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Research Paper

Continuous Measurement of the Lipoxygenase-Catalyzed Oxidation of Unsaturated Lipids Using the Monomolecular Film Technique

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Purpose. This paper presents the first detailed kinetic investigation involving the continuous measurement of the soybean lipoxygenase 1 (LOX1)-catalyzed oxidation of unsaturated lipids using the monomolecular film technique at an argon/water interface.
Materials and Methods. The presence of oxidation products in the monolayer is qualitatively detected, at a constant area, by an increase in the monolayer surface pressure. Alternatively, the rate of lipid oxidation can be measured, at a constant surface pressure, by a backward movement of the mobile barrier, due to the oxidation-dependent increase in the monolayer area.
Results. For instance, the LOX1-catalyzed oxidation of 1,2-di[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine (diC18:2PC) monolayer was found to be characterized by a time dependent increase in the monolayer area, at constant surface pressure. However, the increase in the monolayer area was thought to be caused first by the penetration of the enzyme into the interface, and secondly, by the formation of hydroperoxides at the interface, due to the LOX1-catalyzed oxidation of the diC18:2PC film. The rate of the LOX1-catalyzed oxidation of diC18:2PC film was measured by subtracting the increase in the area due to the LOX1-penetration into the non-oxidizable 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycero-3-phosphocholine (diC18:1PC) film from the increase in the area due to LOX penetration and oxidation of the diC18:2PC film. At a constant optimum surface pressure of 1 mN m^{-1}, similar initial rates of LOX1-catalyzed oxidation are observed with both linoleic acid methyl ester (C18:2) and diC18:2PC. It is worth noting that the surface density of C18:2 acyl chains is also similar in both films. We observed that a phosphatidylcholine (PC) film with two potentially oxidizable chains (e.g., diC18:2PC) is oxidized at a rate which is twice that obtained with a PC containing a single oxidizable chain (e.g., 1-hexadecanoyl-2-[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine).
Conclusions. The enzymatic lipid oxidation seems to occur when the monolayer is in the expanded state. This expanded state may possibly result *in vivo* from the lipolysis of a biomembrane and consequently lipolysis and lipid oxidation are coupled at the membrane level.

KEY WORDS: lipid monolayer; lipoxygenase; oxidation; surface pressure.

INTRODUCTION

Lipid oxidation is characterized by the introduction of a polar oxygen moiety into the hydrophobic chains of unsaturated fatty acids. The occurrence of a hydroperoxy group may influence lipid-lipid and lipid-protein interactions, which leads to structural changes in biological membranes and lipoproteins. In biological systems, lipid hydroperoxides are known to damage biomembranes and are thought to be involved in the development of various chronic diseases such as arteriosclerosis (1), diabetes (2) and other nervous diseases (3). In foods, hydroperoxides are responsible for rancidity, off-flavor and toxicity and decrease the nutritional value.

Various enzymes are known to participate in the enzymatic oxidation of lipids. Among these enzymes, lipoxygenases (LOXs) selectively oxidize polyunsaturated fatty acids containing one or more *cis*,*cis*-1,4-pentadiene system and yield *cis-trans*-conjugated hydroperoxy fatty acids (4,5). LOXs are able to oxidize not only free fatty acids but also other types of carboxylic esters, such as the phospholipid components of biomembranes. LOXs occur widely in animals (6) and plants (7) and play a key role in the formation of biologically active molecules. Soybean (*Glycine max*) seeds contain several LOXs with different pH activity profiles and different regiospecificities (8). The isoenzymes-1 and -2 have been the most thoroughly characterized so far (9) and have been reported to oxidize isolated lipid components of biomembranes (10). Owing to its abundance in soybean seeds, its easy purification, and its stability, soybean LOX1 has been widely used as a model in lipoxygenase research.

