Superoxide Reacts with Nitric Oxide to Nitrate Tyrosine at Physiological pH via Peroxynitrite*

Christopher D. Reiter, Ru-Jeng Teng, and Joseph S. Beckman‡

From the Departments of Anesthesiology, Biochemistry and Molecular Genetics, and Pediatrics, Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, Alabama 35223

Tyrosine nitration is a widely used marker of peroxynitrite (ONOO\textsuperscript{-}) produced from the reaction of nitric oxide with superoxide. Pfeiffer and Mayer (Pfeiffer, S., and Mayer, B. (1998) J. Biol. Chem. 273, 27280–27285) reported that superoxide produced from hypoxanthine plus xanthine oxidase in combination with nitric oxide produced from spermine NONOate did not nitrate tyrosine at neutral pH. They suggested that nitric oxide and superoxide at neutral pH form a less reactive intermediate distinct from preformed alkaline peroxynitrite that does not nitrate tyrosine. Using a stopped-flow spectrophotometer to rapidly mix potassium superoxide with nitric oxide at pH 7.4, we report that an intermediate spectrally and kinetically identical to preformed alkaline peroxynitrite was formed in 100% yield. Furthermore, this intermediate nitrated tyrosine in the same yield and at the same rate as preformed peroxynitrite. Equivalent concentrations of nitric oxide under aerobic conditions in the absence of superoxide did not produce detectable concentrations of nitrotyrosine. Carbon dioxide increased the efficiency of nitration by nitric oxide plus superoxide to the same extent as peroxynitrite. In experiments using xanthine oxidase as a source of superoxide, tyrosine nitration was substantially inhibited by urate formed from hypoxanthine oxidation, which was sufficient to account for the lack of tyrosine nitration previously reported. We conclude that peroxynitrite formed from the reaction of nitric oxide with superoxide at physiological pH remains an important species responsible for tyrosine nitration in vivo.

In the decade since its discovery, nitric oxide has been shown to have multiple physiological actions and is implicated in the pathology of a wide range of diseases. However, nitric oxide itself is neither highly reactive nor particularly toxic, but rather forms secondary oxidants responsible for tissue injury. A major pathway that enhances the toxicity of nitric oxide is the near diffusion-limited reaction with superoxide to form peroxynitrite (ONOO\textsuperscript{-}). One of the most easily identified products of peroxynitrite attack on proteins is 3-nitrotyrosine (2). Nitrotyrosine has been identified in human atherosclerosis, pulmonary and heart disease, acute and chronic kidney rejection, Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (3–9). Over 250 publications in the past 6 years have described nitration in at least 80 different diseases (10). Specific nitrated proteins have been isolated from diseased human tissues, which include manganese superoxide dismutase (6, 11, 12), neurofilament L (13, 14), a key surfactant protein in lung (15), the type II isoform of the sarcoplasmic reticulum calcium ATPase (16, 17), and prostacyclin synthase (18–20). Quantitatively, the amount of nitration in human diseased tissues can be surprisingly high. Circulating levels of free nitrotyrosine as high as 120 μM have been found in septic patients in renal failure (21). From 1 to 3% of tyrosine in spinal cords of amyotrophic lateral sclerosis patients is nitrated (22, 23). The mechanisms by which tyrosine is nitrated in vivo remain an area of active investigation and controversy (2, 24, 25).

Most experiments have used preformed alkaline peroxynitrite, which can be commercially purchased or easily synthesized by a variety of methods (26, 27). Peroxynitrite in alkaline solution is present only in the cis conformation (28, 29), which contributes to the unusual stability of peroxynitrite by preventing the rearrangement of the terminal oxygen to form nitrate (NO\textsubscript{3}\textsuperscript{-}). Curiously, peroxynitrite in the solid state is entirely in the cis conformation (28, 30). The x-ray structure of the tetramethylammonium salt of peroxynitrite has recently been determined and was also in the cis conformation (31). However, it remains possible that nitric oxide plus superoxide reacting at neutral pH might form peroxynitrite in a different conformation or in an excited electronic state that could be substantially less reactive than preformed cis-peroxynitrite (1).

