Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11173–11177, November 1994
Biochemistry

One- and two-electron oxidations of methionine by peroxynitrite

(hydroxyl radical/nitric oxide/superoxide/sulfoxide/ethylene)

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Communicated by Irwin Fridovich, June 15, 1994

ABSTRACT **Peroxynitrite is stable, but its acid, HOONO, either rearranges to form nitrate or oxidizes nearby biomolecules. We report here the reactions of HOONO with methionine and the methionine analog 2-keto-4-thiomethylbutanoic acid (KTBA). These oxidations proceed by two competing mechanisms. The first yields the sulfoxide; the second-order rate constants, k_2, for this process for methionine and KTBA are 181 ± 8 and 277 ± 11 $M^{-1}\cdot s^{-1}$, respectively, at pH 7.4 and 25°C. In the second mechanism, methionine or KTBA undergoes a one-electron oxidation that ultimately gives ethylene. We propose that the one-electron oxidant is an activated form of peroxynitrous acid, HOONO*, that is formed in a steady state mechanism. The ratios of the second-order rate constants for the ethylene-producing reaction (k_2^*) and the first-order rate constant to produce nitric acid (k_N) for methionine and KTBA, k_2^*/k_N, are 1250 ± 290 and 6230 ± 1390 M^{-1}, respectively. Both ceric and peroxydisulfate ions also oxidize KTBA to ethylene, confirming a one-electron transfer mechanism. The yields of neither MetSO nor ethylene are affected by several hydroxyl radical scavengers, suggesting that a unimolecular homolysis of HOONO to HO· and ·NO_2 is not involved in these reactions. HOONO* gives hydroxyl radical-like products from various substrates but displays more selectivity than does the hydroxyl radical; thus, HOONO* is incompletely trapped by typical HO· scavengers. However, a mechanism involving dissociation of HOONO* to caged radicals cannot be ruled out at this time.**

Nature's use of ·NO as a biological signal molecule is remarkable from many perspectives, not the least of which is the fact that ·NO is a toxin. Paradoxically, the toxicity of ·NO involves oxidation reactions, but nitric oxide itself is only a weak oxidant. However, nitric oxide can be converted to a potent oxidant by reaction with superoxide to produce the peroxynitrite ion ($^-$OO—N=O) and its conjugate acid, peroxynitrous acid (HOO—N=O) (1), as shown in Eqs. 1 and 2. Peroxynitrite‡ is capable of oxidizing thiols (2), lipids (3), and the methionine residue at the active site of α1-proteinase inhibitor (4)

$$O_2^{\cdot-} + {}^{\cdot}NO \rightarrow {}^-OONO \qquad [1]$$

$$H^+ + {}^-OONO \overset{K_a}{\rightleftarrows} HOONO \qquad [2]$$

Several lines of evidence indicate that peroxynitrite is formed *in vivo*. For example, superoxide can diminish the effects of ·NO by diverting it to form peroxynitrite (5, 6). For this reason, superoxide scavengers such as superoxide dismutase and thiols can enhance the biological activity of ·NO (5, 7). Immunohistochemical techniques demonstrate that nitrotyrosine residues are present in atherosclerotic human coronary arteries (8); tyrosine is nitrated by peroxynitrite but not by nitric oxide (9).

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The reactions of peroxides with nucleophiles have been intensively studied, and normally either SN2 or one-electron transfer reactions occur (10, 11). Surprisingly, peroxynitrite reacts with methionine in both a one-electron oxidation that leads to ethylene and a two-electron reaction that gives methionine sulfoxide (MetSO), making the study of the HOONO–methionine reaction unusually revealing. We here report a detailed investigation of the reaction of peroxynitrite with methionine itself and with the methionine analog 2-keto-4-thiomethylbutanoic acid (KTBA).

MATERIALS AND METHODS

Synthesis of Peroxynitrite. Peroxynitrite was synthesized by two methods.

Method A. This method involves the reaction of ozone with azide and yields peroxynitrite solutions that are useful for stopped-flow studies (12).

Method B. This method involves the autoxidation of hydroxylamine in alkali (13, 14). Peroxynitrite solutions were purified by freeze fractionation and concentrations were determined spectrophotometrically by measuring the absorbance at 302 nm using an extinction coefficient of 1670 $M^{-1}\cdot cm^{-1}$ (4, 15).

