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Kinetics of Leukotriene A_4 Synthesis by 5-Lipoxygenase from Rat Polymorphonuclear Leukocytes

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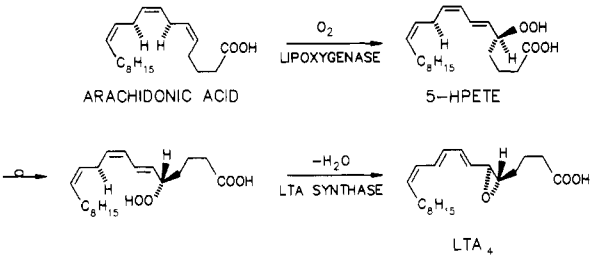
ABSTRACT: When arachidonic acid is added to lysates of rat polymorphonuclear leukocytes, it is oxidized to (5*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (5-HPETE). The 5-HPETE then partitions between reduction to the 5-hydroxyeicosanoid and conversion to leukotriene A_4 (LTA_4). Both steps in the formation of LTA_4 are catalyzed by the enzyme 5-lipoxygenase. When [^{3}H]arachidonic acid and unlabeled 5-HPETE were incubated together with 5-lipoxygenase, approximately 20% of the arachidonic acid oxidized at low enzyme concentrations was converted to LTA_4 without reduction of the specific radioactivity of the LTA_4 by the unlabeled 5-HPETE. A significant fraction of the [^{3}H]-5-HPETE intermediate that is formed from arachidonic acid must therefore be converted directly to LTA_4 without dissociation of the intermediate from the enzyme. This result predicts that even in the presence of high levels of peroxidase activity, which will trap any free 5-HPETE by reduction, the minimum efficiency of conversion of 5-HPETE to LTA_4 will be approximately 20%, and this prediction was confirmed. 5-HPETE was found to be a competitive substrate relative to arachidonic acid, so that it is likely that the two substrates share a common active site.

Leukotrienes are potent biologically active metabolites of arachidonic acid that play a role in inflammation and immediate hypersensitivity (Samuelsson, 1983; Hammarström et al., 1979). The first step in the biosynthetic pathway for leukotrienes is the oxidation of arachidonic acid to 5-HPETE[1] catalyzed by 5-lipoxygenase (Scheme I). The 5-HPETE then partitions between LTA_4 synthesis, catalyzed by LTA synthase, and reduction to 5-HETE (Samuelsson, 1983; Borgeat & Samuelsson, 1979; Skoog et al., 1986b). LTA_4 is the precursor to all of the biologically active leukotrienes, so that the synthesis of the leukotrienes depends critically on the partitioning of 5-HPETE between reduction and LTA_4 synthesis.

Scheme I



Both LTA synthase and 5-lipoxygenase activities have now been shown to be expressed by a single enzyme.[2] The two activities have been shown to copurify from four different sources: potatoes (Shimizu et al., 1984), human leukocytes (Rouzer et al., 1986), porcine leukocytes (Ueda et al., 1986), and murine mast cells (Shimizu et al., 1986). The control of the synthesis of LTA_4 is, therefore, a function of the kinetics

[1] Abbreviations: LTA_4, leukotriene A_4, (5*S*)-*trans*-5,6-oxido-7(*E*),9-(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-5,12-dihydroxy-6(*Z*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid, 5*S* unless otherwise specified; 5-HETE, (5*S*)-5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5,12-diHETEs, 6(*E*)-LTB_4 and 12-*epi*-6(*E*)-LTB_4; 5,6-diHETEs, (5*S*,6*RS*)-5,6-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; PMN, polymorphonuclear leukocytes; 5-LO, 5-lipoxygenase; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

[2] 5-Lipoxygenase will be used in this work to refer to the enzyme with both activities, which will be differentiated as the "lipoxygenase" and "LTA synthase" activities.

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of 5-lipoxygenase. The kinetics of LTA_4 synthesis from both arachidonic acid and 5-HPETE as substrates have been studied with regard to two questions. Is LTA_4 formed from arachidonic acid without release of the intermediate 5-HPETE from the enzyme, and do the lipoxygenase and LTA synthase activities of the 5-lipoxygenase comprise a single or multiple active sites?

