Potato (*Solanum tuberosum* Var. Desiree) Tuber 5-Lipoxygenase Selectivity for the Physicochemical Properties of Linoleic Acid

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The dependence of potato 5-lipoxygenase (LOX) activity on the physicochemical properties of linoleic acid (LA) was studied. pH and β-cyclodextrin (β-CD) were used as tools to investigate the effect of the physicochemical state of LA on LOX kinetic properties *in vitro*. The LA concentration dependence of LOX activity was best fitted by using the Hill equation. It was found that the decrease of LOX activity at high pH corresponds to a pK_a lower than the pK_a of LA; thus, such decrease was assigned to some ionizable side chain group of LOX related to the active center. At a fixed LA concentration, the presence of β-CD led to a decrease in the LOX reaction rate, which was due to its effect on K and the Hill constant since V_{max} was not affected. Experiments in the presence of β-CD revealed that LA monomers were also used as substrate, although less efficiently than aggregates. The different activities exhibited against monomers and aggregates is the reason for the observed apparent substrate cooperativity, which can be interpreted as an aggregate-induced enzyme activation. The effect of β-CD on LOX activity could be explained on the basis of the specific interaction between LA and β-CD and the equations derived for such interaction developed in a previous work.

Keywords: *Potato tuber; 5-lipoxygenase; PUFA; cyclodextrins; fatty acid aggregates; Solanum tuberosum var. Desiree*

INTRODUCTION

Lipoxygenases (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a group of enzymes that catalyze the hydroperoxidation of free and/or esterified polyunsaturated fatty acids (PUFA) containing one or more 1,4-*cis*,*cis*-pentadienyl moieties, such as linoleic, linolenic, or arachidonic acid, by using O_2.

Although most LOX are soluble enzymes, a certain affinity for membranes or aggregates must be intrinsic as some are capable of peroxidizing PUFA-esterified phospholipid bilayers and biological membranes (Kühn et al., 1990; Takahashi et al., 1993; Maccarrone et al., 1994) and free fatty acids bound to detergent micelles (López-Nicolás et al., 1994). For this reason a binding step to a membrane or an aggregate might be considered to take place during enzyme catalysis.

The kinetically well-known soybean LOX isoenzyme I (Schilstra et al., 1992, 1993) has generally been studied taking care to use only the monomeric form of linoleic acid (LA) so that the enzyme's response was clear and the interpretation of results could be simplified (Verhagen et al., 1978; Schilstra et al., 1992). For this, the pH was set high (from 9 to 10), so that LA stays monomeric up to ca. 200 μM, thus preventing the formation of substrate aggregates and so the interaction or binding of LOX to them. However, most LOX display an optimum pH that ranges from 5.5 to 7.5 (Vick and Zimmerman, 1987; Vliegenthart and Veldink, 1982). In these conditions PUFA aggregates at quite low concentrations (from 10 to 50 μM depending on pH), being no longer a monomer and, presumably, in physiological conditions LOX usually acts in the presence of these substrate aggregates. The different characteristics of the reaction at basic pH and at optimum pH, which might seem unimportant from a kinetic point of view (that is, the reaction may progress more quickly or more slowly), is not so in the case of LOX, since the isomer ratio of the reaction product is highly dependent on pH (Vick and Zimmerman, 1987; Vliegenthart and Veldink, 1982; Yamamoto, 1992) and each isomer is a precursor of a different metabolic pathway (Vick and Zimmerman, 1987). Thus, it is relevant to investigate the kinetic properties of LOX in conditions that are closer to the physiological, concerning both pH and aggregation state of the substrate, about which little is known, particularly in the case of plant LOX. The rigorous determination of LOX activity at each pH by monitoring its products would require, however, their identification and quantification, so it is more convenient from a practical point of view simply to monitor the substrate depletion by measuring the oxygen uptake (Berkeley and Galliard, 1976).

Following our observation that potato 5-LOX was able to utilize LA bound to detergent micelles at pH 6.3 (López-Nicolás et al., 1994), we investigated whether the mere aggregation state of its substrate has any effect on the enzyme and discuss to what extent this may have a physiological meaning. For this, experiments were carried out in the absence of detergents. Since pH is a factor that determines not only the protonation but also the aggregation state of fatty acids (Cistola et al., 1988), we used it as a tool for our investigation.

