Decomposition of Sodium Trioxodinitrate (Angeli's Salt) To Hydroxyl Radical: An ESR Spin-Trapping Study

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There is much interest on the preponderant redox form of nitric oxide under physiological conditions. Despite the controversy whether nitric oxide synthase catalyzes the synthesis of nitroxyl anion (NO^-) or nitric oxide ($NO^•$),[1,2] various mechanisms for production of NO^- that may operate under physiological conditions have been reported. Superoxide dismutase can convert $NO^•$ to NO^- [3] and S-nitrosothiols, a class of compounds implicated as playing key roles in a variety of physiological processes, can react with thiols to generate NO^-.[4] In model studies aimed to mimic the biochemistry of NO^-, sodium trioxodinitrate ($Na_2N_2O_3$, Angeli's salt, AS) is often used as a specific NO^- donor.[1,5] The hydrolysis of AS is a complex process that may trigger reactions of alkylation, condensation, and oxidation[6] by mechanism(s) not well understood and which cannot be correlated only with the hydrolysis of AS to NO^-. In this report, we present ESR spin-trapping data which suggest that AS undergoes a pH-dependent decomposition to hydroxyl radical.

The Angeli−Rimini test for aldehydes is based on the AS-dependent formation of hydroxamic acids.[7] The reaction is mechanistically believed to proceed via addition of the hydroxylamine nitrogen of the trioxodinitrate anion to the carbonyl carbon atom of the aldehyde; a subsequent release of NO_2^- and a tautomerization of the CH−N═O structure of the resulting α-nitroso alcohol forms the corresponding hydroxamic acids, which can be detected spectrophotometrically after chelation with $FeCl_3$.[6,7] Depending on the degree of protonation, the stability of AS in aqueous solutions follows the sequence $N_2O_3^{2-} \rightarrow HN_2O_3^- \rightarrow H_2N_2O_3$ ($pK_1 = 3.0$ and $pK_2 = 9.35$[8]). AS is relatively stable in alkaline solutions; its rate of decomposition within the pH interval 3.5−8.5 is rapid and $[H^+]$-independent, proceeding via an intermediate formation of NO^-, and NO_2^- and N_2O as end products. At lower pH values, the decomposition rate increases with increasing acidity with production of $NO^•$.[8] While the structure of AS has been determined to be $[ON{=}NO_2]^{2-}$, there is uncertainty over the exact mechanism(s) of its decomposition in aqueous solutions. Studies with $[^{15}N_2O_3]^{2-}$ have suggested that a nitrite anion is released via N═N cleavage of the nitrogen that is bound to two oxygen atoms.[9] At pH > 4 AS is decomposed, depending on the site of protonation, to either HNO ($pK_a = 4.7$) and NO_2^-, or to NO^- and HNO_2.[8,9] AS can be stabilized by sodium nitrite, presumably due to a recombination of HNO with NO_2^- to $HN_2O_3^-$;[8] at pH < 4, however, sodium nitrite accelerates the decomposition of AS via a HNO_2-dependent consumption of $H_2N_2O_3$.[8] The rate of AS decomposition at pH values less than 3 decreased in the presence of ethanol, suggesting that the hydrolytic process may proceed via a $HO^•$-dependent rate-limiting step.[8] Thermal decomposition of AS in aqueous solutions generates peroxynitrite anion ($ONOO^-$) via the formation of singlet NO^-; upon relaxation of singlet-state NO^-, a triplet state NO^- is formed that reacts with oxygen to form $ONOO^-$.[10]

The physiological effects of NO^- are associated with its potential to selectively oxidize low-molecular weight and protein thiols via a nonradical mechanism, and to form complexes with heme proteins.[1] However, in ESR spin-trapping experiments with

