Nitrosation of 1,2-Phenylenediamine by Peroxynitrite/CO_2: Evidence for a Free Radical Mechanism

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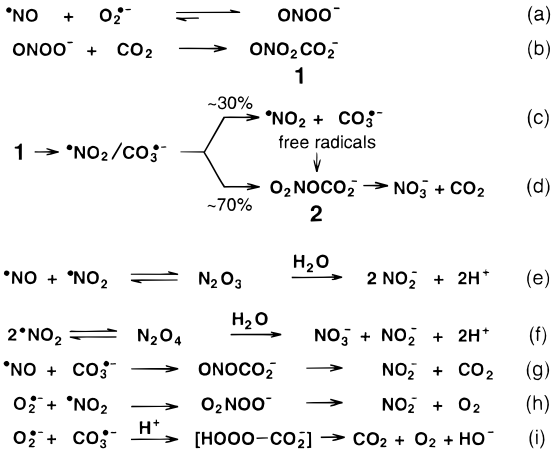
Nitric oxide and *S*-nitrosothiols function as critical signaling species.[1] *S*-Nitrosothiols have a longer half-life[2] than does •NO, and typical concentrations of *S*-nitrosothiols in blood plasma are 3−4 orders of magnitude higher than those of •NO.[3] Thus, nitrosation can lead to the formation of *S*-nitrosothiols, which can serve as carriers of •NO; however, since •NO itself does not nitrosate,[4] the mechanism by which nitrosation occurs in vivo is still unclear.[5] We have recently shown that peroxynitrite (PN),[6] an oxidant formed in the down-regulation of •NO,[7] can nitrosate phenol[8] in a CO_2-dependent pathway. The nitrosating species was suggested to be a nitrosonium ion (NO^+) carrier X−N═O (where X can be −ONO, $-ONO_2$, $-O_2NO_2$, or $-OCO_2^-$) or the free radicals $CO_3^{\bullet-}$ and •NO, all[8] of which can be formed from the reactions of PN[9] and its adduct with CO_2, $ONOOCO_2^-$[10] (Scheme 1). We here present unequivocal evidence for nitrosation of nucleophiles by PN/CO_2 based on the oxidation of 1,2-phenylenediamine (**4**), which gives up to 20 mol % yield of 1,2,3-benzotriazole (**9**). The inhibitory effects of azide support a free radical mechanism for the reaction.

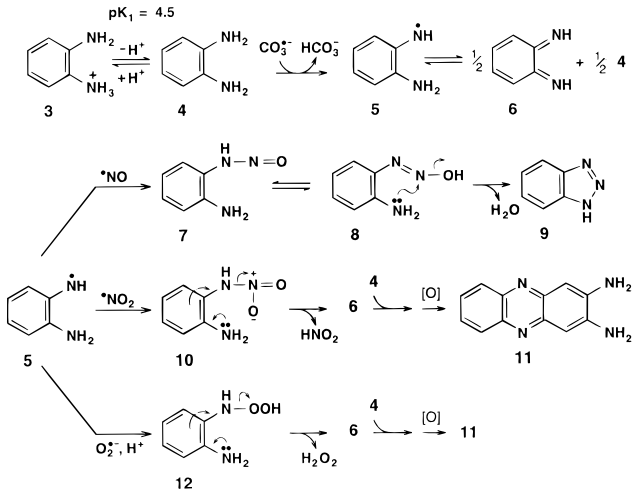
A characteristic probe for nitrosation involves the reaction of a vicinal diamine such as **4** or 2,3-diaminonaphthalene[11] to give a triazole (**9**), formed as a result of an intramolecular nucleophilic displacement on the diazo hydroxide (**8**) by the neighboring amino group (Scheme 2). Reactions of this type give quantitative yields of nitrosation under mildly acidic, neutral, or even somewhat alkaline conditions. Figure 1A shows the typical product profile for the reaction of PN with **4** in the presence of trace amounts of CO_2 (curve a). One of the major products of this reaction,[12] which elutes with a retention time of 8.0 min, has been identified as **9** based on coelution with authentic 1,2,3-benzotriazole (Figure 1A, curve b) as well as GC/MS/EI analysis giving ions at *m*/*z* 119, 91, and 64, corresponding to M^+ ($C_6H_5N_3^{\bullet+}$), and fragmentation to $C_6H_5N^{\bullet+}$ (loss of N_2) and $C_5H_4^{\bullet+}$ (further loss of HCN).

The pH profile of the yields of **9** in the PN/CO_2/**4** system parallels the formation of NO_2^- (in the absence of **4**) (Figure 1B), confirming[8−10] that the nitrosation reaction is mechanistically related to the pathways that produce NO_2^- (Scheme 1, eqs e−h). Like most oxidation[13] and nitration[7,14] reactions mediated by PN/CO_2, the nitrosation reaction levels off with a maximum yield that is only about 0.2 mol/mol of PN used (Figure 1B).[15] This confirms the existence of competing steps[8,10,13,14] in which PN

Scheme 1



Scheme 2



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(6) The term PN refers to the sum of PN anion ($ONOO^-$) and its acid, ONOOH (pK_a 6.8).

