The N-terminal Domain of 5-Lipoxygenase Binds Calcium and Mediates Calcium Stimulation of Enzyme Activity*

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Human 5-lipoxygenase (5-LO) is a key enzyme in the conversion of arachidonic acid into leukotrienes and lipoxins, mediators and modulators of inflammation. In this study, we localized a stimulatory Ca2+-binding site to the N-terminal region of the enzyme. Thus, in a 45Ca2+ overlay assay, the N-terminal 128 amino acids of recombinant human 5-LO (fused to glutathione S-transferase) bound radioactively calcium to about the same extent as intact 5-LO. The glutathione S-transferase fusion protein of the C-terminal part of 5-LO (amino acids 120–673) showed much weaker binding. A model of a putative 5-LO N-terminal domain was calculated based on the structure of rabbit reticulocyte 15-LO. This model resembles β-sandwich C2 domains of other Ca2+-binding proteins. Comparison of our model with the C2 domain of cytosolic phospholipase A2 suggested a number of amino acids, located in the loops that connect the β-strands, as potential Ca2+ ligands. Indeed, mutations particularly in loop 2 (N43A, D44A, and E46A) led to decreased Ca2+ binding and a requirement for higher Ca2+ concentrations to stimulate enzyme activity. Our data indicate that an N-terminal β-sandwich of 5-LO functions as a C2 domain in the calcium regulation of enzyme activity.

The leukotrienes are important mediators in asthma as well as in other inflammatory and allergic disorders. 5-Lipoxygenase (5-LO); arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34 catalyzes two initial steps in the cellular production of leukotrienes. Thus, 5-LO converts arachidonic acid into 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid and subsequently into the unstable epoxide leukotriene A4, which, in turn, is the precursor of the biologically active leukotrienes B4, C4, D4, and E4. Leukotriene B4 stimulates adherence of leukocytes to the vessel wall and is a potent chemotactic agent for these cells. The cysteinyl leukotrienes C4, D4, and E4 increase vascular permeability and are effective constrictors of bronchial smooth muscle. 5-LO also participates in the formation of lipoxins, another group of arachidonate-derived bioactive lipids that are implicated in inflammatory and vascular events.

Calcium is a well known 5-LO activator (for reviews, see Refs. 3–5). In brief, stimuli that elevate the intracellular Ca2+ levels were shown to induce cellular 5-LO activity, and several reports have described Ca2+-induced translocation of 5-LO from the cytosol to cellular membranes. More detailed analyses showed an association primarily with the nuclear envelope, where the membrane-bound 5-LO-activating protein (FLAP) is also found and where the substrate arachidonic acid can be released from membrane lipids by cytosolic phospholipase A2 (cPLA2). The stimulatory effect of Ca2+ is evident also for purified 5-LO. The basal enzyme activity, which is observed in the presence of a membrane fraction or lipids, increases up to 10-fold if micromolar concentrations of Ca2+ are included in the assay mixture. 5-LO catalysis has been shown to occur at the lipid/water interface, and Ca2+-dependent binding of 5-LO to phospholipid vesicles has been reported. By several experimental approaches, we have recently demonstrated that 5-LO binds Ca2+ in a reversible manner (3). A Kd close to 6 μM was determined by equilibrium dialysis, and the stoichiometry of maximum binding averaged around two Ca2+ ions/5-LO molecule. We also showed that binding of calcium increased the hydrophobicity of 5-LO. Thus, a present conception is that calcium stimulates 5-LO activity and leukotriene production by promoting membrane association.

