# A Novel Procedure for Generating both Nitric Oxide and Superoxide in Situ from Chemical Sources at Any Chosen Mole Ratio. First Application: Tyrosine Oxidation and a Comparison with Preformed Peroxynitrite

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The first method for generating 'NO and  $O_2^{\bullet-}$  at any known, constant ratio has been developed. Spermine NONOate and di(4-carboxybenzyl)hyponitrite decay with first-order kinetics and exactly equal rate constants (half-lives of 80 min) at 37 °C and pH 7.5 to give 200 and 40 mol % 'NO and  $O_2^{\bullet-}$ , respectively. Tyrosine oxidation to dityrosine and 3-nitrotyrosine (the major and minor products under the conditions used in these experiments) has been studied (mainly in the presence of CO<sub>2</sub>) using various different ratios of the rates of formation of 'NO and  $O_2^{\bullet-}$ . The 'NO/O<sub>2</sub><sup>•-</sup> = 1.0 product profiles are very similar to those of the products derived from equal amounts of 'NO and  $O_2^{\bullet-}$  generated at a 'NO/O<sub>2</sub><sup>•-</sup> ratio of 1.0 from SIN-1 but are very different from those derived from preformed peroxynitrite. All the experimental results can be explained in terms of free radical chemistry. The product profiles at all the 'NO/O<sub>2</sub><sup>•-</sup> ratios could be satisfactorily simulated provided an important group of reactions which lead to the consumption of dityrosine was included.

## Introduction

Peroxynitrite may be formed in vivo by the diffusioncontrolled coupling of endogenous superoxide and nitric oxide. Its conjugate acid decomposes rapidly ( $t_{1/2} = 0.6$  s at 25 °C)

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow {}^{-}OONO \rightleftharpoons HOONO \rightarrow HNO_3 + HO^{\bullet} + NO_2^{\bullet}$$
 (1)

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giving nitrate together with smaller quantities of the hydroxyl radical and nitrogen dioxide (1-5). The yield of the hydroxyl radical is ca. 28% at pH 6.3 (5) and declines at higher pHs (2, 3). Under physiological conditions, these reactions are superseded by a reaction of the peroxynitrite anion with carbon dioxide ( $k = 3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) (6) to give a short-lived adduct which decomposes to ca. 67% nitrate and ca. 33% carbonate radical anion and NO<sub>2</sub>• (5, 7, 8).

$$OONO + CO_2 \longrightarrow ONOOCO_2 \longrightarrow ['NO_2 CO_3']_{cage} \xrightarrow{67\%} NO_3' + CO_2 (2a)$$

The radicals formed from peroxynitrite are very strong (HO<sup>•</sup>) or moderately strong ( $CO_3^{\bullet-}$  and  $NO_2^{\bullet}$ ) one-electron oxidizing agents. It is, therefore, not surprising that treatment of tyrosine (Tyr) with preformed peroxynitrite

yields dityrosine  $(di-Tyr)^1$  and 3-nitrotyrosine  $(NO_2-Tyr)$ (9) or that nitrotyrosine has been identified in proteins at sites of inflammation such as atherosclerotic lesions (10, 11). The tyrosyl radical intermediate in this reaction has been observed by ESR spectroscopy (12).



Ex vivo reactions of preformed peroxynitrite with biological substrates are of doubtful relevance in vivo where both 'NO and  $O_2^{\bullet-}$  must also be present, either of which may react with the bioradical initially produced by HO•, NO<sub>2</sub>•, or CO<sub>3</sub>•-. The absence of 'NO and O<sub>2</sub>•- can be overcome by using SIN-1 (3-morpholinosydnonimine) which generates both 'NO and O<sub>2</sub>•-. The SIN-1 is first hydrolyzed to SIN-1A which is oxidized by O<sub>2</sub> to SIN-1•+ and O<sub>2</sub>•-, and the SIN-1•+ then spontaneously decomposes, releasing 'NO (*13*). The yields of 'NO and O<sub>2</sub>•- from SIN-1 should be equal, but it seems unlikely that these two radicals are commonly (or ever) produced in equal amounts in vivo. It has also been demonstrated (*13*) that other electron acceptors can compete with O<sub>2</sub> for SIN-

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<sup>&</sup>lt;sup>1</sup> Abbreviations: di-Tyr, dityrosine; NO<sub>2</sub>-Tyr, 3-nitrotyrosine; SIN-1, 3-morpholinosydnonimine; s-NONOate, spermine NONOate; XO, xanthine oxidase.

1A and, in the presence of such oxidants, SIN-1 becomes "just" a source of 'NO. Although the abilities of  $CO_3^{\bullet-}$  and HO<sup>•</sup> to effect one-electron oxidation of SIN-1A were not examined (*13*), there is at least a possibility that these radicals could function in such a way and cause SIN-1 to generate an excess of 'NO over  $O_2^{\bullet-}$ .

Another approach has been to combine a chemical NO source, spermine NONOate (s-NONOate), with an enzymatic  $O_2^{*-}$  source, xanthine oxidase (XO) along with hypoxanthine (*14*, *15*).



Unfortunately, the XO/hypoxanthine (or other substrate) reaction produces both  $O_2^{\bullet-}$  and, in comparable but usually higher yields,  $H_2O_2$ , and the organic products derived from the substrates themselves react with peroxynitrite. Moreover, XO is commonly contaminated with adventitious iron, so that Fenton and Haber–Weiss chemistry may also occur. Furthermore, XO generates a relatively short burst of  $O_2^{\bullet-}$  which cannot easily be matched to the first-order formation of  $\bullet$ NO from the NONOate. Since the products derived from tyrosine depend on the absolute and relative amounts of  $\bullet$ NO and  $O_2^{\bullet-}$  (vide infra), any change in the  $\bullet$ NO/ $O_2^{\bullet-}$  ratio during an experiment will limit the derivable information.

