J|A|C|S
COMMUNICATIONS

Published on Web 08/31/2002

Increased Antioxidant Reactivity of Vitamin C at Low pH in Model Membranes

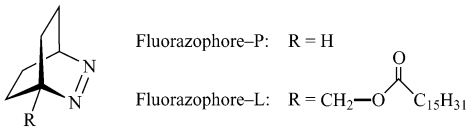
Gabriela Gramlich, Jiayun Zhang, and Werner M. Nau*

Department of Chemistry, University of Basel, Klingelbergstrasse 80, CH-4056 Basel, Switzerland

Received May 16, 2002

The water-soluble vitamin C is a well-known preventive and chain-breaking antioxidant;[1] for example, it recycles the lipid-soluble vitamin E by reducing α-tocopheroxyl radicals in membranes.[2] Additional physiological functions or adverse side effects of vitamin C have also been described.[2d,3] Because of the first pK_a value of 4.04,[4] vitamin C exists at neutral pH as ascorbate monoanion (HA^-), which is generally accepted to act as a stronger antioxidant than the protonated form, ascorbic acid (H_2A).[2,4−8] Nevertheless, many applications of vitamin C in food industry apply to low pH values where H_2A prevails, for example, in canned beverages, preserved fruits, or salad dressings.

We now introduce the fluorescent probe Fluorazophore-L as a model for reactive radicals in membrane systems. Fluorazophore-L[9] is a lipophilic version of the azoalkane 2,3-diazabiyclo[2.2.2]-oct-2-ene (Fluorazophore-P), which has been established as a fluorescent probe for antioxidants.[7] Its fluorescent excited state is quenched by antioxidants through hydrogen transfer and resembles in its reactivity and selectivity alkoxyl and reactive peroxyl radicals. Because of the exceedingly long fluorescence lifetime of Fluorazophore-P (325 ns in aerated water), sizable quenching effects result at physiologically relevant concentrations of chain-breaking antioxidants (micromolar to millimolar).[7] The fluorescence quenching, which can be monitored with high sensitivity and real time resolution, provides a direct measure of the kinetics of the primary redox reaction, a crucial factor for determining the total phenomenological activity of an antioxidant.



Fluorazophore-L combines the long fluorescence lifetime of fluorazophores (125 ns in POPC liposomes under air) with the possibility for incorporation into membrane models.[9] This allows now for the first time to measure directly and even "visualize" antioxidant reactivity at the lipid/water interface by fluorescence; see Figure 1. Until now, long-lived tocopheroxyl and persistent nitroxyl radicals have been employed as probes to assess the interaction of oxidizing species at this interface by using transient absorption[2c,5] and EPR spectroscopy.[2b,6,10] Because of its high excited-state reactivity, Fluorazophore-L may in some respects be a better mimic for the reaction kinetics of reactive lipid peroxyl and alkoxyl radicals and allows one to exploit the numerous advantages of fluorescence for detection. We have now investigated the interaction of this fluorescent probe with the water-soluble vitamin C at model membrane interfaces. Because Fluorazophore-L is amphiphilic, with a polar (3.2 D), nonionic chromophore as headgroup and a palmitoyl tail as the lipophilic part, the reactive fluorophore resides at the lipid/water interface,[9] which is also the presumed location of the peroxyl group of lipid peroxyl radicals.[11] The peroxyl group resembles the fluorophore in its high dipole moment (ca. 2.6 D).[11]

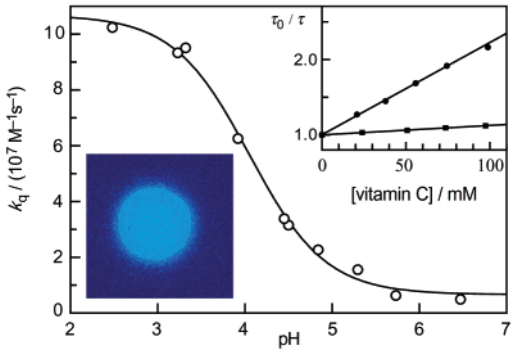


Figure 1. Quenching rate constant (k_q, 5% error) of Fluorazophore-L by vitamin C versus pH in POPC liposomes. Inset on the right: kinetic plots of τ_0/τ versus [vitamin C] (circles, for H_2A at pH 2.7; rectangles, for HA^- at pH 7.0). Inset on the left: fluorescence microscopic image of a large liposome (1 μm diameter) labeled with Fluorazophore-L.

