Oxygenation of Unsaturated Fatty Acids by Soybean Lipoxygenase*

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SUMMARY

Lipoxygenase catalyzes its own destruction during oxygenation of fatty acid substrates. The destruction is first order with respect to enzyme. The rate constant for this inactivation ($k_I$) has a characteristic value for each fatty acid substrate. The values are greater for acids having correspondingly higher degrees of unsaturation. In the presence of product, the enzyme loses activity 10 times faster with both substrates, fatty acid and oxygen, present than with either alone.

We have confirmed that oxygenation of fatty acids by lipoxygenase occurs with a kinetic lag period which can be abolished by adding product hydroperoxides. Product hydroperoxides play an essential role in the over-all autocatalytic reaction. We have further shown that the lag period may be extended as a result of inhibition of the enzyme by polyenoic acid substrates. The previously reported inhibition of lipoxygenase by GSH peroxidase in the presence of glutathione appears to be due to the removal of the hydroperoxide product.

These findings as well as those of others may be accommodated by a kinetic model in which distinct roles exist for both the product and the fatty acid substrate in a manner allowing product binding only at the product site whereas fatty acid substrate may bind at either site.

A number of conflicting reports have appeared on the kinetic lag period with lipoxygenase (5, 7-9); however, most can be reconciled with the earlier report of product activation which indicated that the lag time could be virtually eliminated with high enzyme concentrations (7). In a preliminary report, we showed that lipoxygenase is reversibly inhibited by GSH peroxidase in the presence of GSH (10). This paper presents further evidence that lipoxygenase requires hydroperoxide for activity.

A kinetic model is presented which accounts for our observations and those of others.

METHODS

Materials—All fatty acids were high purity grade obtained from the Hormel Institute and Nuchek Preps. The fatty acids were dissolved in benzene containing 0.3% (w/w) Santoquin (a gift from the Monsanto Company) and stored at $-20^\circ$ Dr. Rodney Boyer generously donated a sample of 1-octyl hydroperoxide. The soybean lipoxygenase used in most experiments was purchased from Sigma Chemical Company and used without further purification; lipoxygenase obtained from Worthington Biochemical Corporation was used, after partial purification, for amino acid analyses. DEAE-Sephadex (A-50) and reduced glutathione were obtained from Sigma Chemical Company. All other chemicals, reagent grade, were obtained from common commercial sources. Deionized water was used for the preparation of all solutions.

Determination of Lipoxygenase Activity—The oxygenation reactions were initiated by the addition of aliquots of solutions of soybean lipoxygenase (0.2 to 1.0 mg per ml) prepared daily in 0.1 M Tris-HCl (pH 9.0) to 1.0-ml suspensions of fatty acid (30 to 260 µM) in 0.1 M Tris-HCl (pH 9.0). Assays of lipoxygenase were performed at room temperature in cuvettes exposed to air by continuously monitoring the absorbance at 234 nm due to the formation of the hydroperoxy-cis-trans conjugated dienoic acid product on a Beckman DU spectrophotometer equipped with a Gilford model 2000 Multiple Sample Absorbance Recorder. A molar extinction coefficient of 28,000 liters per mole-cm (5) was used to convert absorbance readings to moles of product. The commercial enzyme preparation (Sigma) catalyzed the oxygenation of 1.52 µmoles of acid per min per mg of protein in an assay mixture containing 5 to 15 µg of lipoxygenase and 100 µmoles of 9,12-octadecadienoic acid in 1.0 ml of 0.1 M Tris-HCl (pH 9.0).

One unit of lipoxygenase is defined as that amount of enzyme which will catalyze the oxygenation of 1 µ mole of 5,8,11,14-eicosatetraenoic acid per min at room temperature.

Determination of GSH Peroxidase Activity—GSH peroxidase

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† Recipient of a United States Public Health Service Health Science Predoctoral Traineeship.
activity was measured colorimetrically by a modification of the procedure of Jocelyn (11). Aliquots (0.3 ml) of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) were added after different times of incubation of samples containing 590 nmoles of GSH, 50 to 80 μmoles of 15 hydroperoxide 5,8,11,13-cis,cis-octadecatetraenoic acid (2), and 5- to 20-μl aliquots of the GSH peroxidase preparation in 4.0 ml of 0.1 M HCl immediately before addition of 5,5'-dithiobis(2-nitrobenzoic acid). Controls containing no enzyme were run with all samples. The absorbance at 413 nm of the assay solutions was determined on either a Beckman model B or model DU spectrophotometer. The readings were converted to nanomoles of hydroperoxide consumed using an extinction coefficient of 13,000 liters per mole cm for thionitrobenzoate anion (12) and the observation that GSH peroxidase catalyzes the oxidation of 2 molar equivalents of GSH in reducing 1 eq of hydroperoxide (13). One unit of GSH peroxidase activity is defined as the amount of GSH peroxidase which will catalyze the reduction of 1 nmole of hydroperoxide per min under the standard assay conditions.

