AQUEOUS NITRIC OXIDE AUTOXIDATION:
EVIDENCE AGAINST THE PRODUCTION OF NITROGEN DIOXIDE

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Abstract

Nitric oxide (NO) oxidation occurs spontaneously in aerobic environments and potentiates the detrimental effects of NO production in the body. This autoxidation reaction can form reactive nitrogen oxygen species, capable of oxidizing and nitrosating proteins, lipids, and DNA, potentially leading to tissue damage, disease, and cancer. Autoxidation of NO in the gas phase is well-characterized, and produces such intermediates, particularly nitrogen dioxide (NO$_2$) and dinitrogen trioxide (N$_2$O$_3$). The mechanism of NO autoxidation in aqueous media has commonly been assumed to be analogous to the gas phase reaction, with the extension of N$_2$O$_3$ hydrolysis to form nitrite. We propose that NO autoxidation within cells occurs through a mechanism that is fundamentally different from the gas phase reaction, producing variants of the above intermediates that possess distinct nitrosative and oxidative capabilities. Although the rate constant for aqueous NO autoxidation has been determined to be similar to the gas phase reaction, results support a unique mechanism. Using competitive kinetics techniques, new insight into the mechanism of NO autoxidation has been achieved. The characterization of aqueous phase NO autoxidation is thus critical to fully understanding the pathophysiology of NO in biological systems.
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1 Introduction

Many organisms biosynthesize NO as a ubiquitous agent for signaling, blood pressure regulation, and immune response. Nitrogen oxides can also be significant toxins, whether as air pollutants or as potentiators of DNA mutation and cancer [1]. NO autoxidation is a fundamental reaction in aerobic environments that may generate reactive nitrogen oxide species (RNOS) capable of inducing deleterious modifications. Gas and organic phase NO autoxidation is well-characterized; formation of N$_2$O$_3$ occurs via the intermediacy of NO$_2$ [2]. It is commonly assumed that aqueous NO autoxidation parallels the gas phase reaction, with the additional step of N$_2$O$_3$ hydrolysis to nitrite [3].

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow 2\text{NO}_2 \\
\text{NO} + \text{NO}_2 & \leftrightarrow \text{N}_2\text{O}_3 \\
\text{N}_2\text{O}_3 + \text{H}_2\text{O} & \rightarrow 2\text{NO}_2^- + 2\text{H}^+ \tag{1}
\end{align*}
\]

However, quenching studies using reductants such as ferrocyanide have suggested a media-dependent mechanism of NO autoxidation [4]. It is our hypothesis that NO$_2$ is not formed appreciably in aqueous media, and that NO autoxidation within cells occurs through a mechanism that is fundamentally different from the gas phase reaction, producing variants of the above intermediates that possess distinct nitrosative and oxidative capabilities. To
fully understand the deleterious chemistry of NO in biological systems, full characterization of NO autoxidation in various environments is critical.

Due to the bimolecular dependence on NO, aqueous NO autoxidation may be too slow to lead to higher nitrogen oxides in biological systems. However, the reaction can be accelerated by diffusion into membranes where both NO and $O_2$ are more soluble [5]. Aqueous NO autoxidation may also occur during inflammation, when high fluxes of NO are released [6]. To fully understand NO autoxidation, aqueous phase oxidation, nitrosation, and nitration must be understood in reference to membrane phase chemistry. Spectroscopic analysis of the intermediates is challenging at best, but small-molecule trapping agents can be used to selectively probe the intermediates.

Membrane phase NO autoxidation can be effectively mimicked in water by conversion of NO directly to NO$_2$ using a chemical potentiator. 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) [7], a nitronyl nitroxide spin-trap agent, converts NO directly to NO$_2$ in an oxygen transfer reaction [4].

$$\text{NO} + \text{PTIO} \rightarrow \text{NO}_2 + \text{PTI} \quad (2)$$

We have utilized several assays to investigate the kinetics and thermodynamics of aqueous NO autoxidation as well as the identity and potency of the nitrosating and oxidizing intermediates. A series of fluorophores, chromophores, scavengers, and chemical donors of NO were used to investigate
the intermediates of NO autoxidation.
2 Experimental

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich and used without further purification, unless otherwise specified.