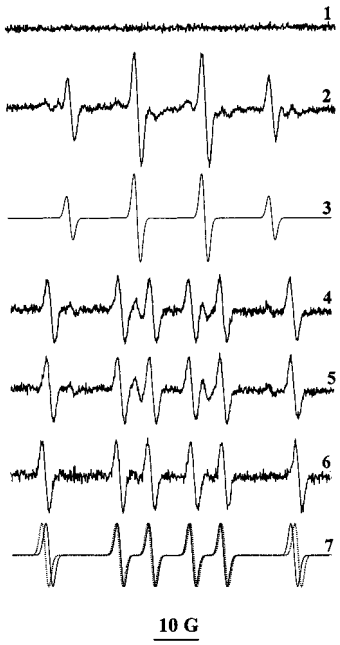

Figure 1. ESR spectra of DMPO nitroxides formed in a solution of AS. ESR measurements and spectra simulations were performed as described in ref 11. DMPO was used at a concentration of 0.1 M in phosphate buffer (pH 7.4, 25 °C). The hyperfine splitting constants (in G) used for simulation of the spectra of the DMPO/$^•$OH, DMPO/HER, and DMPO/$^•CH_3$ nitroxides were as follows: ($A_N = 15.0$; $A_H = 15.0$), ($A_N = 15.8$; $A_H = 22.8$) and ($A_N = 16.3$; $A_H = 23.2$), respectively (11). AS was synthesized as described in (6). All ESR spectra were recorded after 2 min of incubation of the reaction solutions. Spectra **1**, no addition; spectra **2**, plus AS (0.1 mM; a stock solution of AS was prepared in 0.2 M NaOH); spectra **3**, computer simulation of the ESR spectra of DMPO/$^•$OH; spectra **4**, plus ethanol (0.5 M) and AS; spectra **5**, ethanol and AS were added after deoxygenation of all solutions with N_2 for 45 min; spectra **6**, plus DMSO (0.3 M) and AS; trace **7** represents computer simulation of the ESR spectra of DMPO/HER (solid lines) and DMPO/$^•CH_3$ (dashed lines). The ESR spectra in repetitive experiments did not differ more than 10% ($n \geq 3$).

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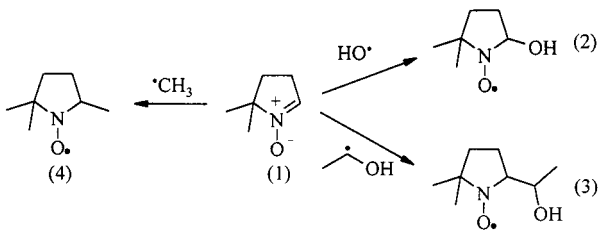
Scheme 1



AS-loaded murine macrophage cells, we observed the formation of radical intermediates (data not shown). This led us to study whether the hydrolysis of AS is associated with the generation of HO•. Figure 1.2 depicts the ESR spectra of a cell-free solution of AS in 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO, **1**; Scheme 1)-containing phosphate buffer (pH 7.3). The appearance of a four-line ESR spectra with hyperfine structure (in G) of $A_N = 15.0$ and $A_H = 15.0$ allows the assignment of the adduct as that formed by addition of HO• to DMPO (Scheme 1, **2**;).[11] Catalase (500−2500U/ml) and superoxide dismutase (30−3000 U/ml) did not affect the AS-dependent formation of DMPO/•OH, suggesting that H_2O_2 and superoxide anion are not involved in this production. A maximal rate of DMPO/•OH production was observed at pH 5 (Figure 2A). The process had a bi-phasic kinetic profile with increasing values over a time period of 10 min. The maximal, steady-state spin concentration of the DMPO/•OH nitroxide was 5.4 μM as determined by double integration of the ESR signal using 4-hydroxyl-1-TEMPO as a standard.[12]

Ethanol and dimethyl sulfoxide (DMSO) are oxidized by HO• to 1-hydroxyethyl radical (HER[11]) and methyl radical, respectively;[13] the hydrolysis of AS in the presence of DMPO and either ethanol or DMSO produced a typical ESR spectra of the DMPO/HER or DMPO/•CH_3 nitroxide (Scheme 1, **3** and **4**, respectively; Figure 1, traces 4 and 5: $A_N = 15.8$ G, $A_H = 22.8$ G; trace 6: $A_N = 16.3$ G, $A_H = 23.2$ G):

AS can generate $ONOO^-$ under aerobic conditions which may affect the ESR spin-trapping measurements (ref 14 and the references therein). Removal of oxygen from the reaction solutions, however, did not decrease the ESR spectra of DMPO/HER (trace 5 compared to trace 4), suggesting that the formation of the nitroxide follows a HO•- rather than a $ONOO^-$-dependent abstraction of α-hydrogen atom from ethanol. The AS-dependent generation of HO• was further supported by determination of the rate constant for the interaction of HO• with DMPO as described in ref 15; we obtained a value of 3.52×10^9 M^{-1} s^{-1}, which is in good agreement with the literature values of $2.1-3.4 \times 10^9$ M^{-1} s^{-1}.[15]

HER has a short lifetime and, after an electron transfer to oxygen, is converted to acetaldehyde. Figure 2B illustrates the accumulation of acetaldehyde in an ethanol-containing solution of AS. It is likely that in this system a considerable part of the generated acetaldehyde reacts with AS to produce acetyl hydroxamic acid (Scheme 2). In support of the latter assumption are the results from the $FeCl_3$ color reaction for hydroxamic acids (Angeli−Rimini test); an addition of $FeCl_3$ into AS-containing solutions of either ethanol or acetaldehyde led to qualitatively equal spectral changes within the visible range (Figure 2C, spectra 2 and 3, respectively). No $FeCl_3$-dependent changes were observed in the absence of ethanol or acetaldehyde (Figure 2C, spectrum 1).