**Purification of Lipoxygenase**—Soybean lipoxygenase purchased from Worthington Biochemical Corporation was partially purified by chromatography on DEAE-Sephadex (A-50) according to a modification of the procedure of Allen (14). All procedures were performed at 0-4°. The commercial enzyme (150 to 250 mg) was dissolved in 10 to 20 ml of 0.12 M NaCl in 0.09 M Tris-HCl (pH 8.5) and dialyzed overnight against two or three 500-ml portions of 0.12 M NaCl in 0.09 M Tris-HCl (pH 8.5). The dialyzed enzyme solution was applied to a column of DEAE-Sephadex (2.2 x 30 cm). The DEAE Sephadex had been previously washed, defined, and equilibrated with 0.12 M NaCl in 0.09 M Tris-HCl (pH 8.5). The column was eluted using a linear gradient (total volume = 300 ml; flow rate = 15 to 20 ml per hour) from 0.12 M NaCl in 0.09 M Tris-HCl (pH 8.5) to 0.18 M NaCl in 0.09 M Tris-HCl (pH 8.5); 8- to 15-ml fractions were collected. Lipoxygenase appeared at approximately the middle of the gradient. Fractions with high lipoxygenase activity were pooled and protein was precipitated with 75% (NH₄)₂SO₄. The precipitated protein was collected by centrifugation at 15,000 x g for 15 min and resuspended in 0.1 M Tris-HCl (pH 8.5). This procedure afforded a 2- to 4-fold purification of the dialyzed lipoxygenase with a 50% recovery of activity. The (NH₄)₂SO₄ precipitate, after dialysis against 0.1 M Tris-HCl (pH 8.5), showed one major and two minor protein-staining bands (15) on disc gel electrophoresis performed essentially according to the procedure of Brewer and Ashworth (16). The major band contained all of the lipoxygenase activity on the gel; however, only 50% of the units could be eluted from the gel after electrophoresis.

**Purification of GSH Peroxidase**—GSH peroxidase was partially purified from rat liver by a procedure similar to that of O'Brien and Little (17). Either male or female Sprague-Dawley rats (175 to 200 g) were fasted 18 to 24 hours and killed by decapitation. The livers were homogenized in 0.1 M sodium citrate buffer (pH 2.2) and stored frozen at -15° until submitted to analysis.

**RESULTS**

We presented evidence in earlier reports (6, 10) that lipoxygenase was destroyed during oxygenation of fatty acid substrates. The velocity of oxygenation decreased to zero before all the fatty acid was consumed and the extent of reaction depended on the quantity of lipoxygenase added to the reaction mixture. If instantaneous velocities are measured during the course of the reac-
The initial lag phase is observed) are lower than the kz values. Measured: 0.050 (7) 0.041 (7) 0.020 (3) 0.023 (2)

Values of kz and ratio of rate to extent measurements are from experiments such as shown in Fig. 1 and kz values are from experiments such as those shown in Fig. 2. Each value is an average of the number of separate experiments indicated in parentheses; for kz and k determinations each experiment involved 4 to 10 individual rate determinations. All values are expressed as min⁻¹.

<table>
<thead>
<tr>
<th>Acid</th>
<th>kz</th>
<th>Ratio of rate to extent</th>
<th>k</th>
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</thead>
<tbody>
<tr>
<td>18:2 (n-6)</td>
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<td>0.041 (7) 0.020 (3) 0.023 (2)</td>
<td></td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>0.040</td>
<td>0.032 (5) 0.013 (3) 0.027 (6)</td>
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<tr>
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<td>0.032 (5) 0.013 (3) 0.050 (7)</td>
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<tr>
<td>20:4 (n-6)</td>
<td>0.023</td>
<td>0.024 (5) 0.018 (3) 0.036 (4)</td>
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<tr>
<td>20:5 (n-3)</td>
<td>0.074</td>
<td>0.070 (7) 0.096 (3) 0.18 (6)</td>
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<tr>
<td>22:6 (n-3)</td>
<td>0.059</td>
<td>0.032 (5) 0.050 (7) 0.15 (6)</td>
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</tr>
</tbody>
</table>

* Values from Reference 6 in which no initial hydroperoxide activation was used.

Summary of rate constants for inactivation of lipoxygenase by various processes

Values of kz and ratio of rate to extent measurements are from experiments such as shown in Fig. 1 and kz values are from experiments such as those shown in Fig. 2. Each value is an average of the number of separate experiments indicated in parentheses; for kz and k determinations each experiment involved 4 to 10 individual rate determinations. All values are expressed as min⁻¹.

<table>
<thead>
<tr>
<th>Acid</th>
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<th>k</th>
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<tr>
<td>18:2 (n-6)</td>
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<td>0.041 (7) 0.020 (3) 0.023 (2)</td>
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<tr>
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<td>20:3 (n-6)</td>
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<tr>
<td>20:4 (n-6)</td>
<td>0.023</td>
<td>0.024 (5) 0.018 (3) 0.036 (4)</td>
<td></td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>0.074</td>
<td>0.070 (7) 0.096 (3) 0.18 (6)</td>
<td></td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>0.059</td>
<td>0.032 (5) 0.050 (7) 0.15 (6)</td>
<td></td>
</tr>
</tbody>
</table>

* Values from Reference 6 in which no initial hydroperoxide activation was used.