The reaction of tyrosine (2 mM) with equal concentrations of 'NO and  $O_2^{\bullet-}$  has also been studied recently by the radiolysis of aerated 50 mM phosphate buffer (pH 7.6) containing 0.38 mM CO<sub>2</sub>, 10 mM nitrite, and 1 M methanol (*16*). Unfortunately, this technique does not permit the generation of 'NO and  $O_2^{\bullet-}$  at ratios other than 1/1. The probable effects of different 'NO/ $O_2^{\bullet-}$  ratios on the yield of NO<sub>2</sub>-Tyr had, therefore, to be estimated by kinetic simulation.

We have overcome the above-mentioned difficulties by combining a clean, chemical source of 'NO (s-NONOate, reaction 4) with a clean, chemical source of  $O_2^{*-}$  (SOTS-1, eq 5) (*17*, *18*).

$$\begin{bmatrix} -O_2C-C_6H_4-CH_2ON= \end{bmatrix}_2 \rightarrow \rightarrow \xrightarrow{O_2} 0.4O_2^{\bullet}$$
(5)  
SOTS-1

The great advantage of this new system is that the decompositions of s-NONOate and SOTS-1 both follow first-order kinetics with rate constants that are exactly equal at 37 °C and pH 7.5. This means that the ratio of the rates of formation of 'NO and O<sub>2</sub>.<sup>-</sup> can be set to any value, not necessarily equal, and this ratio will be maintained until the s-NONOate and SOTS-1 have decomposed completely ( $\geq$ 4.5 h). Although the rates of formation of 'NO and O2'- decline exponentially, this could be overcome by adding "makeup" SOTS-1 and s-NONOate every 20-30 min. We did not bother with this refinement for our first application of this new system which was tested on tyrosine, and the results were compared with those obtained using SIN-1 and preformed peroxynitrite. There are very significant differences in product yields as the ratios of the rates of formation of 'NO and O2'- are changed, and even at a 1/1 ratio, the product yields differ from those obtained

with preformed peroxynitrite. However, all our results can be explained in terms of known radical chemistry.

# **Experimental Procedures**

**Chemicals.** SOTS-1 and di-Tyr were synthesized as described previously (*17, 19*); other chemicals were of the highest purity and used as supplied: Chelex 100, Tyr, and NO (Aldrich), *freshly* manufactured s-NONOate (both Alexis Corp. and Molecular Probes),  $H_2O_2$  (Anachemia), phosphate (BDH),  $O_2$  and  $CO_2$  (Praxair), methanol (EM Science), and  $NO_2$ -Tyr (Sigma).

**Reactions with Preformed Peroxynitrite.** Unless stated otherwise, all reactions were performed at 37 °C, in 50 mM Chelex 100-treated phosphate buffer (pH 7.5). Reaction mixtures were first purged with and then the reactions were carried out under 100%  $O_2$  or 90%  $O_2/10\%$  CO<sub>2</sub> [resulting in 4 mM CO<sub>2</sub>(aq) and 80 mM HCO<sub>3</sub><sup>-</sup>] (*20*). Purging the solutions with CO<sub>2</sub> slightly reduced their pH which was countered by the addition of sodium hydroxide, after which the solution was purged for at least a further 15 min and the pH checked to ensure it was 7.5 before the reaction was started.

Reactions with SOTS-1 and/or s-NONOate. Solutions of SOTS-1 and Tyr (4 mM) were made up at 0 °C and purged for ca. 30 min with the desired gas. This solution was then added to solid s-NONOate and heated to 37 °C with gentle rocking in a water bath while being kept in a sealed system under a slight positive pressure (provided by a small balloon) of the original purging gas throughout the experiment. The solution was not stirred, and gas was not bubbled through it or passed over it during reaction to avoid displacement of the 'NO. Samples were analyzed with a HPLC system fitted with a Spherisorb ODS-2 5a column (Waters). The eluent was 20 mM phosphate buffer (pH 3.0) and methanol [97/3 (v/v) between 0 and 10 min followed by ramping over the course of 2 min to 90/10 (v/v)]. The retention times of Tyr (followed at 276 nm), di-Tyr (followed by fluorescence;  $\lambda_{ex} = 284$  nm,  $\lambda_{em} = 410$  nm), and NO<sub>2</sub>-Tyr (followed at 365 nm) were 6.7, 10.6, and 15.0 min, respectively.

**Kinetic Simulations.** The products formed after 4.5 h by the action of SOTS-1 and s-NONOate on Tyr were modeled on  $10^7$  molecules using the "Chemical Kinetics Simulator" (CKS, version 1.01, IBM), a program which uses a stochastic algorithm to calculate the consequences of a given set of reactions (*21*). A few simulations on  $10^8$  molecules reduced the stochastic "noise" but gave results which were not significantly different.

