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KINETICS OF TROLOX C OXIDATION BY LACTOPEROXIDASE COMPOUND II

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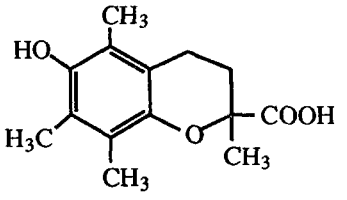
SUMMARY: The lactoperoxidase (LPO) compound II catalyzed oxidation of Trolox C, a vitamin E water-soluble derivative, was studied by rapid scan spectral analysis and stopped-flow kinetic measurements. Our rapid scan spectral analysis clearly indicates that LPO compound II is reduced to native enzyme by Trolox C; hence the reaction is a one-electron redox process. The reaction was investigated at pH's ranging from 3.0 to 7.0. Trolox C is more reactive with LPO-II in acidic solutions. Kinetic and spectroscopic studies demonstrate that LPO has a binding site in the vicinity of the heme for Trolox C. Trolox C exhibits a quantitative 1:1 binding to native LPO in acidic solutions. The binding ability of Trolox C to native LPO decreases with increasing pH. The same trend is observed when the second order rate constants k_{app} for the reaction are plotted against pH. A mechanism of Trolox C oxidation by LPO-II has been proposed in which protonation of an amino acid residue on LPO-II with a pK_e of 2.3 is essential and ionization of the carboxylic acid group on Trolox C accelerates the reaction rate. The second-order rate constants were determined to be $(4.1 \pm 0.5) \times 10^6\ M^{-1}s^{-1}$ for protonated Trolox C oxidation and $(1.9 \pm 0.3) \times 10^7\ M^{-1}s^{-1}$ for deprotonated Trolox C.

The biological activity of vitamin E (α-tocopherol) is generally believed to be due to its antioxidant action to inhibit lipid peroxidation in biological membranes by donating a hydrogen atom to the chain-propagating peroxyl radicals (ROO·) and giving rise to chromanoxyl radicals of the antioxidant (1):

$$ROO\cdot + Toc\text{-}OH \longrightarrow ROOH + Toc\text{-}O\cdot, \qquad (1)$$

where Toc-OH is tocopherol and Toc-O· is the chromanoxyl radical. Trolox C:



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an antioxidant originally designed for food preservation (2), has a chromane structure similar to α-tocopherol but without the inert hydrophobic tail; it is both water and lipid soluble. The oxidation of Trolox C by lactoperoxidase has potential physiological relevance. It could help maintain the catalytic cycle and activity of animal peroxidases, leading to detoxification of hydrogen peroxide formed as a product of inflammation processes. A water-soluble derivative of vitamin E may have more therapeutic value than vitamin E itself (3).

MATERIALS AND METHODS

Lactoperoxidase (LPO) (EC 1.11.1.7, donor-H_2O_2 oxidoreductase) was purchased from Sigma. The R.Z. (A_{412}/A_{280}) of the samples was 0.70-0.85. The enzyme concentration was determined by using a molar absorptivity of 1.12 x 10^5 $M^{-1}cm^{-1}$ at 412 nm (4). LPO-II (λ_{max} 430 nm) stable for 8 min was prepared after mixing 2.0 μM LPO with 4.0 μM H_2O_2 in pH 7.3 and ionic strength 0.11 M phosphate buffer.

Hydrogen peroxide was obtained as a 30% solution from Fisher. The concentration of diluted H_2O_2 stock solution was determined by the peroxidase assay (5). Trolox C was obtained from Aldrich. The solution of Trolox C was freshly prepared just before each experiment. Trolox C was found to be stable in buffers in the pH range from 3.0 to 7.0, monitored at its maximum absorbance at 288 nm. All buffer solutions had an ionic strength 0.11 M, adjusted by adding K_2SO_4 as an inert salt. All solutions were prepared using the deionized water from a Milli-Q water purification system.

Kinetic measurements were performed on a Photal (formerly Union Giken) stopped-flow spectrophotometer Model RA-601. The 1-cm observation cell was thermostated at 25°C for all measurements. A pH-jump method was used for all experiments because of the instability of LPO-II in acidic solutions. For stopped-flow experiments, one drive syringe was filled with LPO-II in 0.01 M phosphate buffer at pH 7.3. Inert salt (K_2SO_4) was used to maintain the ionic strength at 0.11 M. The other syringe was filled with Trolox C in 0.05 M citrate buffers at pH values ranging from 2.8 to 7.0. The ionic strength was also kept at 0.11 M by adding the inert salt. The pH after mixing the solutions in the two syringes were in the range from 3.0 to 7.0. At least a 10-fold excess of Trolox C was used to maintain pseudo-first-order conditions. The reaction was monitored by the disappearance of LPO-II at its maximum absorbance of 430 nm. Between six to eight traces were collected for each substrate concentration. The standard deviation was below 5%. Measurements of relative absorbance at regular time intervals from the recorded exponential traces followed by a nonlinear least-square analysis gave the observed pseudo-first-order rate constants.

