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FACTORS AFFECTING THE INITIAL RATE OF LIPOXYGENASE CATALYSIS

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SUMMARY. The rate of peroxidation of linoleic acid by soybean type-1 lipoxygenase was studied under conditions which assured that the substrate was present as a monomolecular solution and that the first 5% of the reaction was observed. In order to achieve this, the kinetics were carried out at pH 10.0 in borate buffer using linoleic acid and enzyme concentrations of less than 75 μM and 0.2 nM respectively. The initial rate was increased by the presence of added product (13-hydroperoxy-9(Z), 11(E)-octadecadienoic acid) in the substrate solutions in a concentration dependent and saturatable fashion. Product analogues lacking the hydroperoxide group (13-hydroxy-9(Z),11(E)-octadecadienoic acid and 13-methoxy-9(Z),11(E)-octadecadienoic acid) did not evoke this rate enhancing effect. These compounds reduced the initial rate when preincubated with enzyme prior to mixing with substrate. The results indicated that the chemical reactivity of the product was a necessary requirement for its activating effect on the enzyme.

INTRODUCTION. Lipoxygenase is a catalyst for the peroxidation of methylene interrupted cis double bonds in certain naturally occurring polyunsaturated fatty acids. The enzyme is well known in plants, having been isolated in crystalline form from soybeans in 1947. Although the metabolic importance of lipoxygenase in plants has not been fully elucidated, its activity has been associated with several physiological processes and it has been detected in extracts from a variety of sources. The enzyme is no longer thought to be found only in plants. In fact, research interest in lipoxygenase has been greatly stimulated by its recently discovered involvement in various aspects of the metabolism of arachidonic acid in cells of the mamallian circulatory system. Lipoxygenase derived metabolites influence the aggregation of platelets (1), the chemotaxis of neutrophils and eosinophils (2,3), and the

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destruction of reticulocytes (4). They have also been identified as the biosynthetic precursors of slow reacting substance of anaphylaxis in mast cells (5).

In an attempt to provide novel compounds for the study of lipoxygenase pathways in both plants and animals, the synthesis of a series of stabilized product analogues for the enzyme was recently carried out in this laboratory (6). The compounds were designed to be metabolically blocked at the oxidized position by converting the hydroperoxides into the corresponding methyl ethers. These methoxy derivatives retain all of the structural features of the normal product, but lack the intrinsic reactivity of the peroxide bond. Compounds of this type may be useful in probing not only the lipoxygenase reactions, but associated peroxidase and dehydrogenase activities as well. The present report illustrates how the methoxy derivatives of polyunsaturated fatty acids can be utilized in enzyme studies by describing the effects of one such compound on the kinetics of the enzyme from which it was prepared: type-1 lipoxygenase from soybeans.

MATERIALS AND METHODS. Type-1 soybean lipoxygenase (Sigma) was purified using the ion exchange chromatographic step of the isolation reported by Finazzi-Agro *et al.* (7). Isoelectric focusing was done in polyacrylamide slabs using a horizontal electrophoresis cell (Biorad). Ampholytes (LKB) in the range pH 5-7 were employed. The purified type-1 enzyme (14 μg) appeared as a single band after isoelectric focusing and staining with Coomasie blue. Isoelectric focusing with a tenfold greater loading revealed the presence of trace amounts of three proteins with pI values closely related to the type-1 lipoxygenase. The major isoenzyme impurities in the original sample (type-2 and type-3) were completely removed by the purification procedure. The details of the preparation and purification of 13-hydroxy-9(Z), 11(E)-octadecadienoic acid and 13-methoxy-9(Z),11(E)-octadecadienoic acid have been reported elsewhere (6). Linoleic acid was obtained from Nu Chek Prep. The fatty acids were purified by high performance liquid chromatography (silica) before being used for kinetic studies. Samples were collected under an argon atmosphere and the compounds were stored packed in solid carbon dioxide as dilute, argon saturated ethanol solutions.

