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Original Contribution

RAPID SCAVENGING OF PEROXYNITROUS ACID BY MONOHYDROASCORBATE

CHRISTOPHE R. KURZ, REINHARD KISSNER, THOMAS NAUSER, DANIEL PERRIN, and WILLEM H. KOPPENOL
Laboratorium für Anorganische Chemie, Eidgenössische Technische Hochschule Zürich (Hönggerberg), Zürich, Switzerland

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Abstract—The reaction of peroxynitrous acid with monohydroascorbate, over the concentration range of 250 μM to 50 mM of monohydroascorbate at pH 5.8 and at 25°C, was reinvestigated and the rate constant of the reaction found to be much higher than reported earlier (Bartlett, D.; Church, D. F.; Bounds, P. L.; Koppenol, W. H. The kinetics of oxidation of L-ascorbic acid by peroxynitrite. *Free Radic. Biol. Med.* **18:**85–92; 1995; Squadrito, G. L.; Jin, X.; Pryor, W. A. Stopped-flow kinetics of the reaction of ascorbic acid with peroxynitrite. *Arch. Biochem. Biophys.* **322:**53–59; 1995). The new rate constants at pH 5.8 are $k_1 = 1 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 500$ s^{-1} for 25°C and $k_1 = 1.5 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 1 \times 10^3$ s^{-1} for 37°C. These values indicate that even at low monohydroascorbate concentrations most of peroxynitrous acid forms an adduct with this antioxidant. The mechanism of the reaction involves formation of an intermediate, which decays to a second intermediate with an absorption maximum at 345 nm. At low monohydroascorbate concentrations, the second intermediate decays to nitrate and monohydroascorbate, while at monohydroascorbate concentrations greater than 4 mM, this second intermediate reacts with a second monohydroascorbate to form nitrite, dehydroascorbate, and monohydroascorbate. EPR experiments indicate that the yield of the ascorbyl radical is 0.24% relative to the initial peroxynitrous acid concentration, and that this small amount of ascorbyl radicals is formed concomitantly with the decrease of the absorption at 345 nm. Thus, the ascorbyl radical is not a primary reaction product. Under the conditions of these experiments, no homolysis of peroxynitrous acid to nitrogen dioxide and hydroxyl radical was observed. Aside from monohydroascorbate's ability to "repair" oxidatively modified biomolecules, it may play a role as scavenger of peroxynitrous acid. © 2003 Elsevier Inc.

Keywords—Ascorbic acid, Peroxynitrite, Oxoperoxonitrate(1-), Antioxidant defense, Reaction kinetics, Stopped-flow spectrophotometry, EPR, Ascorbyl radical, Homolysis, Free radicals

INTRODUCTION

Peroxynitrite [oxoperoxonitrate(1-) or nitrosodioxidanide] is an inorganic molecule of biological interest. Activated macrophages produce both superoxide and nitrogen monoxide; these react at a nearly diffusion-controlled rate to produce peroxynitrite [1]. The pK_a of peroxynitrite, about 7, depends on the temperature and buffer composition and concentration [2–4]. Although production of peroxynitrite by macrophages is beneficial, its formation elsewhere must be considered harmful [5]. Peroxynitrous acid is likely to play a role in the oxidation of thiols [4,6–8], the nitration of tyrosines [9–13], and the initiation of lipid peroxidation [14,15]. One-electron oxidations and oxygen-transfer have both been reported [16–18]. It is clear from the above that the cytotoxic potential of peroxynitrous acid is quite high. On the other hand, it is possible that peroxynitrite plays a role as a messenger, because its formation takes place at the expense of another signaling molecule, nitrogen monoxide, or because it nitrates proteins like kinases and thereby inhibits their function [19–24].

Has evolution afforded a defense mechanism? Scavenging by antioxidants such as ascorbic acid is one possible approach toward the mitigation of potentially harmful reactions. Ascorbic acid is an important antioxidant in vitro and in vivo [25]. The concentration range of monohydroascorbate ($HAsc^-$) in human tissue varies: about 1 mM in human lung and neurophils [26], 10 mM in neurons [27], 30 to 150 μM in human plasma [28], 7

Address correspondence to: Dr. Willem H. Koppenol, Laboratorium für Anorganische Chemie, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich, Switzerland; Tel: +41 (1) 6322875; Fax: +41 (1) 6321090; E-Mail: koppenol@inorg.chem.ethz.ch.

mM in leukocytes [29], and 1.3 mM in liver [30]. Ascorbic acid has two acidic protons with pK_a values of 4.04 and 11.34 [31]. Rates of oxidation may be strongly pH-dependent in the case with peroxynitrous acid, where the highest reaction rate has been observed at pH 5.8 several years ago [32]. It was concluded that peroxynitrous acid oxidized monohydroascorbate in a bimolecular fashion with a rate constant of 235 M^{-1} s^{-1} [32,33], a rate constant that is too small to allow monohydroascorbate to play a significant role as a scavenger in most tissues. Furthermore, given the observation of the ascorbyl radical by EPR, the oxidation was believed to involve consecutive one-electron steps.

