Calcium Is Not Required for 5-Lipoxygenase Activity at High Phosphatidyl Choline Vesicle Concentrations

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ABSTRACT: 5-Lipoxygenase (5-LO) catalyzes the formation of 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and leukotriene A_4 (LTA_4) from arachidonic acid. Following a rise in intracellular calcium, 5-LO translocates to a membrane where it reacts with arachidonic acid via an 18 kD protein (FLAP). In vitro studies using a vesicle system of phosphatidylcholine (PC) and purified 5-LO were conducted under varying concentrations of PC and calcium. At high PC concentrations, 5-LO partitioned onto the vesicle containing arachidonic acid, resulting in product formation in the absence of calcium. Addition of calcium increased the initial rate of the reaction with a small increase in product accumulation. Dilution experiments in the absence of calcium at high PC concentrations indicated that binding of 5-LO to the vesicles is rapidly reversible. In the presence of calcium, this binding is much more favorable than without calcium. Stimulation of 5-LO activity by dithiothreitol (DTT) was more pronounced at high PC concentrations than at low PC concentrations. The requirement for ATP for maximal activity was independent of vesicle concentration. Inhibitors that functioned in the conditions of low PC with calcium present also inhibited under high PC without calcium. In the presence of PC and calcium and without substrate, the enzyme was unstable and was rapidly and irreversibly inactivated. In high PC without calcium, the enzyme was much more stable but it was still subject to turnover-dependent inactivation. Fluorescence energy-transfer experiments confirmed the kinetic findings that 5-LO could bind to the vesicle in the absence of calcium. These results show that in the absence of calcium, 5-LO can reversibly bind to the vesicle containing arachidonic acid and produce the same amount of product by a similar mechanism as observed with low PC and calcium. Calcium likely causes a conformational change that increases the affinity of the enzyme for the vesicle, but it is not strictly required for enzymatic activity and has no effect on the function of the catalytic site.

5-Lipoxygenase (5-LO)[1] is a 78 kD protein responsible for the oxidation of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequent conversion of 5-HPETE to leukotriene A_4 (LTA_4) as the first two steps of the leukotriene biosynthesis pathway (*1, 2*). 5-Lipoxygenase activity in cell-free preparations is highly calcium dependent and is stimulated by ATP, dithiothreitol (DTT), and phosphatidylcholine (*3, 4*). In intact cells the enzyme is also calcium and ATP dependent and has the additional requirement for a leukocyte protein (5-lipoxygenase activating protein, FLAP) found in the membrane (*5*).

5-LO has been shown to translocate from the cytosol or the nucleus to the nuclear membrane with ionophore challenge in human peripheral blood leukocytes (*6*), in RBL-2H3 cells (*7*), in the human myeloid cell line HL-60 (*8*), and in human alveolar macrophages (*9*). Upon translocation to the membrane and in the presence of FLAP, leukotriene production occurs. Without FLAP, in these cell systems no 5-lipoxygenase activity is observed. This translocation from the cytosol to the membrane can be inhibited by quinoline inhibitors such as MK-886 (*8*). It has been shown that MK-886 binds to FLAP and prevents 5-lipoxygenase from binding to the membrane (*10*).

Initially it was postulated that FLAP was essential for translocation by binding 5-LO to the membrane via a "docking" mechanism (*11*). It was considered that drugs such as MK-886 were binding to a site on FLAP which mediated the interaction of FLAP with 5-lipoxygenase and hence were blocking a protein−protein interaction. However it was shown from immunoblot analysis of osteosarcoma cells expressing only 5-LO and no FLAP that 5-LO still translocated to the membrane fraction with calcium-A23187 stimulation (*12*). As expected, MK-886 did not inhibit this FLAP-independent translocation as the inhibitor was specific for FLAP. No product was observed with the FLAP-free cell system, consistent with the absolute requirement for FLAP for enzyme activity.

Although FLAP was ruled out as necessary for translocation, the requirement for calcium for 5-LO to be found in

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[1] Abbreviations: 5-LO, 5-lipoxygenase; 5-HPETE, 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; LTA_4, leukotriene A_4; FLAP, 5-lipoxygenase activating protein; AA, arachidonic acid; PC, L-α-phosphatidylcholine; 13(S)-HPODE, 13(S)-hydroperoxy-9-*cis*-11-*trans*-octadec dienoic acid; ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; D-57, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine.

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the membrane fractions has remained (*13*). Even in studies in which calcium ionophore challenge was replaced by IgE/antigen stimulation, an increase in calcium concentration was still observed concomitant with 5-LO translocation to the membrane (*14*).

The requirement for calcium in cell-free systems with low phospholipid concentration has correlated well with the cell-based studies (*15*). Calcium in micromolar concentrations under micromolar phosphatidylcholine (PC) concentrations has been observed to be required for 5-lipoxygenase activity (*4*). Under low calcium concentrations extended lag phases were observed in the time course for 5-HPETE production, consistent with the activation requirement of calcium (*4*). Studies with hydrophobic-binding columns have shown that human 5-LO binds to a hydrophobic column only in the presence of calcium. (Percival, M. D., unpublished results.)

The first indication that 5-LO may not absolutely require calcium for activity was the observation that a small amount of product was always produced in the absence of calcium in the cell-free system with low phosphatidylcholine vesicle concentration. This residual activity suggested that 5-LO could bind to the arachidonic acid-containing vesicles without calcium.

