# 5-Lipoxygenase Binds Calcium†

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ABSTRACT: 5-Lipoxygenase (5LO) catalyzes the first two steps in the biosynthesis of leukotrienes and lipoxins and has therefore become an important target for pharmacological treatment of inflammatory disorders. Binding of calcium to 5LO was shown using several different approaches. Human recombinant enzyme was expressed in *E. coli* and purified. Association of $Ca^{2+}$ to 5LO was demonstrated by a calcium-induced mobility shift in gel electrophoresis, by calcium overlay, by gel filtration in the presence of calcium, and by equilibrium dialysis. The two latter methods also showed that calcium binds reversibly to 5LO. Equilibrium dialysis gave a $K_d$ close to 6 $\mu$M; the stoichiometry of maximum calcium binding seemed to average around two $Ca^{2+}$ per 5LO. Similar results were obtained when 5LO was inactivated during equilibrium dialysis, indicating that the calcium binding site(s) is (are) different from the active site. By Triton X-114 partitioning, it was confirmed that calcium increases the hydrophobicity of 5LO.

Human 5-lipoxygenase (5LO)[1] catalyzes the first two steps in the biosynthesis of leukotrienes: oxygenation of arachidonic acid to yield 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and the subsequent dehydration to form the unstable epoxide intermediate 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid. The latter compound (leukotriene $A_4$) is the precursor of the biologically active leukotrienes $B_4$, $C_4$, $D_4$, and $E_4$ (*1*), which are regarded as mediators in asthma and other inflammatory and allergic disorders (*2*).

Calcium stimulates 5LO enzyme activity and membrane association. Regarding intact cells, ionophore A23187 was first shown to stimulate 5LO activity in human PMNL (*3*). Subsequently, the significance of elevated intracellular calcium levels (evoked by ionophores and other stimuli) for activation of 5LO was further elucidated (*4*−*7*). Also in cell-free systems and after purification, calcium stimulates 5LO activity (*8*, *9*). For purified 5LO, the concentration of calcium giving half-maximal activation was 1−2 $\mu$M while full activity was reached at 4−10 $\mu$M (*10*−*12*). When compared, it was found that the calcium concentrations required for stimulation of the two activities of 5LO (oxygenase and $LTA_4$-synthase) were quite similar (*11*, *13*). It should be noted that 5LO has some activity also without addition of calcium (*14*, *15*).

5LO activity in vitro is low unless a membrane fraction or phosphatidylcholine is included in the assay mixture (*9*, *13*, *16*). After homogenization of leukocytes in the presence of calcium, 5LO was recovered in the cell membrane fraction, but not when the homogenization was done with EDTA (*17*, *19*). When intact cells were stimulated to produce leukotrienes (by ionophore, *N*-formylmethionylleucinylphenylalanine, or IgE−antigen complexes), membrane association of 5LO occurred as well (*7*, *18*, *20*, *21*). In studies utilizing rat basophilic leukemia cells, it was determined that extracellular calcium was important for this process (*6*). More detailed subcellular fractionations, and immunohistochemical studies, showed that 5LO primarily binds to the perinuclear membrane, where also most of the 5LO activating protein (FLAP) resides permanently [see, for example, (*22*−*25*)]. Further studies on 5LO activity in the presence of detergents were performed, and it was concluded that 5LO performs its catalysis at a lipid/water interface (*16*, *26*). It was also found that purified 5LO can bind to phosphatidylcholine vesicles in a $Ca^{2+}$-dependent manner (*11*). It would seem that 5LO activation is usually coupled to membrane binding; however, using 5LO inhibitors it was demonstrated that membrane binding can occur also in the absence of 5LO activity (*27*, *28*). Recently, it was published that at high concentrations of phosphatidylcholine (PC), 5LO activity in vitro can be high without addition of $Ca^{2+}$. Nevertheless, $Ca^{2+}$ improved the binding of 5LO to PC vesicles (*12*).

Thus, it is generally agreed that calcium stimulates 5LO activity and leukotriene production by promoting membrane binding. However, an alternative mechanism for stimulation of 5LO activity has also been suggested, i.e., that calcium could form a complex with arachidonic acid (*14*). The amino acid sequence of 5LO does not contain an obvious $Ca^{2+}$ binding motif, but weak homologies to calcium-dependent membrane binding proteins in the annexin family have been

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[1] Abbreviations: 5LO, 5-lipoxygenase (EC 1.13.11.34); 5-H(P)ETE, 5-hydro(pero)xyeicosatetraenoic acid; PMNL, polymorphonuclear leukocytes; $C_{12}E_8$, polyoxyethylene-8-lauryl-ether; PVDF, poly(vinylidene difluoride); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BME, $\beta$-mercaptoethanol; GP, gluthathione peroxidase; SOD, superoxide dismutase; BSA, bovine serum albumin.

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reported (*29*, *30*). In this report we show that 5LO binds calcium.

## EXPERIMENTAL PROCEDURES

*Materials.* All chemicals were of analytical grade and, unless stated otherwise, obtained from Merck. Soybean lipoxygenase, parvalbumin, and detergents were from Sigma. PVDF membranes, bovine serum albumin, and Chelex 100 (100−200 mesh, Na form) were from Bio-Rad, and $^{45}CaCl_2$ (specific activity 10−40 mCi/mg) was from Amersham.

