Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 134-139

STOPPED-FLOW INVESTIGATION OF THE REACTION BETWEEN VITAMIN E RADICAL AND VITAMIN C IN SOLUTION

Kazuo Mukai, Kazuyuki Fukuda, Kazuhiko Ishizu, and Youichi Kitamura

Department of Chemistry, Faculty of Science, Ehime University, Matsuyama 790, Japan

Received May 26, 1987

Kinetic study of the reaction between vitamin E radical and vitamin C has been performed. The rates of reaction of vitamin C (ascorbic acid 1, 6-O-stearyl ascorbic acid 2, and 2,6-O-dipalmitoyl ascorbic acid 3) with vitamin E radical (5,7-diisopropyl-tocopheroxyl) in benzene-ethanol (2:1, v/v) solution have been determined spectrophotometrically, using stopped-flow technique. The second-order rate constants obtained are 549 ± 30 $M^{-1}s^{-1}$ for 1, 626 ± 53 $M^{-1}s^{-1}$ for 2, and 4.84 ± 1.41 $M^{-1}s^{-1}$ for 3 at 25.0°C. The result shows that the ascorbic acid ester 2 having a long-alkyl-chain at 6-position is 1.14 times as reactive as the ascorbic acid 1, whereas the ascorbic acid ester 3 substituted at 2-position is only 0.01 times as reactive as the ascorbic acid 1.

It is well recognized that vitamin E (α-, β-, γ-, and δ-tocopherols) is localized in cellular membranes and have functions as an antioxidant by protecting unsaturated lipids from peroxidation. The antioxidant actions of the tocopherols have been ascribed to the oxidation reaction of phenolic hydroxyl group, producing corresponding tocopheroxyl radicals (1,2). Therefore, several investigators have measured the oxidation rates k_1 of α-tocopherol by peroxyl radicals (LOO·), using different experimental methods such as chemiluminescence, pulse radiolysis, O_2 consumption, and EPR (3-8).

$$\text{LOO}\cdot + \text{Toc} \xrightarrow{k_1} \text{LOOH} + \text{Toc}\cdot \qquad [1]$$

Tappel made the important suggestion that the tocopheroxyl radical formed from tocopherol by removal of the phenolic hydrogen can be reduced back to the starting tocopherol by vitamin C (ascorbic acid 1) (9). Subsequent in vitro experiments showed that the ascorbic acid 1 can indeed reduce the α-tocopheroxyl radical [eq 2] (4,10). Packer et al. have

$$\text{Toc}\cdot + \text{Vit C} \xrightarrow{k_2} \text{Toc} + \text{Vit C}\cdot \qquad [2]$$

measured the rate constants for reactions between α-tocopheroxyl radical and ascorbic acid 1 in aerated water-isopropanol-acetone mixtures, using pulse radiolysis method (4). From measurements at four ascorbic acid concentrations

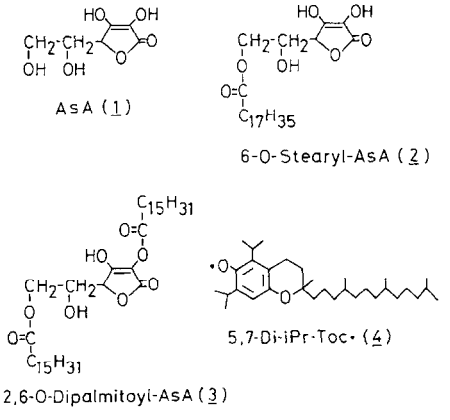
0006-291X/87 $1.50

the rate constant for reaction [2] was (1.55 ± 0.20) x 10^6 $M^{-1}s^{-1}$. Further, the rates of interaction of α-tocopheroxyl radical in liposomes with ascorbic acid 1 in the surrounding aqueous phase have been studied by Scarpa et al., using EPR technique (11). A kinetic rate constant of about 2 x 10^5 $M^{-1}s^{-1}$ was estimated from the above experiments.