We reinvestigated the reaction of monohydroascorbate with peroxynitrous acid and show that the reaction proceeds via two intermediates, of which the first is an adduct in equilibrium with peroxynitrous acid and monohydroascorbate. This first intermediate decays to a second intermediate, which can follow one of two pathways, depending on the ascorbate concentration. At low concentrations the second intermediate forms, remarkably, nitrate and monohydroascorbate; this is the isomerase pathway. At higher ascorbate concentrations, nitrite and dehydroascorbate are formed; this is the oxidation pathway. Simulations show that these processes are competitive with the reaction of peroxynitrite with carbon dioxide.

EXPERIMENTAL PROCEDURES

Reagents

Ascorbic acid, disodium monohydrogen phosphate, and sodium dihydrogen phosphate were purchased from Fluka Chemical Corp. (Buchs, Switzerland) and were analytical grade or better. Potassium superoxide was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), sodium hydroxide was purchased from Siegfried Fabrik (Säckingen, Germany), and nitrogen monoxide was purchased from Linde (Unterschleissheim, Germany). Peroxynitrite was synthesized according to Koppenol and coworkers [34]. The concentration of the solution was determined by measuring the absorbance at 302 nm (ϵ_{302} = 1705 $M^{-1}cm^{-1}$) [35]. Deionized water was purified further by a Millipore Milli-Q unit (Millipore, Bedford, MA, USA). Buffers were prepared from salts and acids. All solutions were freshly prepared and, except for the peroxynitrite solutions, were evacuated for 5 min and then saturated with argon to avoid oxidation of monohydroascorbate by dioxygen. The peroxynitrite solutions were prepared by dilution with 0.010 M sodium hydroxide that was deaerated by evacuation for 5 min prior to its addition to the peroxynitrite stock solution.

Pulse radiolysis

Solutions were irradiated with a 2 MeV electron accelerator (Febetron 705, Titan Systems Corp., formerly Field Emission Corp., San Diego, CA, USA). A solution containing 0.50 mM monohydroascorbate and 0.050 M sodium phosphate buffer (pH 5.8) was saturated with dinitrogen monoxide and then irradiated. Under these conditions, over 90% of the radicals produced initially are, or are converted to, hydroxyl and oxide($^{\bullet}1-$) radicals, which react at a nearly diffusion-controlled rate of 7.0×10^9 M^{-1} s^{-1} [36] with monohydroascorbate to form the ascorbyl radical. An Acton SP300i monochromator (Acton, MA, USA) and a Princeton PI-MAX 512T ICCD camera (Trenton, NJ, USA) were used for the detection of optical signals.

Stopped-flow spectrophotometry

Kinetic experiments were carried out at 25 and 37°C at ambient pressure with an Applied Photophysics SX 17MV stopped-flow spectrophotometer (Leatherhead, Surrey, GB) operated in the symmetric mixing mode. The reaction was initiated by mixing a peroxynitrite solution in 0.010 M sodium hydroxide with a monohydroascorbate solution in 0.10 M phosphate buffer (pH 5.8, mostly $H_2PO_4^-$). The pH, measured at the outlet, was always 5.8. Time-dependent spectra were obtained at 25°C with an OLIS stopped-flow instrument (Bogart, GA, USA) equipped with an OLIS RSM 1000 rapid scanning monochromator set to collect 1000 spectra per second from 320 to 400 nm. All kinetic experiments were repeated on an Applied Photophysics SX 18MV stopped-flow spectrophotometer with identical results.

EPR

Electron paramagnetic resonance spectra were obtained with a Bruker EMX 080 (Bruker, Karlsruhe, Germany) equipped with a microwave-bridge ER 041 XG and the dielectric mixing resonator ER 4117 D-MVT. The instrument settings used in these experiments were: gain = 1×10^5; resolution = 1024 points; measurement field = 3475.3 G; measurement time = 20.9 s; microwave power = 2.012 mW; modulation amplitude = 2 G; and frequency = 9.761 GHz. Data acquisition and analysis were carried out with ACQUISIT software (Bruker). The solutions were injected into the cavity with a KD-Scientific 220 syringe pump (New Hope, PA, USA) at a flow of 1 ml/min per syringe in a ratio 1:1. The solutions were of the same composition as those used in the stopped-flow experiments.