Co-generation of superoxide and nitric oxide is experimentally difficult to control. The products and reactants used to generate superoxide and nitric oxide can competitively inhibit tyrosine nitration (32). For example, urate produced by xanthine oxidase is a strong antioxidant and decreases both dihydrodroadamine oxidation and phenolic nitration by peroxynitrite (33, 34). A second issue is the limited availability of dissolved oxygen in buffer, which is generally in the range of 200–250 μM depending upon ionic strength and temperature. Both xanthine oxidase and SIN-1 can quickly make a solution anaerobic, thereby stopping superoxide production unless special precautions are taken to refresh oxygen supplies (35).

Recently, Pfeiffer and Mayer (1) reported that the co-generation of superoxide from xanthine oxidase and nitric oxide from spermine NONOate yielded far less nitrotyrosine than produced by preformed alkaline peroxynitrite. They confirmed the rate of peroxynitrite formation by monitoring the oxidation of dihydrodroadamine. To explain the apparent lack of nitration, Pfeiffer and Mayer (1) proposed that superoxide and nitric
Nitration by NO and Superoxide

Xanthine oxidase was purchased from CalBiochem (La Jolla, Ca). All other reagents were purchased from Sigma-Aldrich. All water was deionized and had a conductivity greater than 18 megohms. Dimethyl sulfoxide (Me2SO) was dried over 3-Å molecular sieves and used to dissolve potassium superoxide (KO2). Alkaline stock peroxynitrite was synthesized from acidified nitrite and hydrogen peroxide as described previously (38). Generally, hydrogen peroxide was not removed by treatment with manganese dioxide since it did not interfere in the present assays.

To investigate whether peroxynitrite was generated at neutral pH, we developed a stopped-flow method to mix potassium superoxide with nitric oxide in phosphate buffer. We report that this resulted in the stoichiometric formation of peroxynitrite, which gave the same yields of nitrotyrosine as with preformed alkaline peroxynitrite.

MATERIALS AND METHODS

Xanthine oxidase was purchased from CalBiochem (La Jolla, Ca). All other reagents were purchased from Sigma-Aldrich. All water was deionized and had a conductivity greater than 18 megohms. Dimethyl sulfoxide (Me2SO) was dried over 3-Å molecular sieves and used to dissolve potassium superoxide (KO2). Alkaline stock peroxynitrite was synthesized from acidified nitrite and hydrogen peroxide as described previously (38). Generally, hydrogen peroxide was not removed by treatment with manganese dioxide since it did not interfere in the present assays.

To investigate whether peroxynitrite was generated at neutral pH, we utilized an SX.18MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, United Kingdom) to rapidly mix superoxide in the stopped-flow cell was determined by monitoring the apparent extinction coefficient for peroxynitrite decreases from 1.70 m M-1 cm-1 at alkaline pH to 1.36 m M-1 cm-1 at pH 7.4 (40). The differential extinction coefficient was used to calculate the apparent absorption spectrum at pH 7.4. The pH-adjusted extinction coefficient was thus εpH = εpH,met-hemoglobin tetramer to met-hemoglobin (40). The differential extinction coefficient at 401 nm of 52.1 m M-1 cm-1 was used to calculate the amount of met-hemoglobin formed (40). The dissolved nitrate concentration was less than in distilled water due to the increased ionic strength of the buffer. The concentration of nitric oxide after dilution in the stopped-flow tonometer was confirmed by removing a small amount of the solution via a gas-tight Hamilton syringe fitted with a cannula for assay with oxyn-hemoglobin.