The pK_a value of the carboxylic acid group of Trolox C was determined by titration with concentrated NaOH using a Fisher Scientific Accumet pH Meter. The ionic strength of the solution was kept at 0.11 M by adding inert salt (K_2SO_4) to the dilute Trolox C solution. The effect of the small volume of NaOH could be ignored.

Trolox C binding to native LPO experiments were performed on Cary 210 spectrophotometer. Both the reference and sample cuvettes contained LPO (6.47 μM). Trolox C was added only to the sample cuvette, and difference spectra in the range of 370-490 nm were determined in several low pH citrate buffer solutions, ionic strength 0.11 M. The dissociation constants (K_D) were calculated from the plot of 1/ΔA *versus* 1/[Trolox C] where ΔA is the difference in absorbance at maximum absorption of the native enzyme. The number of binding sites near the heme was calculated from ΔA using a Hill plot (6).

RESULTS

Determination of pK_a of Trolox C. The pK_a value of the carboxylic acid group of Trolox C was determined to be 4.03 in the solution of the ionic strength 0.11 M.

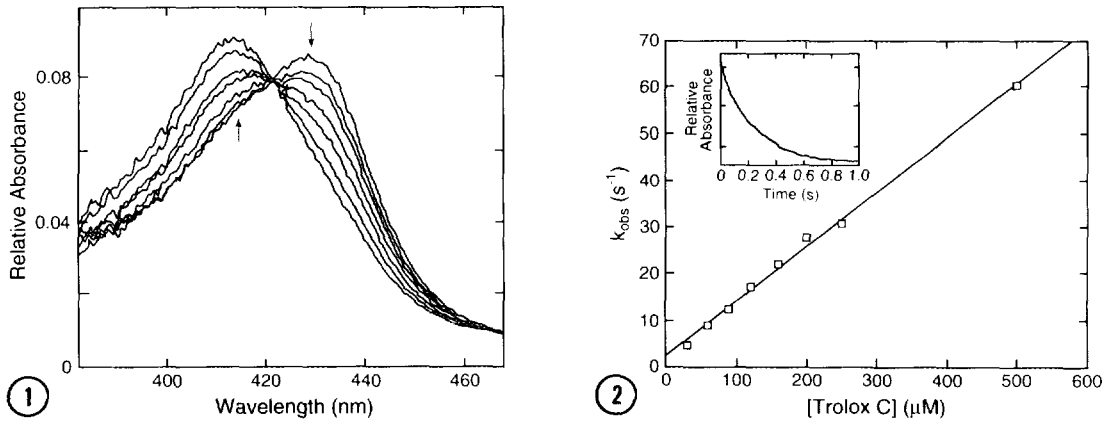

Figure 1. Rapid scan spectrophotometric measurements of the reaction of LPO-II with Trolox C over 166 ms. One drive syringe contained LPO-II by mixing 1.0 μM LPO with 2.0 μM H_2O_2 in phosphate buffer, pH 7.30, total ionic strength 0.11 M; the other contained 30 μM Trolox C in citrate buffer, pH 4.14, ionic strength 0.11 M. Final pH 4.33. The partial conversion of LPO-II to LPO is shown clearly. The arrows indicate the direction of absorbance change with increasing time.

Figure 2. Plot of k_{obs} versus Trolox C concentration for the oxidation of Trolox C by lactoperoxidase compound II. The reactions were carried out in buffer solutions of final pH 4.33, ionic strength 0.11 M. Experimental conditions as in Fig. 1. The inset shows an example of a first order exponential trace (30 μM Trolox C) from which k_{obs} was obtained.

Oxidation of Trolox C by LPO-II. The spectral changes observed during the reaction indicate that LPO-II is reduced directly to native state as shown in Fig. 1. The isosbestic point appearing at 421 nm is proof of an LPO-II to native LPO conversion with no detectable intermediates:

$$\text{LPO-II} + \text{Trolox C} \longrightarrow \text{LPO} + \text{Trolox C}\cdot \qquad (2)$$

Kinetic Measurements. With Trolox C in excess of LPO-II, pseudo-first-order kinetics was observed. One example is shown in the inset of Fig. 2. The corresponding differential rate expression is

$$-d[\text{LPO-II}]/dt = k_{obs}\,[\text{LPO-II}], \qquad (3)$$

where k_{obs} is the observed pseudo-first-order rate constant. The apparent second-order rate constant, k_{app}, is related to k_{obs} by

$$k_{obs} = k_{app}\,[\text{Trolox C}]. \qquad (4)$$

Values of k_{app} were then obtained from the slopes of plots of k_{obs} versus [Trolox C], as shown by an example in Fig. 2. The small intercept is zero within the experimental error. A plot of k_{app} *versus* pH is shown in Fig. 3 for the oxidation of Trolox C by LPO-II.

