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Wink, DA
Darbyshire, JF
Nims, RW
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Reactions of the Bioregulatory Agent Nitric Oxide in Oxygenated Aqueous Media: Determination of the Kinetics for Oxidation and Nitrosation by Intermediates Generated in the NO/O_2 Reaction

David A. Wink,*,† John F. Darbyshire,‡ Raymond W. Nims,† Joseph E. Saavedra,† and Peter C. Ford§

Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702, Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892, and Department of Chemistry, University of California, Santa Barbara, California 97106

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The reaction kinetics of nitric oxide autoxidation in aerobic solutions were investigated by direct observation of the nitrite ion product and by trapping the strongly oxidizing and nitrosating intermediates formed in this reaction. The rate behavior observed for nitrite formation [rate = $k_3[O_2][NO]^2$, $k_3 = (6 \pm 1.5) \times 10^6$ M^{-2} s^{-1} at 22 °C] was the same as found for oxidation of $Fe(CN)_6^{4-}$ and of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and as for the nitrosation of sulfanilamide. There was a slight decrease in k_3 to $(3.5 \pm 0.7) \times 10^6$ M^{-2} s^{-1} at 37 °C. The second-order dependency for NO was observed at NO concentrations as low as 3 μM. The results of the competitive kinetics studies suggest that the key oxidizing intermediates, species which are both strong oxidants and nitrosating agents, are not one of those commonly proposed (NO_2, N_2O_3, NO^+, or O_2NO^-) but are one or more as yet uncharacterized NO_x species.

Introduction

A recent discovery in mammalian biology has been the important physiological role that nitric oxide plays in blood pressure regulation, neurotransmission, macrophage-induced cytostasis and cytotoxicity, and inhibition of platelet aggregation, etc. (*1*, *2*). Furthermore, it has been shown that intermediates derived from aerobic solutions of NO cause mutations in bacterial and mammalian cells, suggesting a possible involvement of endogenously formed NO in a genotoxic mechanism (*3–6*). However, despite evidence that NO has a short half-life in aerobic aqueous systems (*1*, *7–11*), the chemical intermediates and kinetics involved in the aerobic oxidation of this critically important species have not been completely elucidated. Clearly, the quantitative characterization of the reaction between NO and O_2 under biologically relevant conditions is essential to understanding the roles of this bioregulatory agent in living systems. Described here are kinetics studies of this reaction, which demonstrate that there are fundamental differences between the intermediates generated during the oxidation of NO in the gas phase and those generated in the same reaction occurring in aqueous solution.

Materials and Methods

Sodium azide, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD),[1] potassium superoxide, and nitrosonium tetrafluoroborate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Potassium ferrocyanide was purchased from Fisher Scientific Co. (Fairlawn, NJ), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was obtained from Sigma Chemical Co. (St. Louis, MO). The above-named reagents were used without further purification. Nitric oxide gas was purchased from Potomac AirGas (Frederick, MD) and passed through a 10 N KOH solution to remove other NO_x species. Nitric oxide solutions were made by degassing aqueous buffer solutions, followed by introduction of nitric oxide, as described previously (*12*). Nitric oxide concentrations were determined by the oxyhemoglobin method (*13*). Oxygen concentrations were determined with a dissolved oxygen electrode (Orion Research Inc., Boston, MA). No appreciable effect was observed between aqueous buffers treated or untreated with Chelex 100 (Bio-Rad, Richmond, CA).

The kinetics of nitrosation by the NO/O_2 reaction were measured via diazotization of sulfanilamide and subsequent coupling with NEDD to form the azo dye (*14*). Sulfanilamide (25 mM) and NEDD (2.5 mM) were dissolved in 100 mM phosphate buffer (pH 7.4) by stirring for 10 min. The resulting solutions were filtered with a Nylon-66 syringe filter (Rainin, Woburn, MA) to remove any small particulates. Introduction of 10–100 μM NO in this aerobic solution yielded an absorbance band at 496 nm which increased with [NO] such that $\epsilon_{NO} = 950 \pm 130$ M^{-1} cm^{-1}. Addition of acid to the solution resulted in an absorption band at 546 nm indicative of the corresponding azo dye [lit. 545 nm (*14*)]. The absorption at 496 nm was not generated in the presence of as much as 100 mM $NaNO_2$ at pH 7.4.