*Purification of Recombinant Human 5-LO.* 5LO was expressed in *E. coli* and purified using the previously described ATP−agarose affinity chromatography procedure (*31*). The sequence of the plasmid used for expression was confirmed, and the activity of different preparations of purified protein was around 20 $\mu$mol mg$^{-1}$ (10 min)$^{-1}$. To obtain ATP-free and homogeneous 5LO, two additional chromatography steps were added. First, most of the ATP was removed by gel filtration. The eluate from the ATP column (2 mL) was loaded onto a 10-mL column, containing Sephadex G-75 (Pharmacia), and eluted with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl. Second, the protein-containing fractions from G-75 were subjected to anion exchange chromatography on a MonoQ column (Pharmacia). Buffer A was 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 14 mM $\beta$-mercaptoethanol (BME). Buffer B was 20 mM Tris-HCl, pH 7.5, 0.5 M KCl, 14 mM BME. In a gradient from 0 to 100% buffer B, 5LO eluted at about 40% buffer B. To 5LO-containing fractions were added the stabilizing enzymes glutathione peroxidase (GP) and superoxide dismutase (SOD) [0.1 unit/mL (1 unit = 1.5 $\mu$g) and 4.2 units/mL (1 unit = 0.24 $\mu$g), respectively]. Thus, the added amounts of glutathione peroxidase and superoxide dismutase were at most 0.15 $\mu$g/100 $\mu$g of 5LO and 1 $\mu$g/100 $\mu$g of 5LO, respectively. For long-time storage at −20 °C, glycerol to 24% (v/v) was added, and protein aliquots were snap-frozen in a dry ice/ethanol bath.

*Gel Electrophoresis in the Presence of $Ca^{2+}$.* SDS−PAGE (7.5%, w/v, 0.75 mm thick) were prepared according to Laemmli (*32*), except for inclusion of either 1 mM $Ca^{2+}$ or 0.1 mM EGTA both in stacking and in separation gels. After electrophoresis, the gels were stained with Coomassie brilliant blue. Relative migration was calculated as the ratio of distances migrated (from bottom of sample well to center of protein spot).

*$^{45}Ca^{2+}$ Overlay of Protein Blots.* After conventional SDS−PAGE (*32*) using precast gradient gels from Bio-Rad (Ready Gels 4−15%), and electrotransfer onto PVDF membranes, $^{45}Ca^{2+}$ overlay was performed according to le Maire et al. (*33*). The membrane was first washed 3 times for 10 min with 5 mM imidazole buffer (pH 7.4) containing 1 mg/mL octaethylene glycol dodecyl ether ($C_{12}E_8$) and then incubated for 30 min in 15 mL of a solution containing 5 mM imidazole buffer (pH 7.4), 1 mg/mL $C_{12}E_8$, 60 mM KCl, 5 mM $MgSO_4$, and 10 $\mu$M $^{45}CaCl_2$ (specific radioactivity 1 mCi/$\mu$mol). The membranes were washed 3 times with 15 mL of 30% (v/v) ethanol in deionized water, and dried on Whatman filter paper at room temperature for at least 4 h before exposure to Fuji RX film at −70 °C for 35−50 h. In many experiments, two gels with the same set of samples were run and blotted simultaneously. One membrane was Coomassie-stained and the other subjected to $^{45}Ca^{2+}$ overlay.

*Gel Filtration in the Presence of $^{45}Ca^{2+}$.* Calcium binding was also studied by the method of Hummel and Dreyer (*34*). A gel filtration column (Sephadex coarse G-25, 1 × 14 cm) was equilibrated at room temperature with the glycerol-containing buffer in which 5LO was stored after purification [15 mM Tris-HCl, pH 7.5, 11 mM BME, 167 mM KCl, glycerol 24% (v/v)], supplemented with 20 $\mu$M $^{45}CaCl_2$ (specific radioactivity 1 mCi/$\mu$mol). Radioactive calcium was also added to the 5LO sample to the same final concentration as in the column buffer, and the sample was incubated 5−20 min prior to chromatography. 5LO did not lose activity during this preincubation. The sample (5−8 nmol in 450 $\mu$L) was applied, and the column was eluted at 0.1 mL/min. Fractions of 0.3 mL were collected in siliconized tubes, and protein and radioactivity contents were measured.

*Equilibrium Dialysis.* Dialysis tubes (4 cm long) were made from glass tubing (5 mm diameter). The tubes were siliconized (to prevent attachment of proteins) by immersion for 1 min in 5% (v/v) dimethyldichlorosilane in toluene, followed by rinsing twice in ethanol and drying at 60 °C overnight. Dialysis membrane (Spectra/Por, 3500 MWCO) was attached to one end of the tube using a piece of Tygon tubing to hold the membrane firmly in place. Up to nine tubes were tightly fitted into holes in the lid of a 250-mL plastic beaker.

