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Tuning of the Thermochemical and Kinetic Properties of Ascorbate by Its Local Environment: Solution Chemistry and Biochemical Implications

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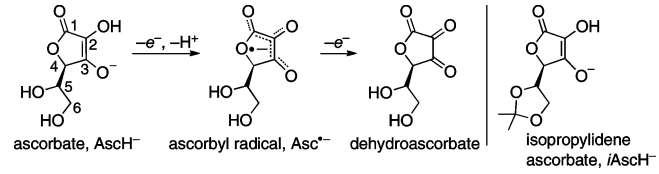
Abstract: Ascorbate (vitamin C) is a ubiquitous biological cofactor. While its aqueous solution chemistry has long been studied, many *in vivo* reactions of ascorbate occur in enzyme active sites or at membrane interfaces, which have varying local environments. This report shows that the rate and driving force of oxidations of two ascorbate derivatives by the TEMPO radical (2,2′,6,6′-tetramethylpiperidin-1-oxyl) in acetonitrile are very sensitive to the presence of various additives. These reactions proceed by the transfer of a proton and an electron (a hydrogen atom), as is typical of biological ascorbate reactions. The measured rate and equilibrium constants vary substantially with added water or other polar solutes in acetonitrile solutions, indicating large shifts in the reducing power of ascorbate. The correlation of rate and equilibrium constants indicates that this effect has a thermochemical origin rather than being a purely kinetic effect. This contrasts with previous examples of solvent effects on hydrogen atom transfer reactions. Potential biological implications of this apparently unique effect are discussed.

Introduction

Ascorbic acid (vitamin C) is a key biological cofactor, necessary for human health,[1] that has a wide range of biochemical roles. It is important as an antioxidant, for instance reducing α-tocopheroxyl radical or oxidized glutathione,[2] and as a cofactor for metalloenzymes such as ascorbate oxidase[3] and cytochrome b_{561}.[4] At physiological pH, it exists predominantly as the ascorbate monoanion, $AscH^-$ (Scheme 1). Oxidation of ascorbate forms the ascorbyl radical $Asc^{\bullet-}$, which has one proton and one electron less than $AscH^-$, and further oxidation gives dehydroascorbate, which can hydrate to the corresponding hemiketal in aqueous solution.[5]

Ascorbate has traditionally been considered a one-electron reductant, but it almost always reacts with loss of both an electron and a proton (Scheme 1) because the neutral ascorbyl radical is a high-energy, highly acidic species [$pK_a(AscH^{\bullet}) = -0.45$].[6] In many cases, ascorbate reacts to transfer the e^- and H^+ in a single kinetic step, in effect as a hydrogen atom. Njus and co-workers have shown that ascorbate reacts as a hydrogen atom donor to oxyl radicals, cytochrome b_{561}, and other

Scheme 1. Redox Chemistry of Ascorbate (with the Carbon Numbering Shown for $AscH^-$)



systems.[7,8] In such cases, the thermochemistry is in some ways better described by the O−H bond dissociation free energy (BDFE), 73.6 ± 1.0 kcal mol^{-1}, for aqueous ascorbate (see below), rather than the redox potential.[9] The standard 0.282 mV potential for ascorbate at pH 7 is mechanistically less informative because it is for the more complex $2e^-/1H^+$ transfer to produce dehydroascorbate.[6]

We recently described oxidations of ascorbate or 5,6-isopropylidene ascorbate ($iAscH^-$, Scheme 1) by oxyl radicals[10] or porphyrin−iron−imidazole complexes[11] and showed that these reactions proceed via a concerted transfer of $H^+ + e^-$ ($H^{\bullet}$) rather than by a stepwise mechanism with initial proton or electron transfer.[10] The ascorbyl radical ($Asc^{\bullet-}$ or $iAsc^{\bullet-}$) is remarkably persistent in "dry" MeCN solvent, decaying over hours at ambient temperatures.[10] This contrasts with the rapid disproportionation of ascorbyl radicals in water (milliseconds

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(8) Njus, D.; Kelley, P. M. *FEBS Lett.* **1991**, *283*, 147–151.
(9) The BDFE is a better predictor of reactivity than the more commonly used bond dissociation enthalpy. See: Mader, E. A.; Davidson, E. R.; Mayer, J. M. *J. Am. Chem. Soc.* **2007**, *129*, 5153–5166.
(10) Warren, J. J.; Mayer, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 7546–7547.
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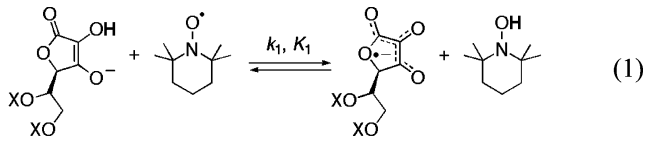
 10.1021/ja102337n

at 10^{-4} M concentrations).[12] The surprisingly long lifetime has allowed us to directly measure equilibrium constants, as described below, and thereby determine the O−H BDFE of $AscH^-$ in MeCN as 67.7 ± 0.8 kcal mol^{-1}. Remarkably, this is 5.9 ± 1.3 kcal mol^{-1} weaker than the aqueous value.

Such a large change in X−H homolytic bond strength with solvent is highly unusual. The studies described here show that this change is not due the bulk properties of the medium such as dielectric (ε). Instead, the dramatic shift in ascorbate homolytic bond strength is predominantly due to the local environment about the ascorbate, especially the presence of hydrogen bond donors. Ascorbate reactions often occur within enzyme active sites or at membrane interfaces that are different from bulk water, so it seems likely that Nature has taken advantage of this unusual property of ascorbate; the last section of this article explores three possible examples.

Results

1. Ascorbate + TEMPO Rate and Equilibrium Constants in Pure Solvents. To examine the intrinsic reactivity of ascorbate derivatives, we have examined their oxidation by the stable TEMPO radical (eq 1, TEMPO = 2,2′,6,6′-tetramethylpiperidin-1-oxyl). These are representative and easily studied reactions that occur rapidly by hydrogen atom transfer to form TEMPOH and the corresponding ascorbyl radical (eq 1), as previously described.[10]



(1)

$AscH^-$ (X = H), $iAscH^-$ (X_2 = isopropylidene)

The studies presented here used the acetonitrile-soluble ascorbate salt with potassium 18-crown-6 ($[K18C6^+]AscH^-$) and the DBU-H^+ salt of the isopropylidene derivative $iAscH^-$ (DBU = 1,2-diazabicyclo[5.4.0]undec-7-ene), avoiding the low-melting and more difficult to handle $^nBu_4N^+$ compounds used in the previous study. $[K18C6^+]AscH^-$ was prepared from potassium ascorbate[13] + 18-crown-6 in MeCN, and $[DBU\text{-}H^+]iAscH^-$ was generated *in situ* from solutions of $iAscH_2$ by adding 1 equiv of DBU. Both of these salts are spectroscopically and kinetically identical to their $^nBu_4N^+$ analogues.

