Rapid Reaction between Peroxonitrite Ion and Carbon Dioxide: Implications for Biological Activity

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Superoxide ion reacts rapidly with nitric oxide in aqueous solutions to form the peroxonitrite (oxoperoxonitrate(1−), ONO_2^-) ion.[1] This anion and its conjugate acid, hydrogen oxoperoxonitrate (ONO_2H), are powerful oxidants[2] which are reported to rapidly oxidize sulfhydryl groups[3] and thioethers[4] and to nitrate and hydroxylate aromatic compounds.[5−7] In biological environments that are capable of simultaneously generating O_2^- and NO, uncontrolled formation of ONO_2^- has been proposed[7] to cause oxidative damage to biological tissues, giving rise to a variety of pathogenic conditions that may include pulmonary[8,9] and coronary[10] diseases, impairment of central motor nervous system function,[11,12] and injury to ischemic tissues accompanying reperfusion with aerobic fluids.[13,14] Peroxonitrite has also been proposed to be an important microbicidal agent generated by phagocytic cells associated with host defense systems.[15,16] However, ONO_2^- is also unstable in carbonate-containing media,[17,18] and low concentration levels of HCO_3^- have been shown to protect *Escherichia coli* from the toxic effects of ONO_2^- in *in vitro* bactericidal assays.[19] We report herein that ONO_2^- reacts rapidly with CO_2, apparently forming an adduct whose composition is $ONO_2CO_2^-$. The rate constant is sufficiently large (3×10^4 M^{-1} s^{-1}) that this must be the predominant pathway for ONO_2^- disappearance in normal physiological fluids, where the total carbonate concentration is typically 25 mM or greater. It is therefore highly unlikely that ONO_2^- itself is damaging to cells, although it might be an obligatory intermediate for forming destructive cellular oxidants, i.e., $ONO_2CO_2^-$ or its decomposition products.

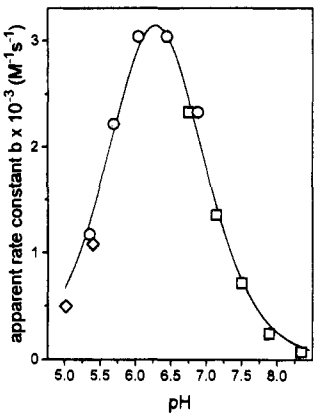

Figure 1. Dependence of the apparent rate constant (b) for reaction between peroxonitrite and carbonate upon solution pH in various buffered media. Conditions: 200 mM phosphate (□); 150 mM pyrophosphate (○); 400 mM acetate (◇). The solid line is the theoretical fit to the data assuming $k_2 = 3 \times 10^4$ M^{-1} s^{-1}; $pK_a = 6.6$; $pK_a' = 5.96$; these constants are defined in the text.