The signal intensity of the ascorbyl radical signal was determined by mixing a freshly prepared cerium(IV) solution (25 μM final concentration) with monohy-

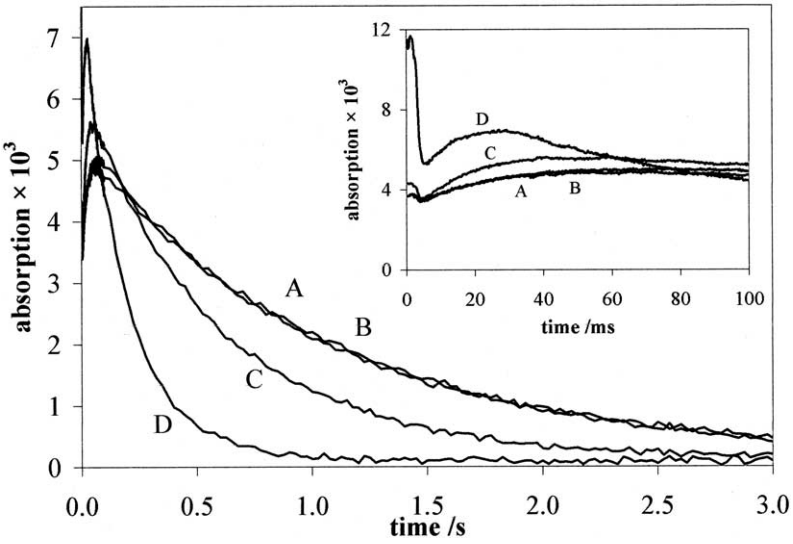


Fig. 1. Time course of the absorption at 345 nm after mixing monohydroascorbate with peroxynitrous acid. Peroxynitrous acid (25 μM) was mixed with monohydroascorbate at the following concentrations: trace A, 250 μM; trace B, 1 mM; trace C, 8 mM; and trace D, 50 mM. All concentrations indicated are those after mixing. All traces were recorded at 345 nm, 25°C, and pH 5.8 (50 mM phosphate buffer). Traces C and D are in agreement with earlier reports [32,33] where higher ONOOH and ascorbate concentrations were used. At lower ascorbate concentrations (traces A and B), no dependence on the monohydroascorbate concentration was observed. Each curve is an average of 12 measurements. Inset: a fast decrease in absorption that depends on the monohydroascorbate concentration was observed during the first 6 ms.

droascorbate at pH 5.8 in 50 mM phosphate buffer. Only a few percent of cerium(IV) reacts with monohydroascorbate due to the competing reaction with phosphate that produces an unidentified, inert cerium-phosphate complex [37]. The concentration of the small amount of ascorbyl radical produced was determined by the rate of its bimolecular decay (2×10^7 M^{-1} s^{-1}) [38].

Ion chromatography

Nitrite and nitrate were determined as described before, except that a Super-Sep IC anion exchanging column (Metrohm, Herisau, Switzerland) with a phthalate solution [2.5 mM phthalate, 5% acetonitrile, pH 4.2 (Tris), $\Lambda \approx 130$ μS/cm] as eluent was used [18,39,40].

Simulation

Kinetics were simulated based on sets of ordinary differential equations with an Excel (Microsoft, Redmond, WA, USA) macro program that implements a two-step implicit Runge-Kutta algorithm of fourth order [41]. This method is more stable than explicit integration techniques but requires more computation time.

RESULTS

Stopped flow studies

Kinetic traces recorded at 345 nm, at 25°C and pH 5.8 after mixing of peroxynitrite with different concentrations of monohydroascorbate (0.25–50 mM), showed an increase in absorption during 20–40 ms followed by a decrease. At monohydroascorbate concentrations higher than 1 mM, the rate of this previously unobserved increase in absorption depends on monohydroascorbate (Fig. 1), while a dependence on peroxynitrous acid is observed over the whole concentration range (not shown). The decrease in absorption, which begins 50–100 ms after mixing, was measured before at higher monohydroascorbate concentrations [32,33]. The experiments at these concentrations (Fig. 1, traces C and D) are in agreement with the cited literature and show a faster decrease of absorption at higher monohydroascorbate concentrations. The rate of decrease is not affected by the peroxynitrous acid concentration.

