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HYDROGEN PEROXIDE : A POTENT ACTIVATOR OF DIOXYGENASE ACTIVITY OF SOYBEAN LIPOXYGENASE

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Hydrogen peroxide, an ubiquitous biologically occurring peroxide, was found to stimulate the dioxygenase activity of soybean lipoxygenase at the physiologically attainable concentration. The increase in enzyme specific activity was directly proportional to hydrogen peroxide concentration up to 0.5 nM. A decrease in the stimulation of dioxygenase activity was observed at higher concentrations. At low enzyme concentration up to 28-fold stimulation was noted when the formation of lipid hydroperoxide was monitored spectrophotometrically. The stimulation was further confirmed by increased oxygen uptake. It is proposed that the mechanism for in vivo activation involves hydrogen peroxide.

Lipoxygenase (lipoxidase, EC 1.13.11.12) is a non-heme iron containing enzyme. Despite being a single protein the enzyme has been reported to have dioxygenase (1), leukotriene synthase (2), and hydroperoxidase (3) activities. In view of the increasing realization of the biomedical importance of end products, the enzymatic process of fatty acid oxygenation is receiving greater attention in recent years. The generation of plethora of intermediates and end products such as hydroperoxy or hydroxy fatty acids, lipoxins, leukotrienes, leukotoxins and other products involving lipoxygenase are implicated in a variety of pathophysiological conditions such as inflammation and hypersensitivity (4,5). Several studies have shown that a kinetic lag phase exists in the dioxygenation reaction (6-8) that can be avoided by the initial addition of hydroperoxide to the

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reaction mixture or by increasing the enzyme concentration (6,9). Funk et al. (9) reported that activation of dioxygenase is a saturable process. It is generally assumed that after the lag phase, the protein exhibits full expression of enzyme activity and the observed dioxygenation rate is taken as maximal velocity.

Hydrogen peroxide, an ubiquitous metabolic product, is constantly formed during various normal biochemical reactions in living cells. Earlier reports indicated that hydrogen peroxide is ineffective as a stimulator of dioxygenase activity of lipoxygeanse (6,10). Recently it was demonstrated that hydrogen peroxide can replace lipid hydroperoxide in the co-oxidation of xenobiotics via the hydroperoxidase activity of soybean lipoxygenase (3). Since, the dioxygenase and peroxidase activities were shown to be associated with the same protein, it was thought important to reinvestigate whether hydrogen peroxide can also activate the dioxygenase activity of the enzyme.

MATERIALS AND METHODS

Soybean lipoxygenase-Type V (737,000 Sigma Units/mg protein; M.W.=108 kdaltons), linoleic acid (99% pure) and 30% hydrogen peroxide were purchased from Sigma Chemical Co., St. Louis, MO.

Dioxygenase activity of lipoxygenase was measured either by oxygen uptake or spectrophotometrically. For spectrophotometric assay, reaction medium (3.0 ml final volume) in the sample cuvette contained indicated concentration of enzyme (0.05-0.5 nM), freshly prepared linoleic acid (0 - 100 uM), hydrogen peroxide (0-3.0 nM) and Tris buffer, pH 9.0. The reference cuvette contained linoleic acid $\pm$ hydrogen peroxide in 50 mM Tris buffer, pH 9.0. All spectral measurements were done using a Gilford Response UV-VIS spectrophotometer at room temperature in open quartz cuvettes. Increase in absorbance at 234 nm was monitored and an extinction coefficient of 25 mM^{-1} cm^{-1} was used for quantitation of hydroperoxylinoleic acid. Oxygen uptake was monitored by a Clark electrode on a YSI Model 5300 biological oxygen monitor under identical assay conditions except that final volume of the reaction mixture was 2.0 ml. Measurements were begun immediately after the addition of enzyme.

Linoleic acid was prepared according to the method of Tappel et al. (11) with some modification. Stated briefly, to a mixture of 50 ul of linoleic acid and 50 ul of absolute ethanol, 10 ml of 50 mM Tris buffer pH 9.0 (deaerated with nitrogen for 10 minutes) and 1 drop of Tween 80 were added and the mixture was

vortexed for 5 seconds. The preparation was immediately poured into a cold beaker and maintained on ice. This resulted in a substrate solution without spectrophotometrically detectable linoleic acid hydroperoxide.

