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Competitive inhibition of the 5-lipoxygenase-catalysed linoleate oxidation by arachidonic and 5-hydroperoxy-eicosatetraenoic acids

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Abstract Linoleic and arachidonic acids are competing substrates for 5-lipoxygenase from barley. When these two substrates are added simultaneously, arachidonic acid acts as a competitive inhibitor of linoleic acid oxidation with K_i of 20 μM, the same value as the Michaelis constant for arachidonate oxygenation by this enzyme (22 ± 3 μM). Linoleic acid hydroperoxide accumulated in the reaction mixture does not inhibit the enzymatic process, while arachidonic acid hydroperoxy product (5-hydroperoxy-6,8,11,14-eicosatetraenoic acid) inhibits it with very low K_i equal to 0.5 μM.

Key words: 5-Lipoxygenase; Linoleic acid; Arachidonic acid

1. Introduction

5-Lipoxygenase (5-LO) catalyses the oxygenation of linoleic acid to 9(*S*)-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9-HPODE) and arachidonic acid to 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) [1,2]. In plants, lipoxygenases have been involved in wounding, disease resistance [3,4] and in the formation of products having characteristic aroma [5].

Although the predominant polyunsaturated fatty acids of plants are generally linoleic and linolenic acids, many other fatty acids, including eicosanoid fatty acids, can serve as substrates. The barley enzyme, 5-lipoxygenase, can convert arachidonic acid with the activity about 15% of that found for linoleic acid as substrate [6].

The use of substrate mixtures to characterise the enzymatic mechanism is of special interest for lipoxygenase enzymes oxidising many fatty acids in plants and animals. The object of the present work is to characterise the 5-lipoxygenase from ungerminated barley in the reaction with two lipid substrates, linoleic and arachidonic acids.

2. Materials and methods

2.1. Isolation of 5-lipoxygenase

5-LO was isolated from barley grains, variety Risk, by the method described previously [7] with some modifications. The crude extract was fractionated with ammonium sulphate (30–60%) and subjected to CM- and DEAE-Sepharose (Pharmacia) chromatography. A column (2.6 × 20 cm) of CM-Sepharose was equilibrated with 0.04 M sodium acetate buffer, pH 4.8 and, after application of the sample, eluted with a linear gradient of sodium acetate (0.04–1 M). A column (1.6 × 20 cm) of DEAE-Sepharose was equilibrated with 0.01 M sodium phosphate buffer, pH 6.8, and, after application of the sample, eluted with a linear gradient of NaCl (0–0.6 M) in the same buffer. The fractions containing 5-LO were collected and kept at +4°C. The specific activity of the enzyme in reaction with 100 μM linoleate was about 50 μmol/min mg.

2.2. Assay of 5-lipoxygenase activity

The activity of the enzyme was assayed spectrophotometrically as described [8]. All kinetic measurements were done using a Shimadzu spectrophotometer in open quartz cuvettes at 25°C in the presence of 0.02% of Lubrol PX. Increase in absorbance at 235 nm was monitored, and an extinction coefficient of 25 $mM^{-1} \cdot cm^{-1}$ was used for quantitation of hydroperoxy products. One unit of lipoxygenase activity corresponds to formation of 1 μmol of HPODE per min in a reaction mixture containing 100 μM linoleate, 0.02% Lubrol PX and 0.2–1 μg/ml of 5-LO in 50 mM sodium phosphate buffer, pH 6.8 at 25°C.

2.3. Assay of 5-lipoxygenase with a mixture of two substrates

The reaction mixture containing 50 mM sodium phosphate buffer pH 6.8, 0.02% of Lubrol PX, 5 mE/ml (1 μg/ml) of barley 5-LO and arachidonate was preincubated for varied time, and the formation of 5-HPETE was monitored spectrophotometrically. Following the preincubation, 40 μM of linoleate was added and the 5-LO-catalysed linoleate oxidation in the presence of arachidonate and its product 5-HPETE was recorded.

2.4. Chemicals

Linoleic and arachidonic acids and Lubrol PX were Sigma products. CM- and DEAE-Sepharose were from Pharmacia.