#### Results

Matching 'NO and O<sub>2</sub>.<sup>-</sup> Formation Rates. Each s-NONOate molecule yields two molecules of 'NO (22), while 0.4 molar equiv of  $O_2^{\bullet-}$  is produced from  $O_2$  by SOTS-1 (17). The rate of decomposition of SOTS-1 has been measured by <sup>1</sup>H NMR spectroscopy and found to be independent of oxygen pressure and pH (data not shown). In contrast, the rate of 'NO release depends on pH (22) because protonation of the s-NONOate is necessary for 'NO release. At 37 °C in 50 mM phosphate buffer, s-NONOate decay, monitored at 252 nm, followed clean first-order kinetics from pH 7.6 to 7.2, and at pH 7.5, the half-life was 4900 s (independent of the presence of SOTS-1, data not shown). This exactly matches the halflife of SOTS-1 under the same conditions (17) [but is, for reasons, e.g., pH, ionic strength, etc., which were not explored, about twice the half-life reported in previous studies at pH 7.4 (23, 24)]. Since reactions 4 and 5 are both first-order and have equal rate constants, the ratio of the rates of formation of 'NO and  $O_2^{\bullet-}$  will be constant throughout an experiment irrespective of the chosen •NO/O<sub>2</sub>•<sup>-</sup> ratio. SIN-1, another source of •NO and O<sub>2</sub>•<sup>-</sup>, decays with a half-life of about 2500 s at 37 °C in phosphate buffer at pH 7.4 (13).



**Figure 1.** Buildup of di-Tyr during the reaction of SOTS-1 (1.25 mM  $\equiv$  0.5 mM  $O_2^{\bullet-}$ ) and s-NONO (0.25 mM  $\equiv$  0.5 mM 'NO) with Tyr (4 mM) in 50 mM phosphate buffer (pH 7.5) at 37 °C under an atmosphere of a 9/1  $O_2/CO_2$  mixture. The inset shows formation of di-Tyr after 4.5 h when SOTS-1 (0.5 mM  $\equiv$  0.2 mM  $O_2^{\bullet-}$ ) and s-NONOate (0.1 mM  $\equiv$  0.2 mM 'NO) react with Tyr (0.4–4.0 mM) under the same conditions: (bars) experimental results (errors of less than  $\pm 15\%$ ) and ( $\bigcirc$  and  $\bigcirc$ ) simulated yields (black and white circles refer to  $k_{-8}$  values of  $4 \times 10^5$  and  $2 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>, respectively; see the text for details). Connecting lines are only a visual aid.

**Reaction of Tyr with**  $O_2^{\bullet-}$ **.** Di-Tyr was not detected after the incubation (4.5 h) of oxygenated SOTS-1 (1.25 mM  $\equiv 0.5 \text{ mM } O_2^{\bullet-}$  in total) with Tyr (4 mM) in the presence or absence of CO<sub>2</sub>.

Reaction of Tyr with 'NO. Incubation (4.5 h) of s-NONOate (0.1 mM  $\equiv$  0.2 mM ·NO total) with Tyr (4 mM) under an O<sub>2</sub>/CO<sub>2</sub> atmosphere (9/1) did not yield detectable quantities of NO2-Tyr or di-Tyr. However, at higher concentrations of s-NONOate and after a lag phase, both di-Tyr and NO<sub>2</sub>-Tyr were formed in steadily increasing quantities. The yield of NO<sub>2</sub>-Tyr went through a maximum of 1.6  $\mu$ M at 5 mM s-NONOate and that of di-Tyr through a maximum of 8.8 µM at 2.5 mM s-NONOate. The formation of NO<sub>2</sub>-Tyr and di-Tyr required  $O_2$ , and the lag phase can be attributed to the slow oxidation of 'NO to NO2' (the one-electron oxidant of Tyr in such systems). Addition of 100  $\mu$ L of buffer which had been purged with  $N_2$  and then saturated with NO to 0.5 mL of Tyr (4 mM, saturated with a 9/1 O<sub>2</sub>/CO<sub>2</sub> atmosphere) also gave NO<sub>2</sub>-Tyr (1.2  $\mu$ M) and di-Tyr (0.8  $\mu$ M).

**Reaction of Tyr with 'NO and O<sub>2</sub>**. Incubation (4.5 h) of SIN-1 (0.5 mM) with Tyr (4 mM) under an  $O_2/CO_2$ atmosphere (9/1) gave 23.9  $\mu$ M di-Tyr and 1.2  $\mu$ M NO<sub>2</sub>-Tyr, while under 100% O<sub>2</sub>, the yield of di-Tyr fell to 3.5  $\mu$ M and only traces of NO<sub>2</sub>-Tyr were produced. Incubation (4.5 h) of s-NONOate (0.25 mM  $\equiv$  a total of 0.5 mM  $\cdot$ NO) and SOTS-1 (1.25 mM  $\equiv$  a total of 0.5 mM O<sub>2</sub><sup>•-</sup>) with Tyr (4 mM) gave 26.5  $\mu$ M di-Tyr and 0.56  $\mu$ M NO<sub>2</sub>-Tyr under an  $O_2/CO_2$  atmosphere, while under 100%  $O_2$ , the yield of di-Tyr fell to 2.9  $\mu$ M and only trace quantities of NO<sub>2</sub>-Tyr could be detected. The yield of di-Tyr increased with the initial Tyr concentration (0.4-4.0 mM, inset of)Figure 1), and therefore, all subsequent reactions were performed using 4 mM Tyr (close to its solubility limit). The buildup of di-Tyr follows first-order kinetics which match the decay kinetics of s-NONOate and SOTS-1 (half-life of 80 min); see Figure 1. The effects of varying the ratio of the rates of formation of 'NO and  $O_2^{\bullet-}$  are shown in Figures 2 and 3, respectively.

**Reaction of Tyr with Preformed Peroxynitrite at 24** °C. Peroxynitrite was synthesized according to a literature method (*25*). This preformed peroxynitrite (0.5 mL, 11 mM in N<sub>2</sub> saturated 25 mM NaOH) was syringe pumped into Tyr [5 mL, 4 mM in a 9/1 O<sub>2</sub>/CO<sub>2</sub> saturated



**Figure 2.** Effect of 'NO concentration on di-Tyr and NO<sub>2</sub>-Tyr (inset) yields after 4.5 h from SOTS-1 [1.25 mM  $\equiv$  0.5 mM O<sub>2</sub><sup>--</sup> (top) and 2.5 mM  $\equiv$  1.0 mM O<sub>2</sub><sup>--</sup> (bottom)] and Tyr (4 mM) in 50 mM phosphate buffer (pH 7.5) at 37 °C under a 9/1 atmosphere of O<sub>2</sub> and CO<sub>2</sub>: (bars) experimental results (errors of less than ±15%) and (•) simulated yields. Connecting lines are only a visual aid.