Peroxynitrite anion was made as follows: a 25-mL basic solution (0.1 M KOH) was degassed, and the solution was transferred to a septum vial containing 53 mg of KO_2, followed by degassing on ice. Nitric oxide was then bubbled through the solution, and after 1 min on ice, the headspace was evacuated to remove excess nitric oxide, which was replaced with argon. The resulting solution gave a broad band at 300 nm in basic solution characteristic of peroxynitrite anion ($OONO^-$) (*15*).

Kinetic modeling simulations were performed with Stella 2.1 software from High Performance Systems (Hanover, NH).

* To whom correspondence should be addressed.
† National Cancer Institute.
‡ National Heart, Lung, and Blood Institute.
§ University of California.

[1] Abbreviations: NEDD, *N*-(1-naphthyl)ethylenediamine dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).

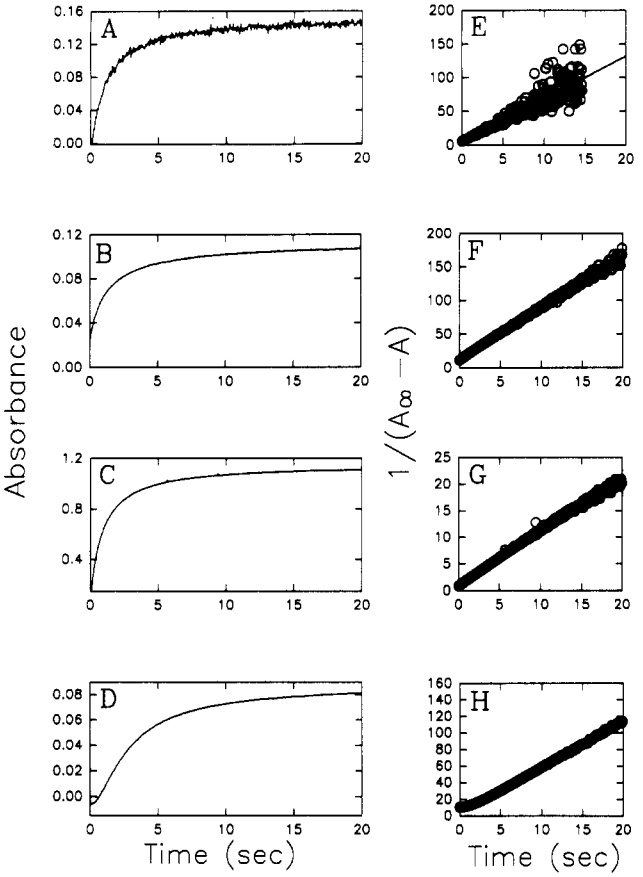

Figure 1. (A) Absorbance changes at 216 nm with time as detected with stopped-flow techniques for a 1.0 mM phosphate buffer (pH 7.4) containing 0.1 mM NO and 1 mM O_2 at 22 °C. (B) Absorbance changes at 420 nm [representing the appearance of $Fe(CN)_6^{3-}$], where the $[Fe(CN)_6^{4-}]$ = 50 mM in 100 mM phosphate buffer (pH 7.4), [NO] = 0.1 mM, and $[O_2]$ = 1.0 mM. (C) Absorbance changes at 600 nm for the appearance of $ABTS^+$, where [ABTS] = 50 mM. (D) Absorbance changes at 500 nm for the appearance of the diazotization product, where sulfanilamide was 25 mM and NEDD was 2.5 mM in 100 mM phosphate buffer (pH 7.4), [NO] = 0.1 mM, and $[O_2]$ = 0.9 mM. Panels E–H are plots of $1/(A_\infty - A)$ vs time for each absorbance change. The slopes of the regression lines are 6.23 s^{-1} [linear correlation (lc) = 0.918] for panel E, 7.59 s^{-1} (lc = 0.998) for panel F, 0.99 s^{-1} (lc = 0.998) for panel G, and 5.7 s^{-1} (lc = 0.998) for panel H.