The inset of Fig. 1 shows that directly after mixing there is a decrease in absorption. This process clearly had started already during the mixing and was difficult to quantify. At 345 nm, monohydroascorbate does not absorb, and the absorption directly after mixing and that after about 40 ms were significantly higher than those expected for peroxynitrous acid alone (see below). These observations indicate that there may be two intermediates, Im_1 and Im_2, of which Im_2 has an absorption maximum at 345 nm.

The transient absorption with a maximum at 345 nm (Fig. 2, traces A and B) has not been observed before. We note that the spectrum of the ascorbyl radical (Fig. 2, trace C), determined by pulse radiolysis of a dinitrogen

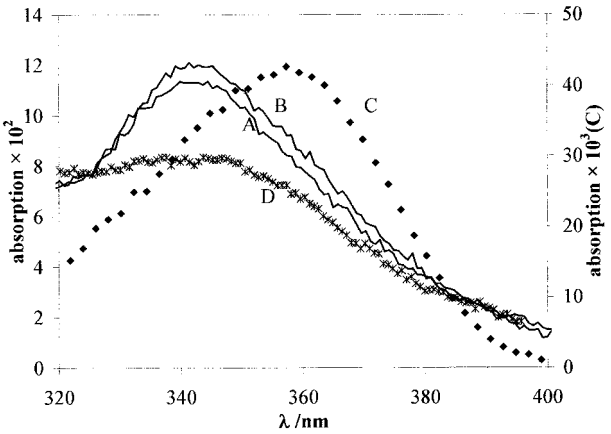


Fig. 2. Absorption spectrum of Im_2 compared with that of peroxynitrous acid and the ascorbyl radical. Absorption spectra of Im_2 at (trace A) 4 and (trace B) 52 ms after mixing 1 mM peroxynitrite with 10 mM monohydroascorbate at pH 5.8 (50 mM phosphate buffer) at 25°C. All concentrations indicated are those after mixing. Controls: (trace C) absorption spectrum of the ascorbyl radical 50 μs after irradiation of a N_2O-saturated 500 μM monohydroascorbate solution at pH 5.8 (50 mM phosphate buffer) with 2 MeV electrons. (Trace D) absorption spectrum of a 1 mM peroxynitrous acid solution at pH 3. Monohydroascorbate had no absorption at 345 nm.

monoxide saturated solution of 0.50 mM monohydroascorbate, showed a maximum at 360 nm, in agreement with the literature [36,42].

The absorption at 345 nm, 4 ms after mixing (Fig. 2, trace A), is about 50% higher than that expected for peroxynitrous acid at zero time. The extinction coefficient of peroxynitrous acid at that wavelength is 78 M^{-1} cm^{-1} (Kissner, 2001, unpublished). Monohydroascorbate itself does not absorb at that wavelength. If we separate the absorption at 345 nm into two components, Δ_1 and Δ_2, where Δ_1 is the absorption 4 ms after mixing and Δ_2 is the maximum absorption at about 50 ms minus Δ_1, then Δ_1 increased linearly with the peroxynitrite concentration up to a 2-fold excess (Fig. 3). Δ_2, which is much smaller than Δ_1, showed a saturation effect at a ratio of [ONOOH]:[$HAsc^-$] = 1:1 (Fig. 3 inset). These observations suggest that a 1:1 peroxynitrite-dehydroascorbate adduct was formed.

The rates of decay of the Im_2 and monohydroascorbate, observed at 345 and 265 nm, respectively, were similar (not shown). Both rates are first order in monohydroascorbate. However, Im_2 was formed during the first 100 ms and its decay lags behind that of monohydroascorbate absorption, which starts to diminish directly after mixing.

From measurements over a wide range of monohydroascorbate concentrations, it is clear that the rates of formation and decay of Im_2 depend on the monohydroascorbate concentration. It is implied that the rate of formation of the first intermediate also depends on the monohydroascorbate concentration. As shown in Fig. 1, Im_2 was already present at very low monohydroascorbate concentrations, while at higher monohydroascorbate concentrations the rate of formation increased with a slope of $k_\alpha = 1.6 \times 10^3$ M^{-1} s^{-1} (Fig. 4). We do not