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ABBREVIATIONS: C16:0-C18:2PC, 1-Hexadecanoyl-2-[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine; C18:1, oleic acid methyl ester; C18:2, linoleic acid methyl ester; diC18:1PC, 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycero-3-phosphocholine; diC18:2PC, 1,2-di[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine; LOX1, soybean lipoxygenase-1.

The kinetics of lipid oxidation in biological systems depend on several factors including the topology of the lipid array in which the oxidation takes place. Lipid monolayers spread at the air/water interface are a convenient model system for characterizing the role played by the molecular organization in oxidation processes. Contrary to other membrane models, it is possible with this model to control the degree of lipid packing and/or surface potential by compressing or expanding the monomolecular film. The monomolecular film technique at the air/water interface has been extensively used at our laboratory to study the hydrolytic action of (phospho)lipases on a variety of substrates (11–13). This technique was also used to study the enzymatic oxidation of pure cholesterol monolayers (14). It has been established that cholesterol and its oxidation product, cholestenone, have different molecular areas and that the oxidation rate can be detected by measuring the increase in the area of a monolayer (14). In a previous paper, we studied the interfacial properties of a series of oxidized and non-oxidized fatty acids by recording the surface pressure and surface potential at the argon/water interface upon film compression (15). The oxidized fatty acids were found to be in a more expanded state than their non-oxidized counterparts. This was interpreted in terms of the bipolar conformations (bola forms) existing at the argon/water interface, which reflect their higher hydrophilic-lipophilic balance (15).

In the present study, we used the monolayer technique for the first time to continuously monitor the enzymatic oxidation of unsaturated lipids. Since oxidized lipids show an increase in their molecular area, the rate of oxidation was measured continuously (at a constant area) in terms of the monolayer surface pressure increase. The rate of lipid oxidation was also recorded continuously (at constant surface pressure) in terms of the backward movement of the mobile barrier resulting from the oxidation-dependent increase in the monolayer area.

MATERIALS AND METHODS

Materials

Purified soybean LOX1 was kindly provided by Dr. G.A. Veldink (Ultrecht University) (10). Linoleic acid methyl ester (C18:2); oleic acid methyl ester (C18:1); 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycero-3-phosphocholine (diC18:1PC); 1,2-di[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine (diC18:2PC) and 1-Hexadecanoyl-2-[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine (C16:0–C18:2PC) were from Sigma.

Monolayer Measurements

The surface pressure was measured using the Wilhelmy method with a thin platinum plate (3.92 cm in perimeter) attached to a Beckman electromicrobalance (model LM-600). The aqueous phase (10 mM Tris/HCl buffer (pH 8)) used was filtred (0.22 μm) and degassed for at least 2 h. All experiments were performed at room temperature under an argon atmosphere to prevent non-enzymatic oxidation as far as possible (16,17).

A cylindrical Teflon trough (volume, 5 ml; area, 7 cm^2) was used to measure the protein penetration into the lipid monolayers. The LOX1 was injected into the aqueous phase stirred continuously at 250 rpm with a magnetic rod in order to homogenize the enzyme solution.

Principle of LOX1-Catalyzed Oxidation of Lipid Monolayers

The use of the monolayer technique to study LOX1-catalyzed oxidation of diC18:2PC monolayers is based on the fact that diC18:2PC and its oxidation products have markedly different mean molecular areas. The principle underlying the oxidation of lipid monolayer by soybean LOX1 is shown schematically in Fig. 1. After spreading diC18:2PC film at the argon/water interface, phospholipid oxidation is initiated by soybean LOX1 injected into the aqueous phase. Water soluble LOX1 penetrates reversibly into the lipid monolayer, giving rise to a LOX1* form. The oxidation products in the monolayer can be detected qualitatively (at constant area) from the increase in the monolayer surface pressure as a function of time (Fig. 1a, right panel). Alternatively, the rate of lipid oxidation in monomolecular films can be recorded continuously (at constant surface pressure) by measuring the backward movement of the mobile barrier due to an enzymatic oxidation-dependent increase in the monolayer area as a function of time (Fig. 1b, right panel).