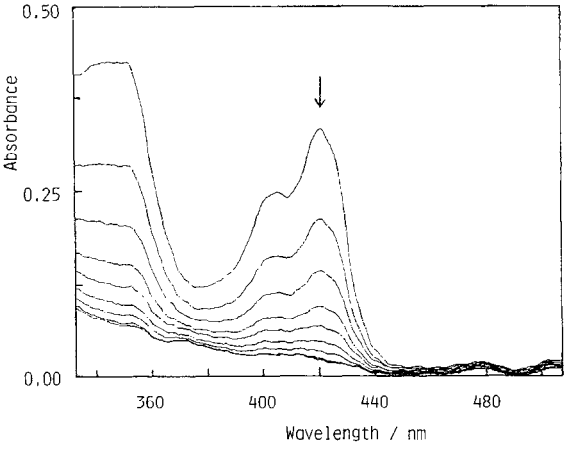
In the present work, we have used a stopped-flow technique to examine the reaction of vitamin C with tocopheroxyl radical (12). The second-order rate constant k_2 for the reaction of vitamin C (ascorbic acid 1, 6-O-stearyl ascorbic acid 2, 2,6-O-dipalmitoyl ascorbic acid 3) with tocopheroxyl radical (5,7-diisopropyl-tocopheroxyl 4) have been determined by following the decrease in absorbance at 421 nm of tocopheroxyl radical (13) (see Fig. 1). As reported in a previous paper, α-tocopheroxyl radical was not stable, and thus the stable 5,7-diisopropyl-tocopheroxyl radical 4 was used for the present work (14).

MATERIAL AND METHODS

Commercial ascorbic acid 1, 6-O-stearyl ascorbic acid 2, and 2,6-dipalmitoyl ascorbic acid 3 were used as received. The 5,7-diisopropyl-tocopheroxyl radical 4 was prepared by the PbO_2 oxidation of corresponding 5,7-diisopropyl-tocopherol in benzene-ethanol (2:1, v/v) solution under nitrogen atmosphere (14).



①



②

Fig. 1. Molecular structures of vitamin C (ascorbic acid 1, 6-O-stearyl-ascorbic acid 2, and 2,6-dipalmitoyl ascorbic acid 3) and vitamin E radical (5,7-diisopropyl-tocopheroxyl 4).

Fig. 2. Change of electronic absorption spectrum of 5,7-diisopropyl-tocopheroxyl radical 4 for the reaction of tocopheroxyl 4 with 6-O-stearyl-ascorbic acid 2 in benzene-ethanol (2:1, v/v) solution at 25.0°C. $[Toc\cdot]_{t=0}$ ca. 0.08 mM and $[Vit\ C]_{t=0}$ 5.60 mM. The spectra were recorded at every 100-msec interval. Arrow indicates decrease (↓) of absorbance with time.

The stopped-flow data were obtained on a UNISOKU stopped-flow spectrophotometer Model RS-450 by mixing equal volumes of benzene-ethanol solutions of tocopheroxyl and vitamin C derivatives. The oxidation reactions were studied under pseudo-first-order conditions, and the observed rate constants, k_{obsd}, were calculated in the usual way using a standard least-squares analysis. All measurements were performed at 25.0 ± 0.5°C.

RESULTS AND DISCUSSION

The 5,7-diisopropyl-tocopheroxyl 4 is comparatively stable in the absence of vitamin C and shows absorption peaks at λ_{max} = 421 nm and 405 nm in benzene-ethanol (2:1, v/v) solution (see Fig. 2) (14). By adding benzene-ethanol (2:1) solution of excess vitamin C to benzene-ethanol (2:1) solution of tocopheroxyl (1:1 in volume), the absorption spectrum of the tocopheroxyl immediately disappeared. Figure 2 shows the example of the result of interaction between 5,7-diisopropyl-tocopheroxyl 4 (ca. 0.08 mM) and 6-O-stearyl ascorbic acid 2 (5.60 mM) in benzene-ethanol (2:1, v/v). It was found that the ascorbic acid 1 and the ascorbic acid ester 2 at 6-positions reacted with tocopheroxyl at similar rate. On the other hand, by reacting tocopheroxyl with 2,6-O-dipalmitoyl ascorbic acid 3, the decrease of absorption at 421 nm of tocopheroxyl was very slow. The time dependence of the decrease in absorbance at 421 nm observed when ca. 0.35 mM benzene-ethanol (2:1) solution of tocopheroxyl is mixed with 21.0 mM benzene-ethanol (2:1) solution of ascorbic acid 1 (1:1 in volume, final concentration of 1 is 10.5 mM) is shown in Fig. 3. The pseudo-first-order rate constants, k_{obsd}, obtained by varying the concentration of ascorbic acid 1 are presented in Table 1. As shown in Fig. 3, the 5,7-diisopropyl-tocopheroxyl shows very