Recently, we reported on the aggregation behavior of PUFA in the presence of CD and showed that these aggregate at higher concentrations due to the formation of 1:1 and 1:2 PUFA/CD inclusion complexes, the CD not affecting the aggregation mode (López-Nicolás et al., 1995; Bru et al., 1995). On the basis of that PUFA/CD interaction model, we showed that soybean LOX-I acting at pH 9.0 utilized exclusively the free, noncomplexed LA as substrate and that the enzymatic conversion of free substrate into products leads the complexes to

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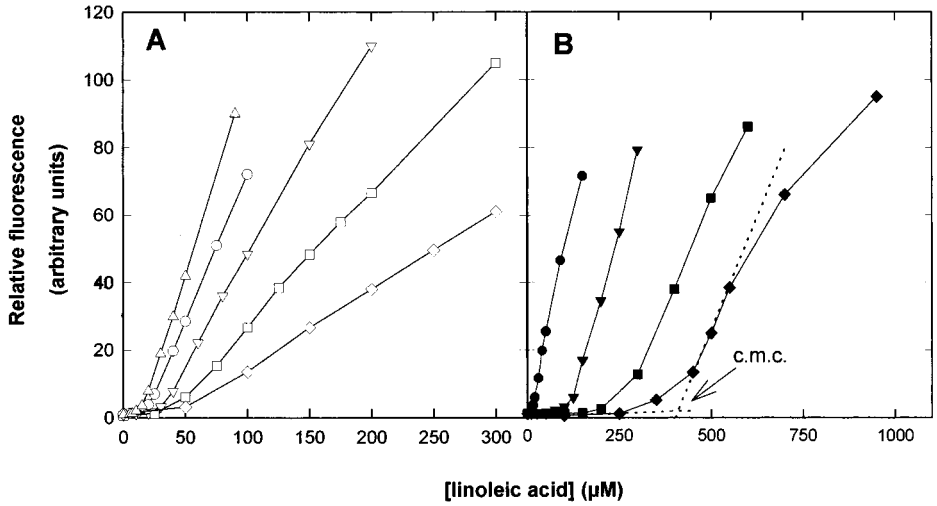

Figure 1. Dependence of relative fluorescence intensity of DPHT at 430 nm (excitation wavelength 358 nm) on LA concentration. (A) Effect of pH: 0.1 M potassium phosphate, pH (△) 6.3, (○) 7.0, (▽) 7.3, (□) 7.6, and (◇) 8.0. (B) Effect of β-CD: 0.1 M potassium phosphate, pH 6.3, in the presence of (●) no β-CD, (▼) 0.5 mM β-CD, (■) 1 mM β-CD, and (◆) 1.5 mM β-CD. The FA cmc was determined graphically as the intercept of the lines that define the increase in fluorescence in the pre- and postmicellar regions, as indicated.

dissociate, thus releasing fresh LA (López-Nicolás et al., 1997). In that way both free and complexed LA would be reacted by LOX. At lower pH values (6.5−8.0) soybean LOX-I was proposed to be able to utilize directly the complexes as substrate (Jyothirmayi and Ramadoss, 1991). The complexing ability of CD makes them particularly suitable for our investigation on the effect of the physicochemical state of PUFA on potato 5-LOX reaction.

The aim of this work was to study whether potato 5-LOX had any preference for the physicochemical properties of the LA substrate, particularly in its protonation and aggregation state, to characterize the response of the enzyme to these substrate forms and discuss the possible physiological implications.

MATERIALS AND METHODS

LA was purchased from Cayman Chemical Co. (Paris, France). β-Cyclodextrin was obtained from Sigma (Madrid, Spain). Diphenylhexatriene (DPHT) was a product from Fluka (Madrid), and tetrahydrofuran was from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity. 5-Lipoxygenase was purified from potato tubers according to the method of Mulliez et al. (1987) [27 μmol of O_2 min^{-1} (mg of protein)$^{-1}$].

Fluorometric Determination of Critical Micelle Concentration (cmc). LA cmc was determined as described elsewhere (López-Nicolás et al., 1995) by means of a fluorescence spectroscopy method described by Chattopadhyay and London (1984) and adapted to FA by Serth et al. (1991). Two milliliter samples contained 0.1 M potassium phosphate or borate buffer, 0.88 μM diphenylhexatriene (supplied in 2 μL of tetrahydrofuran), 1% v/v ethanol, and the required concentrations of CD and FA. The samples were flushed with N_2 and incubated for 30 min in the dark at the desired temperature for equilibration and to prevent photoisomerization of the fluorescent probe. Fluorescence intensity was measured at 430 nm (358 nm excitation wavelength) in a SFM-25 spectrofluorometer (Kontron Instruments, Milan, Italy) equipped with thermostated cells. The relative values of fluorescence were plotted against LA concentration, and the cmc was determined as the intersection between the lines defining the fluorescence intensity in the pre- and postmicellar regions (Figure 1).