To study the nitration of tyrosine, we added 1.04 m M tyrosine to the buffer in the tonometer before adding nitric oxide. There was no detectable nitrotyrosine formation in this solution for as long as 4 h. To study the effects of dissolved CO2, we added a final concentration of 26 m M sodium bicarbonate to sparged buffer before addition of nitric oxide. The bicarbonate-containing buffer was then sparged briefly with helium to remove any oxygen introduced with the bicarbonate. The bicarbonate was then allowed to re-establish equilibrium with free CO2 for 2 min before addition of nitric oxide. The final pH of all buffers was 7.43, which was measured in the stopped-flow effluent. All stopped-flow reactions were performed at 37 °C.

Alkaline stock peroxynitrite was used as a comparison to the nitric oxide/superoxide system by placing alkaline peroxynitrite in the 100-μl drive syringe in place of superoxide. Because peroxynitrite stock solutions are unstable in concentrated Me2SO, we controlled for the effects of dissolved CO2 by adding 0.5 M Me2SO into the 2.5-ml syringe with the buffer. Because the mixing chamber in the stopped-flow is not symmetric with respect to the drive syringes, greater mixing artifacts were apparent during the initial mixing but did not interfere with the last 90% of the reaction course.

Decay of peroxynitrite anion was followed at 302 nm (1.70 m M-1 cm-1). Formation of nitrotyrosine was observed at 430 nm (0.87 m M-1 cm-1 as determined using nitrotyrosine standards in the stopped-flow instrument under identical reaction conditions). Reaction rates were determined using Applied Photophysics stopped-flow software. The spectrum of the intermediate product from the reaction of superoxide and nitric oxide was constructed from consecutive stopped-flow traces between 250 and 450 nm taken in 5-nm steps. These data plus the initial concentrations of superoxide and nitric oxide were used by a single value decomposition algorithm in the kinetic software Pro-K supplied with the Applied Photophysics stopped-flow spectrophotometer to estimate the spectrum and extinction coefficient of the intermediate product generated from superoxide and nitric oxide as a function of wavelength. The spectrum of peroxynitrite decreases at more acidic pH because peroxynitrous acid does not absorb significantly in the visible or UV. The pH of peroxynitrite in phosphate buffer was measured using a glass electrode which was used to calculate the apparent absorption spectrum at pH 7.4 via the Henderson-Hasselbalch equation. The pH-adjusted extinction coefficient is thus εpH = εpH,met-hemoglobin × 10pH/{10pH (1 + 10εpH,met-hemoglobin)} where εpH,met-hemoglobin is the extinction coefficient of peroxynitrite at alkaline pH. At 302 nm, the apparent extinction coefficient for peroxynitrite decreases from 1.7 m M-1 cm-1 (41) at alkaline pH to 1.36 m M-1 cm-1.
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RESULTS

Superoxide reacted with nitric oxide at neutral pH to produce an intermediate product with the same spectrum as preformed alkaline peroxynitrite (Fig. 2). The solid line shown in Fig. 2 is not a fitted curve to the experimental data, but rather the spectrum of cis-peroxynitrite at alkaline pH adjusted to account for the 20% peroxynitrous acid present at pH 7.4. Utilizing all of the experimental data in Fig. 2 and the starting concentration of superoxide, a simple irreversible bimolecular reaction was fitted by a single value decomposition algorithm and yielded an extinction coefficient of 1.34 mM$^{-1}$ cm$^{-1}$ as described above. Dihydrorhodamine oxidation was followed by withdrawing 1-ml aliquots of the xanthine oxidase reaction at timed intervals, adding dihydorhodamine, and then measuring the rate of increase in absorbance at 550 nm. The accumulation of an inhibitor of tyrosine nitration was monitored by adding 0.5 mM peroxynitrite to aliquots taken at progressively longer times following the addition of xanthine oxidase.

