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Peroxynitrite-Dependent Tryptophan Nitration

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Peroxynitrite ($ONOO^-$), the reaction product of superoxide ($O_2^{\bullet-}$) and nitric oxide ($^\bullet NO$), nitrates tyrosine and other phenolics. We report herein that tryptophan is also nitrated by peroxynitrite in the absence of transition metals to one predominant isomer of nitrotryptophan, as determined from spectral characteristics and liquid chromatography−mass spectrometry analysis. At high peroxynitrite to tryptophan ratios, other oxidation products were detected as well. The amount of nitrotryptophan formed from peroxynitrite increased at acidic pH, with an apparent pK_a of 7.8. High concentrations of Fe^{3+}-EDTA were required to enhance peroxynitrite-induced nitrotryptophan formation, while addition of up to 15 μM Cu/Zn superoxide dismutase had a minimal effect on tryptophan nitration. Cysteine, ascorbate, and methionine decreased nitrotryptophan yield to an extent similar to that predicted by their reaction rates with ground-state peroxynitrite, and typical hydroxyl radical scavengers partially inhibited nitration. Plots of the observed rate constant of nitrotryptophan formation vs tryptophan concentration presented downward curvatures. Thus, the kinetics of metal-independent nitration reactions were interpreted in terms of two parallel mechanisms. In the first one, ground-state peroxynitrous acid nitrated tryptophan with a second-order rate constant of 184 ± 11 M^{-1} s^{-1} at 37 °C. The activation enthalpy was 9.1 ± 0.3 kcal mol^{-1}, and the activation entropy was −19 ± 1 cal mol^{-1} K^{-1}. In the second mechanism, ONOOH*, an activated intermediate derived from *trans*-peroxynitrous acid formed in a steady state, was the nitrating agent.

Introduction

Nitric oxide ($^\bullet NO$) reacts with superoxide ($O_2^{\bullet-}$) to produce peroxynitrite anion ($ONOO^-$), which can rapidly protonate (pK_a = 6.8; *1*) to its conjugate acid, peroxynitrous acid (ONOOH). Peroxynitrite[1] is a potent and versatile oxidant that can react with a wide range of target molecules such as sulfhydryls (*1*), methionine (*2*), ascorbate (*3*), DNA (*4*), and lipids (5). Several lines of evidence point at peroxynitrite as a key biomolecule in mediating toxic effects of superoxide and nitric oxide (*1*, *6*−*11*).

In addition to its behavior as a strong oxidant, peroxynitrite can nitrate aromatics such as tyrosine, a reaction that is catalyzed by metal complexes and Cu/Zn superoxide dismutase (*12*−*14*). Detection of nitrotyrosine in proteins with polyclonal and monoclonal antibodies supports the formation of peroxynitrite *in vivo* (*15*, *16*).

The reactivity of peroxynitrite is multifaceted and strongly pH-dependent (*17*). In the pathway of peroxynitrite isomerization to nitrate, the formation of a reactive intermediate derived from *trans*-peroxynitrous acid, ONOOH*, has been postulated (*2*, *18*). This intermediate would be responsible for the hydroxyl radical-like reactivity of peroxynitrite at acidic pH. Controversy exists as to whether the reactant is ONOOH* or hydroxyl ($^\bullet OH$) and nitrogen dioxide ($^\bullet NO_2$) radicals derived from the dissociation of peroxynitrous acid in a cage of water molecules (*19*, *20*). As for the mechanisms of aromatic nitration, it has been proposed that metal complexes catalyze the formation of a nitronium ion (NO_2^+)-like species from *cis*-peroxynitrite. In contrast, the active site of superoxide dismutase would accommodate *trans*-peroxynitrite to promote nitration (*14*). In addition, it has been suggested that nitration of tyrosine proceeds via a radical mechanism with intermediate formation of nitrogen dioxide (*21*).

We report herein the nitration of tryptophan by peroxynitrite and the role of metal complexes and superoxide dismutase on nitration yields. In addition, the kinetics of the metal-independent formation of nitrotryptophan were investigated to provide insight into the molecular mechanisms of peroxynitrite reactivity with tryptophan.

Experimental Procedures

Chemicals. Peroxynitrite was prepared from sodium nitrite and hydrogen peroxide in a quenched-flow reactor as previously described (*1*, *6*). To eliminate excess hydrogen peroxide, peroxynitrite was treated with manganese dioxide. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm in 1 M sodium hydroxide (ϵ = 1670 M^{-1} cm^{-1}; *22*).

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[1] Note: The term peroxynitrite is used to refer to both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH). IUPAC recommended names are oxoperoxonitrate(1−) and hydrogen oxoperoxonitrate, respectively.

