Ascorbic Acid: The Chemistry Underlying Its Antioxidant Properties

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ABSTRACT

Ascorbic acid (vitamin C) is an unusual antioxidant in that it donates a single reducing equivalent, and the radical it forms, monodehydroascorbate, reacts preferentially with radicals instead of with non-radical compounds. This happens because removal of an electron from monodehydroascorbate would create a tricarbonyl structure that is energetically unfavored. Instead of forming this structure, ascorbic acid oxidizes only to monodehydroascorbate, and monodehydroascorbate reacts with other radicals, oxidizing by a mechanism that circumvents formation of this unfavored structure. Ironically, this tricarbonyl compound is commonly and mistakenly cited as the real product of ascorbic acid oxidation. This misconception obscures the chemistry underlying the antioxidant properties of ascorbic acid and diverts attention from significant mechanistic questions.

Key Words: Antioxidant; Ascorbic acid; Dehydroascorbate; Disproportionation; Monodehydroascorbate; Superoxide

Abbreviations: DBM, dopamine β-monooxygenase; GSH, glutathione; GSSG, glutathione disulfide; MDAR, monodehydroascorbate reductase; PAM, peptidyl-glyine α-amidating monooxygenase; TOH, tocopherol (vitamin E)

Ascorbic acid or vitamin C is popularly acclaimed as a potent antioxidant and free-radical scavenger [1.2], yet the chemical basis for its remarkable behavior is poorly or even misunderstood. Crucial to the free-radical scavenging properties of ascorbic acid is the nature of the fully oxidized species, dehydroascorbate (Figure 1, **AH_2O**). It has been known for over 40 years that dehydroascorbate exists as a hydrated and bicyclic hemiketal [3,4], yet the tricarbonyl structure (**A**) continues to be widely cited as the product of ascorbic acid oxidation. **A** is the product expected when two H atoms are abstracted from ascorbic acid in a typical two-equivalent oxidation, but vitamin C is an atypical antioxidant precisely because this does not happen. Kerber [5] argued more than a decade ago that continued reference to the incorrect structure is not justified as a conceptual simplification, because it obscures the true nature of ascorbic acid oxidation. Indeed, our recent work [6] reaffirms the significance of this argument. The incorrect structure **A** exists rarely and fleetingly and has never been detected. The energetic barrier it imposes is responsible for the unique properties of ascorbic acid. We suggest that it be referred to as pseudodehydroascorbate to distinguish it from **AH_2O**, the dehydroascorbate structure that is actually found.

Before addressing the chemistry of ascorbic acid, we will review its biological functions: its role as a reducing agent for enzymatic reactions, its action as an antioxidant eliminating reactive oxygen species, and its role as a scavenger of free radicals. We will also consider pro-oxidant effects caused by redox cycling of metals and quinones. From these examples, two generalizations emerge. First, as a reducing agent, ascorbic acid acts as a donor of single reducing equivalents (H or H^+ + e-) cycling between the fully reduced ascorbic acid and the radical anion, monodehydroascorbate [7]. This makes ascorbic acid itself a good free radical scavenger. Second, monodehydroascorbate reacts preferentially with other radicals. For this reason,

monodehydroascorbate is not just a free radical scavenger but a terminator of free radical chain reactions [8]. Both of these unusual properties are consequences of the fact that loss of two equivalents (H or e-) would lead to the formation of the energetically unfavored pseudodehydroascorbate (**A**). As a consequence, ascorbic acid cycles between $\mathbf{AH^-}$ and $\mathbf{A^{\bullet -}}$, and oxidation to dehydroascorbate ($\mathbf{AH_2O}$) occurs by unusual mechanisms that avoid passing through the unfavored intermediate structure **A** (Figure 1).