The disappearance of peroxonitrite was monitored at 302 nm (pH >6) or 265 nm (pH <6) in a Hi-Tech SF-40 stopped-flow instrument thermostated at 24 ± 0.1 °C. The reaction was initiated by flow-mixing alkaline (pH > 11) solutions of ONO_2^- with buffered $NaHCO_3$; buffer concentrations and acidities were chosen to yield appropriate final pH values. In neutral solutions with HCO_3^- absent, peroxonitrite isomerized to nitrate according to the rate law[7,17,20] $-d[ONO_2]_T/dt = a[ONO_2]_T$, where $[ONO_2]_T = [ONO_2^-] + [ONO_2H]$ and $a = k_1[H^+]/([H^+] + K_a)$. From the pH−rate profile (data not shown), values for the isomerization rate constant (k_1) and acid dissociation constant (K_a) of ONO_2H were determined to be 0.9 ± 0.05 s^{-1} and 2.5×10^{-7} M, respectively, nearly identical to published values.[7,17,20] In neutral solutions, the presence of carbonate accelerated ONO_2^- decay in a concentration-dependent manner. When carbonate was in large excess, this decay was exponential and obeyed the rate law $-d[ONO_2]_T/dt = (a + b[HCO_3]_T)[ONO_2]_T$, where $[HCO_3]_T = [HCO_3^-] + [CO_2]$. The pH dependence of the apparent second-order rate constant (b) exhibited a bell-shaped profile whose maximum appeared at pH ~6.2 (Figure 1). This pH dependence indicates that the actual reactant species are either CO_2 and ONO_2^- or HCO_3^- and ONO_2H, i.e., that the rate-limiting elementary reaction step in the carbonate-dependent pathway corresponds to either (1) $ONO_2^- + CO_2 \rightarrow ONO_2CO_2^-$ or (2) $HCO_3^- + ONO_2H \rightarrow$ products. The corresponding rate law terms for these reactions are $b = k_2/[(1 + [H^+]/K_a)(1 + K_a'/[H^+])]$ and $b = k_2'/[(1 + K_a/[H^+])(1 + [H^+]/K_a')]$, where K_a' is the constant for the CO_2 hydration−dehydration equilibrium, $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$, and k_2 and k_2' are the rate constants for reaction steps 1 and 2, respectively. Under the prevailing conditions, $K_a' = 1.1 \times 10^{-6}$ M.[21] In general, it would not be possible to identify the actual reactants from kinetics alone because the rate laws are homomorphic. However, in neutral solutions the hydration−dehydration equilibrium is slowly established relative to the rate of ONO_2^- decay (at pH 7−8, the equilibration half-time[22] is ~25 s), so that it is possible by conducting pH-jump experiments to distinguish between CO_2 and HCO_3^- as reactants. In one type of experiment, acidic solutions containing dissolved CO_2 were rapidly mixed with alkaline solutions of ONO_2^- to give neutral solutions in which the transitory CO_2 concentrations were much higher than their equilibrium values. A typical result is

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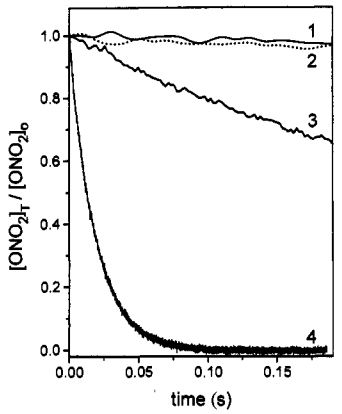

Figure 2. Stopped-flow kinetic traces of peroxonitrite ion decay. Trace 1 (solid): $NaONO_2$ and 5 mM carbonate in NaOH (pH 12) mixed with 400 mM phosphate (pH 7.4). Trace 2 (dashed): $NaONO_2$ in 180 mM phosphate (pH 12) mixed with 220 mM phosphate (pH 5) with no added carbonate. Trace 3: $NaONO_2$ in 20 mM phosphate (pH 12) mixed with 5 mM carbonate in 380 mM phosphate (pH 7.5). Trace 4: $NaONO_2$ in 180 mM phosphate (pH 12) mixed with 5 mM carbonate in 220 mM phosphate (pH 5). For all experiments, 1:1 reactant volumes were mixed, the pH after mixing was 7.6, and the initial ONO_2^- reactant concentration was $\simeq$500 μM.

illustrated in Figure 2. Here, rates of ONO_2^- decay are compared under identical medium conditions (200 mM phosphate, pH 7.6) for 500 μM ONO_2^- with no added carbonate (trace 2), with 2.5 mM HCO_3^- under near-equilibrium conditions (~75 μM CO_2, trace 3), and with ~2.25 mM CO_2 plus 0.25 mM HCO_3^- (trace 4). The rate of reaction increased proportionately with the CO_2 concentration, establishing that ONO_2^- and CO_2 comprise a reactant pair. The possibility that ONO_2H and HCO_3^- also comprise a reactant pair was investigated by a second type of pH-jump experiment in which alkaline solutions containing both CO_3^{2-} and ONO_2^- ions were mixed with acidic solutions containing only buffer to give neutral solutions that were initially devoid of CO_2. Rapid catalysis of ONO_2^- decay was not observed under these conditions (Figure 2, trace 1). Kinetic analyses indicated that the rate of this reaction was controlled by the rates of HCO_3^- dehydration and spontaneous isomerization of ONO_2^-.[23] Thus, direct reaction between the species HCO_3^- and ONO_2H must be slower than these processes. This experiment was possible because CO_3^{2-} was also found to be unreactive toward ONO_2^-, i.e., addition of carbonate to alkaline (pH >11) solutions of ONO_2^- did not accelerate its very slow intrinsic rate of decomposition. Additional evidence indicating that CO_2 and ONO_2^- comprise a reactant pair, but ONO_2H and HCO_3^- do not, is that biphasic kinetics were always observed when solutions containing excess HCO_3^-, but limiting CO_2, were mixed with solutions of ONO_2^- (data not shown). The breakpoints of these kinetic curves corresponded to the amount of CO_2 initially present in the solutions; they indicate that when exposed to ONO_2^- the existing CO_2 is rapidly consumed, following which the reaction becomes limited by the rate of CO_2 generation by dehydration of HCO_3^-.