Fig. 2. Log initial velocity versus time for the aerobic inactivation of lipoxygenase by 13-hydroperoxy-9,11,15-octadecatrienoic acid. All assays were performed as described under "Methods." Hydroperoxide was generated in situ by the additions of 20 μg of lipoxygenase to suspensions of 9,12,15-octadecatetraenoic acid. After this initial quantity of substrate was consumed, 80 nmoles of 9,12,15-octadecatetraenoic acid. Lipoxygenase (2 to 4 μg) was added to a suspension of 9,12,15-octadecatetraenoic acid in the bottom of a Thunberg tube and the reaction was allowed to proceed until the enzyme was completely inactivated but fatty acid substrate still remained. The tube was then closed with the side arm containing 30 to 50 μg of fresh lipoxygenase. The volume of each sample before the beginning of the aspiration procedure was 0.75 ml of 0.1 m Tris-HCl (pH 9.0) in both the bottom and side arm of the Thunberg tube. Air was removed by aspirating for 1 min and the tube was flushed with N₂ which had been passed through a solution of propyl gallate in 0.1 m NaOH. This degassing procedure was repeated 3 times on all samples. At 0 min the contents of each tube were mixed thoroughly. After the indicated pre-inactivation times the tubes were opened, the contents were transferred quickly to a cuvette which was shaken thoroughly, and, initial rates were determined spectrophotometrically as described under "Methods." The estimated quantity of hydroperoxide product and substrate in the bottom of the Thunberg tube and the quantity of lipoxygenase in the side arm at the time of mixing were as follows: (►►), 0 nmoles of 13-hydroperoxy-9,11,15-octadecatetraenoic acid (P), 82 nmoles of 9,12,15-octadecatetraenoic acid (S) and 50 μg of lipoxygenase (E); (►►), OP, 55S and 30 μgE; (►►), 30S, 55S, 40E: (►►), 35P, 50S, 40E; (►►), 45P, 30S, 40E. The first order rate constant for the aerobic inactivation of lipoxygenase in the presence of substrate and product is 0.046 min⁻¹ (average of four experiments); and for anaerobic inactivation in the presence of substrate, 0.012 min⁻¹ (average of three experiments).

Fig. 3. Log initial velocity versus preincubation time for the anaerobic inactivation of lipoxygenase with 9,12,15-octadecatrienoic acid in the presence and absence of 13-hydroperoxy-9,11,15-octadecatrienoic acid. Lipoxygenase (2 to 4 μg) was added to a suspension of 9,12,15-octadecatrienoic acid in the bottom of a Thunberg tube and the reaction was allowed to proceed until the enzyme was completely inactivated but fatty acid substrate still remained. The tube was then closed with the side arm containing 30 to 50 μg of fresh lipoxygenase. The volume of each sample before the beginning of the aspiration procedure was 0.75 ml of 0.1 m Tris-HCl (pH 9.0) in both the bottom and side arm of the Thunberg tube. Air was removed by aspirating for 1 min and the tube was flushed with N₂ which had been passed through a solution of propyl gallate in 0.1 m NaOH. This degassing procedure was repeated 3 times on all samples. At 0 min the contents of each tube were mixed thoroughly. After the indicated pre-inactivation times the tubes were opened, the contents were transferred quickly to a cuvette which was shaken thoroughly, and, initial rates were determined spectrophotometrically as described under "Methods." The estimated quantity of hydroperoxide product and substrate in the bottom of the Thunberg tube and the quantity of lipoxygenase in the side arm at the time of mixing were as follows: (►►), 0 nmoles of 13-hydroperoxy-9,11,15-octadecatrienoic acid (P), 82 nmoles of 9,12,15-octadecatrienoic acid (S) and 50 μg of lipoxygenase (E); (►►), OP, 55S and 30 μgE; (►►), 30S, 55S, 40E: (►►), 35P, 50S, 40E; (►►), 45P, 30S, 40E. The first order rate constant for the aerobic inactivation of lipoxygenase in the presence of substrate and product is 0.046 min⁻¹ (average of four experiments); and for anaerobic inactivation in the presence of substrate, 0.012 min⁻¹ (average of three experiments).
TABLE II

Amino acid composition of lipoxygenase and of lipoxygenase inactivated during oxygenation of 5,8,11,14,17,20-docosahexaenoic acid