Table 1. Quenching Rate Constants by Vitamin C

		k_q/(10^7 M^{-1} s^{-1})	
probe	environment	pH 7.0 (HA^-)	pH 2.7 (H_2A)
Fluorazophore-P	water	205	120
Fluorazophore-L	SDS micelles	2.6	47
Fluorazophore-L	Triton micelles	5.4	11
Fluorazophore-L	POPC liposomes	1.3	10

Fluorescence quenching rate constants for Fluorazophore-L by vitamin C (H_2A at pH 2.7 and HA^- at pH 7.0) were measured by time-resolved and steady-state fluorimetry in neutral micelles (Triton X-100R), anionic micelles (SDS), and liposomes with zwitterionic headgroups (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPC; only time-resolved measurement); see Table 1 and Figure 1.

The second-order quenching rate constants for H_2A and HA^- in liposomes fall generally 1−2 orders of magnitude below those measured for the water-soluble parent Fluorazophore-P in aqueous solution (Table 1). This reflects the partial protection of the fluorophore against reactive solutes when immersed in the hydrophobic environment. Strikingly, however, the rate constants are consistently higher for H_2A than for HA^- with the reactivity difference ranging from a factor of 2 for nonionic Triton X-100R micelles, 10 for POPC liposomes, and up to 20 for SDS micelles. In fact, the rate constant for SDS at low pH falls only a factor of 2 below that measured in aqueous solution, regardless of the well-known protection provided by the micellar environment. The detailed pH rate profile (Figure 1) reveals a rapid increase in reactivity near the pK_a value of H_2A (4.04), both for POPC

* To whom correspondence should be addressed. E-mail: Werner.Nau@unibas.ch.

10.1021/ja026927b CCC: $22.00

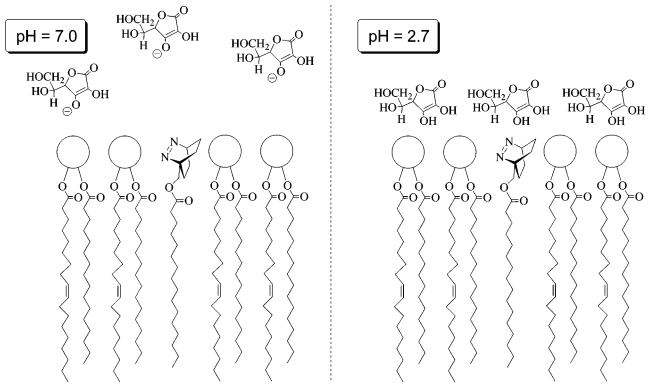


Figure 2. Presumed interaction between singlet-excited Fluorazophore-L and vitamin C at different pH values. Note the association at low pH.

liposomes and for micelles, which demonstrates that the protonation of H_2A is responsible for this effect. Control experiments confirmed that the inherent fluorescence lifetime of Fluorazophore-L is insensitive to pH between pH 2−7.

To rationalize the up to 1 order of magnitude higher reactivity of H_2A versus HA^- in liposomes and micelles, one must recall that the observed rate constants for this bimolecular reaction depend on the intrinsic reactivity *and* on the quencher concentration. The intrinsic reactivity difference, which can be measured for the water-soluble Fluorazophore-P in aqueous solution, cannot be responsible for the observed effect because it shows the expected trend ($k_q = 2.1 \times 10^9$ M^{-1} s^{-1} for HA^- versus 1.2×10^9 M^{-1} s^{-1} for H_2A).[7] This is consistent with the reactivity pattern observed for other reactive oxidizing species such as singlet oxygen ($k_q = 3.1 \times 10^8$ M^{-1} s^{-1} versus 1.9×10^7 M^{-1} s^{-1})[8] and the trichloromethyl peroxyl radical ($k_q = 5.8 \times 10^8$ M^{-1} s^{-1} versus 1.4×10^7 M^{-1} s^{-1}).[12]

We speculate that a local concentration effect applies. The less hydrophilic neutral form of vitamin C has an increased tendency to associate with the surface of the hydrophobic assemblies. The resulting interactions at the interface with the incorporated probe (Figure 2) can be described by a simple kinetic model as an association with a preequilibrium constant K followed by a pseudounimolecular reaction at the interface (k'), that is, $k_q = Kk'$, where k' reflects the intrinsic reactivity.

The known intrinsic reactivity difference in homogeneous solution (factor $^1/_2$) and the experimental difference in k_q values for the heterogeneous model membrane systems (factor 2−20) suggest that the association constant (K) for H_2A is ca. 4−40 times higher than that for HA^-. The absolute value of K must be relatively low, however, because the kinetic plots are linear up to 100 mM of H_2A and no saturation was observed (Figure 1); this precluded a direct determination of the association constant or partition coefficient of H_2A. Note also that the larger pH effect for the negatively charged SDS (factor 20 difference) as compared to the neutral Triton X-100R micelles (factor 2) is expected from the differences in the association constant due to charge repulsion between HA^- and SDS.[10a]