I. Biological actions of ascorbic acid

Ascorbic acid is needed most noticeably for collagen synthesis; defective collagen synthesis is the first sign of scurvy, caused by vitamin C deficiency. The enzymes procollagen-proline dioxygenase and procollagen-lysine dioxygenase (prolyl and lysyl hydroxylase), involved in the cross-linking of collagen chains, require ascorbic acid (Table I). Ascorbic acid does not participate directly in the reactions catalyzed by these enzymes. They incorporate one atom of O_2 into the substrate and use the other to oxidize 2-oxoglutarate to succinate + CO_2. However, these enzymes contain Fe^{2+} as a cofactor, and it has a tendency to oxidize to Fe^{3+} rendering the enzymes inactive. Ascorbic acid is needed to reduce Fe^{3+} back to Fe^{2+} [9] . Although the product of this reaction is often cited as dehydroascorbate, reduction of Fe^{3+} takes a single reducing equivalent from ascorbic acid and should yield monodehydroascorbate as the immediate product [2]. This mechanism is also exhibited by other members of the 2-oxoglutarate-dependent oxygenase superfamily [10]. These include trimethyllysine dioxygenase, which is involved in carnitine synthesis, and other prolyl hydroxylases responsible for post-translational modification of other proteins such as hypoxia-inducible factor (HIF).

Ascorbic acid is also required as a reducing agent for dopamine β-monooxygenase (DBM) and peptidyl-glycine α-amidating monooxygenase (PAM). DBM hydroxylates dopamine

to form norepinephrine. PAM amidates peptide hormones by cleaving two carbons off a glycine residue on the carboxyl terminal. Both enzymes incorporate one atom of molecular oxygen into the substrate and reduce the other to H_2O. Two molecules of ascorbic acid are required, each donating a single hydrogen atom to the formation of H_2O, so these enzymes also convert ascorbate to monodehydroascorbate [11,12,13].

Ascorbate oxidase and ascorbate peroxidase form H_2O by reducing O_2 and H_2O_2 respectively. Both of these enzymes use ascorbate as a single-equivalent donor producing monodehydroascorbate as the product [12,14].

The antioxidant and free-radical scavenging actions of ascorbate result from its non-enzymatic reduction of superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and other radicals (Table I). Ascorbic acid also reduces the tocopheroxyl radical ($TO^{\bullet}$) formed when tocopherol (vitamin E) reduces free radicals in lipid environments [15,16]. These radicals take a single H atom from ascorbic acid, oxidizing it to monodehydroascorbate [17,18].

While generally regarded as an antioxidant, ascorbic acid may also have prooxidant effects. Many of these are caused by reduction of metal ions such as Fe^{3+} and Cu^{2+}, contributing to the pathological effects of and extensive biological protections against these ions free in solution. For example, ascorbate reduces Fe^{3+} to Fe^{2+}, which can then reoxidize producing superoxide, H_2O_2 and hydroxyl radical by Fenton chemistry [17,18,19]:

$$Fe^{3+} + \text{ascorbate} \rightarrow Fe^{2+} + \text{monodehydroascorbate} + H^+$$

$$O_2 + Fe^{2+} \rightarrow O_2^{\bullet-} + Fe^{3+}$$

$$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$$

Finally, ascorbic acid can drive one-equivalent redox cycling of quinones such as menadione with reduction potentials in a suitable range [20,21,22]. Ascorbic acid reduces the quinone (Q) to the semiquinone ($Q^{\bullet-}$) oxidizing to monodehydroascorbate itself. The semiquinone then reoxidizes by reacting with O_2 to yield superoxide. Superoxide is quickly reduced by ascorbate and monodehydroascorbate yielding H_2O_2 as the ultimate product.

$$Q + \text{ascorbate} \rightarrow Q^{\bullet-} + \text{monodehydroascorbate} + H^+$$

$$Q^{\bullet-} + O_2 \rightarrow Q + O_2^{\bullet-}$$

$$O_2^{\bullet-} + \text{ascorbate} + H^+ \rightarrow H_2O_2 + \text{monodehydroascorbate}$$

