Note

Kinetic Study of Lipoxygenase-13-L-hydroperoxylinoic Acid Interaction[†]

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In lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) reaction, the product hydroperoxide acts as an activator of the enzyme.^{1,2)} We studied the interaction between lipoxygenase and the 13-L-hydroperoxylinoic acid, using a fluorescence stopped-flow method and showed that the interaction is considered to be a two-step mechanism involving a fast bimolecular association followed by a slow irreversible unimolecular process.^{3,4)} Recently, De Groot *et al.*⁵⁾ have reported that lipoxygenase has three spectroscopically different forms; a) the native enzyme with ferrous state iron, b) the yellow species with ferric state iron, which is formed upon the addition of a stoichiometric amount of hydroperoxylinoic acid and c) the purple species with ferric state iron, which arises when an excess (3 to 5 molar ratio) of the product is added to the native enzyme or to the yellow species. They suggested that the purple species is an active one during the aerobic reaction of lipoxygenase. In this paper, we studied the interaction between lipoxygenase and 13-L-hydroperoxylinoic acid from the absorbance increase at 330 nm and at 570 nm observed by stopped-flow method.

Lipoxygenase and 13-L-hydroperoxylinoic acid were prepared as described in the previous paper.⁴⁾ A stopped-flow apparatus (Union Giken SF 70) was used for the kinetic study of the interaction from the absorbance change at 330 nm and at 570 nm. All reagents used for the stopped-flow measurement were dissolved in 0.15 M NH_4Cl - NH_4OH buffer or 0.15 M borate buffer, containing 1.25% ethanol.

The time courses of the changes in the absorbance at 330 nm and at 570 nm upon mixing lipoxygenase

and 13-L-hydroperoxylinoic acid observed by the stopped-flow method followed the pseudo-first order kinetics giving a single relaxation time. Figure 1 shows the concentration dependence of the relaxation time τ (the reciprocal of the pseudo-first order rate constant) obtained from the traces of the absorbance changes at 330 nm and at 570 nm, and from the traces of the fluorescence quenching of lipoxygenase. The reciprocal relaxation time ($1/\tau$) is plotted against $[\text{P}]_0 - [\text{E}]_0$ which is well approximated to $(\bar{c}_E + \bar{c}_P)$, where $[\text{P}]_0$, $[\text{E}]_0$, $\bar{c}_E$ and $\bar{c}_P$ designate the initial concentrations of the enzyme and the product, the equilibrium concentrations of the enzyme and the product, respectively. No appreciable systematic difference was observed among the relaxation times obtained from the absorbance changes at two wavelengths and the fluorescence change. The reciprocal relaxation time increases with the increasing concentration of $[\text{P}]_0 - [\text{E}]_0$, but levels off at high concentrations. These results are consistent with a two step mechanism involving a fast bimolecular association process followed by a slow unimolecular process.³⁾

Figure 2 shows the pH dependence of reciprocal relaxation time ($1/\tau$) at the high concentration range, where $1/\tau$ reaches the limiting value of k_2 (the rate

FIG. 1. The Concentration Dependence of Reciprocal Relaxation Time ($1/\tau$).

The $1/\tau$ was obtained from the absorbance changes at 330 nm (○) and at 570 nm (□), and from the fluorescence quenching (△) at pH 9.0 and 15°C in 0.15 M NH_4Cl - NH_4OH buffer, using a stopped-flow method. The reciprocal relaxation time ($1/\tau$) for the yellow- and purple-enzyme species at the low concentrations was obtained by averaging the traces for several times with a digital memory equipment. The sum of the equilibrium concentration $(\bar{c}_E + \bar{c}_P)$ is well approximated to $[\text{P}]_0 - [\text{E}]_0$. The solid line represents the theoretical curve drawn from the equation, $1/\tau = k_2(\bar{c}_P + \bar{c}_E)/(k_{-1}/k_1 + (\bar{c}_P + \bar{c}_E))$, with the values $k_{-1}/k_1 = 3 \times 10^{-5}$ M and $k_2 = 85 \text{ sec}^{-1}$. $[\text{E}]_0 = 3.2 \times 10^{-6}$ M or 4.2×10^{-6} M for measurement of fluorescence quenching. $[\text{E}]_0 = 4.5 \times 10^{-6}$ M or 5×10^{-6} M for measurement of absorbance changes at 330 nm and 570 nm.