Determination of Equilibrium Constants. To determine the equilibrium constants between free and complexed FA, a model involving the sequential binding of two CD molecules to one FA molecule was used (López-Nicolás et al., 1995; Bru et al., 1995). The solution of that complexation scheme led to an expression which relates the total FA cmc in the presence of CD with cmc_0 (the cmc in the absence of CD), the unknown equilibrium constants K_1 and K_2, and the total CD concentration. K_1 and K_2 can be estimated by nonlinear regression (nlr) of the apparent cmc* data versus the total CD concentration. The expression is

$$cmc^* = cmc_0[1 + K_1(CD_f) + K_1K_2(CD_f^2)] \quad (1)$$

where

$$CD_f = \frac{-[1 + K_1(cmc_0)] + \sqrt{[1 + K_1(cmc_0)^2] + 8K_1K_2(cmc_0)(CD_t)}}{4K_1K_2(cmc_0)} \quad (2)$$

The nlr fitting was performed by using a Marquardt algorithm implemented in the SigmaPlot v 5.1 (Jandel Scientific) computer program.

LOX Assay. LOX activity was determined by monitoring O_2 consumption with a Clark type electrode. Samples of 1 mL were prepared as for fluorescence spectroscopy but with omission of the fluorescent probe and tetrahydrofuran; these were shaken vigorously before using to air-saturate and then transferred to the stirred, thermostated oxygraph chamber (Hansatech Ltd., Norfolk, U.K.). The reaction was started by injection of 10 μL of potato 5-LOX (1.7 μg of protein). Quantitative measurements were made by using an oxygen calibration method (Rodriguez-López et al., 1992), and the activity was expressed as the maximal amount of O_2 consumed per minute (maximal slope of reaction progress curves).

Kinetic Analysis. Reaction rate data plotted versus LA concentration were analyzed according to the Hill equation, and kinetic parameters V_{max}, K, and Hill constant, h, were determined by nlr fitting.

$$v = V_{max}[S]^h/(K^h + [S]^h) \quad (3)$$

RESULTS

Aggregation of LA. Effect of pH and β-CD. LA aggregation can be monitored by the fluorescence emission of DPHT, the quantum yield of which increases dramatically when surrounded by an apolar environment, such as that created when LA monomers aggregate. The fluorescence intensity is low in the so-called premicellar region and increases suddenly when

Scheme 1. Equilibria between a Long-Chain FA and CD

$$\text{FA} + \text{CD} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{FA-CD}$$

$$\text{FA-CD} + \text{CD} \overset{K_2}{\rightleftharpoons} \text{FA-CD}_2$$

Table 1. LA/β-CD Inclusion Complex Equilibrium Constants and Kinetic Parameters for Potato 5-LOX in the Absence or Presence of β-CD in 0.1 M Potassium Phosphate Buffer, pH 6.3 at 25 °C

	value ± SME[a]	
parameter	β-CD −	β-CD[b] +
K_1[c] (mM^{-1})		15.7 ± 3.4
K_2[c] (mM^{-1})		1.8 ± 0.4
K (μM)	20.0 ± 0.8	22.2 ± 2.2
V_{max} (nmol of O_2/min)	45.9 ± 0.5	49.1 ± 2.5
h	1.57 ± 0.08	1.37 ± 0.11

[a] The error given by the nlr fitting. [b] Kinetic parameters determined by nlr from the whole set of activity data in the presence of β-CD to eq 3 after transformation of total LA into free LA by using eqs 4 and 5. [c] Equilibrium constants determined according to the method of López-Nicolás et al. (1995).

LA monomers aggregate, thus defining a cmc. Graphically, cmc is determined as the cross-point between the straight lines that define the trend of the fluorescence intensity in the pre- and postmicellar regions.

As shown in Figure 1, the aggregation behavior is affected by both pH and β-CD concentration. An increase in either produces an increase in LA cmc. The effect of pH on the cmc values determined here by the fluorescence method (see Figure 3) is in good agreement with those reported by using surface tension measurements (Verhagen et al., 1978). Recently, we have reported on the aggregation behavior of LA in the presence of CD, and evidence has been presented concerning the formation of 1:1 and 1:2 linoleic acid/β-CD inclusion complexes (López-Nicolás et al., 1995; Bru et al., 1995). As shown in Scheme 1, these complexes are in equilibrium with free β-CD and LA, which is characterized by two equilibrium constants, K_1 and K_2; values for K_1 and K_2 determined at 25 °C and pH 6.3 are given in Table 1.

The aggregation mode of LA was clearly affected by pH since samples of pH 8.0 or higher were transparent in both pre- and postmicellar regions, while samples of lower pH values were transparent in the premicellar region but turbid in the postmicellar region. These observations agree with the description of the aggregation modes of other saturated and unsaturated FA (Cistola et al., 1988) which correspond to dispersed oily droplets at low pH and to micellar aggregates at pH 8 and higher.

