Investigations into Calcium-dependent Membrane Association of 15-Lipoxygenase-1
MECHANISTIC ROLES OF SURFACE-EXPOSED HYDROPHOBIC AMINO ACIDS AND CALCIUM*§

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Among mammalian lipoxygenases the 15-lipoxygenase-1 is somewhat special because of its capability of oxygenating complex lipid-protein assemblies (biomembranes, lipoproteins) and previous investigations have implicated calcium in enzyme/membrane interaction. We investigated the mechanism of calcium-dependent membrane association and obtained the following results. (i) Membrane binding of 15-lipoxygenase-1 involves electrostatic forces as well as hydrophobic interactions of solvent-exposed apolar amino acids (Tyr15, Phe70, Leu12, Trp181, and Leu195), with the hydrophobic core of membrane phospholipids. These sequence determinants of membrane association are clustered at the membrane contact plane of the enzyme that also involves the entrance to the substrate binding pocket. Site-directed mutagenesis of these determinants to negatively charged residues strongly impaired membrane binding. (ii) Calcium at micromolar concentrations (5–50 μM) is required for efficient membrane binding. For direct 15-lipoxygenase/calcium interaction a dissociation constant of 2–5 × 10⁻⁴ M was determined (low affinity binding) and we failed to detect high affinity calcium-binding sites at the enzyme. Reversible low affinity calcium binding induces subtle structural alterations of the enzyme, which did not impact catalytic activity. (iii) Increasing calcium concentrations failed to reverse impairment of membrane binding induced by mutagenesis of the sequence determinants indicating the priority of hydrophobic interactions. Taken together these data suggest that 15-lipoxygenase-1 associates to biomembranes primarily via hydrophobic interactions between surface-exposed apolar amino acid side chains and membrane lipids. Calcium supports membrane binding probably by forming salt bridges between the negatively charged head groups of membrane phospholipids and acidic surface amino acids of the membrane contact plane and this interaction might contribute to overcome repulsive forces.

Lipoxygenases (LOXs) constitute a heterogeneous family of lipid peroxidizing enzymes that catalyze dioxygenation of free and/or esterified polyunsaturated fatty acids to the corresponding hydroperoxy derivatives. In mammals, LOXs are categorized with respect to their positional specificity of arachidonic acid oxygenation (2), but plant physiologists prefer a linoleic acid-related enzyme nomenclature (3). Mammalian LOXs are involved in the biosynthesis of inflammatory mediators, such as leukotrienes (4) and lipoxins (5), but have also been implicated in cell differentiation (6, 7), carcinoma metastasis (8, 9), and atherogenesis (10, 11).

Although LOXs are cytosolic enzymes they have a tendency of membrane association. For the soybean LOX-1 it has been reported that the enzyme binds to biomembranes via calcium-dependent mechanisms (12). Structural modeling suggested the existence of two independent calcium-binding sites, one in the NH₂-terminal β-barrel domain (Glu81, Glu196, and Glu179) the other one in the catalytic domain (Glu372, Asp74, and Glu777). Together with surface-exposed hydrophobic amino acids these calcium-binding sites have been implicated in membrane association of the enzyme. Although the role of calcium ions is still not completely understood they have been suggested to form salt bridges between acidic surface amino acids and negatively charged constituents of the membrane phospholipids (12).

Mammalian LOX isoforms, in particular the human 5-LOX, were also shown to interact with calcium. In vitro activity assays with the purified 5-LOX indicated that the catalytic activity measured in the presence of biomembranes or phospholipid liposomes was strongly augmented in the presence of calcium (13). In various cells an increase in the cytosolic calcium concentration triggers translocation of the enzyme from the cytosol to the nuclear envelope (14, 15). There, the enzyme associates with the 5-lipoxygenase activating protein (FLAP) and the cytosolic phospholipase A₂ that supplies free arachidonic acid by cleaving membrane phospholipids (16). Intracellular membrane binding assays with fusion protein constructs of the human 5-LOX and green fluorescence protein suggested that the NH₂-terminal β-barrel domain of the enzyme appears to play a major role in Ca²⁺-dependent membrane association (17). The human 5-LOX binds calcium reversibly with a moderate affinity (𝐾ᵣ of about 6 μM) and stoichiometry calculations suggested the existence of two specific calcium-binding sites (18). Structural modeling of the enzyme on the basis of the x-ray coordinates for the rabbit 15-LOX (19) suggested that the amino acids interconnecting various β-sheets of the NH₂-terminal domain (Asn44, Asp54, and Glu65) may constitute poten-

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tial calcium ligands (20). In fact, mutagenesis of these residues led to an impaired calcium affinity and to requirement for higher calcium concentrations to stimulate enzyme activity (20). However, as for the soybean enzyme selective calcium binding may not be the only process involved in membrane association. More detailed mechanistic investigations suggested an important role of solvent-exposed tryptophans (Trp<sup>15</sup>, Trp<sup>77</sup>, and Trp<sup>199</sup>) for binding of the human 5-LOX to phosphatidylcholine vesicles (21).