Materials and Methods

Materials. [1-^{14}C]- and [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (which was diluted to 8.7 Ci/mmol) were purchased from New England Nuclear, and unlabeled arachidonic acid was from Nu-Chek Prep. Racemic 5-HPETE was synthesized as described (Maas et al., 1982). Arachidonic acid was purified by reverse-phase and 5-HPETE by normal-phase HPLC before use, and both were stored in ethanol at −78 °C. 5-HPETE concentrations were determined by reduction with glutathione (catalyzed by glutathione peroxidase) and subsequent titration of excess unoxidized glutathione with 5,5′-dithiobis(2-nitrobenzoic acid). The extinction coefficient of the 5-HPETE at 234 nm was calculated to be 24000 M^{-1} cm^{-1}. DEAE-cellulose was type DE-52 from Whatman Biochemicals.

Enzyme Assays. 5-Lipoxygenase was assayed as described (Skoog et al., 1986a). All assays were performed at 37 °C in 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate, 1 mM EDTA, 2 mM ATP, and 2 mM $CaCl_2$ at pH 7.0. Substrate concentrations were as indicated, and radioactive products were analyzed by HPLC with a Radiomatic Flo-One flow radioactivity detector (Skoog et al., 1986a,b). Conversion of unlabeled 5-HPETE to leukotrienes in the presence of low concentrations of [^{3}H]arachidonic acid was measured with a Kratos Spectroflow 773 absorbance detector at 270 nm. Recovery of total radioactivity was used as an internal standard, and detector response was calibrated with [^{14}C]LTB_4 and [^{14}C]-5,12-diHETEs generated enzymatically from [^{14}C]arachidonic acid of known specific activity.

HPLC peaks were identified as described (Skoog et al., 1986a,b). All products observed could be attributed to 5-lipoxygenase activity. In the absence of reducing agents as much as 40% of the 5-HPETE that is formed decomposes to unidentified products during the assay and subsequent analysis (Skoog et al., 1986b). The values given for 5-HPETE concentrations are therefore underestimated, but not to such an extent that they will affect the qualitative interpretation of the results. In the presence of glutathione, 5-HPETE is rapidly reduced by peroxidases (Skoog et al., 1986b) to 5-HETE, which is stable, and no significant amounts of other decomposition products are formed. LTA_4 cannot be measured directly since it is too unstable (Borgeat & Samuelsson, 1979). The concentration of LTA_4 has been inferred from the concentrations of the stable products derived from it. These are LTB_4 and the two isomeric 5,12-diHETEs formed by nonenzymatic hydrolysis of LTA_4. In crude lysates, which contain LTA hydrolase and synthesize LTB_4, these three products account for 80 ± 3% of the possible LTA_4-derived products. In enzyme purified on DEAE-cellulose, which does not contain LTA hydrolase, they account for 73 ± 5%. The remaining products include the isomeric 5,6-diHETEs and possibly unidentified products that comigrate by HPLC with the 5,6-diHETEs (Skoog et al., 1986b). Since all of the products in the 5,6-diHETE peak have not been positively identified, this peak was not included in the quantitation of LTA_4. This underestimation of the LTA_4 does not affect the interpretation of the results. The precision in measuring [^{3}H]LTA_4 was ±5% of the [^{3}H]LTA_4. There was an additional uncertainty in determining background radioactivity of ±0.5% of the total radioactivity, which was significant at low levels of product formation. Results for [^{3}H]LTA_4 formation are presented only if the amounts of LTA_4 are >2% of the total radioactivity. The uncertainty in measuring [^{1}H]LTA_4 from [^{1}H]-5-HPETE was ±0.02 μM (compared, for example, to 11 μM total racemic [^{1}H]-5-HPETE used in the experiment of Figure 1). Oxidation of arachidonic acid is not quantitative in some experiments, even at long reaction times, because the 5-lipoxygenase is unstable and loses activity during the assay.

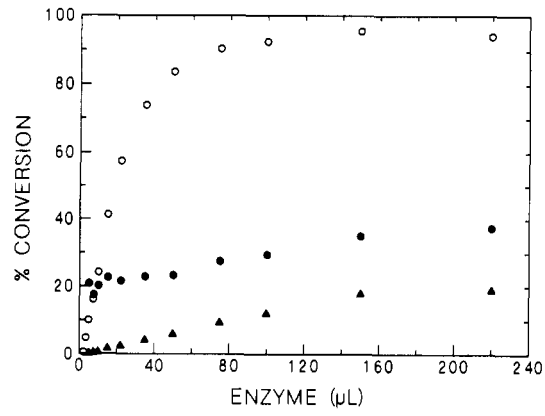


FIGURE 1: Simultaneous conversion of [^{3}H]arachidonic acid and [^{1}H]-5-HPETE to LTA_4. Rat PMN 15000*g* supernatant was partially purified on DEAE-cellulose to remove reducing agents, peroxidases, and contaminating arachidonic acid. [^{3}H]Arachidonic acid, 0.082 μM, and racemic [^{1}H]-5-HPETE, 11.4 μM, were added simultaneously to enzyme to initiate the reaction, which was quenched and analyzed after 5 min. Enzyme concentrations refer to microliters of enzyme, 0.7 mg/mL, in a final 0.5-mL assay volume. (○) Percent of [^{3}H]-arachidonic acid oxidized. (▲) Percent of [^{1}H]-5-HPETE converted to LTA_4, corrected by factor of 2 since the 5-HPETE was racemic. (●) Percent of the [^{3}H]HPETE initially formed from [^{3}H]arachidonic acid that was further converted to [^{3}H]LTA_4.