Effect of pH on Potato 5-LOX Activity. FA in solution exhibit a complex polymorphism, mainly due to their amphiphilic character and their ionic head group (Cistola et al., 1988). When protonated, FA are barely soluble in water and form a more or less dispersed oil phase above cmc. However, deprotonated FA forms transparent solutions, which are truly micellar above the cmc (Small, 1986). In the pH range we have studied, 5.8−9.0, all of these forms occur since the pK_a of LA is 7.9 (Bild et al., 1977).

Figure 2 shows the substrate dependence of potato 5-LOX at different pH values. At pH 7.6 the dependence was clearly nonhyperbolic, and the dependence

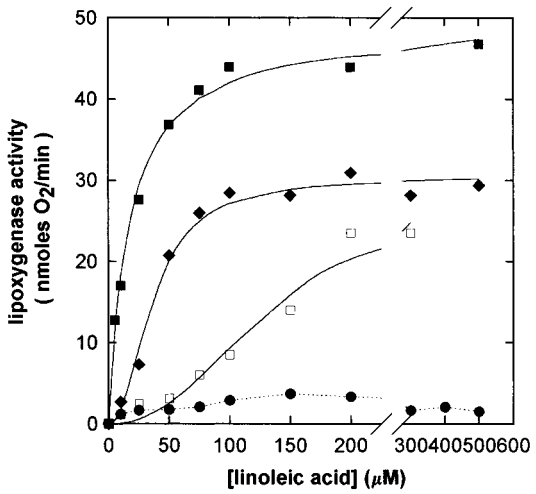

Figure 2. Dependence of oxygen consumption rate of potato 5-LOX on LA concentration at different pH values. The oxygen consumption rate was determined at 25 °C as indicated under Materials and Methods. The solid lines represent the nlr fitting using eq 3. The dotted line is used to join the data at pH 8.0. Symbols are 0.1 M potassium phosphate at pH (■) 6.3, (◆) 7.3, (□) 7.6, and (●) 8.0.

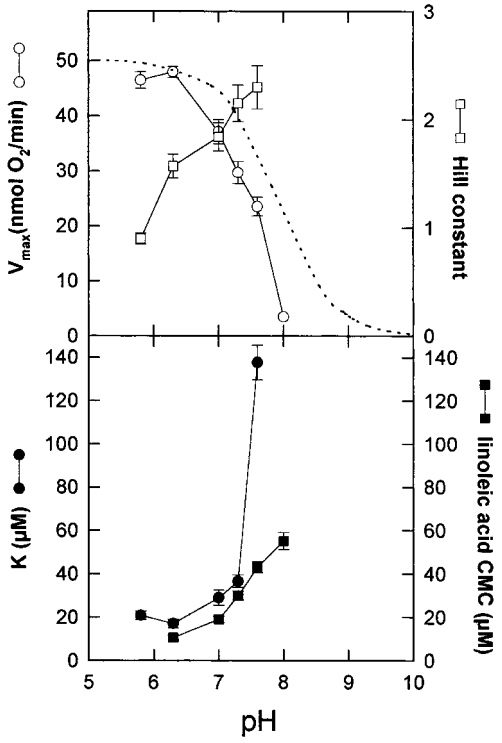

Figure 3. Dependence of potato 5-LOX kinetic parameters and LA cmc on pH. Kinetic parameters V_{max}, K, and Hill constant, h, were determined by nlr fitting using eq 3. V_{max} datum at pH 8.0 is the maximal reaction rate observed. Error bars are those given by the nlr fitting. LA cmc was determined graphically as indicated in Figure 1, and data represent the mean ± SEM of two to three determinations. The dotted line represents the relative protonated LA concentration as calculated by the Henderson−Hasselbalch equation for a pK_a = 7.9: pH = 7.9 + log[LA^-]/[LAH].

appeared more hyperbolic as pH decreased. As shown in Figure 3, the Hill constant, h, ranged from 0.92 ± 0.05 at pH 5.8 to 2.31 ± 0.26 at pH 7.6. The maximum value of the reaction rate at pH 8.0 was 3.5 nmol/min, but the other parameters could not be reliably estimated due to the low enzyme activity. The pH profile with respect to V_{max}, with its optimum pH at 6.3, is qualitatively similar to profiles described when the enzyme was assayed at a single substrate concentration in the pres-

ence of detergents (Mulliez et al., 1987; Jyothirmayi and Ramadoss, 1991). Having a $pK_a = 7.9$, the amount of protonated LA at pH 8.0 is practically 50% of total LA (Figure 3), but at that pH the enzyme's reactivity is almost nil. Since the reactivity decay occurs at lower pH values than deprotonation of LA, it is reasonable to think that the V_{max} profile is mostly dependent on the titration of some enzyme's charged group rather than the LA carboxyl group. Likewise, the largest effect of pH on K occurs previous to significant LA deprotonation levels.