Stopped-flow experiments were carried out with a Hi-Tech Scientific Multi-Mixing stopped-flow spectrophotometer, Model SF-51MX (*16*), with IS 1.0 Rapid Kinetics Software Suite (Hi-Tech Scientific Limited, Salisbury, Wiltshire, England).

Results and Discussion

Nitrite is the product of NO oxidation in aerobic aqueous solution (*9*, *17*, *18*).

$$4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^- \quad (1)$$

We have investigated the reaction of NO (100 μM) plus O_2 (1.0 mM) in pH 7.4 aqueous solution with a stopped-flow spectrophotometer by monitoring absorbance increases at 216 nm ($\Delta\epsilon_{216}$ = 1000 M^{-1} cm^{-1}) due to the formation of nitrite (Figure 1A). A plot of $(Abs_\infty - Abs)^{-1}$ vs time proved to be linear (Figure 1E), consistent with a second-order rate dependence on the limiting reagent, NO. The rate constant k_2 (=slope × $\Delta\epsilon$) so determined was (6.3 ± 1.2) × 10^3 M^{-1} s^{-1}. When the reaction was carried out similarly, but with O_2 (40 μM) as the limiting reagent ([NO] = 0.17–1.7 mM), first-order behavior was observed, and a plot of the resulting k_{obs} values vs $[NO]^2$ was linear with a slope of k_3 = (4.8 ± 1.0) × 10^6 M^{-2} s^{-1} (Figure 2), effectively within experimental uncertainty of the value of $k_2/[O_2]$ = (6.3 ± 1.2) × 10^6 M^{-2} s^{-1} derived from the experiment above. Thus, formation of nitrite ion in this system is governed by a third-order rate law:

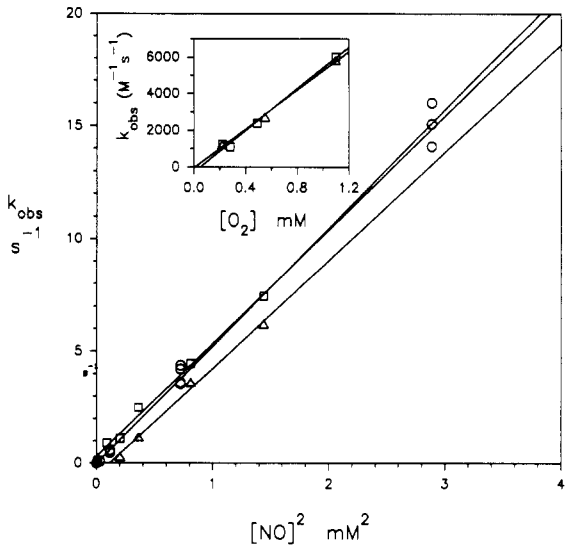

Figure 2. Plot of k_{obs} (s^{-1}) vs $[NO]^2$ (mM^2) for the formation of nitrite (△), $Fe(CN)_6^{3-}$ (□), and $ABTS^+$ (○) at pH = 7.4 and 22 °C when $[O_2]$ = 0.04 mM. The conditions for nitrite formation were 1 mM phosphate buffer (pH 7.4) and $[O_2]$ = 0.04 mM at 22 °C. The conditions for $Fe(CN)_6^{3-}$ and $ABTS^+$ formation were 100 mM phosphate buffer (pH 7.4) and $[O_2]$ = 0.04 mM with the reductants $[Fe(CN)_6^{4-}]$ = 50 mM and [ABTS] = 10 mM, respectively. The slope for nitrite formation is 4.78 × 10^6 M^{-2} s^{-1} (lc = 0.996), that for $Fe(CN)_6^{4-}$ oxidation is 5.08 × 10^6 M^{-2} s^{-1} (lc = 0.997), and that for ABTS oxidation is 5.23 × 10^6 M^{-2} s^{-1} (lc = 0.997). The inset plot represents the oxygen dependence of ferrocyanide oxidation (□), where $[Fe(CN)_6^{4-}]$ = 50 mM and [NO] = 0.04 mM, as well as nitrosation and diazotization of sulfanilamide and NEDD (△), where sulfanilamide was 25 mM, NEDD was 2.5 mM, and [NO] = 0.26 mM. The slopes of the lines were 5.6 × 10^6 M^{-2} s^{-1} and 5.3 × 10^6 M^{-2} s^{-1} for ferrocyanide oxidation and sulfanilamide nitrosation, respectively.