To explain this residual activity, we have postulated that the enzyme can bind to the vesicle containing arachidonic acid without calcium. Calcium alters the binding affinity of the enzyme for the membrane or vesicle. The effect of calcium on the activity of 5-LO could be due entirely to tighter binding of the enzyme to the vesicles, or there could be an activation due to changes at the catalytic site as well.

In this study we asked whether 5-LO activity was dependent only on an altered catalytic activity induced by calcium or on the equilibrium concentration of the enzyme−vesicle system complex. To determine the calcium requirement, the effect of various concentrations of phosphatidylcholine with and without calcium on enzyme activity was studied. In this investigation we find that calcium is not required for in vitro 5-LO activity but affects the partitioning of the enzyme between the vesicle surface and the aqueous phase.

MATERIALS AND METHODS

Materials. Peroxide-free arachidonic acid (AA) was purchased from Cayman Chemical (Ann Arbor, MI). 13-(S)-HPODE was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). L-α-phosphatidylcholine Type III-E (PC): from egg yolk, adenosine 5′-triphosphate (disodium salt, ATP), calcium chloride, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), and potassium phosphate buffer were purchased from Sigma Chemical (St. Louis, MO). *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (triethylammonium salt, D-57) and fura-II calcium titration kit were purchased from Molecular Probes Inc. (Eugene, OR). Human 5-LO was obtained from a baculovirus/insect cell expression system and purified to apparent homogeneity using affinity chromatography on an ATP−agarose column (*4*). To remove ATP, the enzyme was desalted on a Pharmacia fast desalting column using 50 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA as buffer.

Preparation of Arachidonic Acid-Containing Vesicles. Phosphatidylcholine vesicles, 20 mg/mL, containing 16 mM arachidonic acid were prepared by evaporating under argon a solution of 40 μL (100 mg/mL in ethanol) egg-PC and 10 μL (0.32 M in ethanol) peroxide-free arachidonic acid and resuspending with sonication (2 min) in 200 μL (0.05 M, pH 7.4) K-phosphate buffer containing 0.1 mM EDTA.

Determination of 5-Lipoxygenase Activity. The standard 5-LO assay mixture contained 0.05 M K-phosphate buffer, pH 7.4, 0.2 mM ATP, 0.1 mM EDTA, 0.1 mM DTT, 1−10 μL 20 mg/mL PC containing 16 mM AA, and 0−0.3 mM $CaCl_2$. The enzyme was added to 1 mL of the assay mixture and the production of product, 5-HPETE, was followed spectrophotometrically at 238 nm. The initial rate and plateau (total product formed) were measured, and the total activity was calculated from the plateau using $\epsilon = 23\,000$ M^{-1} cm^{-1} (*16*). The $k_{inactivation}$ rate was estimated assuming a first-order decay of the enzyme activity as previously described (*6*).

Fluorescence Energy Transfer Experiments. Vesicles (10 mM, 7.7 mg/mL PC) containing 10% D-57 were prepared by evaporating under argon a solution of egg-PC (20 μL, 130 mM, 100 mg/mL) and D-57 (26 μL, 10 mM in ethanol) and rediluting into 260 μL ethanol. Energy-transfer experiments for translocation were conducted using a 1 mL solution containing 0.1 mM EDTA, 20 μM PC (10% D-57) in 0.05 M K-phosphate buffer, pH 7.4. 5-LO (1 μg) and various amounts of calcium were added and the fluorescence was immediately read with excitation at 283 nm (slit width 5 nm) and emission at 515 nm (slit width 10 nm) on a Perkin-Elmer LS50B luminescence spectrophotometer. The % fluorescence was calculated on the basis of the fluorescence maximum obtained at 0.3 mM calcium. To the same cell was then added 20 μM AA. The initial rate was measured spectrophotometrically at 238 nm, and after the reaction reached completion it was quenched with methanol (250 μL). The total amount of 5-HPETE product formed was measured by HPLC (65:35 CH3CN:H2O, 0.1% HOAc, NovaPak C18 column). Activity was calculated using total product formation, as the initial rate data was not accurate at calcium concentrations below 1 μM. The actual calcium concentration in the reaction mixture was measured by fura-2 fluorescence titration using the kit provided by Molecular Probes Inc.

RESULTS

The Effect of Vesicle Concentration on 5-LO Activity in the Absence of Calcium. The rate of the 5-LO reaction has been shown to be dependent on the ratio of arachidonic acid to phospholipid rather than on the bulk arachidonic acid concentration (*17*). Therefore, for proper comparisons the molar fraction of arachidonic acid was kept constant in all experiments.

