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Short communication

Ascorbic acid possesses labile oxygen atoms in aqueous solution

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Abstract

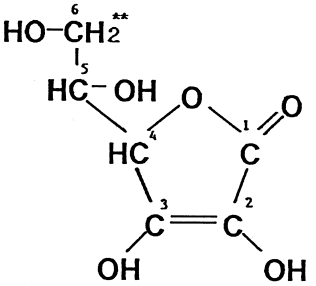
The interaction between ascorbic acid and water was examined using gas chromatographic–mass spectrometric analysis. Structural modifications were analyzed by comparing spectra of *tert*.-butyldimethylsilyl-derivatized ascorbic acid, [6,6-$^{2}H_{2}$]ascorbic acid, and [$^{13}C_{6}$]ascorbic acid with ascorbic acid incubated in ^{18}O-labeled water. The $[M-57]^{+}$ ion of ascorbic acid readily gained a mass increase of m/z 4 on incubation with labeled water, demonstrating an exchange of two atoms of oxygen. Structural analysis showed this mass increase to occur on carbons 2 and 3. These data indicate that ascorbic acid readily exchanges specific hydroxyl oxygen which may account for some of the prooxidant properties of ascorbic acid in solution. © 1998 Elsevier Science B.V.

Keywords: Ascorbic acid; Vitamins

1. Introduction

Ascorbic acid (AA) (vitamin C) is a well known reducing agent both in vivo and in vitro [1–3]. AA consists of a lactone containing enol hydroxyls on carbons 2 and 3 (Fig. 1). It is believed that hydrogen donation is the first step during oxidation of AA to dehydroascorbic acid (DHA), ultimately leading to the loss of two hydrogen atoms from those hydroxyls with conversion of both enols to ketones [4–6]. AA is reported to form DHA in a reversible reaction, through a free radical intermediate [4,6,7]. The hydrogen donation from AA during this conversion is thought to be primarily responsible for the antioxidant properties attributed to AA [4–6].

In contrast to its role as an antioxidant, AA also appears to act as a prooxidant under certain conditions. AA can damage DNA in the presence of oxygen [8] and initiate oxidative cell death in tissue



AA

Fig. 1. Structure of AA. The carbons are numbered. Each hydroxyl is derivatized with TBDMS, adding a net of 456 u to the parent molecule. The (**) marks the site where ^{2}H replaces H in [6,6-$^{2}H_{2}$]AA.

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culture [9,10]. Reactions of AA with metals lead to the production of OH-radical [11] and H_2O_2 [12]. The mechanisms leading to the prooxidant properties of AA are not well defined but may in part be due to the simultaneous formation of the semidehydroascorbic acid free radical [7] during AA oxidation to DHA.

This laboratory has recently shown that AA and DHA interconvert without net oxidation, suggesting nonoxidative lability of the hydrogen atoms on the hydroxyls of carbon 2 and 3 [13]. This hydrogen lability is likely related to the reducing ability of AA. To explain potential prooxidant mechanisms, the lability of AA-hydroxyl oxygens in aqueous solution was likewise examined by mass spectrometric methods.

2. Experimental

2.1. Materials and methods

AA and AA-sodium salt were obtained from Sigma (St. Louis, MO, USA). [$^{13}C_6$]AA (98% ^{13}C) and [6,6$^2H_2$]AA (98% ^2H) were obtained from MSD Isotopes (Montreal, Canada). $H_2{}^{18}O$ (97% ^{18}O) was purchased from Isotec (Miamisburg, OH, USA). Solvents and other reagents, including purified Burdick–Jackson distilled water, were purchased from Fisher Scientific (Pittsburgh, PA, USA). *N*-Methyl-*N*(*tert*.-butyldimethylsilyl) trifluoroacetamide (TBDMS) was obtained from Regis (Morton Grove, IL, USA).