The first structural determination of a mammalian 15-lipoxygenase (6) revealed that, similar to soybean lipoxygenases (7–9), it is composed of two major domains: a C-terminal domain containing the catalytic site and an N-terminal β-barrel domain. It seems reasonable that this is the overall structure also for 5-LO. The capability of 5-LO to bind more than one calcium ion and the calcium-dependent binding to phospholipids make 5-LO functionally similar to a group of calcium-binding proteins known as C2 domain proteins. The C2 domain is a conserved structural motif that forms an eight-stranded anti-parallel β-sandwich, and C2 domains have been identified in some 70 membrane-interacting proteins, including protein kinase C, synaptotagmin, and cPLA2. C2 domains mediate binding to a variety of ligands such as divalent cations, phospholipids, and proteins (for reviews, see Refs. 10–12). In this report, we suggest that the N-terminal domain of 5-LO functions as a Ca2+-binding C2 domain. This is based on a model structure, calcium binding analysis, and site-directed mutagenesis of putative calcium ligands in the 5-LO N-terminal domain.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of analytical grade and obtained from Merck, unless stated otherwise. Calmodulin, imidazole, phosphatidylinositol-phospholipid...
choline (P-35S), soybean lipoxygenase, and detergents were from Sigma. PVDF membranes, bovine serum albumin, and Ready-Gels were from Bio-Rad. Vectors, the GST purification kit, [35S]cysteine (specific activity of 10−40 mCi/mg), ATP-agarose, and other chromatography products were from Amersham Pharmacia Biotech. 17(S)-Hydroxy-
13Z,12Z,15Z-eicosatrien-15-oic acid was synthesized by the manufacturer’s gift (Harvard Bioscience, Cambridge, MA). 

**4Ca**2⁺ Overlay of Protein Blots—After conventional SDS-polyacrylamide gel electrophoresis (20) using 4−15% pre-cast gradient gels from Bio-Rad (Ready-Gels) and electrotransfer onto PVDF membrane, [35S]cysteine was overlayed as described previously (21). Briefly, the membrane was first washed (3 × 10 min) with 5 ml imidazole buffer (pH 7.4) containing 1 mg/ml octaethylen glycol dodecyl ether (C12E8). The membrane was then rinsed for 15 min of octyl p-nitrophenyl imidazole buffer (pH 7.4), 1 mg/ml C12E8, 60 mCi/l, 5 mg MOPS, and 10 μM [35S]cysteine (specific radioactivity of 1 mCi/mmol). The membrane was washed three times with 15 ml of 30% (v/v) ethanol in deionized water and dried on Whatman filter paper at room temperature for 2 h before exposure to Fuji RX film at −70 °C for 35−50 h. In general, two gels with the same set of samples were run and blotted simultaneously. One membrane was Coomassie Blue-stained, and the other was subjected to [35S]cysteine overlay.

**Site-directed Mutagenesis**—Selected amino acid codons (in plasmid pT3-5LO (14)) were mutated using the QuikChange™ kit from Stratagene. The DNA sequences of mutated plasmids were confirmed by sequence analysis as described above.

**Expression and Purification of Wild-type and Mutant 5-LO Proteins**—Wild-type and mutant 5-LO proteins were transformed into E. coli MV1190, and proteins were expressed at 27 °C as described previously (21). 5-LO proteins were purified from 1-liter cultures according to the ATP affinity chromatography procedure (21), followed by MonoQ anion exchange chromatography (3).

For activity assay of crude 5-LO samples, 10-ml cultures were grown. Cells were homogenized by sonication in 1 ml of 50 mM triethanolamine (pH 8.0) in the presence of 2 mM dithiothreitol, 60 μg/ml soybean trypsin inhibitor, and 0.5 mg/ml lysozyme. After centrifugation for 20 min at 16,000 × g, the proteins in the supernatant were precipitated with 60% ammonium sulfate on ice for 45 min. The precipitate was collected by centrifugation for 25 min at 16,000 × g, resuspended in Chelex-treated Tris-HCl (50 mM, pH 7.5), and gel-filtered through Sephadex G-25 (NAP-5 columns, Amersham Pharmacia Biotech) into the same buffer. The expression levels of wild-type and mutant 5-LO proteins were analyzed by Western blotting.

