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FEBS Letters 339 (1994) 89–92

FEBS 13623

FEBS LETTERS

Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite

Evidence for hydroxyl radical production from peroxynitrite

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Received 28 December 1993

Abstract

Peroxynitrite is a highly reactive species, generated from superoxide and nitric oxide. Some effects of peroxynitrite are ascribed to the molecule itself, but decomposition products of the protonated form, peroxynitrous acid, may account for much of its reactivity in biological systems. Suggested products include highly-reactive hydroxyl radicals, but thermodynamic calculations have been used to claim that free hydroxyl radicals cannot be formed from peroxynitrite. We utilized aromatic hydroxylation of phenylalanine as a specific detector of hydroxyl radicals, and found that incubation of phenylalanine with peroxynitrite leads to a small amount of *p*-, *m*- and *o*-tyrosine, specific products of attack by this radical. Products of nitration of phenylalanine and tyrosine were also detected, as was dityrosine. Peroxynitrite decomposition generates several reactive species, including some that can nitrate aromatic rings. Formation of nitro-aromatic compounds may be a useful marker of peroxynitrite generation in biological systems.

Key words: Peroxynitrite; Hydroxyl radical; Phenylalanine; Tyrosine; Nitrotyrosine; Dityrosine

1. Introduction

Peroxynitrite is a reactive species which can be formed in biological systems by the reaction of superoxide anion and nitric oxide [1], at a close to diffusion-controlled rate [2]. Peroxynitrite is capable of damaging some biological molecules directly, e.g. -SH groups and methionine [3]. Indeed, peroxynitrite formation is thought to contribute to inflammatory cell-mediated tissue injury in biological systems [3–6].

Peroxynitrite has been suggested to cause further damage by decomposing into highly-reactive hydroxyl (•OH) radicals [1]. However, Koppenol et al. [7] have claimed that thermodynamic calculations preclude formation of •OH from peroxynitrite. According to these calculations the peroxynitrous acid (ONOOH) is in the *trans* configuration and reacts by first forming a transitory intermediate of higher energy rather than separating into free •OH and $^{\bullet}NO_2$ [7]:

ONOOH <=====> O=N-O.....OH + R -------> ROH• + $^{\bullet}NO_2$

Rapid isomerization of the intermediate leads to the nitrate, thus limiting the yield of hydroxyl radical-like oxidant to a maximum of 25–30% of the added peroxynitrite [1]:

ONOOH <=====> O=N-O.....OH ===> $H^+ + NO_3^-$

In order to investigate the formation of •OH directly, we have used a sensitive and specific assay, aromatic hydroxylation of phenylalanine [8,9]. We show here that (i) •OH is formed and (ii) a reactive nitrating species is also formed that nitrates aromatic ring structures.

2. Materials and methods

2.1. Chemicals

L-Phenylalanine, DL-*para*-, *meta*-, and *ortho*-tyrosine, 3-nitro-L-tyrosine, 4-nitro-L-phenylalanine, uric acid, mannitol, catalase (thymol-free, 10,000–25,000 units/mg protein, from bovine liver), and bovine serum albumin (essentially fatty acid free, BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxynitrite was synthesized as described previously [10], and quantified prior to use by measuring the absorbance at 302 nm, using a molar extinction coefficient of 1670 $M^{-1} \cdot cm^{-1}$ [10]. All other reagents were of the highest purity commercially available.

2.2. Treatment of aromatic amino acids with peroxynitrite

Solutions of L-phenylalanine or DL-tyrosine were prepared in 50 mM

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SSDI 0014-5793(94)00007-I

KH_2PO_4–K_2HPO_4 buffer at various pH values and preincubated for 5 min at 37°C. Peroxynitrite was added in small aliquots (< 20 μl) directly above the surface of the solution, which was rapidly vortexed. Reaction mixtures were incubated for 30 min at 37°C and subsequently kept on ice until analysis. Pilot studies have shown that reactions are essentially complete 1 min after addition of peroxynitrite. Mannitol (100 mM), uric acid (300 μM), BSA (40 mg/ml) or bicarbonate (25 mM) were added to the buffer to give the final concentrations stated before addition of peroxynitrite.

