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Activation of a covalent outer membrane phospholipase A dimer

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The activity of outer membrane phospholipase A (OMPLA) is regulated by reversible dimerization. However, native OMPLA reconstituted in phospholipid vesicles was found to be present as a dimer but nevertheless inactive. To investigate the importance of dimerization for control of OMPLA activity, a covalent OMPLA dimer was constructed and its properties were compared to native OMPLA both in a micellar detergent and after reconstitution in a phospholipid bilayer. Unlike native OMPLA, activity of the covalent OMPLA dimer was independent of type and concentration of detergent in micellar systems. In such systems, the covalent OMPLA dimer invariantly displayed high calcium affinity. In contrast, high calcium concentrations were required to activate a covalent OMPLA dimer when present in intact vesicles. Solubilization of the vesicles increased the affinity for calcium, suggesting that in an intact bilayer the dimer interface is not properly formed. This was supported by the observation that OMPLA variants having an impaired dimeric interface also lacked high affinity calcium binding. A covalent linkage was not able to restore high affinity calcium binding in these variants, demonstrating that a proper dimer interface is essential for optimal catalysis.

Keywords: OMPLA; dimerization; calcium binding; activity regulation.

The outer membrane phospholipase A (OMPLA) is an integral membrane enzyme that catalyses the hydrolysis of acylester bonds in phospholipids using calcium as a cofactor [1]. The enzyme is widespread among Gram-negative bacteria, both in pathogens and nonpathogens. In pathogenic bacteria such as *Campylobacter coli* and *Helicobacter pylori* OMPLA is involved in pathogenesis and virulence [2,3]. In nonpathogenic bacteria the physiological function of OMPLA is less clear. The *Escherichia coli* enzyme has been best studied and is involved in the secretion of bacteriocins, antibacterial peptides that are produced in order to survive under starvation conditions [4,5].

Although OMPLA is constitutively expressed, no phospholipid turnover can be detected under physiological conditions, suggesting that OMPLA resides in the outer membrane in an inactive state. OMPLA activity can be triggered by processes that severely perturb the outer membrane integrity, such as phage-induced lysis [6], spheroplast formation [7], heat shock [8], EDTA treatment [9] and colicin release [4,10,11].

In vitro OMPLA activity is strongly dependent on the experimental conditions such as type of detergent, detergent concentration and protein concentration. It has been shown that activity is regulated by reversible dimerization and that the experimental conditions influence the dimerization equilibrium [12]. Chemical cross-linking on whole cells indicated that OMPLA is present in the outer membrane as a monomer, and that activation by bacteriocin-release protein-induced dimerization [5]. This suggests that *in vivo* dimerization is also part of the regulatory mechanism. However, fluorescence resonance energy transfer experiments on OMPLA reconstituted in vesicles demonstrated that in this situation OMPLA was already dimeric whereas no activity could be detected [13]. These results seem to indicate that dimerization of OMPLA dimers is necessary but not sufficient for activation. Thus, the exact role for dimerization in activation of OMPLA remains to be clarified.

In the present study, we have constructed a well-defined covalent OMPLA dimer to study the importance of dimerization for activity regulation both in a detergent system and after reconstitution in a phospholipid bilayer. The importance of proper packing of the dimer interface was studied using OMPLA variants that were designed to interfere with OMPLA dimerization.

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Abbreviations: $C_{12}E_5$, dodecylpentaethylene glycol ether; C_{12}SB, dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate; C_{16}*P*Cho, hexadecanoylphosphocholine; C_{16}thioglycol*P*Cho, 2-hexadecanoylthio-ethane-1-phosphocholine; $C_{18:1}$*P*Cho, 1,2-dioctadecenoyl-*sn*-glycero-3-phosphocholine; $C_{18:1}$dithio*P*Cho, 1,2-dioctadecenoylthio-*sn*-glycero-3-phosphocholine; $C_{18:1}$dithio*P*Etn, 1,2-dioctadecenoylthio-*sn*-glycero-3-phosphoethanolamine; $C_{18:1}$dithio*P*Gro, 1,2-dioctadecenoylthio-*sn*-glycero-3-phosphoglycerol; DOC, sodium deoxycholate; dithiothreitol, 1,4-dithiotreitol; OMPLA, outer membrane phospholipase A; *pldA*, gene encoding OMPLA.

