Insensitivity of the Rate of Decomposition of Peroxynitrite to Changes in Viscosity; Evidence against Free Radical Formation

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Abstract: Peroxynitrite is a versatile and important biological oxidant that is produced from the reaction of nitric oxide and superoxide radicals. Two mechanisms have been proposed to rationalize oxidation reactions of peroxynitrite. One assumes that HO−ONO can homolize to form the hydroxyl radical and nitrogen dioxide, and that the hydroxyl radical is the proximate oxidant in peroxynitrite systems. The second argues this homolysis is too slow to occur at ordinary temperatures and suggests an excited species, HOONO*, is the proximate oxidant. If the radical mechanism is correct, then peroxynitrite should disappear more slowly in solvents of higher viscosity. This is true because for free radical initiators undergoing single-bond homolysis: (1) cage return is substantial and more of the cages would return to re-form HO−ONO as the viscosity of the medium increases; and (2) diffusion from the radical cage competes effectively with other cage processes. We have studied the disappearance of peroxynitrite at pH 5 and 7 in buffers with and without dioxane (as a control) or up to 30 wt % of the poly(ethylene glycol) (PEG) polymers, PEG 3350 and PEG 8000. These polyethers produce substantial changes in viscosity, raising the viscosity from about 0.89 to about 17 mPa·s. The rate constant for diffusion should decrease by about 10- to 20-fold as the viscosity increases in this interval, and the rate of diffusion from the solvent cage would be predicted to vary accordingly. However, at pH 5, where most of HOONO is undissociated, no change in the rate of disappearance of peroxynitrite is observed with increasing viscosity. At pH 7, a small increase in the observed rate constant is found, but it is likely due to the greater concentration of the undissociated HOONO in the ether-containing solvents resulting from a pK_a shift. Thus, we conclude that the viscosity test does not support a free radical mechanism for the unimolecular decomposition of peroxynitrite.

Introduction

Peroxynitrite, the product that results from the combination of superoxide and nitric oxide, is a unique and fascinating species. It is an important biological oxidant and the first peroxyacid that is known to be produced *in vivo*. The reaction of •NO with superoxide to form peroxynitrite can reduce the biological effects of nitric oxide;[1−4] similarly, the combination of superoxide with •NO can reduce the effects of superoxide.[4,5] However, the production of peroxynitrite replaces superoxide and nitric oxide, both weak and highly selective oxidants, with peroxynitrite, a more versatile and powerful oxidant.[6−9]

There are currently two proposals[10] that explain the oxidizing properties of peroxynitrite, particularly in reactions in which the rate of disappearance of peroxynitrite is zero order in the substrate undergoing oxidation.[11,12] The first, depicted in Scheme 1, postulates that peroxynitrous acid, HO−ONO, undergoes homolysis to form the hydroxyl radical, and that this species is the proximate oxidant.[6,13] The second (Scheme 2) arises from arguments that homolysis of HO−ONO (eq 1-2) at ordinary temperatures is much too slow to be involved in oxidations by peroxynitrite;[7] instead, a reactive form of peroxynitrous acid, HOONO*, is proposed to be the active oxidant.[8,9] A recent review[9] as well as new data[8,14] favor the HOONO* mechanism.

Determining whether peroxynitrite can decompose to give either the reactive hydroxyl radical or a less reactive and more selective species HOONO* is of paramount biological importance. The toxicity of the hydroxyl radical is limited by its high reactivity and consequent short diffusion radius; however, a longer-lived intermediate could reach specific, distant sites, and could selectively react with preferred target molecules.