The rabbit reticulocyte-type 15-LOX (15-LOX-1) also associates to biomembranes in a calcium-dependent manner (22, 23). When the purified enzyme was incubated with submitochondrial membranes in the presence of 0.5 mM calcium the majority of the 15-LOX was recovered from the membrane pellet (23). This membrane binding is a necessary pre condition for oxygenation of membrane lipids but it also activates the free fatty acid oxygenase activity of the enzyme (23). In resting cells the 15-LOX is localized in the cytosol but an increase in cytosolic calcium concentration induces binding to intracellular membranes (23). In contrast to the 5-LOX no preferential binding to the nuclear envelope was observed. Instead, the enzyme was primarily detected at the cytosolic side of intracellular membrane systems and at the internal phase of the plasma membrane (23). To shed light on the molecular basis of calcium-dependent membrane binding we recently explored the importance of selected surface-exposed amino acids and identified Phe<sup>70</sup>, Leu<sup>71</sup>, and Trp<sup>181</sup> as primary determinants of membrane binding (24). However, the mechanistic role of calcium and the contribution of other candidate amino acids remained unclear.

To fill this gap we investigated calcium-dependent membrane association of the rabbit 15-LOX in reconstituted in vitro systems. The study was aimed at defining the structural basis of enzyme/membrane interaction and at exploring the mechanistic role of calcium in this process. Although we confirmed the importance of calcium for membrane association we failed to detect high affinity calcium-binding sites at the enzyme. However, mutagenesis data indicated that hydrophobic interactions of solvent-exposed apolar amino acid side chains with the hydrophobic core of membrane phospholipids are the major driving forces for the formation of 15-LOX-1-membrane complexes.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The chemicals used were from the following sources: (5Z,7Z,11Z,14Z)-eicos-5,8,11,14-tetraenoic acid (arachidonic acid), EDTA, imidazole, and sodium borohydride from Serva (Heidelberg, Germany); ampicillin from Invitrogen (Eggenstein, Germany); kanamycin, glycerol, dithiothreitol, trichloroacetic acid, calcium chloride (99.99%), and sucrose from Sigma; HPLC reference compounds (99.99%) and sucrose in the same buffer) and centrifuged for 15 min at 100,000 g. The supernatant was applied to a 0.5-ml nickel-agarose column (Qiagen, Hilden, Germany) that had been pre-equilibrated with 20 mM imidazole, pH 7.4. The column was washed twice with washing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0) and the adherent proteins were eluted by rinsing the column with elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 200 mM imidazole, pH 8.0). Five 0.25-ml fractions were collected and the LOX activity was assayed in each of these fractions. More than 90% of the activity was recovered in fractions 2–4. These fractions were pooled, diluted 1:50 with 20 mM Tris-HCl buffer, pH 7.4, and loaded onto a Q-Sepharose column (gel bed volume 500 µl; Amersham Biosciences) for further purification by anion exchange chromatography. After loading the column was washed twice with 2 ml of 20 mM Tris-HCl buffer, pH 7.4, and then the enzyme was eluted with 120 mM KCl dissolved in the same buffer. Fractions of 0.25 ml were collected, activity was assayed, and the active fractions were pooled. For storage the enzyme preparation was supplemented with 10% glycerol and stored at −80 °C. For most mutants this procedure yielded about 2 mg of electrophoretically pure protein (Fig. S4, Supplemental Materials) starting from a 3-liter culture.

**Site-directed Mutagenesis**—Site-directed point mutations were performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) and the primary structures of mutated plasmids were confirmed by DNA sequencing. For each mutant, 5–10 clones were screened by restriction mapping and activity assays to identify catalytically active LOX-positive clones. For deletion of the NH<sub>2</sub>-terminal β-barrel domain a Sall restriction site was introduced in front of Cys<sup>155</sup> by PCR. The PCR fragment was digested with Sall and KpnI, ligated into the pQE-9 expression plasmid containing the wild-type 15-LOX and E. coli (M15[prep4]) were transformed with this plasmid. This procedure led to a NH<sub>2</sub>-terminal truncation mutant that lacked the first 114 amino acids (β-barrel truncation mutant). Expression and purification of this truncation mutant was performed as described for the other mutants.

**Arachidonic Acid Oxygenase Activity**—Arachidonic acid oxygenase activity of wild-type and mutant 15-LOX species was assayed by HPLC quantification of the oxygenation products. The assay mixture consisted of a 10 mM Tris-HCl buffer, pH 7.4, and 0.1 mM arachidonic acid as substrate (0.5 ml total assay volume). The reaction was stopped by the addition of 0.5 ml of ice-cold methanol, the hydroperoxydes formed were reduced to the more stable hydroxy compounds with sodium borohydride, and the sample was acidified to pH 3 (acetic acid). After removing the precipitate by centrifugation aliquots of the clear supernatant were injected to reverse phase-HPLC for isolation and quantification of the reaction products.