Incubations and solubilization of volumes greater than 500 μl were performed in plastic conical tubes. Reactions were otherwise carried out on 3–25-μl aliquots in 1.1-ml glass autosampler vials. Temperatures ranged from 20–37°C.

Since reactions run in $H_2{}^{18}O$ were performed on very small volumes, the pH could not be determined for solutions of sodium-AA in $H_2{}^{18}O$ directly. To avoid contaminating the sample with the pH probe, the pH was extrapolated as follows: sodium-AA solutions were made in water, and 25- and 1000-μl aliquots were dried using a Savant (Farmingdale, NY, USA) vacuum centrifuge system. The exact amount of water removed (either 25 or 1000 μl) from the sample was readded either as water (25 or 1000 μl) or $H_2{}^{18}O$ (25 μl). The pH of a 1000-μl sodium-AA sample which had been dried and reconstituted with 1000 μl of water was determined using a Fisher Accumet pH meter. The pH of the 25-μl solutions of sodium-AA in $H_2{}^{18}O$ was extrapolated from the measured pH of the 1000-μl solutions.

Reactions were halted by drying at 20°C for 0.5 to 4 h using a Savant vacuum centrifuge system. To assist in derivatization, samples which originated as sodium-AA had 3 μl of 1 *M* HCl added after drying, and this acid was removed by continued drying.

Dried aliquots were derivatized by adding 10 μl of TBDMS and 20 μl of acetonitrile then incubating the capped samples for 0.5 h at 60°C. Two-μl aliquots were applied to a Hewlett–Packard (Avondale, PA, USA) 5890 gas chromatograph. Gas chromatography was carried out through a splitless Supelco (Bellefonte, PA, USA) 12-m dimethylsiloxane, fused-silica capillary column (0.25 mm I.D.) using a temperature ramp of 30°C/min from 80 to 300°C with helium as a carrier, and mass spectrometry was performed on a Hewlett–Packard 5971A mass spectrometer. The scan mode was used to obtain full spectra (including the $[M-57]^+$ ion) and retention times using a mass range of 215–650. The electron multiplier was at 1600 V.

Experiments were done in triplicate to quadruplicate. Samples of AA or sodium-AA in water were used as controls as indicated. The mean values and standard deviations are shown where indicated. Significance is defined as $P<0.05$ on a two-tailed, unpaired Students *t*-test.

2.2. Results

The structure of AA, with carbons numbered, is shown in Fig. 1. Each hydroxyl is derivatized with TBDMS, adding 114 u per site. The derivatized parent molecule is 632 u. All ^{12}C is replaced with ^{13}C in [$^{13}C_6$]AA. The (*) show where ^2H replaces H in [6,6$^2H_2$]AA.

Solutions of AA, (approximately 5 m*M*, pH 3.2±0.1) in $H_2{}^{18}O$ and in water were incubated at 20° for 42 h. Four-μl aliquots were dried and derivatized in TBDMS. Fig. 2 shows the full mass spectra (*m*/*z* 215–650) from AA in (A) H_2O compared to (B) $H_2{}^{18}O$. Fig. 2C and D show an