**Figure 3.** Effect of  $O_2^{\bullet-}$  concentration on di-Tyr and  $NO_2$ -Tyr (inset) yields after 4.5 h from s-NONOate [0.05 mM  $\equiv$  0.1 mM 'NO (top), 0.2 mM  $\equiv$  0.4 mM 'NO (middle), and 0.4 mM  $\equiv$  0.8 mM 'NO (bottom)] and Tyr (4 mM) in 50 mM phosphate buffer (pH 7.5) at 37 °C under a 9/1 atmosphere of  $O_2$  and  $CO_2$ : (bars) experimental results (errors of less than  $\pm 15\%$ ) and ( $\bullet$ ) simulated yields. Connecting lines are only a visual aid. White circles in the middle panel represent simulated yields of di-Tyr, divided by 4.8, using the reaction mechanism described by Goldstein et al. (*16*).

200 mM phosphate buffer (pH 7.5, increasing to 7.6 during the addition)] which was being rapidly vortexed under a  $9/1 O_2/CO_2$  atmosphere. Yields of di-Tyr (0.1 mM) and NO<sub>2</sub>-Tyr (0.1 mM) were independent of the per-

oxynitrite addition rate (equal volume from 13 s to 136 min) and represent individual yields for both products of 10% based on peroxynitrite (in the absence of CO<sub>2</sub>, the product yields did depend on the addition rate; unpublished results). Preformed alkaline peroxynitrite (0.2 mL, 2 mM and O<sub>2</sub>-saturated) was also mixed with Tyr [0.2 mL, 4 mM in O<sub>2</sub>-saturated 100 mM phosphate buffer (pH 4) to which 6 mM Na<sub>2</sub>CO<sub>3</sub> had been added 2 min prior to use] in a rapid mixing (1 ms) apparatus. The resulting solution (pH 7.5) contained di-Tyr (50  $\mu$ M, 5% yield) and NO<sub>2</sub>-Tyr (170  $\mu$ M, 17% yield). Interestingly, the percentage yields in these very rapid mixing experiments were independent of the peroxynitrite concentration (0.1–1.7 mM, after mixing).

#### Discussion

Tyr is not oxidized to di-Tyr or NO<sub>2</sub>-Tyr by either 'NO or  $O_2^{\bullet-}$  (although it is oxidized slowly by •NO and  $O_2$ following a lag phase during which some of the 'NO is converted into NO<sub>2</sub>·) (16). Tyr is oxidized by preformed peroxynitrite and by peroxynitrite formed in situ from •NO and O<sub>2</sub>•<sup>-</sup>, the principle products being di-Tyr and NO<sub>2</sub>-Tyr. The yields of Tyr oxidation products from peroxynitrite generated in situ from equal quantities of •NO (0.5 mM from s-NONOate) and O<sub>2</sub>•- (0.5 mM from SOTS-1) were in excellent agreement with the yields obtained using 0.5 mM SIN-1 (which also yields 0.5 mM •NO and 0.5 mM O<sub>2</sub>•<sup>-</sup>). Thus, in the presence of CO<sub>2</sub>, the di-Tyr and NO<sub>2</sub>-Tyr yields were 5.3 and 0.1% (s-NON-Oate/SOTS-1) and 4.8 and 0.2% (SIN-1), respectively, while in the absence of CO<sub>2</sub>, the yields of di-Tyr fell to 0.6% (s-NONOate/SOTS-1) and 0.7% (SIN-1) with only traces of NO<sub>2</sub>-Tyr being formed in either system. The essentially equal yields of products from the s-NONOate/ SOTS-1 and SIN-1 experiments which were matched in terms of total 'NO and  $O_2$  ·- production demonstrate that none of the organic radicals generated from SOTS-1 (17)is trapped by the 'NO formed from the s-NONOate, a conclusion consistent with our studies on the absolute kinetics of each step in the process leading to  $O_2^{\bullet-}$  (18). It also proves that SIN-1 provides equal quantities of •NO and  $O_2$ •- under the reaction conditions that were employed.

When Tyr is oxidized by preformed peroxynitrite under an  $O_2/CO_2$  atmosphere (9/1), overall product yields are greater and their distribution differs. Thus, syringe pump addition of preformed peroxynitrite (in total 1 mM) to a rapidly vortexed solution of Tyr (4 mM) gave di-Tyr and  $NO_2$ -Tyr, each in yields of 10% at times from 13 s to 136 min which indicates that the peroxynitrite anion reacts at the alkaline peroxynitrite:  $CO_2$ -buffer interface and not in the bulk solution. In contrast, addition in 1 ms gave di-Tyr and  $NO_2$ -Tyr in yields of ca. 5 and 17%, respectively, which match yields reported by others for the rapid flow mixing of preformed peroxynitrite with Tyr in the presence of  $O_2$  and  $CO_2$ , e.g., 3% di-Tyr and 17%  $NO_2$ -Tyr (9). Under these fast mixing conditions, reaction occurs in the bulk solution.