RESULTS

Two independent assay methods were employed to assess the effects of hydrogen peroxide on the dioxygenase activity of soybean lipoxygenase. From the spectral data given in Fig. 1a, it is evident that a time-dependent increase in absorbance at 234 nm was observed. Concomitantly, the linoleic acid peak at 212 nm decreased. In the presence of hydrogen peroxide the product formation was significantly higher than that observed with enzyme and substrate alone. From the data given in Fig. 1b it is clearly evident that the addition of hydrogen peroxide caused a marked increase (14-fold) in oxygen uptake. Taken together, these results suggested that the dioxygenation reaction was being measured and the increase in absorbance at 234 nm (Fig.1a) was not due to artifacts, such as a change in the turbidity of the reaction mixture etc. However, chemical identity of the products formed during the reaction remains to be established.

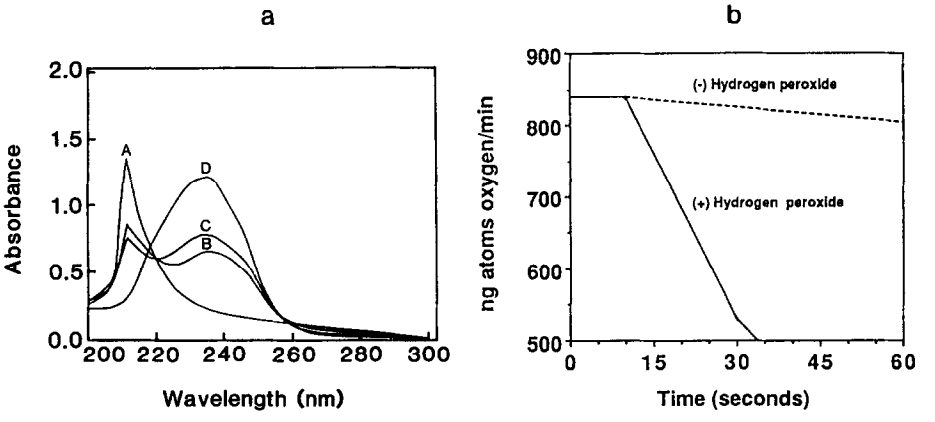

Figure 1. Linoleic acid (70 uM) metabolism by soybean lipoxygenase (0.15 nM) in the presence or absence of hydrogen peroxide (0.5 nM). (a) Spectral analysis: [A] linoleic acid; [B] linoleic acid + enzyme + hydrogen peroxide after 1 min.; [C] linoleic acid + enzyme after 3 min.; [D]: linoleic acid + enzyme + hydrogen peroxide after 3 min. (b) Oxygen uptake (ng atoms/min) in the presence or absence of hydrogen peroxide.

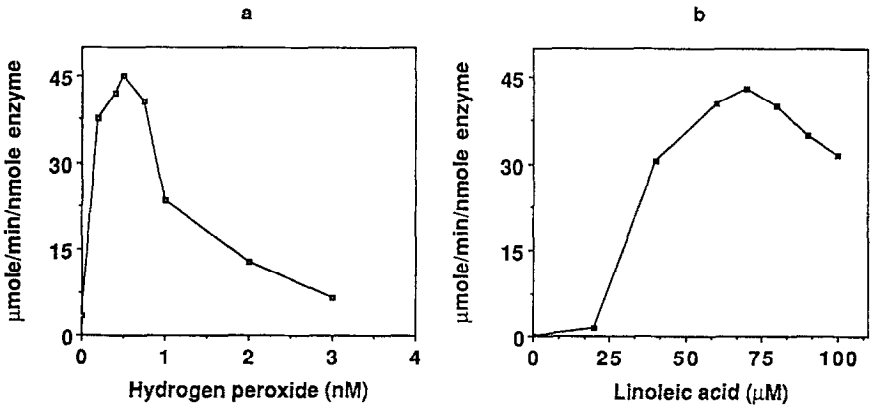

Figure 2. Dioxygenase activity of soybean lipoxygenase (0.15 nM) in the presence of (a) 70 uM of linoleic acid and indicated concentration of hydrogen peroxide; (b) 0.5 nM hydrogen peroxide and indicated concentration of linoleic acid. The spectrophotometric assays were carried out as described in Materials and Methods.