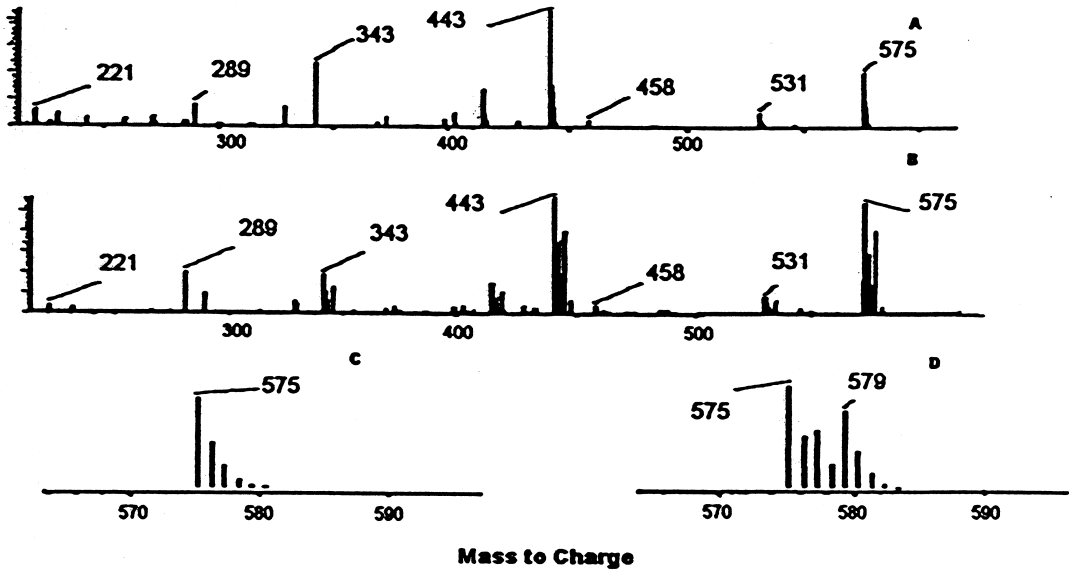

Fig. 2. Total ion chromatogram (m/z 215–650) of TBDMS derivatized: (A) AA and, (B) AA following incubation in $H_2{}^{18}O$ for 42 h. The expanded spectra from 570–582 (i.e., the $[M-57]^+$ ion) is shown for (C) AA and (D) AA following incubation in $H_2{}^{18}O$.

expansion of the $[M-57]^+$ ions (which result from loss of *tert*.-butyl from the derivatizing agent, but no loss of any component from the parent molecules). A comparison of Fig. 2C and D reveals that following incubation in $H_2{}^{18}O$, the $[M-57]^+$ ion of AA increases by 4 u, consistent with the exchange of 2 molecules of ^{18}O for ^{16}O in the ascorbic acid molecule.

Full spectra of TBDMS-derivatized [$^{13}C_6$]AA and [6,6$^2H_2$]AA have previously been published by our laboratory [14]. Fig. 3 shows expanded spectra from AA, [$^{13}C_6$]AA and [6,6$^2H_2$]AA (m/z 286–355 in Fig. 3A–C, m/z 412–540 in Fig. 3D–F). These ions were used to identify fragments containing specific carbon atoms from the parent AA molecule.

Based on Fig. 3, three ions from the spectrum of AA were chosen for analysis. These were m/z 289 (carbons 5 and 6, complete with original oxygen, hydrogens and TBDMS), m/z 343 (carbons 1–4, complete with original oxygen, hydrogens and TBDMS), and m/z 531 (carbons 2–6, from decarboxylation of the $[M-57]^+$ ion). It should be noted that ions m/z 289 and 343 arise from the same fragmentation process, with ionization between carbons 4 and 5.

These ions allow the separation of the oxygen atoms of AA into three groups: the oxygen on carbons 5 and 6 (present on m/z 289 and 531, but not 343), the oxygen on carbons 1 through 4 as a lactone (present on m/z 343, but not m/z 289 and 531), and the oxygen on carbons 2 and 3 (present on m/z 343 and 531, but not 289).

The $[M-57]^+$ ion of AA incubated in $H_2{}^{18}O$ shows the pattern obtained from partial exchange of 2 oxygen atoms with ^{18}O, since all oxygen atoms are retained in this fragment. The relative ratios of ions in the spectrum of AA from m/z 575–581 following incubation in $H_2{}^{18}O$ was then compared to the other ions in the spectrum of AA incubated in $H_2{}^{18}O$, to determine which of the AA-ions m/z 289, 343 or 531 retained the exchanged ^{18}O.