Protein samples (MonoQ-purified 5LO, 15−35 $\mu$g/50 $\mu$L) were added to the dialysis tubes. The plastic beaker contained 200 mL of dialysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 14 mM BME, 2 mM $MgCl_2$, 2−100 $\mu$M $CaCl_2$, and 0.2−0.4 $\mu$M $^{45}CaCl_2$, giving a radioactivity of 0.2−0.4 mCi/L). This buffer was prepared without $MgCl_2$ and $CaCl_2$, autoclaved, and stored in the presence of Chelex 100. On the day of use, the Chelex resin was removed by filtration through a Millipore GVWP filter, and the buffer was completed by addition of $MgCl_2$ and $CaCl_2$. In the experiments where ATP or phosphatidylcholine was included in the buffer, these, too, were added after filtration. Dialyses were carried out at +4 °C for 15−18 h with magnetic stirring. In each dialysis experiment, all samples were performed in triplicate or quadruplicate.

After dialysis, tube contents were well mixed by repeated pipetting and transferred to siliconized Eppendorf tubes. Two aliquots (8 $\mu$L) were used for scintillation counting and three aliquots (4−6 $\mu$L) for protein determination. The amount of bound $Ca^{2+}$ per 5LO was calculated after subtraction of the radioactivity in the buffer outside the dialysis membrane (corresponding to the free $Ca^{2+}$) from the radioactivity of the samples.

The absolute calcium concentrations of the $CaCl_2$ stock solution and selected samples from equilibrium dialysis buffers were analyzed by flame atomic absorption spectrophotometry using a Perkin-Elmer 5000 instrument.

*Protein Assay and Amino Acid Analysis.* Protein concentration was determined as described by Bradford (*35*), using a protein assay kit from Bio-Rad with bovine serum albumin (BSA) as standard. The accuracy of the Bradford assay was evaluated by comparison to analyses of the total amino acid composition of purified 5LO samples. The amino acid analyses were performed by the Protein Analysis Centre at Karolinska Institutet (ninhydrin-technique). The comparison gave a correction factor of 0.8, which was used for

adjustment of all 5LO concentrations determined by Bradford assay.

*Radioactivity Determinations.* Samples (8−100 $\mu$L) were thoroughly mixed with 2 mL of scintillation fluid (Insta-gel II, Packard or OptiPhase, "HiSafe3", Wallac), and radioactivity was determined using a Minibeta liquid scintillation counter (LKB−Wallac).

*Determination of Protein Hydrophobicity by Phase Partitioning.* In the procedure described by Bordier (*36*), the property of the nonionic detergent Triton X-114 to remain in solution at low temperature, but to form giant micelles and undergo phase separation at 30 °C, is utilized. The micelles can be recovered in a detergent phase, with attached hydrophobic proteins. To 5LO samples eluted from the MonoQ column (in 20 mM Tris-HCl, pH 7.5, 14 mM BME, and 220 mM KCl) was added Triton X-114 to a final concentration of 1% (w/v), at +4 °C. The clear protein sample (200 $\mu$L, 0.1−0.2 mg/mL) was overlaid on 300 $\mu$L cushions consisting of 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.06% Triton X-114, on the bottom of a small glass tube. The tube was incubated for 3 min at 30 °C, resulting in clouding of the two phases (micelle formation). The tube was then centrifuged for 3 min at 300*g* in a swinging-bucket rotor prewarmed to 37 °C. After centrifugation, the upper aqueous phase was clear again (devoid of detergent micelles), and beneath the opaque sucrose phase a third detergent phase (containing micelles with bound hydrophobic proteins) constituted a barely visible droplet on the bottom of the tube. For a second step, the upper aqueous phase was aspirated, cooled to +4 °C, and supplied with fresh Triton X-114 to 0.5%. The mixture was then put back on the same sucrose cushion and incubated for 3 min at 30 °C, and the tube was centrifuged. The upper aqueous phase was again removed and transferred to a separate tube. Also most of the sucrose cushion was removed to reach the detergent droplet, which was analyzed by SDS−PAGE. Before the upper aqueous phase was subjected to SDS−PAGE, Triton X-114 was added a third time (2%), to remove residual micelle-bound 5LO. After warming at 30 °C for 3 min and centrifugation (in a separate tube without sucrose cushion), the upper aqueous phase was finally collected for analysis. SDS−PAGE (*32*) was performed using precast gels (Ready Gels 10%, Bio-Rad). The effects of calcium and ATP on the phase partitioning of the protein were studied by addition of $Ca^{2+}$ (40−200 $\mu$M) or ATP (5 mM) to protein samples before the addition of Triton X-114.

## RESULTS

*Gel Electrophoresis in the Presence of $Ca^{2+}$.* Binding of $Ca^{2+}$ to 5LO was indicated by SDS−PAGE with calcium or EGTA present in the gel. This method, with calcium present in the entire gel, was previously used to detect proteins which bind calcium in a reversible manner (*37*). As can be seen in Figure 1, in the presence of calcium (1 mM) 5LO migrated faster (0.93 relative to BSA) than in the presence of 0.1 mM EGTA (0.90 relative to BSA). The migration of BSA in SDS−PAGE is not affected by the presence of calcium or EGTA in the gel (*33*), and no change regarding the migration of $LTA_4$-hydrolase could be observed. This is in accordance with the lack of effect of calcium on $LTA_4$-hydrolase enzyme activity. We could also