As mentioned above, cmc increases with pH, and such an increase parallels that of K, at least up to pH 7.3. The cmc values were always below those of K, and so the K value determined corresponds to an aggregated state of LA. Taking into consideration the LOX operative pH range and LA concentration at which there is maximal activity as compared to cmc, we suggest that a major factor for expression of LOX activity is the aggregation rather than the protonation state of LA, pH being a feature that affects directly the enzyme. These preferences are quite different from those of soybean LOX-I, which prefers LA monomers, and in addition, the aggregation of LA at high concentration has almost no effect on the enzyme activity or is slightly inhibiting (Galpin and Allen, 1977). Moreover, soybean LOX-I displays a good activity even 1 unit of pH below the pK_a of LA (Jyothirmayi and Ramadoss, 1991); thus, pH seems to be a factor influencing mostly the enzyme itself, but for its different pH profile, the groups affected should be different from those of potato 5-LOX.

Effect of β-CD on Potato 5-LOX Activity. Unlike with soybean LOX-I, the preferences of potato 5-LOX for monomers or aggregates are difficult to investigate in detail because of the low cmc of its substrate at the optimum pH. CD may overcome this difficulty as they have been shown to form inclusion complexes with FA (Schlenk and Sand, 1961; Szejtli and Bánky-Elöd, 1975), causing their cmc to increase (see Figure 3) and thus enlarging the apparent monomer concentration range (López-Nicolás et al., 1995; Bru et al., 1995).

Figure 4 shows the substrate dependence of potato 5-LOX at pH 6.3 at different β-CD concentrations. In the presence of β-CD the shape of the velocity vs substrate concentration plots was clearly nonhyperbolic, although as β-CD concentration decreased, the dependence appeared more hyperbolic. As shown in Figure 5, the Hill constant, h, increased from 1.6 ± 0.1 without β-CD to 3.4 ± 0.4 at 1.5 mM β-CD. In contrast to the effect of pH, β-CD did not affect the V_{max}. Both K and cmc increased in parallel, but with a nonlinear dependence on β-CD concentration. As has been shown (López-Nicolás et al., 1995; Bru et al., 1995), the dependence of cmc can be explained according to Scheme 1. In the presence of CD, it is the apparent cmc that changes since the real cmc_0, defined as the concentration of free amphiphile at which the monomer-to-aggregate transition occurs, is independent of β-CD concentration and accounts for the cmc in the absence of β-CD (Junquera et al., 1992). Therefore, the observed cmc is the sum of cmc_0 plus the complexed amphiphile concentration. As deduced from the fluorescence experiments and our previous studies (López-Nicolás et al., 1995), free and complexed monomers of LA exist in the premicellar region, while in the postmicellar region there is the additional presence of LA aggregates. In both regions, the complexed LA simply constitutes a pool of substrate to which enzymes might or might not have direct access (Bru et al., 1995).

As can be seen in Figure 4, the activity data fit eq 3 well in the postmicellar region (see arrows indicating the cmc) but not in the premicellar region, indicating that the observed cooperativity may not respond to the classical schemes of kinetic or allosteric cooperativity (Fersht, 1985). Indeed, the activity versus LA concentration plots can be divided into two parts, one before and the other after the cmc. In each part, a plateau of activity is reached, although at different levels, producing a cooperative effect which might be a reflection of

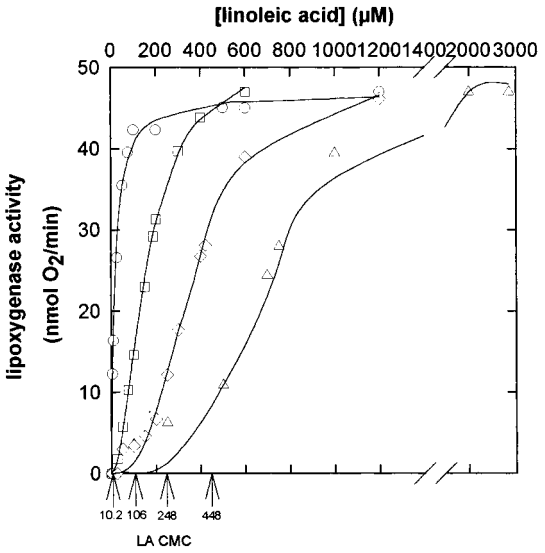

Figure 4. Dependence of oxygen consumption rate of potato 5-LOX on LA concentration at different β-CD concentrations. The oxygen consumption rate was determined at 25 °C as indicated under Materials and Methods. The lines represent the nlr fitting using eq 3. Symbols are 0.1 M potassium phosphate, pH 6.3, in the presence of (○) no β-CD, (□) 0.5 mM β-CD, (◇) 1 mM β-CD, and (△) 1.5 mM β-CD. LA cmc at 0, 0.5, 1, and 1.5 mM β-CD is indicated by arrows together with their respective values.