Lipoxygenase (20 units, 4.8 mg) partially purified as described under "Methods" was added to a suspension of 5,8,11,14,17,20-docosahexaenoic acid (100 micromoles) to give a final volume of 8.0 ml of 0.1 M Tris-HCl (pH 8.5). Aliquots were withdrawn at various times and assayed for remaining lipoxygenase activity. After no activity remained (15 to 20 min), the reaction mixture was cooled to 0°C and 1.0 mmole of NaBH₄ added to reduce hydroperoxides. The sample was allowed to stand in ice for 30 min and then at room temperature for approximately 5 hours. The sample was then exhaustively dialyzed against (a) 90% ethanol, (b) 1.0 mM EDTA, and (c) deionized water. Following the dialysis procedures, the resulting protein suspension was divided into aliquots and subjected to hydrolysis (24 and 48 hours) and amino acid analysis as described under "Methods." Control values were obtained by incubating lipoxygenase with S-octadecenoic acid and carrying this sample through a procedure identical with that outlined above. Results are presented as moles of amino acid per mole of enzyme calculated on the basis of a molecular weight for lipoxygenase of 100,000 daltons (36) and an average amino acid residue weight of 127 daltons. Each set of values has been corrected for serine and threonine destruction. Tryptophan and cysteine were not included in these calculations.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>18:1 (n-9)</th>
<th>22:6 (n-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>Lys</td>
<td>46</td>
<td>47</td>
</tr>
<tr>
<td>His</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>Arg</td>
<td>35</td>
<td>33</td>
</tr>
<tr>
<td>Asp</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>Thr</td>
<td>104</td>
<td>100</td>
</tr>
<tr>
<td>Ser</td>
<td>75</td>
<td>74</td>
</tr>
<tr>
<td>Glu</td>
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<td>82</td>
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<td>Pro</td>
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<tr>
<td>Gly</td>
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<td>Ala</td>
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<td>52</td>
</tr>
<tr>
<td>Val</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Met</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Ile</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>Leu</td>
<td>54</td>
<td>55</td>
</tr>
<tr>
<td>Tyr</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Phe</td>
<td>22</td>
<td>23</td>
</tr>
</tbody>
</table>

Fig. 4. Measurement of the kinetic lag period in the oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase. All assays were performed as described under "Methods." Lipoxygenase was added at 0 min to initiate the reaction. The lag time indicated on the figure was measured from time zero to the time at which a line tangent to the maximum velocity intersects the "time-axis." Initial concentrations of reactants were: (A), 66 PM, 20:4 (n-6), 10 mg per ml enzyme; (B), 132 PM, 20:4 (n-6), 5 mg per ml enzyme; (C) 66 PM, 20:4 (n-6), 5 mg per ml enzyme; and (D) 132 PM, 20:4 (n-6), 5 mg per ml enzyme. The final reaction volume is 1.0 ml of 0.1 M Tris-HCl (pH 9.0). Each curve is derived from at least two individual experiments with continuous recordings of product formation throughout the reaction period.

The oxygenation of fatty acid substrates by lipoxygenase occurs with an observable lag phase under certain conditions. Fig. 4 shows this phenomenon with 5,8,11,14-20:4 and indicates how the lag time was measured in our experiments. The maximum velocities of oxygenation with the substrate and enzyme concentrations used were attained when the concentration of product was 2 to 4 mM. If product was generated in situ before the addition of fatty acid, the lag phase was essentially abolished with product concentrations of 5 mM (Table III). Octylhydroperoxide caused a measurable decrease in the lag time at hydroperoxide concentrations of 40 mM. However, this reduction in lag period occurs with a concomitantly sharp reduction in enzyme activity. Hydrogen peroxide (50 mM) had no effect on the length of the kinetic lag period. We also observed that both 15-hydroxy-5,8,11,13-eicosatetraenoic acid and 13-hydroxy-9,11-octadecadienoic acid will abolish the lag with either 5,8,11,14-eicosatetraenoic acid or 9,12-octadecadienoic acid as substrate; however, the maximum velocity depended only on the nature of the substrate and was independent of the nature of the hydroperoxide product.

The length of the lag period at a fixed concentration of 5,8,11,14-20:4 decreased with increasing enzyme concentration (Table IV). On the other hand, increasing the concentration of 5,8,11,14-20:4 with a fixed enzyme concentration caused a marked
Effect of hydrogen peroxide and alkyl hydroperoxides on lag period in oxygenation of 9,12-octadecadienoic and 5,8,11,14-eicosatetraenoic acids by lipoxygenase

Hydrogen peroxide and 1-octyl hydroperoxide were added at the indicated concentrations to a solution containing the indicated fatty acid substrate. Lipoxygenase (3 µg) and substrate was then added to initiate the reaction and lag time and maximum velocities determined as indicated in Fig. 4. For experiments with product hydroperoxides, the hydroperoxides were generated in situ with lipoxygenase (3 µg) and substrate was then added to initiate the oxygenation. All experiments were performed in a final reaction volume of 1.0 ml of 0.1 M Tris-HCl (pH 9.0).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount</th>
<th>Tube</th>
<th>Hydroperoxide added</th>
<th>Amount</th>
<th>Lag</th>
<th>Maximal velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2 (n-6)</td>
<td>18.2 µM</td>
<td>60</td>
<td>13-Hydroperoxy-9,11-13-Octadeicadienoate</td>
<td>0</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>20.4 µM</td>
<td>60</td>
<td>1-Octyl hydroperoxide</td>
<td>20</td>
<td>1.5</td>
<td>3.1</td>
</tr>
<tr>
<td>H2O2</td>
<td>100</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The lag time could not be precisely estimated because of the uncertain transition from zero to maximal velocity in these instances.

**Table IV**

Effect of lipoxygenase concentration on lag period in oxygenation of 5,8,11,14-eicosatetraenoic acid

All assays were performed as described under "Methods." All samples contained 66 µM 5,8,11,14-eicosatetraenoic acid in a final volume of 1.0 ml of 0.1 M Tris-HCl (pH 9.0). The lag times were measured as indicated in Fig. 4.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lipoxygenase</th>
<th>Lag time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>0.75</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>0.52</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>0.28</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>0.18</td>
</tr>
<tr>
<td>11</td>
<td>21</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*GSH was omitted to indicate no effect with GSH peroxidase alone.

increase in the lag period so that at 204 µM fatty acid the lag was infinite and no oxygenation occurred (Table V).

