBH^{\bullet}]_s = 2.9 \times 10^{-8}$ M. For [Thr-Met] $\leq 1 \times 10^{-3}$ M, the overall decay of $(S \therefore S)^+$ (reaction 31) follows first-order kinetics with $t_{1/2} \leq 1 \times 10^{-5}$ s, corresponding to $k_{31} \geq 6.9 \times 10^4$ s^{-1}.

$$(S \therefore S)^+ \rightarrow products \qquad (31)$$
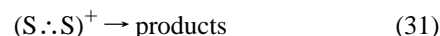
The rate for reaction 31 can be simply expressed as $v_{31} = k_{31}[(S \therefore S)^+]$ and that for reaction 29 as $v_{29} = k_{29}[(S \therefore S)^+] \cdot [CBH^{\bullet}]_s$. If we approximate that $k_{29} \leq 10^{10}$ M^{-1} s^{-1}, it follows that $v_{31}/v_{29} \geq 238$. Thus, reaction 29 will not contribute to the disappearence of $(S \therefore S)^+$ under steady-state photolysis conditions.

The Mechanism. On the basis of the values for $f_{1e,PN}$, we conclude that the efficiency for 1e oxidation of Thr-Met by peroxynitrite increases with decreasing concentrations of Thr-Met. These results are in line with a mechanism displayed in Scheme 3, analogous to reactions proposed by Pryor et al.[9] for the oxidation of Met.

In general ground state $ONOO^-$ should be able to oxidize Thr-Met (reaction 32), although k_{32} is expected to be small.[9,33] However, by analogy to earlier results with Met it appears that, at pH $\leq$ 7.4, it will be predominantly $ONOOH$ which oxidizes Thr-Met to Thr-Met(O) via oxygen transfer in a bimolecular reaction (reaction 33).[9,33] At this point we cannot define to what extent the individual conformers of $ONOOH$,[32] *cis-cis*, *cis-perp*, or *trans-perp* would contribute to reaction 33. In competition with these bimolecular pathways, $ONOOH$ transforms unimolecularly into nitrate according to two independent pathways. The first pathway proceeds via a metastable reactive intermediate, $ONOOH^*$ (reaction 34), which constitutes a precursor not only for the subsequent unimolecular rearrangement into nitrate (reaction 35) but also for the 1e oxidation of Thr-Met (reaction 36) or, potentially, for hydrogen transfer reactions (reaction 37). In the second pathway, recently established,[37] $ONOOH$ directly rearranges into nitrate apparently without the intermediacy of an excited state (reaction 38). Recently, two potential structures of $ONOOH^*$ have been located using density functional theory methods (the Becke3LYP functional and 6-31G* basis set).[41] They are essentially hydrogen-bonded radical pairs ($^{\bullet}OH \cdots$

Scheme 3
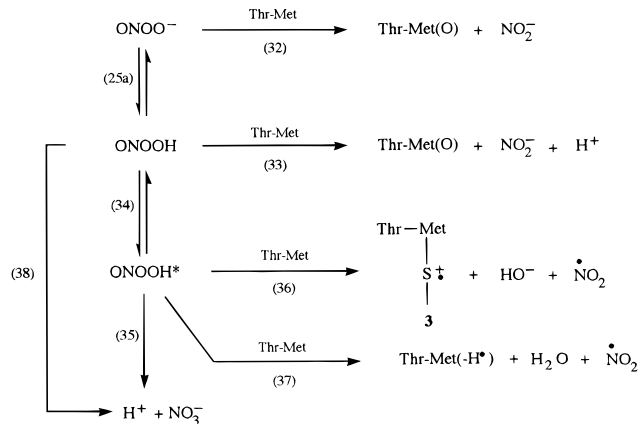
ON•O) with different OH···O bond lengths and show diradical character with degenerate triplet and singlet states. Depending on the level of theory, these hydrogen-bonded complexes are 15.6 or 15.0 kcal/mol higher in energy as compared to ground state *cis,cis*-ONOOH.[41] Interestingly, Tsai et al.[32] also located a triplet instability for *cis*-ONOO⁻ which is 7.9 kcal/mol (MP2/6-311+(d)) or 14.1 kcal/mol (Becke3LYP) higher in energy than singlet *cis*-ONOO⁻. In addition, they located the triplet state of *trans*-ONOO⁻, being 13.0 kcal/mol (MP2/6-311+(d)) or 9.4 kcal/mol (Becke3LYP) higher in energy as compared to singlet *cis*-ONOO⁻. Thus, theoretically both the hydrogen-bonded radical pair[41] and the triplet states[32] (which do not necessarily have to be identical) could account for the 1e oxidation of Thr-Met by peroxynitrite. In addition, the location of these excited states helps to rationalize potential hydrogen abstraction reactions of ONOOH (peroxynitrite was reported to initiate lipid peroxidation[45]) and may serve to explain the fact that we observed additional channels of the reaction of peroxynitrite with Thr-Met at lower Thr-Met concentrations which led to the formation of species with molecular weights of MW(Thr-Met + NO) or MW(Thr-Met + O_2 − 2H) and MW(Thr-Met + NO_3) (see above). These products potentially form after hydrogen abstraction from any C−H bond of Thr-Met (reaction 37) and subsequent net addition of NO_x or O_2.
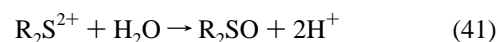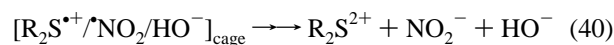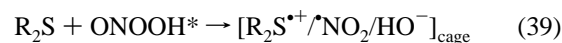
The fact that $f_{acet,PN}$ is higher for lower concentrations of Thr-Met is simply a result of an increasing fraction of unimolecular formation of ONOOH* (reaction 34) at the expense of the bimolecular reaction 33, the rate of which depends on the concentration of Thr-Met.

