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Hypothesis

Vitamins C and E donate single hydrogen atoms in vivo

David Njus and Patrick M. Kelley

Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

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The antioxidant vitamins, C and E, eliminate cytotoxic free radicals by redox cycling. Energetic and kinetic considerations suggest that cycling of vitamin C and vitamin E between their reduced and free radical forms occurs via the transfer of single hydrogen atoms rather than via separate electron transfer and protonation reactions. This may enable these vitamins to reduce many of the damaging free radicals commonly encountered by biological systems while minimizing the reduction of molecular oxygen to superoxide.

Ascorbic acid; Tocopherol; Superoxide anion; Free radical scavenging; Redox; Reaction rate

1. INTRODUCTION

The chemistry and biological effects of vitamins C and E have been extensively studied, and a focus of current interest is the free radical scavenging scheme suggested by Tappel [1]. The hydrophobic vitamin E (tocopherol) reduces free radicals such as lipid hydroperoxyl radicals which arise in biological membranes. In the process, tocopherol is oxidized to the relatively stable tocopheroxyl radical. The tocopheroxyl radical is reduced back to tocopherol by vitamin C (ascorbic acid). The hydrophilic ascorbate also reduces free radicals in aqueous environments. In this way, unpaired electrons are channeled from reactive free radicals to ascorbate. The ascorbate free radical, semidehydroascorbate, is then eliminated by semidehydroascorbate reductase or by disproportionation.

At physiological pH, the free radicals most often encountered by biological systems (ROO•, RO•, OH•), like their reduced forms (ROOH, ROH, H_2O), are uncharged, so reduction of the free radical is formally a hydrogen atom transfer. In terms of mechanism, the reaction could occur either as transfer of a single hydrogen atom or as separate electron transfer and proton equilibration steps. As a general rule, hydrogen atom transfer is the energetically favored mechanism. Separate electron transfer and protonation steps involve unfavorable intermediates, either the reduced anion (e.g. ROO^-) or the protonated radical (e.g. $ROOH^+\cdot$). Consequently, energetic considerations argue that free radical reduction will occur by hydrogen atom transfer.

One-electron donors are essential for free radical scavenging, but such compounds may also reduce molecular oxygen to superoxide. To minimize deleterious effects of superoxide and its products, free radical scavengers must be such that they react rapidly with free radicals but poorly with molecular oxygen. We propose that ascorbate and tocopherol function at physiological pH as donors of single hydrogen atoms. This mechanism enables these vitamins to react efficiently with free radicals but not with molecular oxygen.

2. HYDROGEN ATOM TRANSFER

The argument that ascorbic acid and tocopherol function as donors of single hydrogen atoms may be illustrated by considering the reaction between ascorbate and the Trolox C radical. Trolox C (3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-carboxylic acid) has the same ring structure as tocopherol, but it is water-soluble, so the reaction between the Trolox C radical and ascorbate can be observed in aqueous solution. Ascorbate reduces the Trolox C radical quickly; Davies et al. [2] reported a rate constant of $8.3 \times 10^6\ M^{-1} \cdot s^{-1}$ at pH 7.2. This is comparable to the rate at which ascorbate reduces the tocopheroxyl radical. Packer et al. [3] found a rate constant of $1.55 \times 10^6\ M^{-1} \cdot s^{-1}$ for the latter reaction in 50% isopropanol, 40% water, 10% acetone. Using a phosphatidylcholine liposome system at pH 7.5, Scarpa et al. [4] reported a value of $2 \times 10^5\ M^{-1} \cdot s^{-1}$.

Correspondence address: D. Njus, Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA Fax: (1) (313) 577-6891.

Table I

Thermodynamic parameters

Parameter	Ascorbic Acid			Trolox C		
	Assigned value	Measured values	Ref.	Assigned value	Measured values	Ref.
$E_1^{0\prime}$	+0.33 V	+0.300 V	[18]	+0.47 V	-	-
		+0.330 V	[19]			
		+0.340 V	[20]			
E^{01}	+0.766 V	+0.93 V	[21]	+0.75 V	-	-
		+0.85 - +1.00 V	[22]			
		+0.70 V	[23]			
E^{02}	+0.076 V	+0.015 V	[18]	+0.19 V	0.192	[26]
		+0.050 V	[23]			
pK_1	4.0	4.04	[24]	11.8	11.92	[26]
					11.7	[2]
pK_2	11.3	11.34	[24]	-	-	-
pK_r	-0.45	-0.45	[25]	2.3	2.3	[27]