**Western Blot Analysis**—Protein samples were subjected to SDS-polyacrylamide gel electrophoresis using a Mini-Protein system (Bio-Rad) and the 4−15% pre-cast gels. The separated proteins were then transferred to nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech). Rabbit anti-5-LO antiserum that had been affinity-purified on GST fusion proteins was used to visualize 5-LO-immunoreactive proteins as described previously (21).

**HPLC Assay of 5-LO Enzyme Activity**—5-LO protein was incubated at room temperature in Eppendorf tubes in a total volume of 50 or 100 μl. A substrate mixture containing arachidonate, 13-HPOD, phosphatidylcholine (PC), and ATP in 50 mM Tris-HCl (pH 7.5) was mixed (by immersion in a sonication bath for 1 min) before the addition of EDTA/EGTA, 5-LO protein, and CaCl2. The final concentrations in the incubation solution are specified in each figure legend. Arachidonate, 13-HPOD, and PC were added from stock solutions in ethanol. The final ethanol concentration during the incubation was 3.5% (v/v). After 10 min, the incubation was terminated by the addition of 3 volumes of cold stop solution (67% acetonitrile, 33% water, and 0.2% acetic acid containing a 3.3 μM concentration of the internal standard 17(S)-hydroxy-
5-LO-[5,6,7-3H]-dioleoylphosphatidic acid). The sample was mixed and centrifuged at 15,000 × g for 10 min, and 100 μl of the supernatant was injected onto a C18 reverse-phase HPLC column (Waters Nova Radial Pak) eluted with acetonitrile/water/acetic acid (65:35:0.2, v/v) at 1.2 ml/min. The eluate was monitored at 234 nm, and formation of lipoxygenase products containing a conjugated diene was monitored at 236 nm. The sample was mixed and centrifuged at 15,000 × g for 10 min, and 100 μl of the supernatant was injected onto a C18 reverse-phase HPLC column (Waters Nova Radial Pak) eluted with acetonitrile/water/acetic acid (65:35:0.2, v/v) at 1.2 ml/min. The eluate was monitored at 234 nm, and the enzyme activity was calculated from the sum of the peak areas of 5(12)-hydroxy-6-trans-8,11,14-eicosatrienoic acid and 5(12)-hydroxyeicosatetraenoic acid.

**Cuvette Assay of 5-LO Enzyme Activity**—Incubations were performed in a quartz cuvette at room temperature, and formation of lipoxygenase products containing a conjugated diene was monitored at 236 nm. The incubation solution (total volume of 500 μl) was the same as the HPLC assay, and the concentrations of the various components are given in the figure legends. The reaction was started by the addition of 5-LO (0.25 μg) to the cuvette.

The *N*-terminal Domain of 5-Lipoxygenase Binds Calcium

The initial methionine residue in the recombinant *E. coli* lipoxygenase proteins is designated number 0 throughout the paper. 

The purity of the fusion proteins achieved by this procedure was sufficient for [4Ca]2⁺ overlay experiments. GST without a fusion partner (used as a control) did not appear in the inclusion bodies, but was recovered from the *E. coli* supernatant after lysis and homogenization. GST was affinity-purified using GST-Sepharose (Amersham Pharmacia Biotech) according to the manufacture’s gift.
The N-terminal Domain of 5-Lipoxygenase Binds Calcium