Because the major product of ascorbic acid oxidation is monodehydroascorbate, the principal mechanism for preventing loss of ascorbic acid is reduction of monodehydroascorbate. NADH-dependent monodehydroascorbate reductases are well characterized in plants, which have relatively high levels of ascorbic acid [23]. In animals, NAD(P)H-dependent monodehydroascorbate reduction is achieved by cytochrome b_5 reductase or thioredoxin reductase [24,25,26]. An interesting example of the importance of monodehydroascorbate recycling is the regeneration of ascorbic acid in secretory vesicles [27]. The ascorbate-requiring enzymes dopamine β-monooxygenase and peptidyl-glycine α-amidating monooxygenase are located within secretory vesicles and are needed to synthesize the secreted products: norepinephrine and amidated peptides (Figure 2). Ascorbic acid used by these enzymes produces monodehydroascorbate inside the vesicles. Cytochrome b_{561}, located in the secretory vesicle membrane, reduces intravesicular $\mathbf{A}^{\bullet-}$ and is reduced in turn by cytosolic ascorbate. This forms $\mathbf{A}^{\bullet-}$ in the cytosol, which may then be reduced by cytosolic monodehydroascorbate reductase activities such as cytochrome b_5 reductase.

While ascorbic acid cycles predominantly between the fully reduced form and monodehydroascorbate, complete oxidation to dehydroascorbate does occur. A major pathway is disproportionation of monodehydroascorbate, reaction of two $\mathbf{A}^{\bullet-}$ to form one ascorbate and one dehydroascorbate [28]. Monodehydroascorbate also reacts with other radicals to form dehydroascorbate (Table II). In fact, $\mathbf{A}^{\bullet-}$ reacts with superoxide three orders of magnitude faster than ascorbate itself (Tables IB and IIA) so, despite its lower concentration, monodehydroascorbate may also make a significant contribution to reduction of radical species. Dehydroascorbate thus formed by $\mathbf{A}^{\bullet-}$ oxidation is reduced back to ascorbic acid by dehydroascorbate reductase, which uses glutathione to provide the necessary reducing power [29,30]. This recycling is needed to prevent dehydroascorbate from decomposing to 2,3-diketogulonic acid and other products [1] resulting in the loss of vitamin C. It is apparent that ascorbate oxidizes to monodehydroascorbate and that monodehydroascorbate may then react with itself or other radicals to form dehydroascorbate.

Direct oxidation of ascorbate to dehydroascorbate occurs only in rare circumstances. While many studies of enzymatic or non-enzymatic oxidation of ascorbic acid report dehydroascorbate as the product, they do not test for the formation of monodehydroascorbate. In these cases, it is likely that monodehydroascorbate is the direct product and that dehydroascorbate is then formed by disproportionation.

It should be noted that the rapid disproportionation of monodehydroascorbate is crucial to the antioxidant properties of ascorbic acid. Disproportionation keeps the monodehydroascorbate concentration extremely low thus favoring the oxidation of ascorbic acid.

II. Ascorbic acid is a donor of single hydrogen atoms

It has been known for many years that ascorbic acid is a donor of single reducing equivalents [7,31,32] and normally cycles between its fully reduced form (**AH**$^-$) and the radical anion monodehydroascorbate (Figure 3, **A**$^{\bullet-}$). This is apparent in the above review of its biological functions and is a consequence of the fact that two-equivalent oxidation would form the high-energy product pseudodehydroascorbate (**A**).

How high is the energy barrier presented by the tricarbonyl structure of pseudodehydroascorbate? Tur'yan and Kohen [33] calculated the reduction potential of the **A**/**A**$^{\bullet-}$ pair by analyzing data from polarography and cyclic voltammetry of ascorbic acid. These values (+0.24 V from polarography and +0.22 V from cyclic voltammetry) are about 0.44 V higher than the reduction potential of **AH$_2$O**/**A**$^{\bullet-}$ (-0.21 V, [32]). This implies that the difference in free energy ΔG between **A** and **AH$_2$O** is a truly substantial 10.1 kcal/mol.