2.3. Analysis of phenylalanine and tyrosines

Reaction mixtures were analyzed by spectral scanning between 350 and 500 nm after adjusting the pH to 10–11 [11] and by HPLC [9]. Briefly, separation of *p*-, *m*-, *o*-tyrosine, phenylalanine and various nitroadducts of phenylalanine and tyrosine was carried out using a 5 μ Spherisorb ODS-2 analytical column (250 × 4.6 mm; Alltech, Deerfield, IL), equipped with a 7 μ RP-18 Aquapore guard column (15 × 3.2 mm; Dychrome Inc., Sunnyvale, CA). Chromatographic separation employed an isocratic elution consisting of 500 mM KH_2PO_4–H_3PO_4 (pH = 3.0) with 10% (v/v) methanol at a flow rate of 1 ml · min^{-1} [9]. Detection was accomplished by UV absorbance at 274 nm, using a Waters 484 Tunable UV detector (Waters, Milford, MA), connected to a 486 IBM clone computer using Waters Millennium 2010 v1.10 software. Peaks were identified based on coelution, and quantified by peak area using external standards.

Formation of dityrosine was analysed by measuring fluorescence spectra after 20-fold dilution of the reaction mixture in 50 mM KH_2PO_4–K_2HPO_4 (pH 5.0, 7.4, or 9.0) [12].

3. Results and discussion

3.1. Hydroxyl radical formation from peroxynitrite

Attack of •OH upon phenylalanine produces three specific products, *p*-, *m*-, and *o*-tyrosine. We found that addition of peroxynitrite to solutions of phenylalanine (1 or 5 mM) at pH 7.4 resulted in the generation of *p*-, *m*- and *o*-tyrosine in comparable amounts. Typical chromatograms are shown in Fig. 1. Table 1 indicates the levels of *p*-, *m*- and *o*-tyrosine detected. Using 5 mM phenylalanine, about 10 μM of tyrosines were generated from 1 mM phenylalanine, corresponding to about 1% of the peroxynitrite added. At lower concentrations of phenylalanine or peroxynitrite, the amounts of tyrosines formed are lower but the relative yield is slightly higher. Generally, the amounts of tyrosines generated were proportional to the concentration of peroxynitrite added, but not to the concentration of phenylalanine (over the concentration range tested). Hence it seems that 1 mM phenylalanine is sufficient to trap most of the •OH generated. Formation of hydroxylated products was inhibited by the specific •OH scavenger mannitol (Table 1). However, catalase (1000 units/ml) had no effect, indicating that H_2O_2 played no role in the generation. The hydroxylation of phenylalanine was dependent on pH, being maximal at pH 6–7 and decreased at higher pH values

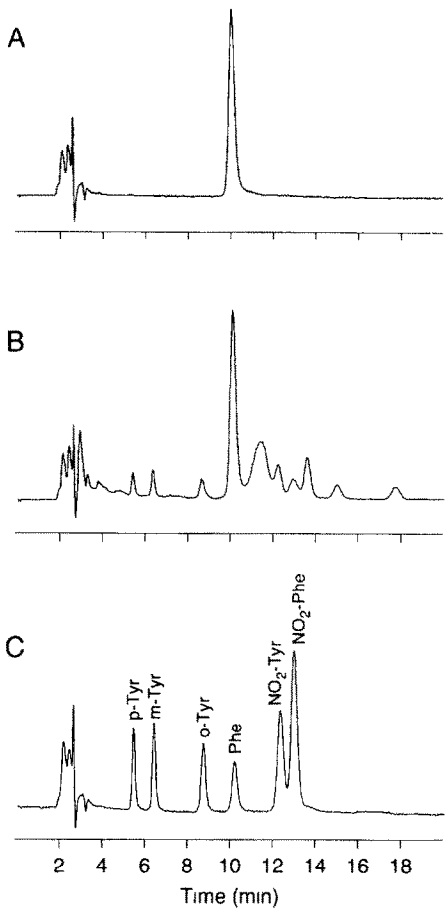

Fig. 1. Hydroxylation and nitration of phenylalanine by peroxynitrite. HPLC chromatograms of 5 mM phenylalanine (A), phenylalanine (5 mM) after incubation with 1.0 mM peroxynitrite (B) and a mixture of 40 μM *p*-tyrosine (*p*-Tyr), 40 μM *m*-tyrosine (*m*-Tyr), 40 μM *o*-tyrosine (*o*-Tyr), 2 mM phenylalanine (Phe), 20 μM 3-nitrotyrosine (NO_2-Tyr) and 20 μM 4-nitrophenylalanine (NO_2-Phe) are shown, demonstrating the formation of both hydroxylated products and nitro-adducts from phenylalanine.