We reported earlier (10) that rat liver GSH peroxidase inhibits oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase in the presence of GSH, dithiothreitol, or cysteine and that this inhibition could be reversed by removal of mercaptan. Table VI indicates the levels of GSH peroxidase required to inhibit lipoxygenase in the presence of GSH. Measurement of the absorbance at 413 nm after addition of 5,5-dithiobis(2-nitrobenzoic acid) to aliquots of oxygenation reaction mixtures completely inhibited by GSH peroxidase indicated that no sulfhydryl consumption was occurring; however, GSH was consumed in systems incompletely inhibited by the peroxidase. GSH peroxidase plus GSH caused an increase in the observed kinetic lag period from less than 1 min to infinity. In addition, the amount of product that must be pro-

**Table V**

Effect of fatty acid substrate concentration on lag period in oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase

All assays were performed as described under "Methods." All reactions were initiated by the addition of 3 µg of lipoxygenase to give a final volume of 1.0 ml of 0.1 M Tris-HCl (pH 9.0) containing the indicated concentrations of 5,8,11,14-eicosatetraenoic acid. The lag times were measured as indicated in Fig. 4.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[µM]</th>
<th>Lag time</th>
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<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>0.24</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1.20</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>1.60</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>1.84</td>
</tr>
<tr>
<td>5</td>
<td>132</td>
<td>4.16</td>
</tr>
<tr>
<td>6</td>
<td>204</td>
<td></td>
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</tbody>
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*The legend to Fig. 4.
TABLE VII

| [GSH (a-60)] | First enzyme | Lag time | Maximi- | Product formed | Second enzyme | Lag time | Maximum velocity
<table>
<thead>
<tr>
<th></th>
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<tr>
<td>(µM)</td>
<td>min</td>
<td>µM/min</td>
<td>µM</td>
<td>µM</td>
<td>min</td>
<td>µM/min</td>
<td>µM</td>
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<tr>
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<td>0</td>
<td>4.1</td>
<td>0</td>
</tr>
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<td>70</td>
<td>1.8</td>
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<td>15</td>
<td>55</td>
<td>0</td>
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<td>94</td>
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<tr>
<td>110</td>
<td>4.4</td>
<td>1.9</td>
<td>14</td>
<td>96</td>
<td>0</td>
<td>3.9</td>
<td>0</td>
</tr>
</tbody>
</table>

**Effect of fatty acid substrate concentration on oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase in presence and absence of hydroperoxide product**

All assays were performed as described under "Methods." The lag times and maximum velocities were measured as indicated in Fig. 4. Lipoxygenase (3 µg) was added to initiate the reactions. After the indicated amounts of product were formed, no further change in absorbance at 234 nm occurred with time, a second aliquot of enzyme (3 µg) was added. The final volume in all assays was 1.0 ml of 0.1 M Tris-HCl (pH 9.0).

Dissolved before the maximum rate occurred was seen to increase with increasing concentrations of GSH peroxidase whereas the attainable maximal rate decreased.

In the absence of any initial product hydroperoxide, increasing the fatty acid substrate concentration at a fixed enzyme concentration not only caused an increase in lag time, but also decreased the maximum velocity attained by the enzyme (Table VII). When product (15 µM) was present initially, the maximum velocity was independent of the substrate concentration at saturating substrate levels.

**DISCUSSION**

**Self-catalyzed Destruction of Lipoxygenase**—The inactivation of soybean lipoxygenase during oxygenation of fatty acid substrates was first described by Theorell et al. (20). Our results show that the velocity of the lipoxygenase-catalyzed reaction decreases as a linear function of substrate utilization with all substrates tested. The inactivation observed during oxygenation of 9,12,15-octadecatrienoic, 8,11,14-eicosatetraenoic, and 5,8,11,14,17-eicosapentaenoic acids is most easily explained as a deleterious breakdown of an active complex resulting from the interaction of enzyme, product, fatty acid, and oxygen (Fig. 5). The involvement of fatty acid in the formation of this metastable complex is suggested by the fact that the rate of decrease in lipoxygenase activity observed in the complete system is generally much faster than the rate of inactivation which occurs when enzyme is incubated aerobically with product in the absence of substrate acid. Only in the case of 17-hydroperoxy-5,8,11,14,18,20-docosahexaenoic acid and 13-hydroperoxy-9,11,15-octadecadienoic acids is the pseudo-first order inactivation (see Table I) of lipoxygenase rapid enough to account for much of the observed destruction of the enzyme during oxygenation of the respective substrate acids. However, a direct inactivation by these products (as well as those of the other substrates tested) may not be occurring at appreciable rates when substrates are also present and the oxygenation process is taking place. (See Equations 18 to 20 below in "Kinetic Formulation for Lipoxygenase".) In addition, the relatively slow inactivation of lipoxygenase which occurs during the anaerobic incubation of 13-hydroperoxy-9,11,15-octadecatrienoic and 9,12,15-octadeca-