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Diethylenetriaminepentaacetic acid (DTPA)[2] (0.1 mM) was added to L-tryptophan and potassium phosphate buffer solutions made from deionized water to avoid metal interference. For the study of superoxide dismutase and metal-catalyzed nitration reactions, solutions were passed through a chelating resin (Sigma, St. Louis, Mo) before use. Solutions of Fe^{3+}−ethylenediaminetetraacetic acid (EDTA) were prepared by adding a 1.1 excess of EDTA to $Fe(NH_4)(SO_4)_2$ dissolved in HCl (*14*). $Fe(NH_4)(SO_4)_2$ was obtained from Fluka (Buchs, Germany). Cu/Zn superoxide dismutase was kindly provided by Grünenthal Inc. (Aachen, Germany). Desferrioxamine was a gift of Ciba-Geigy (Basel, Switzerland). All other chemicals were purchased from Sigma.

Analytical Procedures. Nitrated derivatives of tryptophan were assessed by mass spectrometry. Analysis were performed on an API III triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) in the electrospray mode. Nitrotryptophan was separated from tryptophan by reversed phase high pressure liquid chromatography on a C_{18} capillary column of 320 μm inner diameter at a flow rate of 7 μL min^{-1} into the IonSpray interface. A gradient of 0−100% acetonitrile in 10 mM ammonium acetate was used. Positive-ion mass spectra were recorded with an orifice potential of +60 V.

Ultraviolet and visible absorption spectra were determined with a diode-array spectrophotometer (Milton Roy Spectronic 3000 Array).

Nitration Assays. Reactions were initiated by addition of peroxynitrite and rapid vortexing. The yield of nitrotryptophan was measured by absorbance at 400 nm, and pH of reactions was measured after completion to account for changes caused by the addition of alkaline peroxynitrite. Each data point represents the mean of duplicate measurements which had less than 10% difference, for a representative experiment, unless otherwise specified. Superoxide dismutase, Fe^{3+}-EDTA, and scavengers were added to mixtures 5 min before peroxynitrite to determine their effect on nitration.

Kinetic Studies. The rate of peroxynitrite decomposition in the absence of tryptophan was followed at 302 nm in a stopped-flow spectrophotometer (Applied Photophysics SF.17MV) with dead time of <2 ms. Since tryptophan absorbs at this wavelength, no direct observation of peroxynitrite decay was possible in its presence. Thus, the formation of nitrotryptophan was followed at 400 nm, where there is no interference from the absorbance of tryptophan or peroxynitrite and its decomposition products. Apparent rate constants for nitrotryptophan formation, k_{obs} (s^{-1}), were determined by nonlinear least-squares fitting of stopped-flow data to a single exponential function with a nonzero offset. Reported values are the average of at least 7 separate determinations. The temperature was maintained to within 0.1 °C and the pH was measured at the outlet.

Results

Formation of Nitrotryptophan. Addition of peroxynitrite to tryptophan solutions in potassium phosphate buffer (pH 6−9) resulted in the appearance of a yellow chromophore. Liquid chromatography−mass spectroscopic analysis of reaction mixtures showed the formation of a product with a molecular weight of 249 consistent with the formation of nitrotryptophan (Figure 1a). The addition of 0.5−5 mM peroxynitrite to 10 mM tryptophan resulted in the formation of one product with a retention time of 14 min, suggesting the presence of only one predominant isomer of nitrotryptophan. At higher peroxynitrite concentrations (10 mM) two other nitrotryptophan species with different retention times were evident, indicating the formation of other nitrated isomers. In addition, at high peroxynitrite concentrations (5 and 10 mM) other species were formed such as hydroxytryptophan (Figure 1b) and products with a molecular weight of 236, that could correspond to *N*-formylkinurenine or dihydroxytryptophan (Figure 1c). Thus, it can be inferred that when tryptophan was present in excess (low peroxynitrite to tryptophan ratios), the main derivative formed was a single isomer of nitrotryptophan.