If HOONO undergoes homolysis to form HO• and •NO_2, these

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(11) The term peroxynitrite is used here for the sum of the concentrations of HOONO and its anion $^-$OONO. The systematic names for these species are hydrogen oxoperoxonitrate acid and oxoperoxonitrate(1−), respectively.
(12) We refer here specifically to peroxynitrite decomposition reactions that are kinetically overall first order, that is, first order in peroxynitrite and zero order in oxidizable substrate.
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Scheme 1. The Cage (Free Radical) Mechanism for the Decomposition of Peroxynitrite[a]

$$HOONO \underset{}{\overset{K_a}{\rightleftharpoons}} H^+ + {}^-OONO \quad (1\text{-}1)$$

$$HOONO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [HO^{\bullet}\ {}^{\bullet}NO_2]_{cage} \quad (1\text{-}2)$$

$$[HO^{\bullet}\ {}^{\bullet}NO_2]_{cage} \xrightarrow{k_N} H^+ + NO_3^- \quad (1\text{-}3)$$

$$[HO^{\bullet}\ {}^{\bullet}NO_2]_{cage} \xrightarrow{k_{diff}} HO^{\bullet} + {}^{\bullet}NO_2 \quad (1\text{-}4)$$

$$k_{obs} = \frac{k_1 (k_N + k_{diff})}{k_{-1} + k_N + k_{diff}} \times \left\{\frac{[H^+]}{K_a + [H^+]}\right\} \quad (1\text{-}5)$$

[a] This scheme is simplified from that presented in ref 9.

Scheme 2. The Non-Radical Mechanism for the Decomposition of Peroxynitrite[a]

$$HOONO \underset{}{\overset{K_a}{\rightleftharpoons}} H^+ + {}^-OONO \quad (2\text{-}1)$$

$$HOONO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HOONO^* \quad (2\text{-}2)$$

$$HOONO^* \xrightarrow{k_N} H^+ + NO_3^- \quad (2\text{-}3)$$

$$k_{obs} = \frac{k_1 k_N}{k_{-1} + k_N} \times \left\{\frac{[H^+]}{K_a + [H^+]}\right\} \quad (2\text{-}4)$$

[a] This scheme is simplified from that presented in ref 9.

two radicals (the geminate pair) are produced in a solvent cage.[15−19] In solution, the surrounding solvent molecules create a barrier to diffusive separation of the geminate pair. Since the diffusive barrier is very low (1−3 kcal/mol in ordinary solvents), only very fast reactions can compete with diffusion of the pair apart to form free radicals. In the case of the cage consisting of $[HO^{\bullet}\ {}^{\bullet}NO_2]$, combination to form nitrate or to reform peroxynitrite (cage return) should compete with diffusive separation to form free radicals.[20]

The temperature dependence of the disappearance of HOONO gives an Arrhenius pre-exponential factor *A* equal to 6.2×10^{12} s^{-1}; this *A* value corresponds to a small positive change in activation entropy for the overall disappearance of peroxynitrite (+3 eu).[7] In the gas phase, this entropy of activation is too small to be consistent with homolytic dissociation.[21] In solution, however, the geminate radical pair is formed in a solvent cage and significant cage return can occur, reducing the efficiency (and thus decreasing the entropy) for the disappearance of peroxynitrite. Thus, the recombination of the geminate pair $[HO^{\bullet}\ {}^{\bullet}NO_2]$ to form peroxynitrous acid could reconcile the small Arrhenius pre-exponential value with a free radical mechanism.

Thus, if the free radical mechanism is correct, cage return must be an important process for peroxynitrite.[9,20] In this case, since diffusive separation of the geminate pair is fast enough to compete with nitrate formation, then the viscosity of the solvent will influence the rate of diffusion of the geminate pair apart, and hence, the rate of disappearance of peroxynitrite.[20] That is, in media of higher viscosity, the cage will "live longer" and more of the geminate pairs will combine to reform peroxynitrous acid. Therefore, the free radical mechanism for decomposition of peroxynitrite predicts that the rate of rearrangement of peroxynitrite to give nitrate must be slower in solvents of higher viscosity.[9,20] It is this prediction that we have tested; our data show that viscosity has little or no effect on the rate of disappearance of peroxynitrite in the systems we have studied.

Experimental Section

Materials. Poly(ethylene glycol) (PEG) 3350, PEG 8000, and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma Chemical Co. HPLC-grade dioxane, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from EM Science, A Division of EM Industries, Inc. Sodium acetate was purchased from Mallinckrodt Inc.