**Membrane Binding Assay**—In our standard membrane binding assay 200 µg of SMP were incubated at room temperature with 1 µg of recombinant 15-LOX (wild-type or mutant enzyme species) in 50 mM HEPES buffer, pH 7.4, containing 150 mM NaCl and 1 mM dithiothreitol, pH 7.4 (total assay volume 25 µl). After a 5-min incubation period the membranes were transferred onto 0.1-mM amiloride (in sucrose in the same buffer) and centrifuged for 15 min at 100,000 × g in a Beckman tabletop centrifuge (4°C). The pellet of this centrifugation step represented LOX-loaded SMPs. The supernatant was carefully removed, transferred to a separate tube, and ovalbumin was added to reach a final concentration of 60 µg/ml. The supernatant proteins were precipitated with trichloroacetic acid at a concentration of 10% and spun down by centrifugation for 20 min at 20,000 × g. The supernatant of the centrifugation step was discarded and the two pellets (100,000 × g pellet and 20,000 × g pellet) were reconstituted in 25 µl of 2-fold concentrated electrophoresis loading...
buffer (0.58 m sucrose, 280 m Tris base, 111 m SDS, 1 m EDTA, 0.44 m Serva Blue G250, and 200 mM dithiothreitol). After heating to 95 °C for 5 min, aliquots (4 μl) were loaded onto SDS-PAGE. After electrophoresis the proteins were blotted to a nitrocellulose membrane by a semi-dry blotting technique and the blots were probed with a mouse anti-BCS-His tag antibody (Qiagen). As secondary antibody a peroxidase-conjugated anti-mouse IgG antibody (Sigma) was used. The blots were visualized by treating them with the Western lighting chemiluminescence plus reagent (PerkinElmer Life Sciences).

The intensity of the immunoreactive bands was quantified densitometrically using the Phoretix 1D software package (Phoretix Intl., Newcastle, United Kingdom).

**HPLC Analysis—HPLC was performed on a Shimadzu system connected to an Agilent 1100 diode array detector (Agilent Technologies, Waldbronn, Germany). Reverse phase-HPLC was carried out on a Nucleosil C-18 column (Macherey-Nagel, Düsseldorf, Germany). The 15-LOX preparation exhibiting a specific linoleic acid hydroperoxide substrate binding pocket, is given in orange. A, hydrophobic surface amino acids (more than 30% solvent exposed) are shown in yellow. These residues constitute potential sequence determinants for membrane binding. B, surface-exposed amino acids that are involved in membrane binding as indicated by site-directed mutagenesis are colored in red; residues with minor impact (see Fig. 2) are shown in pink. C, negatively charged surface amino acids (shown in green) are located at the putative site of enzyme/membrane interaction. These residues constitute potential Ca2+ ligands and binding of the positively charged calcium ions to these amino acids may reduce repulsive forces between the negatively charged surface amino acids and acidic membrane phospholipids.

**Calcium Binding Studies in Solution**—For determination of calcium concentrations all vials and pipettes were rinsed with 50% nitric acid and then washed extensively with Tracepure® water (Merck, Darmstadt, Germany). The 15-LOX preparation was incubated for 10 min at room temperature at various calcium concentrations (100, 10, 3.3, 0.33, and 0.1 mM) and then desalted twice by passing them over the same desalting columns. Calcium concentrations of the samples were measured by electrothermal atomic absorption spectroscopy (see Supplemental Materials) on a PE 4100 ZL spectrometer (Überlingen, Germany). Numerical values were determined in triplicate and mean ± S.D. are given.

**Surface Plasmon Resonance Measurements (Biacore Experiments)—**The Biosensor BIACORE X and the sensor chip CM5 (disposable sensor chip, the surface of which was covered with a thin gold layer coated with carboxymethyl-dextran residue for covalent protein immobilization) were purchased from Biacore AB (Uppsala, Sweden). The purified rabbit 15-LOX (native enzyme prepared from rabbit reticulocytes as well as recombinant wild-type and mutant enzyme species) was immobilized on the sensor chip (see Supplemental Materials). The coated sensor chip was placed in the Biacore flow chamber and pre-conditioned with 10 mM Tris-HCl buffer, pH 7.4, using a flow rate of 5 μl/min until a constant baseline of light diffraction was observed. Then, calcium ions (1 mM CaCl2, in 10 mM Tris-HCl buffer, pH 7.4) were pumped over the 15-LOX-loaded sensor chip (5 μl/min) and light diffraction was monitored continuously. When the diffraction index has reached a new steady state calcium was removed by washing the sensor chip with calcium-free 10 mM Tris-HCl buffer, pH 7.4 (5 μl/min for 2–3 min), and removal of calcium was indicated by return of the light diffraction index to baseline levels. Next, the chip was rinsed with 1 mM EDTA to remove traces of calcium and then re-equilibrated with 10 mM Tris-HCl buffer, pH 7.4, to initiate a second round of calcium binding studies (descending Ca2+ concentrations). With this technology a single 15-LOX-coated sensor chip was sufficient to perform a series of binding studies covering Ca2+ concentrations between 8 μM and 1 mM. The light diffraction raw data were analyzed with the BIAevaluation 3.0 software package (steady state method).

**Miscellaneous Methods—**Protein concentration was determined with the Roti-Quant detection system (Roti, Karlsruhe, Germany) that is based on the Bradford method. SMPs, which mainly constitute inside-out vesicles of the inner mitochondrial membrane, were prepared as described previously (26). Fluorescence measurements were carried out at room temperature on a Shimadzu RF5000 PC spectrofluorimeter (Shimadzu, Duisburg, Germany) using 4-ml quartz cuvettes that contained 2 ml of 10 mM Tris-HCl buffer, pH 7.4. An excitation wavelength of 284 nm was selected and the emission spectrum was recorded between 300 and 400 nm. Structural modeling of the rabbit 15-LOX-arachidonic acid complex was carried out with the Hyperchem 5.0 software package. Amino acids, the coordinates of which have not been determined in the crystal structure, were modeled using Swiss-Pdb software followed by energy minimization of the side chains.