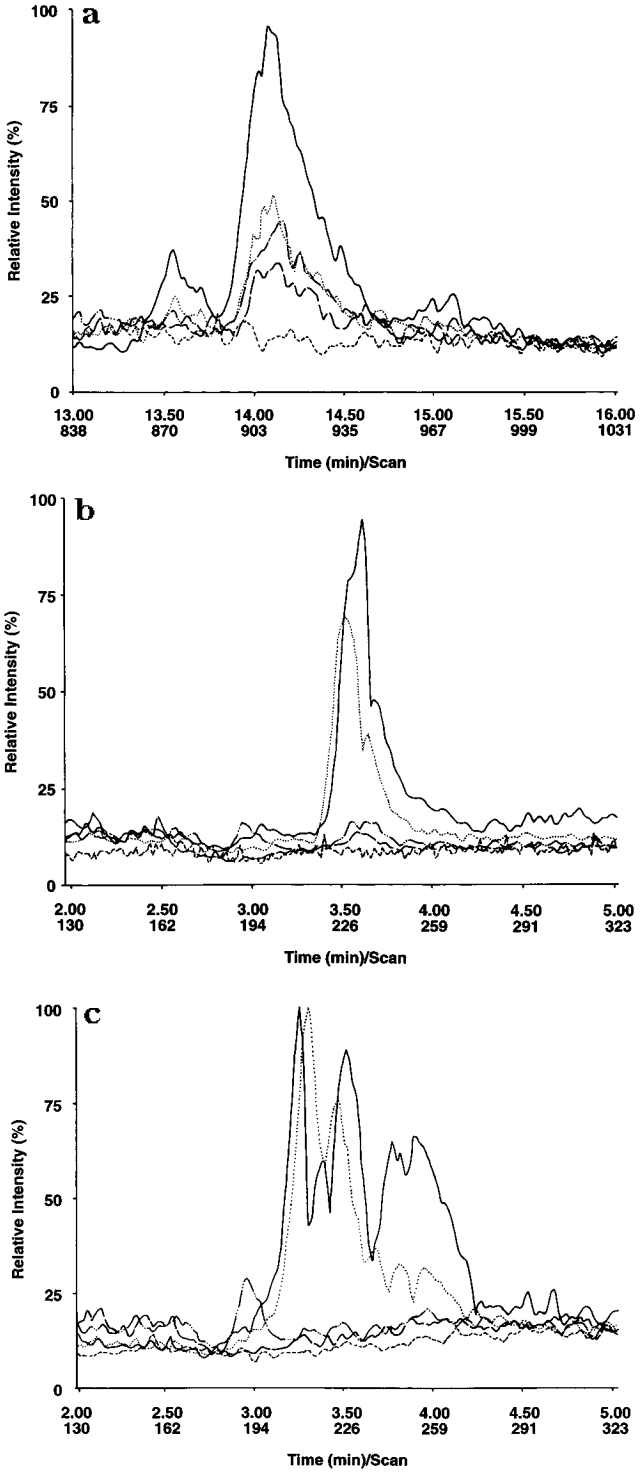


Figure 1. Ion chromatograms of tryptophan derivatives at various peroxynitrite concentrations. Reactions were started by addition of the following concentrations of peroxynitrite: (---) none, (− −) 0.5 mM, (−··−) 1 mM, (···) 5 mM, or (—) 10 mM to 10 mM tryptophan in 0.1 M potassium phosphate, pH 7.4. Samples were then analyzed by liquid chromatography−mass spectrometry as described in the Experimental Procedures. (a) Nitrotryptophan at m/z 250. (b) Hydroxytryptophan at m/z 221. (c) *N*-Formylkinurenine or dihydroxytryptophan at m/z 237. All represent $(M+H)^+$ ions.

[2] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide.

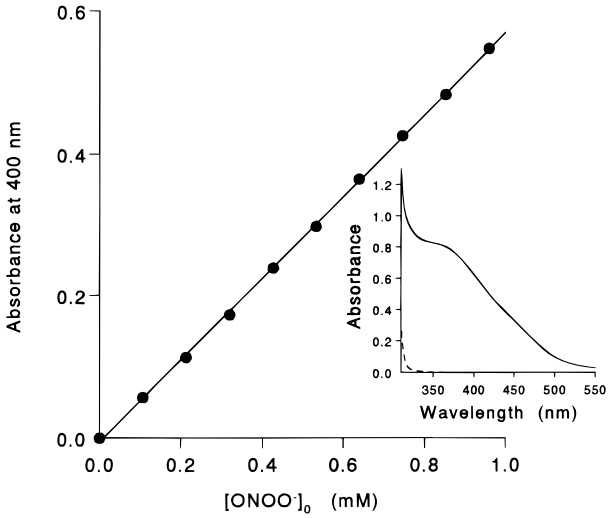


Figure 2. Nitrotryptophan formation as a function of peroxynitrite concentration. Reactions were started by addition of peroxynitrite to 10 mM tryptophan in 0.1 M potassium phosphate buffer, 0.1 mM DTPA, pH 6.03. After 5 min incubation at 37 °C the absorbance at 400 nm was determined. (Inset) Typical spectrum obtained from this reaction mixture with no (---) or 1 mM peroxynitrite (—).