Protein was measured by the method of Schaffner and Weissman (1973) with bovine serum albumin as standard.

Purification of 5-Lipoxygenase. All buffers contained *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate, pH 7.0, 1 mM EDTA, and 5 mM dithiothreitol with NaCl added as indicated. The starting material, which is referred to as the lysate, was the 15000*g* supernatant from sonicates of rat peritoneal PMN (Skoog et al., 1986a). The lysate, 110 mg of protein in 14 mL, was applied to 10 mL of DEAE-cellulose (2.5 × 2 cm) with stirring of the top half of the column to maintain flow rates. The column was washed with 3 mL of buffer and 50 mL of 0.075 M NaCl, and the activity was eluted with 25 mL of 0.175 M NaCl. Recovery of activity was typically 50% with 3-fold purification. This partial purification removed typically 98% of the peroxidase activity in the lysate. The peroxidase activity was inferred from the steady-state levels of 5-HPETE observed in experiments such as that in Figure 2.

Desalting of the lysate on Sephadex G-50 was as described (Skoog et al., 1986b) and typically removed >99% of the endogenous glutathione.

Results

Oxidation of [^{3}H]Arachidonic Acid in the Presence of 5-HPETE. The experiment presented in Figure 1 was designed to test the possibility that arachidonic acid can be converted to LTA_4 without dissociation of the intermediate 5-HPETE to solution. [^{3}H]Arachidonic acid, 0.082 μM, was added simultaneously with 11 μM unlabeled 5-HPETE to enzyme, and the appearance of both [^{3}H]- and [^{1}H]LTA_4 was monitored. In this experiment, the crude rat PMN lysate was

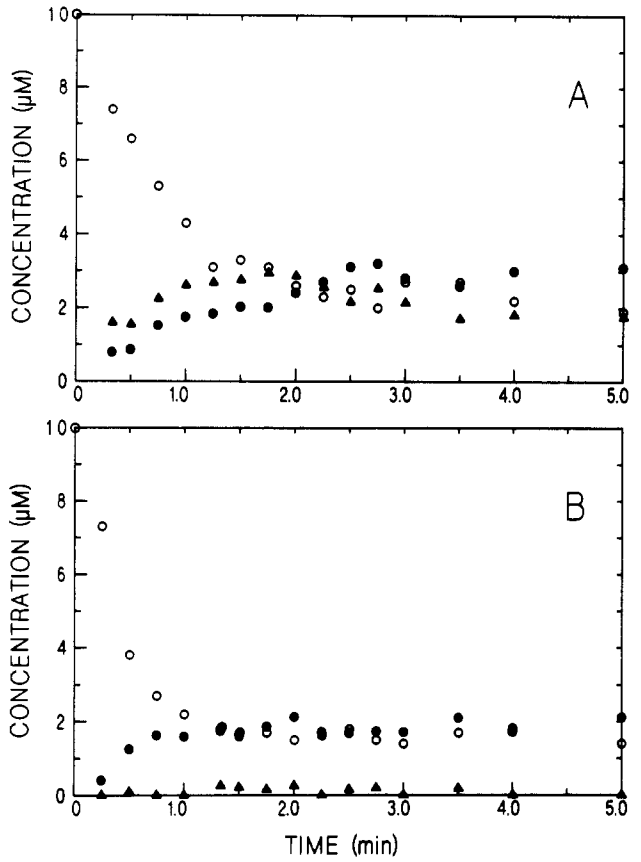


FIGURE 2: Time courses for 5-HPETE and LTA_4 synthesis in the presence and absence of reducing agents. The 15000*g* supernatant of rat PMN lysates was chromatographed on Sephadex G-50 to remove glutathione and other endogenous reducing agents. The time courses for the loss of 10 μM arachidonic acid (○) and formation of 5-HPETE (▲) and LTA_4 (●) are shown. (A) No reducing agent present; (B) 0.2 mM glutathione added. The protein concentration in both experiments was 0.6 mg/mL.