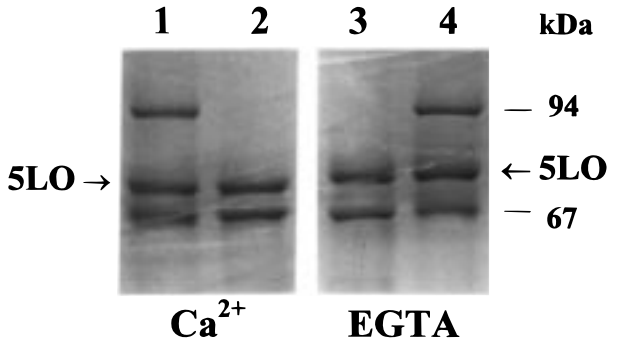


FIGURE 1: Effect of $Ca^{2+}$ on the electrophoretic migration of 5LO. SDS−polyacrylamide gel electrophoresis (7.5%) was performed in the presence of either 1 mM $CaCl_2$ or 0.1 mM EGTA in the gels. Proteins were stained with Coomassie brilliant blue. Lanes 1 and 4, phosphorylase *b*, 5LO, and bovine serum albumin; lanes 2 and 3, 5LO and $LTA_4$-hydrolase. Approximately 1 $\mu$g of each protein was loaded.

confirm that phosphorylase *b* migrated somewhat slower in the presence of calcium (phosphorylase *b*/BSA 0.71) than in the presence of EGTA (phosphorylase *b*/BSA 0.73), as published (*37*). For proteins that migrate faster in the presence of $Ca^{2+}$, it has been suggested that $Ca^{2+}$ binding, even in the presence of SDS, causes formation of a more compact molecular species that migrates at a faster rate (*33*, *37*). The presence of zinc (1 mM) in the gel did not affect the migration of 5LO (not shown).

*$^{45}Ca^{2+}$ Overlay after Protein Blots.* $Ca^{2+}$ binding of 5LO was demonstrated by $^{45}Ca^{2+}$ overlay of protein blots, a method first described by Maruyama et al. (*38*). In the modified protocol used in this study (*33*), the membrane is incubated in pH 7.4 buffer containing $MgSO_4$ (5 mM) and KCl (60 mM); in this milieu, $Ca^{2+}$ binding is expected to be specific (*39*). Octaethylene glycol dodecyl ether was also present in the buffer; this detergent is believed to aid renaturation of the transferred SDS-coated protein. The autoradiograph in the lower panel of Figure 2 visualizes the binding. 5LO (0.5 nmol) gave about the same signal as 2 nmol of the positive control protein, parvalbumin, which is known to bind 2 mol of $Ca^{2+}$/mol of protein (*40*). Similar signal intensity was observed using 0.5 nmol of calmodulin (data not shown). Neither soybean lipoxygenase, bovine serum albumin, nor recombinant human $LTA_4$-hydrolase showed any binding of $Ca^{2+}$ (Figure 2). Instead, these non-calcium binding proteins gave bright spots on the overlay membrane, probably by reducing the background binding. Interestingly, the stained proteins in the "kaleidoscope" standard all bound $Ca^{2+}$, presumably due to the attached chromophore. $Zn^{2+}$, in 10-fold excess to $Ca^{2+}$, could not compete in the overlay experiments, although $Zn^{2+}$ has been shown to inhibit 5LO enzyme activity (*10*). By this method, it was also demonstrated that human 5LO purified from leukocyte buffy coats could bind calcium. Equal amounts (0.4 nmol) of purified recombinant and native 5LO samples gave autoradiography spots of similar intensity (data not shown).

*Gel Filtration in the Presence of $^{45}Ca^{2+}$.* The "Hummel−Dreyer method" (*34*) was carried out as described by Ackers (*41*). The obtained gel filtration pattern was compatible with a reversible interaction between 5LO and $Ca^{2+}$ (Figure 3). Thus, the 5LO protein which was excluded from the G-25 column (eluted with the void) bound to $^{45}Ca^{2+}$, resulting in