(7) •NO decomposes in vivo primarily through the reactions with $O_2^{\bullet-}$ and oxyhemoglobin, both reactions being extremely rapid and giving PN, which is then trapped by the reaction with CO_2. For example, see: Beckman, J. S.; Koppenol, W. H. *Am. J. Physiol.* **1996**, *271*, C1424. Radi, R. *Chem. Res. Toxicol.* **1996**, *9*, 828. Lymar, S. V.; Hurst, J. K. *J. Am. Chem. Soc.* **1995**, *117*, 8867. Uppu, R. M.; Squadrito, G. L.; Pryor, W. A. *Arch. Biochem. Biophys.* **1996**, *327*, 335. Houk, K. N.; Condroski, K. R.; Pryor, W. A. *J. Am. Chem. Soc.* **1996**, *118*, 13002. Squadrito, G. L.; Pryor, W. A. *Free Radical Biol. Med.* **1998**, *11*, 718.

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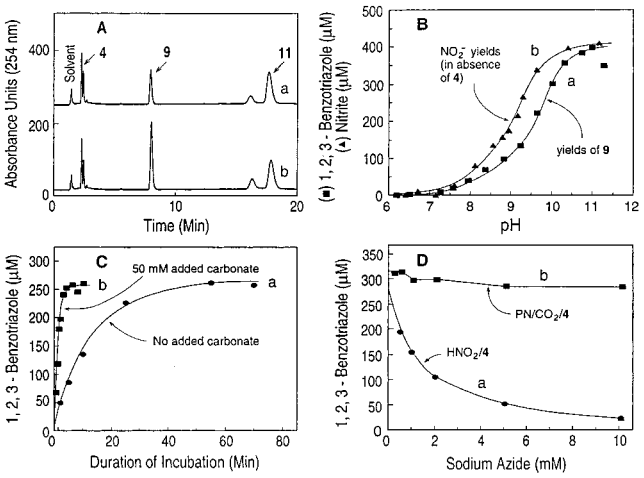

Figure 1. Nitrosation of **4** and the yields of NO_2^- in the PN/CO_2 and HNO_2 reactions at pH 3.7−11.3. (A) Reversed phase HPLC analysis of the products of PN/CO_2 reaction with **4**: (a) 2 mM PN plus 2 mM **4** in the presence of 20 mM added carbonate at pH 9.8; and (b), reaction mixture (a) spiked with 1,2,3-benzotriazole (0.28 mM, added after the decay of PN). (B) Effect of varying the pH (6.1−11.3) on the yields of **9** and NO_2^- in the PN/CO_2 reaction performed using 2 mM PN, 20 mM added carbonate, and (a) 0 or (b) 2 mM **4**. (C) Buildup of **9** in reactions of 2 mM PN with 2 mM **4** in the (a) absence or (b) presence of 50 mM added carbonate at pH 10.0. (D) Yields of **9** from the nitrosation of 2 mM **4** in the presence of 0.2−10 mM N_3^- in assay systems that use (a) 0.3 mM NO_2^- at pH 3.7 and (b) 2 mM PN plus 20 mM carbonate at pH 9.8. Assays at pH 6.1−11.3 performed in 2 mL of 0.1 M phosphate buffer that also contained 0.1 mM DTPA. Assays at pH 3.7 performed in 2 mL of 1.5 M acetate buffer. Analysis on 20-μL aliquots of reaction mixtures was performed using a Luna 5F C18 column (150 × 4.6 mm) and a mobile phase consisting of 0.1% CF_3CO_2H/30% MeOH in water at a flow of (A) 1 or (B−D) 1.5 mL/min. The eluent was monitored at 254 nm. Nitrite was estimated according to the Griess reaction,[8] and all samples, except C were analyzed after complete decomposition of PN.[8]

mainly gives rise to the biologically innocuous products NO_3^- and regenerated CO_2 (Scheme 1, eqs b−d, f, and i). A relatively small portion of the intermediates [which presumably serve as precursors for NO_2^- and O_2 (Scheme 1, eqs e−i)] are involved in oxidations, nitrations, and nitrosations. The data in Figure 1C demonstrate that CO_2 acts as a catalyst in the formation of reactive intermediates for nitrosation, since the buildup of **9** is much faster in reactions performed with added carbonate (curve b) than those that contain only adventitious carbonate (curve a).[7,14]