Pfeiffer and Mayer (*15*) first reported that the reaction of Tyr with peroxynitrite formed in situ (both from SIN-1 and from s-NONOate and XO/hypoxanthine) gave substantially lower yields of NO<sub>2</sub>-Tyr (yields of di-Tyr were not given) than in its reaction with preformed peroxynitrite.<sup>2</sup> Under CO<sub>2</sub>-free conditions, 4 and ~0.07% NO<sub>2</sub>-Tyr were obtained using preformed and in situ peroxynitrite, respectively, and in the presence of CO<sub>2</sub>, the yield of NO<sub>2</sub>-Tyr increased using preformed peroxynitrite (by ca. 70%) but was unchanged using in situ-generated peroxynitrite<sup>3</sup> (15). This led to the suggestion that peroxynitrite formed in situ was "different" from "authentic" (i.e., preformed) peroxynitrite. This is, of course, highly unlikely. However, in its reaction with Tyr, peroxynitrite formed slowly in situ will appear to be different from a bolus addition of an equal quantity of preformed peroxynitrite (both in the presence and in the absence of CO<sub>2</sub>) because of the different rates of radical formation.<sup>4</sup> Thus, with a bolus addition, particularly in the presence of CO<sub>2</sub>, the initial concentrations of Tyr-O<sup>•</sup> and NO<sub>2</sub><sup>•</sup> will be very high, making their combination very favorable [k(NO2+Gly-Tyr-O') =  $3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ] (26) and resulting in a NO<sub>2</sub>-Tyr yield of  $\sim 17\%$  ( $\sim 5\%$  di-Tyr). However, the slow (4.5 h) in situ formation of peroxynitrite in the presence of CO<sub>2</sub> involves only very low radical concentrations, and most of the NO<sub>2</sub><sup>•</sup> that formed will react with Tyr to generate Tyr-O•  $[k(NO_2 + Gly - Tyr) = 3 \times 10^5 M^{-1} s^{-1}]$  (26). As a consequence, the yield of NO<sub>2</sub>-Tyr is drastically reduced (to 0.1–0.3%). Under "intermediate" conditions, the yield of NO<sub>2</sub>-Tyr falls between these extremes, e.g., 10% for syringe pump addition of preformed peroxynitrite. A less important reason for product differences between in situ-generated and preformed peroxynitrite is that in the former case reactions of Tyr-O<sup>•</sup> with O<sub>2</sub><sup>•</sup> and 'NO become more probable and may contribute to lower NO<sub>2</sub>-Tyr and di-Tyr yields (vide infra).

Tyr oxidation by in situ-generated peroxynitrite gave maximum yields of di-Tyr and a local maximum of NO<sub>2</sub>-Tyr at a ratio of the rates of formation of 'NO to O<sub>2</sub><sup>•-</sup> of 1.0<sup>5</sup> (see Figures 2 and 3), the yield of di-Tyr decreasing as the total quantities of 'NO and O<sub>2</sub><sup>•-</sup> increased; viz.,  $['NO]_{total} = [O_2^{•-}]_{total} = 0.1, 0.2, 0.4, 0.5, 0.8, and 1 mM resulted in di-Tyr yields of 6.0, 6.0, 5.7, 5.3, 4.2, and 3.8%, respectively, while NO<sub>2</sub>-Tyr yields remained at ca. 0.1%. This decrease in di-Tyr yield is caused by further reactions of di-Tyr (see below). Even at equal total 'NO and <math>O_2^{•-}$  concentrations of 0.2 mM, the further reactions of di-Tyr resulted in the di-Tyr yield increasing almost linearly with the initial Tyr concentration (up to its solubility limit, 4 mM; see the inset of Figure 1).

Product profiles for the CO<sub>2</sub>-mediated reaction (Figures 2 and 3) are easy to understand qualitatively. Thus, for a constant total yield of  $O_2^{\bullet-}$  (Figure 2), di-Tyr was not detected in the absence of •NO, but in the presence of •NO, peroxynitrite is formed and Tyr is converted to di-Tyr and NO<sub>2</sub>-Tyr which reach maxima at equal total •NO and  $O_2^{\bullet-}$  concentrations. However, as the quantity of •NO is further increased, it traps Tyr-O• causing the

 $<sup>^2</sup>$  The maximum yield of NO<sub>2</sub>-Tyr was obtained with \*NO alone (with O<sub>2</sub>) and decreased upon addition of O<sub>2</sub>- $^-$  (15). The explanation for this result is unclear.

 $<sup>^3</sup>$  However, in our experiments,  $\rm CO_2$  increased the yields of  $\rm NO_2\textsc{-}Tyr$  from in situ-generated peroxynitrite.

<sup>&</sup>lt;sup>4</sup>  $\gamma$ -Radiolysis provides slow, constant, and equal rates of formation of 'NO and  $O_2$ <sup>--</sup> (16). In the presence of CO<sub>2</sub>, the yields of NO<sub>2</sub>-Tyr were reduced from 0.6 to 0.2% as the radiation intensity was decreased. Yields of di-Tyr were not reported.

<sup>&</sup>lt;sup>5</sup> The oxidation of dihydrorhodamine by separately generated 'NO and O<sub>2</sub><sup>--</sup> has also been reported to be at a maximum when the 'NO/ O<sub>2</sub><sup>--</sup> ratio equals 1.0, and it was suggested "that excess production of one radical over the other may act as an endogenous modulator of ONOO<sup>--</sup>..." (14). However, the postulated 'NO-induced and O<sub>2</sub><sup>--</sup>induced decompositions of ONOOH appear to be unknown reactions, and they certainly are not required to explain the maximum in the yields of di-Tyr and NO<sub>2</sub>-Tyr at a 'NO/O<sub>2</sub><sup>--</sup> ratio of 1.0, vide infra.