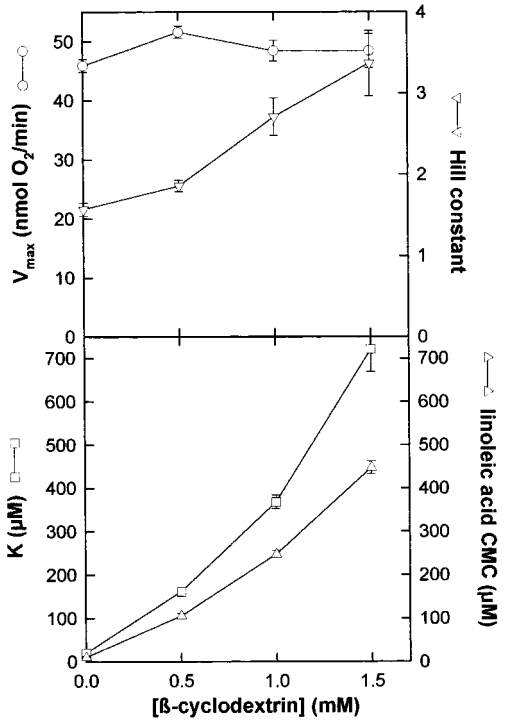

Figure 5. Dependence of potato 5-LOX kinetic parameters and LA cmc on β-CD concentration. Kinetic parameters V_{max}, K, and Hill constant, h, were determined by nlr fitting using eq 3. Error bars are those given by the nlr fitting. LA cmc was determined graphically as indicated in Figure 1, and data represent the mean ± SEM of two to three determinations.

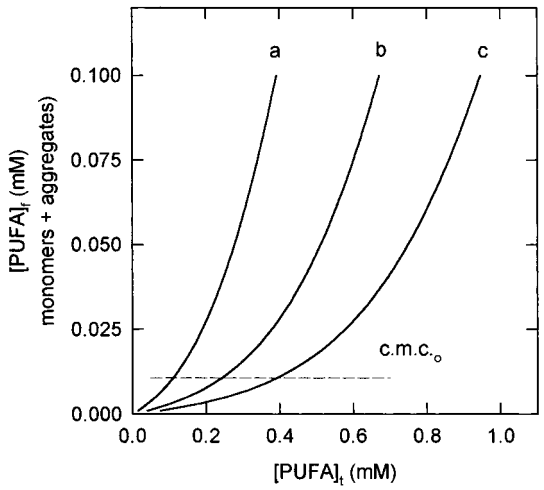

Figure 6. Mnemogram for the conversion of $[PUFA]_t$ into $[PUFA]_f$. For each fixed CD_t (a, 0.5 mM β-CD; b, 1 mM β-CD; and c, 1.5 mM β-CD), values are given to $[PUFA]_f$ to obtain the corresponding $[PUFA]_t$ by using eqs 4 and 5.

the cooperative aggregation of LA at the cmc. This phenomenon is well correlated to the β-CD-induced cmc shift. It is clear from the data that potato 5-LOX undergoes an activation process when the substrate monomer-to-aggregate transition occurs.

Kinetic Analysis in CD. In aqueous solution, CD sequester part of the LA to form soluble inclusion complexes, thereby reducing the concentration of the free FA. If K_1 and K_2 are known, then the total LA concentration, $[PUFA]_t$, and the free LA concentration, $[PUFA]_f$, are readily interconvertible through the equation

$$[PUFA]_t = [PUFA]_f[1 + K_1(CD_f) + K_1K_2(CD_f^2)] \quad (4)$$

where

$$CD_f = \frac{-[1 + K_1[PUFA]_f] + \sqrt{[1 + K_1[PUFA]_f]^2 + 8K_1K_2[PUFA]_f(CD_t)}}{4K_1K_2[PUFA]_f} \quad (5)$$

For the sake of simplicity the conversion of $[PUFA]_t$ into $[PUFA]_f$ can be carried out by a graphical method instead of obtaining the explicit expression of $[PUFA]_f$ as a function of $[PUFA]_t$. Thus, giving values to $[PUFA]_f$, the corresponding values of $[PUFA]_t$ are obtained for each fixed CD_t. A plot of $[PUFA]_f$ versus $[PUFA]_t$, such as that shown in Figure 6, provides the graphical tool to readily convert $[PUFA]_t$ into $[PUFA]_f$ for each fixed CD_t. This can be used to test whether potato 5-LOX is utilizing only the free FA or can also convert the complexed.