Rate constants for the oxidation of $[K18C6^+]AscH^-$ by TEMPO (eq 1) have been determined in MeCN, DMSO, and water (and in MeCN with various additives, see below). The reactions in MeCN and DMSO were performed under pseudo-first-order conditions of excess TEMPO (15−180 equiv), observing the growth of the ascorbyl radical optical band at 377 nm by using UV/vis stopped-flow spectrophotometry (Figure 1). SPECFIT global analysis software was used to fit the spectra over the entire observed spectral range (320−550 nm).[14] The data follow a first-order kinetic model over >4 half-lives. A plot of the derived pseudo-first-order rate constants as a function of [TEMPO] is linear (see Supporting Information), with slopes that are independent of starting $[AscH^-]$. These

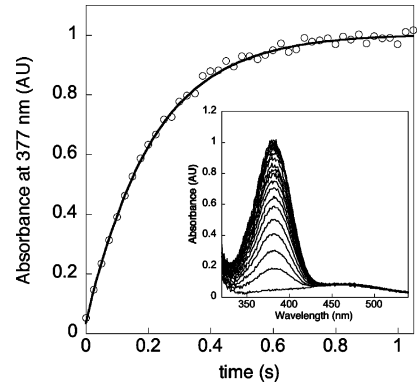


Figure 1. Kinetic data (○) for reaction of 0.28 mM $AscH^-$ with 8.7 mM TEMPO in DMSO and a first-order global fit (—) from SPECFIT. Inset: Stopped-flow UV/vis spectra over 1.1 s for this reaction; the broad absorption centered at 460 nm is due to TEMPO.

slopes give bimolecular rate constants $k_{1,MeCN}$ = 500 ± 50 M^{-1} s^{-1} and $k_{1,DMSO}$ = 100 ± 10 M^{-1} s^{-1} (see Tables 1 and 2, below). The rate constants (and equilibrium constants, see below) are unaffected by the presence or absence of 0.1 M $^nBu_4NPF_6$. Unless specifically noted, all rate and equilibrium constants were determined at 298 K without added electrolyte. Rate constants for oxidations of isopropylidene ascorbate ($iAscH^-$) by TEMPO have been measured similarly, under a wide range of conditions, extending the previous report[10] (see Tables 1 and 3, below).

In MeCN and DMSO, with <10 equiv of TEMPO, reaction 1 proceeds to an equilibrium mixture of the four species. The position of equilibrium can be measured by using the stopped-flow instrument to do a spectrophotometric titration. Reactions are complete after a few seconds, and the position of the equilibrium can be determined before there is significant decay of the ascorbyl radical product. Equilibrium constants were calculated by plotting $[TEMPOH][Asc^{\bullet-}]/[AscH^-]$ vs [TEMPO]. These plots show good linearity, validating the mass balance assumption and the equilibrium treatment (see Supporting Information). The derived equilibrium constant for $[K18C6^+]$-$AscH^-$ + TEMPO, $K_{1,MeCN}$, is 0.14 ± 0.02 ($\Delta G^\circ_{1,MeCN}$ = 1.3 ± 0.1 kcal mol^{-1}), which is within error of the previously measured K for $^nBu_4N^+AscH^-$, 0.12 ± 0.04.[10] Equilibrium constants for analogous reactions were measured similarly and are given in Tables 2 and 3.

Rate constants have also been measured in water (pH 7.1, 100 mM phosphate buffer) for both $AscH^-$ and $iAscH^-$. In water, the ascorbyl radical optical band is not observed because disproportionation of $Asc^{\bullet-}$ is fast[15] compared to the initial reaction of $AscH^-$ + TEMPO. Therefore, these reactions were done with excess ascorbate (10−42 mM) and monitored using the disappearance of the optical band for TEMPO (4.3 mM). The data were fit using SPECTFIT to a model with an initial bimolecular hydrogen atom transfer step followed by $Asc^{\bullet-}$ disproportionation (k_{disp} = 1 × 10^6 M^{-1} s^{-1} in 0.045 M phosphate buffer, pH 7[15]), yielding k_{1,H_2O} = 2.4 ± 0.3 M^{-1} s^{-1} and k_{1i,H_2O} = 5.5 ± 0.5 M^{-1} s^{-1}. The former rate constant is in excellent agreement with a recent determination at more acidic pH for the reduction of TEMPO by ascorbate.[16] The corresponding equilibrium constants cannot be determined in water, again due to the rapid ascorbyl radical disproportionation.

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Table 1. Ascorbate Bond Dissociation Free Energies (BDFEs) and Rate and Equilibrium Constants for Their Reactions with TEMPO (Eq 1) in Pure Solvents[a]

reagent	solvent	k (M^{-1} s^{-1})	K_1	BDFE[b]
$AscH^-$	MeCN	500 ± 50	0.14 ± 0.02	67.7 ± 0.8
$AscH^-$	DMSO	100 ± 10	0.9 ± 0.1	67.6 ± 1.0
$AscH^-$	H_2O	2.4 ± 0.3	$\sim 1.2 \times 10^{-3}$ [c]	73.6 ± 1.0
$iAscH^-$	MeCN	1720 ± 150	1.2 ± 0.2	66.5 ± 0.8
$iAscH^-$	DMSO	250 ± 20	0.85 ± 0.10	67.5 ± 1.0

[a] At 298 K. [b] BDFE values in kcal mol^{-1}. Determined for equilibrium measurements with TEMPO (eq 1) except as noted. [c] Calculated from $BDFE_{water}(AscH^-)$ and $BDFE_{water}(TEMPOH)$ (see below).

2. Ascorbate Bond Dissociation Free Energies in Pure Solvents. The free energy change of eq 1 in pure solvents ($\Delta G°_1$) is the difference in O−H BDFEs of the ascorbate and TEMPOH in that solvent. Thus, the measured values for K_1 ($= e^{-\Delta G°_1/RT}$) and the well-known TEMPO-H BDFEs (66.5 ± 0.5 kcal mol^{-1} in MeCN,[17] 67.5 ± 1.0 kcal mol^{-1} in DMSO[18]) provide the ascorbate O−H BDFEs in those solvents (Table 1). The bond strength of ascorbate in water is derived using a well-established procedure[18,19] from the known pK_a and $E_{1/2}$ values:[6] $BDFE(X-H) = 1.37pK_a + 23.06E_{1/2} + C_G$ [$C_G(H_2O) = 57.6$ kcal mol^{-1} with $E_{1/2}$ vs NHE[18b]). The iAscH$^-$ BDFE in MeCN derived from this equation[10] is within error and less precise than that derived from K_1, so the latter is given in Table 1.[20]

For both ascorbate and isopropylidene ascorbate, the O−H BDFEs are the same in MeCN and DMSO, with that for $AscH^-$ about 1 kcal mol^{-1} stronger than $iAscH^-$. The O−H BDFE of ascorbate is about 6 kcal mol^{-1} larger in water than in the polar organic solvents, as noted above. These solution BDFEs are related to the gas-phase BDFE of ascorbate by the free energies of solution of $AscH^-$, Asc$^•$, and H$^•$, and about half of the difference between the organic solvents and water can be ascribed to differences in $\Delta G°(H^•)_{solv}$.[21] Still, the 6 kcal mol^{-1} strengthening of the ascorbate O−H bond between MeCN/DMSO vs H_2O is significantly larger than would be expected. For substrates with one hydroxylic moiety, the change in BDFE from MeCN to water is typically +2−4 kcal mol^{-1} and from DMSO to water is about +1−3 kcal mol^{-1} (see Supporting Information).

3. Rate and Equilibrium Constants in Mixed MeCN/H_2O Solvent. To probe the substantial difference in BDFEs for ascorbate in acetonitrile versus water, reaction 1 has been studied in acetonitrile/water mixtures. The effects of other "additives" are explored in the next section.

Scheme 2. Kinetic Model Used To Fit Reaction 1 in the Presence of Water or Other Additives

$$\text{R-AscH}^- + \text{TEMPO} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{R-Asc}^{•-} + \text{TEMPOH}$$

$$2\ \text{R-Asc}^{•-} \xrightarrow{k_P} \text{products}$$

Table 2. Rate and Equilibrium Constants for Ascorbate ($AscH^-$) + TEMPO (Eq 1)[a]

solvent	χ_{water}	k_1	K_1
MeCN	3×10^{-5} [b]	500 ± 50	0.14 ± 0.02
MeCN/H_2O	2.2×10^{-3}	410 ± 50	0.10 ± 0.01
MeCN/H_2O	3.6×10^{-3}	370 ± 40	0.080 ± 0.008
MeCN/H_2O	7.2×10^{-3}	280 ± 70	0.048 ± 0.005
MeCN/H_2O	7.2×10^{-3}	300 ± 60	0.052 ± 0.005
MeCN/H_2O	1.2×10^{-2}	260 ± 50	0.035 ± 0.004
H_2O	1	2.4 ± 0.3	ND
DMSO	2×10^{-4} [b]	100 ± 10	0.9 ± 0.1

[a] At 298 K. Rate constants in M^{-1} s^{-1}. [b] χ_{water} in "dry" solvent from Karl Fischer titration.