The effect of increasing vesicle concentration under constant molar fraction of arachidonic acid without calcium was investigated. 5-Lipoxygenase was added to standard buffer containing ATP, DTT, and EDTA with various concentrations of PC vesicles containing a constant molar fraction of arachidonic acid. The results (Figure 1) show an increase in 5-LO activity as the vesicle concentration increases. At high vesicle concentration (Figure 1, line d), the total 5-HPETE formed was the same as the standard 5-LO assay conducted under low PC conditions with calcium

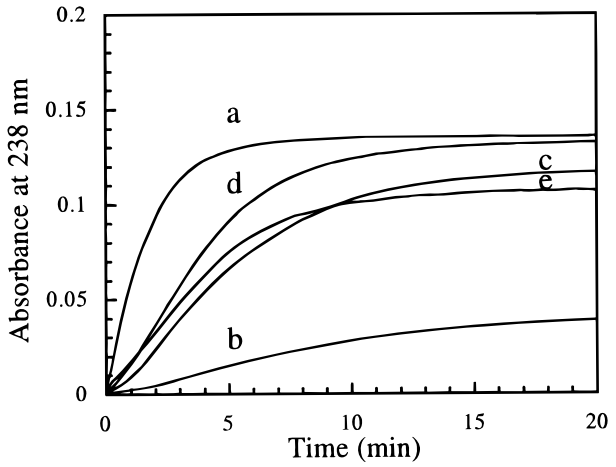

FIGURE 1: Effect of vesicle concentration on 5-LO activity in the absence of calcium. Aliquots from a stock solution of vesicles containing arachidonic acid ([PC] = 27 mM, [AA] = 16 mM) were added to 1 mL of standard reaction buffer less calcium. The reaction was initiated with 1 nM active 5-LO, and the production of 5-HPETE followed at 238 nm; (a) control with calcium (0.3 mM), 16 μg/mL PC, (b) 27 μg/mL PC, (c) 135 μg/mL PC, (d) 270 μg/mL PC, (e) 540 μg/mL PC.

(Figure 1, line a) as previously optimized (*4*). Inactivation during the time course of the enzyme reaction still occurred under high PC without calcium, although the rate of inactivation was slower under these conditions than under the standard conditions with low PC and calcium. LTA_4 levels were also measured at the end of the reaction by HPLC (*18*) at high PC concentrations (200 μg/mL PC, 160 μM arachidonic acid, 0 or 0.3 mM calcium), and no significant change was observed with and without calcium present (data not shown).

The Effect of Calcium on 5-LO Activity under Various Vesicle Concentrations. To characterize any activation due to calcium alone, the effect of calcium on 5-LO activity under low (12 μg/mL), medium (100 μg/mL), and high (200 μg/mL) PC with constant molar fraction of arachidonic acid was studied. The results from the time courses are shown in Figure 2A and 2B as measurements of the total product formed (plateau) and the initial rate, respectively. At low PC concentration, calcium causes a 10-fold increase in the activity of the enzyme as measured by initial rate. As the concentration of vesicle increases, the stimulation by calcium is less and the differences in both the plateaus and initial rates with and without calcium become neglible. Note that higher PC/arachidonic acid concentrations with calcium result in significant rate decreases.

High Vesicle Concentration: Effect of DTT. It has been previously shown that the presence of DTT and EDTA stimulates 5-LO activity by about 20% under low PC conditions with calcium present (*4*). To characterize the activity of 5-LO under conditions of high PC without calcium, the enzyme's activity with and without DTT was measured. As shown in Figure 3, the activation of 5-LO by DTT is dependent on vesicle concentration. In the absence of DTT, 5-LO maintains about 80% of its activity when the vesicle concentration is kept low (Figure 3, line b versus line a), while at high concentration of vesicle in the absence of DTT, 5-LO maintains only about 25% of the activity observed in the presence of DTT (Figure 3, line d versus line c). This suggests that DTT may be activating 5-LO by

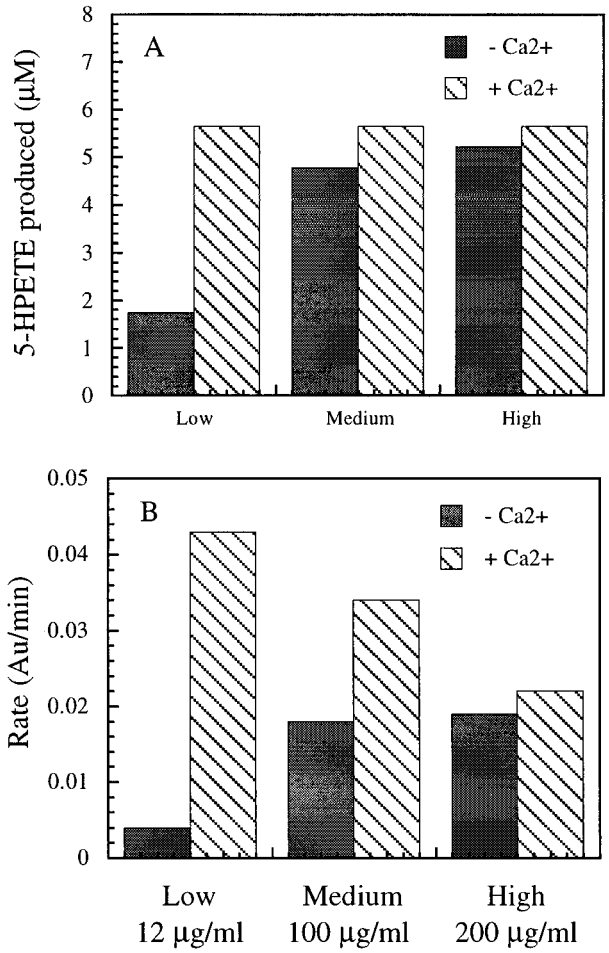

FIGURE 2: Effect of calcium on 5-LO activity in low, medium, and high vesicle concentration. The activity of 5-LO was measured under low (12 μg/mL), medium (100 μg/mL), and high (200 μg/mL) PC concentrations with constant molar ratio of AA (1.6 PC:AA) in standard reaction buffer with and without calcium (0.3 mM). The reaction was initiated by addition of 1 nM active 5-LO, and the total production (Plot A) and initial rate (Plot B) of 5-HPETE produced was followed at 238 nm.

reducing inhibitory oxidation products (*18*) from either the PC or the arachidonic acid.