By applying the above, the total LA concentration of the experiments in the presence of β-CD (see Figure 4) was transformed into free LA concentration and replotted as shown in Figure 7. As a result, almost all data fell in one slightly sigmoidal curve, which coincided very well with the curve obtained in the absence of β-CD. This suggests that β-CD does not induce any effect in the enzyme that did not exist before but simply exaggerates those induced by the substrate's aggregation behavior. Indeed, as seen in Table 1, the kinetic parameters were almost independent of the presence of β-CD when analyzed as a function of free LA (monomers plus aggregates). Since enzyme activity responds to the concentration of free LA, it can be concluded that the effective concentration of substrate is that of LA nonsequestered by β-CD.

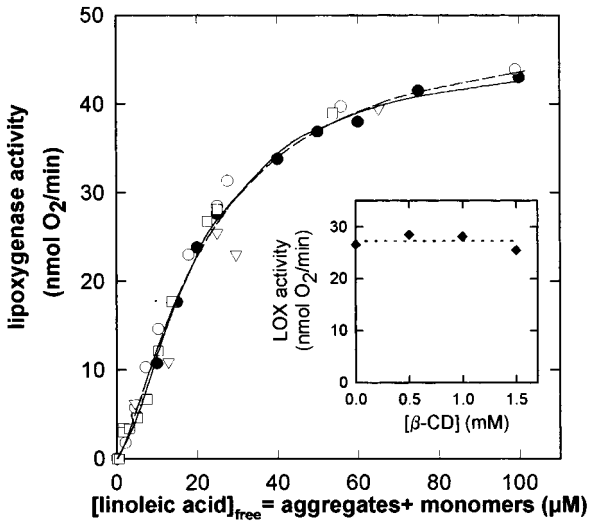

Figure 7. Dependence of oxygen consumption rate of potato 5-LOX on free LA concentration (monomers + aggregates) in the presence of β-CD. The substrate axis, which in Figure 4 represents the total substrate concentration, has been transformed into free substrate concentration by using eqs 4 and 5. Continuous line represents the nlr fitting of data in the absence of β-CD (●) to eq 3. Dotted line represents the nlr fitting of the whole set of data in the presence of β-CD [(○) 0.5, (□) 1, and (△) 1.5 mM] to eq 3. (Inset) CD assay. Conditions of total β-CD and total LA were set so that the free LA concentration had a constant value of 25 μM.

Consequently, an experiment can be designed, that, by analogy with the mixed micellar assay (López-Nicolás et al., 1994), we may call the CD assay, in which different initial conditions can be set so that the concentration of free LA in equilibrium remains constant. When potato 5-LOX was tested in such conditions that free LA was 25 μM, the activity was independent of the total LA concentration, as seen in the inset of Figure 7, further demonstrating that the complexes cannot be used by potato 5-LOX. However, in the presence of β-CD, much more than 25 μM of LA was transformed. This result suggests that there is a pathway to transform the LA included in β-CD, which most probably represents the dissociation of the complexes as the free LA is converted (Bru et al., 1995).

DISCUSSION

Preferences for the Physical Form of the Substrate and Activation of Potato 5-LOX. The critical importance of knowing the preferences of LOX for the different physical forms of its substrate has recently been stated because this is the only way to correctly analyze the kinetic data (De Wolf, 1991; Ford-Hutchinson et al., 1994) and thus perform correct extrapolations to physiological conditions. The preference of soybean LOX-I for monomeric LA has long been known (Galpin and Allen, 1977), although, rather than being a common case, it is an exception when the pH range at which most LOXs act and the low concentration of monomers at those pH values are taken into consideration. It seems interesting, therefore, to investigate such preferences to get insight into the physiological roles of this enzyme in both animals and plants. Potato 5-LOX serves as an excellent model because of its pH action range and its abundant availability in potato tubers (Pinsky et al., 1971; Berkeley and Galliard, 1976; Sekiya et al., 1977; Mulliez et al., 1987).