Rate constants for ascorbate ($AscH^-$) oxidation by TEMPO were measured under pseudo-first-order conditions, as described above, in solutions with the mole fraction of water (χ_{water}) from $\sim 3 \times 10^{-5}$ ("dry" acetonitrile, see Methods) to 0.012. These values correspond to concentrations of water up to 200 mM, or 0.1−720 equiv of water per ascorbate. Qualitatively, the reaction becomes substantially slower in the presence of even quite small amounts of water. Second-order rate constants for reactions with $\chi_{water} < 0.003$ were determined by simple pseudo-first-order kinetic analysis, as above. However, in reactions with higher water concentrations, the decay of the ascorbyl radical is more appreciable and had to be treated explicitly when fitting the data. Above $\chi_{water} = 0.012$, the decay is too rapid to extract meaningful kinetic results. Fortunately, the isopropylidene derivative iAsc$^{•-}$ decays more slowly so that experiments up to $\chi_{water} = 0.028$ could be analyzed. The kinetic data were globally fit with a model of a second-order approach-to-equilibrium step followed by the second-order decay of the ascorbyl radical (Scheme 2).[22] In all cases, the spectrum of ascorbyl radical was fixed in SPECFIT to the known spectrum in pure acetonitrile,[10] which is reasonable since the λ_{max} and ε of the radicals are similar in pure MeCN (377 nm/3900 ± 200 M^{-1} cm^{-1})[10] and H_2O (360 nm/3500 ± 200 M^{-1} cm^{-1}).[23]

Equilibrium constants for these reactions with added water are also determined using Scheme 2, with K_{eq} given by the ratio of the rate constants k_1/k_{-1}. This is best done for reactions where a limited amount of TEMPO is used. For reactions with $\chi_{water} < 0.003$, analysis with the model in Scheme 2 gives the same values of k_1 and K_1 as obtained from the pseudo-first-order plots and plots of [TEMPOH][Asc$^{•-}$]/[$AscH^-$] vs [TEMPO] in MeCN (Table 1). The derived rate and equilibrium constants for reactions with added water are given in Tables 2 and 3 and shown graphically in Figure 2. To pick one example, the rate constant for the iAscH$^-$ reaction is a remarkable 3 times slower in the presence of just $\chi_{water} = 0.014$, and the equilibrium constant is 8 times smaller.

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(18) The BDFE in DMSO is derived from the known[18a] $E_{1/2}$ and pK_a values using $BDFE(X-H) = 1.37pK_a + 23.06E_{1/2} + C_G$, with $C_G = 71.1$ kcal mol^{-1} (ferrocene$^{+/0}$ electrochemical reference).[9,18b] (a) Bordwell, F. G.; Liu, W.-Z. *J. Am. Chem. Soc.* **1996**, *118*, 10819–10823. (b) Mader, E. A. Hydrogen atom transfer reactions of iron and cobalt alpha-diimines: A study of intrinsic and thermodynamic effects. Ph.D. Thesis, University of Washington, 2007.

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(20) Bond dissociation free energies derived from thermochemical cycles typically have errors of ±1−2 kcal mol^{-1}.

(21) A more detailed discussion of $\Delta G_{solv}°(H^•)$ in different solvents can be found in ref 18b. Briefly, $\Delta G_{solv}°(H^•)$ is well approximated by $\Delta G_{solv}°(H_2)$, which is (in kcal mol^{-1}) 5.12 in MeCN, 5.61 in DMSO, and 8.98 in water.[18b] Thus, the solvation of H$^•$ contributes +3.9 kcal mol^{-1} to the difference in BDFEs between MeCN and H_2O (3.4 kcal mol^{-1} for DMSO vs H_2O). This is only one aspect of the solvation, however, and $\Delta G_{solv}°(XH)$ is not necessarily close to $\Delta G_{solv}°(X^•)$ for hydroxylic substrates ROH such as ascorbate, which can form strong hydrogen bonds with H-bond-accepting solvents.

(22) The second step, Asc$^{•-}$ disproportionation, was fit to the kinetic model as a bimolecular process but could be mechanistically more complicated. The derived values for k_1 and k_{-1} do not appear to be very sensitive to the value of the disproportionation rate constant.

(23) The λ/ε given for aqueous ascorbate is the average of the values reported in ref 12 and the following: Schuler, R. H. *Radiat. Res.* **1977**, *69*, 417–433.

Table 3. Rate and Equilibrium Constants for Isopropylidene Ascorbate (*i*AscH$^-$) + TEMPO (Eq 1)[a]

solvent	χ_{water}	k_{1i}	K_{1i}
MeCN	3×10^{-5} [b]	1720 ± 150 [c]	1.2 ± 0.2 [c]
MeCN/H_2O	1.5×10^{-3}	1290 ± 100	0.65 ± 0.07
MeCN/H_2O	3.6×10^{-3}	1020 ± 100	0.43 ± 0.06
MeCN/H_2O	7.2×10^{-3}	780 ± 80	0.38 ± 0.04
MeCN/H_2O	7.2×10^{-3}	745 ± 80	0.25 ± 0.03
MeCN/H_2O	1.2×10^{-2}	525 ± 70	0.13 ± 0.02
MeCN/H_2O	1.4×10^{-2}	560 ± 70	0.15 ± 0.02
MeCN/H_2O	2.8×10^{-2}	240 ± 50	0.065 ± 0.006
H_2O	1	5.5 ± 0.4	ND
DMSO	2×10^{-4} [b]	250 ± 20	0.85 ± 0.10

[a] At 298 K. Rate constants in M^{-1} s^{-1}. [b] χ_{water} in "dry" solvent from Karl Fischer titration. [c] Reference 10.

4. Effects of Other Additives on Rate and Equilibrium Constants. Additives other than water also have a large effect on the rate and equilibrium constants for reaction 1 in MeCN. These experiments were done with *i*AscH$^-$ for experimental convenience (see above). We have examined the effects of adding ethylene glycol ($HOCH_2CH_2OH$), glycerol [$HOCH_2$-$CH(OH)CH_2OH$], dimethoxyethane (DME, $MeOCH_2CH_2$-OMe), diethanolamine [$(HOCH_2CH_2)_2NH$], *N*-acetyl-leucine-methyl ester [MeC(O)NHCH(^{i}Bu)C(O)OMe], DMSO, choline chloride ($HOCH_2CH_2NMe_3^+Cl^-$), tetramethylammonium chloride ($Me_4N^+Cl^-$), and, as noted above, ^{n}Bu$_4$N$^+$PF$_6^-$. These were chosen to include both hydrogen bond donors and acceptors, molecules with biological-type functional groups, and charged species. Acidic compounds, such as $NH_4^+Cl^-$, cannot be used because they would protonate the ascorbate substrate under these conditions. For each additive, k_{1i} and K_{1i} were determined as described above.

Most of the additives cause a decrease in rate and equilibrium constants, consistent with the effect of added water (Table 4 and Figure 3). The exceptions are DME, diethanolamine, $Me_4N^+Cl^-$, and ^{n}Bu$_4$N$^+$PF$_6^-$, which have little or no effect on the reaction. The lack of any change with 100 mM ^{n}Bu$_4$N$^+$PF$_6^-$ is in striking contrast to the effect of only 6.5 mM choline chloride ($\chi = 3.4 \times 10^{-4}$), which decreases k_{1i} and K_{1i} by factors of 1.6 and 2.9, respectively. DMSO has a much weaker effect than other additives: at $\chi = 7.7 \times 10^{-3}$, DMSO lowers k_{1i} by a factor of 1.3, while ethylene glycol causes a 5.7-fold rate decrease.