High Vesicle Concentration: Effect of ATP. The stimulation of 5-LO by ATP has been reported many times (*1*), the extent of stimulation usually being on the order of 2-fold (*4*). This activation has been reported to be dependent on the presence of calcium (*19*). To test whether ATP stimulation does require calcium, the activity of desalted 5-LO with and without ATP under high PC and no calcium conditions was measured. Under these conditions a maximal stimulation (Figure 4) of approximately 2-fold was observed at 0.2 mM ATP, indicating that calcium does not need to be present for ATP stimulation.

High Vesicle Concentration: Effect of 13-HPODE. Kinetic lags have been observed for both the soybean 15-LO (*20*−*22*) and the human 5-LO (*23*). This lag is believed to be due to the time required for the enzyme to be oxidized from the inactive reduced Fe(II) form to the active oxidized Fe(III) form. Addition of fatty acid hydroperoxides will oxidize the enzyme rapidly to the active Fe(III) form and reduce or eliminate the initial lag (*24*). The time course for product formation under high PC conditions without calcium is shown in Figure 5 and shows this kinetic lag. Addition

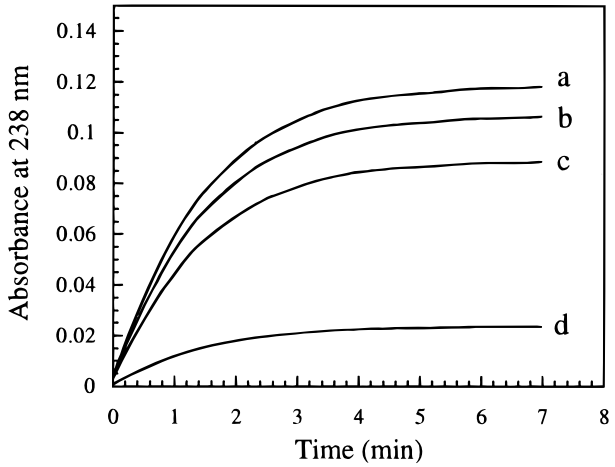

FIGURE 3: Effect of DTT on 5-LO activity in low and high vesicle concentration. To standard reaction buffer containing 0.1 mM EDTA and 0.2 mM ATP was added PC, calcium, and DTT as indicated: (a) 12 μg/mL PC, 0.3 mM calcium, 0.5 mM DTT; (b) 12 μg/mL PC, 0.3 mM calcium, 0 mM DTT; (c) 200 μg/mL PC, 0 mM calcium, 0.5 mM DTT; (d) 200 μg/mL PC, 0 mM calcium, 0 mM DTT. After 5 min, 1 nM active 5-LO was added and the reaction initiated 1 min later by the addition of 20 μM AA and 0.3 mM calcium.

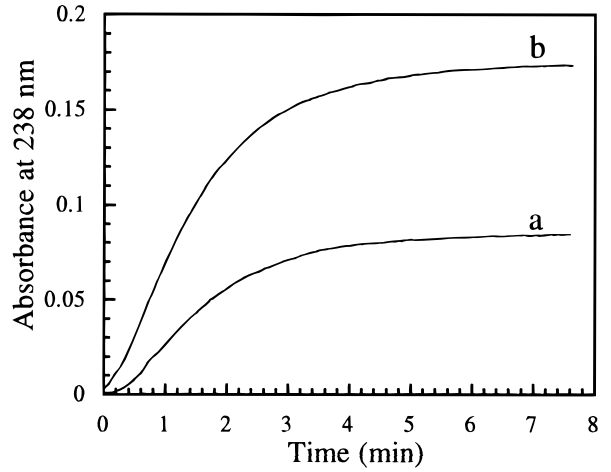

FIGURE 4: Effect of ATP on 5-LO activity at high vesicle concentration without calcium. To buffer containing 200 μg/mL PC and 160 μM AA without calcium was added (a) 0 μM ATP and (b) 0.2 mM ATP. The reaction was initiated with 1 nM active 5-LO, and the production of 5-HPETE was followed at 238 nm.

of 3 μM 13-HPODE considerably reduced the lag, suggesting the same mechanism for 5-LO activation [Fe(II) to Fe(III)] occurs in both the absence and presence of calcium.