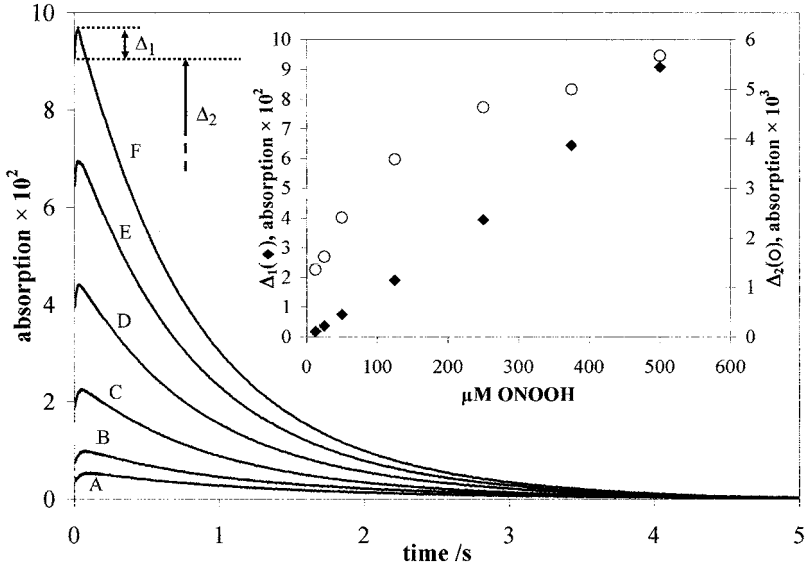


Fig. 3. Kinetics of the formation of Im_2 as a function of the peroxynitrous acid concentration. Ascorbic acid (250 μM) was mixed with peroxynitrous acid at the following concentrations: trace A, 25 μM; trace B, 50 μM; trace C, 125 μM; trace D, 250 μM; trace E, 375 μM; and trace F, 500 μM. Inset: absorption after mixing monohydroascorbate (250 μM final concentration) with variable concentrations of peroxynitrous acid final concentrations separated into Δ_1 (♦ absorption 4 ms after mixing) and Δ_2 (○ absorption difference between Δ_1 and maximal absorption after about 50 ms).

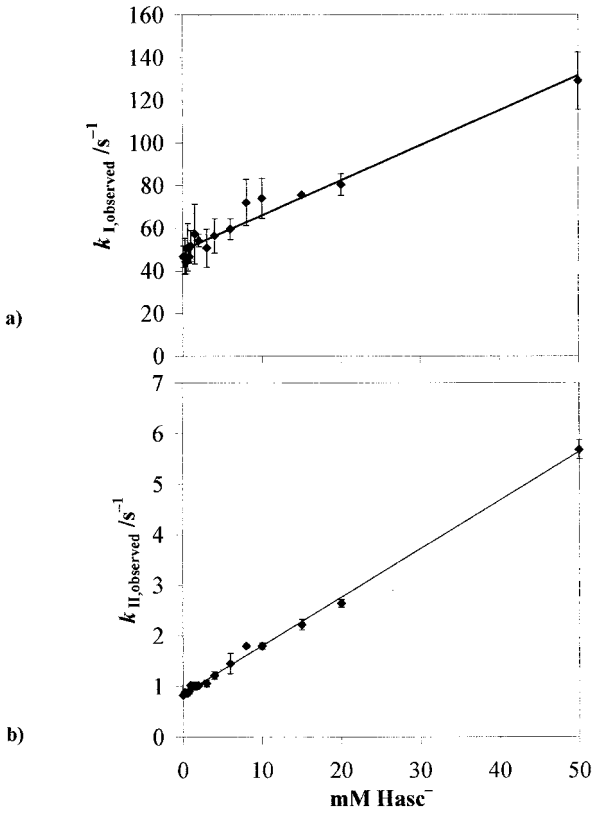


Fig. 4. Formation (a) and decay (b) rate constants of Im_2. Measurements were carried out at 345 nm under pseudo-first-order condition. Conditions: 25 μM peroxynitrite, 50 mM phosphate buffer, pH 5.8, T = 25°C. The error bars represent the 95%, (2s) error margin. All concentrations indicated are those after mixing.

wish to imply that the intercept at zero monohydroascorbate concentration is real. Firstly, under this condition there is no reaction and there are no intermediates. Secondly, the points in Fig. 4a are based on absorbances between 5–15 ms, which may well contain contributions from Im_1. The error bars only show that the measurements were reproducible. It seems likely, though, that directly after mixing there was already a significant amount of Im_2. The decay of the intermediate has a monohydroascorbate-independent, k_β = 0.85 s^{-1} and a dependent rate constant, k_γ = 95 M^{-1} s^{-1} (Fig. 4b).