Preparation of Peroxynitrite. Peroxynitrite was synthesized using the reaction of ozone with azide;[22] the resulting peroxynitrite solutions are more useful for stopped-flow studies than older preparation methods and are free of hydrogen peroxide and trace metals. Peroxynitrite solutions were concentrated by freeze fractionation and the concentrations were determined spectrophotometrically by measuring the absorbance at 302 nm using an extinction coefficient of 1670 M^{-1} cm^{-1}.[23]

Kinetic Measurements. Kinetics of the decomposition of peroxynitrous acid in the presence of PEG 3350, PEG 8000, or dioxane were studied at pH 5 and 7. The pH dependence of the decomposition of peroxynitrous acid in 7.5 wt % dioxane−water was studied from pH 4 to 8.5. Acetate buffers were used from pH 4 to 5.5 and phosphate buffers were used from pH 6 to 8.5. The final concentrations of buffers were 250 mM and contained 0.25 mM DTPA. The ionic strength was held at 0.75 M using NaCl.

The kinetic measurements were followed by monitoring the disappearance of peroxynitrite versus time at 302 nm on a stopped-flow spectrophotometer manufactured by Kinetic Instruments, Inc. (Ann Arbon, MI) and On-Line Instrument Service (Jefferson, GA) with a mixing time of less than 1.5 ms. The stock peroxynitrite solution was diluted to the appropriate concentration with water (pH 11, NaOH) or water containing the desired amount of PEG 3350 or PEG 8000 (pH 11, NaOH) immediately before loading onto the stopped-flow spectrophotometer. Equal volumes of the peroxynitrite solution and a buffer solution, or a buffer solution containing PEG 3350, PEG 8000, or dioxane, as required, were mixed in the cell of the stopped-flow apparatus. Reactions were carried out at 25.0 ± 0.1 °C. The pH measured at the outlet was less than 0.1 pH units higher than the initial pH of the buffers. The observed rate constant was extracted from a fit of the change in absorbance versus time to a single-exponential decay using the software provided with the stopped-flow spectrophotometer.[8] The viscosities of the dioxane mixtures were determined on a Brookfield digital viscometer Model LVTDCP, and those of the PEG mixtures were calculated in accordance with standard procedures.[24]

Results and Discussion

The effect of viscosity on the rate of disappearance of free radical initiators that undergo single-bond homolysis is well-known and is usually observed as a decrease in the rate of decomposition of the initiator with increasing solvent viscosity.[18,20] This occurs since recombination of the geminate pair of radicals to reform the initiator competes with diffusion of the caged pair of radicals, and more cage return occurs at higher viscosity.[18,20] In contrast, the rates of most chemical reactions are usually insensitive to changes in viscosity. Thus, for an

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initiator such as HO−ONO that undergoes the homolysis of just one bond, changes in viscosity should produce greater amounts of cage return and a decrease in the rate of decomposition of the initiator.[18,20]

Scheme 1 depicts a free radical mechanism for the decomposition of peroxynitrite and the corresponding observed rate constant, k_{obs}. For the viscosity test to be unambiguous, the rate constant for diffusion of the geminate pair out of the solvent cage (k_{diff}) must be larger than the rate constant for formation of nitrate from the cage (k_N). Additionally, as can be seen from eq 1-5, cage return (k_{-1}, reformation of HOONO in the cage) must be large enough such that modulation of k_{diff} by means of a viscosity enhancer will result in $k_N + k_{diff}$ approaching or exceeding the value of k_{-1}. These requirements are likely to be met for cases in which a free radical initiator undergoes single-bond homolysis.[18,20] In organic solvents, recombination of the geminate pair and diffusion out of the cage can have quite similar rates. For example, for acetyl peroxide in octane at 80 °C, k_{-1} is 6×10^9 s^{-1} and k_{diff} is 8.3×10^9 s^{-1}.[20] The effects that water, a polar and hydrogen-bonding solvent, could have on a cage consisting of [HO$^\bullet$ $^\bullet$NO$_2$] have been discussed.[9]

We have followed the disappearance of peroxynitrite at 302 nm in buffers of pH 5 and 7 using methods previously described.[7−9] Two poly(ethylene glycols) (PEG), PEG 3350 and PEG 8000, were used to increase the viscosity of the aqueous solutions; solutions containing comparable weight fractions of dioxane were used to control changes in general solvent properties other than viscosity that might be produced by the polyethers.