High Vesicle Concentration: Effect of Inhibitors. There are two classes of compounds which inhibit 5-LO by two different mechanisms. The hydroquinones are representatives of the redox class of inhibitors causing the reduction of the active Fe(III) enzyme to the inactive Fe(II) enzyme (*25*, *26*). The phenylpyridines and lignans are nonredox, direct inhibitors binding to both the reduced and oxidized forms of the enzyme and preventing reaction with arachidonic acid (*27*). To test whether inhibitor binding to 5-LO is influenced by calcium, the IC50's of various redox and nonredox inhibitors were determined under high PC conditions with no calcium and under low PC conditions with calcium. Inhibitor time course patterns and IC50's (measured as molar fraction in vesicle) were not altered by

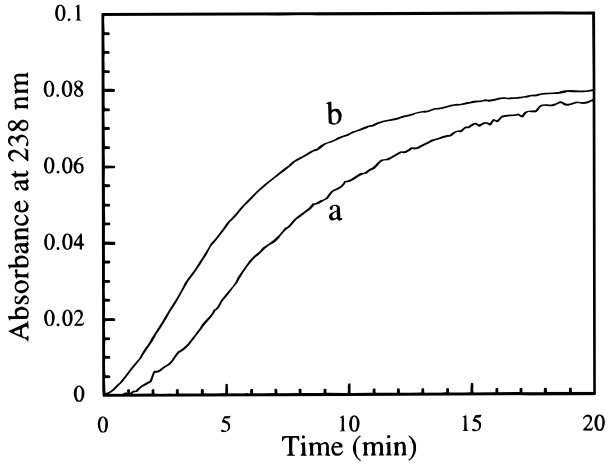

FIGURE 5: Effect of 13-HPODE on 5-LO activity in high vesicle concentration without calcium. To buffer containing 200 μg/mL PC and 160 μM AA without calcium was added (a) no additions and (b) 3 μM 13-HPODE. The reaction was initiated with 1 nM active 5-LO, and the production of 5-HPETE was followed at 238 nm.

eliminating calcium, indicating that calcium is not required for inhibitor binding (data not shown).

Equilibrium Binding to the Vesicle. In a vesicle system, the enzyme can partition on and off the vesicle rapidly. We propose that calcium prevents the enzyme from coming off the vesicle once it binds to it. To test this hypothesis, a split-cell experiment was conducted in which the enzyme was added to one side of the cell containing PC and arachidonic acid with and without calcium. The other compartment of the cell contained only buffer without PC, arachidonic acid, or calcium. The initial activity was measured, then the cell was inverted thus diluting the reaction mixture 2-fold with buffer, and the time course measurements were continued. If the enzyme binds tightly to the vesicle, dilution in a split cell should not cause any noticeable change in rate since the 2-fold dilution of the enzyme would be exactly compensated by the doubling of the light path. If, however, the enzyme rapidly dissociates from the vesicle upon dilution, then a 2-fold dilution should cause the rate to decrease as the new equilibrium level of enzyme bound to vesicles is established. The results of these experiments are shown in Figure 6A and 6B. Under high PC conditions without calcium, the activity was decreased upon dilution (Figure 6A), indicating that 5-LO binding to the vesicle is rapidly reversible. In the presence of calcium (Figure 6B), no change in activity was observed upon dilution, as expected since in the presence of calcium, 5-LO is partitioned fully toward binding to the vesicles both before and after dilution by inverting the split spectrophotometer cell.

High Vesicle Concentration: Effect on Stability of Enzyme. In vitro, 5-LO is rapidly irreversibly inactivated in the presence of low concentrations of PC and calcium (*4*, *18*). To test whether inactivation of 5-LO under high PC still requires calcium, 5-LO was preincubated with and without calcium and initial rates were measured at various time points. Under conditions of high PC without calcium, efficient conversion of arachidonic acid in the vesicle to 5-HPETE is observed, implying translocation to the vesicle has occurred. As shown in Figure 7, under high PC conditions the enzyme is reasonably stable, losing only about