Our in vitro findings point to a sizable association of the uncharged H_2A to neutral or negatively charged surfaces of micelles and liposomes. The resulting higher local concentration of H_2A as compared to HA^- results in an apparently increased bimolecular reaction rate constant. The enhanced antioxidant reactivity of vitamin C toward reactive oxidizing species in heterogeneous systems at low pH may play a biological role for acidified tissues.[13] However, the implications for food processing and conservation may be more important. Because rapid autoxidation of vitamin C occurs in solution at neutral pH,[4] many applications require low aqueous pH (or dry conditions).[1,3] Under these conditions, the more stable protonated form (H_2A) prevails. On the other hand, there are also numerous applications[1] of vitamin C in lipid-containing foods such as salad dressings, cured meat, and oil-baked potato chips where interactions between vitamin C and lipid radicals must come into play. Our results suggest that H_2A essentially forms a protective coating around lipid surfaces (Figure 2, right). Interestingly, the antioxidant actually accumulates where antioxidant activity is required. This finding may also be important for the understanding of the so-called "polar paradox",[3] a phenomenological observation, that polar antioxidants, for example, H_2A, are more active in bulk oil systems, whereas nonpolar antioxidants, for example, ascorbyl palmitate, are more active in emulsified systems. This was tentatively related to differences in the surface-to-volume ratio.

In summary, we suggest that the role of vitamin C as an antioxidant and scavenger of *lipid* radicals has been underestimated. H_2A can display a higher antioxidant reactivity than HA^- under certain conditions, in particular toward the most reactive and potentially most damaging radicals localized in membranes. The conclusion drawn from previous studies involving much less reactive radicals,[2c,5,6,10] namely, that the antioxidant reactivity of vitamin C is reduced at low pH, cannot be generalized.

Acknowledgment. This work was supported by the Swiss National Science Foundation and by the NRP 47 "Supramolecular Functional Materials". We thank the group of Prof. W. Meier for the dynamic light scattering experiments. This work is dedicated to Prof. W. Adam on the occasion of his 65th birthday.

Supporting Information Available: Experimental details and specialized discussion on pH-dependent reactivity (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Liao, M.-L.; Seib, P. A. *Food Chem.* **1988**, *30*, 289−312. (b) Frei, B.; England, L.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6377−6381. (c) Newsome, R. L. *Food Technol.* **1987**, *9*, 163−168.
(2) (a) Packer, J. E.; Slater, T. F.; Willson, R. L. *Nature* **1979**, *278*, 737−738. (b) Noguchi, N.; Niki, E. *Free Radical Res.* **1998**, *28*, 561−572. (c) Bisby, R. H.; Parker, A. W. *Arch. Biochem. Biophys.* **1995**, *317*, 170−178. (d) Podmore, I. D.; Griffiths, H. R.; Herbert, K. E.; Mistry, N.; Mistry, P.; Lunec, J. *Nature* **1998**, *392*, 559.
(3) (a) Porter, W. L. *Toxicol. Ind. Health* **1993**, *9*, 93−122. (b) Frankel, E. N.; Huang, S.-W.; Kanner, J.; German, J. B. *J. Agric. Food Chem.* **1994**, *42*, 1054−1059.
(4) Khan, M. M. T.; Martell, A. E. *J. Am. Chem. Soc.* **1967**, *89*, 4176−4185.
(5) Mukai, K.; Nishimura, M.; Kikuchi, S. *J. Biol. Chem.* **1991**, *266*, 274−278.
(6) Craescu, C. T.; Baracu, I.; Grecu, N.; Busca, L.; Niculescu-Duvaz, I. *Rev. Roum. Biochim.* **1982**, *19*, 15−23.
(7) Nau, W. M. *J. Am. Chem. Soc.* **1998**, *120*, 12614−12618.
(8) Bisby, R. H.; Morgan, C. G.; Hamblett, I.; Gorman, A. A. *J. Phys. Chem. A* **1999**, *103*, 7454−7459.
(9) Gramlich, G.; Zhang, J.; Winterhalter, M.; Nau, W. M. *Chem. Phys. Lipids* **2001**, *113*, 1−9.
(10) (a) Liu, Y.; Liu, Z.; Han, Z.; Chen, P.; Wang, L. *Prog. Nat. Sci.* **1991**, *1*, 297−306. (b) Liu, Y.-C.; Liu, Z.-L.; Han, Z.-X. *Rev. Chem. Intermed.* **1988**, *10*, 269−289.
(11) Barclay, L. R. C. *Can. J. Chem.* **1993**, *71*, 1−16.
(12) Neta, P.; Huie, R. E.; Maruthamuthu, P.; Steenken, S. *J. Phys. Chem.* **1989**, *93*, 7654−7659.
(13) Halliwell, B.; Zhao, K.; Whiteman, M. *Free Radical Res.* **2000**, *33*, 819−830.

JA026927B