**RESULTS**

**Identification of Novel Sequence Determinants Involved in Membrane Binding—**It has been reported before that the rabbit 15-LOX is capable of oxygenating biomembranes (27, 28) and lipoproteins (29) and that calcium ions are required for effective membrane oxygenation (23). More recently, two clusters of hydrophobic amino acids have been implicated in membrane binding (24). However, there was evidence for the importance of other structural elements and the mechanistic role of calcium ions has not been investigated. Inspecting the surface of the rabbit 15-LOX we observed additional hydrophobic amino acids, the side chains of which are largely solvent-exposed (Fig. 1A). Rotating the three-dimensional model of the enzyme-substrate complex (Fig. S1, Supplemental Materials) we found that most of these residues are clustered on one side of the enzyme molecule. Interestingly, this part of the protein surface also contains the immediate entrance to the substrate-binding cleft. For this study we first performed membrane binding assays with the recombinant wild-type 15-LOX and single point mutants, in which the most prominently solvent-
exposed hydrophobic amino acids were exchanged to negatively charged residues. From Fig. 2A it can be seen that the majority (some 80%) of the wild-type rabbit 15-LOX added to the membrane binding assay was recovered from the membrane fraction; only a minor share was found in the membrane-free supernatant. In contrast, the majority of the Y15E and L195E mutants was detected in the supernatant fraction. Similar results were obtained with W181E, F70H, and L71K (Fig. 2B), which have previously been implicated in membrane binding assays of wild-type 15-LOX, various single point mutants, and the NH2-terminal truncation mutant (catalytic domain), in which the \( \beta \)-barrel domain has been deleted. As measure for the membrane binding capacity the S/P ratios (quotient of unbound (supernatant) versus membrane bound (pellet) shares) were calculated (see panel A). Thus, large bars (W181E) indicate low membrane binding capacities, small bars (wild-type) strong membrane binding. C, membrane binding assay carried out with selected 15-LOX multiple mutants. The mutants tested are specified above the Western blots and the S/P ratios are indicated below. It should be pointed out that the membrane binding studies shown in this figure were carried out in the absence of exogenous calcium.

To further reduce the membrane binding capacity of the rabbit 15-LOX multiple mutants were created, in which the sequence determinants for membrane binding were mutated simultaneously. From Fig. 2C it can be seen that the triple mutant Y15E/F70H/L71K exhibited a strongly reduced membrane binding capacity. The double mutant W181E/L195E was almost completely recovered from the supernatant fraction indicating a low membrane binding capacity. Similar results were obtained for the quadruple mutant Y15E/F70H/L71K/L195E. It should be stressed at this point that all surface mutants created were enzymatically active although some of them exhibited a slightly reduced specific activity (Table I and Supplemental Materials). However, even those mutants retained their positional specificity, which is proof that catalysis proceeded entirely enzyme-controlled.

Summarizing our mutagenesis data one may conclude that the solvent-exposed hydrophobic amino acids Tyr\(^{15}\), Phe\(^{70} \), Leu\(^{71} \), Trp\(^{181} \), and Leu\(^{195} \) constitute sequence determinants for the membrane binding activity of the rabbit 15-LOX. These sequence determinants are localized in both, the NH2-terminal \( \beta \)-barrel domain and COOH-terminal catalytic domain (Fig. 1). Thus, membrane binding of functional rabbit 15-LOX constitutes a concerted action of both structural subunits and this conclusion is consistent with the results of previous truncation studies (24).

**Calcium Impacts Membrane Binding of 15-LOX**—It has been reported before that membrane binding of the native rabbit 15-LOX requires calcium (22, 23). However, it remained unclear whether calcium primarily binds to the LOX, to the membranes, or to both. It is well known that biomembranes effectively bind calcium via electrostatic interactions with negatively charged phospholipid head groups (30, 31). In fact, most biomembrane preparations contain large amounts of calcium when prepared in the absence of EDTA. We found that our routine SMP preparations contain sufficient amounts of calcium to bind 15-LOX in the absence of exogenous calcium (Fig. 2). However, after washing the membranes in the presence of millimolar concentrations of EDTA LOX binding was strongly reduced. In fact, we found that in the absence of exogenous calcium the vast majority of the 15-LOX was recovered from the supernatant fraction (Fig. 3A). Addition of exogenous calcium in concentrations higher than 5 \( \mu \)M considerably improved membrane binding. In contrast, at calcium concentrations lower than 5 \( \mu \)M there was no major alteration in membrane binding capacity of the enzyme. Our data (Fig. 3A) suggest that calcium concentrations between 5 and 50 \( \mu \)M are required for effective membrane binding of the rabbit 15-LOX.

The results of our mutagenesis studies and the relevance of calcium ions suggested that formation of enzyme-membrane complexes proceeds via a concerted action of hydrophobic (sequence determinants) and electrostatic (calcium) binding forces. To explore their relative importance we attempted to reverse mutagenesis-induced impairment of membrane binding by increasing the calcium concentrations. As indicated in Fig. 3B even high concentrations of calcium (5 mM) did not significantly improve membrane binding of the 15-LOX mutants. Thus, hydrophobic interactions may be considered the primary driving forces of membrane translocation.