Table 1
Aromatic hydroxylation of phenylalanine by peroxynitrite

	p-Tyr (μM)	*m*-Tyr (μM)	*o*-Tyr (μM)
1 mM phenylalanine			
+ 1.0 mM peroxynitrite	3.1 ± 0.4	3.5 ± 0.5	3.8 ± 0.8
+ 1.0 mM peroxynitrite + 100 mM mannitol	0.5	0.5	< 0.5
5 mM phenylalanine			
+ 0.5 mM peroxynitrite	2.6 ± 0.5	2.8 ± 0.2	2.6 ± 0.4
+ 1.0 mM peroxynitrite	5.3 ± 0.4	6.0 ± 0.5	5.9 ± 0.8
+ 1.0 mM peroxynitrite + 100 mM mannitol	2.2	2.0	2.6
+ 1.0 mM peroxynitrite (pH 6.0)	3.8	3.3	6.4
+ 1.0 mM peroxynitrite (pH 8.0)	0.7	1.1	< 0.5
+ 1.0 mM peroxynitrite (pH 9.0)	< 0.5	< 0.5	< 0.5

Phenylalanine was dissolved in 50 mM KH_2PO_4–K_2HPO_4 buffer (pH 7.4) and incubated with the indicated concentrations of peroxynitrite for 30 min at 37°C. Aliquots were injected directly on the HPLC column and assayed for tyrosines as described in section 2. Data are expressed as mean ± S.E. from three separate incubations, or as single observations.

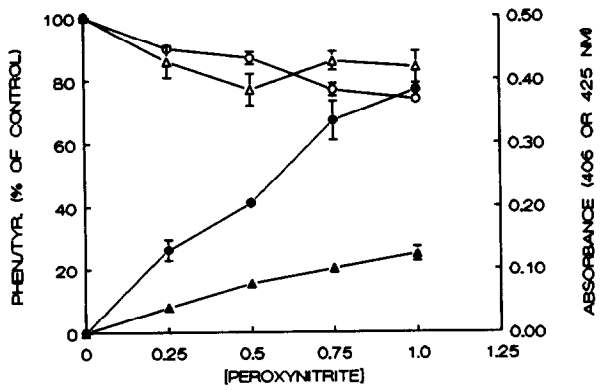

Fig. 2. Reaction of peroxynitrite with phenylalanine and tyrosine. The reaction was followed by measuring the absorbance between 350 and 500 nm as described in section 2. Phenylalanine and tyrosine were quantified by HPLC as described in section 2. Open symbols: levels of phenylalanine (circles) or tyrosine (triangles) after incubation with peroxynitrite. Closed symbols: product formation from phenylalanine (A_{max} at 406 nm) and tyrosine (A_{max} at 425 nm). Mean values ± S.E. from three experiments are shown.

(Table 1). Our data suggest that, as originally proposed [1] and in contradiction to [4], peroxynitrous acid does decompose to form small amounts of •OH.

3.2. Formation of nitro-adducts and dityrosine

Addition of peroxynitrite to phenylalanine also leads to formation of other products, of which some coeluted with 3-nitrotyrosine and 4-nitrophenylalanine (Fig. 1). Formation of nitroadducts from phenylalanine was also studied by spectral analysis, showing increased absorbance between 350 and 500 nm, with a maximum at 405 nm. Peroxynitrite induced a concentration-dependent increase in absorbance at 406 nm, which paralelled the decrease in phenylalanine concentration (Fig. 2). Other unidentified peaks in the chromatogram could be isomers of nitrotyrosine or nitrophenylalanine as well as other products such as dihydroxy-, dinitro-, nitro-dihydroxy-, dinitro-hydroxy-adducts. We attempted to identify some of these products by adding peroxynitrite to *p*-, *m*- or *o*-tyrosine, 4-nitrophenylalanine or 3-nitrotyrosine and found that some products are probably nitroadducts from *m*- or *o*-tyrosine.