The results of the experiments designed to evaluate the effect of increasing concentrations of hydrogen peroxide are presented in Fig. 2a. The hydrogen peroxide concentration that caused maximal stimulation of about 20-fold in dioxygenase activity was 0.5 nM. Higher concentrations (up to 3 nM) resulted in a lower extent of enzyme stimulation. Further increase of hydrogen peroxide concentration resulted in enzyme inhibition (data not shown). Fig.2b represents the effects of 0.5 nM hydrogen peroxide on the rates of dioxygenase reaction in the presence of different linoleic acid concentration. The data indicated an increase in the extent of dioxygenase activity stimulation when linoleic acid concentration was increased up to 70 uM. Further increase in the substrate concentration (70-100 uM) resulted in a gradual decline of the stimulation.

The data presented in Table 1 indicate that the magnitude of stimulation was inversely proportional to the enzyme concentration (28 fold stimulation with 0.05 nM enzyme as compared to an approximately 7 fold stimulation with 0.5 nM protein).

Table 1. Stimulation of soybean lipoxygenase by hydrogen peroxide

Enzyme (nM)	H_2O_2 (0.5 nM)	Dioxygenase Activity*	Fold Stimulation
0.05	-	2.32	
0.05	+	64.16	28
0.10	-	2.76	
0.10	+	58.64	21
0.15	-	2.24	
0.15	+	44.64	20
0.20	-	2.12	
0.20	+	39.20	19
0.30	-	2.00	
0.30	+	27.17	14
0.50	-	2.49	
0.50	+	16.58	7

*Specific activity is expressed in μmoles of linoleic acid hydroperoxide formed/min/nmole enzyme. The spectrophotometric assays were carried out in the presence of 70 μM linoleic acid as described in Materials and Methods.

DISCUSSION

Although many attempts have been made in the past to understand the phenomenon of hydroperoxide-caused activation of dioxygenase activity of lipoxygenase, the question of specificity of peroxide has remained far from being resolved. Earlier de Groot *et al*. (12) observed that 13-linoleic acid hydroperoxide was effective in activating the enzyme whereas 9-linoleic acid hydroperoxide was not. According to Haining and Axelrod (6) only hydroperoxides of compounds which are substrates for dioxygenase serve as activators whereas, hydrogen peroxide, cumene hydroperoxide and t-butyl hydroperoxide were ineffective as activators and could not reduce the lag period. They also reported that Tween 20, 40, 60, and 80 reduced the lag period but only, at much higher concentration and by a mechanism different than that of linoleic acid hydroperoxide. In addition, calcium, ATP and other endogenous factors have also been shown to stimulate dioxygenase activity (13,14).

This is, to our knowledge the first report on the stimulation of dioxygenase activity of lipoxygenase by physiologically attainable concentration of hydrogen peroxide. In spite of the presence of active catalase and peroxidases, some tissues acccumulate this endogenously generated peroxide. For example, steady state concentration of hydrogen peroxide ranging between 1.0 nM-100 nM have been reported for liver (15).

Our results are in agreement with the earlier reports on inactivation of the enzyme by high concentrations of hydrogen peroxide (6,10,16). However, the evidence gathered in this study indicates that hydrogen peroxide at subnanomolar concentrations actually can serve as an activator. Whether hydrogen peroxide alone activates the enzyme or linoleic acid hydroperoxide is also required, cannot be answered at present since it is difficult to prepare linoleic acid free of its hydroperoxide. The mechanism of activation of dioxygenase activity by hydrogen peroxide seems to be different from that of linoleic acid hydroperoxide in view of the following: (i) if the activation is due to linoleic acid hydroperoxide alone then the reaction velocity should increase with time. But this does not occur; (ii) furthermore, the initial rates with extensively autooxidized linoleic acid alone were found to be lower than those in the presence of hydrogen peroxide (data not shown). Linoleic acid hydroperoxide-catalyzed activation was reported to be a saturable process that requires high linoleic acid hydroperoxide concentration (0.15 nM of enzyme in presence of 10 uM linoleic acid hydroperoxide, i.e. 66,666 fold in excess of the enzyme) (9). In contrast to this, on a molar basis, our results (molar ratio of enzyme:hydrogen peroxide is 1.33:1; Fig. 2a) indicate that hydrogen peroxide is approximately 50,000 times more effective than linoleic acid hydroperoxide in stimulating dioxygenase activity of soybean lipoxygenase. Apparently, the observed phenomenon of hydrogen

peroxide-caused activation of dioxygenase activity is not uniquely restricted to the soybean enzyme alone but extends to the mammalian enzymes as well, since, similar synergistic response was observed with rat liver and lung lipoxygenase (details to be published elsewhere). In view of these findings we would like to propose hydrogen peroxide as the trigger for the in vivo activation of lipoxygenases.

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