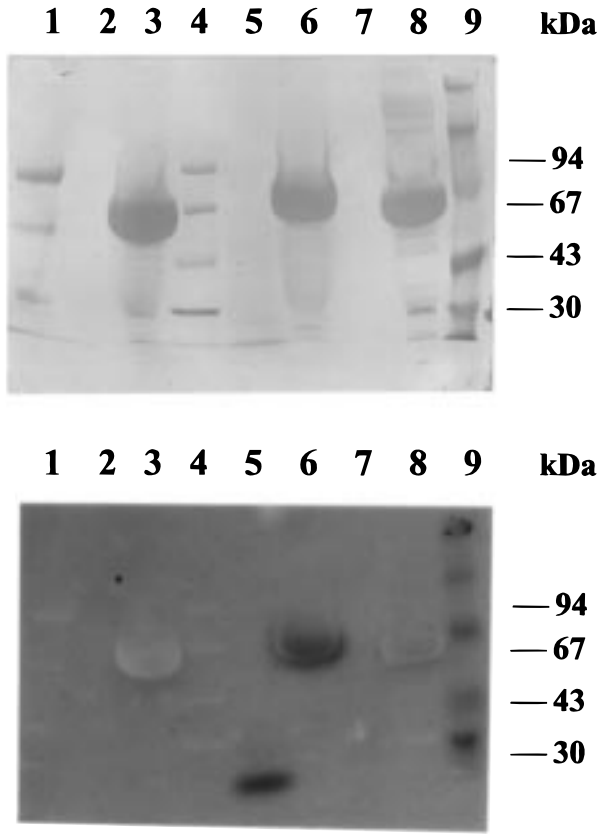


FIGURE 2: $^{45}Ca^{2+}$ overlay of blotted proteins. After electrophoresis of the same set of samples on two SDS−polyacrylamide gels (4−15%), proteins were electrotransferred onto PVDF membranes. One membrane was Coomassie stained (upper panel), and the other membrane was subjected to $^{45}Ca^{2+}$ overlay followed by autoradiography (lower panel). Lane 1, soybean lipoxygenase, 0.11 nmol; lanes 2 and 7, blanks; lane 3, $LTA_4$-hydrolase, 0.75 nmol; lane 4, molecular weight standard (Pharmacia, low molecular weight); lane 5, parvalbumin, 2 nmol; lane 6, 5LO, 0.51 nmol; lane 8, bovine serum albumin, 0.71 nmol; lane 9, molecular weight standard (Bio-Rad, prestained kaleidoscope).

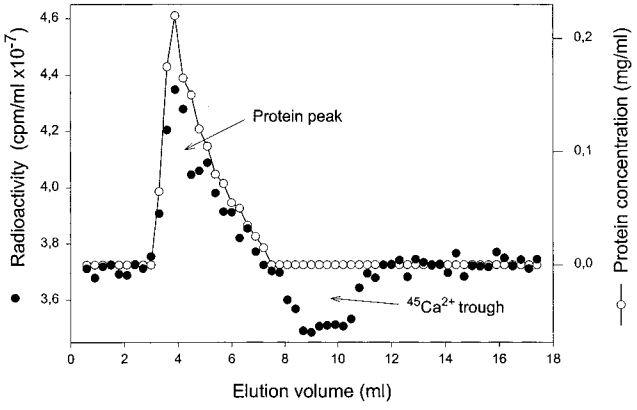


FIGURE 3: Gel filtration of 5LO in the presence of $^{45}Ca^{2+}$. A Sephadex G25 column (1 × 14 cm) was equilibrated with a buffer containing 15 mM Tris-HCl, pH 7.5, 11 mM $\beta$-mercaptoethanol, 169 mM KCl, glycerol 24% (v/v), and 20 $\mu$M $^{45}CaCl_2$. Purified 5LO (5.5 nmol in 450 $\mu$L) was preincubated in the same buffer, and applied. Fractions (0.3 mL) were collected and analyzed for protein content (open circles) and radioactivity (filled circles).

a radioactivity peak concordant with the protein peak. This peak was followed by a $^{45}Ca^{2+}$ radioactivity trough due to depletion of $^{45}Ca^{2+}$, since ligand was bound and carried ahead with the protein. In the experiment shown in Figure 3, the elution buffer contained glycerol (24% v/v). In other chromatographies excluding glycerol, the same pattern was obtained. In two independent experiments, the stoichiometry (ratio of radioactivity and protein in the 5LO peak) of the $Ca^{2+}$−5LO complex was 1.21 (no glycerol) and 0.94 (with glycerol). A control sample of 5LO, with the same concentration of $^{45}Ca^{2+}$ (20 $\mu$M) kept at +4 °C for the time of the experiment (3 h), retained 75% of its original enzyme activity.

*Equilibrium Dialysis of 5LO against $^{45}Ca^{2+}$.* A total of 19 dialysis experiments were performed, in which aliquots of purified recombinant human 5LO (typically 15−35 $\mu$g in 50 $\mu$L) were dialyzed against buffer containing radioactive calcium in different concentrations up to 70 $\mu$M. Every experiment resulted in a hyperbolic curve, typical of saturation binding. Without addition of 5LO to the dialysis tube, there was no accumulation of radioactive calcium. The stoichiometry at maximum $Ca^{2+}$ binding seemed to center around 2 (see Figure 4A which shows the mean saturation curve obtained from 8 experiments). However, in different experiments there was a variation in the level of the plateaus of the binding curves, as demonstrated by the substantial error bars. Several changes of the conditions were tried, but the variability was still present. We tested omission of $MgCl_2$ or BME from the dialysis buffer, or the effect of having a stabilizing detergent (CHAPS, 1 mM) present. CHAPS has proven helpful in maintaining 5LO activity during protein concentration treatments. The age of the isotope did not influence the variability ($^{45}Ca^{2+}$ decays to $Sc^{3+}$ which can bind to calcium binding sites). Neither was there any correlation to 5LO protein concentration. Dialyses were made with or without addition of 5LO stabilizing agents [GP and SOD, see ref (*42*)] to the dialysis tubes. Without GP and SOD, 5LO enzyme activity decreased during the dialysis to less than 10%; with stabilizing agents, activity was well preserved (more than 75% remaining activity). However, with both active and inactive 5LO, calcium binding was the same. This indicated that the $Ca^{2+}$ binding site(s) is (are) separated from the active site.