RESULTS

Molecular Modeling—A model of the N-terminal 114 amino acids of 5-LO, based upon the crystal structure of rabbit reticulocyte 15-LO (6), is presented in Fig. 1. In reticulocyte 15-LO, the N-terminal β-barrel is composed of eight β-strands, the last ending at residue 111. Since the sequence identity is 34% and only few insertions/deletions occur between amino acids 1 and 111 in 15-LO and amino acids 1 and 114 in 5-LO (see Fig. 3), the structures are expected to be homologous (cf. Ref. 22), and molecular modeling was therefore expected to give a plausible structure (cf. Refs. 23 and 24). In support of the model, residues not conserved between 5-LO and 15-LO, as well as gaps, are found mainly in the loop regions, which connect the β-strands. Our 5-LO model, which may be described as a β-sandwich (Fig. 1 (upper) and Fig. 2), resembles reported structures of C2 domains, e.g. in cPLA₂ (Fig. 1, lower) (Protein Data Bank codes 1RLW (25) and 1BCI (26)). In the 5-LO model cross at an angle close to 45°, whereas the β-sheets in the cPLA₂ C2 domain are more parallel. Another difference is that the 5-LO model did not exhibit any of the two distinct topologies that have been described for C2 domains (10). There is no pronounced sequence similarity between 5-LO and cPLA₂ (Fig. 3); nevertheless, the 5-LO β-sandwich model is similar to the calcium-binding C2 domains, and also the 5-LO model structure contains apparent ligand-binding loops. This encouraged us to examine the 5-LO N-terminal region as a potential Ca²⁺-binding domain.

Calcium Binding of Truncated Lipoxygenase Proteins Fused to Glutathione S-Transferase—5-LO and its two putative domains (the N-terminal β-sandwich, 5-LO(N), and the C-terminal domain, 5-LO(C)) were expressed as GST fusion proteins in E. coli (Fig. 4A). All three fusion proteins were recovered in insoluble inclusion bodies after cell homogenization. The ability of the fusion proteins to bind Ca²⁺ was determined by ⁴⁵Ca²⁺ overlay, performed directly on the solubilized inclusion bodies without further protein purification (Fig. 4B). Calmodulin, which can bind four Ca²⁺ ions/protein molecule, was used as a positive control for the ⁴⁵Ca²⁺ overlay procedure (Fig. 4B, lane 7). However, in our hands, calmodulin (up to 0.5 nmol) blotted onto PVDF membrane stained weakly (or not at all) with Coomassie Blue. GST/5-LO gave the same response as recombinant 5-LO without a GST tag (lanes 4 and 5). GST alone did not bind Ca²⁺ (lane 3), confirming that the calcium binding of GST/5-LO was caused by the 5-LO part of the fusion protein. GST/5-LO(N) bound Ca²⁺ as strongly as the entire GST/5-LO fusion protein, whereas GST/5-LO(C) gave a much weaker response (lanes 1, 2, and 4). Thus, 0.7 nmol of GST/5-LO(C) did not give a detectable signal, but 2.1 nmol of GST/5-LO(C) gave about the same response as 0.7 nmol of GST/5-LO (lanes 2, 5, and 8). We consider the response of GST/5-LO(C) as weak, but it may be significant since comparable amounts of other proteins that do not bind calcium gave bright spots, probably by reducing the background binding (3). In a control experiment, the calcium binding for GST/5-LO(N) could be competed by the addition of cold CaCl₂ to the ⁴⁵Ca²⁺-containing overlay buffer, indicating that the calcium binding visualized by the overlay technique was not unique for the ⁴⁵Ca isotope (data not shown).

The calcium binding of the N-terminal part of 5-LO was compared with that of the corresponding regions of 12-lipoxygenases. GST fusion proteins of the N-terminal parts (cf. Fig. 2) of rat 12-LO (amino acids 0–110) and of human platelet 12-LO (amino acids 0–109) were expressed in E. coli and recovered in inclusion bodies. In Fig. 5, the calcium binding of these 12-LO fusion proteins is compared with that of the corresponding regions of 12-lipoxygenases. GST/5-LO(N) and GST/5-LO(C) gave the same response as 0.7 nmol of GST/5-LO (lanes 2, 5, and 8). We consider the response of GST/5-LO(C) as weak, but it may be significant since comparable amounts of other proteins that do not bind calcium gave bright spots, probably by reducing the background binding (3). In a control experiment, the calcium binding for GST/5-LO(N) could be competed by the addition of cold CaCl₂ to the ⁴⁵Ca²⁺-containing overlay buffer, indicating that the calcium binding visualized by the overlay technique was not unique for the ⁴⁵Ca isotope (data not shown).