$$d[NO_2^-]/dt = k_3[O_2][NO]^2 \quad (2)$$

These data confirm the results of earlier studies conducted at millimolar concentrations of NO and O_2 in water (8.8 × 10^6 M^{-2} s^{-1}; *18*) and in CCl_4 (k_{NO_2} = 2.8 × 10^6 M^{-2} s^{-1}, $d[NO_2]/dt = k_{NO_2}[O_2][NO]^2$; *19*).

The reaction of NO and O_2 under these conditions generates an intermediate that rapidly oxidizes $Fe(CN)_6^{4-}$ and ABTS to $Fe(CN)_6^{3-}$ and $ABTS^+$, respectively. These reactions can be monitored at 420 nm ($\Delta\epsilon_{420}$ = 1000 M^{-1} cm^{-1}, Figure 1B,F) and 600 nm ($\Delta\epsilon_{600}$ = 6600 M^{-1} cm^{-1}, Figure 1C,G), respectively. Under limiting [NO], these reactions display second-order kinetics with k_2 values of (7.2 ± 1.4) × 10^3 and (6.5 ± 1.2) × 10^3 M^{-1} s^{-1}, respectively, the latter values being within experimental uncertainty of those observed above for nitrite formation. Similarly, when these reactions are carried out under limiting $[O_2]$, the k_{obs} values again prove to be dependent on $[NO]^2$, with the respective k_3 values, (5.1 ± 1.0) × 10^6 and (5.2 ± 1.0) × 10^6 M^{-2} s^{-1} (Figure 2), again being equivalent to the values for nitrite formation. Since the trapping agents are *not* oxidized by O_2, NO_2^-, or anaerobic NO, the rate data clearly imply that $Fe(CN)_6^{4-}$ and ABTS are both being oxidized by intermediate species formed subsequent to the rate-limiting step in the sequence leading to NO oxidation by O_2 in aqueous media.

As noted above, the NO/O_2 system has been shown to deaminate nucleosides (*3*, *4*) and, therefore, has the potential to cause genotoxicity. This activity has been attributed to nitrosation of the exocyclic amino groups of the nucleosides. In this context, the components of the Greiss reaction [a colorimetric assay for nitrite which involves the nitrosation under acidic conditions of sulfanilamide by NO^+ donors followed by diazotization and coupling with NEDD to form an azo dye (*14*)] were used to examine the kinetics of nitrosation by intermediate(s) generated during the NO/O_2 reaction in aqueous solution at physiological pH. Addition of 100 μM NO to a 0.9 mM O_2/100 mM phosphate solution (pH 7.4) containing 25 mM sulfanilamide and 2.5 mM NEDD generated an absorption at λ_{max} = 496 nm, indicative of the characteristic azo product resulting from nitrosation (Figure 1D). Preliminary stopped-flow studies under limiting [NO] show an increase in absorption at 500 nm similar to that of the formation of nitrite and oxidation of $Fe(CN)_6^{4-}$ and ABTS observed above in Figure 1. A plot of $(A_\infty - A)^{-1}$ versus time was linear (Figure 1H, $\Delta\epsilon_{NO}$ = 950 ± 150), giving a $k_2 = (6.0 \pm 0.7) \times 10^3\ M^{-1}\ s^{-1}$. As shown in Figure 2 inset, an $[O_2]$ dependence was observed such that $k_3 = (5.3 \pm 0.5) \times 10^6\ M^{-2}\ s^{-1}$. Thus, it appears that this nitrosation, which may serve as a model for the deamination of nucleic acids by NO, also proceeds via the formation of the same intermediate(s) as the oxidation reactions described above.