partially purified on DEAE-cellulose before use. This purification removed glutathione and glutathione-dependent peroxidases, so that the 5-HPETE was stable over the assay period. It also removed arachidonic acid, which is present in the lysate at a concentration of approximately 10 μM. The two substrates were incubated with varying enzyme concentrations for 5 min, and the reaction was then quenched and assayed for product formation. [^{3}H]-5-HPETE is assumed to account for all of the initial product formed from [^{3}H]arachidonic acid (Skoog et al., 1986b), and formation of [^{3}H]LTA_4 is expressed as the percent of the initially formed [^{3}H]-5-HPETE that is further converted to LTA_4. Conversion of [^{1}H]-5-HPETE to [^{1}H]LTA_4 is also expressed as percentage of the [^{1}H]-5-HPETE initially present and is corrected by a factor of 2 since the [^{1}H]-5-HPETE used is racemic.

If the [^{3}H]-5-HPETE initially formed is released to solution before it is converted to LTA_4, then it must mix with the [^{1}H]-5-HPETE and cannot be converted to LTA_4 more rapidly than the unlabeled 5-HPETE. If, however, [^{3}H]-5-HPETE is committed to LTA_4 synthesis before it dissociates from the enzyme, then synthesis of [^{3}H]LTA_4 will be independent of [^{1}H]LTA_4 synthesis. The results of Figure 1 clearly indicate that the latter is the case. At low enzyme concentrations approximately 20% of the [^{3}H]-5-HPETE is converted to LTA_4 with no detectable conversion of [^{1}H]-5-HPETE. At higher enzyme concentrations, synthesis of [^{3}H]LTA_4 increases, and this is accompanied by a parallel formation of [^{1}H]LTA_4. At these high enzyme concentrations, therefore, there must be sufficient LTA synthase activity that conversion

Table I: Inhibition of 5-Lipoxygenase by 5-HPETE[a]

HPETE (μM)	[^{3}H]LTA_4 (% of product at $E = 0$)[b]	lipoxygenase activity, $E_{1/2}$ (μL)[c]	V_{max}/K_m ratio[d]
0	18	2.9	
3.8	20	8.6	31
7.1	18	20	34
11.4	20	16	32
19.0	22	58	
21.6	15	52	31

[a] [^{3}H]Arachidonic acid and varying concentrations of unlabeled 5-HPETE were incubated together with 5-lipoxygenase as described for Figure 1. [b] Conversion of [^{3}H]arachidonic acid to [^{3}H]LTA_4 at limiting low enzyme concentrations was determined by extrapolation of the percent conversion of [^{3}H]-5-HPETE to [^{3}H]LTA_4 to zero enzyme concentration with data plotted as in Figure 1 (closed circles). [c] 5-Lipoxygenase activity for oxidation of [^{3}H]arachidonic acid was determined from plots as in Figure 1 and expressed as $E_{1/2}$ values (see text). [d] V_{max}/K_m values for [^{3}H]arachidonic acid oxidation compared to conversion of unlabeled 5-HPETE to LTA_4 are the ratios of the slopes of the lines for data plotted as in Figure 3 (see text).

of the 5-HPETE that is free in solution to LTA_4 becomes significant.

Similar results have been obtained at arachidonic acid concentrations from 0.04 to 4 μM and 5-HPETE concentrations from 0 to 21 μM and with crude lysate.

Synthesis of LTA_4 Is Insensitive to Rate of Reduction of 5-HPETE. When sonicates of rat PMNs are desalted on Sephadex G-50 to remove reducing agents, it is possible to observe high levels of 5-HPETE as an intermediate in the transformation of arachidonic acid to LTA_4. The steady-state level of 5-HPETE can then be modulated by addition of a reducing agent, such as glutathione, back to the enzyme (Skoog et al., 1986b). In this way it is possible to compare the rate of LTA_4 synthesis to the steady-state concentration of 5-HPETE (Figure 2). The filled triangles in Figure 2 represent the detectable 5-HPETE concentration, and the filled circles represent LTA_4. The results in Figure 2A show that 5-HPETE accumulates to a maximum concentration of as much as 3 μM in the absence of glutathione when the initial concentration of arachidonic acid is 10 μM. In contrast, in the presence of glutathione (Figure 2B) no 5-HPETE is detected within the limits of sensitivity (0.1 μM). There is little difference in the initial rate of LTA_4 synthesis under these two conditions, despite the large difference in 5-HPETE concentration. In the first minute, during which time 70–80% of the arachidonic acid is turned over, approximately 2 μM LTA_4 is synthesized in both panels A and B of Figure 2. In the absence of glutathione, conversion of 5-HPETE to LTA_4 continues from 2 to 5 min, but at a rate that is significantly slower than the initial rate during the period when oxidation of arachidonic acid is occurring (Figure 2A). For example, compared to 2 μM LTA_4 formed in the first minute of reaction, an increase in concentration of only 1 μM is observed in the subsequent 4 min.