Trolox C Binding to Native LPO. Native LPO binds Trolox C in acidic solutions which

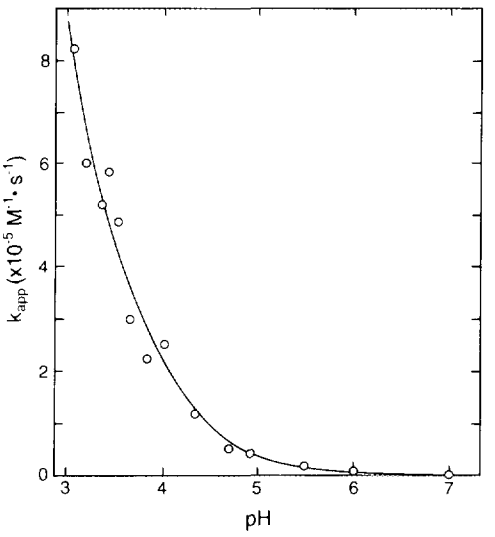

Figure 3. The rate-pH profile for the reaction of LPO-II with Trolox C at 25°C and ionic strength 0.11 M. The experimental points and the computer fitted curve are shown.

results in a decrease in absorbance at the Soret maximum. Results of Hill plots (data not shown) indicate 1:1 binding within 10% error, and the binding increases with decreasing pH. The dissociation constants (mM) are 0.56 ± 0.04, 4.0 ± 0.3 and 12.1 ± 0.5 at pH's 2.93, 3.18 and 3.38. At pH higher than 3.38, the binding is weak and the difference spectra could not provide accurate data.

DISCUSSION

Our rapid scan spectral analysis of the changes in the enzyme oxidation states clearly indicates that the oxidation of Trolox C catalyzed by LPO-II is a one-electron redox process. The oxidation of Trolox C is similar to that of vitamin E and the reaction results in phenoxyl radical formation (7-10). The spectral changes observed upon binding of Trolox C to native LPO suggest that the binding site of Trolox C is near the heme. The extent of binding of Trolox C to native LPO as a function of pH indicates that an amino acid residue with a small pK_a value in the vicinity of the heme is part of the binding site for Trolox C. It has been reported from this group that LPO-II has an amino acid residue of pK_a 2.3 near the heme (7). The simplest mechanism which will account for the experimental data is one in which the protonated LPO-II with a pK_e of 2.3 reacting with both the protonated and deprotonated Trolox C as follows:

$$\begin{array}{ccc} \text{LPO-II} & & \\ \uparrow\downarrow\ K_e & & \\ \text{HLPO-II}^+ + \left\{ \begin{array}{l} \text{H}^+ \uparrow\downarrow\ K_a \quad \text{HS}^+ \xrightarrow{k_1} \text{product} \\ \text{S} \xrightarrow{k_2} \text{product} \end{array} \right. & & \end{array} \qquad (5)$$

The k_{app}-$[H^+]$ relationship corresponding to eq. 5 is:

$$k_{app} = \frac{k_1}{(1+K_a/[H^+])(1+K_e/[H^+])} + \frac{k_2}{(1+[H^+]/K_a)(1+K_e/[H^+])} . \quad (6)$$

Using nonlinear least-square analysis, the experimental k_{app}-pH data points shown in Fig. 3 were fitted to eq. 6 by using fixed values of $pK_a = 4.03$ and $pK_e = 2.3$ (11). The best-fit kinetic parameters are $k_1 = (4.1 \pm 0.5) \times 10^6\ M^{-1}s^{-1}$ and $k_2 = (1.9 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$. The curve computed on the basis of the best-fit parameters is also shown in Fig. 3.

In conclusion the oxidation of Trolox C by LPO-II takes place first by binding to a site near the heme. The tighter binding of Trolox C at lower pH facilitates the reaction. The binding process is followed by hydrogen atom transfer from the phenolic hydroxyl group of Trolox C to LPO-II. A simple although speculative explanation of our results is that the carboxylate group of Trolox C binds to the acidic form of the LPO-II group with pK_e of 2.3.

Lipid peroxidation of iron-loaded liposomes has been found to increase with decreasing pH (12). The myeloperoxidase-H_2O_2-Cl^- system, a key part of the phagocytic process, displays maximum activity at about pH 5 (13, 14). Furthermore a decrease to pH 4 occurs in the vacuole upon phagocytosis (15). Our k_{app}-pH profile shows that Trolox C is more reactive at lower pH, which correlates with the trend in lipid peroxidation. Trolox C is reported to be the most efficient antioxidant in a series of analogues with a similar chromane structure to α-tocopherol (2).

This work can be compared to the oxidation of *p*-cresol by LPO-II, in which the second-order rate constants were studied over the pH range 2.1-11.2 by the same technique (11). The second-order rate constant for *p*-cresol oxidation by LPO-II is greater than the rate constant for Trolox C oxidation over the pH range 3.0-7.0. The difference in the pH-rate profiles for these two substrates may be a result of different binding sites. Contrary to the Trolox C results, protonation of an amino acid residue with pK_e 2.3 results in a decreasing rate of reaction of LPO-II with *p*-cresol. The bigger chromane ring of Trolox C compared to the single benzene ring of *p*-cresol may result in some steric hindrance for Trolox C to attain the same position relative to the heme active site as *p*-cresol.

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