THE SELF-CATALYZED DESTRUCTION OF LIPOXYGENASE*

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SUMMARY

Lipoxygenase (E.C. 1.99.2.1) from soybean catalyzes its own destruction in the presence of both substrates, oxygen and fatty acid. The apparent first order rate constant for this inactivation was 0.050 min^{-1} in the presence of 9,12-octadecadienoate and 0.32 min^{-1} with 5,8,11,14-eicosatetraenoate.

Lipoxygenase is a dioxygenase capable of stereospecific attack (1) upon selected fatty acid substrates (1,2). A mechanism of action has been proposed by Tappel et al. (3) and further insight was provided by the isotope enrichment noted by Hamberg and Samuelsson (1). In designing experiments to help elucidate the mechanism by which oxygen adds to unsaturated fatty acids, we noted that lipoxygenase behaved somewhat like a substrate in that it limited the extent, as well as the rate, of the reaction. Such enzyme inactivation may occur during isolation or handling and thus explain losses in lipoxygenase activity such as observed by Dillard et al. (4) and may also account for the differences in specific activities of the purified enzyme noted by Allen (5). This phenomenon of an enzyme-limited extent of reaction is likely to affect kinetic measurements with soybean lipoxygenase particularly with substrates containing three or more double bonds.

EXPERIMENTAL

Soybean lipoxygenase was obtained from Sigma Chemical Company. The commercial enzyme preparation catalyzed the oxygenation of 1.52 μmole of

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acid per minutes per mg protein in an assay mixture containing 5-15 µg lipoxygenase and 100 nmoles of 9,12-octadecadienoic acid in 1.0 ml of 0.1 M Tris·HCl (pH 9.0). All fatty acids were high purity grade obtained from the Hormel Institute except 8,11,14-eicosatrienoic acid which was generously donated by the Upjohn Company. The fatty acids were dissolved in benzene containing Santoquin* (0.3% w/w) and stored at -20°. All other chemicals were reagent grade.

The oxygenation reactions were initiated by the addition of aliquots of solutions of soybean lipoxygenase (0.2-1.0 mg/ml) in 0.1 M Tris·HCl (pH 9.0) to 1.0 ml suspensions of fatty acid in 0.1 M Tris·HCl (pH 9.0). Assays for lipoxygenation were performed at room temperature in cuvettes exposed to air by continuously monitoring the absorbance at 234 mµ on a Beckman DU spectrophotometer equipped with a Gilford Model 2000 Multiple Sample Absorbance Recorder. A molar extinction coefficient of 28,000 l/mole-cm (3) was used to convert absorbance readings to moles of product.

RESULTS AND DISCUSSION

Our preliminary studies on lipoxygenase with 5,8,11,14-eicosatetraenoic acid indicated that the reaction velocity decreased to zero before all the fatty acid appeared to be oxidized. We could not attribute this effect to an incorrect ε_{234}, an impure substrate, or production of a competitive inhibitor during reaction since further addition of substrate with concomittant dilution of the reaction mixture produced no further extent of reaction. However, if lipoxygenase was added to the reaction mixture, product formation occurred at a rate which (at saturating substrate levels) was directly proportional to the amount of fresh enzyme added. This phenomenon is illustrated in Fig. 1 for 9,12-octadecadienoic acid and 8,11,14-eicosatrienoic acid. It should be noted that the lag observed by others (4,6,7) does not appear in Fig. 1. This lag, which is proportional to substrate concentration, can be eliminated when hydroperoxide is present in the initial reaction mixture (7). Therefore, to achieve consistent

