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Excess substrate inhibition of soybean lipoxygenase-1 is mainly oxygen-dependent

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Abstract Soybean lipoxygenase-1 kinetics are known to show product and substrate inhibition. With linoleic acid as the substrate and using a simple Michaelis-Menten formulation, we have shown that K_{ss}, the substrate inhibition constant was increased by more than five-fold when initial oxygen concentration was increased from 228 to 1140 μM. Excess substrate inhibition is in fact almost avoided at high initial oxygen concentration. This modification seems correlated with enzyme saturation with oxygen relative to linoleic acid, as reflected by alterations of the substrate conversion rate. Possible implications for the enzyme kinetics are discussed.

Key words: Soybean lipoxygenase-1; Oxygen; Excess substrate inhibition; Linoleic acid

1. Introduction

Lipoxygenase (EC 1.13.11.12) is a non heme iron enzyme that catalyzes the dioxygenation of polyunsaturated fatty acids containing one or more (Z, Z)-pentadiene systems into enantiomeric hydroperoxide fatty acid with (Z, E)-diene conjugation (for reviews, see refs. [1–4]). While animal lipoxygenase roles in inflammatory process or cell membrane maturation have been well documented [5–7], the roles of plant lipoxygenases, as soybean lipoxygenase-1, is not yet totally elucidated [2].

In both animal and plant lipoxygenases, the enzyme is present either as Fe(II) or as Fe(III) form. Treatment of the iron(II), inactive lipoxygenase with an equimolar amount of the hydroperoxide product results in the oxidation of the cofactor, leading to the Fe(III), active form [8]. Thus the reaction product is an activator of lipoxygenase activity [9,10], however, a competition between product and substrate for the active ferric enzyme form causes a product inhibition [11]. Such a competition also occurs for the inactive ferrous enzyme, resulting in the observed substrate inhibition [12].

Soybean lipoxygenase-1 activity is furthermore very sensitive to many factors, as polyols [13] and ions [14,15], but on account on its high enzymatic efficiency ($k_{cat}/K_M = 5\times10^6$ M^{-1} s^{-1}), dioxygenation rate remains significative, even under unfavourable conditions.

In this paper we report an investigation into the important relationship between dioxygenation rate and concentration of the cosubstrate oxygen. All the experiments were carried out with soybean lipoxygenase-1 as catalyst and linoleic acid (LA) as substrate. We especially focused on enzyme inhibition by the substrate, as indicated by the inhibition constant estimated with a simple Michaelis-Menten relation between maximum rate and linoleic acid concentration. The influence of oxygen concentration on this inhibition is reported and the resulting implications for lipoxygenase mechanism discussed.

2. Materials and methods

Soybean lipoxygenase-1 was purified according to the procedure described in ref. [16], as modified by ref. [17], and stored at −20°C under N_2.

Linoleic acid (Sigma Chemical Co., 99%) was stored at −20°C, as a 100 mM solution in 9×10^{-3} N NaOH +0.7% (v/v) Tween 20 (Sigma Chemicals Co.), prepared at 4°C, under anaerobic conditions.

2.1. Kinetic measurements

Dioxygenation reaction was performed at 25°C, in a 0.1 M $Na_4P_2O_7$, pH 9.0 buffer, containing 35×10^{-3} g/l Tween 20 (final concentration) and the desired substrate concentration. The concentration of hydroperoxides in these solutions, measured through the absorbance at 234 nm ($\varepsilon = 25\,000$ M^{-1} cm^{-1}), was less than 0.6% of the linoleic acid (LA) concentration. The buffer was saturated with air or O_2 bubbling, oxygen concentration in the buffer was 228 and 1140 μM, respectively. The reaction was followed by a polarographic method convenient to measure pO_2 variations [18]. A Clark electrode covered with a propylene membrane (Radiometer, Denmark) was used in a 100 ml hermetic glass reaction vessel purchased from Tacussel. Reaction buffer volume was 20 ml. Final soybean lipoxygenase-1 concentration was 9.3 nM.

2.2. Kinetic parameter estimations

Lipoxygenase dioxygenation reaction kinetics sometimes present a lag phase [8]. In these cases, the reaction starts with a low initial reaction rate, which is followed by a higher one (r_{max}). But the two rates are not concomitant, unlike usually observed with classical Michaelis-Menten enzymes. In this study, possible lag phases at the beginning of the reaction were neglected, so that dioxygenation rates were determined on the basis of the maximum rate of dioxygenation (r_{max}), rather than the initial reaction rate. Analysis of r_{max} as a function of linoleate concentration has been shown to conform to steady-state approximation [19], so that kinetic studies based on a simple Michaelis-Menten formulation expressed as a function of r_{max} are justified. In this study, kinetic parameters were estimated by a least square non-linear fit of the following Michaelis-Menten formulation with substrate inhibition: $r_{max} = V_m \times ([LA]/(K_M + [LA] + ([LA]^2/K_{ss}))$, with the program Minim 3.0.3 (R.D. Purves, Pharmacology Department, University of Otago, P.O. Box 913, Dunedin, New Zealand). V_m is the maximal rate, and K_M, and K_{ss} are the dissociation constants relative to enzyme affinity for linoleate, and inhibition of the enzyme by this fatty acid, respectively.