GC and HPLC Analyses. Systems used 0.5 M sodium phosphate buffer (pH 7.4), methionine varying from 1 to 17.5 mM as needed, 0.5 mM peroxynitrite, and the other components as required. Ethylene was analyzed by GC, and methionine and MetSO were analyzed by HPLC (14). Ethylene was also analyzed from reactions of the one-electron oxidants ceric ammonium nitrate and ammonium peroxodisulfate (10 mM) with KTBA (10 mM).

NMR Analysis. KTBA was measured by ^1H NMR on a Bruker AC 200 spectrometer operating at 200 MHz using *tert*-butanol as an internal standard (14).

Reaction Kinetics. Kinetics measurements used a stopped-flow system that has been described elsewhere (14, 15). The rate of peroxynitrite disappearance was followed at 302 nm maintaining the temperature at 25°C ± 0.1°C. The observed rate constant, k_{obs}, was extracted from a fit of the change in absorbance versus time to a first-order decay.

Kinetics Analysis. As discussed below, there are two possible mechanisms for oxidations by peroxynitrite: one involves dissociation of HOONO to form HO· and ·NO_2, and the other involves an excited species that has hydroxyl radical-like properties (15). The mechanism for decomposition of peroxynitrite must be written in terms of an intermediate (HOONO* or HO·) rather than a transition state [as had been done before (1, 15–18)]. Although it is not possible to definitely rule out the hydroxyl radical on the basis of

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; KTBA, 2-keto-4-thiomethylbutanoic acid; MetSO, methionine sulfoxide.

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‡The term peroxynitrite is used to refer to the sum of both $^-$OONO and its conjugate acid HOONO. When a specific species is referred to, it is named peroxynitrite ion or peroxynitrous acid.

published data (see ref. 19 and references cited therein), we choose to formulate our kinetics analysis in terms of a metastable intermediate, HOONO*, because, as shown below, our scavenger data cannot be explained by the published rate constants for the hydroxyl radical. We assume that HOONO* is formed in a steady state according to Eqs. **3–5** and that it gives hydroxyl radical-like products but reacts with more selectivity than does the hydroxyl radical itself (19)

$$H^+ + {}^-OONO \overset{K_a}{\rightleftarrows} HOONO \quad [3]$$

$$HOONO \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} HOONO^* \quad [4]$$

$$HOONO^* \overset{k_N}{\rightarrow} H^+ + NO_3^- \quad [5]$$

In this mechanism, k_{obs} for the decomposition of peroxynitrite is given by Eq. **6**, where K_a is the acidity constant for HOONO and has the value 1.58×10^{-7} M at 25°C (14, 19)

$$k_{obs} = \left(\frac{k_1 k_N}{k_N + k_{-1}}\right)\left(\frac{[H^+]}{K_a + [H^+]}\right). \quad [6]$$

Eqs. **7** and **8** are required to account for the reactions of substrate S with ground-state HOONO and/or with HOONO*, resulting in a total of five rate constants. The value of k_{obs} in terms of these rate constants at 25°C and pH 7.4 is given by Eq. **9**, where a, b, c, and d are defined in Eqs. **10–13**

$$HOONO + S \overset{k_2}{\rightarrow} \text{products} \quad [7]$$

$$HOONO^* + S \overset{k_2^*}{\rightarrow} \text{products} \quad [8]$$

$$k_{obs} = \frac{a(1 + b[S])}{1 + \frac{ab}{c}[S]} + d[S]. \quad [9]$$

$$a = \left(\frac{k_1 k_N}{k_N + k_{-1}}\right)\left(\frac{[H^+]}{K_a + [H^+]}\right). \quad [10]$$

$$b = \frac{k_2^*}{k_N}. \quad [11]$$

$$c = \frac{k_1[H^+]}{K_a + [H^+]}. \quad [12]$$

$$d = \frac{k_2[H^+]}{K_a + [H^+]}. \quad [13]$$

The value of k_{obs} at pH 7.4 for the reaction of S with HOONO was calculated from a fit of the data to Eq. **9** using TABLECURVE (Jandel Scientific, San Rafael, CA) (see Fig. 1). The mechanism presented here can be further expanded to account for ionization of HOONO* and different reactivities of HOONO* and its ion, as well as for different stoichiometries for its reaction with different substrates should that become necessary.