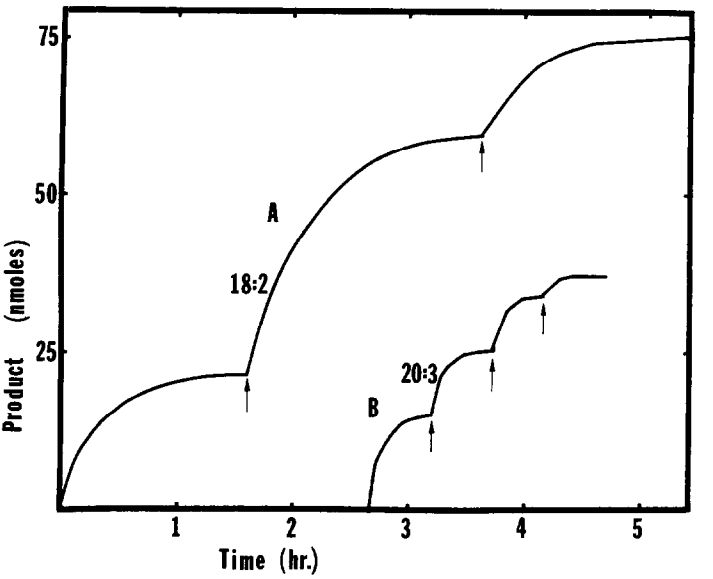

Fig. 1. Product formation versus time for lipoxygenation of 9, 12-octadecadienoic and 8,11,14-eicosatrienoic acids.

Curve A: The sample initially contained 76 nmoles of 9,12-octadecadienoic acid and 4 nmoles of 15-hydroperoxy-5,8,11,13-eicosatetrenoic acid (to eliminate the lag observed at low enzyme concentrations) in 1.0 ml of 0.1 M Tris·HCl (pH 9.0). The reaction was initiated by the addition of 2.5 µl of a solution of soybean lipoxygenase (0.4 mg/ml). After no further change in absorbance occurred lipoxygenase was added as indicated. Curve B: The sample contained 37.5 nmoles of 8,11,14-eicosatrienoic acid and 4 nmoles of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid. The reaction was initiated with 5.0 µl of lipoxygenase solution (0.4 mg/ml). Further additions were made as indicated.

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kinetic results over the wide range of substrate concentrations used in these studies, lipid hydroperoxide was added where indicated to ensure fully activated enzyme in the assay system. The addition of heat-treated lipoxygenase (80° for 10 min) or bovine serum albumin (50 µg) either to the initial substrate solution or to the reaction mixture after the rate had dropped to zero did not result in further reaction. This indicates that the enzyme-limiting oxygenation is probably not due to the utilization of a free heat-stable cofactor present in the enzyme solutions or to the loss of the small amount of active enzyme by absorption to the walls of the cuvette observed by Allen (5).

TABLE I

Acid	Enzyme µg	Substrate µM	Rate (nmoles/min)	Extent (nmoles)	100 x rate/extent	Avg.
	1	46	0.72	39	1.8	
18:2(n-6)	1	92	0.72	38	1.9	2.0
	2	92	1.84	76	2.3	
	2	42	0.48	28	1.7	
20:2(n-6)	2	84	0.44	24	1.8	1.9
	4	84	0.96	45	2.1	
	2	45	1.9	32	5.9	
18:3(n-3)	2	90	2.0	36	5.6	6.2
	4	90	4.3	61	7.0	
	2	72	0.32	6.0	5.3	
18:3(n-6)	2	144	0.24	6.4	3.8	4.8
	4	144	0.64	12.0	5.3	
	10	37	2.3	26	8.9	
20:3(n-3)	10	74	2.3	27	8.5	9.5
	20	74	5.5	49	11	
	2	47	0.36	5.6	6.4	
20:3(n-6)	2	94	0.24	6.0	4.0	5.9
	4	94	0.88	12	7.3	
	10	50	0.95	8.5	11	
20:4(n-6)	20	50	2.4	16	15	13
	20	100	1.6	12	13	
	10	24	1.9	11	17	
20:5(n-3)	20	24	4.7	23	20	19
	20	48	5.2	25	21	
	3	32	2.3	10	23	
22:6(n-3)	6	32	4.9	18	27	23
	6	64	5.7	31	18	

Rates and Extents of Lipoxygenation of Different Fatty Acids

All measurements were made under the reaction conditions described in the text. Lipoxygenase was added to the substrate solution to initiate the reaction, and the initial rate measured. After no further change in the absorbance at 234 mµ was observed, more substrate was added. In no case did the reaction proceed until another aliquot of lipoxygenase was added, and in all cases the reaction then proceeded.