2.2 Production of NO and RNOS

Amine-based diazeniumdiolates (NONOates) were used as NO donors because their rate of decomposition is a function of the amine component, dependent only upon pH and temperature [8]. Diethylamine NONOate (DEA/NO) and spermine NONOate (SPER/NO), which have half-lives of 2.5 and 37 min at pH 7.4 and 37°C [8], can be used in this regard.

\[
\text{DEA/NO} + \text{H}^+ \rightarrow 2\text{NO} + \text{NH}_2\text{Et}_2
\]  

(3)

2.3 Instrumentation

UV-visible spectroscopy was performed with a Hewlett-Packard 8452A diode-array spectrophotometer. Fluorescence spectroscopy was performed with a AMINCO-Bowman Series 2 spectrometer.
2.4 Fluorometric Assays

In PBS (pH 7.4), PTIO, 2,3-diaminonaphthalene (DAN), or tyrosine, and DEA/NO were added in order for a total solution volume of 1 mL. The samples were covered and incubated at 37°C for 1 h and their volume doubled with 1 mL of 10 mM NaOH for DAN assays and 1 mL of water for tyrosine assays. For DAN assays, excitation and emission wavelengths were 363 and 420 nm, respectively with a 4 nm bandpass and PMT voltage of 575 V. Similarly, for tyrosine assays, excitation and emission wavelengths were 275 and 300 nm, respectively with a 4 nm bandpass and PMT voltage of 510 V. The PMT voltage and emission scale were set such that the most fluorescent solution of each experiment was at 60% of the instrument’s detection maximum. Experiments were performed in triplicate.

2.5 Colorometric Assays

NONOates react directly with 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and cannot be used for the oxidative assay. Stock solutions of NO were used instead. A sealed Wheaton vial with 10 mL of 100 mM PBS was dearated with argon for 30 min. NO was then passed through this solution after passing through two spargers containing 1 M NaOH and 10 mM NaOH. The concentration of this NO solution was determined by ABTS oxidation ($\epsilon_{660} = 12,000 \text{ M}^{-1}\text{cm}^{-1}$) [4]. In 970 µL, 940 µL, and 910 µL of 5 mM ABTS, 30, 60, and 90 µL of NO solution was added via Hamilton
glass syringe while simultaneously vortexing. The absorbance values were measured at 660 nm immediately after injection.
3 Results

3.1 Nitrosation

Nitrosation of DAN results in formation of a fluorescent triazole, providing a sensitive fluorescent marker for nitrosation [9].

\[ \text{N}_2\text{O}_3 + \text{DAN} \rightarrow \text{NO}_2^- + \text{NAT} + \text{H}^+ \]  \hspace{1cm} (4)

Under complete trapping conditions the reaction in the aqueous phase is

\[ 4\text{NO} + \text{O}_2 + 2\text{DAN} \rightarrow 2\text{NO}_2^- + 2\text{NAT} + 2\text{H}^+ \]  \hspace{1cm} (5)

and in the gas phase

\[ \text{NO} + \text{PTIO} + \text{DAN} \rightarrow \text{NO}_2^- + \text{NAT} + \text{PTI}. \]  \hspace{1cm} (6)

The conditions for maximum nitrosation of DAN in Eqn. (6) were investigated using varying amounts of PTIO (Fig. 1). Exposure of DEA/NO to DAN and varying PTIO forms a bell-shaped curve. Without PTIO, the reaction proceeds solely through Eqn. (5). As PTIO is titrated, the pathway shifts towards Eqn. (6), leading to a peak at \( \approx 5 \mu \text{M} \) PTIO where nitrosation dominates. This peak corresponds to the optimal concentration of PTIO to produce maximum nitrosative yield, and was used in later DAN assays. A decrease in the nitrosation of DAN occurs when stoichiometry favors NO
Figure 1: Nitrosation of DAN by PTIO potentiation. DEA/NO (5 μM, solid), DAN (5 μM), and PTIO (0-20 μM) were incubated in PBS (pH 7.4, 50mM DTPA) for 1 h at 37°C. Volumes were doubled with 10 mM NaOH after incubation, and the triazole yield measured.

consumption by PTIO, limiting N$_2$O$_3$ yield. It is seen that in the absence of PTIO nitrosation does occur, but the nitrosating agent is less potent than N$_2$O$_3$ from the mimicked gas phase reaction.