**Calcium Binding at 15-LOX-1 Is a Low Affinity Process**—Although our mutagenesis data suggested hydrophobic interactions as primary driving forces of membrane association the importance of calcium ions must not be neglected. To shed light on the role of calcium in 15-LOX/membrane interaction we first tested whether the enzyme directly interacts with calcium ions suggested that formation of enzyme-membrane complexes proceeds via a concerted action of hydrophobic (sequence determinants) and electrostatic (calcium) binding forces. To explore their relative importance we attempted to reverse mutagenesis-induced impairment of membrane binding by increasing the calcium concentrations. As indicated in Fig. 3B even high concentrations of calcium (5 mM) did not significantly improve membrane binding of the 15-LOX mutants. Thus, hydrophobic interactions may be considered the primary driving forces of membrane translocation.

**Calcium Binding at 15-LOX-1 Is a Low Affinity Process**—Although our mutagenesis data suggested hydrophobic interactions as primary driving forces of membrane association the importance of calcium ions must not be neglected. To shed light on the role of calcium in 15-LOX/membrane interaction we first tested whether the enzyme directly interacts with calcium ions and whether this interaction may be considered a high affinity process. For this purpose we carried out surface plasmon resonance experiments, a method that is usually employed to characterize the formation of receptor-ligand complexes. This method is based on the finding that ligand binding to a receptor is associated with subtle structural alterations, which can be measured by changes in light diffraction (see Supplemental Materials). To start these experiments we first immobilized the native and recombinant 15-LOX species as well as some mu-
Membrane Binding of 15-LOX

Impact of calcium ions on arachidonic acid oxygenase activity and positional specificity of 15-LOX species

The arachidonic acid oxygenase activity of the wild-type 15-LOX and selected mutants was assayed in the presence and absence of 1 mM calcium as described under “Experimental Procedures.” Activities were assayed in duplicate (mean ± error range are given) and the absolute arachidonic acid oxygenase activity of the wild-type enzyme in the absence of exogenous calcium was set 100%.

| Enzyme species | Relative arachidonic acid oxygenase activity | 15-HETE/12-HETE ratio |
|----------------|---------------------------------------------|-----------------------|
|                | −Ca\(^{2+}\) | +Ca\(^{2+}\) | −Ca\(^{2+}\) | +Ca\(^{2+}\) |
| Wild-type (recombinant) | 100 ± 2 | 101 ± 2 | 13 ± 1 | 20 ± 1 |
| Y15E           | 61 ± 20 | 61 ± 20 | 19 ± 4 | 21 ± 1 |
| F70H           | 84 ± 5  | 53 ± 9  | 11 ± 1 | 20 ± 1 |
| L71K           | 64 ± 4  | 50 ± 2  | 21 ± 1 | 21 ± 1 |
| W181E          | 54 ± 3  | 34 ± 1  | 9 ± 1  | 14 ± 1 |
| L195E          | 42 ± 1  | 42 ± 1  | 19 ± 1 | 16 ± 1 |
| Y15E + F70H + L71K | 111 ± 2 | 112 ± 2 | 13 ± 1 | 18 ± 1 |
| Y15E + F70H + L71K + L195E | 28 ± 4 | 29 ± 4 | 15 ± 1 | 17 ± 1 |

Fig. 3. Impact of calcium concentrations on 15-LOX/membrane interaction. First, SMPs were washed three times in the presence of 1 mM EDTA to remove endogenous calcium. Then wild-type and mutant 15-LOX species were incubated with EDTA-washed SMP in the presence and absence of exogenous calcium and membrane binding assays were carried out as described under “Experimental Procedures.” A, Western blot analysis of 15-LOX-1 recovered from the membrane pellet (P) and from the membrane-free supernatant (S) of the membrane binding assay. The calcium concentrations added to the binding assay are indicated above the blots. B, impact of high calcium concentrations on membrane binding capacity of mutant 15-LOX species exhibiting an impaired membrane binding activity. −Ca\(^{2+}\), no exogenous calcium; +Ca\(^{2+}\), 5 mM exogenous calcium. It should be stressed that the standard binding assay used for these experiments (unwashed SMP) contains sufficient calcium to allow membrane binding of the wild-type enzyme (82% membrane bound share).

Tants exhibiting a reduced membrane binding capacity on a gold-coated matrix. These sensor chips were then equilibrated with different calcium concentrations and alterations in light diffraction were quantified in real-time mode. From Fig. 4A it can be seen that calcium induces alterations in light diffraction and that the extent depended on the concentration of calcium ions. Washing the LOX chips with calcium-free buffer (removal of calcium) reversed these alterations completely indicating the reversibility of calcium binding.