Effect of 5-HPETE Concentration on Lipoxygenase and LTA Synthase Activities. In a series of experiments similar to that of Figure 1, it was observed that 5-HPETE inhibits the oxidation of arachidonic acid. The effects of 5-HPETE on the lipoxygenase and LTA synthase activities were therefore compared in order to determine whether the effect is equal for both activities. The results are presented in Table I. As an estimate of lipoxygenase activity, we chose the amount of enzyme required to give 50% oxidation of arachidonic acid, hence the $E_{1/2}$ values of Table I (see also footnote 3). The

$E_{1/2}$ values were corrected by substracting the observed "threshold concentration" of enzyme below which no turnover of arachidonic acid was observed. An increase in $E_{1/2}$ indicates inhibition, and the observed values span a 20-fold range.

In Figure 1 it was shown that approximately 20% of the 5-HPETE that is formed from arachidonic acid is converted to LTA_4 before it can dissociate from the enzyme. It is evident from the data in the second column of Table I that the fraction of [^{3}H]-5-HPETE converted to LTA_4 is not dependent on the concentration of exogenous unlabeled 5-HPETE.

Rat neutrophil 5-lipoxygenase, in common with the other mammalian lipoxygenases, is unstable under the assay conditions and loses activity with a half-life of approximately 1 min. In addition, there is normally an initial lag in the turnover of arachidonic acid, which is also a general phenomenon for the lipoxygenases. For these reasons it is difficult to measure initial rates and to characterize the inhibition by 5-HPETE by classical steady-state kinetic techniques. We have taken advantage, therefore, of competitive techniques to measure relative rate constants for turnover of arachidonic acid and 5-HPETE. These techniques are insensitive to the kinetic lag and loss of enzyme activity. The analysis is analogous to the measurement of isotope effects by competition methods (O'Leary & Baughn, 1972). For two substrates S_1 and S_2 that correspond to arachidonic acid and 5-HPETE, respectively, substrate turnover is defined by eq 1–3. In these equations

$$\frac{d[S_1]}{dt} = -V_1[ES_1] \quad (1)$$

$$\frac{d[S_2]}{dt} = -V_2[ES_2] - V_2'[E'S_2] \quad (2)$$

$$K_1 = \frac{[E][S_1]}{[ES_1]} \qquad K_2 = \frac{[E][S_2]}{[ES_2]} \qquad K_2' = \frac{[E'][S_2]}{[E'S_2]} \quad (3)$$

ES_1 and ES_2 represent binary complexes between enzyme and the two substrates, E′ represents enzyme inhibited for turnover of arachidonic acid, and $E'S_2$ represents a possible productive complex between inhibited enzyme and 5-HPETE. In our case, the concentration of arachidonic acid is very low, and the 5-HPETE concentration is high (see below) relative to the K_m values so that these limiting conditions were used to simplify the derivations. Calculating $d[S_1/d[S_2]$ and integrating gives the result in eq 4. The superscript 0 denotes initial concentration.

$$\frac{\ln([S_1]/[S_1^0])}{\ln([S_2]/[S_2^0])} = \frac{V_1/K_1}{V_2/K_2}\left(1 + \frac{V_2'[E_T']}{V_2[E_T]}\right)^{-1} \quad (4)$$

[3] There is theoretical justification for plotting log (percent unreacted arachidonic acid) vs. enzyme concentration as in Figure 3, although this is not critical to the interpretation of the results. The observed loss of enzyme activity under our assay conditions is first order in enzyme and independent of arachidonic acid concentration in the range 0.04–1 μM. Since the concentration of arachidonic acid is below its K_m (Aharony & Stein, 1986), the oxidation of arachidonate should be first order with respect to both substrate and enzyme. Having thus defined the form of the rate equations for loss of enzyme activity and substrate turnover, it is straightforward to derive that plots of log [arachidonate] vs. [enzyme] should be linear and that the slope of the line should be proportional to V_{max}/K_m for arachidonic acid oxidation and inversely proportional to the rate of loss of enzyme activity. The values of $E_{1/2}$ presented in Table I are equal to (log 2)/slope. The deviation from linearity at high enzyme concentrations is due at least in part to the presence of impurities, probably stereoisomers, present in the arachidonic acid. These impurities comigrated with arachidonic acid on HPLC but were not substrates for either 5-lipoxygenase or soybean lipoxygenase. There is a threshold enzyme concentration below which no turnover of arachidonic acid is observed, but the cause of this phenomenon is unclear.