We repeated the xanthine oxidase/spermine NONOate co-generation experiments of Pfeiffer and Mayer (1) at 22 °C using 10 milliunits of xanthine oxidase, 1 mM hypoxanthine, 1 mM tyrosine, and 1 mM spermine NONOate in 50 mM potassium phosphate, pH 7.4. The activity of xanthine oxidase was measured using xanthine and 1 unit was defined as the amount of enzyme needed to produce 1 μmol of urate min$^{-1}$ at 25 °C in 50 mM potassium phosphate, pH 7.4. Urate formation was measured at 292 nm (ε = 1.1 × 10$^4$ M$^{-1}$ cm$^{-1}$) (43). Superoxide was measured by the reduction of cytochrome c at 550 nm as described above. Dihydrorhodamine oxidation was followed by withdrawing 1-ml aliquots of the xanthine oxidase reaction at timed intervals, adding dihydorhodamine, and then measuring the rate of increase in absorbance at 550 nm. The accumulation of an inhibitor of tyrosine nitration was monitored by adding 0.5 mM peroxynitrite to aliquots taken at progressively longer times following the addition of xanthine oxidase.

Carbon dioxide present in the buffer accelerated the decomposition of the intermediate product from superoxide and nitric oxide (Fig. 2) and increased the yield of nitrotyrosine by 3–4-fold to the same extent as preformed peroxynitrite (Table I). Reducing the initial concentration of nitric oxide resulted in a proportional decrease in the formation of peroxynitrite and nitrotyrosine. In the absence of superoxide, even saturated solutions of nitric oxide mixed with 100% oxygen-saturated phosphate buffer containing 1 mM tyrosine did not yield detectable nitrotyrosine after as long as 2000 s. Me$_2$SO, necessary to dissolve superoxide, did not interfere with nitration by peroxynitrite in the presence or absence of carbon dioxide as previously reported (45, 46).

Consistent with the results of Pfeiffer and Mayer (1), we have observed little nitration of free tyrosine using hypoxanthine plus xanthine oxidase with spermine NONOate to generate superoxide and nitric oxide. Hypoxanthine, xanthine oxidase, and spermine NONOate tested separately did not interfere with nitration by bolus additions of peroxynitrite. However, hypoxanthine is oxidized to urate by xanthine oxidase and urate is known to inhibit peroxynitrite-mediated nitration (47). Under our assay conditions using 10 milliunits of xanthine oxidase, urate was formed from hypoxanthine at a rate of 5.2 μM min$^{-1}$ and superoxide was produced at 6.7 μM min$^{-1}$. Hypoxanthine donates four electrons to xanthine oxidase, which under our experimental conditions resulted in 32% univalent reduction of oxygen to superoxide. The remaining 68% was consumed by direct divalent reduction of oxygen to form hydrogen peroxide as previously demonstrated by Fridovich (43). When bolus additions of preformed peroxynitrite were made to the xanthine oxidase reaction, tyrosine nitration was progressively inhibited as urate accumulated in the assay system (Fig. 5). Addition of 50–100 μM urate into the stopped-flow system also strongly inhibited tyrosine nitration to an equal extent with either preformed peroxynitrite or the combination of superoxide and nitric oxide (not shown).

Pfeiffer and Mayer (1) quantified peroxynitrite formation from xanthine oxidase plus spermine NONOate by measuring the rate of dihydorhodamine oxidation only at the start of the assay. The oxidation of dihydorhodamine is known to be strongly inhibited by urate (33). Immediately after the addition of xanthine oxidase, the rate of dihydorhodamine oxidation

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**Fig. 2.** Comparison of the spectrum of the intermediate product of the reaction of superoxide (100 μM) and nitric oxide (100 μM) with that of alkaline stock peroxynitrite. Peroxynitrite was generated in situ as an intermediate by rapidly mixing potassium superoxide dissolved in Me$_2$SO with nitric oxide in buffer. The spectrum of this intermediate (solid) was constructed from consecutive stopped-flow traces recorded at wavelengths from 250 to 450 nm in 5-nm steps as described under "Materials and Methods" (mean ± S.D. from three separate kinetic runs at each wavelength). The solid line is the spectrum of preformed alkaline peroxynitrite after adjustment for the decreased absorption expected due to 20% protonation of peroxynitrite at pH 7.4 (see "Materials and Methods").