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FIG. 2. The pH Dependence of the Reciprocal Relaxation Time ($1/\tau$) Obtained from the Absorbance Change at 330 nm and 570 nm at 15°C.

A solid line is the theoretical titration curve with pK 8.6. The reciprocal relaxation time ($1/\tau$) will correspond to the rate constant k_2 in this system. $[E]_0 = 5.5 \times 10^{-5}$ M and $[P]_0 = 1.26 \times 10^{-3}$ M in 0.15 M borate buffer containing 1.25% ethanol. ○, 330 nm; □, 570 nm.

constant for the unimolecular process). This plot is consistent with a titration curve with a pK of about 8.6, which agreed with the pH dependence of the rate constant k_2 obtained from the fluorescence quenching.⁴⁾

It may be reasonable, therefore, to consider that those changes in the absorbances and the fluorescence may result from an identical step or otherwise arise from different steps operating at an equal rate. Egmond *et al.*⁶⁾ have suggested that the fluorescence changes are directly coupled to the absorbance changes at 330 nm, which are related to change of the iron in lipoyxygenase from the ferrous state to the ferric one.⁵⁾ So it is reasonable to think that both the absorbance change at 330 nm and fluorescence quenching arise from an identical step (the oxidation of iron in lipoyxygenase) and their relaxation times therefore become essentially identical. On the other hand, De Groot *et al.*⁶⁾ obtained the purple-coloured enzyme species (absorption maximum at 570 nm) by the addition of a 3-fold excess of the product hydroperoxide to the yellow-coloured enzyme, which had been obtained after treatment of the native enzyme with an equimolar amount of the product. They also reported that the purple species gradually loses its purple colour and becomes yellow again on standing (10 min at room temperature or 1 hr at 0°C).⁵⁾ It is reasonable to think that the absorbance of lipoyxygenase at 570 nm increases independently from the change in the formal change of the iron in the protein. From these results, following scheme can be proposed;

where ROOH, $E(Fe^{2+})$, $E(Fe^{3+})$ and $E(Fe^{3+})ROOH$ are the product, the native enzyme which is highly fluorescent, the yellow enzyme species which is less fluorescent, and the purple enzyme species which is an active enzyme and less fluorescent, respectively. The trace of the absorbance change at 570 nm could not be observed by the stopped-flow method when the yellow enzyme species were mixed with the product, possibly because k_3 is very large. Since the unimolecular process is the rate-determining step, *i.e.* $k_2 \ll k_1$, k_{-1} , k_3 and k_{-3} , the relaxation time derived from the absorbance change at 570 nm (production of purple enzyme species) must be almost equal to that at 330 nm (production of yellow enzyme species). The rate constants at pH 9.0 and 15°C in 0.15 M NH_4Cl-NH_4OH buffer containing 1.25% ethanol were estimated from the result of Fig. 1 (k_2 and k_{-1}/k_1) and from the dependency of absorbance change at 570 nm as observed

FIG. 3. Titration of Lipoyxygenase with 13-L-Hydroperoxylinoleic Acid Observed from the Absorbance Change at 570 nm with the Stopped-flow Method.

The amount of the absorbance change at 570 nm (ΔA) in 0.15 M NH_4Cl-NH_4OH buffer at pH 9.0 and 15°C was evaluated from the trace of stopped-flow upon mixing lipoyxygenase (9×10^{-8} M) and 13-L-hydroperoxylinoleic acid at various concentrations, considering the dead time of the apparatus (1.5 msec). The concentration of 13-L-hydroperoxylinoleic acid is approximated to $[P]_0 - [E]_0$, as one hydroperoxide molecule was possibly consumed to convert one native lipoyxygenase molecule to the yellow species. The dissociation constant of the purple species (k_{-3}/k_3) in 0.15 M NH_4Cl-NH_4OH buffer at pH 9.0 and 15°C was estimated to be 1.3×10^{-4} M from this plot.

by the stopped-flow method on the concentration of the product, *i.e.* titration of lipoxygenase with 13-L-hydroperoxylinoleic acid, the result of which is shown in Fig. 3 (k_{-3}/k_3);

$$k_{-1}/k_1 = 3 \times 10^{-5} \text{ M}, \quad k_2 = 85 \text{ sec}^{-1} \quad \text{and} \quad k_{-3}/k_3 = 1.3 \times 10^{-4} \text{ M}$$

When we were in preparation of the manuscript of this study, a similar kinetic study was reported in detail by Egmond *et al.*,⁷⁾ which did not contain the essential discrepancy with our result.

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