A total of nine polyunsaturated fatty acids were tested to see whether this enzyme-limited extent of reaction occurred with all substrates (Table I). In all cases examined the extent of reaction was incomplete in the presence of small amounts of enzyme. The extent of reaction with a given amount of enzyme was compared with the rate of reaction to see if both measures of lipoxygenase action responded equally to different levels of added catalyst. The amount of active lipoxygenase is expressed in terms of catalytic units

rather than mass, since the dilute enzyme solutions underwent losses in specific activity during the course of a day's work. The results in Table I indicate that each substrate is consumed at a different rate in accord with observations of Holman et al. (8). Interestingly the amount of reaction that a given amount of enzyme can produce also differs for different substrates. These differences are illustrated in the last column of Table I as the ratio x 100 of the catalytic rate over the extent obtained by that amount of catalyst. These ratios for each acid were found to be independent of both enzyme and substrate concentration over the range where the initial rate was proportional to enzyme concentration.

A 10-fold range in ratios was observed among the different substrates, with the more highly unsaturated acids producing much less product per observed unit of catalytic activity. In comparing isomers of octadecatrienoic

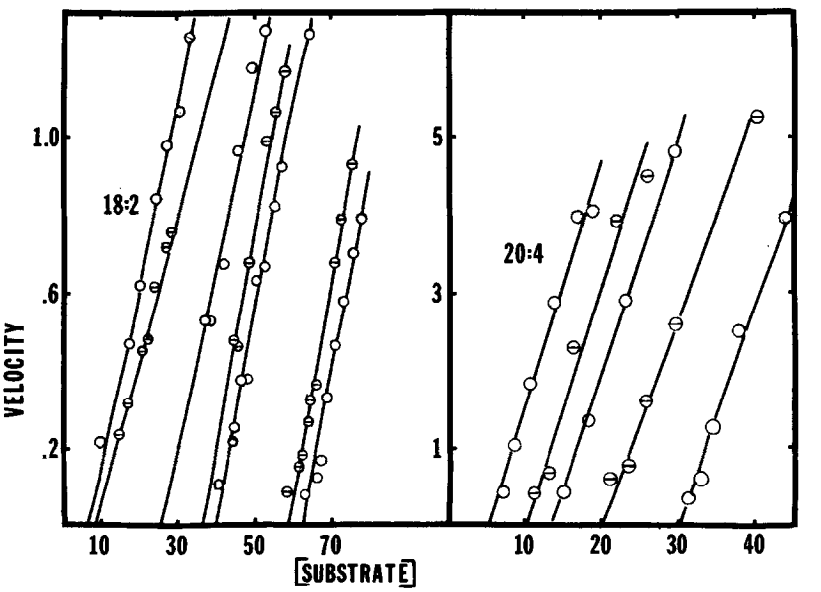

Fig. 2. Velocity versus substrate concentration for the lipoxygenation of 9,12-octadecadienoic and 5,8,11,14-eicosatetraenoic acids.

The instantaneous rate at various substrate concentrations was determined from Gilford tracings similar to those depicted in Fig. 1. All points are from samples which exhibited maximal velocity both upon initiating the reaction and when further enzyme additions were made after the velocity had dropped to zero. The velocity is expressed as nmoles/ml/min and the substrate concentration as nmoles/ml.

acid and eicosatrienoic acid, the (n-3) compounds are observed to have higher ratios of rate to extent than the corresponding (n-6) compounds.

A preliminary kinetic analysis was provided by measuring the instantaneous velocity for many time points during the reaction and plotting them against the substrate concentration at that time as shown in Fig. 2. The decreased rate with decreasing substrate does not follow the classical rectangular hyperbola but appears to be quite linear during the course of the reaction. The families of parallel lines indicate that the rate by which the velocity decreased with respect to substrate may have a characteristic value for each compound. The average of the slopes are 0.050 min^{-1} for 9,12-octadecadienoic acid and 0.32 min^{-1} for 5,8,11,14-eicosatetraenoic acid. We have found that the decrease in catalytic activity occurs more rapidly during oxygenation of substrate than during aerobic preincubation of lipoxygenase with the hydroperoxide product. In addition, we have observed no significant inactivation during anaerobic preincubation with substrate. The present results are sufficient to indicate that the following general scheme may account for the observed enzyme-dependent incomplete extents of reaction.

$$\text{E-active} + S + O_2 \rightleftharpoons [EO_2S] \begin{array}{l} \nearrow \text{E-active} + P \\ \searrow \text{E-inactive} \end{array}$$

In such a situation each mole of ES-complex has a certain probability of either forming product or destroying itself. The mechanism for these processes are currently under investigation.

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