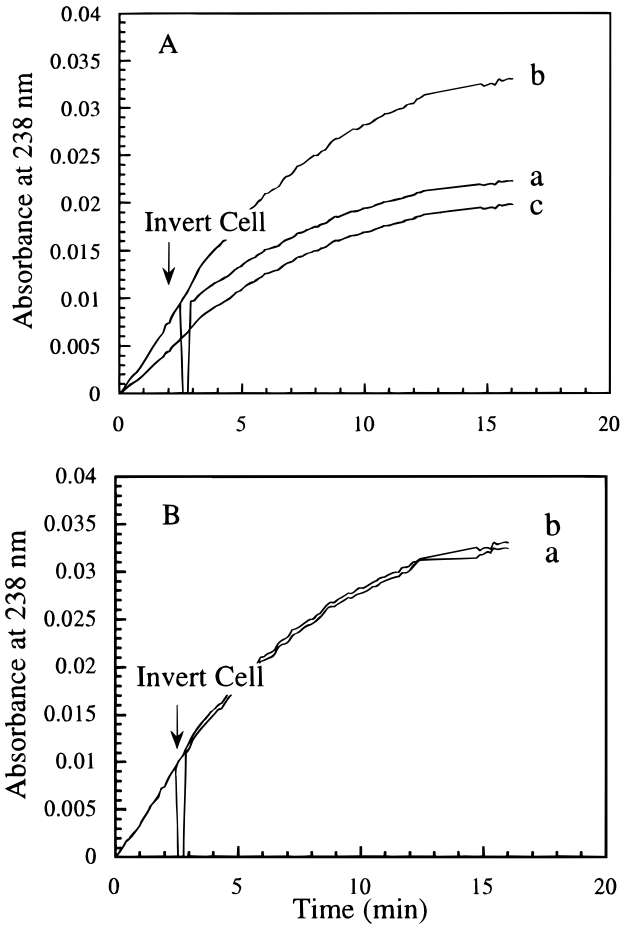

FIGURE 6: Reversibility of 5-LO binding to the vesicle under high vesicle concentrations. The progress curves for production of 5-HPETE were compared using a divided quartz cuvette with side A containing 1 mL of buffer with 1.7 μM 13-HPODE, 100 μg/mL PC, 80 μM AA, and 0 mM calcium (Panel A) or 0.3 mM calcium (Panel B). Side B contained 1 mL of the above buffer without PC or AA. (a) Active 5-LO (1 nM) was added to side A and quickly mixed with a cuvette stick; the reaction was followed at 238 nm for 2.5 min. The cell was then removed and inverted to mix sides A and B together, and the absorbance measurements were continued. (b) 100% control was measured using the same conditions as (a), only the cell was not inverted. (c) Fifty percent control was measured using the same conditions as (b), except with 50 μg/mL PC and 40 μM AA.

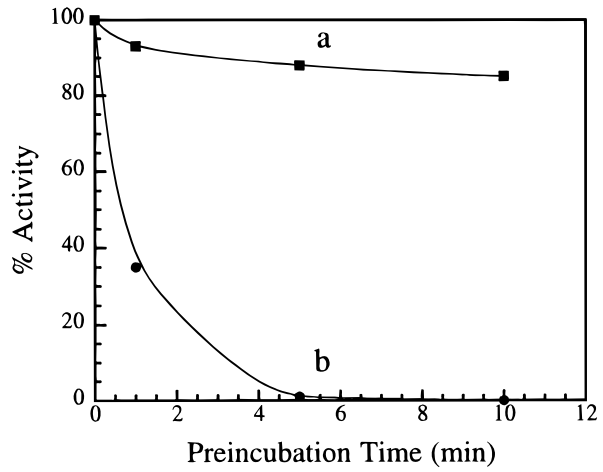

FIGURE 7: Stability of 5-LO under high vesicle concentration with and without calcium. 5-LO (1 nM) in 0.05 M K-phosphate buffer at pH 7.4 with 0.2 mM EDTA was incubated with 100 μg/mL PC and 0 mM calcium (a, ■) or 0.3 mM calcium (b, ●). At various times the reaction was initiated by the addition of 80 μM AA, and the production of 5-HPETE was followed at 238 nm. Percent activity was calculated on the basis of the total production of product.

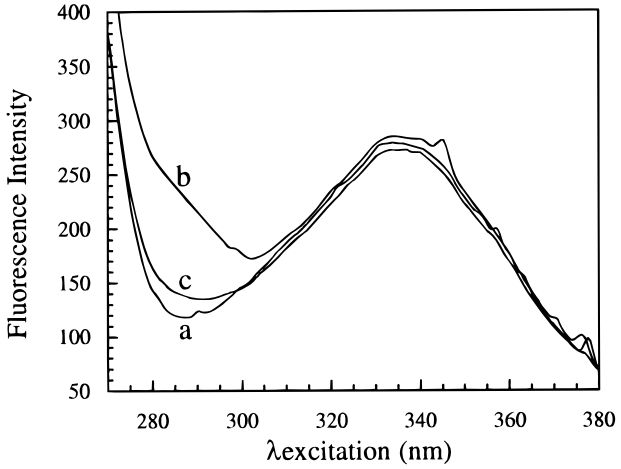

FIGURE 8: Binding of 5-LO to vesicles under low PC concentration with calcium followed by energy-transfer fluorescence. A fluorescence excitation scan (slit width 5 nm) was taken at $\lambda_{em} = 515$ nm (slit width 10 nm) of 5 μg/mL 5-LO in (a) K-phosphate buffer (0.05 M, pH 7.4) and 0.1 mM EDTA containing 12 μg/mL PC with 10% D-57, (b) after addition of 0.3 mM calcium, (c) after further addition of 0.5 mM EDTA.

20% of its activity after 10 min. Only when calcium is added does the enzyme rapidly inactivate.

Evidence of Enzyme Binding to a PC Vesicle in the Presence of Calcium. The binding of a protein to a lipid can be monitored using fluorescence energy transfer. The fluorescence of PC vesicles labeled by spiking with dansyl-PE (D-57) can be enhanced as energy flows from tryptophan in the protein to the dansyl moiety of the lipid. This is monitored by following the excitation spectrum of the dansyl group. The excitation of the dansyl at 283 nm will show additional contributions corresponding to absorption by the protein energy donor when the protein and vesicle are within 20 Å (*28*). Shown in Figure 8 is the excitation spectrum of low concentration (20 μg/mL) mixed PC vesicles containing 10% D-57 (Figure 8, trace a). Addition of 5-LO did not change the excitation spectrum, but upon addition of calcium, a greater excitation at 280 nm was observed as the enzyme bound to the vesicles and energy transfer from the protein to the dansyl-PE occurred (Figure 8, trace b). When EDTA was added, the dansyl excitation signal returned to baseline indicating that the enzyme can dissociate from the vesicle when calcium is removed (Figure 8, trace c).