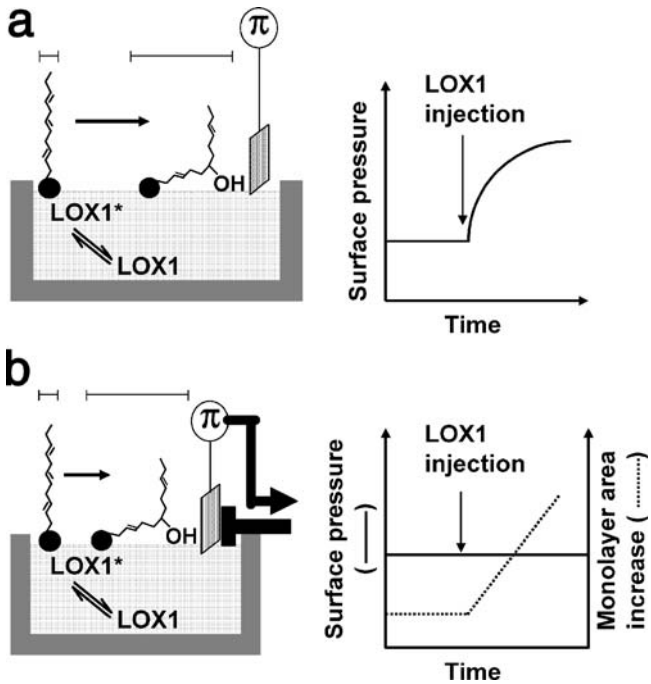

Fig. 1. Principle of the measurement of the rate of oxidation of a lipid monolayer by LOX1, using the monomolecular film technique at an argon/water interface. (a) The oxidation products in the monolayer were detected qualitatively (at constant area) by measuring the increase in the monolayer surface pressure with time. (b) Alternatively, the rate of lipid oxidation in the monolayer was recorded continuously (at constant surface pressure, π) by measuring the backward movement of the mobile barrier resulting from an oxidation-dependent increase in the monolayer area.