These shifts in k_{1i} and K_{1i} presumably result from the additive binding to or in some way differentially solvating the ascorbate and/or ascorbyl radical. To probe such a binding, optical spectra of ascorbate in the presence of ethylene glycol or choline chloride have been examined, but no changes were observed. ^{1}H NMR spectra, however, show downfield shifts of the resonance for the proton at C4 for both AscH$^-$ and *i*AscH$^-$ (see Scheme 1 for numbering) with either ethylene glycol or choline chloride. The addition of choline chloride has a qualitatively larger effect on the chemical shift at lower concentrations than ethylene glycol, consistent with the above results that choline chloride has the larger effect on k_1 and K_1.[24]

5. Other Reactions of TEMPO, TEMPOH, and Ascorbate. In principle, the changes in k_1 and K_1 with increasing water or other additives could be due primarily to changes in the ascorbate/ascorbyl radical couple, to changes in the TEMPO/TEMPOH couple, or to changes in both couples. To resolve this question, hydrogen atom transfer reactions of TEMPO, TEMPOH, and ascorbate with other reagents have been studied in MeCN as a function of added water.

5.1. Reactions with Phenoxyl Radicals. *i*AscH$^-$ and TEMPOH each rapidly and completely transfer H$^\bullet$ to the 2,6-di-*tert*-butyl-4-methoxyphenoxyl radical (^{t}Bu$_2$(MeO)ArO$^\bullet$). In pure MeCN, both reactions have $K_{eq} = 3 \times 10^5$ based on the relevant BDFEs.[25] The rate constants in MeCN with various amounts of added water are shown in Figure 4 and given in Supporting Information Table S1. For TEMPOH + ^{t}Bu$_2$(MeO)ArO$^\bullet$, the rate constant at $\chi_{water} = 0.028$ is slower than that in pure MeCN by only 13%. Similarly, the rate constant for TEMPOH + ^{t}Bu$_3$PhO$^\bullet$ ($K_{eq} = 5 \times 10^7$)[17] is reduced by only 15% at $\chi_{water} = 0.028$. In contrast, k_{1i} for TEMPO + *i*AscH$^-$ decreases by *a factor of 7* at this water concentration (Table 3). The mild dependence of the TEMPOH rate constants on small amounts of water is the expected behavior for a hydrogen atom transfer reaction (see below). These reactions do not show the unusual solvent effect.

In contrast, reactions of *i*AscH$^-$ with ^{t}Bu$_2$(MeO)ArO$^\bullet$ are substantially slowed by added water, as shown in Figure 4. For instance, this reaction is slower by 38% at $\chi_{water} = 0.0038$ vs pure MeCN. This reduction is remarkably similar to the decrease of 35% for TEMPO + *i*AscH$^-$ at the same χ_{water}. This water concentration is *7 times less* than the amount that causes only a 13−15% decrease in the TEMPOH reactions. In sum, the reaction of ascorbate with ^{t}Bu$_2$(MeO)ArO$^\bullet$ shows the same unusual slowing with added water, but the reactions of TEMPOH do not. The similarity between the water effects for the two reactions of *i*AscH$^-$, with ^{t}Bu$_2$(MeO)ArO$^\bullet$ and with TEMPO, suggests that this effect is due to the ascorbate rather than the hydrogen abstractor.

5.2. Hydrogen Atom Transfer from a Ruthenium Complex to TEMPO. Added water could also potentially slow k_1 and k_{1i} by forming a hydrogen bond to the R$_2$N−O$^\bullet$ moiety of TEMPO, which is a moderately strong hydrogen bond acceptor.[26] To test this possibility, we have explored the effect of added water on the ability of TEMPO to remove H$^\bullet$ from (acac)$_2$Ru^{II}(py-imH)[27] [acac = 2,4-pentanedionato, py-imH = 2-(2′-pyridyl)imidazole]. This reaction has been previously examined in pure MeCN, where $\Delta G^\circ = -4.4 \pm 0.1$ kcal mol^{-1} ($K_{eq} = 1800 \pm 200$) and $k = (1.4 \pm 0.1) \times 10^3$ M^{-1} s^{-1}.[27b] Between $\chi_{water} = 3 \times 10^{-5}$ and 7.2×10^{-3}, the measured rate constant is within the 7% error bars of the value in pure MeCN (see Supporting Information). At $\chi_{water} = 2.8 \times 10^{-2}$, there is a decrease in k by a factor of 1.4. In contrast, this concentration of water reduces k_{1i} by a factor of 7.2 (Table 3).

5.3. Hydrogen Atom Transfer from ^{t}Bu$_2$NOH to TEMPO. The effect of added water on the reaction of TEMPO with ^{t}Bu$_2$NOH has also been explored. Experimentally, this reaction is most conveniently studied in the reverse direction, ^{t}Bu$_2$NO$^\bullet$ + TEMPOH, but since the reaction is close to isoergic, the approach-to-equilibrium kinetics yield the forward and reverse rate constants as well as the equilibrium constant. In pure MeCN,

(24) Similar NMR shifts are observed for *i*AscH$^-$ + choline chloride. A plausible quantitative analysis of the AscH$^-$ ^{1}H NMR shifts is given in the Supporting Information.

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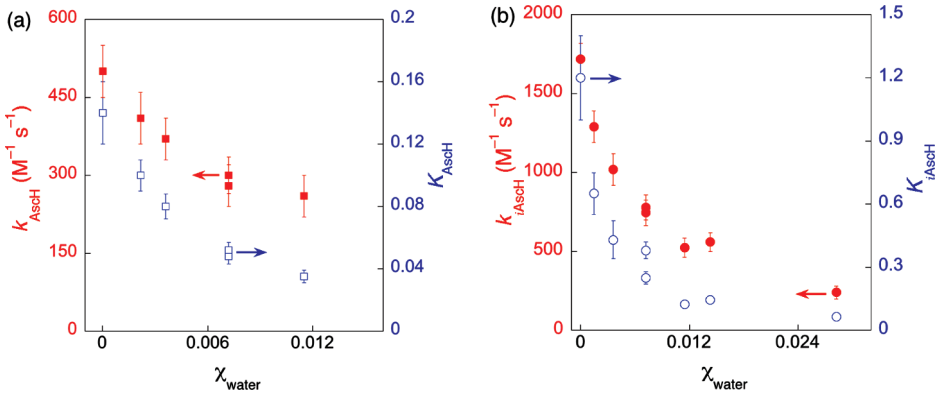

Figure 2. Plots of rate constants [red solid symbols, left axes] and equilibrium constants [blue open symbols, right axes] as a function of mole fraction of added distilled water in MeCN for the reactions of (a) ascorbate with TEMPO (k_1, K_1) and (b) isopropylidene ascorbate with TEMPO (k_{1i}, K_{1i}).

Table 4. Rate and Equilibrium Constants for *i*AscH$^-$ + TEMPO (Eq 1) in MeCN with Additives[a]

additive	χ in MeCN	k_{1i}	K_{1i}
none	*b*	1720 ± 150	1.2 ± 0.2
ethylene glycol	7.0×10^{-4}	1170 ± 80	0.65 ± 0.09
ethylene glycol	7.7×10^{-3}	300 ± 40	0.085 ± 0.010
glycerol	5.1×10^{-5}	1500 ± 100	0.87 ± 0.10
DME	4.8×10^{-3}	1680 ± 130	1.2 ± 0.2
diethanolamine	2.7×10^{-4}	1600 ± 120	0.90 ± 0.10
N-AcO-Leu-OMe[c]	3.0×10^{-3}	1150 ± 100	0.35 ± 0.05
DMSO	7.7×10^{-3}	1360 ± 100	0.50 ± 0.05
DMSO	2.8×10^{-2}	1100 ± 100	0.40 ± 0.05
choline chloride[d]	5.5×10^{-5}	1410 ± 120	1.1 ± 0.2
choline chloride	3.4×10^{-4}	1100 ± 100	0.46 ± 0.06
$Me_4N^+Cl^-$	9.5×10^{-4}	1550 ± 150	0.9 ± 0.1
$^nBu_4N^+PF_6^-$	5.2×10^{-3}	1720 ± 150	1.2 ± 0.2

[a] At 298 K. Rate constants in M^{-1} s^{-1}. [b] $\chi_{H_2O} \simeq 3 \times 10^{-5}$ present in "dry" MeCN. [c] *N*-Acetyl-leucine-methyl ester (MeC(O)NHCH(^{t}Bu)C(O)-OMe). [d] $HOCH_2CH_2NMe_3^+Cl^-$.

based on the reported results,[28] TEMPO + ^{t}Bu$_2$NOH has $\Delta G^\circ = -1.3 \pm 0.1$ kcal mol^{-1} ($K_{eq} = 9.0 \pm 1.5$) and $k = 17.3 \pm 3.6$. We find that addition of water to $\chi = 1.4 \times 10^{-2}$ has essentially no effect on either the forward or the reverse rate constants, or the equilibrium constant. At $\chi_{water} = 2.8 \times 10^{-2}$ there is no change in rate constant for TEMPO + ^{t}Bu$_2$NO$^\bullet$, although k for the reverse reaction and the equilibrium constant both decrease by about 15%. Thus, neither the reaction of TEMPO with (acac)$_2$Ru^{II}(py-imH) nor TEMPO + ^{t}Bu$_2$NOH show, the substantial effect of added water that is observed for TEMPO + ascorbates. This effect of additives is thus indicated to be a property of ascorbate rather than TEMPO.