It is worthwhile to consider the implications of the present observations vis-a-vis the expected reactivities of NO under physiologically relevant conditions, where maximal concentrations of NO in the cellular microenvironment are estimated to be in the range of 0.45–10 μM (*18–22*). The mechanistic requirement that NO oxidation be second-order in [NO] allows one to predict half-lives of 1–500 s in air-saturated aqueous solution ($[O_2]$ = 220 μM, $t_{1/2} = 1/(c_0k)$) (*23*). The validity of this prediction was tested by carrying out the oxidation of ABTS at low NO concentrations. When a solution (pH 7.4, 22 °C) containing 5 mM ABTS plus 220 μM O_2 was rapidly mixed with a solution of NO (3–10 μM) by means of stopped-flow techniques, the absorbance increase at 600 nm, indicative of ABTS oxidation, followed second-order kinetics with a k_2 of $(5.0 \pm 0.8) \times 10^6\ M^{-2}\ s^{-1}$, identical to the above. The first half-life under these conditions ranged from 10 to 333 s. At 37 °C, similar second-order behavior was observed, although the resulting k_3 was slightly smaller, $(3.5 \pm 0.5) \times 10^6\ M^{-2}\ s^{-1}$.[2] In a previous report, little or no difference was detected in the observed rate between 15 and 30 °C in aqueous solution (*18*).

It has been widely speculated, in analogy with the better characterized gas-phase mechanism, that NO_2 is a key reactive intermediate formed in the oxygenation of NO in aqueous media or in vivo (*4*, *7*, *24*, *25*) and, furthermore, that NO_2 is a species responsible for oxidative damage by such systems (*26*). The role of NO_2 in the aqueous oxidation may be tested by carrying out competition kinetics, since the second-order rate constants for the reactions of NO_2 with NO ($1.1 \times 10^9\ M^{-1}\ s^{-1}$) and $Fe(CN)_6^{4-}$ ($3 \times 10^6\ M^{-1}\ s^{-1}$) have been determined under analogous conditions (*17*, *27*). A series of experiments was carried out using the following two initial [NO], 0.17 and 1.2 mM, and with initial $[O_2]$ = 1 mM and 40 μM, respectively, and the total absorbance change at 420 nm [corresponding to the formation of $Fe(CN)_6^{3-}$] was determined. If NO_2 were the intermediate intercepted by $Fe(CN)_6^{4-}$, plots of $[Abs_{420}]^{-1}$ vs $[Fe(CN)_6^{4-}]^{-1}$ would give X_m values equal to the ratio $k_{NO}[NO]/k_{Fe}$.[3] However, instead of the predicted X_m values 62 and 440 mM, both plots (Figure 3A) gave X_m values of ~2 mM. Hence, we conclude that NO_2 is not the species being intercepted by $Fe(CN)_6^{4-}$ under these conditions.