As shown in the inset of Fig. 1, a rapid absorption decay took place within 4 ms after mixing. Control experiments without peroxynitrite, but with added nitrite or nitrate, showed no transient at 345 nm, as don't experiments in which peroxynitrite is mixed with buffer, which confirms that an intermediate was formed as a product of the reaction of monohydroascorbate with peroxynitrous acid. Furthermore, we observed no influence of nitrite, nitrate, or dioxygen on the kinetics (not shown).

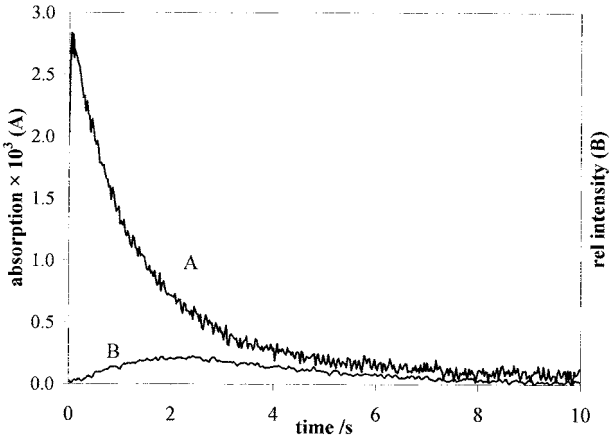


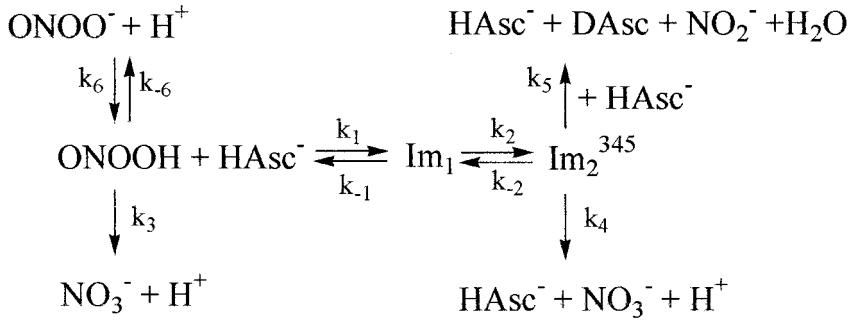
Fig. 5. (Trace A) Comparison of the decay at 345 nm with (trace B) an EPR trace of the ascorbyl radical formation and decay. Trace B, an average of 10 measurements, increases concomitant with the decrease of trace A, an average of 12 measurements. Measurements were made at initial concentrations of 25 μM peroxynitrite and 250 μM monohydroascorbate in 50 mM phosphate buffer at pH 5.8. All concentrations indicated are those after mixing.

EPR studies

We observed a small signal with a g value of 2.005, which is due to the ascorbyl radical. This signal was not detected without monohydroascorbate or peroxynitrite. Directly after mixing 25 μM peroxynitrite and 250 μM monohydroascorbate, no EPR signal was seen; instead, this signal rose for approximately 2 s and, subsequently, disappeared by second-order kinetics within 10 s (Fig. 5, trace B). Compared with the optical kinetic trace recorded at 345 nm (Fig. 5, trace A), it is clear that the concentration of ascorbyl radicals increased while that of Im_2 decreased. The total ascorbyl radical concentration formed during the reaction was estimated at only 60 nM. Because the ascorbyl radical decays by second-order kinetics [36,38], its concentration could be estimated to be 20 nM at t = 2 s. Special care was taken to avoid contamination by dioxygen and transition metals, which also produce ascorbyl radicals [43].

Product analysis

When 25 μM peroxynitrite and increasing amounts (25 μM to 50 mM) of monohydroascorbate were mixed, nitrite increased concomitant with a decrease in nitrate. At 2 mM monohydroascorbate, equal amounts of nitrite and nitrate were produced (not shown), and, at concentrations higher than 10 mM, saturation was observed. At lower monohydroascorbate concentrations, most of the peroxynitrous acid was converted to nitrate. Without monohydroascorbate, peroxynitrous acid yielded already 13% nitrite at pH 5.8, in agreement with a previous report [39].



Scheme 1.