The rate constant for reaction between ONO_2^- and CO_2 calculated from a series of pH-jump experiments is $k_2 = (2.9 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$; the value determined from the fit to the pH–rate profile (Figure 1) was $k_2 \simeq 3.0 \times 10^4\ M^{-1}\ s^{-1}$. These values are comparable to the largest rate constants reported for reaction of ONO_2^- with organic compounds[3-7] and indicate that carbonate should effectively scavenge any peroxonitrite ion generated in biological fluids. This follows because the CO_2 concentration in these environments is at least 10^2-fold greater than that of other potential reactants. This high reactivity can be attributed to strong electrophile–nucleophile interactions between the electron-deficient central carbon atom in CO_2 and the nucleophilic terminal oxygen atom of the ONO_2^- peroxo group. In contrast, the absence of detectable reactivity between ONO_2H and HCO_3^- is attributable to their considerably reduced nucleophilic and electrophilic character. Although the electron-withdrawing N=O substituent reduces the peroxo group nucleophilicity relative to other peroxo anions,[20] e.g., HO_2^-, it also reduces its basicity, allowing the reactant pair ONO_2^- and CO_2 to be present simultaneously in neutral solutions at concentration levels that are sufficiently high to ensure a large overall reaction rate between them. In contrast, more basic peroxides that might form analogous peroxocarbonates, e.g., HO_2^-, would be considerably less reactive toward CO_2 because they exist primarily in their unreactive conjugate acid forms (H_2O_2) in neutral solutions. Formation of ONO_2^- might therefore serve the unique function of allowing entry into a pathway for generating peroxide-based toxins that is not accessible by H_2O_2 itself in physiological environments.

The chemistry of the putative $ONO_2CO_2^-$ adduct is unexplored; however, homolytic cleavage of the weak peroxo O–O bond would give rise to the toxic HCO_3 radical,[24] and heterolytic cleavage would yield NO_2^+, which is a highly reactive nitrating agent. Formation of either of these products appears to be thermodynamically feasible.[25] We have also recently found that carbonate is an effective catalyst for ring nitration of aromatic compounds by ONO_2^- in neutral aqueous media. The actual nitrating agent in these reactions appears to be the $ONO_2CO_2^-$ intermediate (or ONO_2CO_2H).[26] In any event, the results presented here clearly demonstrate that considerations of the biological function of ONO_2^- must include its reaction with CO_2.

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(23) Under these conditions, CO_2 is in a steady state and ONO_2^- disappearance follows the rate law $[ONO_2]_T = [ONO_2]_0 \exp(-at) - (R/a)[1 - \exp(-at)]$, where $[ONO_2]_0$ is the initial peroxonitrite concentration and R is the rate of HCO_3^- dehydration under the prevailing conditions.[22] The first term on the right side of the equation is for spontaneous isomerization of ONO_2^-, and the second is for its reaction with CO_2.

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(26) From the kinetics and yield of carbonate-catalyzed nitration of fluorescein by ONO_2^-, the lower limit for the lifetime of the nitrating agent is estimated to be $t_{1/2} > 120$ ns. This value is almost 2 orders of magnitude greater than the estimated lifetime of NO_2^+ in water ($t_{1/2} \simeq 1.4$ ns),[27] eliminating NO_2^+ as the nitrating agent and leaving $ONO_2CO_2^-$ or its protonated form as the most likely alternative.

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