![Fig. 5. Kinetic model for the self-catalyzed destruction of lipoxygenase.](http://www.jbc.org/)

Theoretically, this $k_2$ value may be approximated by dividing the initial oxygenation rate by the final extent of product formation since:

$$\frac{v}{P_{\text{t,ini}}} = \frac{k_3[E_0]}{k_2} \left( \frac{K_M}{[S]_t} + 1 \right)$$

We noted that the ratio of rate to extent measurement for $k_2$ agreed well with the slopes determined from velocity versus substrate concentration plots in systems where hydroperoxide product was included initially in the reaction mixture. On the other
hand, the maximal velocity of oxygenation attained with a given acid is consistently lower when no product is initially present in the reaction mixture than when initial low concentrations of product are present. In such a situation, some enzyme is consumed while producing the levels of product needed to reach maximal rate and thus the eventual observed maximal rate is less. Therefore, the ratio of rate to extent values determined in systems containing no product initially were always lower than ratio of rate to extent values determined in systems that initially contained product.

The chemical nature of this self-destruction phenomenon observed with lipoxygenase is not clear. Comparison of the amino acid composition of active and inactivated enzyme did not allow us to draw any conclusions about the destruction of any particular residue. Based on the analyses with partially purified enzyme, methionine is the only residue of which there are less than 20 per lipoxygenase molecule. Detection of a loss of 1 residue in 20 requires a resolution of better than 5%, which we did not achieve. However, it seems reasonable that a deleterious modification of at least 1 active site amino acid residue does occur. A potentially related inactivation of lipoxygenase by 5,8,11,14-eicosatetraenoic acid has been reported by Downing et al. (21) who suggested that modification of an active site histidine residue resulted in loss of lipoxygenase activity. The pH-activity profile for oxygenation of 9,12-octadecadienoic acid by lipoxygenase indicates a catalytically active group with pK = 6.5 to 6.8 (11) which could be a histidine residue. Histidine is readily modified by active oxygen (22) related to the mechanism discussed below.

Homolytic cleavage of the oxygen-oxygen bond in hydroperoxides occurs easily and yields the highly reactive hydroxyl radical (23) which might modify the lipoxygenase. The mechanism presented here in the discussion involves such radicals in a manner that reconciles the greater instability of the protein when both substrates are present with the hydroperoxide. In addition, nonenzymatic formation of malondialdehyde from polyunsaturated hydroperoxides (24) provides another reactive species which along with the hydroxyl radical is capable of reacting with a number of amino acids. Somewhat similar self-catalyzed enzyme destructions have been described in the case of citrate lyase (25), threonine deaminase (26), and 3-hydroxyanthranilate oxidase (27). In this last-named instance the self-destruction seems to be due to oxidation of Fe$^{2+}$ to Fe$^{3+}$ during the reaction.

**Product Activation of Lipoxygenase**—The oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase may be completely and reversibly inhibited by GSH peroxidase in a process requiring the presence of mercaptans. No inhibition occurs when the GSH peroxidase is heated. This inhibition may be caused by a GSH-dependent binding of some catalytically active group on the lipoxygenase molecule by the peroxidase. However, inhibition by a protein-protein interaction is inconsistent with the relatively small difference in the levels of GSH peroxidase required to give partial (20 to 40%) versus complete reduction in the maximum rate (Table VI). Furthermore, the lower maximum rates that we noted in the presence of intermediate levels of GSH peroxidase are closely predicted by Equation 6 on the basis of the observed substrate consumption and therefore may be attributed to a self-catalyzed loss of lipoxygenase by the process noted as $k_5$. This would mean that GSH peroxidase had no direct effect on the maximal velocity, but only on the rate of attaining that velocity.

The concept of a threshold level for GSH peroxidase inhibition is consistent with a delicate balance in rates of hydroperoxide formation and destruction and the ability of the hydroperoxide to function autocatalytically. Such a balance would explain the fact that GSH peroxidase causes a marked increase in the kinetic lag period and the fact that measureable GSH consumption occurs in systems incompletely inhibited by GSH peroxidase but does not occur with higher levels of the peroxidase.

We do not know whether a very slow product-independent enzymatic process or an autooxidative process produces the first few molecules of hydroperoxide needed for lipoxygenase to initiate its autocatalytic activity. That very slow initial process appears to be the step that is so easily counteracted by the minute amounts of glutathione that we assume are consumed during GSH peroxidase inhibition.

We have found, in agreement with others (7, 28), that hydroperoxides which abolish the kinetic lag period are products of the lipoxygenase oxygenation process. The apparent obligatory role of product in the activity of lipoxygenase, the marked increase in the kinetic lag time caused by GSH peroxidase, and a similarity in structures of the hydroperoxides which caused removal of the kinetic lag period suggest that a binding site exists for hydroperoxide product. The concept of binding site for product distinct from that for substrate is compatible with the mixed inhibition results of Mitsuda et al. (29). The common structural feature of all hydroperoxy acids which have been shown to reduce the kinetic lag time is an (n-7) trans (n-9) cis diene grouping.