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**Fig. 3.** Comparison of the decomposition of peroxynitrite generated at physiological pH and alkaline stock peroxynitrite in the absence of carbon dioxide. Peroxynitrite decay (dashed traces) was followed in the stopped-flow spectrophotometer at 302 nm and production of nitrotyrosine (solid lines) at 430 nm. The reaction of 140 μM nitric oxide plus 140 μM superoxide anion is shown by inverted triangles and 140 μM peroxynitrite by open squares. Traces are averages of 5 to 14 individual reactions.
Nitration by NO and Superoxide

Nitration of tyrosine by NO and superoxide was studied to understand the reaction mechanisms and the role of peroxynitrite.

**TABLE I**

| Peroxynitrite   | Rate of tyrosine | Yield nitrotyrosine |
|-----------------|------------------|---------------------|
| Decay           | nitration        |                     |
| No CO₂ O²⁻/NO   | 1.8 ± 0.13 (10)  | 1.6 ± 0.42          | 5.3 ± 0.4          |
| O²⁻/NO         | 1.9 ± 0.05 (5)   | 1.7 ± 0.15          | 5.1 ± 0.4          |
| With CO₂ ONOO⁻ | 7.1 ± 9.4 (9)    | 7.7 ± 11            | 14.1 ± 1.1         |
| ONOO⁻          | 75 ± 3.5 (10)    | 83 ± 5.8            | 15 ± 1.0           |

was 1.4 \( \mu \text{M/min}\), which corresponds to 4.8 \( \mu \text{M/min}\) peroxynitrite after correction for the 30% efficiency of trapping peroxynitrite (48). When dihydrorhodamine was added after the xanthine oxidase reaction had progressed for as little as 5 min, its oxidation was more strongly inhibited by the accumulation of urate than was tyrosine nitration (Fig. 5).

**DISCUSSION**

Peroxynitrite was produced from nitric oxide reacting with superoxide at neutral pH with essentially 100% efficiency. Furthermore, peroxynitrite formed at neutral pH nitrated tyrosine in the same yield and rate as preformed alkaline peroxynitrite. Physiologically relevant concentrations of carbon dioxide-catalyzed tyrosine nitration and accelerated the isomerization of peroxynitrite to nitrate equally well for the product of superoxide plus nitric oxide as for preformed peroxynitrite. For all practical purposes, nitric oxide plus superoxide generated the same products at neutral pH as preformed alkaline peroxynitrite.

Peroxynitrite is known to be entirely in the cis conformation at alkaline pH (28), whether produced from either nitrite plus hydrogen peroxide or from nitric oxide plus superoxide (49). cis-Peroxynitrite is also formed by gaseous nitric oxide reacting with either solid potassium superoxide (26) or tetramethylsuperoxide dissolved in liquid ammonia (41). To explain the apparent lack of nitration observed in their co-generation experiments at neutral pH, Pfeiffer and Mayer (1) suggested that nitric oxide plus superoxide form peroxynitrite in an unreactive trans conformation, which rearranged to nitrate without forming a nitrating species. However, the \( \text{pK}_a \) of superoxide is 4.7 and the \( \text{pK}_a \) of peroxynitrite is 6.8, which are both below 7.4. Because the bulk of the reactants and the product have the same ionization state as at alkaline pH, they would be expected to react in the same manner at neutral pH. In a second publication, Pfeiffer and Mayer (50) subsequently concluded that a conformation differing from cis-peroxynitrite was unlikely to account for the apparent lack of nitration in their system.