As can be seen from eq 1-5 in Scheme 1 and eq 2-4 in Scheme 2, the expression for k_{obs} includes a term that involves the acidity constant, K_a, for peroxynitrite. Two cases must be considered, one in which k_{obs} changes with changes in the acidity constant, K_a, for peroxynitrite and one where it does not. If a concentration of protons is chosen that is large, then $[H^+]/\{K_a + [H^+]\}$ is approximately unity; in this case, k_{obs} is not affected by any change in the K_a of peroxynitrite due to the addition of the ether solvents. This is true around pH 5 since the pK_a of peroxynitrite will be near 7 in these aqueous solutions and $[H^+]/\{K_a + [H^+]\}$ will be about unity. Figure 1 shows that the observed rate constant for the disappearance of peroxynitrite at pH 5 is unchanged as the weight percent of PEG 3350, PEG 8000, or dioxane in the solvent was increased, despite the fact that (as can be seen from the upper horizontal axis) there is a very substantial change in the viscosity of the solutions, from 0.89 to 17.9 mPa·s for PEG 3350, and from 0.89 to 16.9 mPa·s for PEG 8000. In contrast, the viscosity of the dioxane−water solutions changes very little, even as substantial amounts of dioxane are added; again, and quite strikingly, k_{obs} does not change from any solvent property of dioxane.

The second case occurs for data obtained near pH 7, which is close to the pK_a of HOONO.[8,9] Figure 2 shows data comparable to those in Figure 1 but obtained near pH 7, and there is a slight increase in the values of k_{obs} as the amounts of PEG or dioxane are increased; this *increase* in k_{obs} is due to a change in the K_a for peroxynitrite. The decrease in the ion-supporting properties of these solvents as the amount of ether or polyether is increased will increase the pK_a of HOONO, since the undissociated acid will become more stable relative to the anion in less polar solvents. To correct for this effect, the pK_a of HOONO was measured in 7.5 wt % dioxane−water.

Figure 3 depicts the pH dependance of the rate of decomposition of peroxynitrite in 7.5 wt % dioxane−water. A least-squares computer fit gives a pK_a of 7.4, about 0.6 pK_a units higher than in the absence of dioxane,[8,9] indicating, as expected,