Table 1. Goldstein et al.'s Proposed Mechanism for the Action of NO<sup> $\cdot$ </sup> and O<sub>2</sub><sup> $\cdot-$ </sup> on Tyr in the Presence of CO<sub>2</sub>

|   | $k^{a,b}$   |
|---|---|
| $O_2^{\bullet-} + NO^{\bullet} \rightarrow -OONO$         | $4.3 - 6.7 	imes 10^9$  |
| $-OONO + CO_2 \rightarrow -O_2COONO$                      | $3	imes 10^4$   |
| $^{-}O_{2}COONO \rightarrow NO_{3}^{-} + CO_{2}$          | $>1 	imes 10^3 c$   |
| $^{-}O_{2}COONO \rightarrow NO_{2} + CO_{3} -$            | $>2	imes 10^3 c$  |
| $Tyr + CO_3^{\bullet-} \rightarrow Tyr - O^{\bullet}$     | $4.5 	imes 10^7$  |
| $Tyr + NO_2^{\bullet} \rightarrow Tyr - O^{\bullet}$      | $3.2 	imes 10^5$  |
| $Tyr-O^{\bullet} + O_2^{\bullet-} \rightarrow products$   | $1.5 	imes 10^9$  |
| $Tyr-O^{\bullet} + NO_2^{\bullet} \rightarrow NO_2$ -Tyr  | $1.3 	imes 10^9$  |
| Tyr-O• + NO <sub>2</sub> • $\rightarrow$ other products   | $1.7 	imes 10^9$  |
| 2Ťyr-O• → di-Tyr  | $2.3 	imes 10^8 d$  |
| $2O_2^{\bullet-} (+2H^+) \xrightarrow{\bullet} H_2O_2$    | $2	imes 10^5$   |
| $NO^{\bullet} + NO_2^{\bullet} \rightleftharpoons N_2O_3$ | $1.1 	imes 10^9$ (f), $8.4 	imes 10^4$ (r)                            |
| $N_2O_3 (+H_2O) \rightarrow 2NO_2^-$                      | $2 \times 10^3$ , $1.9 \times 10^6$ [HCO <sub>3</sub> <sup>-</sup> ], |
|   | $8 \times 10^{5}$ [phosphate]   |
| $NO_2 + O_2 = O_2 NO_2$                                   | $4.5 \times 10^9$ (f), $1.1$ (r)                                      |
| $^{-}O_2NO_2 \rightarrow O_2 + NO_2^{-}$                  | 1.3   |
| $2NO_2 \rightarrow N_2O_4$                                | $4.5 	imes 10^8$ (f), $6.9 	imes 10^3$ (r)                            |
| $N_2O_4 (+H_2O) \rightarrow NO_3^- + NO_2^-$              | $1 	imes 10^3$  |
| $2NO^{\bullet} + O_2 \rightarrow 2NO_2^{\bullet}$         | $2.9	imes10^6$  |
| Tyr-O• + NO• ⇐ Tyr-ONO                                    | $1	imes 10^9$ (f), $1	imes 10^3$ (r)                                  |
| $Tyr-ONO \rightarrow products$                            | $0.18^{e}$  |

<sup>*a*</sup> Rate constants in units of s<sup>-1</sup>, M<sup>-1</sup> s<sup>-1</sup>, and M<sup>-2</sup> s<sup>-1</sup> for first-, second-, and third-order reactions, respectively. <sup>*b*</sup> These rate constants were taken from ref *16* and relate to room temperature. We have not attempted to "adjust" them to 37 °C. <sup>*c*</sup> -O<sub>2</sub>COONO yields of 67% (NO<sub>3</sub><sup>-</sup> + CO<sub>2</sub>) and 33% (NO<sub>2</sub> + CO<sub>3</sub> -). <sup>*d*</sup> In our simulations, the yield of di-Tyr in this reaction was assumed to be 35%. <sup>*e*</sup> In our simulations, this rate constant was increased to 2 s<sup>-1</sup>.

Table 2. Additional Reactions Used in Our Simulation of the Action of NO<sup> $\circ$ </sup> and O<sub>2</sub><sup> $\circ-$ </sup> on Tyr in the Presence of CO<sub>2</sub>

|  | K <sup>u</sup>   |
|--|--|
| $SOTS-1 \rightarrow 0.4O_2^{\bullet-}$   | $1.5	imes 10^{-4}b$                                      |
| $s-NONO \rightarrow 2NO^{\bullet}$   | $1.5	imes 10^{-4}b$                                      |
| $Tyr-O^{\bullet} + di-Tyr \Rightarrow Tyr + di-Tyr-O^{\bullet}$                  | $1 \times 10^{7}$ (f), $^{c}4 \times 10^{5}$ (r) $^{c}$  |
| $di/tri-Tyr + CO_3^{\bullet-} \rightarrow di/tri-Tyr-O^{\bullet}$                | $1.1	imes 10^9c$   |
| $di/tri-Tyr + NO_2 \rightarrow di/tri-Tyr-O \rightarrow$                         | $8	imes 10^6c$   |
| $di/tri-Tyr-O^{\bullet} + NO_2^{\bullet} \rightarrow NO_2^{\bullet}(di/tri-Tyr)$ | $1.3	imes 10^9d$   |
| di/tri-Tyr-O• + $O_2$ •- $\rightarrow$ products                                  | $1.5	imes 10^9d$   |
| di/tri-Tyr-O• + NO• ➡ di/tri-Tyr-ONO   | $1 \times 10^{9}$ (f), $^{d} 1 \times 10^{3}$ (r) $^{d}$ |
| di/tri-Tyr-ONO → products  | $2^d$  |
| di-Tyr-O• + Tyr-O• → tri-Tyr   | $2.3	imes 10^8d^*e$                                      |
| 2di/tri-Tyr-O• → poly-Tyr  | $2.3	imes 10^8d$   |
| tri-Tyr-O• + Tyr-O• → poly-Tyr   | $2.3	imes10^8d$  |
|  |  |