The raw data of the surface plasmon resonance studies were used to quantify the affinity of the rabbit 15-LOX for calcium ions. For this purpose the diffraction indices measured were plotted versus the different calcium concentrations (Fig. 4B) and the dissociation constants for the different enzyme species were calculated. For the native wild-type enzyme a numerical value of 4.8 \(\times\) 10\(^{-4}\) M was obtained. This \(K_D\), which reflects the average affinity of all calcium-binding sites present in the 15-LOX, suggests a low affinity process. Similar experiments were carried out for the recombinant wild-type enzyme and for two 15-LOX mutants (NH\(_2\)-terminal truncation mutant, quadruple mutant of the membrane binding sequence determinant) that exhibit reduced membrane binding capacity (Fig. 2). From Table II it can be seen that for all these enzyme species similar \(K_D\) values were obtained. In contrast, the \(K_D\) for elastase, which is known to bind calcium with a higher affinity (32), was by almost 1 order of magnitude lower (6.6 \(\times\) 10\(^{-5}\) M). It should be stressed that the numeric \(K_D\) determined for elastase with the surface plasmon resonance approach was comparable with that obtained by equilibrium dialysis ranging between 1.4 and 7.3 \(\times\) 10\(^{-5}\) M depending on the experimental conditions (33). These data indicate the validity of our methodological approach.

Calcium Binding Induces Subtle Structural Alterations in 15-LOX—Calcium binding to proteins may induce structural...
alterations and such changes can be sensitively quantified by measuring the tryptophan fluorescence. It is well known that the quantum yield of tryptophan fluorescence increases in more hydrophobic microenvironments (34). In other words, an increase in tryptophan fluorescence indicates structural alterations resulting in a restriction of water accessibility to tryptophan residues. Inversely, a reduced tryptophan fluorescence suggests improved water accessibility. For SLO1 a biphasic structural response to increasing calcium concentrations has been described (12). At low concentrations (lower micromolar range) an increase in tryptophan fluorescence was observed, whereas at higher concentrations (millimolar range) fluorescence was impaired. We observed a similar biphasic behavior for the rabbit enzyme (Fig. 5). However, the structural alterations induced appear to be rather subtle because neither the enzymatic activity nor the positional specificity of the enzyme were dramatically impacted by calcium binding (Table I and Supplemental Materials). Although some mutants (F70H, L71K, and W181E) exhibited a somewhat reduced arachidonic acid oxygenase activity in the presence of 1 mM calcium the alterations were not really dramatic. It may well be, that addition of calcium alters the physicochemical state of the substrate (formation of fatty acid calcium soaps) and this effect may contribute to the gradual decrease of the catalytic activity. Investigating the impact of calcium on the positional specificity of arachidonic acid oxygenation we found that in the presence of calcium the 15-HETE/12-HETE ratio tended to be somewhat higher. However, under all experimental conditions the share of 12-HETE did not exceed 10%.

**Lack of High Affinity Calcium-binding Sites**—Although the Biacore experiments indicated that calcium binding at the rabbit 15-LOX constitutes a low affinity process the experimental data do not rule out the existence of high affinity calcium-binding sites. SLO1 (12) and the human 5-LOX (20) were suggested to contain such high affinity binding sites but mammalian 15-LOXs have not been investigated in this respect. To address this problem we employed a dual strategy. First, we performed a structure-based amino acid alignment between soybean and rabbit 15-LOXs (35) and found that the high affinity calcium-binding sites identified for SLO1 are not conserved in the rabbit enzyme (Fig. 6). Next, we superimposed the primary structure of the human 5-LOX and failed to observe conservation of those amino acids implicated in calcium binding of this LOX isoform. These data suggested that the rabbit 15-LOX might lack high affinity calcium-binding sites. To test this hypothesis experimentally we loaded the native enzyme with different calcium concentrations, removed the unbound calcium by two consecutive steps of gel filtration, and quantified the LOX-bound calcium by atomic absorption spectroscopy. If one or two high affinity calcium-binding sites are present (tight calcium binding) one would expect a molar calcium/enzyme ratio of 1/1 or 2/1, respectively, after removal of

![Fig. 4. Biacore experiments studying calcium binding at recombinant wild-type 15-LOX.](image)

**Table II**

| Enzyme species | $K_d$ (M) |
|----------------|----------|
| Native 15-LOX-1 | $4.8 \times 10^{-4}$ |
| Recombinant 15-LOX-1 | $1.9 \times 10^{-4}$ |
| Catalytic domain | $3.7 \times 10^{-4}$ |
| Y15E + F70H + L71K + L195E | $2.1 \times 10^{-4}$ |
| Elastase | $6.6 \times 10^{-5}$ |
unbound calcium. From Table III it can be seen that the de-salted enzyme preparation (no calcium loading) contained about 0.3 mol of calcium/mol of enzyme. Similar values were obtained when the enzyme was pre-loaded with increasing calcium concentrations. These data suggest that calcium ions completely dissociate from the enzyme when unbound calcium is removed suggesting the lack of tight calcium binding. These results are in line with the data obtained by the surface plasmon resonance studies. Here the light diffraction index returns to baseline after washing the calcium-treated LOX with calcium free buffer.