Parameters apply to protonation and electron transfer steps as indicated in Fig. 1. $E_1^{0\prime}$ is the midpoint potential for the overall one-electron reaction at pH 7.0. For ascorbate, assigned values were chosen to agree with measured values and to satisfy the following theoretical relationships:

$$E_1^{0\prime} = E^{02} + (RT/F)\ln\{(1 + [H^+]/K_2 + [H^+]^2/K_2K_1)/(1 + [H^+]K_r)\}$$
$$E^{01} = E^{02} + (RT/F)(\ln 10)(pK_2 - pK_r)$$

Assigned values for Trolox C were chosen similarly using the corresponding equations.

The thermodynamic parameters for both ascorbic acid and Trolox C are now known (Table I), so it is possible to analyze the mechanism by which ascorbate reduces the Trolox C radical. In this reaction, the electron acceptor must be the neutral radical of Trolox C (TO•) because the rapid rate of reaction observed at pH 7.2 [2] rules out participation of the radical cation $TOH^+\cdot$. The minor species $TOH^+\cdot$ has a high midpoint potential ($E^{01} = +0.75$ V) and would be readily reduced to TOH, but its relative concentration at pH 7.2 is so low that the rate constant would have to exceed the diffusion controlled limit ($10^{11}\ M^{-1}\cdot s^{-1}$) to account for the observed rate of reaction.

The 3 protonation states of ascorbic acid (Fig. 1) must be considered as potential electron donors to TO•. The minor species (AH_2 and A^{2-}) are unlikely, however, because they would have to reduce TO• at a rate close to the diffusion limit (Table II). This is especially improbable for AH_2 which is a very poor electron donor. Consequently, TO• is most likely reduced by the ascorbate monoanion. The predicted rate of outer-sphere electron transfer between TO• and either AH^- or A^{2-} can be calculated using the Marcus theory for electron transfer reactions in solution [5]. In either case, the predicted rate constants are far too small to account for the observed rate of reaction (Table II). It is not likely that the self-exchange rate constant for TO•/TO^- is larger than the estimate used here but, even if it were, it would have to greatly exceed the diffusion limit to account for the observed reaction rate. This implies that ascorbate reacts with TO• by a non-outer-sphere mechanism.

The midpoint potential for one-electron donation by ascorbate at pH 7.0 ($E_1^{0\prime} = +0.330$ V) suggests that

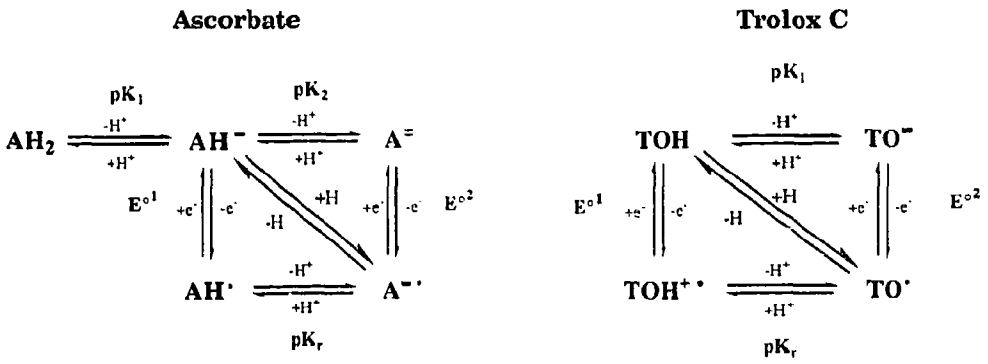

Fig. 1. Proton/electron transfer diagrams for ascorbate and Trolox C. Protonation reactions are shown horizontally and electron transfer reactions are arranged vertically. Fully oxidized forms are not germane to this discussion and are not shown. Abbreviations are: AH_2, ascorbic acid; AH^-, ascorbate monoanion; A^{2-}, ascorbate dianion; $A^-\cdot$, semidehydroascorbate radical anion; AH•, semidehydroascorbate neutral radical; TOH, Trolox C; TO^-, Trolox C anion; TO•, tocopheroxyl neutral radical; $TOH^+\cdot$, tocopheroxyl radical cation.