Kinetic studies were carried out in borate buffer (0.1 M) at pH 10.0. At this elevated pH, the enzyme was not indefinitely stable. At the start of these studies, duplicate runs were routinely carried out successively. If the duplicate sample received prolonged preincubation (while the original sample was running), absorbance values at corresponding times were always fractionally lower in the second experiment. Since the progress curves were relatively complex, no attempt was made to extrapolate these small effects

due to prolonged preincubation to zero time. Instead a strict schedule was adhered to in the preparation of the kinetic samples to insure that the effect of pH on the enzyme was the same in every instance. Fatty acid solutions were prepared by aliquot transfer of stock ethanol solutions into volumetric flasks. The solvent was removed under a stream of nitrogen and the fatty acid was dissolved in air saturated buffer. All kinetic analyses were carried out in a compartmented cell. The enzyme was preincubated either with buffer or test solution for 10 min at 25° in one compartment, while the substrate with or without modifier was present in the second compartment. The reaction was initiated by gently inverting the cuvette. The formation of product was followed spectrophotometrically at 234 nm.

RESULTS AND DISCUSSION. There appears to be no consensus in the literature concerning the kinetic features of soybean lipoxygenase catalysis. Distinctively non linear initial rates were the most prominent characteristics of early reports in this area (8). This was manifest as a kinetic lag phase which could be overcome by treatment with product. This led to the idea that the enzyme was activated chemically by the hydroperoxide. At least one complicating factor in these studies was the low critical micelle concentration for linoleic acid, the typical substrate, under the conditions of the experiments. Galpin and Allen have shown that many early kinetic results were more a reflection of the solution properties of the substrate than specific enzymic effects (9). According to Verhagen et al., the critical micelle concentration for linoleate at pH 9 is even lower than previously thought (60 μM as opposed to 150 μM) (10). Even taking account of these considerations, however, a variety of results and associated conclusions have been reported from different laboratories for experiments carried out at about the same time. Accounts of nonlinear initial rates contrast with descriptions of progress curves which are straight lines in the vicinity of the origin (11,12). A similar situation exists for the effects of modifying agents on the kinetics. For example, different properties have been ascribed to the alcohol obtained from the chemical reduction of the product. This compound has been reported to be either an activator (11) or an inhibitor of product activation (13). These observations have led to discussions concerning the role, if any, the product and alcohol play in the expression of optimal activity by the enzyme.

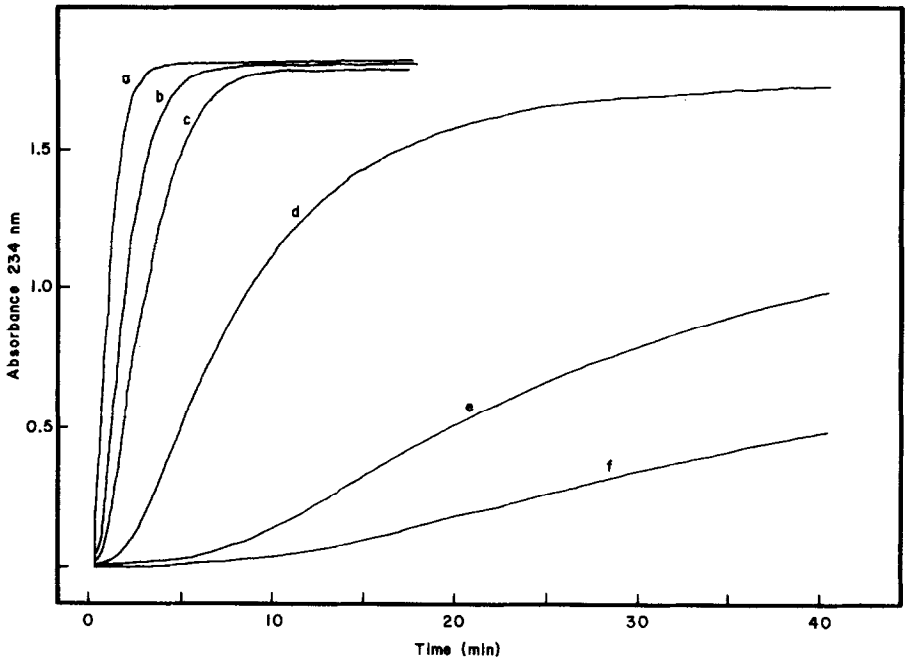

Figure 1. Progress curves of catalysis for various concentrations of type-1 lipoxygenase: a) 6.0 nM, b) 3.0 nM, c) 1.5 nM, d) 0.75 nM, e) 0.40 nM, f) 0.15 nM. Substrate, linoleic acid, 72 μM; pH 10.0, sodium borate buffer, 0.1 M; 25.0°.