Treatment of 1 mM *p*-tyrosine with peroxynitrite results in a concentration-dependent increase in absorbance (350–500 nm) with a maximum at 425 nm, with a corresponding decrease in *p*-tyrosine (Fig. 2), indicative of formation of 3-nitrotyrosine [10]. Analysis by HPLC showed that 3-nitrotyrosine is the major product formed. Addition of 1 mM peroxynitrite to 1 mM *p*-tyrosine lead to generation of 53 ± 11 μM (mean ± S.E.) 3-nitrotyrosine (n = 3), corresponding to about 5% of the parent compound. Formation of 3-NO-tyrosine was inhibited by 40 ± 2% (mean ± S.E.) by 100 mM mannitol, whereas aromatic hydroxylation of phenylalanine was decreased by 56% using 5 mM phenylalanine and by > 85% using 1 mM phenylalanine. This suggests that mannitol is less effective in scavenging the nitrating species. Nitration of tyrosine by peroxynitrite has been demonstrated to be mediated by transition metal ions catalyzing formation of NO_2^+, a powerful nitrating species. However, $^{\bullet}NO_2$ is also capable of forming nitroadducts from tyrosine [11].

Reaction of peroxynitrite with *p*-tyrosine leads to the formation of a fluorescent product (Ex. 320 nm, Em. 410 nm; Fig. 3). The fluorescence signal was dependent on pH, consistent with the presence of dityrosine [12]. This further demonstrates the formation of radicals from peroxynitrite capable of generating tyrosyl radicals, which combine to form dityrosine. Alternatively, the $^{\bullet}NO_2$ formed may be responsible for giving dityrosine by abstracting a proton [11].

3.3. Inhibition of aromatic hydroxylation and nitration by plasma constituents

We studied the inhibitory effect of 300 μM uric acid and found that the aromatic hydroxylation of phenylalanine by peroxynitrite is inhibited > 70% and the nitration of phenylalanine or tyrosine is almost completely inhibited (> 95%). Addition of BSA (40 mg/ml) also inhibits nitration of both phenylalanine and tyrosine > 95%, but does not affect the aromatic hydroxylation of phenylalanine by peroxynitrite. Bicarbonate (25 mM) completely prevents aromatic hydroxylation and nitration of phenylalanine by peroxynitrite, but interestingly the yield of 3-nitrotyrosine and dityrosine after reaction of peroxynitrite with tyrosine is increased by around twofold.

3.4. Conclusions

We have demonstrated formation hydroxylated products after reaction of peroxynitrite with phenylalanine by trapping the 'hydroxyl radical'. Hydroxylated and/or nitrated products from phenylalanine and/or tyrosine might serve as indicators of peroxynitrite formation in

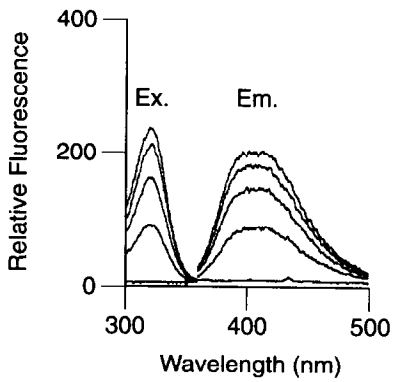

Fig. 3. Formation of dityrosine after reaction of 1 mM tyrosine with peroxynitrite. Fluorescence was measured by recording excitation spectra from 300–360 nm and emission spectra from 360–500 nm. Lower to upper traces represent spectra after incubation with 0, 0.25, 0.5, 0.75 and 1.0 mM peroxynitrite, respectively.

biological systems. However, competing reactions in plasma with urate, protein sulfhydryls or bicarbonate may diminish the aromatic hydroxylation and/or nitration of phenylalanine and/or tyrosine.

Acknowledgements: The authors wish to thank NIH (grants HL47628 and HL07013) and the Arthritis and Rheumatism Council (UK) for research support.

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