DISCUSSION

Earlier work

The reaction of peroxynitrous acid with monohydroascorbate has already been studied by Bartlett and coworkers [32] and by Squadrito and coworkers [33]. They concluded that the reaction was first order in peroxynitrous acid and in monohydroascorbate with a rate constant of 235 M^{-1} s^{-1}. The rapid increase in absorption during the first 100 ms after mixing (Fig. 1) was not seen by these workers because vibrations, generated when the flow was stopped, prevented accurate measurements during 50 ms after mixing. Based on the observation of the EPR spectrum of the ascorbyl radical, Bartlett and coworkers [32] concluded that this radical was a product. We found only a very small EPR signal, and the corresponding ascorbyl concentration would amount to much less than 1% of the peroxynitrous acid concentration. The intermediate Im_2, which exhibited an absorbance maximum at 345 nm, is thus not identical to the ascorbyl radical, which has an absorption maximum at 360 nm (Fig. 2).

Above pH 5.8 the reaction proceeds more slowly, as was reported earlier [32]. A manuscript on the reaction of peroxynitrite with monohydroascorbate at nearly neutral pH is in preparation.

Intermediates

The important findings of this study are that the absorbance at 345 nm about 5 ms after mixing is higher than that expected for peroxynitrous acid alone, the absorbance continued to rise for about 0.1 s and then decreased, and both processes are ascorbate-dependent. The increase in absorbance was also dependent on the peroxynitrous acid concentration. Our attempt to fit these observations to a reaction scheme that included a single intermediate was not successful. It appeared necessary to invoke a second intermediate, appearing prior to Im_2, as shown in Scheme 1. Evidence for the first intermediate is dual: (i) at high concentrations of ascorbate, a decrease in absorption was observed directly after mixing (Fig. 1 inset), and (ii) at that time, there appeared to be already a significant amount of Im_1. We have no data that would provide information regarding the precise structures of these intermediates. As shown in the inset of Fig. 3, the absorption difference Δ_2 showed a saturation effect at [ONOOH] > [$HAsc^-$]. From this, as suggested by Bartlett and coworkers [32], we conclude that Im_2 is a 1:1 adduct of peroxynitrous acid and monohydroascorbate. We already mentioned that Im_2 is not the ascorbyl radical, as the EPR signal of the ascorbyl radical increased while the concentration of Im_2 decreased (Fig. 5).

Kinetics and mechanism

We postulate that Im_1 is formed very rapidly from monohydroascorbate and peroxynitrous acid, that Im_2 is formed from Im_1, and that the former decays spontaneously and by reaction with a second monohydroascorbate molecule. Since the concentration of the second intermediate (Im_2) increased with that of peroxynitrous acid (Fig. 3), we conclude that Im_1 and Im_2 are in equilibrium. We observed the reaction at 345 nm, the absorbance maximum of Im_2; we assume that Im_1 also absorbs at this wavelength. The rates of formation of Im_1 and Im_2 increased with the monohydroascorbate concentration, as shown in Fig. 4a. These rates are extracted from the increase in absorption 5–15 ms after mixing and are subject to a considerable error. We assume that, when the monohydroascorbate concentration approaches zero, k_I in Fig. 4a is zero. We cannot verify that, because below 500 μM monohydroascorbate no significant rise was observed. The rate constant for the increase in absorption at 345 nm at monohydroascorbate concentrations larger than 500 μM (Fig. 4a) is 1.6×10^3 M^{-1} s^{-1}.

The decay of Im_2 is according to the following rate law:

$$k_{II,\,observed} = k_\beta + k_\gamma\,[\text{ascorbate}] \text{ with } k_\beta = 0.85\ s^{-1} \text{ and } k_\gamma = 95\ M^{-1}\ s^{-1}$$

Table 1. Values Used for the Simulation of the Reaction of Peroxynitrous Acid with Monohydroascorbate at 345 nm and 25°C

Rate constant	Rate value	Remark	Compound	Absorption coefficient	Initial concentration
k_1	1×10^6 M^{-1} s^{-1}	a	$HAsc^-$	0 M^{-1} cm^{-1}	250 μM–50 mM
k_1	5×10^2 s^{-1}	a	HOONO	82 M^{-1} cm^{-1}	25 μM
k_2	40 s^{-1}	a	$ONOO^-$	685 M^{-1} cm^{-1}	–
k_2	5 s^{-1}	a	NO_3^-	0 M^{-1} cm^{-1}	–
k_3	1.2 s^{-1}	b	NO_2^-	5 M^{-1} cm^{-1}	–
k_4	0.85 s^{-1}	c	DAsc	0 M^{-1} cm^{-1}	–
k_5	1×10^2 M^{-1} s^{-1}	c	Im_1	250 M^{-1} cm^{-1}	–
k_6	$1 \times 10^{10\text{-pH}}$ s^{-1}	d	Im_2	310 M^{-1} cm^{-1}	–
k_6	1.58×10^3 s^{-1}	e			

a = determined by simulation; b = Kissner et al. [33]; c = rate constant determined by decay of Im_2; d = rate constant for protonation; and e = rate constant determined by pK_a [43].