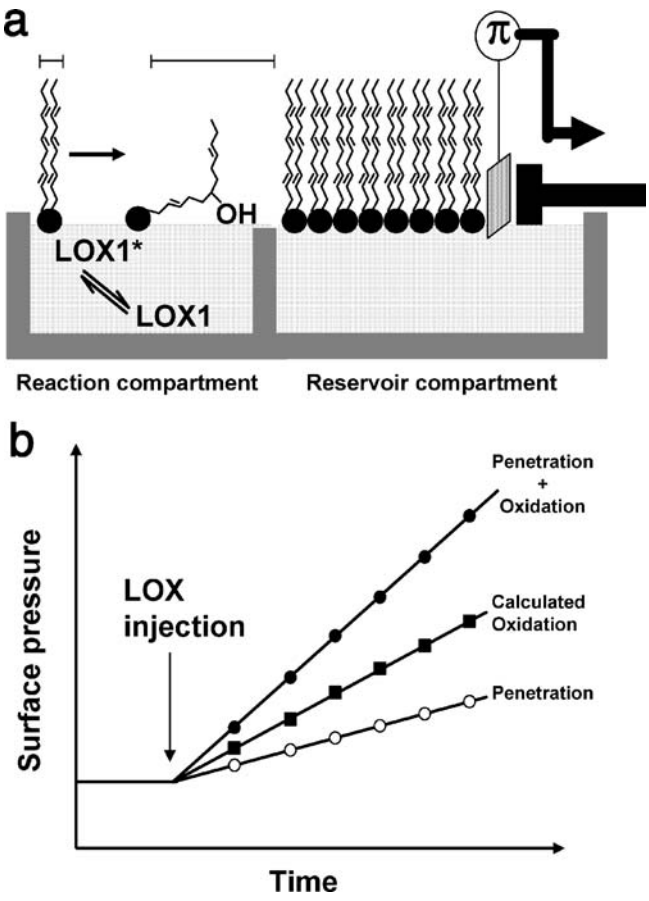

Fig. 2. Penetration of a LOX1 molecule into the interface followed by the LOX1-catalyzed oxidation of lipid monolayers. (a) A monolayer of unsaturated lipid was spread and maintained at a constant surface pressure. LOX1 was injected into the aqueous phase of the reaction compartment under an argon atmosphere. (b) The increase in monolayer area at constant surface pressure, resulting from the LOX1-catalyzed oxidation (—■—), was calculated by subtracting the kinetic curve of the LOX1 penetration into the lipid film (—○—) from the kinetic curve of the LOX1 penetration and catalysis in the lipid film (—●—).

Oxidation Kinetics

The barostat technique was used here, with a "zero-order" trough (11) composed of a reaction compartment (volume 135 ml, area 108.58 cm^2) and a reservoir compartment (area 367 cm^2) communicating by means of three narrow surface channels. The bulk of the reaction compartment was stirred continuously with two magnetic rods at a rate of 250 rpm. A lipid solution (around 10 μl at 1 mg ml^{-1} in chloroform) was spread onto the buffer surface. The monolayer was allowed to stabilize for 5–10 min, and was then compressed to a preselected end point surface pressure (ranging from 1 to 25 mN m^{-1}; barrier speed 10 mm min^{-1}). Constant surface pressure was maintained automatically by the computer controlled barrier movement throughout the experiment. Once the monolayer had stabilized at the selected surface pressure for 5 min, soybean LOX1 (ranging from 0 to 0.8 μg ml^{-1}, final concentration) was injected into the reaction compartment. The rate of lipid oxidation in the monolayer was recorded continuously (at a constant surface pressure) by measuring the backward movement of the mobile barrier resulting from the enzymatic oxidation-dependent increase in the monolayer area (Fig. 2a). This increase in the monolayer area is thought to result not only from the formation of hydroperoxides at the interface due to the oxidation of the lipid film, but also from the penetration of the protein into the interface. The real oxidation rate of lipid films was therefore measured by subtracting the kinetic curve due solely to the LOX1 penetration into the unsaturated lipid film from the kinetic curve corresponding to the sum of LOX1 penetration and oxidation-dependent catalysis in the unsaturated lipid films (Fig. 2b).

RESULTS AND DISCUSSION

Soybean LOX1 Penetration into a Preformed Monomolecular Lipid Film

Since the variations in surface pressure reflect both the penetration and the catalytic activity of LOX1, it is necessary to study first the penetration process into non-LOX1 substrate lipid films. To measure the penetration capacity of soybean LOX1 into monomolecular films, the protein was injected below lipid films of C18:1 or diC18:1PC maintained at a fixed initial surface pressure ($\pi_{initial}$), keeping the area constant. An increase with time in the surface pressure was observed due to the penetration of the LOX1 into the lipid film. The maximum increase in the surface pressure $\Delta\pi = \pi_{final} - \pi_{initial}$ results both from the affinity of the LOX1 for the lipid/water interface and from its intrinsic tensioactivity.

The penetration characteristics of soybean LOX1 were determined using non-substrate lipid monolayers, e.g., C18:1 and diC18:1PC (Fig. 3). At various initial surface pressures ($\pi_{initial}$), soybean LOX1 (0.37 μg ml^{-1}, final concentration) was injected into the aqueous phase, and the variations in surface pressure ($\Delta\pi$) due to the penetration of the enzyme into C18:1 or diC18:1PC monolayers was recorded. The linear dependency between $\Delta\pi$ and $\pi_{initial}$ is a general

