Spin Trapping Of Nitric Oxide (•NO) by *aci*-Nitromethane in Aqueous Solutions

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Nitric oxide (•NO) is a free radical that exhibits important cellular bioregulatory functions.[1,2] It is produced *in vivo* from arginine by the enzyme NO synthase.[2] Nitric oxide also plays an environmental role as an air pollutant. Recently, several different approaches have been described to detect and quantify •NO production by electron paramagnetic resonance (EPR) spectroscopy. These include formation of paramagnetic complexes with proteins and Fe^{2+},[3,4] reactions with nitronyl nitroxides,[5] trapping by nitrone and nitroso spin traps,[6] and reaction with a cheletropic compound tetramethyl-*o*-quinodimethane.[7]

We have recently shown[8] that in the presence of strong alkali (0.5 M NaOH) $•NO_2$ reacts with the *aci*-form of nitromethane (NM), $CH_2{=}NO_2^-$, to generate a characteristic, relatively stable spin adduct, $^-O_2N{-}•CH{-}NO_2^-$. In this communication we examine the ability of the *aci* form of NM to react with •NO in alkaline solutions and characterize the resultant adducts.

The EPR spectrum obtained by passing •NO gas[9] through an aqueous solution of 0.5 M NaOH containing NM is shown in Figure 1A. A simulated spectrum (not shown) of the adduct assuming coupling to two inequivalent nitrogen atoms and one hydrogen atom (Table 1) was in excellent agreement with the experimental spectrum , suggesting that the structure of the observed radical is $^-ON{=}CHNO_2^{•-}$. This radical could be derived from the primary spin adduct, $O{=}NCH_2NO_2^{•-}$, by dissociation of a single βH at high pH followed by a shift of the negative charge from the carbon atom to the oxygen atom, to give a radical dianion, $^-ON{=}CHNO_2^{•-}$ (eq 1). The EPR signal of this species

$$CH_2{=}NO_2^- + •NO \rightarrow O{=}NCH_2NO_2^{•-} \xrightarrow{-H^+} O{=}N\bar{C}HNO_2^{•-} \rightleftarrows {}^-ON{=}CHNO_2^{•-} \quad (1)$$

is very persistent (*vide infra*). No EPR signal was observed at pH 10 or below. However, the addition of strong base (2 M NaOH) to a sample purged with •NO at pH ca. 10, to raise the pH above 12, generated a strong EPR signal from $^-ONCHNO_2^{•-}$.

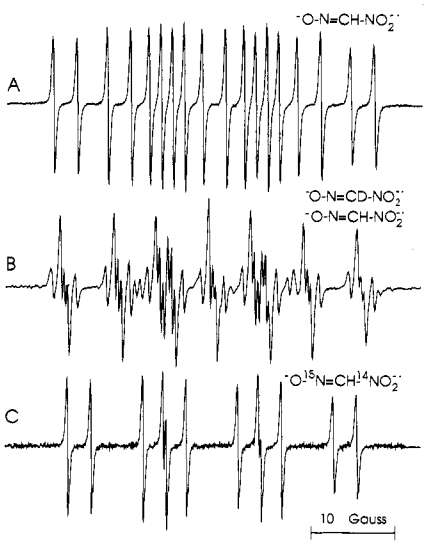

Figure 1. EPR spectra produced by passing •NO gas through deaerated solutions of nitromethane (50 mM) in 0.5 M NaOH (A) and 0.5 M NaOD (B). (C) Same as A but using ^{15}N-labeled nitric oxide.

Table 1. Hyperfine Splitting Constants of Spin Adducts Formed by Addition of •NO to *aci*-NM in NaOH (NaOD) (0.5 M) Solutions

	hyperfine splitting constants (G)		
radical adduct	a_N^{NO2}	a_N^{NO}	a_H^{β}
$^-O_2N{-}•CHNO_2^-$	9.65 (2N)[a]		4.12[a]
$^-ONCHNO_2^{•-}$	11.56	6.5	2.83
	11.58	9.12 (^{15}N)	2.83
$^-ONCDNO_2^{•-}$	11.58	6.48	0.43 (D)
$^-O_2CNO_2^{•-}$	14.38[b]		

[a] EPR spectrum of the $^-O_2N{-}•CHNO_2^-$ radical was produced by UV photolysis of $NaNO_2$ in 0.5 M NaOH in the presence of NM.[8] [b] From ref 13.

Similarly, the weak signal observed when NM solutions were gassed with •NO in the pH range 10–11 intensified markedly upon further alkalinization to pH >12.

The EPR spectrum observed when the experiment was repeated in alkaline (0.5 M NaOD) D_2O solution (Figure 1B) contained contributions from $^-ONCHNO_2^{•-}$ and a second adduct, exhibiting a single deuterium splitting, which was identified as $^-ONCDNO_2^{•-}$. A simulated spectrum using hyperfine splitting constants (hfsc's) (Table 1) for these two adducts indicated a relative concentration ratio of 15:85. The a_N^{NO2} splittings in these two radicals were identical, indicating that this parameter is not affected by deuteration. Gassing of NM in NaOH solution (0.5 M) with ^{15}N-labeled nitric oxide produced the spectrum shown in Figure 1C, in which the triplet from splitting on ^{14}N in the ^{14}NO adduct (Figure 1A) has been replaced by a doublet due to splitting on ^{15}N in the ^{15}NO adduct.