On account of the low initial product concentration (see above), the influence of product inhibition on the kinetics was neglected.

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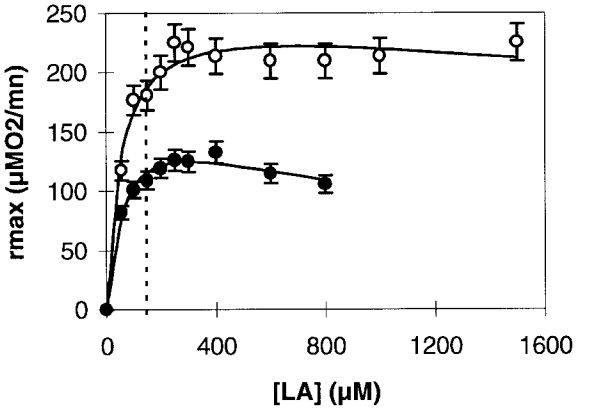

Fig. 1. Maximum rate (r_{max}) as a function of linoleic acid (LA) concentration for the reaction of soybean lipoxygenase-1 with 128 µM (full circles) or 1140 µM (open circles) initial oxygen concentration. Full lines represent simulated values with kinetic constants as described in Table 1. Dashed vertical line represents critical micellar concentration (CMC) for linoleate.

3. Results and discussion

As seen in Fig. 1, soybean lipoxygenase-1 kinetics in an air saturated pyrophosphate buffer (initial $[O_2] = 228$ µM) show a weak substrate inhibition. The estimated value of K_M (Table 1), slightly higher than those obtained with a spectrophotometric method (usually in the range of 20 µM, see ref. [9]), is in good agreement with previously reported observations, that stirring used in the polarographic method induces a three-fold increase of K_M [20].

The estimated value of K_{ss} in this case is also much larger than previous published ones (also usually in the range of 20 µM, refs. [9,12]). Excess substrate inhibition appears to be partly depending on the organization of linoleic acid (LA) in the reaction medium, especially on its distribution between micellar and non-micellar phases. For example, without co-solubilization of LA with detergents, dioxygenation rate decreases only over [LA] = 3.5 mM [21]. In a two-phase system (borate buffer/hexane), excess substrate inhibition is avoided until [LA] = 100 mM in the organic phase [22]. Tween 20 has been shown to inhibit the reaction only at concentrations over 0.05 g/l [19]. In our conditions ([Tween 20] = 0.035 g/l), the detergent is thus not an inhibitor. Linoleate CMC in our conditions has been determined to 150 µM (estimated through platinum-blade measured surface tensions). Excess substrate inhibition is not related to physical distribution of LA between micellar and non-micellar phases, as shown by the continuity of rmax values on both sides of CMC (Fig. 1).

Furthermore, the unusual value for K_{ss} in air-saturated buffer is related to the fact that only maximal rates of dioxygenation were taken into account in this study, possible lag phases being neglected (cf. Section 2). In order to specifically study the lag phase, other authors usually observe initial rates, rather than maximal ones. These different approaches experimentally determine different rates which are consequently used with different enzymatic rate expressions. In our case, analysis of r_{max} as a function of linoleate concentration based on a simple Michaelis-Menten formulation is justified [19].

Lipoxygenase catalysis also sometimes show suicide inactivation, depending on substrate, or enzyme isoform [23]. However, Soybean Lipoxygenase-1 isoform is not inactivated with linoleic acid as substrate [24]. Furthermore, addition of substrate at the end of the reaction allows the dioxygenation reaction to start again (see below), demonstrating that the enzyme is not irreversibly inactivated.

Moreover, soybean lipoxygenase-1 appears very sensitive to ions [15]. Depending on the ions used in the buffer, the activity of the enzyme varies significantly. While phosphate ion has a very unfavourable influence, enzyme activity in pyrophosphate buffer (buffer used in this study) is very high. The reaction medium used in this study can thus be considered as an optimized one, where activity is maximal. This might also account for the observed high K_{ss} value.