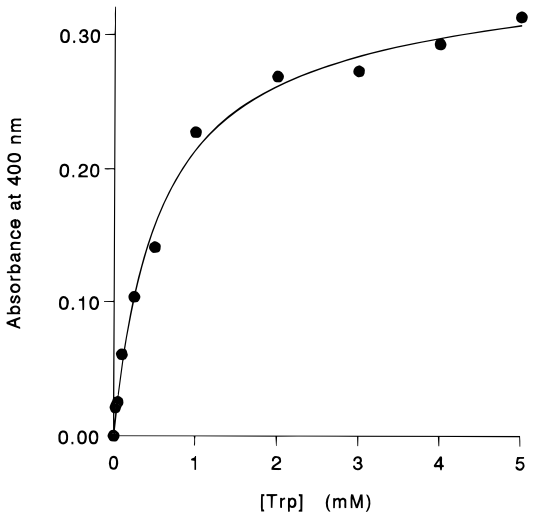


Figure 3. Nitrotryptophan formation as a function of tryptophan concentration. Reactions were initiated by addition of 0.5 mM peroxynitrite to tryptophan in 0.1 M potassium phosphate buffer, 0.1 mM DTPA, pH 6.04, at 37 °C. After 5 min incubation the absorbance at 400 nm was determined.

The amount of nitrotryptophan formed increased linearly as a function of added peroxynitrite concentration (Figure 2). The spectrum of the nitrated product in the 310−550 nm range (inset of Figure 2), when compared with the literature, was compatible with the predominant isomer formed being 6-nitrotryptophan (*23*−*26*). The dependence of the amount of nitrotryptophan formed with the concentration of tryptophan is shown in Figure 3. Assuming the isomer formed was 6-nitrotryptophan, from its known absorption coefficient at 400 nm of 5.2 $mM^{-1}\,cm^{-1}$ (*25*) the yield of nitration could be calculated as 12% with respect to the initial peroxynitrite concentration with 0.5 mM peroxynitrite and 5 mM tryptophan, at pH 6.0.

The yield of nitrotryptophan was greater at acidic pH (Figure 4) and could be described by the Henderson−Hasselbalch equation, with an apparent pK_a of 7.79 ± 0.05, with significant extents of nitrotryptophan formed even at alkaline pH. Similar results were obtained when

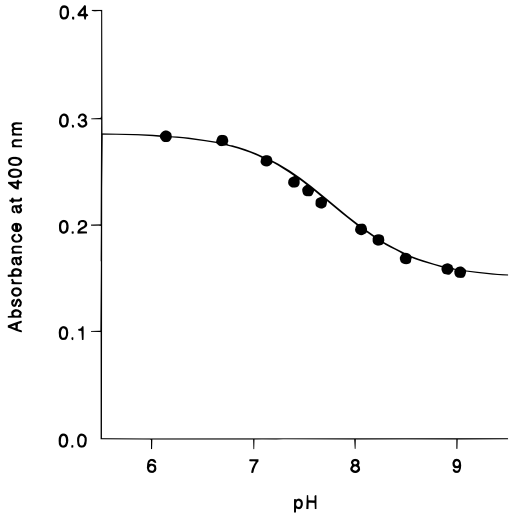


Figure 4. Influence of pH on tryptophan nitration. Peroxynitrite (0.5 mM) was added to 5 mM tryptophan in 0.067 M potassium phosphate buffer, 0.1 mM DTPA, adjusted to pH 6−10, at 37 °C. After 5 min incubation, the absorbance at 400 nm was determined. The solid line was fitted by nonlinear regression using the Henderson−Hasselbalch equation.

Table 1. Influence of Fe^{3+}-EDTA and Cu/Zn Superoxide Dismutase on Nitrotryptophan Yields [a]

addition	absorbance at 400 nm
none	0.231 ± 0.004
Fe^{3+}-EDTA (mM)	
0.1	0.286 ± 0.011
0.5	0.418 ± 0.001
1	0.455 ± 0.016
2	0.521 ± 0.030
superoxide dismutase (μM)	
0.5	0.245 ± 0.002
5	0.238 ± 0.002
10	0.245 ± 0.002
15	0.239 ± 0.006

[a] Peroxynitrite (0.5 mM) was added to 5 mM tryptophan and Fe^{3+}-EDTA or bovine Cu/Zn superoxide dismutase in 0.1 M potassium phosphate buffer, pH 7.35. After 5 min incubation at 37 °C, the absorbance at 400 nm was determined. Corrections were made for the intrinsic absorbance of the iron complex at 400 nm. Each value represents the mean ± SD of triplicate measurements. All values were significantly different ($p < 0.05$) from the control (no addition).

the pH of the different reaction mixtures was adjusted to a value of 6.8 before absorbance measurement; and no major changes in the spectrum of the nitrated product occurred in the range of pHs tested (data not shown). The pH dependence of nitration yield can only be attributed to peroxynitrite, since the pK_a of the nitrogen in the indole ring is too high to influence reactivity.

Influence of Fe^{3+}-EDTA and Superoxide Dismutase. Fe^{3+}-EDTA promoted the nitration of tryptophan (Table 1), but to a much smaller extent than the nitration of 4-hydroxyphenylacetic acid as reported by Beckman *et al.* (*14*). Superoxide dismutase induced a marginal effect (∼5% increase) on tryptophan nitration, in contrast to the 60% increase on the nitration of 4-hydroxyphenylacetic acid with similar micromolar concentrations of superoxide dismutase (*14*). Controls showed that superoxide dismutase nitration did not interfere with our determination.