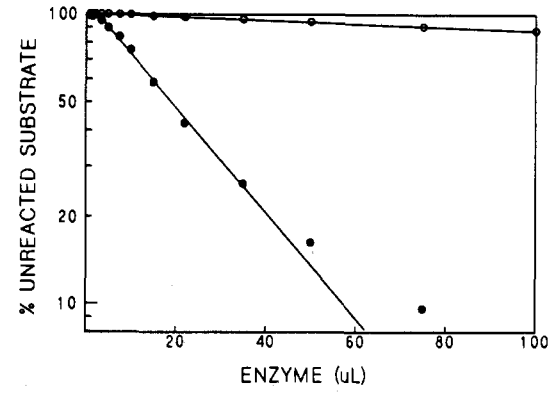
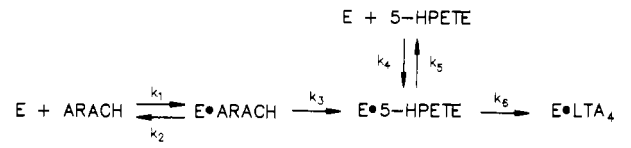


FIGURE 3: Determination of 5-lipoxygenase and LTA synthase activities in mixtures of [^{3}H]arachidonic acid and 5-HPETE. The data are taken from Figure 1. Points are percent (●) [^{3}H]arachidonic acid and (○) unlabeled 5-HPETE remaining after 5-min reaction in the presence of varying enzyme concentrations.

Equation 4 shows that the ratio of $\ln([S_1]/[S_1^0])$ to $\ln([S_2]/[S_2^0])$ at any point in the reaction will be proportional to the V_{max}/K_m values for the two substrates. If 5-HPETE affects both the lipoxygenase and 5-HPETE activities equally, then $V_2' = 0$, and no change in the V_{max}/K_m ratios will be observed. Otherwise, the apparent V_{max}/K_m ratio will vary as the 5-HPETE concentration, and therefore the $[E_T']/[E_T]$ ratio, is increased. The required values of log (fraction unreacted substrate) can most easily be determined from the slopes of the lines obtained when the data are plotted as in Figure 3, and the results are presented in the last column of Table I. There is clearly no dependence of the result on the initial concentration of 5-HPETE. With the data of Table I the analysis is sensitive enough to detect as low as 1% reaction of 5-HPETE via the inhibited enzyme relative to active enzyme (i.e., if the V_2'/V_2 ratio is 0.01 and the $[E_T']/[E_T]$ ratio is 20, then the apparent V_{max}/K_m ratio would change by an easily detectable factor of 1.2 from the value in the absence of added 5-HPETE). Also, these arguments are symmetrical in that oxidation of arachidonic acid by enzyme inhibited for LTA synthesis would also be detected.

The above arguments do not depend on the type of inhibition that the 5-HPETE produces and would be valid for either reversible or irreversible inhibition. It is possible to show that a significant fraction of the observed inhibition is reversible, however. Although 5-lipoxygenase loses activity rapidly and irreversibly during an assay, at high enzyme concentrations the rate of substrate turnover can be faster than the rate of irreversible loss of enzyme activity. For example, at enzyme concentrations sufficient to give at least 95% conversion of arachidonic acid, the rate of turnover is at least 3 times faster than the rate of loss of activity. By using high enzyme concentrations, therefore, it is possible to measure the reversible component of the inhibition independent of any irreversible component. Arachidonic acid turnover was measured at high enzyme levels at 2 and 10 μM 5-HPETE, concentrations at which the $E_{1/2}$ values differed by a factor of 2.9. Turnover of arachidonic acid was first order for at least two half-lives, and the measured half-lives were 5.2 and 12.3 s, respectively, a difference of a factor of 2.4. The inhibition by 5-HPETE is therefore largely reversible in that the differences in $E_{1/2}$ values can be almost entirely accounted for by differences in the rate of substrate turnover rather than the rate of loss of enzyme activity. Although the reversible inhibition has not been further characterized as competitive as opposed to noncompetitive or uncompetitive, etc., it is reasonable to assume that it is competitive. Since 5-HPETE is a substrate for

Scheme II



5-lipoxygenase, it is probable that the 5-HPETE follows saturation kinetics and that the binding that is observed is binding at the LTA synthase active site to form the Michaelis complex.