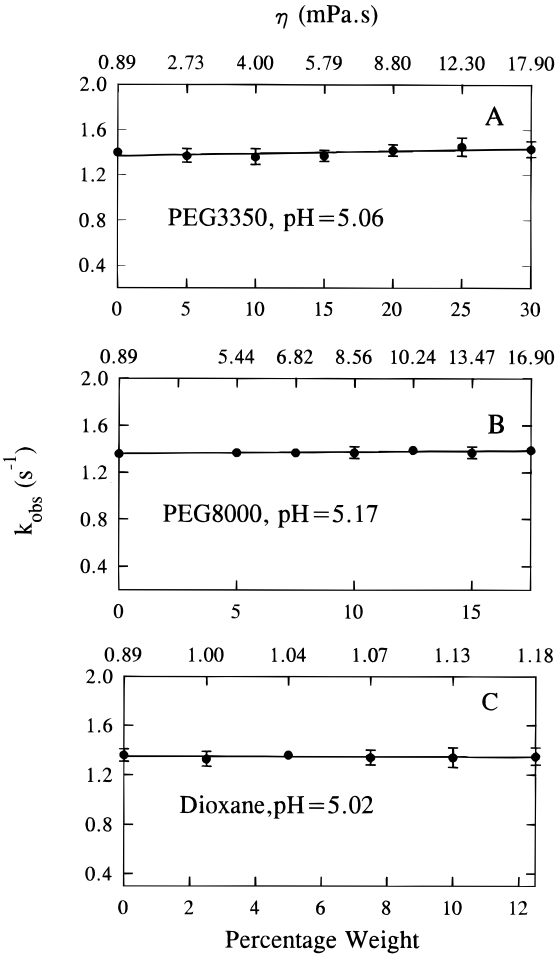

Figure 1. Peroxynitrous acid decomposition in the presence of poly(ethylene glycol) (PEG) or dioxane near pH 5 and at 25 °C. Each data point represents an average of five determinations. (A) PEG 3350: An appropriate amount of peroxynitrite was mixed with solutions containing 0, 5, 10, 15, 20, 25, or 30 wt % of PEG 3350 in 250 mM acetate buffer, pH 5.06. (B) PEG 8000: An appropriate amount of peroxynitrite was mixed with solutions containing 0, 5, 7.5, 10, 12.5, 15, or 17.5 wt % of PEG 8000 in 250 mM acetate buffer, pH 5.17. (C) Dioxane: The final concentrations of dioxane were 0, 2.5, 5.0, 7.5, 10, or 12.5 wt %. Reactions were carried out in 250 mM acetate buffer, pH 5.02.

that HOONO becomes a weaker acid in the less polar ether-containing solvents. Thus, the small increase on the rate of peroxynitrite disappearance in the presence of the polyethers can be interpreted as a consequence of the medium effect on the acidity constant of peroxynitrite; that is, the slight increase in k_{obs} seen in Figure 2 appears to result from changes in solvent properties other than viscosity.

If the cage mechanism is correct, k_{obs} can be expressed as given in Scheme 1.[8,9] If the value of 7.4 is used for the pK_a of HOONO rather than 6.77, the data for the point at 7.5 wt % dioxane correct to virtually a flat line, as shown by the dashed line in the bottom panel of Figure 2, suggesting that even at pH 7, viscosity plays little role. As explained above, this type of correction is unimportant at pH 5, where k_{obs} becomes insensitive to changes in the pK_a of peroxynitrite.

Both PEG and dioxane can be expected to be good scavengers of the hydroxyl radical. However, it is important to recognize that possible reactions of *free* radicals with dioxane or PEG cannot influence the rate of disappearance of peroxynitrite. That is true, since if HOONO homolyzes to give HO$^\bullet$, the reaction of PEG or dioxane with the HO$^\bullet$ could occur only *after* the HO$^\bullet$ diffuses out of the cage.[15−19] In fact, it is the inability of scavengers to trap caged radicals that is the operational definition