Site-directed Mutagenesis of Residues in the 5-LO N-terminal Domain.—In analogy to documented C2 domain proteins, we expected the Ca²⁺-chelating ligands in 5-LO to be located in the loops that connect the β-sheets. Several ligand candidates are present. Among these, Asp and Glu residues are depicted in gray in the model (Fig. 1, upper) and marked with arrows in the 5-LO sequence (Fig. 3). Four mutant proteins were produced in which Ca²⁺ ligand candidates present in each of the four loops located on the side of the β-sandwich opposite to the N terminus were mutated to Ala (Table I). In E. coli cells grown at 27 °C, all four mutants showed about the same expression...
levels as wild-type 5-LO as judged by Western blotting (Fig. 6B). The dose responses for Ca\(^{2+}\) stimulation of the 5-LO enzyme activities of the mutants, compared with that of wild-type 5-LO, are shown in Fig. 6A. The loop 2 mutant showed a clearly reduced Ca\(^{2+}\) response, but also mutations in the other loops shifted the response slightly toward higher Ca\(^{2+}\) concentrations. Comparison primarily with cPLA\(_2\) (see Fig. 3) led us to focus on the loops on the side opposite to the N terminus. However, the loop between the fourth and fifth \(\beta\)-strands, on the other side of the \(\beta\)-sandwich, was also subjected to mutagenesis. This mutant (D58A/E59A/E60A/E63A) responded to Ca\(^{2+}\) stimulation as did wild-type 5-LO (data not shown).

The loop 2 mutant was studied further. This protein was expressed in 1-liter scale and purified using the previously

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**TABLE I**

| Mutants of putative Ca\(^{2+}\)-chelating ligands in the N-terminal region of 5-LO |
|-----------------------------------------------|---------------------------|
| **Ca\(^{2+}\)**-ligand candidates | Mutations |
|-----------------------------------------------|-------------|
| Loop 1 Asp\(^{16}\), Asp\(^{39}\) | D18A/D19A |
| Loop 2 Asp\(^{43}\), Asp\(^{44}\), Glu\(^{46}\) | N43A/D44A/E46A |
| Loop 3 Asp\(^{78}\), Asp\(^{79}\) | D78A/D79A |
| Loop 4 Asp\(^{106}\), Glu\(^{108}\) | D106A/E108A |
The N-terminal Domain of 5-Lipoxygenase Binds Calcium

Fig. 6. Ca\(^{2+}\) stimulation of the enzyme activities of crude wild-type and mutant 5-LO proteins and expression levels in E. coli. A, NAP-5 eluates (20 µl) of crude protein preparations were incubated for 10 min at room temperature in Chelex-treated Tris-HCl (50 mM, pH 7.5) containing 100 µM arachidonate, 250 µg/ml PC, 10 mM 13-HPOD, 2 mM EGTA, and the indicated concentrations of CaCl\(_2\), in a total volume of 50 µl. Production of 5(S)-hydro(pero)xyeicosatetraenoic acid was determined by HPLC. Values are means ± S.D. from three separate incubations. The mean absolute values of the maximum activity for the different samples were as follows: wild-type enzyme, 196 nmol/mg/10 min; loop 1 mutant (loop 1 mut), 171 nmol; loop 2 mutant, 99 nmol; loop 3 mutant, 176 nmol; and loop 4 mutant, 151 nmol. B, aliquots (20 µl) of the NAP-5 eluates were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis as described under “Experimental Procedures.” E. coli cells transformed with plasmid without insert were used as a negative control (Neg ctrl).