RESULTS

Peroxynitrite Isomerization to Nitrate. The decomposition of peroxynitrous acid was fitted to a first-order decay equation, giving a k_{obs} at 25°C and pH 7.4 equal to 0.26 ± 0.01 s^{-1}, in agreement with the value 0.25 ± 0.04 s^{-1} previously reported (15).

The Reactions of Peroxynitrite with Methionine and KTBA. Peroxynitrite converts methionine to a mixture of ethylene and MetSO. The average material balance of the data in Table 1 (the sums of the yields of MetSO and ethylene plus recovered methionine) equals 102% ± 5% of the starting amount of methionine. The rate of reaction of peroxynitrite was studied in the presence of 10- to 50-fold excess methionine or KTBA (14). The analogous reaction with KTBA was studied by the same techniques. Fig. 1 shows k_{obs} as a function of the concentration of methionine or KTBA.

The Effect of Ferric Ion and Chelators. Chelators that complex adventitious metals are without effect on the yields of ethylene or MetSO. The experiments summarized in Table 1 (top six lines) show that the presence of 0.5 mM EDTA or diethylenetriaminepentaacetic acid (DTPA) does not affect the yields of either ethylene or MetSO. Furthermore, results are independent of the method of preparation of the peroxynitrite.

The addition of ferric EDTA increases the yield of MetSO. Table 1 (top six lines) shows that the addition of 0.5 mM ferric EDTA increases the yield of MetSO from 517 ± 15 to 793 ± 22 nmol. (Yields increase from 93% ± 3% to 99% ± 4% based on methionine.) The presence of ferric EDTA reduces the yield of ethylene (since more MetSO is formed). Ethylene yields decrease from 44 ± 4 (8.5% ± 0.8%) to 41 ± 3 (5.2% ± 0.5%) nmol.

Ethylene from Methionine and KTBA and Formation of the Hydroxyl Radical. A classical probe for oxygen radicals is the production of ethylene from methionine derivatives such as

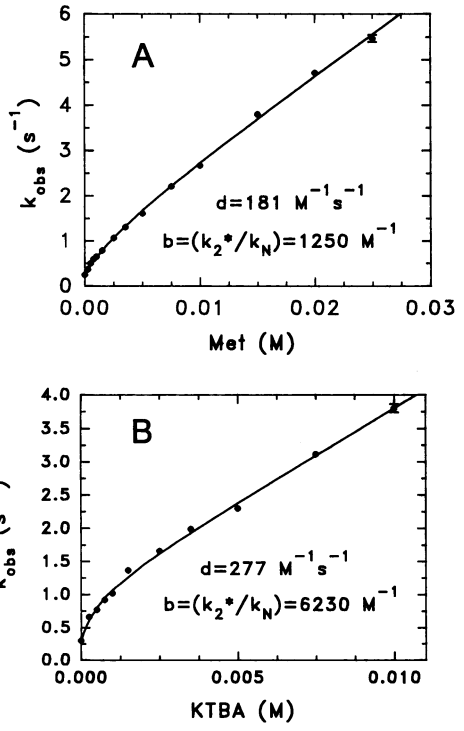

FIG. 1. Peroxynitrite decomposition in the presence of substrates. Plots give d and k_2^*/k_N by computer fit of the data to Eq. **9**; note that k_2 can be calculated from the value of d by using Eq. **13**. Each data point represents an average of six determinations. Error bars are shown only where they are larger than symbols. (*A*) Reaction of methionine with peroxynitrite. Peroxynitrite was added to buffered solutions containing 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.5, 3.5, 5.0, 7.5, 10.0, 15.0, 20.0, or 25.0 mM methionine; $d = 181$ $M^{-1}s^{-1}$; $k_2^*/k_N = 1250$ M^{-1}; if $pK_a = 6.8$, $k_2 = 902$ $M^{-1}s^{-1}$. (*B*) Reaction of KTBA with peroxynitrite. Peroxynitrite was added to buffered solutions containing 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.5, 3.5, 5.0, 7.5, or 10.0 mM KTBA; $d = 277$ $M^{-1}s^{-1}$; $k_2^*/k_N = 6230$ M^{-1}; if $pK_a = 6.8$, $k_2 = 1380$ $M^{-1}s^{-1}$.