The monohydroascorbate-independent pathway yields nitrate and monohydroascorbate; the decay rate constant k_β is smaller than the isomerization rate constant of peroxynitrous acid at pH 5.8, which is another indication that at least one intermediate is present. From the faster decay of Im_2 at increased monohydroascorbate concentrations and the product analysis, we conclude that Im_2 interacts with another monohydroascorbate molecule and produces dehydroascorbate, ascorbate, and nitrite.

Kinetic traces of the reaction between peroxynitrous acid and monohydroascorbate were simulated for 25 and 37°C based on Scheme 1. These simulations were carried out for variable monohydroascorbate concentrations at fixed concentrations of peroxynitrous acid, and reasonable to good agreement with the experimental results was found. The rate constants and extinction coefficients used in the simulation are listed in Table 1. Importantly, the forward rate constants for the reaction of peroxynitrous acid with monohydroascorbate at pH 5.8 are 1×10^6 M^{-1} s^{-1} and 1.5×10^6 M^{-1} s^{-1} at 25 and 37°C, respectively. The rate constants for the backward reaction are 5×10^2 s^{-1} and 1×10^3 s^{-1} at 25 and 37°C, respectively (not shown). From these rate constants, it can be calculated that, at 1 μM peroxynitrous acid and 500 μM monohydroascorbate, every second peroxynitrous acid molecule is scavenged by an ascorbate. While the agreement between simulation and experiment is satisfactory, we do not exclude the possibility of improvements to this model.

Homolysis or one-electron oxidation?

In the literature, contrasting views are expressed with respect to the issue of peroxynitrous acid to nitrogen dioxide and hydroxyl radicals [18,39,44–48]. Both hydroxyl and nitrogen dioxide radicals react with high rate constants of 7.0×10^9 M^{-1} s^{-1} [36] and 3.5×10^7 M^{-1} s^{-1} [49], respectively, with monohydroascorbate and yield the ascorbyl radical. The reaction of peroxynitrous acid with low concentrations of monohydroascorbate takes place during 3 to 4 s (Fig. 1), which is also the time span of the isomerization of peroxynitrous acid to nitrate at 25°C.

Under these conditions, we estimate from our EPR experiments that the maximal concentration of ascorbyl radical formed during the reaction is about 20 nM. This concentration is too small to be part of the main reaction path. It is possible that the ascorbyl radicals observed are not produced from the reaction between monohydroascorbate and peroxynitrous acid at all, but from the oxidation of monohydroascorbate by dioxygen, a reaction catalyzed by transition metals present as trace contamination [43,50].

A mechanism consisting of two fast sequential one-electron oxidations, one by peroxynitrous acid and one by nitrogen dioxide, can be excluded, because both oxidations would yield ascorbyl radicals.

It is conceivable that the ascorbyl radical was not observed because it reacted rapidly with another peroxynitrous acid. In that case there would have been an increase in ascorbyl radicals at higher monohydroascorbate concentrations. No such increase was observed.

Physiological relevance

Can ascorbate compete with carbon dioxide at physiological pH and temperature? The reaction of peroxynitrite with carbon dioxide proceeds with a rate constant of 5.8×10^4 M^{-1} s^{-1} at 37°C [51]. If we assume that in a very short time 10 μM peroxynitrite were formed in a cell, then the product distribution of rate constants and concentrations for removal via the carbon dioxide or the monohydroascorbate pathways can be simulated by adding the reaction of peroxynitrite with carbon dioxide to Scheme 1. A simulation for 1 mM carbon dioxide, 100 μM monohydroascorbate (human serum), and 10 μM peroxynitrite, at pH 7.3 and 37°C, showed that monohydroascorbate diverted only about 3.5% of the peroxynitrite (data not shown). At ascorbate concentrations of 10 mM, as found in neurons, peroxynitrite was trapped to an

extent of 67%. If the reaction between peroxynitrite and carbon dioxide involves an equilibrium [40], then monohydroascorbate will, even at low monohydroascorbate concentrations, curtail the reaction with carbon dioxide.

Our studies were carried out at pH 5.8, which may be relevant to infections where the pH may be significantly lower than 7.3. Under such conditions, monohydroascorbate at physiological concentrations may provide protection against peroxynitrous acid-induced oxidation and nitration [52–54]. A study of the pH dependence on the reaction of monohydroascorbate and peroxynitrite is in progress.

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