described ATP-agarose procedure (21). The purified loop 2 mutant showed a markedly decreased Ca\(^{2+}\) response compared with wild-type 5-LO. When activity was determined in the presence of high concentrations of PC (250 µg/ml) and arachidonate (100 µM), similar patterns were seen in the presence or absence of 1 mM ATP (Fig. 7). It is important to note that the given concentrations of arachidonate and PC would be present if these lipids were truly dissolved in the incubation mixtures. Instead, micelles were formed, and the indicated concentrations reflect the compositions of these micelles. Without ATP (Fig. 7A), the activity of wild-type 5-LO reached a maximum at ~2–10 mM added Ca\(^{2+}\), whereas the loop 2 mutant reached maximum activity first at 100 mM Ca\(^{2+}\). With ATP present (Fig. 7B), higher added calcium concentrations were required to reach maximum enzyme activation. We believe that one reason for this is the chelation of calcium by ATP. For wild-type 5-LO, the activity then decreased substantially at 100 µM and above when determined in the absence of ATP (Fig. 7A). Possibly, this could be related to the effects of Ca\(^{2+}\) on the aggregation of PC and arachidonate at high levels (27), an effect that might have reduced the activity also of the loop 2 mutant at the highest Ca\(^{2+}\) concentrations. With ATP present (Fig. 7B), this tendency was less pronounced. At the highest concentration of Ca\(^{2+}\) (1 mM), the activities of wild-type 5-LO and the loop 2 mutant tended to become quite similar. Also at a low PC concentration in combination with a high concentration of Ca\(^{2+}\), the specific activities of the protein preparations in Fig. 7 were similar: 21 and 16 µmol/mg/10 min for wild-type 5-LO and the loop 2 mutant, respectively (25 µg/ml PC, 100 µM arachidonate, 10 µM 13-HPOD, 1.9 mM CaCl\(_2\), 1.2 mM EDTA, 5 mM ATP, and 15 mM 2-mercaptoethanol). When the stimulatory effect of ATP alone was determined (25 µg/ml PC, 100 µM arachidonate, 1 mM EDTA, and 0–1 mM ATP, without the addition of Ca\(^{2+}\)), there was no significant difference between the loop 2 mutant and the control (5.1 and 4.2 nmol of 5-hydro(pero)xyeicosatetraenoic acid/mg of protein/10 min, respectively, at 1 mM ATP).

It was described previously that the effect of Ca\(^{2+}\) on 5-LO activity was most prominent in assays employing low concentrations of both PC and arachidonate, conditions that give a low basal activity (4, 28). We determined enzyme activity over time (cuvette assay, 5-min incubations) in the presence of low concentrations of PC (25 mM) and arachidonate (20 µM) (Fig. 8). Under these assay conditions, apparently Ca\(^{2+}\) did not affect the aggregation of PC and arachidonate since 1 mM Ca\(^{2+}\) gave as high activity for wild-type 5-LO as did 10 µM Ca\(^{2+}\). For the loop 2 mutant, both the initial rate and final amount of product were reduced compared with the control, best seen at 10 µM calcium (Fig. 8A). However, at a high calcium concentration (1 mM), the loop 2 mutant gave a time course that was similar in character to the time course obtained for wild-type 5-LO at 10 µM calcium. Also, substrate inhibition was observed both for the wild-type enzyme and for the loop 2 mutant (data not shown). With wild-type 5-LO, 50 mM arachidonate gave higher activity than did 100 µM in the cuvette assay at 10 µM Ca\(^{2+}\) and 25 µg/ml PC. The same was observed with the loop 2 mutant, although at higher Ca\(^{2+}\) concentrations (100 µM and 1 mM). These features indicate that the loop 2 mutations did not directly affect the catalytic site of 5-LO.