We also examined whether 5LO stimuli other than calcium could influence the calcium binding. Addition of phosphatidylcholine [PC, 250 $\mu$g/mL as such, or sonicated (PC-vesicles)] to the dialysis buffer (both inside and outside the membrane) did not change the variability of the $Ca^{2+}$ binding stoichiometry (see Figure 4B). As a control, PC alone (no 5LO) in the dialysis tube did not result in accumulation of radioactivity. Thus, in contrast to other $Ca^{2+}$ binding proteins which also bind phospholipids in a $Ca^{2+}$-dependent manner, e.g., the annexins and C2 region proteins [for a review, see (*43*)], the presence of phospholipids did not appear to influence 5LO's affinity for $Ca^{2+}$. However, when ATP (1 mM) was present during dialysis, the results appeared to become somewhat more stringent (see Figure 4C). With ATP present, there was less variation; however, the curve plateau did not quite reach 2 $Ca^{2+}$/5LO.

When all dialysis data (from experiments with and without PC or ATP) were compiled, the data could be nicely fitted to an equation representing a model with only one type of calcium binding site (see Figure 5). Thus, nonlinear regression calculations using average values from all 19 dialysis experiments gave a binding constant ($K_d$) of 6 $\mu$M and a maximum binding ($B_{max}$) of 2.2 mol/mol (regression coef-

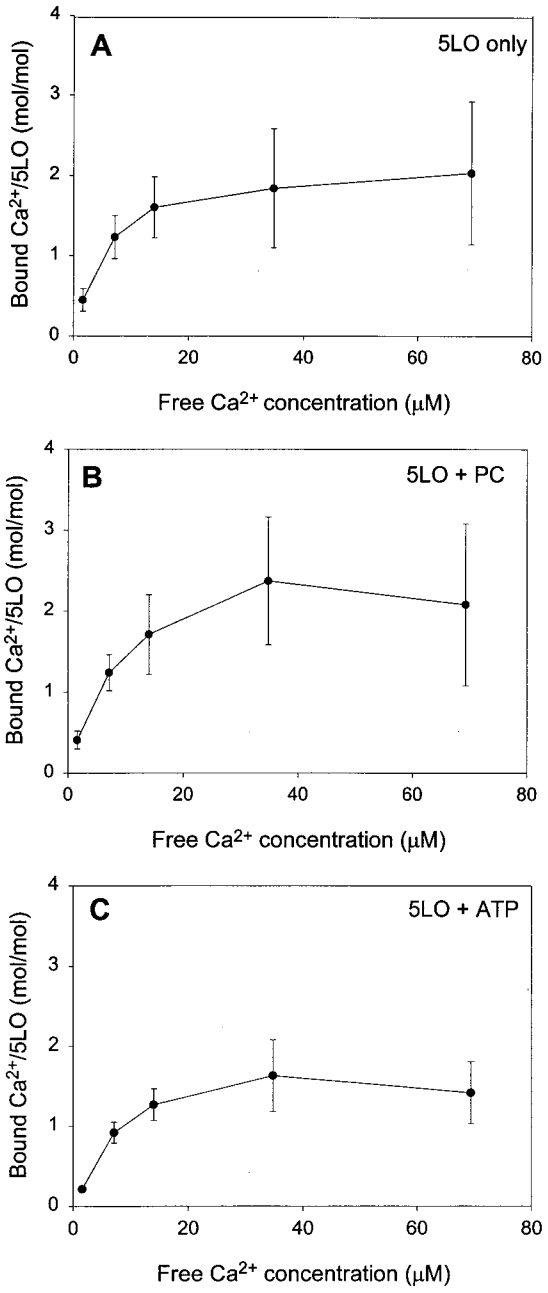


FIGURE 4: Binding of $^{45}Ca^{2+}$ to 5LO under various conditions. 5LO (0.35−0.50 nmol in 50 $\mu$L) was subjected to equilibrium dialysis against the indicated concentration of free $Ca^{2+}$ in 200 mL of 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 14 mM $\beta$-mercaptoethanol, and 2 mM $MgCl_2$. The graphs are mean saturation curves from 8 experiments without further additions (A), from 7 experiments with phosphatidylcholine (250 $\mu$g/mL) in the dialysis buffer (B), and from 4 experiments with ATP (1 mM) in the dialysis buffer (C). Each experiment comprised three or four parallell samples at each concentration of free $Ca^{2+}$. After dialysis, two aliquots of each sample were analyzed for protein and radioactivity. The error bars indicate standard deviations.

ficient 0.996). It was also attempted to fit the data to models containing two or more different types of calcium binding sites; however, the "one-site model" gave the best fit.