As shown in Fig. 4, fragments related to the parent AA ions of m/z 343 and 531 both retained the exchanged ^{18}O, similar to the $[M-57]^+$ ion. The fragment related to m/z 289 lost this label. Therefore, the oxygen on carbons 5 and 6 are not exchangeable, and the 2-oxygens on carbon 1 (one of which is shared with carbon 4 in the lactone ring) are not exchangeable, demonstrating that the remaining oxygen (on carbons 2 and 3) are the labile atoms.

Since these previous experiments were performed at low pH (3.2), solutions were then made from

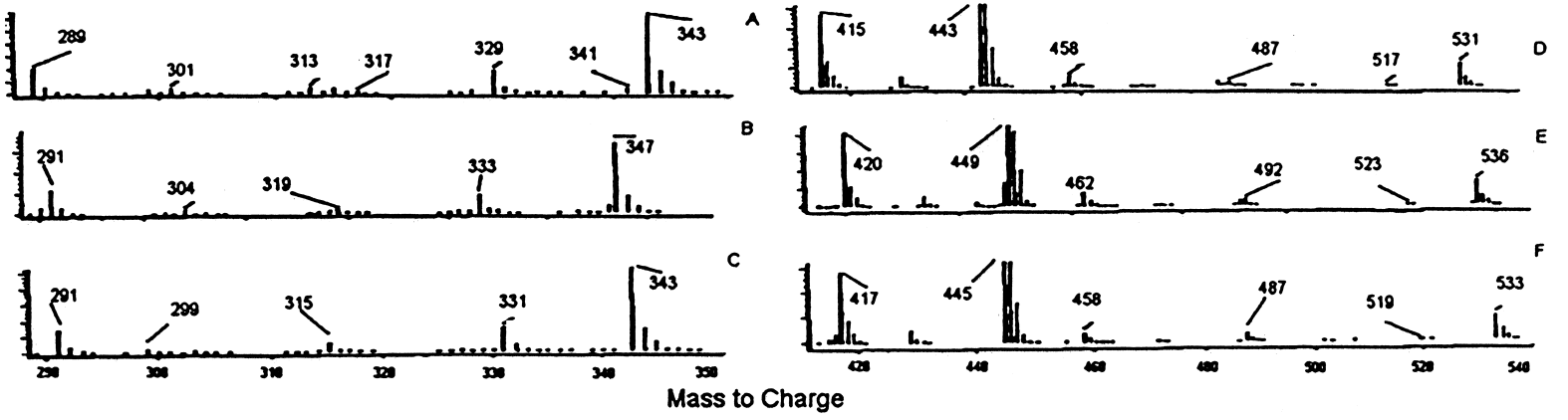

Fig. 3. Expanded spectra of TBDMS derivatized: (A) AA (*m*/*z* 286–355); (B) [$^{13}C_6$]AA (*m*/*z* 286–355); (C) [6,6$^2H_2$]AA (*m*/*z* 286–355); (D) AA (*m*/*z* 412–540); (E) [$^{13}C_6$]AA (*m*/*z* 412–540); and, (F) [6,6$^2H_2$]AA (*m*/*z* 412–540).