That is, if the product stimulates the enzyme, is the stimulation a result of structural interactions (i.e. sensitivity to the configuration in the olefinic system), or is it due to the reactivity of the hydroperoxide bond? The stabilized product analogue, 13-methoxy-9(Z),11(E)-octadecadienoic acid, provides a means for further investigating these possibilities.

In order to control the nature of the substrate and modifier solutions, the kinetic experiments were carried out at pH 10.0. Total fatty acid concentrations were kept below the critical micelle concentration for linoleic acid in order to eliminate the possibility of aggregation. The effect of various enzyme concentrations on the time course of product formation is shown in Figure 1. These experiments established that in order to observe the initial rate of the process, it was necessary to use a substantially lower concentration of the enzyme than had been previously employed (9,11-13). At the elevated levels, the rate was sufficiently high

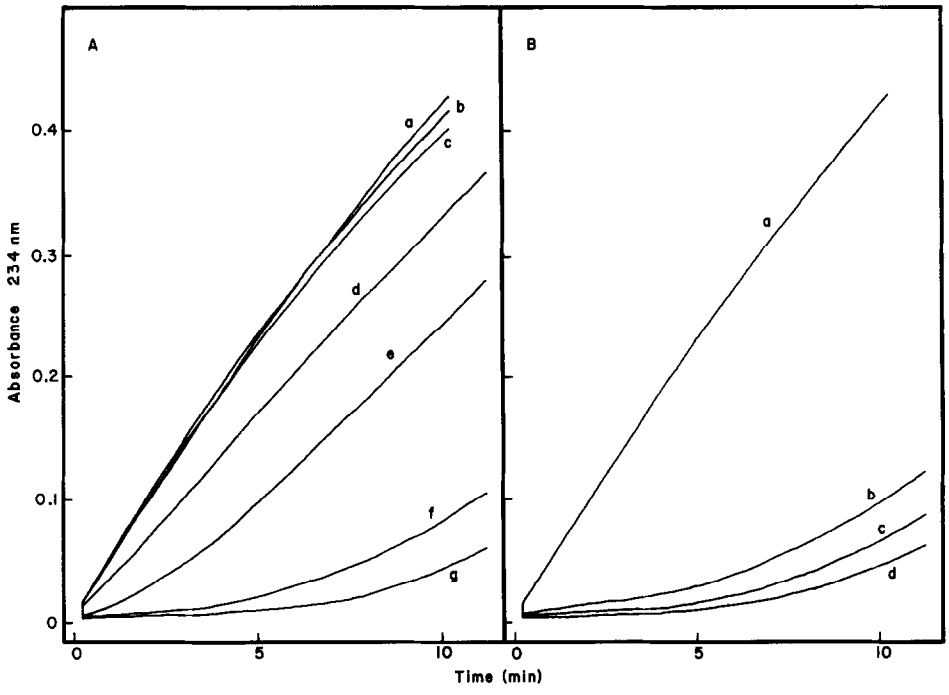

Figure 2. The instantaneous effect of product and product analogue concentrations on the initial rate of catalysis. Substrate, linoleic acid, 72 μM; enzyme, 0.15 nM; pH 10.0, sodium borate buffer, 0.1 M; 25.0°. A. Product: a) 15 μM, b) 38 μM, c) 75 μM, d) 5.0 μM, e) 1.0 μM, f) 0.50 μM, g) none. B. a) Product, 15 μM; b) ether, 15 μM; c) alcohol, 15 μM; d) none.

that the first 5-10% of reaction was completed by the time measurements had begun. In any experiment where the conventional initial portion of the reaction could be observed, the traces were distinctively non linear. The curves were sigmoidal in nature, typical of autocatalytic processes. Since this general shape could also have resulted from substrate inhibition, the effect of added product was also investigated. When present in the substrate solution before mixing with the enzyme, the instantaneous effect of the product on the initial portion of the reaction curve was to increase the rate in all cases (Figure 2). The saturating level for the activating effect of the product for the enzyme was around 10 μM under these conditions.

Catalysis was stimulated at least by a factor of 10^2 when a comparison of the true initial rate in the absence and presence of product was made.