From density functional calculations done using the Gaussian series of programs [34] at the CBS-QB3 level of theory, we found ΔG(cor) between **A** and **AH$_2$O** to be 11.81 kcal/mol [6]. The difference between **A** and unhydrated dehydroascorbate (compound **1** in Figure 1) is 8.71 kcal/mol. Based on similar density functional calculations with a different correction for solvent effects, DiLabio and Wright [35] calculated a similar value (ΔG = 8.2 kcal/mol) for the difference between **A** and **1** (Figure 1). Free energies from these density functional calculations are thus in good agreement with the electrochemical analysis. It is notable that **1** and **AH$_2$O** are much closer in energy to each other than to pseudodehydroascorbate, so their interconversion by the addition/abstraction of H_2O is likely reversible with the equilibrium favoring **AH$_2$O**. Therefore, as concluded by DiLabio and Wright [35], most of the energy driving **A**$^{\bullet-}$ oxidation comes from hemiketal formation. H_2O addition makes an important but lesser contribution.

The ratio of dehydroascorbate (**AH_2O**) to pseudodehydroascorbate (**A**) at equilibrium may be calculated from the free energy difference ($K_H = \exp[\Delta G/RT]$) and ranges from 4 x 10^8 from density functional calculations to 2 x 10^7 from the electrochemical data. It should be noted that Tur'yan and Kohen [33] reported a value of 6 x 10^6 calculated using a slightly different value for the **AH_2O /$A^{\bullet-}$** reduction potential. In any case, the concentration of **A** is vanishingly small, and **A** presents a considerable energy barrier to the oxidation of monodehydroascorbate by outer-sphere electron transfer. It is for this reason that ascorbic acid functions as a one-equivalent donor and cycles between ascorbic acid and monodehydroascorbic acid.

At physiological pH, the ascorbate monoanion (**AH-**) is the predominant reduced form of ascorbate (Figure 3). The concentration of negative charge on the dianion $\mathbf{A^{2-}}$ opposes its formation, although it makes the dianion a strong electron donor. For monodehydroascorbate, the radical anion ($\mathbf{A^{\bullet-}}$) predominates. $\mathbf{A^{\bullet-}}$ is an unusually stable radical because the unpaired electron is delocalized around the ring. The protonated radical $\mathbf{AH^{\bullet}}$ has a higher energy because protonation restricts the resonance possibilities of that unpaired electron. This makes the ascorbate monoanion $\mathbf{AH^-}$ a relatively poor e- donor as reflected in the reduction potential of the $\mathbf{AH^{\bullet}/AH^-}$ couple (+0.766 V). The ascorbate dianion $\mathbf{A^{2-}}$ is a good e- donor (the reduction potential of the $\mathbf{A^{\bullet-}/A^{2-}}$ couple = +0.076 V) but is an insignificant fraction of total ascorbate except at high pH. At neutral pH, therefore, interconversion of $\mathbf{AH^-}$ and $\mathbf{A^{\bullet-}}$ is favored, making ascorbic acid a good donor of single H atoms. H may be transferred directly or by concerted electron/proton (e-/H^+) transfer. Nevertheless, this property allows ascorbic acid to reduce many free radicals (Table I). At the same time, ascorbic acid is a poor e- donor, so it is less effective at prooxidant actions such as reducing metal ions or converting O_2 to $O_2^{\bullet-}$ [7] .

III. Monodehydroascorbate oxidizes to dehydroascorbate by reacting with other radicals

Monodehydroascorbate reacts with other radicals much faster than it reacts with fully oxidized or fully reduced compounds (Table II). The poor reactivity of monodehydroascorbate with fully reduced compounds is unusual, as radicals typically react with such compounds to form another radical leading to propagation of free radical chain reactions. The rapid oxidation of $\mathbf{A}^{\bullet-}$ by other radicals is incompatible with outer-sphere electron transfer, because that would form the tricarbonyl structure **A**.