Membrane mimetic systems such as liposomes (Riendeau et al., 1993) and mixed micelles (López-Nicolás et al., 1994) have served to analyze the kinetic properties of LOX acting on a surface-distributed PUFA and have demonstrated that it is the mole fraction of substrate rather than the overall concentration that determines the reaction rate. Unfortunately, the above *in vitro* systems do not permit us to evaluate to what extent the FA which is partitioned toward the aqueous phase is utilized by the enzyme, because the partitioning is not known. Although both free and micelle/bilayer-bound forms of substrate were always present, the free concentration was neglected. The use of CD in an *in vitro* system makes it possible to distinguish between the utilization of monomeric or aggregated substrate, and our results for potato 5-LOX clearly show the preference for the latter. Such a preference is manifested kinetically so that two phases can be clearly distinguished by using β-CD as the substrate concentration increases: the premicellar region, where the enzyme uses monomers, and the postmicellar region, where the enzyme uses aggregates. The result is an apparent cooperativity probably generated by the cooperativity of LA aggregation (see Figure 1). The observed kinetics can be interpreted as a phenomenon of aggregate-induced activation of potato 5-LOX, which is a feature described for other water soluble enzymes acting on hydrophobic or membrane-bound substrates such as phospholipase A_2 (Verger and de Haas, 1976; Dennis, 1983; Menashe et al., 1986) and cholesterol oxidase (Randolph et al., 1988). Increases in LOX activity in wounded potato tubers in response to arachidonic acid, an exogenous potato LOX substrate, not accompanied by changes in LOX protein levels have been reported (Bostock et al., 1992), thus supporting the idea that LOX can be activated by its substrates.

The pH value and LA concentration ranges (in the absence of CD) at which activation occurs led us to consider the possible physiological meaning of this new feature of potato 5-LOX. In plants, the release of PUFA from membranes before LOX triggers the so-called linoleyl (-enyl) cascade appears to be a committed step in phospholipid metabolism related to growth regulation (Leshem, 1987). In potato tubers there is an active acyl hydrolase (Galliard, 1971) that may supply abundant PUFA for 5-LOX. Concentrations as low as 10−20 μM would be enough to form oily aggregates at physiological pH values and thus to activate the abundant 5-LOX. Note the potato tuber is the plant tissue with the highest content in LOX reported (Pinsky et al., 1971)

Characterization of Potato 5-LOX Reaction in the Presence of β-CD. The equilibrium constants for the formation of the complex were determined at the cmc* point, at which the equilibrium is fundamentally established between monomeric and aggregate LA and LA/CD complexes. A further increase of LA concentration will originate more aggregates, but these will not produce more complexes if the pathway through monomeric LA is a committed step in the complex formation. The establishment of a new equilibrium between aggregated and complexed LA is therefore required for the excess LA to form complexes. Scheme 1 assumes that free LA is a homogeneous pool with respect to complex formation, and so it is assumed that the equilibrium constants determined at the cmc* point are valid for the whole LA concentration range.

The excellent agreement between activity data treated with these equilibrium constants to make them independent of CD concentration and with the activity data measured in the absence of CD indicates that the above assumptions are quite correct, giving support to the conclusion that the true substrate was the free and not the complexed form. The activity of soybean LOX-I has been studied in some detail in aqueous media containing CD (Jyothirmayi and Ramadoss, 1991). The enzyme was found to carry out the reaction in this medium, and the results were interpreted to mean that the PUFA/CD complex was the LOX substrate, although no direct evidence such as we provide in this study with potato 5-LOX was presented to prove that soybean LOX-I was acting on the complexes. Bearing in mind that LOX must recognize the specific structure of its ligands, the structural arrangement of PUFA caged by one or two CD molecules (López-Nicolás et al., 1995; Bru et al., 1995; Jyothirmayi et al., 1991) should be recognizable by LOX if the reaction would involve the utilization of complexes, although this seems very unlikely. The masking effect of the physical and chemical properties of substances when included in CD is well documented (Uekama et al., 1982; Koizumi et al., 1987; Lin et al., 1988). On the other hand, we demonstrated that, at least at pH 9.0, soybean LOX-I did not utilize complexes as substrates but only free monomeric LA (López-Nicolás et al., 1997). The data obtained with potato LOX in this work at pH 6.3 indicate that complexes are not substrates as well, but only the free (monomeric or aggregate) LA. Strangely, in the same study as mentioned above (Jyothirmayi and Ramadoss, 1991), potato 5-LOX displayed very low activity in CD-containing media in a pH range from 4 to 9. Although these results are apparently contradictory with our findings, it should be noticed that we observed very low activity when LA monomers were used as substrate, that is, in nonactivating conditions. Perhaps, LA monomers are the only species of free LA occurring in their experimental conditions.

ABBREVIATIONS USED

PUFA, polyunsaturated fatty acid; LOX, lipoxygenase; CD, cyclodextrin; FA, fatty acid; cmc, critical micelle concentration; nlr, nonlinear regression; LA, linoleic acid; DPHT, diphenylhexatriene.

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