Stopped-Flow Kinetic Analysis. Plots of the apparent rate constant of nitrotryptophan formation (k_{obs}) vs tryptophan concentration presented downward curvatures (Figure 5). It can be assumed that the concen-

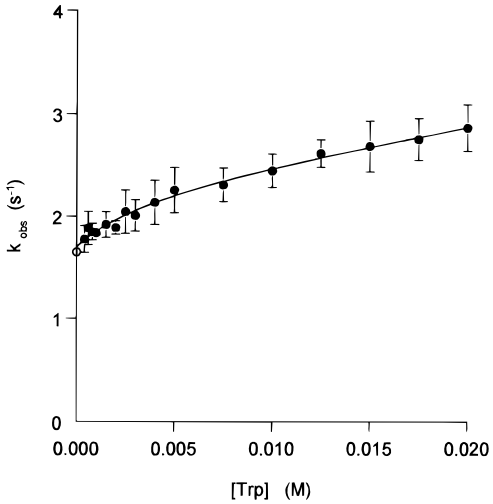


Figure 5. Apparent rate of nitration (k_{obs}) as a function of the concentration of tryptophan. Peroxynitrite (0.2 mM) was mixed with tryptophan in 0.05 M potassium phosphate buffer, 0.1 mM DTPA, pH 7.02, at 37 °C. The increase in absorbance at 400 nm was fitted to a single exponential function. The open symbol represents the decrease in absorbance at 302 nm in the absence of tryptophan.

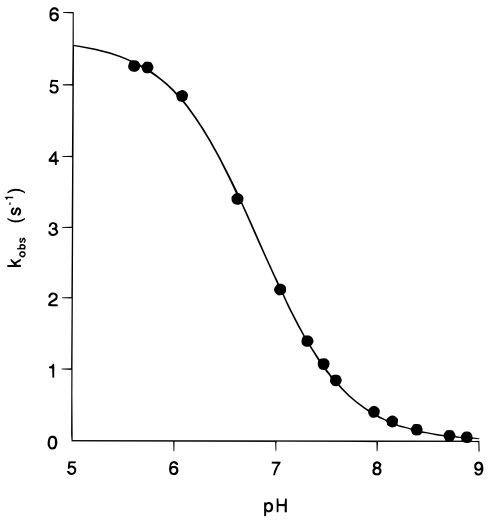


Figure 6. pH dependence of the apparent rate of tryptophan nitration (k_{obs}). Peroxynitrite (0.5 mM) was mixed with 5 mM tryptophan in 0.1 M potassium phosphate buffer, 0.1 mM DTPA, at 37 °C. The increase in absorbance at 400 nm fitted a single exponential function. The error bars are smaller than symbols.

tration of tryptophan remained constant throughout the reaction even at the lowest concentrations assayed (2-fold excess of tryptophan with respect to the initial amount of peroxynitrite added) due to the low yields of nitration. One possible interpretation for the curvature in this figure can be inferred by a reaction pathway analogous to that of peroxynitrite reaction with methionine (*2*). Thus, kinetic analyses were formulated assuming that nitrotryptophan can be formed both from the direct reaction of tryptophan with ground-state peroxynitrous acid, and with the energized intermediate, ONOOH*, formed in a steady state. The latter, besides yielding the nitrated product, can isomerize to nitrate.

$$\mathrm{ONOOH} \overset{K_A}{\rightleftharpoons} \mathrm{H^+ + ONOO^-} \quad (1)$$

$$\mathrm{ONOOH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ONOOH^*} \quad (2)$$

$$\mathrm{ONOOH^*} \xrightarrow{k_N} \mathrm{H^+ + NO_3^-} \quad (3)$$

$$\mathrm{ONOOH + Trp} \xrightarrow{k_T} \mathrm{NO_2\text{-}Trp + H_2O} \quad (4)$$

$$\mathrm{ONOOH^* + Trp} \xrightarrow{k_0} \mathrm{NO_2\text{-}Trp + H_2O} \quad (5)$$

In excess of tryptophan, the nitrated product would be formed as an exponential function of time:

$$[\mathrm{NO_2\text{-}Trp}] = [\mathrm{ONOO^-}]_0(F/P)(1 - e^{-Pt}) \quad (6)$$

where F and P are:

$$F = \left(\frac{k_0k_1[\mathrm{Trp}]}{k_{-1} + k_N + k_0[\mathrm{Trp}]} + k_T[\mathrm{Trp}]\right)\frac{[\mathrm{H^+}]}{K_A} \quad (7)$$

$$P = \left(\frac{k_Nk_1 + k_0k_1[\mathrm{Trp}]}{k_{-1} + k_N + k_0[\mathrm{Trp}]} + k_T[\mathrm{Trp}]\right)\frac{[\mathrm{H^+}]}{K_A + [\mathrm{H^+}]} \quad (8)$$

According to this scheme, P would represent the exponential coefficient determined by stopped-flow spectroscopy for the appearance of nitrotryptophan, k_{obs}, at 400 nm. Accordingly, it represents the rate of disappearance of peroxynitrite. Thus, the exponential increase in absorbance at 400 nm reflects the rate of peroxynitrite decay.