The spectral intermediate produced by nitric oxide plus superoxide was identical to alkaline cis-peroxynitrite (Fig. 2). We have shown that the absorption spectrum of trans-peroxynitrite in a frozen argon matrix is significantly blue-shifted compared with cis-peroxynitrite (51). While aqueous solvents will alter the absorption spectrum, one would still expect a spectral difference between cis- and trans-peroxynitrite (52, 53).

Despite nitric oxide plus superoxide produce an intermediate that is both spectrally and kinetically identical to preformed cis-peroxynitrite, the intermediate is almost certainly in the cis-conformation.

Why should nitric oxide plus superoxide react to preferentially form cis-peroxynitrite? This is likely a consequence of the unusual reaction between two antibonding \( \pi \) orbitals on nitric oxide and superoxide coming together to form a new bonding \( \sigma \) orbital between the nitrogen and oxygen in peroxynitrite (Fig. 6). The bonding interactions between the \( \pi \) orbitals perpendicular to the plane of cis-peroxynitrite further stabilize the formation of cis-peroxynitrite. This gives the N=O bond a partial \( \pi \) bonding character, which greatly limits the isomerization of cis-peroxynitrite to trans-peroxynitrite and helps make cis-peroxynitrite kinetically stable. The barrier to isomerization is estimated to be 26 kcal mol\(^{-1}\) (42). In contrast, trans-peroxynitrite has an antibonding \( \pi \) orbital across the central N=O bond, which destabilizes the trans conformation. The greater stability of the N=O bond in cis-peroxynitrite may explain why it is preferentially formed from nitric oxide and superoxide.

**Inhibition by Oxygen Depletion**—The continuous generation of superoxide and nitric oxide to produce a low steady state.
concentration of peroxynitrite is an experimentally challenging problem. It requires a careful balancing of conditions to prevent substrate depletion while minimizing interference by products from the reactions under study. One major limiting but frequently overlooked substrate necessary for the formation of superoxide is oxygen. In solution, the concentration of oxygen in air-saturated buffers is about 250 μM at 25 °C and 200 μM at 37 °C. Oxygen depletion is likely to have played a significant role in the xanthine oxidase experiments of Pfeiffer and Mayer (1), since the complete oxidation of 1 mM hypoxanthine to urate by xanthine oxidase requires 2 mM oxygen. Most of their experiments were performed with 28 milliunits ml⁻¹ of xanthine oxidase, which will consume 28 μM O₂ min⁻¹ and therefore deplete dissolved oxygen in about 9 min. They noted that the apparent production of superoxide began to diminish after only 1–3 min (1), using an assay combining cytochrome c to measure superoxide and oxy-hemoglobin to measure nitric oxide. In this particular assay, oxygen levels were more effectively maintained because cytochrome c reduces superoxide back to oxygen, while oxy-hemoglobin will release oxygen as the solution becomes anaerobic. Consequently, oxygen depletion was even more rapid in their tyrosine nitration experiments lacking cytochrome c and oxy-hemoglobin.

The amount of superoxide released from xanthine oxidase is also strongly dependent upon oxygen concentration. Xanthine oxidase reduces a substantial fraction of oxygen directly to hydrogen peroxide with the fraction escaping as superoxide being critically dependent upon substrate and oxygen concentration (43). As the concentration of oxygen decreases, the efficiency of producing superoxide decreases as more oxygen is reduced to hydrogen peroxide (43). The commercial preparation of xanthine oxidase as well as storage conditions also affect the efficiency of superoxide production. Using a different source of xanthine oxidase, we were able to generate a flux of 6–7 μM O₂ min⁻¹ with 10 milliunits ml⁻¹ xanthine oxidase compared with a flux of 5 μM O₂ min⁻¹ from 28 milliunits ml⁻¹ xanthine oxidase used by Pfeiffer and Mayer (1).