3. Results and discussion

3.1. Substrate specificity and pH-effects in the 5-lipoxygenase catalysis

The pH optimum for linoleic acid oxygenation is 6.8, and the activity does not drop markedly at higher or lower acidity. The optimum pH value does not change when substrate concentration is varied or different concentrations of a nonionic detergent Lubrol PX are added (data not shown).

Arachidonic acid is metabolished by this enzyme less effectively (the ratio of activities towards 60 μM of linoleic and arachidonic acids is about 20 at pH 6.8), and the pH optimum of arachidonic acid oxidation exhibits a dependence on the concentration of this fatty acid substrate (Fig. 1). Similar shifts in the optimum pH observed for arachidonate oxygenation by human 5-LO [9] were explained in terms of a local pH change in the process of utilising highly amphiphilic substance like arachidonate; it is assumed that the medium around the substrate is acidified at increasing substrate concentration. We used this concept to analyse our data and converted the observed velocities of arachidonate oxygenation to intact activities at different arachidonate concentrations. The data had been transformed as described [9], using the equations

$$\log(V_{obs}) = \log(V_{int}) - \log(1 + [H^+]/K_{d1} + K_{d2}/[H^+]),$$
$$pH_{obs} = (pK_{d1} + pK_{d2})/2$$

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Abbreviations: 5-LO, 5-lipoxygenase; 9-HPODE, 9(*S*)-hydroperoxy-10*E*,12*Z*-octadecadienoic acid; [5-HPETE], 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LA, linoleic acid; AA, arachidonic acid.

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where $pK_{d2} - pK_{d1} = 0.45$, as determined from the observed pH profiles (Fig. 1). V_{obs} is the observed and V_{int} is the intrinsic, pH-independent velocity, K_{d1} and K_{d2} are the dissociation constants for protonated groups at the active site. The calculated intrinsic velocities as a function of arachidonate concentration are shown in Fig. 2B. The intact activity of barley 5-lipoxygenase with arachidonic acid is about 25% of that with linoleic acid. The data at 5–50 μM arachidonate fit well to the Michaelis-Menton equation (Fig. 2C) and give K_m value of 22 ± 3 μM.

3.2. *Activation and inhibition of the enzyme by fatty acid substrate hydroperoxides*

Linoleic and arachidonic acids are competing substrates for 5-LO from barley. When added together to the enzyme, they give a mixture of products, 9-HPODE and 5-HPETE. The extinction coefficients for 5-HPETE and 9-HPODE are equal, allowing one to determine the total concentration of the lipoxygenase oxidation products in the presence of both substrates in the reaction mixture. When the reaction is started with two substrates added simultaneously, arachidonic acid acts as a competitive inhibitor of linoleic acid oxidation (Fig. 3) with K_i of about 20 μM. When added several minutes before linoleate, arachidonic acid can serve both as an activator and an inhibitor of the 5-LO oxygenation of linoleic acid (Fig. 4). What is the reason for this difference?

Fatty acid hydroperoxides are known to be activators of lipoxygenases [10]. The progress curves for the dioxygenation reactions show a characteristic increase in rate immediately after the start, due to accumulation of the hydroperoxy products. Fig. 4 illustrates the reaction rate dynamics associated with accumulation of 5-HPETE in reaction mixture. Low concentrations of 5-HPETE activate 5-lipoxygenase, whereas at concentration above 1–2 μM it inhibits the enzyme; hence the reaction rate gradually decreases with increasing product concentration. Dixon replot of the data (Fig. 4) gives K_i of 0.5 μM for the binding of one 5-HPETE molecule to the enzyme. The same inhibition constant was obtained for other concentrations of arachidonic acid (data not shown).