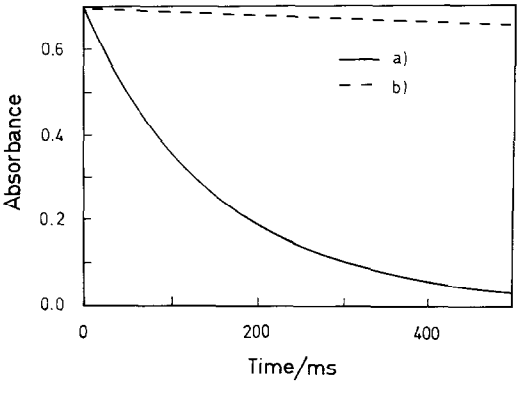


Fig. 3. a) The decay of 5,7-diisopropyl-tocopheroxyl radical 4 for the reaction of tocopheroxyl 4 with ascorbic acid 1 in benzene-ethanol (2:1, v/v) solution at 25.0°C.
$[Toc\cdot]_{t=0}$ ca. 0.17 mM and $[Vit\ C]_{t=0}$ 10.5 mM. At 421 nm.
b) The natural decay of 5,7-diisopropyl-tocopheroxyl radical 4 without ascorbic acid 1 in benzene-ethanol (2:1, v/v) solution.

Table 1. Pseudo-first-order (k_{obsd}) and second-order (k_2) rate constants for reaction of tocopheroxyl radical (4) with vitamin C (1, 2, and 3) in benzene-ethanol (2:1, v/v) solution at 25.0°C

	[Vit C]/mM	k_{obsd}/s^{-1}	$k_2/M^{-1}s^{-1}$
Ascorbic acid 1	2.65	2.06	
	4.42	3.12	
	5.29	3.44	549 ± 30
	6.30	3.82	
	8.04	5.01	
	10.49	6.83	
6-O-stearyl ascorbic acid 2	2.77	2.65	
	5.60	4.63	
	8.24	5.61	626 ± 53
	10.85	8.24	
	13.31	9.08	
2,6-O-dipalmitoyl ascorbic acid 3	2.97	2.83×10^{-2}	
	5.34	5.01×10^{-2}	
	7.71	6.45×10^{-2}	4.84 ± 1.41
	12.28	7.71×10^{-2}	

slow natural decay in benzene-ethanol (2:1) solution. Therefore, the pseudo-first-order rate constant, k_{obsd}, for tocopheroxyl bleaching is given by eq [3]

$$k_{obsd} = k_0 + k_2 [Vit\ C] \qquad [3]$$

where k_0 is the rate constant for natural decay of tocopheroxyl in the medium and k_2 is the second-order rate constants for reaction of tocopheroxyl with added vitamin C. These rate parameters are obtained by plotting k_{obsd} against [Vit C], as shown in Fig. 4. The second-order rate constants k_2 obtained for ascorbic acid 1 is 549 ± 30 $M^{-1}s^{-1}$, and k_0= 0.58 s^{-1}.

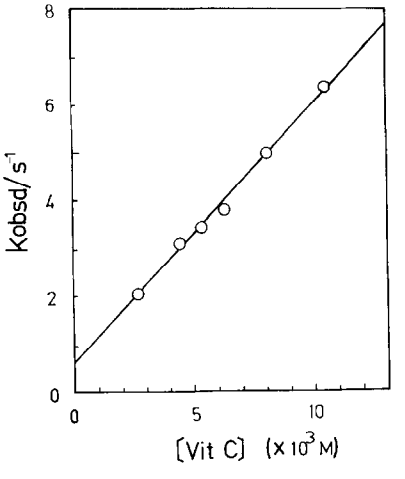


Fig. 4. The dependence of the pseudo-first-order rate constant, k_{obsd}, on the concentration of ascorbic acid 1 in benzene-ethanol (2:1, v/v) solution.

Similar measurements were performed for the reaction of tocopheroxyl with 6-O-stearyl ascorbic acid 2, and 2,6-dipalmitoyl ascorbic acid 3. The pseudo-first-order decay of tocopheroxyl radical took place, and plots of k_{obsd} vs. [Vit C] according to eq [3] yielded rate constants k_2 for the reaction of tocopheroxyl with 2 and 3. These data are summarized in Table 1. The k_2 values obtained are 626 ± 53 $M^{-1}s^{-1}$ for 2 and 4.84 ± 1.41 $M^{-1}s^{-1}$ for 3, respectively.