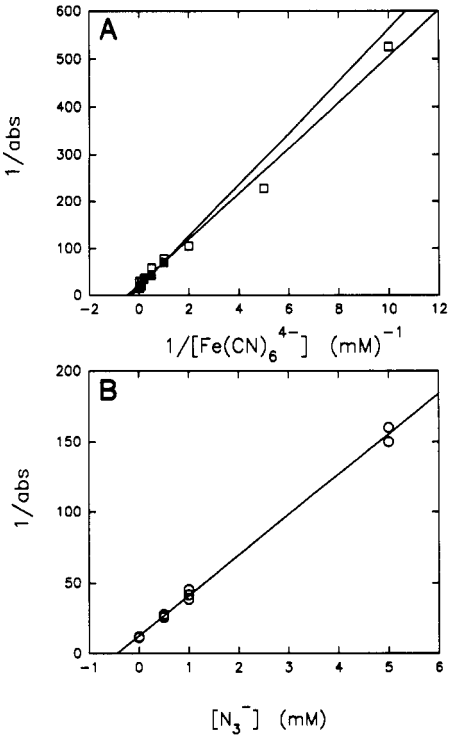

Figure 3. (A) Double-reciprocal plot of the changes in absorbance at 420 nm with varying $Fe(CN)_6^{4-}$ concentration when [NO] = 1.2 mM and $[O_2]$ = 0.04 mM (□) and when [NO] = 0.12 mM and $[O_2]$ = 1 mM (■) with a $1/X_{int}$ = 0.48 mM. (B) The reciprocal of the change in absorbance at 420 nm (appearance of ferricyanide) with varying azide concentration, with an x intercept of 0.437 M. The conditions were 10 mM phosphate buffer (pH 7.4), with [NO] = 0.17 mM and $[O_2]$ = 1 mM.

From a biological perspective, the most important feature of the NO/O_2 reaction is that the reactive intermediates generated are capable of both oxidation and nitrosation. To examine the relationship between these pathways, a known NO^+ acceptor (*28*, *29*), sodium azide, was used to quench the oxidation of $Fe(CN)_6^{4-}$ in a competition study. When the azide concentration was varied from 0.1 to 10 mM, with $[Fe(CN)_6^{4-}]$ = 20 mM, [NO] = 0.12 mM, and $[O_2]$ = 1 mM, a decrease in the amount of $Fe(CN)_6^{3-}$ formed was observed. A plot of 1/Abs vs $[N_3^-]$ is linear, with $X_i = 0.43 \pm 0.05$ mM (Figure 3B).[4] These results allow one to evaluate the relative selectivity between different reaction pathways. Since the slopes of the plot in Figure 3A are independent of [NO], it can be surmised that the competing pathway is unimolecular (e.g.,

[2] While somewhat surprising, the small negative E_a parallels similar behavior in the gas-phase kinetics which is not yet fully explained. In a multistep mechanism, a negative E_a is often the result of a reversible equilibrium, with a negative $\Delta H°$ occurring prior to the rate-limiting step.

[3] X_m is equal to the negative reciprocal of the abscissa intercept (X_{int}). In this case, the numerical value of X_m is equal to the concentration of $Fe(CN)_6^{4-}$ at which half the intermediate is trapped by this quenching agent to give $Fe(CN)_6^{3-}$.

[4] X_i is equal to the negative of the abscissa intercept (X_{int}). In this case, the numerical value of X_i is equal to the concentration of N_3^- at which half the $Fe(CN)_6^{3-}$ is quenched and where $k_{azide}[N_3^-] = k_{Fe}[Fe]$.

involving protonation or hydrolysis). From the X_m (Figure 3A) and X_i (Figure 3B) values, the relative rate constants k_{azide}:k_{Fe}:k_H can be calculated as (2×10^4):500:1.

This relative selectivity can be used to test other possible intermediates. One possible candidate would be N_2O_3 derived from rapid trapping of NO by NO_2 via the mechanism described in the following equations:

$$NO + O_2 \rightleftharpoons {}^{\bullet}O_2NO \xrightarrow{NO} 2NO_2 \quad (3)$$

$$NO_2 + NO \rightarrow N_2O_3 \xrightarrow{H_2O} 2NO_2^- + 2H^+ \quad (4)$$

The published rate constant for the reaction of azide with N_2O_3 is 2×10^9 M^{-1} s^{-1} (*28*) and that for the hydrolysis of N_2O_3 is 1000 s^{-1} (*17*), a ratio of 2×10^6 M^{-1}. This ratio is 100-fold larger than one observed for the trapping of the NO/O_2-reactive intermediate in the experiments described above. This argues against the intermediate being N_2O_3 and reinforces a similar conclusion drawn from results of the deamination of nucleosides by aerobic NO, for which it was noted that addition of nitrite for the purpose of generating N_2O_3 dramatically increased the extent of deamination (*3*). Kinetic modeling of the experiment described in Figure 3B, assuming the intermediacy of NO_2 and N_2O_3 (formed by rapid reaction of NO and O_2) and using published rate constants (*17*, *27*), predicted that at 10 mM [N_3^-] only 40% of the observed $Fe(CN)_6^{3-}$ formation should be quenched. Instead, complete quenching was observed, a discrepency which appears to preclude the mechanism described by eqs 3 and 4.