The pH dependence of k_{obs} is shown in Figure 6. The rate of appearance of nitrotryptophan increased at acid pH with a pK_a of 6.83 ± 0.01, in agreement with eq 8.

At relatively high concentrations of tryptophan (linear portion of eq 8), k_{obs} for the formation of nitrotryptophan would be

$$k_T[\mathrm{Trp}]\frac{[\mathrm{H^+}]}{K_A + [\mathrm{H^+}]} \quad (9)$$

Thus, in order to determine k_T, the second-order rate constant of peroxynitrite reaction with tryptophan, k_{obs} was determined at different pH values. The slopes of the plots of k_{obs} as a function of tryptophan (k'_T, inset of Figure 7) were plotted against $[H^+]/(K_A+[H^+])$. Thus, from the slope of Figure 7, k_T was calculated to be 184 ± 11 s^{-1} M^{-1} at 37 °C.

The temperature dependence of the second-order rate constant of tryptophan with peroxynitrite, k_T, in the range of 10−50 °C, was studied at pH 7.0. Since relatively high concentrations of tryptophan were used, the apparent second-order rate constants were calculated from the slopes of the plots of k_{obs} as a function of tryptophan concentration (inset of Figure 8). After making pH corrections according to eq 9, considering that the equilibrium constant of peroxynitrite ionization, K_A, does not change with temperature (*18*, *28*), k_T was calculated for each temperature. The activation enthalpy for the second-order nitration of tryptophan was 9.1 ± 0.3 kcal mol^{-1} and the activation entropy was −19 ± 1 cal mol^{-1} K^{-1} according to the Eyring plot shown in Figure 8.

Effect of Scavengers. As shown in Table 2, nitration yield was decreased by cysteine, ascorbate, and methionine to the extent predicted by their reaction rates with ground-state peroxynitrite, assuming simple competition kinetics (*1*−*3*). Typical hydroxyl radical scavengers such

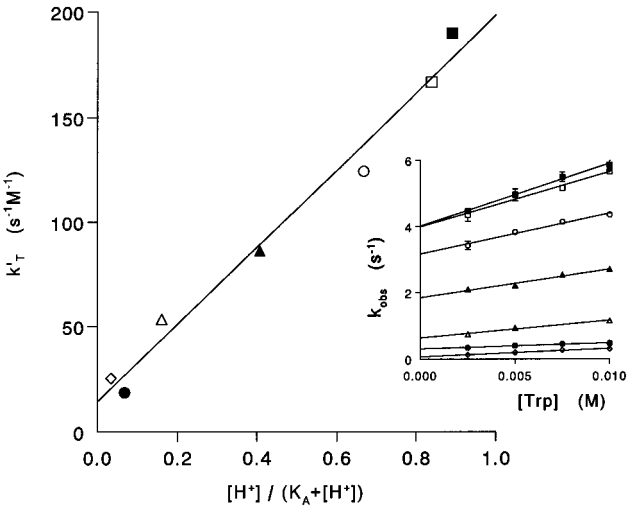


Figure 7. Second-order rate constant (k_T) for peroxynitrite nitration of tryptophan. Peroxynitrite (0.5 mM) was mixed with tryptophan in 0.05 M potassium phosphate buffer, 0.1 mM DTPA, at 37 °C, pH 5.89 (■), 6.09 (□), 6.50 (○), 6.96 (▲), 7.52 (△), 7.94 (●), and 8.25 (◊). The k_{obs} was plotted against tryptophan concentration (inset), and the slopes, k'_T, were plotted against $[H^+]/(K_A + [H^+])$.