Table 1. Effect of metal chelators and radical scavengers on yields of MetSO and ethylene from the reaction of methionine with peroxynitrite

Added scavenger	Added chelator	Ethylene, nmol		MetSO, nmol	
		$-Fe^{3+}$ EDTA	$+Fe^{3+}$ EDTA	$-Fe^{3+}$ EDTA	$+Fe^{3+}$ EDTA
None	None	46 ± 5	40 ± 3	528 ± 14	803 ± 24
None*	None	46 ± 1	43 ± 4	510 ± 10	783 ± 20
None	DTPA	42 ± 4	ND	520 ± 10	ND
None*	DTPA	43 ± 3	ND	535 ± 15	ND
None	EDTA	44 ± 2	ND	505 ± 20	ND
None*	EDTA	45 ± 5	ND	504 ± 17	ND
Benzoate	None	47 ± 4	41 ± 4	548 ± 25	782 ± 15
Mannitol	None	50 ± 6	42 ± 5	532 ± 10	792 ± 12
DMSO	None	51 ± 7	41 ± 4	556 ± 28	774 ± 20
Ascorbate	None	0	0	128 ± 10	65 ± 6
Trolox	None	12 ± 2	9 ± 1	543 ± 15	826 ± 20
Benzoate†	None	41 ± 2	32 ± 2	516 ± 10	790 ± 11
DMSO†	None	43 ± 2	31 ± 1	527 ± 20	797 ± 10
Mannitol†	None	41 ± 2	31 ± 1	532 ± 18	808 ± 25
Mannitol‡	None	41 ± 2	32 ± 2	522 ± 8	810 ± 20

All data were obtained with peroxynitrite from method A unless indicated otherwise. For brevity, only results obtained with peroxynitrite prepared by method A are shown for experiments in the presence of scavengers, but identical data were obtained when peroxynitrite was prepared by either method (12, 14). ND, not determined. All samples contained 1.0 mM methionine, 1.0 mM leucine (internal standard), 0.5 mM peroxynitrite, and the other components as indicated. Concentrations of scavengers were 20 mM unless otherwise indicated, and concentrations of chelators were 0.5 mM. Each value represents average of two experiments ± SD. Fe^{3+}/EDTA was 0.5 mM.

*Peroxynitrite prepared by method B.

†Concentrations of benzoate, dimethyl sulfoxide (DMSO), and mannitol were 200, 200, and 250 mM, respectively.

‡Concentration of mannitol was 500 mM.

KTBA (20) or methional (21), as originally introduced by Beauchamp and Fridovich (22). The reaction of peroxynitrite with KTBA at pH 7.4 gives substantial yields of ethylene (Table 2). DTPA, EDTA, and ferric EDTA do not significantly affect the yields of ethylene from KTBA.

Effects of Radical Scavengers. Neither the yields of MetSO nor those of ethylene are affected by the hydroxyl radical scavengers benzoate, mannitol, or dimethyl sulfoxide (DMSO) in either the presence or absence of ferric EDTA (although more MetSO is formed in the presence of ferric EDTA), as shown in Table 1. The effects of ascorbate and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) are discussed below.

The Mechanism of Ethylene Formation. The production of ethylene from methionine analogs involves an initial one-electron oxidation (22). To test this, we examined the reactions of KTBA with peroxodisulfate and ceric ions, both potent one-electron oxidants. Indeed, we find that when KTBA (10 mM; 2 ml) is allowed to react with ceric ions or peroxodisulfate (10 mM; 2 ml), 274 ± 20 or 70 ± 7 nmol of ethylene is formed, respectively.

As explained in *Materials and Methods*, the kinetics analysis is formulated in terms of a metastable intermediate HOONO* capable of oxidizing a variety of substrates to products similar to those derived from the hydroxyl radical. The decomposition of peroxynitrite to form nitrate (Eqs. 3–5) and the reactions of peroxynitrite with methionine to give MetSO or ethylene (Eqs. 14 and 15), occur simultaneously.

$$\text{HOONO} + \text{Met} \xrightarrow{k_2} \text{MetSO} + \text{HONO} \quad [14]$$

$$\text{HOONO*} + \text{Met} \xrightarrow{k_2^*} \text{CH}_2{=}\text{CH}_2 + \text{other products.} \quad [15]$$

The rate laws for the formation of MetSO and ethylene are described by Eqs. 16 and 17

$$\frac{d[\text{MetSO}]}{dt} = k_2[\text{HOONO}][\text{Met}]. \quad [16]$$

$$\frac{d[\text{CH}_2{=}\text{CH}_2]}{dt} = k^*_2[\text{HOONO*}][\text{Met}]. \quad [17]$$