Discussion

1. Overview. Most biochemical reactions of ascorbate, as is known[4,7] but not always appreciated, initially form the ascorbyl radical and therefore involve loss of e$^-$ *and* H$^+$ or, equivalently, H$^\bullet$. These reactions are best described as hydrogen atom transfer (HAT) (or concerted proton−electron transfer[25]). The results reported here show that small changes in the reaction medium cause surprisingly large changes in the kinetics and thermodynamics of the H-atom transfer reactions of ascorbates with TEMPO and with ^{t}Bu$_2$(MeO)ArO$^\bullet$. The reaction of the potassium-18-crown-6 salt of ascorbate with TEMPO is 1.9 times slower and 4.0 times less favorable in MeCN with *ca.* 1 mol % water (~200 mM water) versus the reaction in pure MeCN. With the more experimentally tractable isopropylidene ascorbate derivative, [DBU-H]$^+$*i*AscH$^-$, the presence of this amount of water decreases k_{1i} and K_{1i} by factors of 3.3 and 9.6, respectively. This effect is not limited to water: the addition of *ca.* 1 mol % ethylene glycol, choline chloride, or *N*-acetyl-leucine-methyl ester to the MeCN solvent causes k_{1i} to fall by more than a factor of 2 and K_{1i} to drop by at least a factor of 8.

The results reported here show that the changes observed in rate and equilibrium constants with various additives are due to changes in the reactivity of ascorbate, not TEMPO or TEMPOH. The reactions of AscH$^-$ + TEMPO, *i*AscH$^-$ + TEMPO, and AscH$^-$ + ^{t}Bu$_2$(MeO)ArO$^\bullet$ all show this remarkable effect. In contrast, none of the reactions of TEMPO or TEMPOH show substantial changes in their rate or equilibrium constants upon addition of water, except for the reactions of TEMPO with the ascorbates. The TEMPO/TEMPOH couple appears to behave like a typical oxyl radical, while the ascorbate/ascorbyl radical couple is anomalous. This conclusion is supported by the unusual solvent dependence of the BDFE of ascorbate, as discussed above and in the Supporting Information).

2. Kinetic and Thermodynamic Results Indicate That Ascorbate Is Strongly Affected by Local Solvation. The large variations in rate and equilibrium constants for reaction 1 cannot be explained by variation of the bulk properties of the solvent, such as the dielectric constant ϵ, nor by a common kind of kinetic solvent effect observed for other HAT reactions (see below). The presence of water in the small amounts used here ($\chi_{water} < 0.03$) has virtually no affect on ϵ.[29] Furthermore, the effects of the other additives on reaction 1 are also inconsistent with changes in ϵ. For example, the dielectric constants of pure MeCN (37.5) and ethylene glycol (37.7) are nearly identical,[30] yet a small amount of ethylene glycol in the MeCN solvent ($\chi = 7.0 \times 10^{-4}$, 13.5 mM) reduces k_{1i} by 32% and K_{1i} by 48%. With the ionic material choline chloride ($HOCH_2CH_2NMe_3^+Cl^-$), addition of only 13 equiv ($\chi = 3.4 \times 10^{-4}$, 6.5 mM) reduces k_{1i} and K_{1i} by factors of 1.6 and 2.9, respectively. This is not due to an ionic strength or ion pairing effect, since a much larger concentration of an inert salt, 100 mM $^nBu_4N^+PF_6^-$, has no effect on the rate or equilibrium constants. The equilibrium constant for *i*AscH$^-$ + TEMPO

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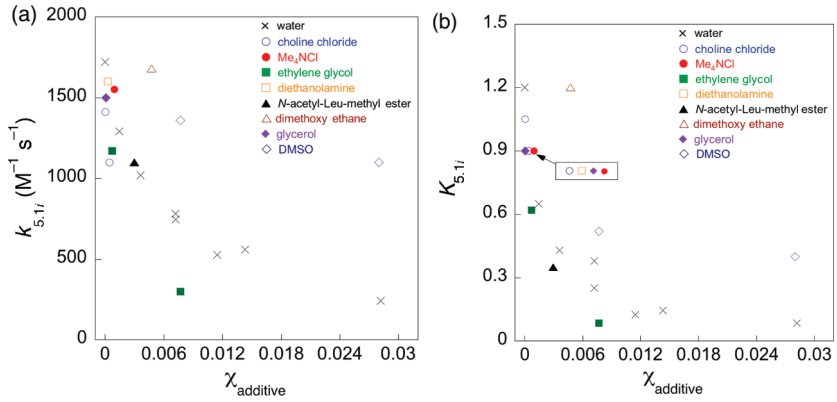

Figure 3. Plots of (a) rate constants [k_{1i}] and (b) equilibrium constants [K_{1i}] for the reaction of isopropylidene ascorbate with TEMPO (eq 1) in MeCN as a function of mole fraction of various additives. Error bars have been omitted for clarity, see Tables 24.

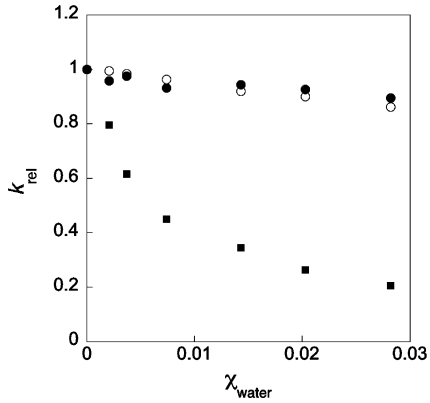

Figure 4. Relative rate constants, $k_{rel} = k(\chi_{water})/k(\chi_{water} = 0)$, as a function of mole fraction water in MeCN for TEMPOH + 2,4,6-tri-*tert*-butylphenoxyl (○), TEMPOH + 2,4-di-*tert*-butyl-4-methoxyphenoxyl (●), and *i*AscH$^-$ + 2,4-di-*tert*-butyl-4-methoxyphenoxyl (■).

decreases by 1800% as water concentration is raised to $\chi_{water} = 0.028$. This decrease is vastly larger than would be predicted from ideal solution behavior, since the reaction is 2.5 kcal mol^{-1} less favorable in pure H_2O vs pure MeCN (see below) and 2.8% of 2.5 kcal mol^{-1} would imply a barely noticeable change in K_{1i} of 12%.

If the large variations in rate and equilibrium constants with small amounts of additives are not due to changes in the bulk properties of the solvent, then they must be due to differences in the local environment in which the HAT reaction occurs. As shown below, it is the presence of H-bond donors in the local environment that is causing the observed effect.