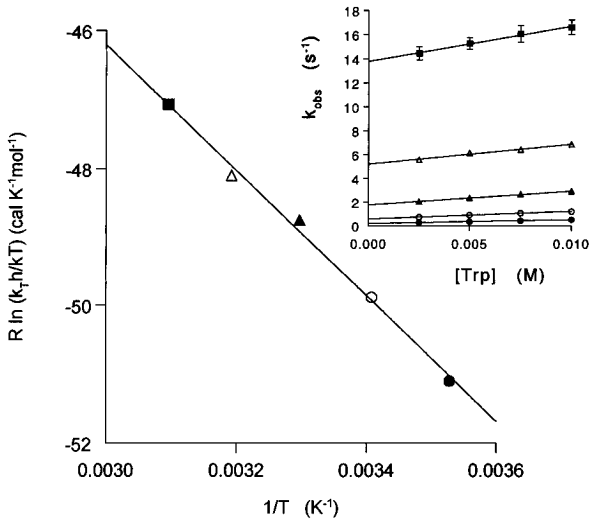


Figure 8. Eyring plot of the second-order rate constant for peroxynitrite nitration of tryptophan. Peroxynitrite (0.5 mM) was mixed with tryptophan in 0.05 M potassium phosphate buffer, 0.1 mM DTPA, pH 7.0, at 10.3 (●), 20.3 (○), 30.1 (▲), 40.1 (△), and 49.9 (■) °C. The k_{obs} was plotted against tryptophan concentration (inset). To determine k_T at each temperature, the slopes were multiplied by $(K_A + [H^+])/[H^+]$.

as mannitol, ethanol, and dimethyl sulfoxide (DMSO), at a concentration of 100 mM, partially decreased the amount of nitrotryptophan formed. Desferrioxamine, postulated to react with ground-state *trans*-peroxynitrous acid (*27*), decreased the absorbance at 400 nm by 46%. Bicarbonate, on the other hand, increased it by 160%.

No nitrotryptophan was formed due to the decomposition products of peroxynitrite, as shown by controls where the order of addition of the reactants was reversed and tryptophan was added 5 min after total decomposition of peroxynitrite in the buffer (reverse order addition experiment).

Discussion

Peroxynitrite reaction with tryptophan yields nitrotryptophan as the predominant product, in a transition metal-independent mechanism.

Table 2. Effect of Scavengers on Nitrotryptophan Yields[a]

addition	absorbance at 400 nm
none	0.261 ± 0.006
reverse order addition	0.005 ± 0.001
cysteine (1)	0.056 ± 0.003
ascorbate (10)	0.061 ± 0.002
methionine (10)	0.120 ± 0.003
mannitol (100)	0.212 ± 0.002
ethanol (100)	0.211 ± 0.001
dimethyl sulfoxide (100)	0.191 ± 0.008
desferrioxamine (2)	0.112 ± 0.001
bicarbonate (10)	0.421 ± 0.001

[a] Peroxynitrite (0.5 mM) was added to 10 mM tryptophan and the indicated mM concentration of scavenger in 0.1 M potassium phosphate buffer, pH 7.03. After 5 min incubation at 37 °C, the absorbance at 400 nm was determined. Each value represents the mean ± SD of triplicate measurements. All values were significantly different (p <0.05) from the control (no addition).

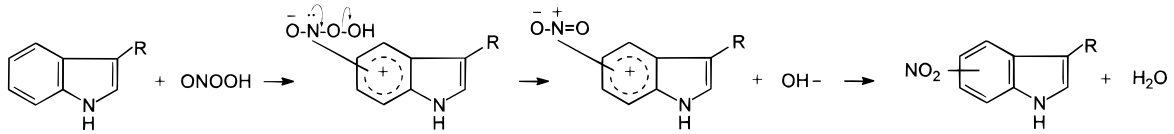
The curvatures in plots of k_{obs} vs tryptophan concentration were interpreted assuming that both ground-state peroxynitrous acid, ONOOH, and the highly reactive intermediate, ONOOH*, were able to nitrate tryptophan.

The scavenger data are fully consistent with both species being nitrating, since, firstly, substrates known to react directly with ground-state peroxynitrite decreased nitration yield as predicted from their rate constants; and, secondly, hydroxyl radical scavengers, which inhibit the hydroxyl radical-like reactivity attributed to ONOOH* (*6*, *19*, *28*), partially reduced nitrotryptophan formation.

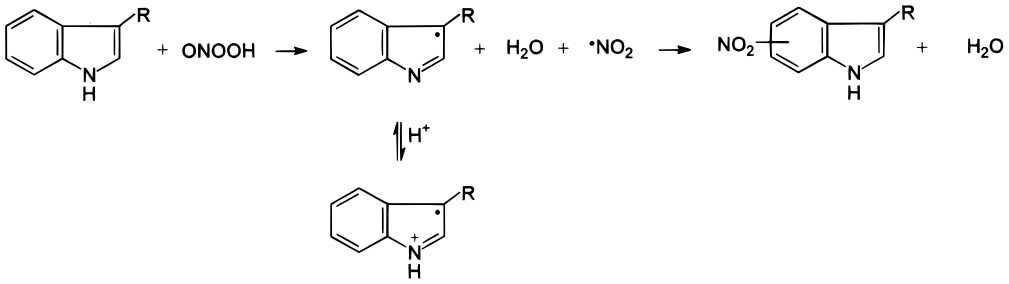
In regard to the peroxynitrous acid-dependent, second-order nitration, at least two alternative mechanisms can be proposed. In the first place, nitration of tryptophan could occur via addition of a nitronium ion-like species to the indole ring and elimination of a proton in an aromatic electrophilic substitution (Scheme 1). The nitrating species is not likely to be free nitronium ion, since theoretical considerations rule out the spontaneous heterolytic cleavage of peroxynitrite into nitronium and hydroxyl ions on grounds that the reaction free energy variation would be about +13 kcal mol^{-1} in water at pH 7, and the energy necessary for the initial charge separation would be approximately 45 kcal mol^{-1} (*18*). Alternatively, nitration could occur through abstraction of a hydrogen atom to give tryptophanyl radical in equilibrium with its cation radical (pK_a 4.3; *29*), followed by addition of nitrogen dioxide, either in steps or concertedly (Scheme 2). In this sense, the one-electron oxidation of tryptophan is thermodynamically possible, since the reduction potential $E^{\circ\prime}(ONOO^-,2H^+/{}^\bullet NO_2,H_2O)$, +1.4 V (*18*), is higher than $E^{\circ\prime}$(tryptophanyl radical,H^+/tryptophan), +1.0 V (*30*).