**Kinetic Formulation for Lipoxygenase**—We noted that the specific activity of lipoxygenase was reduced and the length of the kinetic lag period increased as the concentration of fatty acid was increased. These effects of high fatty acid substrate concentrations were qualitatively similar to the effects of GSH peroxidase on lipoxygenase and suggested that high concentrations of fatty acid caused a decrease in the level of the functional enzyme-product complex. One explanation is that substrate prevented formation of the enzyme-product complex by being bound at the product binding site. Including this concept of binding site for product distinct from that for substrate is compatible with the mixed inhibition results of Mitsuda et al. (29). The common structural feature of all hydroperoxy acids which have been shown to reduce the kinetic lag time is an (n-7) trans (n-9) cis diene grouping.
fashion; (c) $S$ may effectively bind to the product site but $P$ may not bind effectively to the substrate site; (d) the affinity of $S$ for the substrate site is higher than the affinity of $S$ for the product site; and (e) all species are in equilibrium with one another. Using these assumptions we calculated the rate expression for the above model as follows:

$$v = k_4 [EPS]$$  

(8)

$$K_s = \frac{[E][S]}{[ES]}$$  

(9a)

$$K_p = \frac{[E][P]}{[EP]}$$  

(9b)

$$K_{ps} = \frac{[RP][S]}{[EPS]}$$  

(9c)

$$K_{sp} = \frac{[ES][P]}{[EPS]}$$  

(9d)

$$K_{ss} = \frac{[ES][S]}{[ESS]}$$  

(9e)

From the equilibrium Equations 9a to 9e and $E_0 = E + ES + EP + EPS + ESS + E^* + E^*$ we obtain:

$$[E] = \frac{[E][S]}{K_s} + \frac{[E][P]}{K_p} + \frac{[E][P][S]}{K_{ps}K_{sp}} + \frac{[S][P]}{K_{ss}K_{sp}}$$  

(10)

so that:

$$[E] = \frac{[E_0 - E^* - E^*]}{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[P][S]}{K_{ps}K_{sp}} + \frac{[S][P]}{K_{ss}K_{sp}}}$$  

(11)

From Equations 8, 9a, and 9d:

$$v = k_4 [EPS] = \frac{k_4[E][P][S]}{K_{ps}}$$  

(12)

Which, when combined with Equation 11, gives:

$$v = \frac{k_4[E_0 - E^* - E^*]}{[P][S] + \frac{K_{ps}}{K_p} + \frac{K_{sp}}{K_p} + \frac{K_{ss}[S]}{K_K_{ps}}} + 1$$  

(13)

Equation 13 rearranges to:

$$v = \frac{k_4[E_0 - E^* - E^*]}{[P][S] + \frac{K_{ps}}{K_p} + \frac{K_{sp}}{K_p} + \frac{1 + [S]}{K_{ss}K_p} + 1}$$  

(14)

And from Equations 3b and 14 we find that:

$$v = \frac{k_4[E_0 - E^* - k_4P][P][S]}{1 + \frac{K_{ps}}{[P]} + \frac{K_{sp}}{[P]} + \frac{1 + [S]}{K_{ss}K_p} + 1}$$  

(15)

Rate Equation 15 predicts that no velocity is observed if $P$ is zero since the denominator of the equation is infinite in this situation. This prediction is consistent with the results discussed in "Product Activation of Lipoxygenase." Equation 15 also indicates that the velocity will become independent of the concentration of $P$ when $P$ is much greater than $K_{sp}$. In such a case, the equation may be simplified to Equation 16.

$$v = \frac{k_4[E_0 - E^* - k_4P][P][S]}{K_{ps} + 1}$$  

(16)

In this situation, the $K_{ps}$ term would be the same as the apparent $K_M$. We determined an apparent $K_M$ (and thus $K_{ps}$) of approximately 1 to 5 $\mu M$ for 5,8,11,14-eicosatetraenoic acid.

With various concentrations of 5,8,11,14-eicosatetraenoic acid used in our assay system, it was observed that the velocity is independent of the product concentration when the concentration of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid is greater than approximately 2 to 4 $\mu M$. We estimated that the values for $K_p$ and $K_{sp}$ must be no greater than 1 $\mu M$ so that Equation 15 would tend to be reduced to Equation 16 when the concentration of hydroperoxide product is greater than 3 $\mu M$.

During the early part of the oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase, $E^*$ and $E^*$ are essentially zero. Using this initial velocity restriction, Equation 15 may be rearranged to give:

$$v = \frac{k_4[E_0 - E^* - E^*]}{[P][S] + \frac{K_{ps}}{[P]} + \frac{K_{sp}}{[P]} + \frac{1 + [S]}{K_{ss}K_p} + 1} = k_4[E_0] - V_{max}$$  

(17)

Using Equation 17 and data from a series of experiments (Table VII) at constant enzyme concentration in which $v$, $[S]$, and $[P]$ were known, we used a series of successive approximations of $K_p$, $K_{ps}$, and $K_{ss}$ to find those compatible with a constant value for $V_{max}$. Our requirements were that $V_{max}$ should be equal or greater than $v$ and values of the dissociation constants should be on the same order of magnitude which we qualitatively predicted as discussed above. One set of estimates which allowed a close fit to both Equation 17 and our qualitative observations was $K_{ps} = K_p = K_{sp} = 1 \mu M$ and $K_{ss} = 50 \mu M$. $K_1$ which equals $K_sK_{ps}/K_{sp}$ was then calculated to be 1 $\mu M$.