<sup>*a*</sup> See footnote a in Table 1. <sup>*b*</sup> See the text. <sup>*c*</sup> Arbitrary value; see the text. <sup>*d*</sup> Reaction assumed to have the same rate constant as for Tyr-O<sup>•</sup>. <sup>*e*</sup> See footnote d in Table 1, di-Tyr being replaced with tri-Tyr.

yields of di-Tyr and NO<sub>2</sub>-Tyr to decrease. The di-Tyr yield does not decrease to zero but to the steady background level produced using s-NONOate alone. The NO<sub>2</sub>-Tyr yield decreases to a minimum and then increases (as  $2^{\circ}NO + O_2 \rightarrow 2NO_2^{\circ}$ ) to a second maximum, which can exceed the first maximum, and then decreases again (as  $^{\circ}NO + NO_2^{\circ} \rightarrow N_2O_3$ ). Similarly, at a constant total yield of  $^{\circ}NO$  (Figure 3), the di-Tyr and NO<sub>2</sub>-Tyr yields increase on addition of  $O_2^{\circ-}$  to a maximum at equal total  $O_2^{\bullet-}$  and  $^{\circ}NO$  concentrations, and then decrease eventually reaching zero when the total concentration of  $O_2^{\circ-}$  far exceeds that of  $^{\circ}NO$ , where all Tyr-O radicals are trapped by  $O_2^{\circ-}$  ( $k = 1.5 \times 10^9$  M<sup>-1</sup> s<sup>-1</sup>) (27).

**Simulations.** The action of SOTS-1 and s-NONOate on Tyr (4 mM) in the presence of  $O_2$  (1 mM) and a  $CO_2/HCO_3^-$  mixture (4 and 80 mM, respectively) for 4.5 h was initially modeled using the reaction mechanism of Goldstein et al. (*16*) (Table 1) and the first two reactions in Table 2 which reflect the exponential decay of SOTS-1 and s-NONOate. However, to fit our experimental re-

sults, one rate constant was changed and new reactions which consume di-Tyr were included (see Table 2). Goldstein et al. only modeled NO<sub>2</sub>-Tyr yields but noted that in  $\gamma$ -radiolysis reactions the di-Tyr yield was not linear, suggesting subsequent reactions of di-Tyr to form higher derivatives.

The reaction between Tyr-O<sup>•</sup> and <sup>•</sup>NO (last two reactions in Table 1) is complex and not well understood. It appears to be reversible (*28, 29*), and C- and O-nitrosotyrosine may interconvert with the latter hydrolyzing to Tyr and nitrite (*30*). Reactions 6 and 7 were used for modeling purposes.

$$Tyr-O^{\bullet} + NO^{\bullet} \rightleftharpoons TyrONO$$
 (6)

$$TyrONO \rightarrow products \tag{7}$$

Goldstein et al. used a  $k_6$  of  $1 \times 10^9$  M<sup>-1</sup> s<sup>-1</sup>, a  $k_{-6}$  of 1000 s<sup>-1</sup> (actual value suggested to be <1000 s<sup>-1</sup>), and a  $k_7$  of 0.18 s<sup>-1</sup> [actual value suggested to be  $> k_{-6}/(5 \times 10^3)$ ]. However, with these values, the simulation overestimated experimental di-Tyr yields in the absence of O<sub>2</sub><sup>•-</sup> and therefore gave a false product profile as the total O<sub>2</sub><sup>•-/\*</sup>NO concentration ratio was raised from 0 to 1; for an example, see Figure 3 (middle). A much better fit to experiment was achieved using a  $k_7$  of 2 s<sup>-1</sup>.

The coupling of para-substituted phenoxyl radicals yields a large number of products (*31*), e.g., 10 products in the oxidation of *p*-cresol with ferric chloride (*32*), and its oxidation by horseradish peroxidase and  $H_2O_2$  gave 8 times as much of the ortho-para coupled product as of the ortho-ortho coupled analogue of di-Tyr (*33*). Some oxygen-carbon coupled products are also expected. Our di-Tyr yields were best simulated assuming two Tyr-O<sup>•</sup> radicals combine to give a 35% yield of di-Tyr and that (unlike di-Tyr, vide infra) the other 65% of the products do not further react.

Phenoxyl radicals very rapidly abstract phenolic hydrogen atoms (*34*, *35*) and the TyrO<sup>•</sup>/di-Tyr reaction is expected to be fast with a rate constant in the range  $10^{6}-10^{8}$  M<sup>-1</sup> s<sup>-1</sup>. Reaction 8 will be reversible with  $k_{8} > k_{-8}$  because the O–H bond in di-Tyr is weaker than in Tyr (DFT calculations predict a difference of 1.2 kcal mol<sup>-1</sup>).<sup>6</sup>

$$Tyr-O^{\bullet} + di - Tyr \rightleftharpoons Tyr + di - Tyr-O^{\bullet}$$
(8)

Since reaction 8 is fast, the equilibrium between Tyr-O<sup>•</sup> and di-Tyr-O<sup>•</sup> is much more important for simulation than the absolute rate constants. Consequently,  $k_8$  was arbitrarily set at  $10^7 \text{ M}^{-1} \text{ s}^{-1}$  and a value of  $4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  was used for  $k_{-8}$ .<sup>7</sup> The CO<sub>3</sub><sup>•-</sup> and NO<sub>2</sub><sup>•</sup> radicals will also oxidize di-Tyr faster than Tyr. Under the simulated conditions, effectively all CO<sub>3</sub><sup>•-</sup> reacts with either Tyr or di-Tyr, and since their phenoxyl radicals are in equilibrium, the relative rates of the CO<sub>3</sub><sup>•-</sup> reaction are unimportant. (The same simulated results were obtained assuming di-Tyr reacted with CO<sub>3</sub><sup>•-</sup> 100 times faster or slower than Tyr!) The absolute rate of the reaction of di-Tyr with NO<sub>2</sub><sup>•</sup> will influence the NO<sub>2</sub><sup>•</sup> steady-state concentration and, therefore, the yield of NO<sub>2</sub>-Tyr. On the basis of reaction 8, the CO<sub>3</sub><sup>•-</sup> and NO<sub>2</sub><sup>•</sup> reactions with

<sup>&</sup>lt;sup>6</sup> E. Johnson and J. S. Wright, private communication.