Other possible intermediates are the $OONO^-$ and the nitrosyl cation (NO^+). Preliminary studies of the reaction of $Fe(CN)_6^{4-}$ with $OONO^-$ show that the oxidation of $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$ is not appreciably quenched in the presence of 15 mM azide. Thus, the peroxynitrite anion does not behave in the manner seen for the key oxidant in the NO/O_2 reaction, which was intercepted by N_3^-. The nitrosyl cation (NO^+) might be a more likely candidate as a nitrosating agent. This species would also be expected to undergo hydrolysis to nitrite, and to nitrosate amines or azide and to oxidize $Fe(CN)_6^{4-}$ in aqueous solution. The possible role of NO^+ was examined by competition studies in which solid BF_4NO was dissolved in pH 7.4 aqueous solutions containing varying amounts of $Fe(CN)_6^{4-}$ and N_3^-, and the relative rates of Fe oxidation and hydrolysis to NO_2^- as quenched by N_3^- were compared. From these data, the relative rate constants k_{azide}:k_{Fe}:k_H = 15:25:1 were obtained. Although the comparisons clearly suffer from some potential problems, owing to surface catalysis during dissolution of the BF_4NO, the marked differences in the k_{azide}:k_{Fe} ratio from that seen above for the NO/O_2 reaction system argue strongly against NO^+ being the key intermediate.

The above experiments provide evidence against NO_2, N_2O_3, ONO_2^-, or NO^+ being the key intermediate responsible for the strongly oxidizing and nitrosating properties of the intermediate generated in the reaction of NO with O_2. We are thus left with a mechanism involving novel $N_xO_y^{n-}$ intermediates which differ from those intermediates proposed for the gas-phase NO/O_2 reaction mechanism. Correlation between the gas-phase and solution rate constants (*18*, *19*) suggests that the intermediates in the rate-limiting steps are common between the two systems. Guillory and Johnston reported strong evidence for the intermediacy of the peroxynitrite radical in the gas-phase reaction (*30*), while others have suggested that the key intermediate is the NO dimer (*19*, *31*). However, it appears that either intermediate would further react to produce a novel reactive intermediate(s).

The chemical reactivity of NO and O_2 provides insight into the bioregulatory functions of NO, as well as its cytotoxic and genotoxic effects. Similarities between the observed rate expressions for protic (this work and ref 18) versus nonprotic (*19*) solutions suggest that, regardless of the hydrophilicity or lipophilicity of the biological media, these kinetics will govern the half-life of NO. The second-order (NO) dependency of this reaction dictates that the half-life of NO be inversely proportional to its concentration, and the total amount of reactive intermediates produced in NO autoxidation be proportional to the square of its concentration. In the course of various bioregulatory activities (such as blood pressure lowering, neurotransmission, and inhibition of platelet aggregation) relatively low levels of NO are generated; thus even in the presence of high concentrations of O_2, there should be sufficient time for NO to reach target sites such as guanylate cyclase without being consumed by oxygen. However, stimulated macrophages are estimated to generate as much as 1000 times higher concentrations of NO (*25*, *32*), under which conditions a large flux of reactive intermediates must be generated, resulting in cytotoxicity or cytostasis (*1*). As NO diffuses from these sites, the flux of the reactive intermediates would be expected to decrease and the half-life of NO would be expected to increase. This increased half-life would allow detoxication of this radical species by other pathways, possibly via the reaction of NO with oxyhemoglobin or other oxyhemoproteins to yield nitrate, a product not otherwise formed in the NO/O_2 reaction in aqueous solution.

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