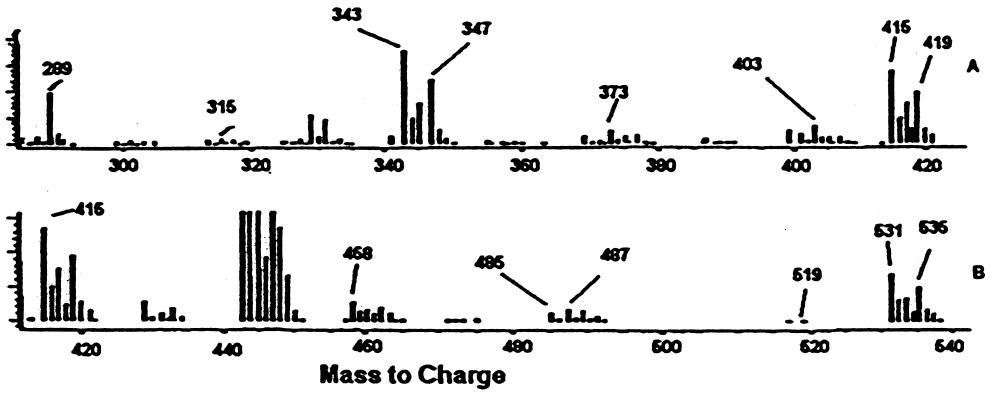

Fig. 4. Expanded spectra of TBDMS derivatized: (A) AA following incubation in $H_2{}^{18}O$ (m/z 286–355); and (B) AA following incubation in $H_2{}^{18}O$ (m/z 412–540).

sodium-AA (pH 6.8±0.1) to approach physiologic pH. Although pH 6.8 is slightly below physiologic pH, by using sodium ascorbate, no buffers needed to be added, eliminating possible side reactions due to metals in buffers [15,16]. Samples of AA pH 6.8 were then incubated at 37°C in either water or $H_2{}^{18}O$, up to 4 h, with hourly removal of aliquots. Data was expressed by examining the quantity of m/z 579 (which comes from both natural isotope abundance and exchange of 2-labeled oxygen atoms).

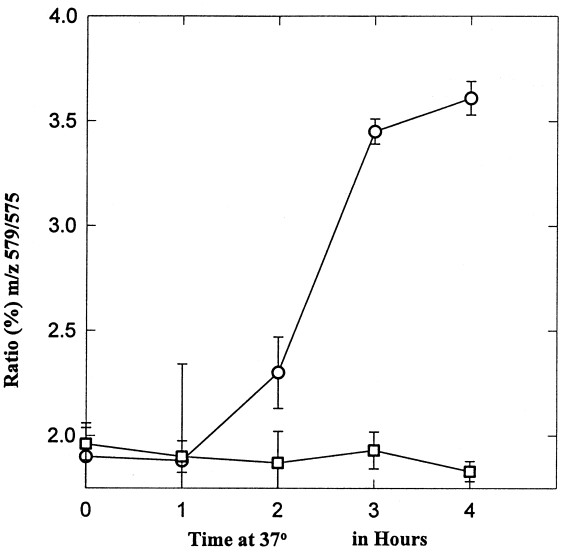

Fig. 5. Ratio of m/z 579 to m/z 575 (AA+2-^{18}O to AA) at pH 6.8 following incubation at 37°C for the specified time intervals. The error bar represents the standard deviation. The open squares show AA incubated in water while the open circles show AA incubated in $H_2{}^{18}O$.

As shown in Fig. 5, there was a significant increase ($P<0.0000001$ in 4-h $H_2{}^{18}O$ incubations relative to either 0-time in $H_2{}^{18}O$, or 4 h in water) in the relative amount of m/z 579 to 575 following incubation of neutral pH solutions at 37°C demonstrating that oxygen exchange occurred under these near physiologic conditions.

2.3. Discussion

The structure of AA suggests that enol-keto interconversion could occur at either carbons 2 or 3. This allows water to attack the slightly positive carbon, ultimately leading to hydroxyl donation. Alternatively, there could be rapid equilibration between DHA and AA [13], with the water addition occurring on DHA as it forms a bicydic hemiketal [17]. Oxidation of organic molecules can be defined as the gain of oxygen (−2) or a loss of hydrogen (−1) while reduction is the converse, either a loss of oxygen (+2) or a gain of hydrogen (+1) [18]. Under this definition, a prooxidant (which is reduced during the reaction) could either accept hydrogen (+1) or donate oxygen (+2) or a hydroxyl (OH−) (net of +1). Data in this report demonstrates the lability of oxygen on carbons 2 and 3 of the AA molecule, providing a mechanism where AA can donate oxygen or hydroxyl, potentially resulting in some prooxidant effects.

AA is an important substance in biology. Human life depends on the constant replenishment of an exogenous source. Better understanding of the fun-

damental behavior of this molecule will hopefully lead to improved utility of this substance.

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