Solvent effects on HAT reactions have been examined in many systems, particularly the effects of solvent polarity and viscosity on selectivity.[31] For HAT reactions involving C−H bonds, solvent effects are usually very small[32] (which is why gas-phase C−H bond strengths can be used to rationalize reactions in solution). For hydroxylic substrates (ROH), Ingold, Litwinienko, and co-workers have found substantial kinetic solvent effects (KSEs) due to hydrogen bonding from the substrate to the solvent, ROH···Solv.[33] Their KSE model indicates that only the non-hydrogen-bonded substrate is reactive toward HAT, so reactions are slower in solvents that are better hydrogen bond acceptors. They gauge the H-bond-accepting ability of solvent using the hydrogen bond basicity parameters (β_2^H) developed by Abraham.[34] This model does predict the slower rates for reaction 1 in the better H-bond-accepting DMSO ($\beta_2^H = 0.78$) vs MeCN ($\beta_2^H = 0.44$) (Tables 2 and 3). However, the large rate deceleration with the addition of small amounts of water is not consistent with the KSE model. Since MeCN is a slightly better H-bond acceptor than H_2O (β_2^H(MeCN) = 0.44 > β_2^H(H_2O) = 0.38), the KSE model predicts that H-transfer rate constants should be unaffected by small amounts of water. These results show that the major effects of additives on the reactions of ascorbate are not due to a hydrogen bonding interaction that blocks the transfer of the ascorbate hydrogen. If this were the case, ethylene glycol and DME would be expected to show very similar effects on the rate constants (k_1). Thus, ascorbate shows a different type of solvent effect on its HAT reactivity that is distinct from the Ingold KSE model for HAT reactions.

It is striking that small amounts of water and other additives affect *both* the rates of reaction and the equilibrium constants. In fact, the equilibrium constants change substantially more than the rate constants. This contrasts with the KSE model, which does not correlate with the (ground-state) reaction thermodynamics because it involves hydrogen bonding only with the reactants and not with the products. Brønsted plots of ln(k) vs ln(K) for the ascorbate and isopropylidene ascorbate reactions at different water concentrations are linear (Figure 5). Figure 5b also includes values for all of the other additives, showing that water and the other materials all behave very similarly—a remarkable and unexpected result. The slopes of the plots are the Brønsted α values, or equivalently, $\Delta\Delta G^{\ddagger}/\Delta\Delta G°$. For AscH$^-$, $\alpha = 0.48 \pm 0.03$. For *i*AscH$^-$, the slope is 0.61 ± 0.04 including all additives, and a very similar 0.60 ± 0.05 for just the MeCN + H_2O reactions of *i*AscH$^-$. A combined plot with all the data shows a very strong linear trend among all the data (*i*AscH$^-$ and *i*AscH$^-$), with a slope of 0.58 ± 0.04 and a correlation coefficient of 0.96 (Figure 5c).

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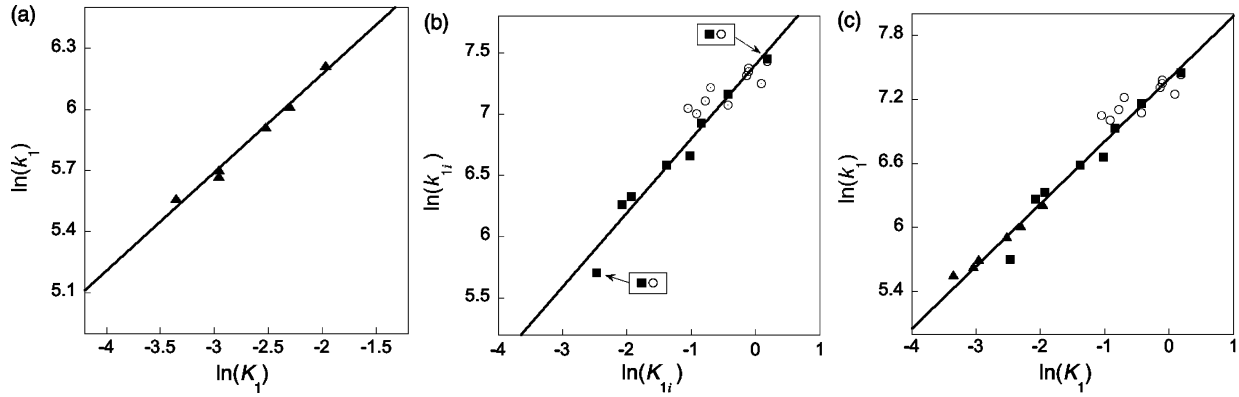
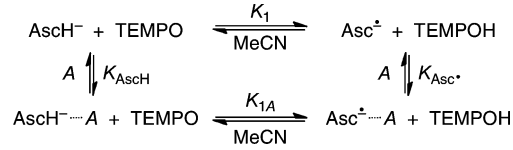


Figure 5. Plots of rate constants $\ln(k_1)$ versus equilibrium constants $\ln(K_1)$ for the reaction of (a) $AscH^-$ in MeCN with added water [▲, $R^2 = 0.99$), (b) *i*$AscH^-$ with TEMPO in MeCN with different additives [■, reactions with added water, $R^2 = 0.93$; □, reactions with other additives], and (c) the aggregate data set ($R^2 = 0.96$).

Rate/driving force relationships in organic HAT reactions have long been studied,[35] although we are unaware of an example such as this with small amounts of additives. Traditionally these correlations have used reaction enthalpies, but we have found that free energies are more appropriate.[9] In addition, we have found that a Marcus theory formalism connecting k and K is very valuable for understanding HAT reactions.[36,37] For reactions such as these with small driving forces ($\Delta G° \ll \lambda/2$), Marcus theory predicts a linear Brønsted plot with a slope of 0.5,[38] which is in excellent agreement with the observed results. *Thus, the observed changes in rate constant stem predominantly from the changes in the driving force of the reactions.*

3. Local Solvation or Binding Shifts the Thermochemistry of Ascorbate. Small amounts of water and other materials strongly shift the equilibrium constant for the reaction of ascorbate and isopropylidene ascorbate with TEMPO. As shown in the previous section, this is due to the local solvation or binding of the additive to the ascorbate and/or ascorbyl radical. The diverse range of additives gives insight into the nature of this binding or solvation. Ethylene glycol at 0.8% markedly lowers both k_{1i} and K_{1i} (factors of 5.7 and 20, respectively), while DME has essentially no effect at a similar mole fraction. Ethylene glycol and DME are similar in size and are comparable hydrogen bond acceptors,[39] but only the former can be a hydrogen bond donor. Thus, it is the hydrogen-bond-*donating* character of the material that is critical. This is again distinct from Ingold's KSE observations, where the hydrogen-bond-*accepting* character of the solvent is the key contributor to the rate constant for hydrogen atom transfer. The importance of hydrogen bond donation is also indicated by the very small effect of DMSO, a good hydrogen bond acceptor, and by the much larger effect of choline chloride ($HOCH_2CH_2NMe_3^+Cl^-$) vs $Me_4N^+Cl^-$. All of the results reported here are consistent with hydrogen bond donation from the additive to ascorbate playing the key role in the observed changes in k and K.

Scheme 3. Effect of Additives *A* on the Reaction of $AscH^-$ with TEMPO



The effect of additives on the equilibrium constants can be analyzed using a thermochemical cycle such as Scheme 3, although this is probably a simplification because the binding is probably not well described by a 1:1 model. In this model, the difference in the measured equilibrium constants, K_{1A}/K_1, is equal to the difference in binding constants for the additive A with $AscH^-$ and $Asc^{\bullet -}$ ($K_{Asc\bullet}/K_{AscH}$). The binding constants K_{AscH} and $K_{Asc\bullet}$ in this analysis are analogous to the K_d values for $AscH^-$ and $Asc^{\bullet -}$ binding to a protein (see next section). Qualitatively, when an additive (or enzyme binding pocket) binds ascorbate more tightly than the ascorbyl radical, the HAT step will be less favorable ($K_{1A} < K_1$). Since these equilibrium constants are related to the O−H BDFE of the ascorbate, hydrogen-bond-donating environments effectively increase the BDFE. A more detailed discussion of BDFEs is given in the Supporting Information.