We investigated the development and stability of the NM adduct with •NO. A strong EPR signal from $^-ONCHNO_2^{•-}$ in 0.5 M NaOH was observed less than 1 min after gassing with •NO. Kinetic measurements showed that the signal intensity doubled during the first 2 h of incubation and then decreased. After 16 h signal intensity was approximately equal to that of the initial signal and showed the presence of only one radical, $^-ONCHNO_2^{•-}$.

We have already mentioned that the EPR signals of samples prepared at pH <11 and then alkalinized increased markedly with time. The question arises as to whether this increase is due to degradation of the spin trap, slow trapping of the remaining •NO, or base-catalyzed transformation of products formed at pH <11. The first possibility was readily eliminated because in a sample prepared in 0.5 M NaOH using the ^{15}N-labeled •NO, and left for several hours, only the $^-O^{15}NCHNO_2^{•-}$ radical was

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(9) Before use, nitric oxide (98.5%, Aldrich Chemical Co., Milwaukee, WI) was passed through a saturated NaOH solution to remove acidic nitrogen oxide impurities. Samples for EPR experiments were prepared by first passing N_2 (2 min) through solutions (50 mM) of NM, then •NO (1–2 min) with N_2 above the sample. In some experiments N_2 was bubbled again (1 min) to remove unreacted •NO. When buffers were used, their pH was constantly monitored during gassing. These precautions were necessary because •NO, in contact with O_2 and water, causes strong acidification, which may hinder both spin adduct formation and EPR detection.

observed. This indicates that the adduct is derived entirely from the •NO gas and that degradation of the spin trap in 0.5 M NaOH cannot be a source of •NO radicals. The signal increase also cannot be due to the trapping of •NO by *aci*-NM because unreacted •NO had been removed by gassing the sample with N_2 prior to the EPR measurements.

The remaining possibility is that the spin adducts are slowly being generated from nonradical precursors[10] formed at lower pH. These precursors are likely to be formed from the primary adduct, $O{=}NCH_2NO_2^{\bullet-}$, by the addition of a second molecule of •NO. The primary adduct contains the nitroso moiety and therefore can itself function as a spin trap. These processes are currently under investigation in our laboratory.

We employed two different approaches to determine whether •NO radicals accumulated in solutions at near physiological pH could be detected using nitro compounds. We used (i) α-nitrotoluene, $PhCH_2NO_2$, whose *aci* form has a pK_a of 6.8 (ref 11), and (ii) NM in strong base to which a •NO-saturated pH 7 buffer was added. When solutions of α-nitrotoluene, in pH 7–12 buffers or in 0.5 M NaOH, were gassed with •NO, no EPR signals were observed. The inability of α-nitrotoluene to form a stable radical adduct may indicate that, unlike NM, deprotonation of the HC(NO) group in $PhHC(NO)NO_2^{\bullet-}$ does not occur, presumably due to the stabilizing effect of the adjacent aromatic ring. In the second approach we gassed an air-free pH 7.0 solution (25 mM phosphate buffer) with •NO (5 min) and then mixed with an equal volume of 0.5 M NaOH containing NM (50 mM). EPR measurements (not shown) clearly indicated the presence of the $^-ONCHNO_2^{\bullet-}$ radical adduct. This experiment demonstrates the feasibility of using NM to detect •NO produced at physiological pH.

A characteristic feature of the *aci*-NM adduct with •NO is its markedly smaller a_N^{NO2} value,[12] 11.56 G, when compared to adducts with other radicals, which are in the range 22–26 G.[14,15] Although the $^-ON{=}CHNO_2^{\bullet-}$ adduct shares this feature with other nitroalkyl radicals, $^-O_2N{-}^{\bullet}CHNO_2^-$ and $^-O_2CNO_2^{\bullet-}$ (see Table 1), its EPR spectrum possesses a unique splitting pattern that is different from that of the adduct with the $^{\bullet}NO_2$ radical.[8,14] We never observed the latter adduct when detecting •NO. The $^-ONCHNO_2^{\bullet-}$ radical was not oxidized to $^-O_2NCHNO_2^{\bullet-}$ during gassing with O_2, nor did the reaction of •NO with oxygen in the presence of *aci*-NM generate the $^-O_2NCHNO_2^{\bullet-}$ radical.[16]

Our results demonstrate that *aci*-NM can be a useful analytical probe for the EPR detection of nitric oxide radicals in aqueous solutions. The EPR spectra of the resultant adducts are of high intensity and possess characteristic hfsc's which facilitate radical identification. While •NO does not need to be generated in alkaline solutions, alkaline conditions are needed to produce the *aci*-nitro forms of the spin traps and to deprotonate the primary spin adduct. The latter process requires an especially high pH but yields very persistent spin adducts. It is hoped that design of •NO traps based on nitro compounds possessing more acidic β-methylene hydrogens may make it possible to trap the •NO radicals produced *in situ* at near neutral pH.

(10) It is known that •NO forms unstable complexes with certain nucleophiles (Hrabie, J. A.; Klose, J. R.; Wink, D.; Keefer, L. K. *J. Org. Chem.* **1993**, *58*, 1472–1476). Formation of similar complexes with *aci*-nitroalkanes has not been reported and is currently under investigation in our laboratory.

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(12) This small splitting on nitrogen of the nitro group may indicate that the nitro group π-electron system is strongly coupled to the π-electron system of the rest of the molecule.[13] Such coupling may occur only in adducts with dissociated βH.

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(16) This observation strongly suggests that free $^{\bullet}NO_2$ radical is not an intermediate during autoxidation of •NO in aqueous solution.