DISCUSSION

A variety of plant and mammalian LOXs are capable of binding to biomembranes and calcium appears to be an important cofactor (36–40). Because most LOXs sequenced so far contain an NH2-terminal C2-like domain it has been speculated that this structural element may function as phospholipid-binding domain anchoring the enzyme in the lipid bilayer in a calcium-dependent manner (20, 21). It should, however, be stressed that the structure of the β-barrel domains in LOXs do not exactly match the topology of the C2-domains in phospholipases (20) and thus, there might be differences in the functionality of this structural element. Membrane interaction has been studied in detail for several C2-domain carrying enzymes and two principle interacting forces have been suggested (41, 42): (i) hydrophobic interactions between surface-exposed apolar amino acid side chains and the hydrophobic core of the membrane phospholipid bilayer and (ii) electrostatic interaction between surface-exposed charged amino acids with inversely charged head groups of the phospholipid molecules. For certain proteins (phospholipase A2) the hydrophobic interactions appear to be dominant (41, 42), for others ionic bondings might prevail (42). For phospholipase A2 it has been shown that two calcium binding loops of the C2-domain may penetrate into the hydrophobic core of the phospholipid bilayer establishing an anchor for docking the C2-domain onto the membrane (43). For the human 5-LOX a similar mechanism has been suggested. In fact, high affinity calcium binding appears to be involved in membrane binding (18, 20) and recent mutagenesis studies suggested an important role of solvent-exposed tryptophans (21). Thus, for the human 5-LOX too, a concerted action of hydrophobic and ionic interactions appears to be important for calcium-dependent membrane translocation. As for the phospholipase A2 the NH2-terminal β-barrel domain may play a crucial role in this process.

In contrast, the mechanism of membrane binding of the rabbit 15-LOX appears to be somewhat different. Although we also observed a calcium dependence and an involvement of solvent-exposed hydrophobic amino acid residues our experimental data suggest several mechanistic differences. (i) Although the NH2-terminal β-barrel domain appears to be involved in membrane binding it may not be essential (24). (ii) Sequence determinants for membrane binding are not restricted to the NH2-terminal β-barrel domain but also occur at the surface of the catalytic domain. (iii) The rabbit 15-LOX does not contain high affinity calcium-binding sites. However, low affinity calcium binding to enzyme, membranes, or both appears to be involved in membrane association. (iv) Hydrophobic interactions may constitute the primary driving forces of membrane/enzyme interaction. However, ionic forces may also contribute and thus, a mixture of hydrophobic and electrostatic interactions is responsible for membrane binding.

The priority of hydrophobic interactions opens up the question about the mechanistic role of calcium. It is well documented in the literature (22, 23) that calcium is required for membrane/15-LOX interaction. Here we found that calcium concentrations between 5 and 50 μM are required for effective membrane binding. As a possible mechanism of how calcium may impact membrane association one may assume that the positively charged ions may weaken repulsive forces between negatively charged phospholipid head groups and acidic surface amino acid side chains of the membrane contact plane of the enzyme. If one inspects the surface of this part of the 15-LOX structure, which involves regions of the NH2-terminal β-barrel and the COOH-terminal catalytic domains, one detects several surface-exposed negatively charged amino acids (Fig. 1C). The bivalent calcium ions may not only shield these negative charges but may even form salt bridges with the phosphate moieties of the membrane phospholipids supporting membrane binding. In this scenario it may not be of major importance whether calcium is primarily bound at the enzyme or at the surface of the membrane phospholipids. Biomembranes and phospholipid vesicles are capable of binding calcium via ionic interactions with their negatively charged phosphate groups (30, 31). For dioleoylphosphatidate/dioleoylphosphatidylycholine multilamellar vesicles (2:5 mol %) calcium was shown to neutralize these negative charges on the liposome surface and thus, induces vesicle fusion and aggregation (44).

Our mutagenesis studies identified five major sequence determinants for membrane binding of the rabbit 15-LOX. To find out whether some of these residues might be important for membrane binding of other LOX-isoforms a multiple sequence

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**Fig. 5. Biphasic calcium-dependent alterations in tryptophan fluorescence of the rabbit 15-lipoxygenase.** The rabbit 15-LOX was purified from a reticulocyte-rich hemolysate to apparent electrophoretic homogeneity as described under “Experimental Procedures.” The pure enzyme was rebuffed into a calcium-free Tris-HCl buffer, pH 7.4, and the tryptophan fluorescence emission spectrum (excitation wavelength 284 nm) was recorded between 300 and 400 nm. 

A, Cu2+-dependent increase in tryptophan fluorescence in the lower micromolar range. 

B, impairment of tryptophan fluorescence in the higher micromolar range of calcium.
Alignment was carried out (Fig. S6, Supplemental Materials). This alignment indicates that for some determinants (Tyr 15, Phe70-Leu71, and Tyr 195) the hydrophobic character of the amino acids was conserved among most LOX isoforms. However, at position 181 (Trp181 for the rabbit enzyme) we found positively charged residues (Lys) in several isoforms. Interestingly, a W181K exchange in the rabbit 15-LOX enzyme (24) only slightly impaired the membrane binding properties (80% membrane-bound share for the wild-type enzyme, 60% membrane-bound share for W181K). A much more pronounced effect was observed when a negatively charged Glu was introduced (21% membrane-bound share). These data suggest that an additional positive surface charge only gradually impaired the membrane binding capacity. In contrast, an additional negative surface charge induced more dramatic effects. The molecular reasons for the different effects of positively and negatively charged residues have not been studied in detail but it may be related to the calcium dependence of membrane binding. It is important to stress that our amino acid alignment (based on sequence information only) does not consider three-dimensional structural aspects. Thus, from the alignment it cannot be concluded whether or not the amino acids, which align with the sequence determinants of the rabbit enzyme, are actually surface exposed in other LOX isoforms. Nevertheless, it might be possible that membrane binding of other LOX isoforms might also involve sequence determinants identified for the rabbit enzyme. On the other hand, for other LOX species additional structural elements may play a role. For instance, the human 5-LOX contains tryptophan residues that have been implicated in membrane binding (21). In summary one may conclude that the functional importance of structural elements with potential impact for membrane binding must be determined experimentally for the various LOX isoforms.