The difference in nitrosation kinetics between the two phases was quantified using a Michaelis-Menten kinetic assay at fixed DEA/NO and PTIO concentrations and varying DAN concentrations. The DAN concentrations were spaced linearly in reciprocal-space so that the reaction kinetics that vary most with changes in [DAN] would be oversampled (Fig. 2), providing a detailed profile of the reaction kinetics.

A Lineweaver-Burk analysis provides a convenient way to quantify this
Figure 2: Nitrosation of DAN as a function of its concentration. DEA/NO (5 μM), ± PTIO (5 μM), and DAN (0-20 μM), were incubated for 1 h at 37°C. Volumes were doubled with 10 mM NaOH after incubation, and the triazole yield measured.

difference. The $X_M$ constant, determined from the $-X^{-1}_{int}$, will be equal to

$$X_M = [NO] \frac{k_{hyd}}{k_{DAN}},$$

(7)

where $k_{hyd}$ is the rate constant of hydrolysis of $\text{N}_2\text{O}_3$, and $k_{DAN}$ is the rate constant of DAN nitrosation by $\text{N}_2\text{O}_3$. A double-reciprocal plot of the NAT yield versus [DAN] allows for the determination of $X_M$ in the presence and absence of PTIO. A 14-fold difference is found in $X_M$ values (Fig. 3). If the autoxidation intermediates were the same, it would be expected that the $X_M$ values would be the same. This stark difference indicates a fundamental difference in the mechanism of aqueous NO autoxidation.
Figure 3: Lineweaver-Burke analysis of DAN nitrosation. $X_M$ values were determined to be 2.2 and 28.0 $\mu$M in the presence and absence of PTIO, respectively.

To qualitatively probe the intermediates for nitrosation and oxidation in the aqueous phase, small molecule kinetic traps were introduced to scavenge $\text{NO}_2$ and $\text{N}_2\text{O}_3$. Reduced glutathione (GSH) is a ubiquitous physiological antioxidant. The thiol scavenges both $\text{NO}_2$ as an oxidant and $\text{N}_2\text{O}_3$ as a nitrosant [10].

\[
\text{GS}^- + \text{NO}_2 \rightarrow \text{GS}^- + \text{NO}_2^-
\]

\[
\text{GS}^- + \text{N}_2\text{O}_3 \rightarrow \text{GSNO} + \text{NO}_2^-
\] (8)

In addition, the chemical reagent azide reacts selectively with $\text{N}_2\text{O}_3$.

\[
\text{N}_3^- + \text{N}_2\text{O}_3 \rightarrow \text{N}_2\text{O} + \text{NO}_2^- + \text{N}_2
\] (9)
Figure 4: Trapping of DAN nitrosation by scavengers. DEA/NO (5 μM), DAN (20 μM), PTIO (5 μM, right), and scavengers (10-1000 μM) were incubated for 1 h at 37°C. Volumes were doubled with 10 mM NaOH after incubation, and the triazole yield was measured.

GSH does not show a difference in scavenging profile for NO autoxidation intermediates in the presence or absence of PTIO due to unspecificity for scavenging oxidating or nitrosating intermediates (Fig. 4). However, there is a varied capacity of $N_3^-$ to inhibit DAN nitrosation, further emphasizing distinct nitrosating species between the two phases. Trapping with the antioxidant ascorbate (Fig. 5) shows no difference in scavenging profile.