The difference in binding to $AscH^-$ and $Asc^{\bullet -}$ is presumably due to the difference in charge distribution in the two anions, as indicated in the drawings in Scheme 1. Ascorbate has a single enolate oxygen that formally carries the negative charge, while the ascorbyl radical has a more delocalized triketone-like structure. This is reflected in the difference in basicity of $AscH^-$ and $Asc^{\bullet -}$ (pK_a values of the conjugate acids are 4.0 and −0.45, respectively[6,8]). This issue has been addressed by Costanzo et al. using DFT calculations and molecular dynamics simulations. They show that all three exocyclic oxygen atoms carry significant negative charge in both $AscH^-$ and $Asc^{\bullet -}$, with all the atomic charges being smaller in the radical.[40] Molecular dynamics simulations indicate that in aqueous solution, the enolate oxygen of $AscH^-$ is the preferred hydrogen bonding site. Oxidation to the radical causes the loss of 0.6 of a hydrogen bond at this oxygen, consistent with the change in calculated charges. Thus, these calculations appear to support the conclusion that hydrogen bond donors bind more strongly to $AscH^-$ than to $Asc^{\bullet -}$.

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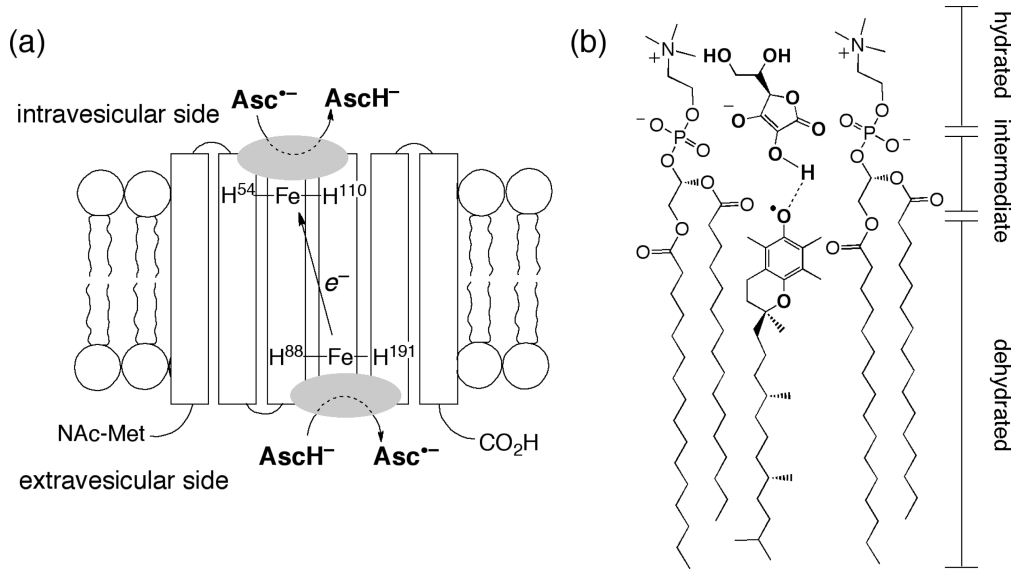

Figure 6. (a) Proposed structural model for bovine cytochrome b_{561}, modified from ref 44, showing the ascorbate/ascorbyl radical conversions on both sides of the membrane. Fe = iron-heme and H = histidine. (b) Depiction of a local environment for the ascorbate reduction of α-tocopheroxyl in a lipid bilayer, as adapted from refs 49 and 52. The bars at the right delineate the regions having different levels of hydration (H-bond-donating water).

Two other reports have described unusual solvent effects on HAT reactions of ascorbate.[16,41] The reduction of PhNO by ascorbate is 40% slower in 1:1 water/dioxane than that in pure water, which is twice as large as the maximum KSE-predicted shift in k from pure water to pure dioxane.[41] In addition, the H_2O/D_2O kinetic isotope effect increases from 2.4 to 8.5 from water to water/dioxane. While these effects could be a result of bulk polarity changes, it seems likely that the issues of local solvation implicated here are also playing a role. A very recent study of the reduction of TEMPO by ascorbate in water/dioxane mixtures reported large kinetic isotope effects indicative of hydrogen tunneling.[16] Tunneling has also been implicated in other HAT reactions of TEMPO.[27b,28] It is not clear how tunneling is contributing to the local solvent effect discussed here. However, it seems unlikely that tunneling effects play a dominant role, given the very strong Brønsted correlations between rate and equilibrium constants. We also note that Amorati et al. have reported a solvent effect that is distinct from Ingold's KSE model.[42] They found that the kinetics and thermodynamics of HAT from hydroquinones to 2,2′-diphenylpicrylhydrazyl radical (DPPH) or peroxyl radicals are affected by hydrogen bonding to the nontransferring hydrogen of the hydroquinone. Such a "remote" hydrogen bonding effect may be analogous to what is reported here, but their study involved hydrogen bond acceptors, whereas the ascorbate reactions above are modulated by hydrogen bond *donors*.

4. Biological Implications. The results reported here show that changes in the hydrogen-bond-donating environment can have a substantial impact on the reactivity of ascorbate. In particular, hydrogen-bond-donating environments increase the ascorbate O−H bond strength, making ascorbate a poorer reductant. Given the wide range of biological processes involving ascorbate, it seems likely that Nature has taken advantage of this apparently unique feature. For example, do ascorbate binding pockets in certain enzymes have hydrogen-bond-donating or hydrogen-bond-accepting character that would thermodynamically favor formation of ascorbate or ascorbyl, respectively? Below we briefly discuss three *in vivo* processes in which modulation of the thermochemistry of ascorbate by the local H-bonding environment could play an important role.

Cytochrome b_{561} is a transmembrane protein, found in both plants and animals, that is thought to mediate ascorbate recycling across the membrane (Figure 6a).[43,44] *In vitro* studies show that it can catalyze the reduction of the ascorbyl radical to ascorbate. In mammalian chromaffin b_{561}, the putative $Asc^{\bullet-}$ binding site on the intravesicular side of the membrane is fairly hydrogen-bond-donating, with the sequence -xYSLHSWxGx- (x is a hydrophobic residue).[43] On the extravesicular side of the lipid bilayer, the putative $AscH^-$ binding site has fewer hydrogen-bond-donating groups (-ALLVYRVFR-), as well as a well conserved lysine residue (Lys85), proposed to play an electrostatic role in binding.[43] Based on the results presented here, the H-bonding environment of the intravesicular $Asc^{\bullet-}$ binding pocket thermodynamically favors formation of $AscH^-$, and the extravesicular $AscH^-$ binding pocket favors the reverse reaction. While the mechanism of $AscH^-/Asc^{\bullet-}$ interconversion may be more complicated than the simple HAT reactions studied here,[45] it is likely that the kinetic barriers reflect the energetics, so this effect should facilitate the reduction of $Asc^{\bullet-}$ on the intravesicular side of the membrane and the oxidation of $AscH^-$ on the extravesicular side.

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Ascorbate peroxidase (APX) has an ascorbate binding pocket that is well characterized.[46] It contains several hydrogen bonding interactions (*e.g.*, Lys30, Arg172, and the heme-propionate), and is fairly exposed to solvent. This pocket thus appears to hold ascorbate in an environment closer to that of aqueous solution, with a high bond strength (high one-electron/one-proton redox potential). The aqueous $1H^+/1e^-$ ascorbate potential is 0.33 V at pH 7,[8] somewhat above the outer-sphere $Fe^{II/III}$ potential of the heme in APX of +0.206 V.[47] Since the catalytic cycle of APX requires an iron(III) resting state,[48] it is important that the bound ascorbate not be any more reducing than aqueous ascorbate. Thus, the enzyme has apparently evolved to have a strongly hydrogen-bond-donating binding site for ascorbate to avoid reduction of the resting ferric state.