We recently reported that millimolar concentrations of Mg\(^{2+}\)
The N-terminal Domain of 5-Lipoxygenase Binds Calcium

Fig. 8. Stimulation of 5-LO enzyme activity by Ca$^{2+}$ and Mg$^{2+}$ and time courses for purified wild-type and loop 2 mutant 5-LO proteins. Purified protein (250 ng) was added to a cuvette containing 500 µl of Chelex-treated Tris-HCl (50 mM, pH 7.5) containing 20 µM arachidonate, 25 µg/ml PC, 2.5 µM 13-HPOD, 1 mM dithiothreitol, 1 µM EDTA, 1 mM EGTA, and the indicated concentrations of CaCl$_2$ (A) or MgCl$_2$ (B). Wild-type 5-LO concentrations are Roman, and loop 2 mutant (loop2 mut) concentrations are italic.

can also stimulate 5-LO (4). In Fig. 8B, the Mg$^{2+}$ stimulation of wild-type 5-LO and the loop 2 mutant is shown. Interestingly, the Mg$^{2+}$ stimulation of the loop 2 mutant was drastically impaired, supporting the concept that Mg$^{2+}$ and Ca$^{2+}$ bind to the same site on 5-LO. The MgCl$_2$ solution used to study magnesium activation was analyzed for Ca$^{2+}$ contamination (4). At 5 mM Mg$^{2+}$, the [Ca$^{2+}$] was below 0.15 µM. Since at least 1 µM Ca$^{2+}$ is required for significant 5-LO activation (Fig. 7A), the activation obtained with Mg$^{2+}$ was not due to Ca$^{2+}$ contamination.

Finally, to confirm that the reduced response to calcium activation of the loop 2 mutant was due to reduced calcium binding capacity, this protein was subjected to $^{45}$Ca$^{2+}$ overlay (10 µM Ca$^{2+}$). As shown in Fig. 9, association of Ca$^{2+}$ with purified loop 2 mutant protein (0.6 and 0.9 nmol) was practically undetectable, whereas normal 5-LO gave a definite response. This correlated well with the activity data, particularly at low concentrations of PC and arachidonate (Fig. 8). Similar results were obtained for another batch of the loop 2 mutant; when 2 nmol was loaded, no clear signal was detectable. Thus, the GST fusion of the C-terminal domain of 5-LO (compare above) apparently had a higher affinity for Ca$^{2+}$ than the loop 2 mutant (which contains the C-terminal domain). In conclusion, it is unclear whether the C-terminal part of native 5-LO truly binds Ca$^{2+}$.

**DISCUSSION**

5-Lipoxygenase enzyme activity is stimulated by calcium, and 5-LO was found to bind calcium reversibly with a $K_d$ of $-6$ µM (3). Here we show that an N-terminal part of 5-LO (amino acids 0–128), which contains the putative 5-LO β-barrel (or β-sandwich, amino acids 1–114), can bind calcium, approximately to the same extent as the intact enzyme. A model of the 5-LO N-terminal domain was created based on the structure for rabbit reticulocyte 15-LO (6). This model (Fig. 1) was strikingly similar to calcium-binding C2 domains, e.g. in cPLA$_2$, suggesting both functional and structural similarities between 5-LO and C2 domain proteins. A corresponding sequence similarity between 5-LO and cPLA$_2$ was not observed (Fig. 2).

Several amino acid residues located in the loops between the β-strands might function as calcium ligands in 5-LO. Mutations of residues in loop 2 of the 5-LO β-sandwich resulted in clear reductions both in Ca$^{2+}$ binding and in the calcium activation dose response. This connects the calcium affinity of the 5-LO β-sandwich to the calcium stimulation of enzyme activity; and based on these observations, we suggest that the putative β-sandwich of 5-LO functions as a calcium-binding C2 domain. C2 domains are of two different topologies with different β-strand connectivities (10). Interestingly, the 15-LO β-barrel domain (6) and, consequently, the 5-LO model do not fit exactly with either of the two topologies. In 15-LO and the 5-LO model, one of the four-stranded β-sheets consists of β-strands 4, 1, 6, and 7, and the other β-sheet consists of β-strands 3, 2, 5, and 8. Thus, in relation to C2 domain topology II (e.g. in cPLA$_2$ and phospholipase Cδ-1), β-strands 6 and 8 have switched positions in the lipoxygenases.