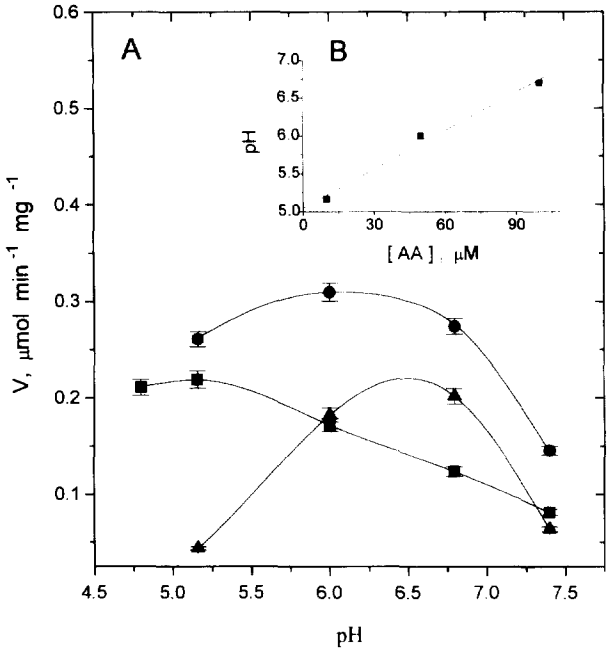

Fig. 1. (A) pH dependence of 5-HPETE synthetase activity measured with 10 μM (■), 50 μM (●) and 100 μM (▲) of arachidonate. The buffers used were 50 mM MES pH 5–6; 50 mM sodium phosphate pH 6–7; 50 mM Tris-HCl pH 7–8. The concentration of 5-LO was 45 mE/ml. (B) Dependence of the optimum pH value on arachidonate concentration. The solid line is a computer fit to the linear equation pH = 5.04 + 0.17[AA] ($R^2 = 0.99$).

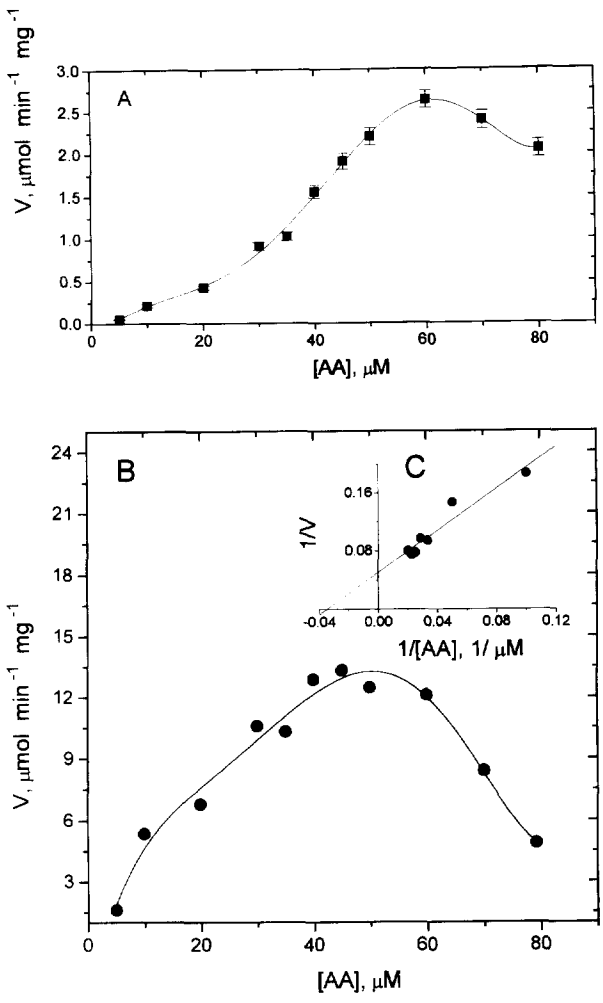

Fig. 2. (A) Initial rates of 5-LO catalysed oxidation of arachidonate as a function of substrate concentration. 5-LO concentration was 36 mE/ml. (B) Intrinsic velocity of the 5-LO-catalysed oxidation of arachidonate as a function of arachidonate concentration. (C) A double-reciprocal replot of Fig. 2B.

Complex interplay between substrates and products in lipoxygenase catalysis gives rise to the situation when the reaction velocity depends on the sequence in which the substrates are added to the enzyme. When the substrates are added simultaneously, they both are converted to corresponding products, but arachidonate is converted much slower at the pH optimal for linoleate. In this case, the activating hydroperoxide is 9-HPODE. In the presence of arachidonic acid, the enzymatic conversion of linoleate is inhibited with K_i of 20 μM, the value equal to the Michaelis constant for arachidonic acid. Arachidonic acid, not 5-HPETE, inhibits the overall reaction rate by competing with linoleic acid for the active site.