The ascorbate reduction of the α-tocopheroxyl radical, to give the ascorbyl radical and α-tocopherol (vitamin E), is perhaps one of the most discussed and studied reactions of ascorbate. This simple HAT reaction *in vitro* is very well documented,[49,50] though its *in vivo* importance is debated.[51] α-Tocopherol is membrane bound, likely with its phenolic moiety near the water−lipid interface.[49] Conversely, ascorbate is hydrophilic and does not significantly partition into lipids, although there is evidence that it can localize in the polar lipid headgroups at the surface of the membrane.[52] This membrane surface region is polar but less hydrated and contains fewer H-bond donors than the cellular bulk (Figure 6b).[53] On the basis of our results, ascorbate at the surface of the membrane is predicted to act as a stronger H-atom donor (reductant) to tocopheroxyl radical than ascorbate in aqueous solution.[54] In addition, the O−H bond in α-tocopherol should be a little stronger in the polar headgroup region than in the bulk lipid.[55] Thus, the "intermediate" region at lipid−water interfaces appears to be an ideal location for Nature to maximize the driving force for, and therefore the rate of, H-transfer from $AscH^-$ to α-tocopheroxyl. Rapid reduction of α-tocopheroxyl is valuable to minimize its activity as a pro-oxidant.[56]

Conclusions

Rate and equilibrium constants for the oxidation of ascorbate by the aminoxyl radical TEMPO (eq 1) in acetonitrile are very sensitive to small amounts of various additives. The results are similar for the potassium 18-crown-6 salt of ascorbate ($AscH^-$) and the more soluble DBU-H^+−isopropylidene ascorbate ($i$$AscH^-$). Addition of water up to 1−3 mol % causes decreases by factors of 2−7 in k_1 and almost 4−20 in K_1. For $AscH^-$, the 4.5-fold decrease in K_1 at only 1.2 mol % water is a quarter of the total change from pure MeCN to pure water. Other hydrogen-bond-donating additives such as glycerol, choline chloride, and *N*-acetyl-leucine-methyl ester have similar effects. On the other hand, the hydrogen bond acceptors DME and DMSO do not significantly affect the reaction outside of normal kinetic solvent effects. Thus, the changes in rate and equilibrium constants are much larger than can be explained by changes in bulk solvent properties such as the dielectric constant or ionic strength. In addition, since the changes in k and K_{eq} are due to hydrogen bond donors but not acceptors, this effect is clearly distinct from Ingold's kinetic solvent effect model for hydrogen atom transfer reactions.

The changes in rate constant directly parallel the changes in equilibrium constant, with Brønsted plots, ln(k) vs ln(K), being linear with slopes close to the theoretical value of 0.5 (0.48 for $AscH^-$ and 0.61 for $i$$AscH^-$). Therefore, the changes in rate are predominantly a consequence of the changes in the driving force for the reactions. This contrasts with previously described kinetic solvent effects in oxyl radical reactions, which are purely kinetic effects due to hydrogen bonding to the transferring hydrogen atom. The results show that ascorbate is a stronger reductant—better able to donate a proton and an electron (or $H^\bullet$)[4,7,8]—in local environments with fewer hydrogen bond donors. The change in reducing power of ascorbate is also indicated by the change in bond strength (BDFE) in pure solvents (Table 1). The unusually large solvation/binding effect of ascorbate is suggested to derive from the substantial differences in charge distribution between ascorbate and the more delocalized ascorbyl radical. This study also indicates that the reactivity of ascorbate can, in many cases, be best understood using the free energy for loss of both a proton and an electron ($H^\bullet$).

The tuning of the properties of ascorbate by its local environment is likely to be of importance in many of the biological actions of ascorbate. Polar, protic environments favor ascorbate, while environments without hydrogen bond donors favor the ascorbyl radical. Three examples illustrate different ways in which Nature may have utilized this unique property, to facilitate ascorbate recycling by cytochrome b_{561}, to avoid unwanted side effects in ascorbate peroxidase, and in the regeneration of α-tocopherol by ascorbate. As new reactivity and structural information emerges about ascorbate-utilizing proteins, specific hydrogen bonding interactions should be considered with respect to the reactivity of the enzyme.

Methods

Physical Techniques and Instrumentation. UV/vis stopped-flow measurements were obtained using an OLIS RSM-1000 instrument. ^{1}H NMR spectra were recorded on a Bruker Avance 300 or 500 MHz spectrometer. All reactions were performed in the absence of air using standard glovebox and high-vacuum techniques.

Materials. Reagent-grade solvents were purchased from Fischer Scientific, unless otherwise noted. All other chemicals were purchased from Aldrich. Anhydrous acetonitrile was purchased from Honeywell Burdick and Jackson, sparged with Ar, and plumbed directly into a N_2-filled glovebox with stainless steel tubing. The supplier specification for water content in the MeCN is 5 ppm ($\chi_{water} = 1.5 \times 10^{-5}$ M); our independent measurement by Karl Fischer

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titration was 10 ppm. Benzene and DMSO were dried using a Grubbs-type catalyst in a Seca solvent system. Deuterated solvents were obtained from Cambridge Isotope Laboratories. TEMPOH, 2,4,6-tri-*tert*-butylphenoxyl, and 2,6-di-*tert*-butyl-4-methoxyphenoxyl were prepared according to the literature.[25] $(acac)_2Ru^{II}$(py-imH) was prepared as previously described.[27a] Deionized water was distilled before use in kinetic experiments. 18-Crown-6 was recrystallized from acetonitrile and dried under vacuum. Choline chloride was recrystallized from absolute ethanol and dried under vacuum. Glyme was freshly distilled from sodium/benzophenone ketyl. *N*-Acetyl-leucine-methyl ester was prepared from leucine-methyl ester (Aldrich) by the method of Applewhite,[57] recrystallized from ethanol/pentane, and stored in a N_2-filled glovebox. Ethylene glycol was distilled from $MgSO_4$, and then a small amount of Na^0 was added. The solvent was stirred overnight and finally was freshly distilled before use. Glycerol was added to an equal volume of *n*-butanol and rotated slowly in an ice bath until crystals formed. The crystals were collected by vacuum filtration, washed with ice-cold acetone, and dried under vacuum. Diethanolamine was distilled and crystallized from its melt.

Preparation of Potassium 18-Crown-6 Ascorbate. Potassium ascorbate was prepared according to the literature.[13] A swivel-frit assembly was charged with 0.5 g (2.3 mmol) of potassium ascorbate and 0.6 g (2.3 mmol) of 18-crown-6. The system was evacuated, and approximately 20 mL of CH_3CN was freshly vacuum-transferred from CaH_2. The resulting yellow solution was stirred for 1 h and filtered. The solvent was removed under vacuum to give a yellow solid, which was recrystallized from CH_3CN/Et_2O and washed once with ~10 mL of Et_2O. ^1H NMR (DMSO-d_6): δ 3.24 (m, 1H), 3.34 (m, 1H), 3.45 (m, 1H), 3.55 (s, 24 H), 3.85 (d, 1H).

Stopped-Flow Kinetics. In a typical procedure, solutions of *i*$AscH_2$ + DBU (0.68 mM each) and TEMPO (1.3−71 mM) in MeCN were prepared and loaded into gastight syringes inside a N_2-filled glovebox. Mixtures containing additives, except for water, were also prepared in the glovebox. The syringes were removed from the glovebox individually for each stopped-flow run. When necessary, freshly distilled and degassed water was quickly transferred from a N_2-filled two-neck flask to both syringes (*i*$AscH^-$ and TEMPO) via a gastight microliter syringe. The stopped-flow syringes were capped and inverted twice to mix thoroughly and immediately placed onto the stopped-flow. A minimum of flow kinetic runs were collected at each [TEMPO]. The data were analyzed using SPECFIT software as described in the text.

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Supporting Information Available: Pseudo-first-order plots for reaction 1 in DMSO and MeCN; equilibrium constant/mass balance plots for reaction 1 in MeCN and DMSO; Benesi−Hildebrand plots for $AscH^-\cdots$additive formation constants; reactions of TEMPOH or *i*$AscH^-$ with phenoxyl radicals; reaction of TEMPO with $(acac)_2Ru^{II}$(py-imH); reaction of $^tBu_2NO^\bullet$ with TEMPOH; calculations of BDFE for TEMPOH. This material is available free of charge via the Internet at http://pubs.acs.org.

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