Discussion

The study of [^{3}H]LTA_4 formation from [^{3}H]arachidonic acid in the presence of unlabeled 5-HPETE revealed that a significant fraction (19%) of the [^{3}H]-5-HPETE intermediate is converted to [^{3}H]LTA_4 before it dissociates from the enzyme (Figure 1). This conclusion is based on the observation that the specific radioactivity of the [^{3}H]LTA_4 formed at low enzyme concentrations was not diluted by the added unlabeled 5-HPETE. This finding, incidentally, is kinetic evidence that 5-lipoxygenase and LTA synthase activities must be expressed by a single enzyme and corroborates the observation that these two activities copurify (Shimizu et al., 1984, 1986; Rouzer et al., 1986).

5-Lipoxygenase will also convert free 5-HPETE to LTA_4, but this reaction is relatively slow. The inefficiency of free 5-HPETE as a substrate has been noted qualitatively previously (Rouzer et al., 1986). The results in Table I indicate that the difference in V_{max}/K_m for oxidation of arachidonic acid compared to conversion of free HPETE to LTA_4 is a factor of 32. The following argument demonstrates that this difference in rates reflects a slower rate of association of 5-HPETE with the enzyme. The relative V_{max}/K_m values for the two substrates are determined by the rate of substrate binding and the partitioning of bound substrate between dissociation and reaction to give product (Scheme II, eq 5),

$$\frac{k_1 k_3}{k_2 + k_3} = 32\frac{k_4 k_6}{k_5 + k_6} \qquad (5)$$

assuming the chemical steps in both reactions are irreversible.

The lipoxygenase reaction involves abstraction of a proton (or hydrogen atom), and a primary kinetic isotope effect on V_{max}/K_m has been demonstrated for the reaction [soybean lipoxygenase, Hamberg (1984) and Egmond et al. (1973); platelet 12-lipoxygenase, Hamberg and Hamberg (1980)]. This indicates that the chemical step is at least partly rate determining, i.e., $k_2 \geq k_3$. We have measured the partitioning of bound 5-HPETE between dissociation and converson to LTA_4 (Figure 1, Table I), and the observed product ratio equals the ratio of the respective rate constants, $k_5 = 4k_6$. By substitution of these relative rate constants into eq 5, it can be calculated that the rate constant for binding of arachidonic acid to the enzyme is at least 13-fold faster than the rate of binding of 5-HPETE ($k_1 > 13k_4$).

Since both 5-lipoxygenase and LTA synthase reactions are expressed by a single enzyme, the question arises whether the two activities are expressed by the same or different active sites. Preliminary results are available that are consistent with but do not prove a single active site. Thus, both activities require Ca^{2+} and ATP for activity, and the two activities are lost in parallel upon heat treatment or incubation with arachidonic acid (Rouzer et al., 1986; Shimizu et al., 1986; Ueda et al., 1986; Jakschik & Kuo, 1983). In general, the observation of parallel inhibition of two activities by competitive inhibitors is considered a useful criterion that the two activities are expressed by a single active site. It has been demonstrated that known inhibitors of 5-lipoxygenase do inhibit both activities (Ueda et al., 1986; Shimizu et al., 1986). The mechanisms of inhibition of 5-lipoxygenase by the inhibitors used—cirsiliol, 5,6-methano-LTA_4, and AA-861—however have not been extensively characterized. In addition, because of the complexity of the kinetics of the 5-lipoxygenase reaction, it is not straightforward even to measure the enzyme activity. It is difficult, therefore, to interpret the available data quantitatively. For these reasons, we have chosen to compare in detail the effects of 5-HPETE on the two activities, since this peroxide must bind at the active site for both activities. In addition, we have used competitive methods, i.e., measuring both activities with both substrates present at the same time, in order to avoid the complexities of the steady-state kinetics of the enzyme. The results (Table I) show that the lipoxygenase activity of 5-lipoxygenase was inhibited by up to 95% over the concentration range of 5-HPETE studied but the apparent V_{max}/K_m values for the lipoxygenase relative to the LTA synthase activities varied by less than 3% over this range. This result is interpreted (see Results) to show that binding of 5-HPETE to the LTA synthase active site blocks reaction of arachidonic acid at the lipoxygenase active site and is evidence for a single active site.