This result indicated that a revival of the idea of product activation as a fundamental aspect of lipoxygenase kinetics was in order. Conclusions based upon measurements in the so called steady state portion of the progress curves while they may have been valid, pertained only to effects obtained after this initial event. It would appear that lipoxygenase is capable of existing in either of two distinctive forms of differing activity with the conversion between them being controlled by the product. This possibility is consistent with the observed formation of different colored forms of the enzyme upon treatment with its product (14,15). There are at least three possible explanations for the observed catalysis in the absence of added product. 1) Regardless of the experimental precautions, some autoxidation could have resulted in a low level of the product in the substrate solution before the experiment was initiated with enzyme. 2) A trace of lipoxygenase isoenzyme without a distinctive product activation may have remained in the sample even after extensive purification. 3) The enzyme itself could have catalyzed the reaction at different rates (a hysteretic effect), with a slow preliminary turnover supplying the product for activation.

Neither the alcohol nor the ether had the instantaneous activating effect on the enzyme that the product displayed (Figure 2). At concentrations in excess of those required for a saturating effect of the product, there was little or no change in the progress curves compared to untreated controls. This indicated that the presence of the peroxide bond was required in order to produce the dramatic rate enhancements seen with the product. The product analogues were not completely without effect on the enzyme system. Preincubation of either the alcohol or ether with the enzyme before treatment with a mixture of the substrate and product led to reduced rates for the progress curves in a concentration dependent fashion (Figure 3). It is interesting to note that the modifiers did not influence the ability of the product to eliminate the lag phase. Rather, they produced uniformly decreased rates without any indication of sigmoidicity. This, along with the observation that these

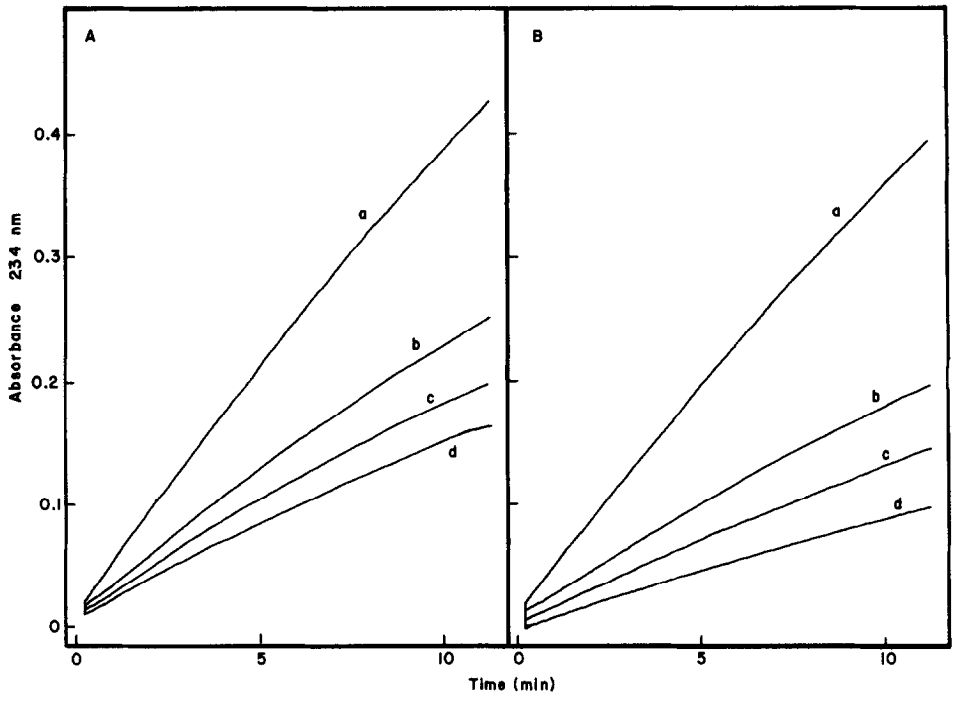

Figure 3. The effects of preincubation of the alcohol and ether on the product activated catalysis. Substrate, linoleic acid, 72 μM; enzyme, 0.15 nM; product, 15 μM; pH 10.0, sodium borate buffer, 0.1 M; 25.0°. A. Alcohol: a) none, b) 4 μM, c) 16 μM, d) 40 μM. B. Ether: a) none, b) 4 μM, c) 16 μM, d) 40 μM.

compounds do not prolong the lag phase in the absence of added product, indicated that their inhibitory effect was not a result of competition with peroxide for an activating site on the enzyme.

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