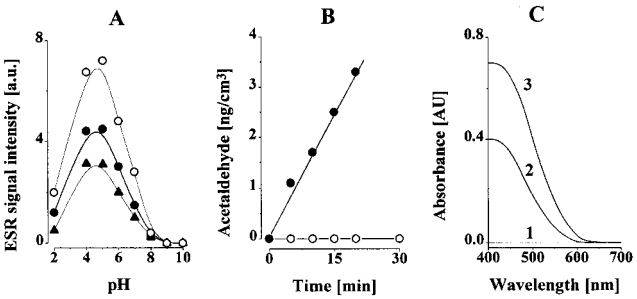
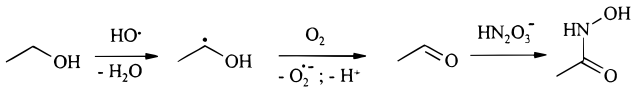
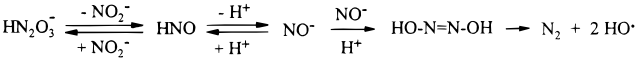


Figure 2. pH-dependent hydrolysis of AS to hydroxyl radical. A. ESR signal intensity of AS in DMPO-containing phosphate buffer as a function of pH. (●) AS; (○), AS plus NaCl (1 M); (▲), AS plus $NaNO_2$ (2 mM). B. GC-monitored accumulation of acetaldehyde in AS (0.3 mM)- and ethanol (0.5 M)-containing phosphate buffer (0.1 M, pH = 5). GC (Hewlett-Packard 5840A gas chromatograph) measurements were conducted as described in ref 17; (●) plus AS; (○) minus AS. C. Effects of $FeCl_3$ (0.02 mM) on the visible spectra of a solution of AS (0.3 mM, spectra **1**), AS plus ethanol (0.5M, spectra **2**), and AS plus acetaldehyde (0.01 M, spectra **3**) in 0.1 M phosphate buffer (pH = 5). The reaction solutions were kept for 4 min at 20 °C prior to the addition of $FeCl_3$.

Scheme 2



Scheme 3



Several mechanisms may account for the AS-dependent generation of HO•. It is anticipated that NO^- can dimerize to *cis*-hyponitrous acid ($H_2N_2O_2$[8]), which is unstable and decomposes via a chain-reaction mechanism that is most likely initiated by an azo-type homolytic fission (HO−N═N−OH → N_2 + 2 HO•[16]). The ability of NaCl (but not $NaClO_4$; data not shown) to stimulate the production of DMPO/•OH in AS-containing solutions suggests that $H_2N_2O_2$ is a potential source of hydroxyl radical (Figure 2A); NaCl has been shown to catalyze the decomposition of $H_2N_2O_2$.[16] Contrary to NaCl, $NaNO_2$ inhibited the AS-dependent production of DMPO/•OH (Figure 2A), presumably by decreasing the concentration of $H_2N_2O_2$ via displacing the equilibrium between $HN_2O_3^-$, HNO, and the endogenous NO_2^- in favor of $HN_2O_3^-$ [8] (Scheme 3):

At certain pH values, the AS-dependent generation of HO• may reflect the occurrence of more complex reactions,[8] or as a reviewer has pointed out, HO• can be preceded by an electron exchange between $HN_2O_3^-$ and $H_2N_2O_3$ (or $N_2O_3^{2-}$ and $HN_2O_3^-$).

Independently of the exact mechanism(s), two immediate implications of the AS-dependent generation of HO· may be envisaged: (1) Contrary to NO^-, hydroxyl radical is a species that interacts indiscriminately, in a diffusion-controlled manner with low-molecular weight compounds, lipids, and proteins present in cells. In AS solutions, the formation of HO• parallels the formation of NO^-, suggesting that the use of Angeli's salt as a NO^- donor in biological systems should be accompanied by appropriate controls for discrimination of NO^-- from HO•-dependent effects, and (2) AS can convert primary alcohols to the corresponding hydroxamic acids, extending in this way the application of Angeli−Rimini test to the detection of alcohols.

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