Inhibition by Urate—However, even when oxygen concentrations were maintained, we found that tyrosine nitration was still greatly reduced in the xanthine oxidase system compared with preformed peroxynitrite due to the accumulation of urate. After less than 10 min, sufficient urate was generated by xanthine oxidase to suppress most of the tyrosine nitration resulting from bolus additions of 0.5 mM peroxynitrite (Fig. 5). Urate reacts with peroxynitrite forming a variety of derivatives (54), some of which can behave as nitrovasodilators (47). Urate has been long recognized as an antioxidant in human plasma (55) and is a major protective factor against peroxynitrite-dependent injury in experimental autoimmune encephalitis in mice (56).

Under our experimental conditions, 1.2 mol of urate were produced per mole of peroxynitrite (Fig. 5). Because tyrosine nitration by peroxynitrite is only about 4% efficient in the absence of carbon dioxide, xanthine oxidase plus spermine NONOate produced 30 mol of urate/mol of nitrotyrosine that would be formed by peroxynitrite in the absence of urate. In the experiments using 28 milliunits ml⁻¹ xanthine oxidase to produce 5 μM O₂ min⁻¹, approximately 100 mol of urate were produced per mol of nitrotyrosine expected to be generated in the absence of urate.

Recently, Pfeiffer and Mayer (50) suggested that urate was not a major complication in their original study, because the addition of uricase did not permit tyrosine nitration. However, uricase could not keep urate concentrations sufficiently low to allow tyrosine nitration under their reaction conditions because uricase has a relatively low specific activity and will be rendered ineffective by oxygen depletion by the xanthine oxidase system. Oxygen depletion was a factor affecting nitration in other superoxide generating systems used in their second publication as well.

Urate is known to strongly suppress dihydrorhodamine oxidation by peroxynitrite (33). Pfeiffer and Mayer (1) measured peroxynitrite formation in the xanthine oxidase/spermine NONOate system by the oxidation of dihydrorhodamine. The measured initial velocity was consistent with the expected flux of peroxynitrite. However, they only measured peroxynitrite formation at the start of the reaction before urate had begun to accumulate. We found that oxidation of dihydrorhodamine added after the xanthine oxidase/spermine NONOate reaction had progressed for as little as 10 min was suppressed to a greater extent than tyrosine nitration by preformed peroxynitrite (Fig. 5). Consequently, the accumulation of urate was sufficient to account for the apparent lack of nitration. Sawah et al. (57) have recently shown similar yields of tyrosine nitration were obtained with peroxynitrite as with nitric oxide generated by a NONOate plus superoxide generated by xanthine oxidase. The assay was conducted only for 5 min to avoid oxygen depletion and used pterin as a substrate for xanthine oxidase. We have obtained similar results using their experimental conditions.

Reactions with Me₂SO—Because potassium superoxide is unstable in aqueous solvents, we used anhydrous Me₂SO. The final concentration of Me₂SO in the stopped-flow mixing experiments was 0.5 M. We had previously shown that high concentrations of Me₂SO and other hydroxyl scavengers do not substantially affect phenolic nitration by peroxynitrite (45), which was confirmed in the present study. Me₂SO is an effective scavenger of hydroxyl radical and possibly nitrogen dioxide (58), which can be produced during the homolysis of peroxynitrous acid (59). About 25–35% of peroxynitrite decomposes to form free hydroxyl radical and nitrogen dioxide. Hydroxyl radical will rapidly oxidize tyrosine to tyrosine radical. Tyrosine radical can either dimerize to form dityrosine or recombine with nitrogen dioxide to yield nitrotyrosine. However, the failure of up to 0.5 M Me₂SO or other hydroxyl radical scavengers to affect nitration suggests that tyrosine nitration may be more subtle than simple radical chemistry would suggest (45, 46). We have previously suggested that an intermediate involving peroxynitrous acid attacks tyrosine by two rapid consecutive one-electron oxidation steps to form nitrotyrosine without necessarily forming free hydroxyl radical and nitrogen dioxide (42, 45). Otherwise, hydroxyl radical scavengers should have at
least partially inhibited nitration by preventing the formation of tyrosine radical by hydroxyl radical.