Both ionic and free radical mechanisms have been postulated to explain nitrosation of nucleophiles by PN/CO_2.[8] We find that azide ions inhibit nitrosation of **4** by nitrous acid (Figure 1D, curve a) but not by PN/CO_2 (curve b). The ability of N_3^- to inhibit nitrosation in the HNO_2 reaction indicates that azide can trap protonated nitrous acid (H_2O^+−NO) and/or N_2O_3. Since N_3^- does not inhibit the PN-mediated nitrosation, it is likely that NO^+-donors (including $ONONO_2$, ONO_2NO_2, and $ONOCO_2^-$) are not the nitrosating agents in the PN/CO_2/**4** system. We suggest the mechanism for nitrosation of **4** by PN/CO_2 involves an initial H-atom abstraction or one-electron oxidation from **4** by $CO_3^{\bullet-}$, followed by the reaction of the product **5** with $^\bullet NO$ (Scheme 2).[16,17] In our system, $^\bullet NO$ is formed from the dissociation of PN ($ONOO^- \rightleftharpoons {}^\bullet NO + O_2^{\bullet-}$; $k = 0.017\ s^{-1}$)[9] or the oxidation of $ONOO^-$ by $CO_3^{\bullet-}$ to give $ONOO^\bullet$ and $^\bullet NO + O_2$.[8] The continued formation of **9** in the presence of N_3^- suggests that $^\bullet N_3$, formed from the reaction of N_3^- with $CO_3^{\bullet-}$, oxidizes **4** to form **5**,[18] and that intermediate **7** is not scavenged by N_3^-.[19]

In biological milieu where $^\bullet NO$ occurs at levels[3,20] of 3×10^{-9} to 3×10^{-6} M, a likely mechanism for nitrosation is the oxidation of the target molecule (e.g., phenols, indoles, and thiols)[21] by $CO_3^{\bullet-}$, followed by the combination of the resulting organic radical ($R^\bullet$) with $^\bullet NO$. The autoxidation of $^\bullet NO$ to $^\bullet NO_2$ is extremely slow under physiological conditions,[5] and a likely source for $^\bullet NO_2$ in vivo is the dissociation of $ONOOCO_2^-$.[7,10,13] Thus, in biological milieu generally [$^\bullet NO$] > [$^\bullet NO_2$]. This, together with the fact that the rate constants for the reactions of $^\bullet NO$ and $^\bullet NO_2$ with most $R^\bullet$ are comparable,[22] suggests that the combination of $R^\bullet$ with $^\bullet NO$ is more likely than with $^\bullet NO_2$. Thus, by these one-electron mechanisms, in vivo nitrosations should be more common than nitration. The reason nitration,[7,14] but not nitrosation (Figure 1), predominates in vitro using bolus additions of PN at physiological pH could be the low concentrations of $^\bullet NO$ from the dissociation of $ONOO^-$ [9] and/or the oxidation of $ONOO^-$ by $CO_3^{\bullet-}$,[8] where $^\bullet NO_2$ is produced from the dissociation of $ONOOCO_2^-$.[7,10,13]

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(15) The maximum yield of nitrosation is somewhat lower than the cage-escaped free radicals from $ONOOCO_2^-$,[13] and one of the referees suggested that $O_2^{\bullet-}$ could lower the yields of **9** by reacting with the radical **5**. We believe that the products resulting from this radical−radical reaction are H_2O_2 and **6**; the latter could then condense with **4** to form **11** (Scheme 2). This explains why we find significant yields of **11** (in addition to **9**) in reactions of PN/CO_2 with **4** in our alkaline solutions (Figure 1A).

(16) In the absence of added carbonate (Figure 1C, curve a), the oxidation of **4** to **5** can be accomplished, in part, by $HO^\bullet$, the latter presumably is formed from the homolysis of ONOOH.[9]

(17) We do propose the formation of several kinds of NO^+-donors (e.g., N_2O_3, N_2O_4, and $ONOCO_2^-$) in the PN/CO_2 reaction in the absence of **4** or other reactive substrate, RH (Scheme 1, eqs e−g). In the presence of a sufficiently high concentration of RH, we suggest that $CO_3^{\bullet-}$ reacts with RH forming $R^\bullet$, which then reacts with $^\bullet NO$ (Scheme 2).

(18) Both $CO_3^{\bullet-}$ and $^\bullet N_3$ react with aromatic primary amines at comparable rates. For example see: Chen, S.-N.; Hoffman, M. Z.; Parsons, G. H., Jr. *J. Phys. Chem.* **1975**, *79*, 1911. Alfassi, Z. B.; Schuler, R. H. *J. Phys. Chem.* **1985**, *89*, 3359.

(19) The inhibitory effect of N_3^- on the PN/CO_2-mediated nitrosation of phenol[8] presumably results from scavenging of intermediates including phenyl nitrite and/or 4-nitrosocyclohexa-2,5-dienyl-1-one.

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