*Can monodehydroascorbate ($\boldsymbol{A}^{\bullet-}$) oxidize to dehydroascorbate ($\boldsymbol{AH_2O}$) without forming the tricarbonyl structure **A**?* The reaction of ascorbic acid with singlet oxygen provides an example of how this might happen. By carrying out the reaction at -85°C in methanol, Kwon *et al.* [36,37] were able to stabilize and identify intermediates in the reaction of singlet oxygen with ascorbic acid. They found that $^1O_2$ adds to ascorbic acid at either the C2 or C3 position forming the respective hydroperoxides (Figure 4A, **2** and **3**). Although **3** is favored over **2** by a 2:1 ratio, the two compounds can interconvert, and **2** cyclizes slowly to form hydroperoxydehydroascorbate. Because this experiment was done in methanol, exchange of H_2O_2 for H_2O to form dehydroascorbate did not occur. Dehydroascorbate could be formed, however, by reducing hydroperoxydehydroascorbate with dimethylsulfide.

One can imagine that reaction of monodehydroascorbate with superoxide could occur by a similar mechanism (Figure 4B). Monodehydroascorbate may react with superoxide to form a hydroperoxy compound analogous to that formed when ascorbate reacts with $^1O_2$. Monodehydroascorbate ($\mathbf{A}^{\bullet-}$) will not cyclize because, like protonation to $\mathbf{AH}^{\bullet}$, cyclization restricts delocalization of the unpaired electron. Coupling with $O_2^{\bullet-}$, however, eliminates the unpaired electron and allows the monodehydroascorbate unit to undergo cyclization. Then H_2O_2

may dissociate exchanging for H_2O. This yields dehydroascorbate (**AH_2O**) in its bicyclic form and avoids passage through the high-energy intermediate structure **A**.

Density functional calculations confirm that intermediate steps in this reaction are energetically feasible [6]. Monodehydroascorbate should add superoxide at the C2 position. This permits ring closure. In aqueous solution, exchange of H_2O for hydrogen peroxide yields dehydroascorbate already in the bicyclic form. Whether the hemiketal **1** is an intermediate or not is an open question. Energy considerations do not preclude formation of **1** followed by H_2O addition to form **AH_2O**.

It is well known that monodehydroascorbate disproportionates rapidly, two $\mathbf{A}^{\bullet-}$ forming one ascorbate and one dehydroascorbate. Again, if formation of dehydroascorbate required passage through the intermediate **A**, rapid disproportionation would not occur. Similar to the reaction of $\mathbf{A}^{\bullet-}$ with $O_2^{\bullet-}$, one can imagine a mechanism in which two $\mathbf{A}^{\bullet-}$ form a dimer with no unpaired electrons. In a landmark study of the kinetics of monodehydroascorbate disproportionation, Bielski *et al*. [28] concluded that monodehydroascorbate first forms a reversible dimer, which then yields ascorbic acid and dehydroascorbate upon attack by either H^+ or H_2O. While they did not suggest a structure for this dimer, we propose that it has the structure **4** (Figure 5). Formation of this dimer allows one monodehydroascorbate unit to rearrange forming the bicyclic structure of dehydroascorbate. The two units then dissociate yielding ascorbic acid and dehydroascorbate.

Density functional calculations confirm that this mechanism is energetically realistic [6]. The energetically favored structure of the monodehydroascorbate dimer has the two units linked at their C2 positions (Figure 5). Dimer formation eliminates the unpaired electrons via internal electron transfer from one unit to the other and permits ring closure in the donor unit. Following

ring closure, the products may then separate as ascorbic acid and dehydroascorbate with the latter already in the bicyclic form.

The oxidation of ascorbic acid to dehydroascorbate has always been regarded as a reversible two-equivalent reaction [38]. Moreover, the interconversion of isotopically labeled ascorbate and dehydroascorbate has been well documented [39]. How do we reconcile this with the barrier imposed by pseudodehydroascorbate? Simple two-equivalent oxidation of ascorbic acid or reduction of dehydroascorbate would require passage through pseudodehydroascorbate as an intermediate. However, disproportionation together with the reverse reaction, comproportionation, would permit equilibration of ascorbate, monodehydroascorbate and dehydroascorbate without forming the high-energy intermediate **A**. We know that disproportionation of $\mathbf{A}^{\bullet-}$ occurs quickly. The free-energy changes calculated for each step in the proposed disproportionation mechanism are small suggesting that the process is also reversible (Figure 5). This means that, comproportionation, the reaction of dehydroascorbate and ascorbate to form monodehydroascorbate, may also occur, albeit more slowly, and disproportionation/comproportionation may account for the observed equilibration of oxidized and reduced forms of ascorbic acid.