**Tyrosine Nitration in Proteins**—The mechanisms of tyrosine nitration studied with just free tyrosine dissolved in simple phosphate buffers have been used to make deductions about how tyrosine is nitrated in proteins in vivo (32, 50, 60). In simple solutions, radical processes can certainly be the major cause of nitration and dimerization by peroxynitrite. However, thiol and other biological molecules are efficient inhibitors of such radical processes that will greatly reduce their contribution to nitration of proteins in cells (2, 32, 45). Prütz et al. (61) noted that small amounts of thiols or ascorbate completely block tyrosine nitration in model peptides by nitrogen dioxide. While large amounts of nitrogen dioxide, acidified nitrite, or nitryl chloride can nitrate free tyrosine (46), they are known to be extremely inefficient at nitrating tyrosine in proteins (3, 10). For example, nitryl chloride (NO2Cl) produced by the reaction of hypochlorous acid with nitrate does not give detectable nitration of proteins unless 2–3 millimolar concentrations of each are added (62). Consequently, the competing targets and endogenous antioxidants present in vivo effectively prevent nitration by nitrogen dioxide and nitryl chloride, but only partially suppress nitration by peroxynitrite because a single peroxynitrite molecule can carry out the complete 2 electron oxidation of tyrosine necessary to form nitrotyrosine (2). As the number of competing targets increases, peroxynitrite becomes more likely to be the predominant nitrating agent in vivo. In complex biological systems, peroxynitrite also becomes increasingly selective for modifying specific tyrosine residues on certain proteins in complex biological systems (14). Increased vulnerability to nitration by peroxynitrite can in part be traced to subtle differences in the local tyrosine environment imposed by the three-dimensional structure of the protein (63). Certain tyrosines appear to be more susceptible because the protein environment substantially decreases the phenoxyl pKa (63). Metals within enzymes can also facilitate the nitration of neighboring tyrosines (45, 64).

There is strong evidence that endogenous peroxynitrite formation from superoxide and nitric oxide is responsible for nitration of certain cellular proteins in vivo. Endogenous nitration of manganese superoxide dismutase (6, 12), prostacyclin synthase (18, 19), calcium ATPase (16, 65), and neurofilament L (13) in vivo has been demonstrated by several laboratories. These same proteins are nitrated when cells are exposed to low concentrations of nitric oxide and superoxide generated simultaneously, but not when exposed to nitric oxide alone, nitrite, or only oxygen radical generating systems.

Endogenous nitration of tyrosine resulting from the co-generation of nitric oxide and superoxide has been demonstrated to result in extensive tyrosine nitration by activated macrophages (66) and in motor neurons (66, 67). Previously, we have shown that alveolar macrophages activated to produce only nitric oxide did not nitrate tyrosine analogs. However, stimulation of superoxide production along with nitric oxide resulted in the expected amount of nitrotyrosine (66). Cultured embryonic motor neurons induce neuronal nitric oxide synthase when deprived of certain trophic factors before undergoing apoptosis (67). Both nitric oxide synthase inhibitors and the intracellular delivery of superoxide dismutase via liposomes prevents apoptosis and greatly reduced the accumulation of nitrotyrosine in these cells (68, 69). Tyrosine nitration in trophic factor-deprived motor neurons treated with nitric oxide synthase inhibitors was restored when <100 nM nitric oxide was exogenously generated. This same extracellular concentration of nitric oxide did not nitrate tyrosine in motor neurons cultured with trophic factors (70). In none of these cells did <40 μM nitrite with or without equimolar hydrogen peroxide lead to the formation of nitrotyrosine (68). These results strongly support a major role for the formation of peroxynitrite from nitric oxide and superoxide as a major source of nitrating tyrosine in proteins when produced at exceedingly low fluxes inside of cells.

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