Table II

Rate constants calculated for reactions between Trolox C radicals (TO·) and the different protonation states of ascorbic acid

Donor	Acceptor	Rate constants ($M^{-1} \cdot s^{-1}$)	
		Necessary	Predicted
AH^-	TO·	8.3×10^6	1.7
A^{2-}	TO·	1.0×10^{11}	2.6×10^7
AH_2	TO·	1.3×10^{10}	–

Necessary rate constants are those required to yield the rate of reaction observed at pH 7.2 (k_{obs} = 8.3×10^6 $M^{-1} \cdot s^{-1}$ [2]). Predicted rate constants were calculated using the cross relation from the Marcus theory for electron transfer reactions in solution [5]. The cross relation gives the rate constant for electron transfer from species 1 to species 2 (k_{12}) in terms of the self-exchange rate constants for each species (k_{11} and k_{22}), the equilibrium constant for the reaction (K_{12}) and a collision factor (f_{12}):

$$(1) \qquad k_{12} \sim (k_{11}k_{22}K_{12}f_{12})^{1/2}$$

Equilibrium constants were calculated from the midpoint potentials of the reacting species (Table I):

$$(2) \qquad K_{12} = \exp\{(E_2^0 - E_1^0)(F/RT)\}$$

Collision factors were evaluated using the equation below taking Z as 10^{11} $M^{-1} \cdot s^{-1}$.

$$(3) \qquad \log(f_{12}) = (\log K_{12})^2/(4 \log\{k_{11}k_{22}/Z^2\})$$

Self-exchange rate constants for ascorbate were taken as 1×10^6 $M^{-1} \cdot s^{-1}$ for both the $A^{2-}/A^- \cdot$ and $AH^-/AH \cdot$ couples [15]. The self-exchange rate constant for $TO^-/TO \cdot$ was evaluated as 1×10^7 $M^{-1} \cdot s^{-1}$. This value is in the range expected for such compounds [28] and may be calculated from the rate of reaction of the Trolox C radical anion with the catechol monoanion [26] at pH 13.5 using a value of $1.6 \times 10^6 M^{-1} \cdot s^{-1}$ for the self-exchange rate constant of the catechol monoanion [15].

ascorbate is a good electron donor especially given that the reduced species (AH^-) is present in great excess over the oxidized form ($A^- \cdot$). This potential, however, applies to the overall reaction in which both an electron and a proton are lost. When the ascorbate monoanion (AH^-) loses only an electron, it forms the energetically unfavored neutral free radial ($AH \cdot$). Consequently, the midpoint reduction potential is quite high (E^{01} = +0.766 V; Table I), and AH^- is a very poor electron donor. As a result, purely electron transfer between the ascorbate monoanion and TO· is not energetically favored and should not occur. The midpoint potential of the donor couple ($E^{0\prime}$ for $AH^-/AH \cdot$ = +0.766 V) is considerably higher than that of the acceptor couple (E^{02} for $TO \cdot/TO^-$ = +0.19 V; Table I). Hydrogen atom transfer, however, is energetically favored because E_1^{01} for the $AH^-/A^- \cdot$ couple (+0.33 V) is lower than that for $TO \cdot/TOH$ (+0.47 V). Thus, thermodynamic arguments suggest that the ascorbate monoanion reduces the Trolox C radical via hydrogen atom transfer (Fig. 2A).

Similar logic applies to all reactions between ascorbate, tocopherol and other free radicals that have the same charge as their reduced compounds at physiological pH. In fact, ascorbate may even act as a hydrogen atom donor rather than as an electron donor in enzyme-mediated reactions. Cytochrome b_{561} is a secretory-vesicle membrane protein that maintains intravesicular ascorbate by equilibrating the internal and external ascorbate/semidehydroascorbate couples [6]. In terms of reaction with ascorbate, cytochrome b_{561}, which is adapted to react with ascorbate, contrasts with cytochrome *c*, which is not. Ascorbate reduces cytochrome *c* relatively slowly at pH 7.0 but the rate is greatly accelerated by raising the pH. This is consistent with the ascorbate dianion acting as the donor for outer-sphere electron transfer to cytochrome *c* [7]. The rate of reduction of cytochrome b_{561} by ascorbate, however, is only slightly pH-dependent. Moreover, ascorbate reduces cytochrome b_{561} ($E^{0\prime}$ = +0.14 V [8]) much more rapidly than it reduces cytochrome *c* ($E^{0\prime}$ = +0.262 V [9]) despite the fact that cytochrome *c* has the higher reduction potential. This is consistent with reduction of cytochrome b_{561} by an inner-sphere mechanism in which the cytochrome oxidizes the ascorbate monoanion directly to the semidehydroascorbate anion via concerted proton and electron transfer [10]. This emphasizes the concept that ascorbate at physiological pH is a poor electron donor but a good donor of single hydrogen atoms.