To substantiate the determinations of 5LO concentration used in the calculations, the total amino acid composition of two purified 5LO samples was analyzed. The values of 5LO amounts obtained by amino acid analysis turned out to be 0.81 ± 0.02 of the values obtained by the Bradford protein assay. Consequently, a factor of 0.8 was used to adjust all

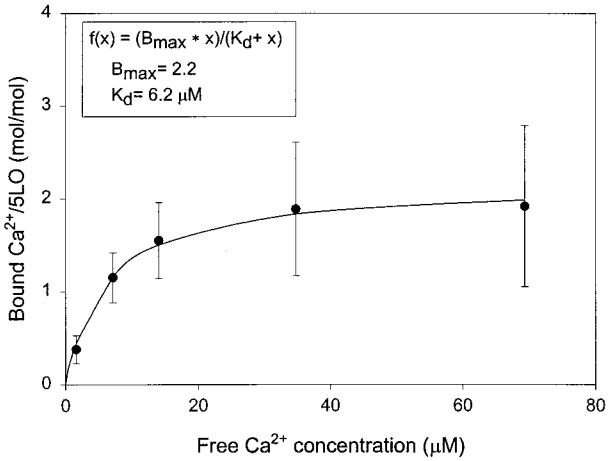


FIGURE 5: Regression analysis of equilibrium dialysis data. Data points (with standard deviations) are mean values from 19 equilibrium dialysis experiments (compare to Figure 4). The graph represents the equation of a hyperbolic binding curve (compare insert) with $K_d = 6.2$ $\mu$M and $B_{max} = 2.2$, as obtained from nonlinear regression analysis performed using SigmaPlot, $R = 0.996$.

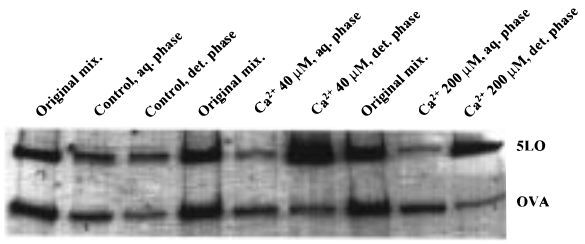


FIGURE 6: Effects of $Ca^{2+}$ on the hydrophobicity of 5LO determined by phase partitioning. A mixture of 5LO (35 $\mu$g) and ovalbumin (OVA, 40 $\mu$g) in 200 $\mu$L of MonoQ elution buffer (20 mM Tris-HCl, pH 7.5, 14 mM $\beta$-mercaptoethanol, and 220 mM KCl) was subjected to Triton X-114 phase partitioning as described under Experimental Procedures, in the absence (control) or presence of 40 or 200 $\mu$M $CaCl_2$. The sample volumes were as follows: original mixtures before partitioning, 200 $\mu$L; aqueous phases, 150−200 $\mu$L; and detergent phases, 30−60 $\mu$L. Aliquots (12 $\mu$L) of the samples were analyzed by SDS−polyacrylamide gel electrophoresis (10% gels). Protein bands were Coomassie stained.

protein concentrations determined by the Bradford assay in this study.

*Determination of Protein Hydrophobicity by Phase Partitioning.* Since calcium has been shown to promote membrane association of 5LO, we studied the influence of $Ca^{2+}$ on the hydrophobicity of 5LO. Triton X-114 phase partitioning, a procedure originally designed for purifying integral proteins (*36*), was used. In a calcium-free solution, 5LO behaved as a hydrophilic protein; i.e., most of the 5LO was recovered in the aqueous phase after phase partitioning. This was the case also for the control protein ovalbumin (Figure 6). When 40 $\mu$M $CaCl_2$ was added to the protein solution before partitioning, most of the 5LO was instead found in the detergent phase; thus, calcium had induced binding to the detergent micelles. The addition of calcium did not change the behavior of ovalbumin. Addition of ATP (1 mM, in the absence of calcium) rather improved the hydrophilicity of the proteins, and again both 5LO and ovalbumin were recovered in the aqueous phase (not shown). Please note that the volumes of aqueous and detergent phases are different; the 5LO:ovalbumin ratios of separate samples in Figure 6 should be compared. These results confirm that binding of

calcium to 5LO leads to increased hydrophobicity and affinity to membrane structures.

The influence of Triton X-114 on 5LO activity was tested in our standard 5LO activity assay (*31*). In the presence of 1% Triton X-114, the enzyme retained 27% of its activity (60% at 0.5% and 23% at 2%). This indicates that calcium could increase the hydrophobicity also of denatured 5LO.

## DISCUSSION

After the observation of a calcium-induced mobility shift in SDS−polyacrylamide gel electrophoresis (Figure 1), direct evidence for calcium binding of 5LO was obtained using different techniques. In $^{45}Ca^{2+}$ overlay experiments, 5LO gave signals comparable to well-known calcium binding proteins such as parvalbumin (Figure 2) and calmodulin (data not shown). The overlay experiments were performed with other salts present in the incubation buffer (KCl and $MgSO_4$) which improves the specificity of the method (*33*, *38*, *39*). Most of the data were obtained with recombinant 5LO expressed in *E. coli*, but with $^{45}Ca^{2+}$ overlay the calcium binding was shown also for 5LO purified from human leukocytes. $Zn^{2+}$ has been shown to inhibit 5LO activity (*10*); however, there was no effect of $Zn^{2+}$ on calcium binding as demonstrated by $^{45}Ca^{2+}$ overlay, and $Zn^{2+}$ did not induce a 5LO mobility shift in gel electrophoresis.