### Table III

Quantification of calcium binding to 15-LOX-1 after preincubation with an excess of calcium.

The native 15-LOX-1 was purified from rabbit reticulocytes as described under "Experimental Procedures." The enzyme was rebuffered into 10 mM Tris-HCl and then preincubated for 10 min in the presence of different calcium concentrations. The enzyme solution was desalted again by 2 consecutive steps of gel filtration and calcium concentration was determined by atomic absorption spectroscopy (see "Experimental Procedures").

| Preincubation | After desalting |
|---------------|-----------------|
| mol Ca²⁺/mol LOX | 0.35 ± 0.01 |
| 0.5 | 0.4 ± 0.01 |
| 1.5 | 0.3 ± 0.03 |
| 15 | 0.3 ± 0.07 |
| 50 | 0.3 ± 0.05 |
| 500 | 0.4 ± 0.01 |

This alignment was carried out (Fig. S6, Supplemental Materials). This alignment indicates that for some determinants (Tyr₁⁵, Phe⁷⁰-Leu⁷¹, and Tyr₁⁹⁵) the hydrophobic character of the amino acids was conserved among most LOX isoforms. However, at position 181 (Trp¹⁸¹ for the rabbit enzyme) we found positively charged residues (Lys) in several isoforms. Interestingly, a W181K exchange in the rabbit 15-LOX enzyme (24) only slightly impaired the membrane binding properties (80% membrane-bound share for the wild-type enzyme, 60% membrane-bound share for W181K). A much more pronounced effect was observed when a negatively charged Glu was introduced (21% membrane-bound share). These data suggest that an additional positive surface charge only gradually impaired the membrane binding capacity. In contrast, an additional negative surface charge induced more dramatic effects. The molecular reasons for the different effects of positively and negatively charged residues have not been studied in detail but it may be related to the calcium dependence of membrane binding. It is important to stress that our amino acid alignment (based on sequence information only) does not consider three-dimensional structural aspects. Thus, from the alignment it cannot be concluded whether or not the amino acids, which align with the sequence determinants of the rabbit enzyme, are actually surface exposed in other LOX isoforms. Nevertheless, it might be possible that membrane binding of other LOX isoforms might also involve sequence determinants identified for the rabbit enzyme. On the other hand, for other LOX species additional structural elements may play a role. For instance, the human 5-LOX contains tryptophan residues that have been implicated in membrane binding (21). In summary one may conclude that the functional importance of structural elements with potential impact for membrane binding must be determined experimentally for the various LOX isoforms in separate studies.

Three sequence determinants (Tyr₁⁵, Phe⁷⁰, and Leu⁷¹) are located at the surface of the NH₂-terminal β-barrel domain and two of them (Trp¹⁸¹ and Leu¹⁹⁵) are constituents of the catalytic domain. Thus, both domains appear to be important for...
membrane binding and this conclusion is consistent with previous truncation studies (24). Interestingly, all sequence determinants are clustered at one site of the enzyme molecule and this part of the protein surface also involves the entrance to the substrate binding pocket (Fig. 1). Other regions of the enzyme surface are virtually free of solvent-exposed hydrophobic amino acids. However, not all surface-exposed apolar residues of the membrane contact plane are important for membrane binding. In fact, our mutagenesis data (Fig. 2B) indicated that Tyr<sup>292</sup> and Phe<sup>412</sup> may not play a major role for membrane binding although their side chains are solvent exposed to a similar extent as Tyr<sup>15</sup> or Leu<sup>195</sup> (Fig. 1B). It might be possible that these two residues are somewhat dislocated from the immediate membrane contact plane of the enzyme and thus their contribution may be minimal.

Taken together our experiments indicate that calcium binding to 15-LOXs may be considered a reversible low affinity process. Although calcium is required for membrane association hydrophobic interactions appear to be the driving forces of membrane association. The NH<sub>2</sub>-terminal β-barrel domain is involved in membrane association but the COOH-terminal catalytic domain also contains sequence determinants for membrane binding. It is hypothesized that low affinity calcium binding to enzyme, membrane, or both weakens repulsive forces between negatively charged phospholipid head groups and acidic amino acid side chains localized at the membrane contact plane of the enzyme. Thus, binding of 15-LOXs to biomembranes appears to be a complex process involving both, the NH<sub>2</sub>-terminal and COOH-terminal enzyme subunit as well as calcium ions.

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Investigations into Calcium-dependent Membrane Association of 15-Lipoxygenase-1: MECHANISTIC ROLES OF SURFACE-EXPOSED HYDROPHOBIC AMINO ACIDS AND CALCIUM
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