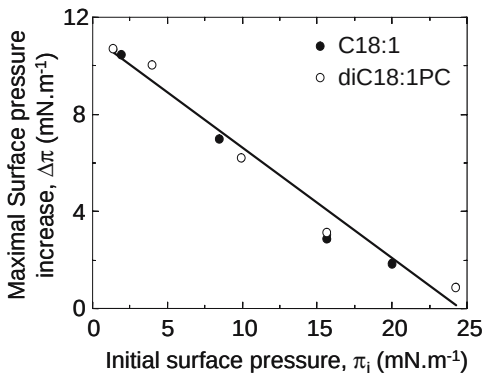

Fig. 3. LOX1 penetration into C18:1 and diC18:1PC monolayers. Maximum surface pressure increase ($\Delta\pi$) reached after LOX1 (0.54 μg ml^{-1}, final concentration) injection under a C18:1 film (•) or a diC18:1PC film (○) spread at various initial surface pressures (π_i). The buffer was 10 mM sodium acetate (pH 5) for C18:1 film and 10 mM Tris–HCl (pH 8) for diC18:1PC film.

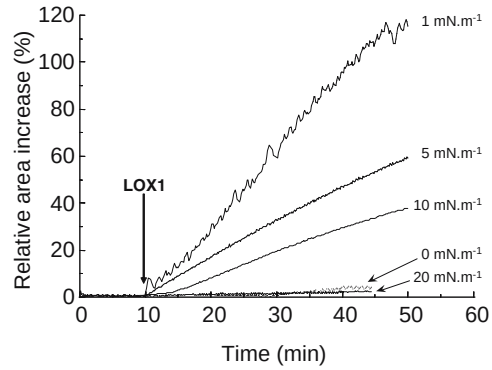

Fig. 4. Kinetics of LOX1-catalyzed oxidation of diC18:2PC monolayers spread at various surface pressures. The diC18:2PC monolayers were spread at a predetermined surface pressure (indicated in mN m^{-1}) in a zero-order trough and LOX1 (0.37 μg ml^{-1}, final concentration) was injected into the magnetically stirred reaction compartment. The surface pressure of the monolayer was kept automatically constant by the barrier movement, which was recorded continuously as a function of time. The buffer was 10 mM Tris–HCl (pH 8).

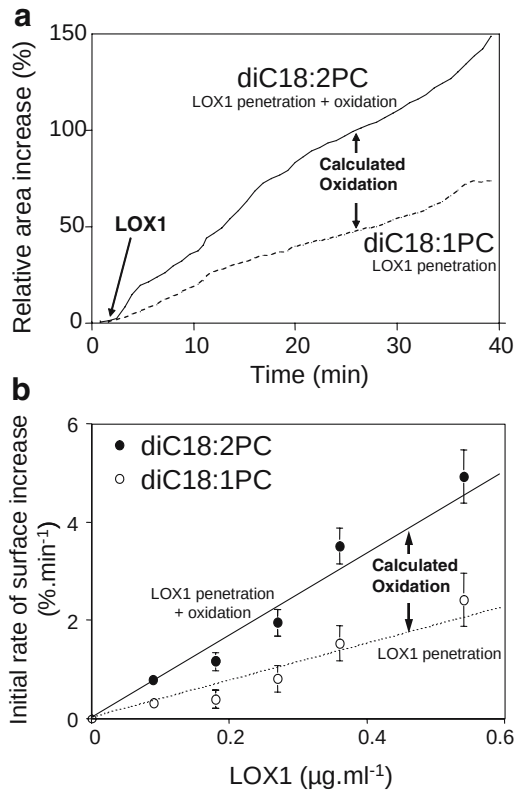

Fig. 5. LOX1 penetration into diC18:1PC film and LOX1 penetration and catalysis of a diC18:2PC film. (a) Kinetic curves of the penetration of LOX1 into a diC18:1PC film and the LOX1-catalyzed oxidation and penetration rate of a diC18:2PC film. A monolayer of diC18:1PC or diC18:2PC was spread and kept at a constant surface pressure of 1 mN m^{-1}. LOX1 (0.37 μg ml^{-1}, final concentration) was injected into the aqueous phase composed of 10 mM Tris-HCl buffer (pH 8) in the reaction compartment under an argon atmosphere. The expansion of the monolayer area at constant surface pressure was recorded as a function of time. (b) LOX1 penetration into diC18:1PC film and LOX1 penetration and catalysis of a diC18:2PC film as a function of the enzyme concentration.