Recently, Takahashi et al. have reported that fatty acid esters of ascorbic acid at either 6- or both 5- and 6-positions were effective as antioxidant and suppressed the oxidation of methyl linoleate, whereas those having ester group at 2-position did not act as antioxidant (15). In other words, fatty acid esters of ascorbic acid at either 6- or both 5- and 6-positions act as chain-breaking antioxidant but that they are no more active as oxygen radical scavenger when ascorbic acid is esterified at 2-position. The present result shows that both the ascorbic acid 1 and ascorbic acid esters 2 having a long-alkyl-chain at 6-position can reduce the tocopheroxyl to tocopherol. The reaction rate for 2 is 1.14 times faster than that for 1. Further, the present result suggests that the 3-OH group in 3 can also regenerate the tocopheroxyl to tocopherol, but the rate of generation is very slow. The k_2 value for 3 (4.84 ± 1.41 $M^{-1}s^{-1}$) is two order of magnitude lower than those for 1 (549 ± 30 $M^{-1}s^{-1}$) and for 2 (626 ± 53 $M^{-1}s^{-1}$).

As described in a previous section, Packer et al. have reported absolute second-order rate constants k_2 for the reaction of vitamin C with α-tocopheroxyl radicals, using the pulse radiolysis method (4). The k_2 value was (1.55 ± 0.20) x 10^6 $M^{-1}s^{-1}$ in water-isopropanol-acetone mixtures. Further, Scarpa et al. have measured the second-order rate constants k_2 for the reaction between α-tocopheroxyl radical incorporated into dimyristoyl PC liposomes and vitamin C in surrounding aqueous phase, by EPR technique (11). A kinetic rate constant of about 2 x 10^5 $M^{-1}s^{-1}$ was obtained. This value is only one order of magnitude lower than that reported by Packer et al. for the same reaction in a homogeneous solution. On the other hand, by comparing the k_2 value (1.55 x 10^6 $M^{-1}s^{-1}$) obtained by Packer et al. with that (5.49 x 10^2 $M^{-1}s^{-1}$) of the present work, the former is about three order of magnitude higher than the latter. There are several factors that might be expected to influence the large differences in the above reaction rates, such as solvent, temperature, and the nature of the attacking tocopheroxyl radical, etc. However, it is not clear at present which factors are most important.

REFERENCES

1. Mukai, K., Tsuzuki, N., Ouchi, S., and Fukuzawa, K. (1982) Chem. Phys. Lipids 30, 337-345.

2. Craw, M.T. and Depew, M.C. (1985) Rev. Chem. Intermediates 6, 1-31.
3. Kharitonova, A.A., Kozlova, Z.G., Tsepalov, V.F., and Gladyshev, G.P. (1979) Kinet. Katal. 20, 593-599.
4. Packer, J.E., Slater, T.F., and Willson, R.L. (1979) Nature 278, 737-738.
5. Burlakova, Ye.B., Kukhtina, Ye.N., Ol'khovskaya, I.O., Sarycheva, I.K., Sinkina, Ye.B., and Khrapova, N.G. (1980) Biophysics 24, 989-993.
6. Burton, G.W., Page, Y.Le., Gabe, E.J., and Ingold, K.U. (1980) J. Am. Chem. Soc. 102, 7791-7792.
7. Burton, G.W. and Ingold, K.U. (1981) J. Am. Chem. Soc. 103, 6472-6477.
8. Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L., and Ingold, K.U. (1985) J. Am. Chem. Soc. 107, 7053-7065.
9. Tappel, A.L. (1968) Geriatrics 23, 96-105.
10. Niki, E., Tsuchiya, J., Tanimura, R., and Kamiya, Y. (1982) Chem. Letter 789-792.
11. Scarpa, M., Rigo, A., Maiorino, M., Ursini, F., and Gregolin, C. (1984) Biochim. Biophys. Acta 801, 215-219.
12. Mukai, K., Watanabe, Y., Uemoto, Y., and Ishizu, K. (1986) Bull. Chem. Soc. Jpn. 59, 3113-3116.
13. Mukai, K., Watanabe, Y., and Ishizu, K. (1986) Bull. Chem. Soc. Jpn. 59, 2899-2900.
14. Mukai, K., Takamatsu, K., and Ishizu, K. (1984) Bull. Chem. Soc. Jpn. 57, 3507-3510.
15. Takahashi, M., Niki, E., Kawakami, A., Kumasa, A., Yamamoto, Y., Kamiya, Y., and Tanaka, K. (1986) Bull. Chem. Soc. Jpn. 59, 3179-3183.