Activation parameters for the second-order nitration of tryptophan are very similar to the activation parameters for the oxidation of ascorbate by peroxynitrite (*3*). In addition, the enthalpy of activation is similar to the one reported for cysteine oxidation by peroxynitrite (*18*), implying that a similar mechanism is operative and that the transition states have characteristics in common. In terms of Scheme 1, these similarities in the activation parameters may indicate that reaction of ground-state peroxynitrous acid with tryptophan leads to an activated complex in which either oxidations or nitrations can occur. Alternatively, they may reflect that the second-order nitration of tryptophan is governed by a rate-determining electron transfer step followed by addition of nitrogen dioxide as proposed in Scheme 2. The large

Scheme 1



Scheme 2



and negative entropy typical of bonded complexes could be indicative of hydrogen bonding between peroxynitrite and the indolic nitrogen in tryptophan.

The mechanism of bicarbonate enhancement of nitration yield remains to be elucidated, although the fast direct reaction of peroxynitrite with carbon dioxide in equilibrium with bicarbonate could yield a strong oxidant or nitrating species (*31*, *32*).[3]

cis-Peroxynitrite anion is relatively stable, while protonation to *cis*-peroxynitrous acid promotes isomerization to the more labile *trans*-peroxynitrous acid (*17*, *18*, *33*). The fact that the yield of nitration decreased at alkaline pH with a pK_a of 7.8, which has previously been attributed to ionization of *trans*-peroxynitrous acid (*17*), could suggest nitrotryptophan formation was mediated by the *trans* rotamer. Nevertheless, the relatively small enthalpy of activation could indicate that the reaction involves *cis*-peroxynitrous acid. Whether the isomerization from the *cis* to the *trans* rotamer is a slow step remains controversial (*20*). This appeared not to be the rate limiting step in the present study, since peroxynitrite-induced nitrotryptophan formation fitted a single exponential.

Undoubtedly, the position of the incoming nitro group will be determined by the electron density distribution in the tryptophan molecule, due to the electrophilic character of the nitronium ion-like species or alternatively the oxidizing species. The most reactive position in indole is the carbon in 3. This position being occupied, as in tryptophan, nitration would occur in the carbon in 2. However, the spectral data obtained for the peroxynitrite-dependent reaction suggests nitration occurred in the benzenic ring, on agreement with previous reports for other nitrating agents. This is consistent with the concept that electrophilic attack on the pyrrole ring is impeded by the proximity of the $-NH_3^+$ group of free tryptophan (*23*, *26*, *34*, *35*).

Nitration of tryptophan will likely have biological implications. In the first place, nitrotryptophan formation can inhibit formation of tryptophan-derived neurotransmitters (i.e., serotonin) or interfere with their action. Secondly, nitration of critical tryptophan residues in proteins, as suggested by preliminary data of our group, may interfere with function, both by affecting critical catalytic motifs or via deformation of three-dimensional protein structure. Also, tryptophan nitration may serve as a revealing indicator for the contribution of nitric oxide to oxidative mechanisms, regardless of the exact nature of the nitrating species. However, although nitrogen dioxide can also nitrate tryptophan as well as tyrosine (*26*), its contribution to the formation of nitroderivatives *in vivo* is likely to be minor. The slow rate of formation of nitrogen dioxide *in vivo*, as well as the rapid reaction of nitric oxide with superoxide and heme proteins, greatly diminishes the potential contribution of nitrogen dioxide in aromatic nitration. Therefore, the potential formation of nitrotryptophan in proteins opens perspectives for a new footprint of peroxynitrite production *in vivo*.

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[3] A. Denicola, B. A. Freeman, and R. Radi, manuscript in preparation, and H. Ischiropoulos, personal communication.

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