In the absence of arachidonic acid and 5-HPETE, the rate

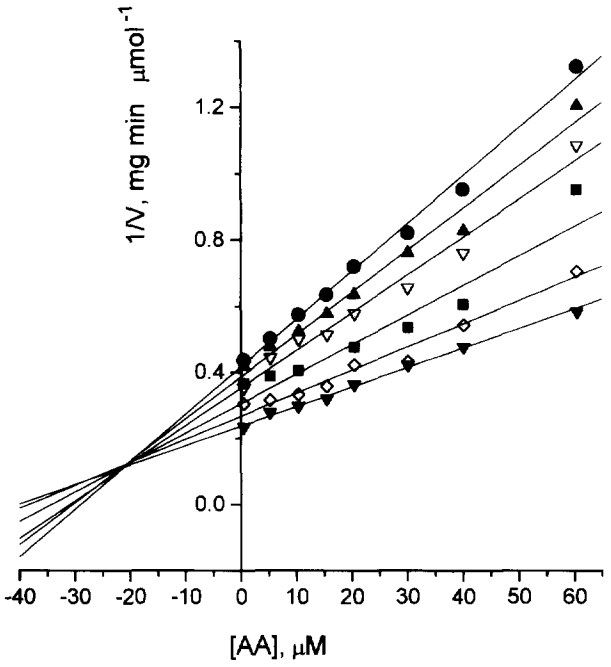

Fig. 3. A Dixon plot for the inhibition by arachidonate of the 5-LO catalysed oxidation of linoleic acid. Linoleate concentrations (μM): (●) 10; (▲) 20; (▽) 30; (■) 40; (◇) 60; (▼) 100. The reaction was initiated by adding 3.8 mE/ml of 5-LO to the mixture containing both substrates.

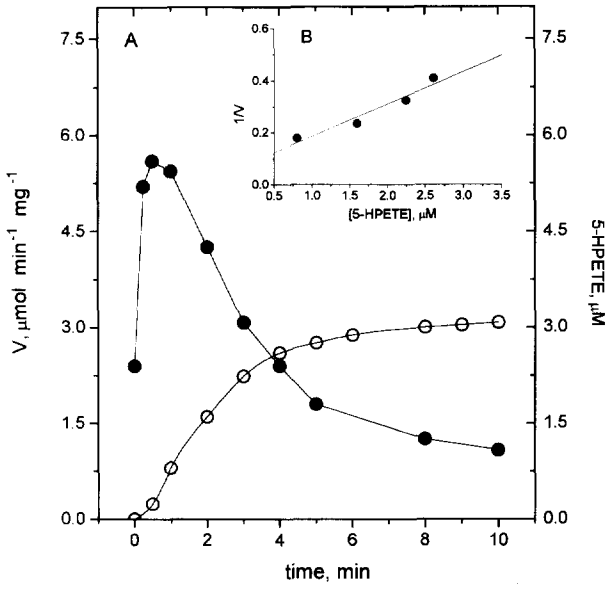

Fig. 4. (A) The effect of 5-HPETE on the barley 5-LO catalysed linoleate oxidation. Initial velocity of linoleate oxidation by 5-LO (●) and 5-HPETE formation (○) during the preincubation of the enzyme with 20 μM arachidonate. A mixture of 5 mE/ml of barley 5-LO and 0.02% Lubrol PX in 50 mM sodium phosphate, pH 6.8, was preincubated with arachidonate and then 40 μM linoleate was added. (B) A replot of the descending part of the velocity versus time curve.

of linoleic acid transformation keeps nearly constant after an initial lag period. The accumulated 9-HPODE does not inhibit the enzymatic process (data not shown). When 5-HPETE is formed, the rate of LA oxygenation exhibits an initial rise followed by a decline at [5-HPETE] > 1 μM (Fig. 4). The tight binding of 5-HPETE to this enzyme may be of importance for subsequent transformation of this intermediate to leukotrienes. It is not the case with 9-HPODE.

Thus, arachidonic acid is a poor substrate for 5-LO from ungerminated barley at pH 6.8, the optimum pH for linoleic acid oxygenation. Arachidonic acid or its hydroperoxide can interfere markedly with the conversion of linoleic acid. The high binding affinity for this eicosanoid substrate and its product results in the inhibition of the overall reaction catalyzed by lipoxygenase.

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