Activity versus Translocation. The optimal amount of calcium required for enzyme activity has been previously determined under low PC conditions (*4*). To correlate the calcium-induced activity to enzyme translocation, a titration of calcium versus fluorescence energy transfer and activity was conducted. As shown in Figure 9, both the translocation (indicated by the fluorescence signal) and activity (indicated byproduct formation) followed the same titration curve.

Evidence of Enzyme Binding to PC Vesicles in the Absence of Calcium. An experiment similar to that described above was conducted using high concentrations of mixed PC (200 μg/mL, 260 uM) containing 1% D-57. As shown in Figure 10, addition of 5-LO caused an increase in the fluorescence excitation spectrum at 283 nm of the mixed PC vesicles

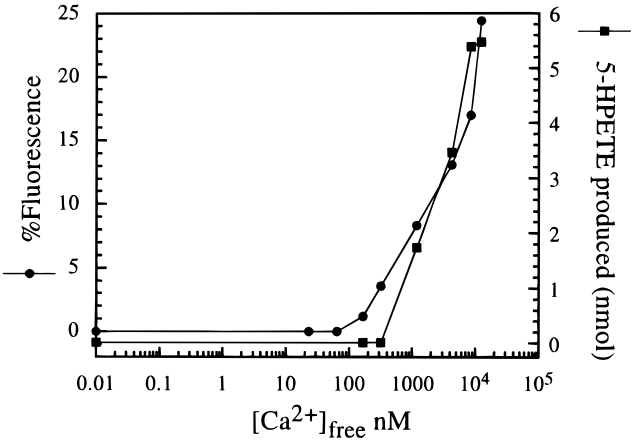

FIGURE 9: Titration of calcium on 5-LO activity and translocation followed by energy-transfer fluorescence. To 1 mL reaction buffer containing 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM ATP, 12 μg/mL PC with 10% D-57 was added 1 μg/mL 5-LO. Various amounts of calcium were added, and the fluorescence at λ_{ex} = 283 nm (slit width 5 nm) and λ_{em} = 515 nm (slit width 10 nm) was monitored and plotted as % fluorescence (●). To the same cell was then added 20 μM AA, and the total amount of 5-HPETE product formed was measured by HPLC (■). The free calcium concentration in the reaction mixture was measured by fura-2 fluorescence titrations.

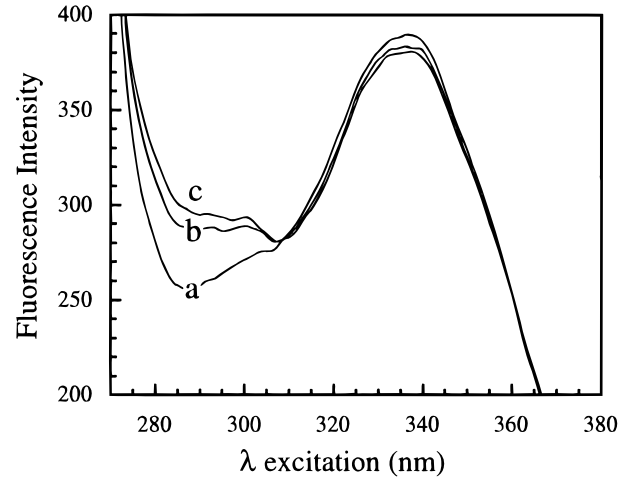

FIGURE 10: Binding of 5-LO to vesicle under high PC concentration without calcium followed by energy-transfer fluorescence. A fluorescence excitation scan was taken at λ_{em} = 515 nm in (a) K-phosphate buffer (0.05M, pH 7.4) and 0.1 mM EDTA containing 200 μg/mL PC with 1% D-57, (b) after addition of 2 μg/mL 5-LO, (c) after further addition of 0.3 mM calcium.

(Figure 10, trace b versus trace a). Addition of calcium caused only a minor increase in fluorescence signal (Figure 10, trace c). This is direct evidence that the enzyme can bind to the vesicle when no calcium is present. Note that the overall energy-transfer efficiency was reduced under high PC, as expected since the dansyl surface concentration was diluted 10-fold compared to the previous experiment which used 20 μM PC and 10% dansyl. The effect of the FLAP inhibitor MK-886 on the binding to the vesicle under high PC conditions was measured. MK-886 is believed to inhibit the production of leukotrienes by preventing 5-LO from binding to the membrane by blocking an arachidonic acid binding site on FLAP (*29*). As expected, MK-886 did not prevent the binding of the enzyme to the PC vesicle under high PC conditions with or without calcium (data not shown). This is in agreement with cell systems in which binding of the enzyme to the membrane under calcium ionophore challenge was observed in the presence of MK-886 and in the absence of FLAP (*10*).

DISCUSSION

The results presented here demonstrate that 5-lipoxygenase can translocate from the aqueous phase to the lipid−water interface of PC vesicles in the absence of calcium and that the same total amount of product can be produced both in the presence and absence of calcium. These results by themselves suggest that binding of calcium to 5-LO does not cause a change in the catalytic site to make it more active, but simply makes the translocation of 5-LO to the lipid−water interface more favorable. Other results which support this model are (1) the approximately 2-fold rate increase caused by ATP both at the low PC concentration in the presence of calcium and at high PC without calcium, (2) the similar effect of added 13-HPODE in decreasing lags in the time course of product formation under both sets of conditions, and (3) the similar sensitivity to inhibitors both in the presence and absence of calcium.