The stimulatory effect of calcium on 5-LO activity in vitro depends on the presence of phospholipids (4), and it was shown that calcium increases the hydrophobicity of 5-LO (3, 4, 29). The general view is that the effect of calcium also on cellular 5-LO activity is related to membrane association (for a review, see Ref. 5). Calcium-induced membrane association is a feature typical of many proteins containing C2 domains (10, 11), and we presume that the putative 5-LO β-sandwich domain also functions in this manner. Membrane interactions have been studied in detail for some C2 domain proteins. The phospholipid head group specificity differs substantially between the cPLA$_2$ C2 domain, which prefers zwitterionic phospholipids, and the synaptotagmin I C2A domain, which prefers acidic phospholipids (30, 31). Two different membrane binding mechanisms have been proposed for these domains: the cPLA$_2$ C2 domain penetrates into the hydrophobic core of membranes, whereas binding is highly electrostatic for the synaptotagmin I C2A domain (25, 26, 32–36). 5-LO, similar to cPLA$_2$, was activated by PC, but 5-LO could associate also with phosphatidylethanolamine and phosphatidylserine (29, 37, 38).

Other lipoxygenases can also be activated by calcium and associate with membrane structures (39–47), and similarities to C2 domain proteins have been discussed for soybean lipoxy-
The protein kinase C isoenzyme family is divided into the class with calcium stimulation mediated by the N-terminal domain (Asp43) that was important for calcium ion. A complete determination of all Ca\(^{2+}\) binding capacities of the N-terminal parts of 12-LO has been shown, but there was no effect on the purified enzymes (48), and purified recombinant platelet 12-LO was active without calcium (49). It has also been reported that calcium may activate 12-LO in the cytosol from rat basophilic leukemia cells, but not 12-LO in subcellular fractions from platelets (40, 50). Calcium (10 \(\mu\)M) added to platelet or human erythrocytes cell homogenates caused a redistribution of 12-LO in the cytoplasm and for lipid body lipoxygenase from cucumber cotyledons (46, 47). It may be speculated that N-terminal domains of all lipoxygenases function as phospholipid-binding C2 domains, but with different calcium requirements. We compared the calcium binding capacities of the N-terminal parts of 12-lipoxygenases (human platelet 12-LO and rat 12-LO) with that of 5-LO. In the 45Ca\(^{2+}\) overlay assay (10 \(\mu\)M Ca\(^{2+}\)), association of Ca\(^{2+}\) with the N-terminal domains could be detected only with considerably higher protein amounts than those needed for the 5-LO N-terminal domain. The effect of calcium on 12-LO enzyme activities is not entirely clear. Calcium activation of crude 12-lipoxygenases (both platelet and leukocyte types) has been shown, but there was no effect on the purified enzymes (48), and purified recombinant platelet 12-LO was active without calcium (49). It has also been reported that calcium could activate 12-LO in the cytosol from rat basophilic leukemia cells, but not 12-LO in subcellular fractions from platelets (40, 50). Calcium (10 \(\mu\)M) added to platelet or human erythrocytes cell homogenates caused a redistribution of 12-LO in the cytosol and for lipid body lipoxygenase from cucumber cotyledons (46, 47). It may be speculated that N-terminal domains (Asn43, Asp44, and Glu46) that were important for calcium binding capacities of the N-terminal parts of 12-LO are involved in chelation of more than one calcium ion. A complete determination of all Ca\(^{2+}\) ligands in other calcium-binding proteins (51). 

The protein kinase C isoform family is divided into the class with calcium stimulation mediated by the N-terminal domain (Asn43, Asp44, and Glu46) that were important for calcium ion. A complete determination of all Ca\(^{2+}\) ligands in other calcium-binding proteins (51). 

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