Urate, a potent antioxidant in blood plasma [10], may selectively reduce NO$_2$ due to its high oxidation potential.

$$\text{NO}_2 + \text{UH}_2^- \rightarrow \text{UH}^- + \text{H}^+ + \text{NO}_2^-$$

(10)

Due to low-solubility in neutral buffer, ten-fold lower concentrations of urate were used in determining its scavenging profile of DAN nitrosation. In the
Figure 5: Trapping of DAN nitrosation by ascorbate. DEA/NO (5 μM), DAN (20 μM), PTIO (5 μM, right), and ascorbate (10-1000 μM) were incubated for 1 h at 37°C. Volumes were doubled with 10 mM NaOH after incubation, and the triazole yield measured.

presence of PTIO, urate completely quenched nitrosation of DAN at maximal urate concentration (Fig. 6). However, under aqueous conditions, no scavenging occurs. This finding significantly emphasizes the lack of free NO₂ produced by aqueous NO autoxidation kinetics.

3.2 Oxidation

The oxidative profile of NO₂ can be probed by monitoring its one-electron oxidation reaction with ABTS. ABTS is useful as an indicator of oxidants, for its cation radical, ABTS⁺, absorbs distinctly between 400-800 nm. The rate of formation of ABTS⁺ corresponds to NO autoxidation, indicating
that oxidation occurs subsequent to the rate-limiting step. ABTS and NO thus compete for NO₂⁻, allowing for comparison between measured ABTS⁺ formation and calculated values obtained by reported rate constants. Assuming NO₂⁻ to be the sole oxidant present, the yield of nitrite would depend only on this competitive association with NO or ABTS. Double reciprocal plots of ABTS⁺ yield against varied ABTS concentrations at constant NO were analyzed (Lineweaver-Burk analysis). In parallel to probing the nitrosation kinetics, the oxidation kinetics are observed between two pathways involving aqueous kinetics and membrane phase kinetics:

\[
2 \text{NO} + \text{O}_2 + 2 \text{ABTS} \rightarrow 2 \text{NO}^- + 2 \text{ABTS}^+ \quad (11)
\]
Figure 7: Oxidation of ABTS; effect of PTIO potentiation. A bolus amount of NO solution (45 μM) was added to ABTS (0-2 mM), PTIO (0-200 μM) in PBS 7.4 at room temperature. Absorbance of ABTS$^+$ was monitored at 660 nm.

for the aqueous phase reaction, and

$$\text{NO} + \text{PTIO} + \text{ABTS} \rightarrow \text{NO}_2^- + \text{ABTS}^+ + \text{PTI}$$ (12)

for the membrane phase. Thus, ABTS provides a colorimetric probe for oxidation. A Lineweaver-Burk analysis again provides a quantitative method of comparing NO autoxidation kinetics. The $X_M$ value is

$$X_M = [\text{NO}] \frac{k_{\text{hyd}}}{k_{\text{ABTS}}},$$ (13)

where $k_{\text{hyd}}$ is the rate constant of hydrolysis of $\text{N}_2\text{O}_3$ , and $k_{\text{ABTS}}$ the rate constant of ABTS oxidation. Figure 7 shows that as PTIO is titrated, the $X_M$ value decreases. This shows that the oxidant produced in the aqueous
profile is much weaker than NO$_2$, now excluded from the aqueous profile. It, too, enhances the idea that N$_2$O$_3$ may be a capable oxidant.

3.3 Nitration

Tyrosine residues in proteins are be nitrated by N$_2$O$_3$ in vivo. Studies have shown the correlation between nitrated tyrosine residues and Alzheimer’s disease [11]. Thus, it is critical to elucidate the nitration profile of NO autoxidation intermediates under aqueous and membrane phase.