However, there are also significant differences in behavior in the presence and absence of calcium, at least some of which could be ascribed to changes in the catalytic site. The major differences in behavior reported here are the following. (1) In the presence of high PC concentrations most of the 5-LO is at the lipid−water interface (as indicated by fluorescence energy transfer measurements) and turnover-independent inactivation is very much slower in the absence than in the presence of calcium. It has been reported elsewhere (*18*), and been confirmed by us, that direct 5-LO inhibitors strongly retard this inactivation, so it presumably involves some function of the catalytic site. (2) The maximum rate achievable in the presence of high PC concentrations without calcium is less than half that achieved at low PC concentrations with calcium. (3) The rate of turnover-dependent inactivation is considerably slower at high PC concentrations without calcium than at low PC concentration with calcium. This is a direct consequence of the catalytic rate differences. (4) Increasing PC concentration with calcium present decreases the rate without changing the total amount of product formed, while increasing PC concentration over the same range in the absence of calcium increases both the rate and the amount of product formed. (5) At high PC concentrations without calcium, where fluorescence energy transfer measurements indicate that most of the 5-LO is bound to the vesicles, the 5-LO dissociates in less than 10 s after dilution. In the presence of calcium, 5-LO remains bound to the vesicles even upon dilution for over 10 min unless EDTA is present.

The five points above can be rationalized without having to postulate a calcium-induced change in the catalytic site of 5-LO if one takes into account the tight binding of 5-LO to a PC vesicle in the presence of calcium, the rapid exchange of 5-LO from vesicle to vesicle at high PC concentration without calcium, the exchange of 5-HPETE among vesicles, and the requirement of 5-HPETE or other fatty acid hydroperoxide to maximally activate 5-LO.

We will first consider the stability of the enzyme in the presence of high PC concentrations unless calcium is present. It has been shown that the PC/calcium-induced inactivation of 5-LO can be considerably retarded by active-site directed

5-LO inhibitors or by treatment of the PC with reducing agents or with glutathione and glutathione peroxidase (*18*). This suggests that the PC/calcium-induced inactivation is due to some reactive radical species generated by the action of 5-LO on hydroperoxides present in the PC. When calcium is present, a given 5-LO molecule remains bound to the PC vesicle containing the reactive species and is likely to be damaged by it. In the absence of calcium, even though the 5-LO is active and mostly bound to vesicles at high PC concentrations, the enzyme does not remain bound to the vesicle in which it has generated the reactive species. The reactive species may be quenched before another 5-LO molecule binds to a vesicle containing them.

The rapid exchange of 5-LO molecules among vesicles in the absence of calcium can also account for the observation that the maximum rate achievable at high PC concentrations is less that half that obtained at low PC concentrations with calcium present. It has been well documented that a certain concentration of 5-HPETE or other fatty acid hydroperoxide is necessary to maintain 5-LO in its active Fe(III) oxidation state (*24*). In the presence of calcium, 5-LO remains bound to the same vesicle long enough for the concentration of 5-HPETE in that vesicle to increase to sufficient levels to maintain the enzyme in its active form. In the absence of calcium, a given 5-LO molecule does not remain bound to the same vesicle long enough to generate enough 5-HPETE in that vesicle to maximally activate the 5-LO. The inhibitory effect of higher PC concentrations on the rate in the presence of calcium cannot be accounted for by inactivation of the enzyme, at least up to 200 μg/mL PC, because the total amount of product generated is not affected by PC concentration in the 12−200 μg/mL range. The rate-retarding effect of increasing PC concentrations in the presence of calcium can be accounted for by an increase in the rate of exchange of 5-HPETE among vesicles as PC concentration increases. Oxygenated lipids are known to exchange between vesicles at much faster rates than saturated lipids, and vesicle−vesicle collision accelerates this exchange process (*30*).

Thus, all of the major differences observed in the presence and absence of calcium can be accounted for simply in terms of calcium causing a change in the affinity of 5-LO for PC vesicles.

In cells, translocation from the cytosol to the nucleus can also occur without calcium by in vivo recruitment and in vitro adherence (*31*). However, calcium stimulation is still required for 5-LO activation on the nuclear membrane where both FLAP and arachidonic lipids are found. There is no evidence that 5-LO can rapidly reverse off the nuclear membrance once bound to it. Instead, the enzyme suffers a similar fate to in vitro 5-LO under PC/calcium conditions and is inactivated in the cell under calcium-ionophore challenge (*13*).

It is not possible on the basis of available results to say with certainty whether 5-LO binding to PC vesicles is rapidly reversible in the presence of calcium, as it clearly is in the absence of calcium. However, the effect of calcium on the turnover independent inactivation of 5-LO at high PC concentrations suggests, as indicated above, that in the presence of calcium 5-LO dissociates relatively slowly, once bound to a PC vesicle. This could be due to an unfavorable dissociation of calcium once the 5-LO is at the lipid interface. It is possible that the conformation of 5-LO which has high affinity for PC and is stabilized by calcium must convert to the low-affinity conformation in order to dissociate at a rapid rate. Furthur speculation on this point is not justified by available data, so this question must remain an interesting challenge for future studies to address.

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