<sup>&</sup>lt;sup>7</sup> To fit the experimental yields from Tyr (4 mM) at progressively higher equal total NO and O<sub>2</sub><sup>--</sup> concentrations,  $k_{-8}$  was optimized only to the nearest 1 × 10<sup>5</sup> M<sup>-1</sup> s<sup>-1</sup>.

di-Tyr were arbitrarily assumed to be 25 times faster than their reactions with Tyr. In radical-radical reactions, di-Tyr-O<sup>•</sup> was assumed to react at the same rate as Tyr-O<sup>•</sup>. The combination of Tyr-O<sup>•</sup> and di-Tyr-O<sup>•</sup> was simulated to yield 35% tri-Tyr, the reactivity of which was assumed to be the same as that of di-Tyr. Higher combinations of "poly-Tyr" were formed in such low yield that their further reactions were not considered.

The close agreement<sup>7</sup> between the simulated and experimental di-Tyr yields shown in Figures 2 and 3 (inset in Figure 1 discussed below) indicates that the kinetic model fulfills its purpose; i.e., it accounts for the experimental results. Experimental and modeled NO<sub>2</sub>-Tyr yields show the same trend, and there is a reasonable agreement in absolute yields at low total O2.- to 'NO concentration ratios. As this ratio approaches 1, the model overestimates the experimental yield and the data could be better modeled using a lower yield of NO<sub>2</sub>-Tyr from Tyr-O $\cdot$  + NO<sub>2</sub> $\cdot$  than the 45% proposed by Goldstein et al. (16) and used in our simulations.<sup>8</sup> However, it seems more likely that the model underestimates the competition for NO<sub>2</sub>• which is quite extraordinary; of its 33% yield from peroxynitrite (reaction 2b), 32.9% experimentally (32.8% by simulation) "disappears" and only 0.1% (0.2%) ends up as NO<sub>2</sub>-Tyr!

The experimental di-Tyr yields from Tyr (4 mM) decreased at progressively higher equal total O2.- and •NO concentrations. Even when  $[O_2^{\bullet-}]_{total} = [\bullet NO]_{total} =$ 0.2 mM, the di-Tyr yield increased with the initial Tyr concentration; see the inset in Figure 1. These facts might have suggested that not all the oxidizing radicals were trapped by Tyr. However, the simulations clearly show that the di-Tyr yield is dependent on the equilibrium between Tyr-O<sup>•</sup>/di-Tyr and Tyr/di-Tyr-O<sup>•</sup> (reaction 8). Reducing the Tyr concentration or raising the di-Tyr concentration shifts the equilibrium in favor of di-Tyr-O' which lowers the final di-Tyr yield. The effect of the initial Tyr concentration on di-Tyr yield could be simulated perfectly by reducing  $k_{-8}$  from  $4 \times 10^5$  to  $2 \times 10^5$  $M^{-1}$  s<sup>-1</sup> (see the inset in Figure 1), but the yields of di-Tyr from 4 mM Tyr and increasing equal total O<sub>2</sub><sup>•-</sup> and 'NO concentrations were then more poorly simulated. Possibly undescribed reactions play a minor role in product formation, or some rate constants are not quite optimal.

It should be noted that all Tyr product-forming reactions in the simulation involve free radicals. It is therefore unnecessary to involve nonradical Tyr nitrating species such as  $O_2 NOCO_2^-$  (*36*). Furthermore, although a report that sulfhydryls inhibit CO<sub>2</sub>-free peroxynitritemediated Tyr nitration (37) has been used as evidence against nitration via  $CO_3^{\bullet-}$  [because  $CO_3^{\bullet-}$  reacts more rapidly with Tyr (Table 1) than with cysteine,  $k = 3.2 \times$  $10^5 \text{ M}^{-1} \text{ s}^{-1}$ ] (*39*), this report appears to be in error. In our hands, cysteine protected Tyr from nitration by preformed peroxynitrite and CO<sub>2</sub> and the level of this protection was 3 times greater than that calculated assuming that the cysteine acted solely as a competitive scavenger for CO<sub>3</sub><sup>--</sup>. This is due to cysteine's facile reduction of phenoxyl radicals (40) leading to a diminution of all Tyr-O'-derived products.

# Conclusions

We consider the overall quality of the fit of simulated and experimental yields over such a wide range of total  $NO/O_2$ . concentration ratios to be a most gratifying proof that the products of the reaction of Tyr with peroxynitrite (preformed or generated in situ) in the presence of  $CO_2$ can be qualitatively and quantitatively accounted for by known free radical chemistry. We therefore conclude that the products formed in the Tyr reactions are formed *solely* by free radical reactions.

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 $<sup>^{8}</sup>$  The true yield may be slightly higher than 45% since preformed peroxynitrite yields 17% NO<sub>2</sub>-Tyr, more than half the 33% theoretical maximum yield (reactions 2b and 3a).

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