An important result is that we are able to exactly quantify the extent of 1e oxidation of Thr-Met by means of measuring acetaldehyde formation. Thus, at pH 7.4 only 1.5% and 1.8% of the reaction of peroxynitrite with Thr-Met proceeded via 1e oxidation in anaerobic and aerobic atmospheres, respectively. This result is quite different from earlier findings with the free amino acid Met[9] where, on the basis of ethylene measurements, at least 8% of the reaction of Met with peroxynitrite afforded 1e oxidation at a substrate concentration of [Met] = 1 × 10⁻³ M. In addition to ethylene, the oxidation of Met by peroxynitrite

yields significant amounts of methional (e.g., 6.2% for 1 × 10⁻³ M Met), another 1e oxidation product of Met. At pH 6.0 there was a higher extent of 1e oxidation for the reaction of peroxynitrite with Thr-Met where, e.g., $f_{1e,PN}$ = 0.14 for 1.75 × 10⁻⁴ M Thr-Met (i.e., 14% of the reaction of peroxynitrite proceeded via 1e-oxidation). At present, we cannot explain this higher efficiency at pH 6.0 as compared to pH 7.4 but note that maximum 1e oxidation yields were also observed for the reaction of peroxynitrite with dimethyl sulfoxide and 2,2′-azinobis(3-ethyl-1,2-dihydrobenzothiazoline-6-sulfonate) (ABTS) at pH 6.0.[46]

One interesting question to pursue in future experiments would be whether ONOOH* can also perform 2e oxidations (sulfoxide formation) in its reaction with Met residues. For example, one could envisage two consecutive 1e oxidation reactions: the first 1e oxidation process between ONOOH* and R_2S would lead to a sulfide radical cation and •NO_2 which, within a solvent cage, could react to yield a sulfide dication (reactions 39 and 40). The latter would react with water to form sulfoxide (reaction 41). Charge stripping experiments in the gas phase have, in fact, demonstrated the possibility of removal of an electron from $R_2S^{•+}$.[47]

$$R_2S + ONOOH^* \rightarrow [R_2S^{•+}/{}^•NO_2/HO^-]_{cage} \quad (39)$$

$$[R_2S^{•+}/{}^•NO_2/HO^-]_{cage} \longrightarrow R_2S^{2+} + NO_2{}^- + HO^- \quad (40)$$

$$R_2S^{2+} + H_2O \rightarrow R_2SO + 2H^+ \quad (41)$$

We conclude that the reactions of peroxynitrite with Met residues might show quite different results depending on whether Met is present as a free amino acid or embedded in a peptide or protein. Comparable observations have been made when Met and Met-containing peptides were subjected to the oxidation by hydroxyl radicals[24,48] and ³CB*.[12,20] Future studies must now show whether and under what conditions peroxynitrite will react with protein-bound Met residues via 1e-oxidation and whether such reactions occur *in vivo*.

Experimental Section

See the Supporting Information.

Supporting Information Available: Experimental section (4 pages). See any current masthead page for ordering and Internet access instructions.

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