A final example of adduct formation is the non-enzymatic reduction of dehydroascorbate to ascorbate by glutathione (GSH). Recognizing that a one-electron reaction yielding two radicals would not explain the observed rate, Winkler *et al*. [40] proposed a two-electron mechanism involving a glutathione-dehydroascorbate adduct. Specifically, they suggested that nucleophilic addition of GS- to the C2 of dehydroascorbate forms a thiol adduct. Then reduction by a second glutathione results in formation of GSSG and ascorbic acid. Although they showed dehydroascorbate with the incorrect pseudodehydroascorbate structure, their mechanism could

also account for reduction of **AH_2O** to **AH^-** without forming pseudodehydroascorbate. This raises the intriguing possibility that adduct formation may occur in other reactions providing a more general mechanism for both oxidation of **$A^{\bullet-}$** and reduction of **AH_2O** while avoiding the kinetic barrier imposed by **A**.

IV. CONCLUSIONS

It is evident that ascorbic acid acts primarily as a donor of single hydrogen atoms, and the radical anion monodehydroascorbate reacts mainly with radicals. Together these properties account for the remarkable antioxidant actions of ascorbic acid. To understand the chemistry underlying these properties, we have proposed mechanisms accounting for the propensity of monodehydroascorbate to disproportionate and to react with other radicals such as superoxide. A crucial aspect of this is the energetic barrier presented by the high-energy tricarbonyl structure **A**, that we have termed pseudodehydroascorbate. Future research should address the two questions we have posed: 1) How high is the energy barrier presented by the tricarbonyl structure of pseudodehydroascorbate? 2) Can monodehydroascorbate (**$A^{\bullet-}$**) oxidize to dehydroascorbate (**AH_2O**) without forming the tricarbonyl structure **A**? To truly understand the antioxidant behavior of ascorbic acid, it is necessary to accept the known fact that dehydroascorbate has the bicyclic and hydrated structure **AH_2O** and that the frequently cited pseudodehydroascorbate is biologically significant not because of its presence but because of its absence.

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FIGURE LEGENDS

Figure 1. Oxidation of ascorbic acid. Kinetic considerations argue that monodehydroascorbate oxidizes to dehydroascorbate (**AH_2O**) without passing through pseudodehydroascorbate (**A**) as an intermediate. The hemiketal **1** has a much lower energy than **A** and could be an intermediate in the formation of **AH_2O**.

Figure 2. Regeneration of intravesicular ascorbic acid by monodehydroascorbate reductase and cytochrome $_{b561}$. DBM = dopamine β-monooxygenase; Cyt b561 = cytochrome b_{561}; MDAR = monodehydroascorbate reductase activity.

Figure 3. Oxidation and protonation states of ascorbic acid. E^2_{m7} is from Iyanagi *et al*. [32] and $E°_{A/A^{\bullet-}}$ is the average of values reported by Tur'yan and Kohen [33]. All other reduction potentials and pK_a's are consensus values tabulated in Njus and Kelley [7].

Figure 4. Dehydroascorbate formation from hydroperoxide adducts. A) Mechanism of ascorbic acid reaction with singlet oxygen ($^1O_2$) yielding hydroperoxydehydroascorbate [36] . B) Hypothesized mechanism of monodehydroascorbate reaction with superoxide ($O_2^{\bullet-}$) yielding H_2O_2 and dehydroascorbate. Values of ΔG (kcal/mol) and pK_a's are from Tu *et al*. [6].

Figure 5. Hypothesized mechanism of monodehydroascorbate disproportionation yielding ascorbic acid and dehydroascorbate. Values of ΔG (kcal/mol) and pK_a's are from Tu *et al*. [6].