The partitioning in arachidonic acid turnover between release of 5-HPETE and LTA_4 synthesis was also quantitated as a function of the concentration of added 5-HPETE. If there were only one active site so that binding of 5-HPETE would be competitive with arachidonic acid, then arachidonic acid would only react with free enzyme, and no effect of 5-HPETE on the partitioning would be observed. If there were two active sites so that 5-HPETE would not necessarily be competitive, then binding of added 5-HPETE to the LTA synthase site might block LTA_4 synthesis but not oxidation of arachidonic acid. The partitioning would then vary in favor of 5-HPETE release. The observation that there is no dependence of the partitioning on 5-HPETE concentration is, therefore, a further indication that 5-HPETE and arachidonic acid bind competitively and evidence for a single active site.

The efficiency of LTA_4 synthesis is determined first by the partitioning of 5-HPETE between dissociation from 5-lipoxygenase and conversion to LTA_4. Ultimately, however, the efficiency is determined by the rate of reuptake of free 5-HPETE by 5-lipoxygenase relative to the rate of its reduction. The major pathway for reduction of 5-HPETE appears to be via glutathione-dependent peroxidases (Skoog et al., 1986b), and the effect of this reducing agent on LTA_4 synthesis is presented in Figure 2. Unpurified 5-lipoxygenase was used in this experiment with the expectation that the in vitro conditions would most closely resemble the situation in whole cells. As expected, an initial rapid formation of LTA_4 equal to about 20% of the products was observed. Further slow conversion of 5-HPETE to LTA_4 was observed in the absence of glutathione but was blocked in its presence. Quantitatively similar partitioning has also been observed for synthesis of LTA_4 in lysates of human and guinea pig PMN (data not shown). The data in Figure 2, therefore, predict that the intracellular peroxidase activity should be sufficiently high relative to 5-lipoxygenase activity that the efficiency of LTA_4 formation from arachidonic acid should be only approximately 20% in intact PMNs. This is not the case, however. The efficiency of formation of LTA_4 from endogenous arachidonic acid in human neutrophils has been shown to be 60–80% (Williams et al., 1985; Sun & McGuire, 1984; de Laclos et al., 1984), and we have found a similar value for metabolism of exogenous arachidonic acid by rat neutrophils (Skoog et al., 1986a). To

the extent that a comparison of the lysed and intact cell systems is valid, there are several possible explanations for this discrepancy. It is possible that the level of 5-HPETE generated is sufficient to deplete glutathione, the principal reducing agent for peroxides. This is unlikely, however, because the intracellular concentration of glutathione is about 5 mM (Egan & Gale, 1985) and the total intracellular concentration of 5-lipoxygenase products from endogenous arachidonic acid reaches only about 0.08 mM (Sun & McGuire, 1984; calculated assuming 5×10^8 cells/g wet weight). It is possible that the 5-HPETE that is formed in intact cells is segregated from the peroxidases so that the rapid rate of reduction of 5-HPETE in vitro is not indicative of the in vivo rate. It is also possible that the intrinsic partitioning of 5-HPETE on the enzyme between dissociation and conversion to LTA_4 is different for 5-lipoxygenase in its native intracellular state and the relative inefficiency observed in vitro is an artifact. We cannot at present provide evidence for either of these possibilities.

Registry No. LTA_4, 72059-45-1; 5-HPETE, 70608-72-9; 5-LO, 80619-02-9; arachidonic acid, 506-32-1.

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Purification and Amino Acid Sequence of Chicken Liver Cathepsin L

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ABSTRACT: Cathepsin L was purified from chicken liver lysosomes by a two-step procedure. Cathepsin L exhibited a single band of M_r 27 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, presented a high affinity for the substrate Z-Phe-Arg-NMec, was very unstable at neutral pH, and was inhibited by Z-Phe-Phe-CHN_2. The complete amino acid sequence of cathepsin L has been determined and consists of 215 residues. The sequence was deduced from analysis of peptides generated by enzymatic digestions and by chemical cleavage at methionyl bonds. Comparison of the amino acid sequence of cathepsin L with those of rat liver cathepsins B and H and papain demonstrates a striking homology among their primary structures.

Lysosomal proteinases play an important role in intracellular protein catabolism (Barrett & Kirschke, 1981). Among lysosomal proteinases, cathepsin L (EC 3.4.22.15), because of its high proteolytic activity, probably makes a major contribution to intralysosomal proteolysis. Cathepsin L has been purified from rat (Kirschke, 1977), rabbit (Okitani et al., 1980; Mason et al., 1984), chicken (Wada & Tanabe, 1986), and human (Pagano & Engler, 1982; Mason et al., 1985) liver and exhibits general characteristics including high affinity for the substrate Z-Phe-Arg-NMec,[1] inhibition by Z-Phe-Phe-CHN_2,

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