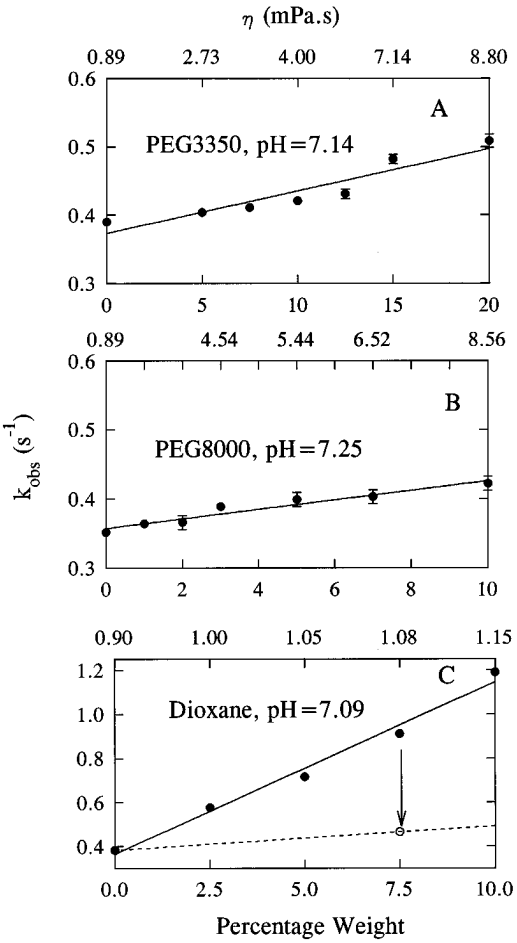

Figure 2. Peroxynitrous acid decomposition in the presence of poly(ethylene glycol) (PEG) or dioxane near pH 7 and at 25 °C. Each data point represents an average of five determinations: (●) experimental data, (○) rate constant corrected for the pK_a change due to changing the solvent from water to 7.5 wt % dioxane−water. The pK_a for peroxynitrite changes from 6.77[8,9] to 7.40 in 7.5% dioxane (see Figure 3). (A) PEG 3350: Peroxynitrite was mixed with solutions containing 0, 5, 7.5, 10, 12.5, 15, or 20 wt % of PEG 3350 in 250 mM phosphate buffer, pH 7.14. (B) PEG 8000: Peroxynitrite was mixed with solutions containing 0, 1, 2, 3, 5, 7, or 10 wt % of PEG 8000 in 250 mM phosphate buffer, pH 7.25. (C) Dioxane: Peroxynitrite was mixed with solutions containing 0, 2.5, 5.0, 7.5, or 10 wt % of dioxane in 250 mM phosphate buffer, pH 7.09.

of a cage, and distinguishes the cage from a meta-stable intermediate that, in contrast to the cage, can be trapped.[18,19] Thus, the reactions of the *free* hydroxyl radical with components of the reaction medium are late events that cannot affect the rate of disappearance of peroxynitrite (which is what we observe and measure).

If PEG or dioxane were able to scavenge the radicals in the solvent cage, k_{obs} around pH 5 should increase considerably.

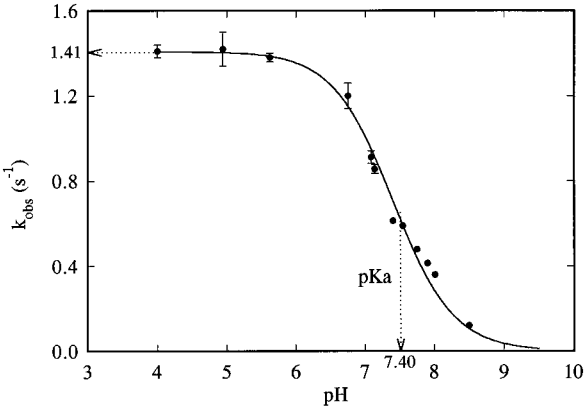

Figure 3. The rate of peroxynitrite decomposition as a function of pH in 7.5 wt % dioxane−H_2O at 25 °C. The first-order rate constants at various pH were determined by following the disappearance of peroxynitrite at 302 nm. Buffers are 250 mM acetate or phosphate containing 0.25 mM DTPA, fixing the ionic strength at 0.75 M using NaCl. Each data point represents an average of five determinations. Notice that the pK_a of HOONO is 7.40 in this system.

This is true because trapping of the caged radicals would limit the process governed by k_{-1} (eq 1-2). Notice, however, that k_{obs} at pH 5, shown in Figure 1, remains unchanged. Dioxane does not increase the viscosity but has a reactivity toward HO• that is similar to that of PEG. Thus, there can be no reaction of *caged* HO• with dioxane; the reaction of *free* HO• radicals with dioxane does not influence the rate of disappearance of peroxynitrite. Similarly, k_{obs} is not changed by the presence of PEG at pH 5.

In summary, peroxynitrite was subjected to the viscosity test in order to assess the likelihood of its decomposition to form free radicals. Contrary to results obtained with many free radical initiators that undergo single-bond homolysis, the rate of decomposition of peroxynitrite is insensitive to changes in viscosity. These results suggest that free radicals are not produced during the spontaneous self-decomposition of peroxynitrite. That is, reaction 1 does not occur. Of course,

$$\mathrm{HOONO} \longrightarrow \mathrm{HO^{\bullet}} + {}^{\bullet}\mathrm{NO_2} \qquad (1)$$

peroxynitrite may give rise to radicals, such as nitrogen dioxide, in the presence of one-electron donors; e.g., reaction 2 can occur.[8,9]

$$\mathrm{X:} + \mathrm{HOONO} \longrightarrow \mathrm{X^{+}:} + \mathrm{HO^{-}} + {}^{\bullet}\mathrm{NO_2} \qquad (2)$$

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