When dioxygenation reaction is initiated with high oxygen concentration (Fig. 1), K_M for LA is not changed. K_{ss} in this case is very large, and actually excess substrate inhibition is almost avoided (Table 1). Under these conditions, K_M and V_m were also estimated without excess substrate inhibition, i.e. with least square non-linear fit of $r_{max} = V_m \times ([LA]/(K_M + [LA]))$, as 38 ± 8 µM and 232 ± 7 μMO_2/min, respectively. The similarity of these values with those estimated in Table 1, indicates an almost disappearance of excess substrate inhibition.

Fig. 2 shows LA conversion rate, calculated as the rate between the total transformed [LA] when minimum pO_2 is reached and the initial [LA], assuming stoichiometry of 1 between O_2 consumption and LA transformation. It is clearly apparent from these results, that with 228 µM initial [O2], O_2 becomes limiting for LA conversion above [LA] = 200–250 µM, whereas at 1140 µM initial [O_2], oxygen is never limiting, LA conversion rate being 100% throughout the used [LA] range. At 228 µM initial [O_2], LA conversion rates decrease for [LA] > 200–250 µM because the dioxygenation reaction reaches a steady state where LA is not completely transformed. This steady state can be evidenced by re-injecting oxygen in the buffer at the end of the reaction (i.e. when no more pO_2 variations are observed). A new dioxygenation reaction is under this condition immediately initiated (data not shown). Whereas at 1140 µM initial [O_2], the reaction stops because all LA has been transformed, and thus an equilibrium is reached. These data indicate that soybean lipoxygenase-1 is not saturated with O_2 in air bubbled buffers (initial [O_2] = 228

Table 1
Kinetic constants for the dioxygenation reaction of soybean lipoxygenase-1 with linoleic acid (LA), in pyrophosphate buffer, 100 mM, pH 9.0, 25°C

	Initial oxygen concentration (µM)	
	228 (air bubbling)	1140 (oxygen bubbling)
V_m ± S.D. (μMO_2/mn)	175 ± 16	257 ± 18
K_M ± S.D. (µM)	62 ± 15	53 ± 14
K_{ss} ± S.D. (mM)	1.51 ± 0.50	8.49 ± 5.67

Values estimated by least square non-linear fit of $r_{max} = V_m \times ([LA]/(K_M + [LA] + ([LA]^2/K_{ss}))$. S.D. represents standard deviation.

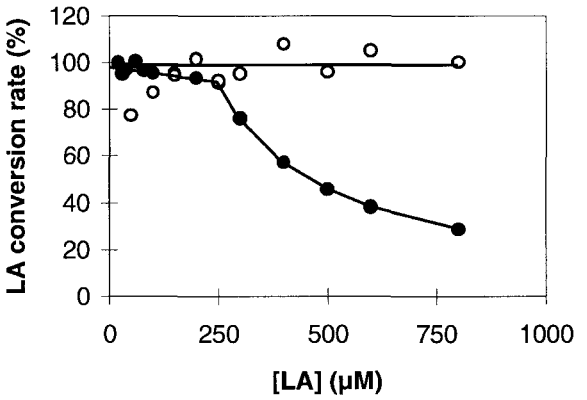

Fig. 2. Linoleic acid (LA) conversion for the reaction of soybean lipoxygenase-1 with 128 μM (full circles) or 1140 μM (open circles) initial oxygen concentration. Conversion rate is calculated as described in text.

μM), saturation being reached at 228 μM ≪ initial $[O_2] < 1140$ μM.

Excess substrate inhibition in soybean lipoxygenase-1 dioxygenation reaction thus appears largely depending on $[O_2]$, and could be related to oxygen limitation when $[S]_{initial} > [O2]_{initial}$. This indicates that excess substrate inhibition is relevant to enzyme saturation with O_2 relative to substrate concentration. It has already been reported, that excess substrate inhibition would rely on modifications of lipoxygenase affinity for O_2 [12]. If the currently accepted hypothesis is assumed, that excess substrate inhibition is caused by substrate fixation on the ferrous, inactive enzyme form [9,11], our results suggest that such a complex could alter enzyme affinity for O_2. Two cavities are present on the surface of lipoxygenase, leading to the internal active site. In the first published 3D structure of the enzyme [25], it has been proposed that cavity I would be largely hydrophobic, and representing the way of O_2 penetration into the enzyme, cavity II being responsible for fatty acid penetration. It has nevertheless been assumed since [26], that cavity I is not hydrophobic, as seen by its high water molecules content, most of which form hydrogen bonds with the protein. It has been proposed instead that oxygen also penetrates through cavity II, or through another way. Alteration of oxygen penetration by substrate fixation is thus a possibility that would account for the results presented here.

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