property of protein penetration (18). Extrapolation to $\Delta\pi = 0$ gives a measure of the penetration capacity of the protein into the lipid monolayer. The critical surface pressure for soybean LOX1 penetration into a C18:1 or diC18:1PC film was thus estimated: it corresponds to the extrapolated initial surface pressure (around 25 mN m^{-1}) beyond which no increase in the surface pressure occurred (Fig. 3).

Oxidation of Lipid Monolayers by LOX1 at a Constant Surface Pressure

In order to determine the surface-pressure dependency of the LOX1 enzymatic activity on diC18:2PC films, the monolayers were spread and compressed to various initial surface pressures ranging from 1 to 20 mN m^{-1}. The increase in monolayer area was recorded as a function of time after the injection of the LOX1 into the reaction compartment (Fig. 4). The LOX1-catalyzed oxidation of diC18:2PC films was characterized by a time- and surface pressure-dependent increase in the monolayer area. At constant surface pressures of 20, 15, 10, 5, or 1 mN m^{-1}, the LOX1 injection (0.37 μg ml^{-1}, final concentration) into the aqueous phase caused the diC18:2PC film to expand by about 5, 10, 50, 90, or 140%, respectively, after 30 min. This increase in the monolayer area was probably due to the penetration of the LOX1 molecules into the interface as well as the LOX1-dependent oxidation of the diC18:2PC films. Interestingly, the adsorption of LOX1 at the argon/water interface (0 m N m^{-1}) was found to be negligible (Fig. 4).

As shown in Fig. 4, the soybean LOX1-catalyzed oxidation of pure diC18:2PC film was faster at low surface pressures, which indicates that the acyl chains containing *cis*-

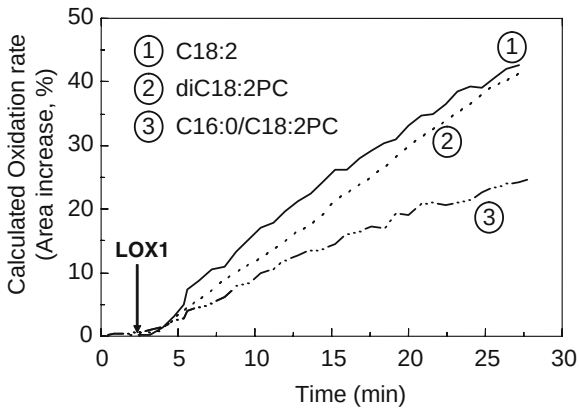

Fig. 6. Kinetic curves of the LOX1-catalyzed oxidation of C18:2 methyl ester, diC18:2PC and C16:0/C18:2PC monolayers. A monolayer of either C18:2 methyl ester or diC18:1PC or diC18:2PC was spread and kept at a constant surface pressure of 1 mN m^{-1}. At zero time, LOX1 (0.37 μg ml^{-1}, final concentration) was injected into the aqueous phase, composed of 10 mM Tris-HCl buffer (pH 8), in the reaction compartment under argon atmosphere. The expansion of the monolayer area at a constant surface pressure was recorded as a function of time. The increase in the monolayer area reflects the LOX penetration and catalysis in either C18:2 methyl ester or diC18:2PC or C16:0/C18:2PC films. The LOX penetration was measured separately and subtracted using either C18:1 methyl ester or diC18:1PC or C16:0/C18:1PC films, respectively.