3-nitrotyrosine (NT) fluoresces very weakly [12], while tyrosine (Tyr) will fluoresce near 300 nm upon excitation at 275 nm. Thus, the decrease in tyrosine fluorescence may be used as a marker for nitration with the following scheme:

$$2\text{NO} + \text{O}_2 + 2\text{Tyr} \rightarrow 2\text{NT}$$  \hspace{1cm} (14)

for the aqueous phase reaction, and

$$\text{NO} + \text{PTIO} + \text{Tyr} \rightarrow \text{NT} + \text{PTI}$$  \hspace{1cm} (15)

for the membrane phase. The reaction kinetics are analyzed qualitatively by holding DEA/NO and PTIO constant while varying Tyr concentration. The
Figure 8: Nitration of tyrosine by PTIO potentiation. DEA/NO (5 μM), tyrosine (0-10 μM), and PTIO (0, 10, 20 μM) were incubated in PBS (pH 7.4, 50 mM DTPA) for 1 h at 37°C. Volumes were doubled with H₂O after incubation, and change in tyrosine signal measured at λ_em = 300 nm upon excitation at 275 nm.

low concentration region is again oversampled to obtain a detailed profile of the reaction kinetics. In the presence of 20 μM PTIO, tyrosine is clearly nitrated, and in absence of an NO₂ source, nitration does not occur. This result shows that nitration chemistry in vivo is likely driven by membrane phase chemistry. A Lineweaver-Burk analysis could not be performed due to only minimal changes in signal in the aqueous phase reaction.
4 Discussion

There are several results that support the hypothesis that free NO\textsubscript{2} is not produced in aqueous solutions. The first is the asymmetry of the PTIO peak test. The second is the scavenging studies with urate. If free NO\textsubscript{2} was produced from aqueous autoxidation, urate would scavenge it, as is seen in the membrane phase reaction. The third is the ABTS oxidation profile of titrated PTIO. If the oxidizing intermediate were the same, the same $X_M$ value would be found for each PTIO concentration. Finally, it is seen that aqueous autoxidation in the presence of tyrosine does not produce significant nitrotyrosine, though the mimicked membrane phase reaction does. Therefore, NO\textsubscript{2} is not produced by aqueous autoxidation.

Similarly, multiple results support the hypothesis that there is a distinct nitrosating agent produced in aqueous solutions, which is verified here. The first indication is the DAN nitrosation PTIO profile. Had the nitrosating agent been the same, it would be expected that the triazole yield would not be significantly different between the aqueous and membrane phase intermediates. Second, Lineweaver-Burk analysis quantifies this difference (Fig. 3). It is seen that the $X_M$ value is 14-fold different between the two phases. Quenching studies of DAN nitrosation with azide in the presence of absence of PTIO shows a different profile. Because azide is N\textsubscript{2}O\textsubscript{3} selective, this suggests further a difference in the nitrosating intermediate between the aqueous and membrane phase autoxidation.
What are the identities of the RNOS produced from aqueous phase autoxidation? One possible scheme is that instead of two NO\textsubscript{2} being produced from Eqn. (1), N\textsubscript{2}O\textsubscript{4}, held in aqueous cage, is produced. N\textsubscript{2}O\textsubScript{4} would show distinct decrease in reactivity due to solvent effects. The distinct nitrosating agents may be isomers of N\textsubscript{2}O\textsubscript{3} of different stability and reactivity. The isomer formed in aqueous media, which is less reactive and thus more stable, may be the symmetric molecule O=\textsubscript{N}O=\textsubscript{N}O, while the membrane phase isomer may be O=\textsubscript{N}NO\textsubscript{2} [13]. Thus, the following mechanism is proposed for NO autoxidation in aqueous media.

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow \text{N}_2\text{O}_4 \\
2\text{NO} + \text{N}_2\text{O}_4 & \iff 2\text{N}_2\text{O}_3 \\
2\text{N}_2\text{O}_3 + 2\text{H}_2\text{O} & \rightarrow 4\text{NO}_2^- + 4\text{H}^+
\end{align*}
\]

In conclusion, NO\textsubscript{2} is not formed appreciably from aqueous autoxidation and the isomer of N\textsubscript{2}O\textsubscript{3} is less reactive. Therefore, nitration of proteins and nitrosative stress is not a result of *aqueous* NO autoxidation. Thus, the deleterious reactions of the signaling agent NO is restricted to non-aqueous components.
References