Equilibrium dialysis and gel filtration in the presence of $^{45}Ca^{2+}$ also demonstrated $Ca^{2+}$ binding, and that the binding was reversible. These methods also give estimates of the stoichiometry of $Ca^{2+}$ binding. Data from a series of equilibrium dialysis experiments could be fitted to a binding curve (Figure 5) with a $K_d$ of 6 $\mu$M and a $B_{max}$ of 2.2 mol of $Ca^{2+}$/mol of protein. This $K_d$ compares reasonably to the concentrations of $Ca^{2+}$ needed for activation of 5LO in vitro [half-maximal activation at 1−2 $\mu$M; full activity at 4−10 $\mu$M (*10*−*12*)]. It should be pointed out that in the hyperbolic curves (Figure 4) there was a variability in the saturation levels, resulting in stoichiometries between approximately 1:1 and 3:1 $Ca^{2+}$ per 5LO. A number of different experimental conditions did not change this variability (presence of $Mg^{2+}$, BME, or detergent; 5LO protein concentration; active/inactive 5LO; age of isotope). The presence of PC also did not affect the dialysis results, but in the presence of ATP (1 mM), the results seemed more uniform. In two gel filtration experiments carried out with a $^{45}Ca^{2+}$ concentration of 20 $\mu$M (Figure 3), the 5LO protein appearing in the protein peak carried 1.24 and 0.96 mol of $Ca^{2+}$/mol of protein, indicating a stoichiometry of 1:1. However, we tend to favor the results obtained from equilibrium dialysis (19 experiments with samples in triplicate). Many calcium binding proteins can bind several ions (*44*), and the $B_{max}$ of 2.2 mol/mol (Figure 5) might reflect a true stoichiometry of 2:1.

A $K_d$ of 6 $\mu$M indicates that purified 5LO has about the same affinity for $Ca^{2+}$ as free calmodulin (*45*), as some of the annexins (*44*), and as the C2 domain of cytosolic phospholipase $A_2$ (*46*). Interestingly, the calcium affinity of calmodulin apparently increases when calmodulin is associated with a target protein, and thus calmodulin-regulated enzymes can react to calcium at concentrations below 1 $\mu$M (*45*). Possibly, a similar association of 5LO with some intracellular protein could increase the affinity of 5LO for calcium, thus explaining the observation that for stimulation of 5LO in PMNL an intracellular calcium concentration of approximately 200 nM seems sufficient (*5*) while 1−2 $\mu$M calcium is required for half-maximal activation of purified 5LO.

Two stretches in sequences of 5LOs showed weak similarity to a 17 amino acid consensus sequence reported for the annexins (*29*, *30*). For the annexins, calcium stimulates binding to phospholipids, and the presence of phospholipids also increases the affinity to calcium (*44*). 5LO is similar to the annexins in the sense that calcium promotes membrane association, but from our data, the presence of phospholipid does not stimulate calcium binding of 5LO. Another difference between 5LO and annexins is that 5LO is stimulated only by PC (*11*, *13*, *47*). This seems in line with 5LO binding best to PC (*11*), although it has also been claimed that 5LO should bind best to phosphatidylserine (*47*). The annexins prefer acidic phospholipids and do not bind to PC (*44*). It was previously published that the presence of calcium should induce dimerization of 5LO (*48*). We performed high-resolution gel filtrations (on Superose 12) in the absence of calcium, with calcium (1 mM) present in the 5LO sample, and with calcium (0.5 mM) present in the elution buffer (data not shown). In all cases, 5LO migrated as a monomer, and we could not confirm a calcium-induced dimerization of 5LO.

Incubation of purified 5LO with calcium (20 $\mu$M) for 3 h did not lead to inactivation. This agrees with published data showing that 5LO was inactivated in the presence of both $Ca^{2+}$ and PC, but not with $Ca^{2+}$ only (*49*). Vice versa, the calcium binding was not dependent on the catalytic activity of 5LO since the equilibrium dialysis results were the same when 5LO activity was maintained during the dialysis by the presence of GP and SOD, as in the absence of these agents. In the absence of stabilizing agents, exposure to oxygen gradually inactivates 5LO (*10*, *42*). The inactivation mechanism involves loss of the central prosthetic iron of the lipoxygenase, which can be expected to lead to a severe change in the structure. Thus, binding of $Ca^{2+}$ to 5LO appears to be independent of higher-order structure around the iron-containing catalytic center of 5LO, possibly indicating that the calcium binding site(s) is (are) well separated from the active site. We confirmed the effect of calcium on membrane binding of 5LO using a Triton X-114 phase partitioning assay, in which the presence of calcium promoted transfer of 5LO from a hydrophilic phase to a detergent micelle phase (Figure 6). In this assay, the presence of the detergent inactivated 5LO (27% remaining activity). Nevertheless, calcium-induced binding to micelle structures was evident. This, and the finding that the effect of $Ca^{2+}$ on the electrophoretic mobility of 5LO occurred in the presence of SDS, may indicate that calcium-induced membrane association of 5LO is not caused by a minor conformational change, which would probably be obscured in the presence of a denaturing detergent. Also, as described, for example, for cytosolic phospholipase $A_2$ (*46*, *50*), calcium could act by changing the charge on a region of 5LO, in a so-called electrostatic switch, thus allowing for membrane association of the calcium binding region of 5LO.

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