The contribution of the terms $E^*$ and $E^*$ to Equation 15 may be estimated in the following manner:

$$\frac{d[E^*]}{dt} = k_4[E][P] = \frac{k_4[E][P]}{K_p}$$  

(18)

Using the values of the dissociation constants estimated above and the $k_4$ and $k_4$ values for 5,8,11,14-eicosatetraenoic acid from Table I, we see that even with a relatively low concentration of 5,8,11,14-eicosatetraenoic acid (30 $\mu M$) and a reasonably high concentration of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (50 $\mu M$) formation of $E^*$ would be slow relative to formation of $E^*$. Furthermore, the rate of $E^*$ formation will decrease with increasing $[S]$.

$$\frac{d[E^*]}{dt} = 1.25 [E]$$  

(19)

$$\frac{d[E^*]}{dt} = 320 [E]$$  

(20)

This indicates that the inactivation of lipoxygenase by a $k_4$ process is less significant during the oxygenation of fatty acid substrate.

**Lipoxygenase Reaction Mechanism**—A model for the possible intermediates in the lipoxygenase reaction should include an enzyme-bound hydroperoxide, a fatty acid substrate, and oxygen in a way that allows no detectable reaction unless all three components are present with enzyme.

A principal difficulty in comprehending the mechanism is in
discerning the way by which the molecular oxygen can reach an intermediate-activated state in the absence of other energy-generating processes. The recognition of the essentiality of hydroperoxide in the process may be a good indicator that the hydroperoxide will react with oxygen to form a tetroxide transition state (I) (30) (see Fig. 7) in a manner somewhat analogous to a reverse of the superoxide dismutase reaction (31). Formation of the transition state (I) is reversible to hydroperoxide and triplet ground state oxygen, \( \Sigma_2 \). In the transition state (I) the four oxygen atoms must all lie in a plane with the carbon and hydrogen out of the plane if the oxygen is formed in its first excited state (30). The excited oxygen, \( \Delta \Sigma_2 \), is 22 kcal above its ground state energy in transition state (I) indicating that a substantial amount of energy would be required to form this planar transition state. Some of this orientational energy could conceivably be supplied by the double bond of an enzyme-bound substrate interacting with the oxygen atoms leading to production of a peroxide metastable intermediate (II) from the planar tetroxide transition state without free singlet state oxygen actually being formed. Significant in this regard is the estimation that the interaction of long hydrocarbon chains may yield 10 to 20 kcal per mole (32). Both the high energy planar-oriented tetroxide and the peroxide can be regarded as energetically equivalent to (and perhaps in equilibrium with) singlet state oxygen. Such reactive intermediates might also react with other compounds in a manner that explains the "cooxidation" phenomenon reported for lipoxygenase (33). In addition, these intermediates do not contradict the isotope enrichment studies of Hamborg and Samuelsson (2) but rather emphasize that removal of the \( L-(n-8) \) proton is the first irreversible covalent bond cleavage limiting the rate of substrate consumption. Peroxide, tetroxide, and singlet state oxygen may all revert spontaneously to precursor molecules (30, 34, 35).

The peroxide intermediate, which forms readily from active singlet state oxygen, has been proposed as a preferred intermediate for the formation of hydroperoxides from alkenes (35). Breakdown of peroxide intermediate (II) to hydroperoxide could occur with an enzymically-directed stereospecific removal of the \( L-(n-8) \) hydrogen from the adjacent methylene carbon. This results in the formation of a new \( \text{trans} \) double bond adjacent to the hydroperoxide group. The catalytic function of lipoxygenase in this mechanism would then be in the positioning of the \( (n-6) \) double bond of the substrate in such a way that the driving force for formation of peroxide would be available and in favoring the removal of the \( L-(n-8) \) hydrogen by the peroxide.

As mentioned earlier, alkoxy and hydroxy radicals such as those formed in conjunction with intermediate (II) (as well as any of the "active oxygen" intermediates) could account for the lipoxygenase self-destruction (\( k_2 \)) process. Both this planar tetroxide mechanism and other radical chain mechanisms could not be distinguished on the basis of the occurrence of a free radical electron paramagnetic resonance signal. However, peroxide intermediate (II) may be susceptible to attack by an appropriate nucleophile such as \( N_3 \) (35) so that its existence might be detected. Excited singlet state oxygen has been suggested as a possible intermediate in the action of quercitinase, a dioxygenase form \( \text{Aspergillus flavus} \) (36, 37). Chan (38) has recently introduced evidence suggesting that singlet state oxygen can be produced during the oxygenation of ethyl-9,2-octadecadienoate by lipoxygenase. Since the planar tetroxide is energetically similar to singlet state oxygen, the presence of either intermediate does not clearly exclude the presence of the other.

Although the physiological role of lipoxygenase activity in plants is not clear, this report and others (2, 10) provides several mechanistic similarities for the plant dioxygenase and that catalyzing the first step in prostaglandin synthesis.

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