In the presence of excess methionine, this mechanism predicts that the yield of ethylene divided by the yield of MetSO should give Eq. 18. For simplicity, we have omitted a correction factor that includes yield, stoichiometry, and possible pH dependence of the reaction of HOONO* with methionine to give ethylene, since present experimental data do not require it

$$\frac{[\text{MetSO}]_f}{[\text{CH}_2{=}\text{CH}_2]_f} = \frac{k_2(k_N + k_{-1})}{k^*_2 k_1} + \frac{k_2}{k_1}[\text{Met}]_0. \quad [18]$$

Table 2. Ethylene yields for reaction of KTBA with peroxynitrite under different conditions

Added compound	Ethylene yield, %
None	76 ± 3
None*	78 ± 4
FE^{3+}EDTA	78 ± 4
DTPA	87 ± 8
EDTA	74 ± 2

Peroxynitrite was prepared by method B. Samples contained 1.0 mM KTBA unless stated otherwise, 0.5 mM HOONO, and, where indicated, 0.5 mM FE^{3+}EDTA, DTPA, or EDTA. Reactions were initiated by addition of peroxynitrite, by means of a syringe, to the head-space vials containing the other components. Values are averages of two measurements ± SD. Ethylene yields were calculated based on KTBA consumed as measured by NMR.

*Concentration of KTBA was 2.0 mM.

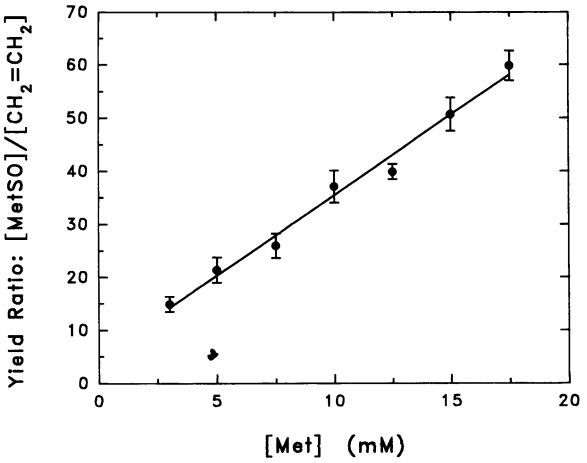

FIG. 2. Plot of the ratio of the yields of MetSO divided by the yields of ethylene versus the initial concentration of methionine (Eq. **18**) at pH 7.4 and 25°C. MetSO was analyzed by HPLC and ethylene was analyzed by GC (14). Each data point represents an average of three independent determinations. Error bars are SDs derived from three determinations.

Thus, a plot of the yield of MetSO divided by the yield of ethylene versus the initial concentration of methionine, $[Met]_0$, should give a straight line. This plot is shown in Fig. 2, and the data fit a straight line ($r^2 = 0.99$).

DISCUSSION

Mechanistic Considerations—An Activated Form of Peroxynitrous Acid. The peroxynitrite anion is stable. However, peroxynitrous acid rapidly isomerizes to nitric acid (ref. 19 and references therein). Oxidations by peroxynitrite have been formulated as involving either the hydroxyl radical (1, 3, 23–26) or an activated species with reactivity like the hydroxyl radical (4, 15, 19, 27). If HOONO homolyzes to form a hydroxyl radical and $^{\bullet}NO_2$, these radicals would be produced in a solvent cage, and the caged radical pair (the geminate pair) then would undergo diffusive separation to give free radicals (19, 28–30). Since caged radicals cannot be scavenged (28–30), this mechanism explains the apparent residual yield of nitrate that is formed even in the presence of very high concentrations of radical scavengers (19, 23, 27). However, the inactivation of α1-proteinase inhibitor by peroxynitrite (4) and the oxidation of methionine (Table 1) show little or no change in the presence of hydroxyl radical scavengers. These experiments were performed with scavenger concentrations that were calculated, using the known rate constants for reaction of $^{\bullet}OH$ with the scavengers (31), so that all of the hydroxyl radicals, if they were present, would react with the scavenger, and oxidation of α1-proteinase inhibitor or methionine would not occur. Thus, these reactions do not appear to involve free radicals (however, see ref. 19).