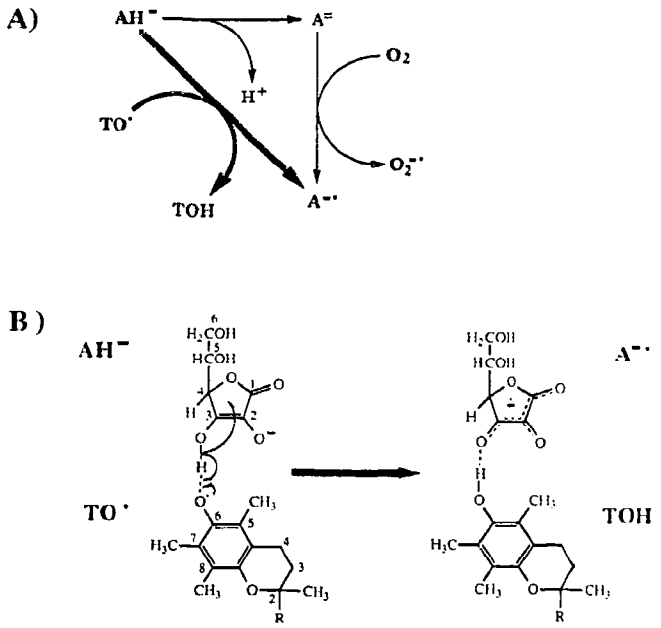

Fig. 2. (A) Alternative mechanisms of ascorbate oxidation. Molecular oxygen oxidizes the ascorbate dianion by an 'outer-sphere' mechanism. Trolox C (and vitamin E) oxidize the ascorbate monoanion directly by hydrogen atom transfer. (B) Hypothesized mechanism of hydrogen atom transfer.

3. SUPEROXIDE GENERATION

The significance of hydrogen atom transfer in free-

radical scavenging is apparent when the problem of superoxide generation is considered. In theory, free radicals could be reduced by purely electron donors of low potential. Low potential one-electron donors, however, will reduce molecular oxygen to superoxide and initiate production of a variety of damaging oxygen radicals. Given cellular O_2 concentrations of 0.02–0.2 atm, $O_2^{-}\cdot$ concentrations of 10^{-11}–10^{-12} M [11], and a standard reduction potential of −330 mV [12,13], the reduction potential of the $O_2/O_2^{-}\cdot$ couple should lie in the range between +0.24 and +0.36 V. Consequently, any one-electron donor with a reduction potential below about +0.2 V will generate superoxide if it can react with O_2.

Ascorbate, the terminal electron donor in the free-radical scavenging chain, has the lowest reduction potential, so its reactivity with molecular oxygen is the most critical. Rate constants for reduction of O_2 by ascorbate are very small (Table III). At low pH, the ascorbate monoanion reduces O_2 very slowly but still at a rate which exceeds that predicted for outer-sphere electron transfer. Thus, the ascorbate monoanion may reduce O_2 by hydrogen atom transfer but the rate is so slow that it is not significant above pH 7. At physiological pH and above, the rate of O_2 reduction is inversely proportional to $[H^+]$ indicating that O_2 is reduced mainly by the ascorbate dianion [14]. The rate constant for reduction of O_2 by A^{2-} is that predicted for outer-sphere electron transfer by the Marcus theory (Table III and [15]). Consequently, hydrogen atom transfer, which facilitates the reduction of Trolox C by the ascorbate monoanion, does not enhance reduction of O_2 by ascorbate. Instead, O_2 reduction is minimal because it occurs mainly via an outer-sphere reaction with the very small fraction of ascorbate in the dianion form (Fig. 2A).