9,12 double bonds of the phospholipid were properly oriented and accessible at the interface. At high surface pressures, diC18:2PC molecules were tightly packed and the acyl chains containing *cis*-9,12 double bonds were presumably forced out from the interface, making it difficult for the enzyme to reach its site of action. These results suggest that soybean LOX1 may be able to oxidize lipids in biological membranes in the expanded state. To measure the LOX1-catalyzed oxidation accurately, independently from the penetration of the enzyme into the lipid monolayer, we used monolayers of DiC18:2PC (substrate) and DiC18:1PC (non-substrate) at a surface pressure of 1 mN m^{-1}. The increase in the monolayer area was recorded as a function of time after injecting soybean LOX1 into the reaction compartment. The rate of LOX1-catalyzed oxidation of diC18:2PC film was measured by subtracting the rate of LOX1 penetration into diC18:1PC film from the kinetic curve of LOX1 penetration and catalysis on diC18:2PC film.

As shown in Fig. 5a, at a constant surface pressure of 1 mN m^{-1}, injecting LOX1 (0.37 μg ml^{-1}, final concentration) into an aqueous phase covered with a diC18:1PC monolayer caused the film to expand by 50% within 30 min. This increase in monolayer area was due solely to the penetration of the enzyme into the interface. In the case of a diC18:2PC monolayer, the corresponding increase in the area was 110% within 30 min (Fig. 5a). This increase in area is thought to be caused not only by the formation of hydroperoxides at the interface due to the LOX1-catalyzed oxidation of the diC18:2PC film, but also by the penetration of the enzyme into the interface. The real rate of LOX1-catalyzed oxidation of a diC18:2PC film was measured by subtracting the kinetic curve of the LOX1 penetration into a diC18:1PC film from the kinetic curve corresponding to the LOX1 penetration and catalysis in a diC18:2PC film (Fig. 5).

The initial rate of diC18:2PC oxidation by soybean LOX1 at a constant surface pressure of 1 mN m^{-1} was measured as a function of the enzyme concentration (Fig. 5b). The diC18:2PC monolayer oxidation rates were found to be linearly dependent on the soybean LOX1 concentration.

The kinetic curves of LOX1-catalyzed oxidation of C18:2, diC18:2PC, and C16:0–C18:2PC films are presented in Fig. 6. Control experiments were performed during this study with C18:1, diC18:1PC and C16:0–C18:1PC films as controls for C18:2, diC18:2PC, and C16:0–C18:2PC films, respectively. As shown in Fig. 6, at a constant surface pressure of 1 mN m^{-1}, similar initial rates of LOX1-catalyzed oxidation were obtained with C18:2 and diC18:2PC films. It is worth noting that the surface density of the acyl chains was also similar in C18:2 and diC18:2PC films. The LOX1-catalyzed oxidation rate was twice as fast on a diC18:2PC film as on a C16:0–C18:2PC film. It is worth noting that a PC film containing two oxidizable fatty acids (diC18:2PC) was oxidized at approximately twice the rate of a PC film with a single oxidizable chain (C16:0–C18:2PC). It is generally agreed that free polyunsaturated fatty acids are the most efficient substrates for LOX (5). However, other studies have provided experimental evidence that fatty acids esterified in phospholipids can undergo a specific process of oxidation by LOX (19–21). Pérez-Gilabert *et al.* (22) recently established that the soybean LOX1 is able to oxidize diC18:2PC, yielding an intermediate and a final reaction product. In the intermediate product, only one of the two linoleoyl chains (either *sn*1 or *sn*2) was oxidized. In the final product, both linoleic acyl chains were converted into hydroperoxides (22).

Studies in which lipid monolayers were used as the enzyme substrate have yielded some information as to how the topology and physicochemical properties of the interface may influence lipid oxidation. We have reported that the enzymatic lipid oxidation seems to occur when the monolayer is in the expanded state. Under physiological conditions, one can imagine that upon enzymatic hydrolysis, the lipolytic products will be captured by natural acceptors such as serum albumin leading to a membrane pressure decrease (expanded state). This expanded state may possibly result *in vivo* from the lipolysis of a biomembrane and consequently lipolysis and lipid oxidation are coupled at the membrane level.

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