The data presented in Table 1 could be rationalized in terms of a reactive species, HOONO*, that is more selective and less reactive than free $^{\bullet}OH$ itself (19). Thus, at present, the caged radical mechanism can explain the apparent residual yield of nitrate (1, 23, 27) but not the scavenger data, and the activated HOONO* mechanism can rationalize the scavenger data but not the apparent residual yield of nitrate that is formed even in the presence of scavengers. Despite this uncertainty, we have chosen to present the data in this manuscript in terms of an activated form of peroxynitrous acid, HOONO*, which is formed in a steady state. This mechanism explains the curvature in Fig. 1 and the fact that the best-fit straight lines extrapolated from studies at high substrate concentrations have intercepts that do not agree with the value of k_{obs} obtained in the absence of substrate. The extent of the curvature observed in plots like Fig. 1 depends on the substrate reactivity and the concentrations of substrate chosen for study; in some cases, the plot may mislead the investigator into believing that the data are satisfactorily fit by a straight line. However, our current data predict that most substrates show curvature in plots like Fig. 1.

The Production of Ethylene. The plot shown in Fig. 2 implies that the reactions giving MetSO and ethylene must have very different mechanisms. This can be accommodated by Eqs. **3–5** and **14** and **15**, involving the formation of an activated form of peroxynitrous acid, HOONO*, that either decomposes to form nitrate or performs a one-electron oxidation to give ethylene. KTBA also is oxidized to ethylene by peroxodisulfate and/or ceric ions, evidence that ethylene is produced from an initial one-electron transfer reaction.

The difference in kinetic order between the formation of MetSO and ethylene means that the ratio [MetSO]/[ethylene] decreases as the initial concentration of methionine is lowered. Both MetSO residues (32, 33) and ethylene (34) are produced *in vivo*. Ethylene also is present in exhaled breath from humans (35, 36); the source of this ethylene is not known, but it is conceivable that some could result from the HOONO–methionine reaction.

The amounts of ethylene produced from either methionine or KTBA are only slightly reduced by metal chelators and radical scavengers (Tables 1 and 2), suggesting that both reactions have similar mechanisms. The presence of ferric EDTA causes a significant increase in the yields of MetSO (Table 1). Thus, the decomposition of HOONO to form nitrate and the reaction with methionine to give ethylene are affected less by metal ions than is the formation of MetSO. This is expected, since displacement reactions often are catalyzed by Lewis acids, whereas unimolecular reactions are less influenced.

Scavenger Data. The yields of MetSO and ethylene are not affected by three typical hydroxyl radical scavengers (Table 1), leading us to formulate the principal pathway leading to ethylene as involving HOONO* rather than hydroxyl radicals.

Trolox and ascorbate deserve separate discussion. Trolox quenches the formation of ethylene better than do all the scavengers we have studied except ascorbate (14). Since HOONO* can react either with methionine to give ethylene or with Trolox, Trolox lowers the yields of ethylene. Since Trolox does not appreciably change the yields of MetSO (Table 1), it must react only slowly with ground-state HOONO. Ascorbate reacts both with HOONO* and with HOONO. At the 20-fold excess ascorbate used in these experiments, the reaction of ascorbate with peroxynitrite is 5-fold faster than the methionine–peroxynitrite reaction, and ascorbate sacrificially destroys HOONO before it can react with methionine.

CONCLUSIONS

Peroxynitrous acid/peroxynitrite converts methionine to MetSO by a two-electron oxidation and to ethylene by a one-electron pathway. An activated, metastable intermediate on the pathway for conversion of peroxynitrous acid to nitric acid, HOONO*, is postulated. This intermediate reacts with potential substrates and scavengers with more selectivity than does the hydroxyl radical but gives product profiles that are similar to those found in reactions mediated by the hydroxyl radical itself. This mechanism rationalizes the inability of several typical hydroxyl radical scavengers to reduce the yields of some of the products examined. It does not, however, provide an explanation for the apparent residual yield of nitrate even in the presence of scavengers, which

would require a caged radical mechanism (19). Until conclusive scavenger experiments can be done, however, the metastable intermediate mechanism presented here is a somewhat more likely rationale of the facts than is the cage mechanism.

We thank Dr. Willem Koppenol for use of his stopped-flow spectrophotometer and for many useful discussions; we are also grateful for very helpful discussions with Drs. Joseph Beckman and Bruce Freeman. The assistance of Dr. R. Cueto and Mr. J. N. Lemercier is greatly appreciated. This work was supported by a grant from the National Institutes of Health.

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