The importance of minimizing the rate of O_2 reduction may be appreciated by considering the following example. At 250 μM O_2, pH 7.4, and 1 mM ascorbate (~10^{-7} M dianion), superoxide will be generated at a rate of 2×10^{-9} M/s. This rate of superoxide generation is apparently not a problem probably because $O_2^{-}\cdot$ is eliminated by superoxide dismutase or ascorbate. Suppose, however, that free-radical reduction were accomplished using a purely electron donation mechanism. To be an effective scavenger of very low concentrations of free radicals, the electron donor would have to be present at a reasonably high concentration. If an electron donor equivalent to the ascorbate dianion were present at a concentration of 1 mM, then oxygen reduction would occur at a rate of 2×10^{-5} M/s. Obviously, this would not only strain superoxide elimination mechanisms; it would quickly make the system anoxic! Even with superoxide dismutase and catalase acting to recycle some of the oxygen, O_2 depletion would occur with a half-time of ~30 seconds.

Table III

Rate constants for reduction of oxygen by ascorbate

Acceptor	Donor	Rate constant ($M^{-1}\cdot s^{-1}$)		
		Observed	Ref.	Predicted
O_2	AH^-	5.9×10^{-4}	[29]	7.2×10^{-7}
		7×10^{-4}	[14]	
O_2	A^{2-}	70	[14]	70
		200	[15]	

Predicted rate constants were calculated using the cross relation from Marcus theory [5] as described in the legend of Table II. The self-exchange rate constant for $O_2^{-}\cdot/O_2$ was taken as 100 $M^{-1}\cdot s^{-1}$ [30-31], and the midpoint reduction potential was corrected to −0.160 V expressing both $[O_2]$ and $[O_2^{-}\cdot]$ in molar concentrations [12].

4. H ATOM TRANSFER MECHANISM

According to the above concept, vitamins C and E will scavenge free radicals that are readily reduced by hydrogen atom donors. Because the mechanism of hydrogen atom transfer determines what will react in this way and what will not, it is important to consider the possibilities. An obvious one is suggested by the fact that the Trolox C radical reacts with ascorbate preferentially via hydrogen atom transfer but that O_2 does not. The 6-oxyl of the Trolox C radical might be expected to hydrogen bond to the protonated 3-hydroxyl of ascorbate (Fig. 2B). Simple electronic rearrangement then would result in transfer of both the electron and the proton to Trolox C. Evidence in support of this mechanism is that isopropyl groups on carbons 5 and 7 of tocopherol inhibit the electron transfer process, presumably by sterically hindering the approach of ascorbate to TO· [16]. Molecular oxygen, which is nonpolar, would not be expected to interact with ascorbate by this mechanism, so hydrogen atom transfer should be greatly slowed. A polarity requirement is also indicated by the fact that Trolox C reacts rapidly with the $CCl_3O_2\cdot$ radical but not with $CCl_3\cdot$ [2].

Free radical scavengers must react spontaneously and very rapidly with the most frequently encountered free radicals. In biological systems, undesirable free radicals are commonly formed by extraction of hydrogen atoms from biomolecules. Hydrogen atom abstraction often forms carbon-centered radicals, and ascorbate and tocopherol would not be expected to reduce these radicals by the above mechanism. However, these typically react very quickly with molecular oxygen to form peroxyl radicals. For example, lipid peroxidation occurs via a chain propagation cycle [17] which begins when a hydrogen atom is abstracted to form a carbon-centered radical R·. This reacts very rapidly with O_2 to give a peroxyl radical (ROO·), and that radical may then abstract a hydrogen atom from another lipid molecule to form the hydroperoxide (ROOH) and a new R·. Chain termination commonly occurs by

reduction of ROO • . The spontaneous incorporation of O_2 to form peroxyl radicals may be crucial to the effectiveness of the tocopherol/ascorbate free radical scavenging system. Oxygen, which presents a problem by limiting the reducing power of the free-radical scavenging system, may also provide the solution by forming polar peroxyl radicals.

In summary, the reaction between ascorbic acid and Trolox C is a paradigm for the free-radical scavenging reactions of vitamins C and E. Destructive free radicals commonly encountered by biological systems (ROO •, RO •, and OH •) are uncharged and must be reduced to an uncharged species. Therefore, a donor of single hydrogen atoms is likely to be the most effective free radical scavenger for these compounds. Moreover, a hydrogen atom donor can be chemically isolated from oxygen/superoxide so that reducing power is maintained and superoxide is not generated. The hypothesis suggested here is that tocopherol and ascorbate are unique as compounds that satisfy these requirements under physiological conditions.

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