Enzyme: outer membrane phospholipase A (EC 3.1.1.32).

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MATERIALS AND METHODS

Chemicals

DNA restriction enzymes were purchased from New England Biolabs. Oligonucleotides were bought from Microsynth. Research grade dodecyl-*N*,*N*′-dimethyl-1-ammonio-3-propanesulfonate (C_{12}SB) was obtained from Fluka

and purified as described previously [14]. The synthesis of hexadecanoylphosphocholine (C_{16}*P*Cho) has been described by van Dam-Mieras *et al.* [15]. 2-Hexadecanoylthio-ethane-1-phosphocholine (C_{16}thioglycol*P*Cho) was synthesized according to Aarsman *et al.* [16]. 1,2-Dioctadecenoyl-*sn*-glycero-3-phosphocholine ($C_{18:1}$*P*Cho) was obtained from Avanti. Dodecylpentaethylene glycol ether ($C_{12}E_5$) and sodium deoxycholate (DOC) were obtained from Fluka and SDS from Serva. 1,2-Dioctadecenoylthio-*sn*-glycero-3-phosphocholine ($C_{18:1}$dithio*P*Cho), 1,2-dioctadecenoyl-thio-*sn*-glycero-3-phosphoethanolamine ($C_{18:1}$dithio*P*Etn) and 1,2-dioctadecenoylthio-*sn*-glycero-3-phosphoglycerol ($C_{18:1}$dithio*P*Gro) were synthesized in our laboratory according to standard procedures and displayed only a single spot upon chromatographic analysis on HPTLC Kieselgel Platten (Merck) using chloroform/methanol/water (65 : 25 : 4, v/v) as the solvent system. All other chemicals were of the highest purity available.

Bacterial strains and plasmids

The *E. coli* K12 strain DH5α was used in the cloning procedures; *E. coli* CE1433 is a *pldA*$^-$ derivative of BL21(DE3) and was used as a host strain for expression. Plasmid pND5 was constructed by Ubarretxena *et al.* [13] and encodes OMPLA containing a His26 → Cys mutation. Plasmid pRK21 is a pND1 [17] derivative that contains the *pldA* gene with several silent mutations introduced to facilitate cloning procedures. pRK21 was used as a DNA template for the introduction of dimer interface mutations by QuikChange™ site-directed mutagenesis (STRATAGENE). In the primer sequences, the mutations are depicted in bold type. A restriction site (underlined) was introduced with the primers to facilitate screening. The following oligonucleotides were used: RK60 (5′-GCATGACAATCCGTTCACG**GCG**TATC**CGTACG**ACACCAACTACC-3′) and its complement RK61 for the introduction of the Leu32 → Ala mutation (restriction site *Bsi*WI), RK62 (5′-GGATGAAGTAAAGTTTC**AAGCTTCCGC**AGCATTTCCGC-3′) and its complement RK63 for the introduction of the Leu71/73 → Ala mutation (restriction site *Hin*dIII). RK64 (5′-CCAATAGCGAAGAGA**GCT**CACCG**ATG**CGTGAAACCAACTACG-3′) and its complement RK65 for the introduction of the Phe109 → Met mutation (restriction site *Sac*I), and RK66 (5′-CGGTGTTGGGTGCGTC**GTATAC**G**GCG**AAATCCTGGTGGC-3′) and its complement RK67 for the introduction of the Gln94 → Ala mutation (restriction site *AccI*). For the combination of the Leu32 → Ala and Leu71/73 → Ala mutations, both plasmids were digested with *Spe*I and *Hin*dIII, and the Leu71/73 → Ala mutation was cloned into the vector containing the Leu32 → Ala mutation. All other mutations were subcloned into pRK21 using *Mfe*I and *Hin*dIII. The relevant part of the sequences was subsequently verified by DNA sequencing.

Purification of proteins and construction of dimeric protein

The OMPLA variants were overexpressed without their signal sequence resulting in the accumulation of inclusion bodies by induction with isopropyl thio-β-D-galactoside in *E. coli* BL21(DE3). The inclusion bodies were folded and purified essentially as described previously [14]. To prevent oxidation of the sulfydryl groups, the His26 → Cys variant was purified in the presence of 5 mM 1,4-dithiothreitol.

His26 → Cys OMPLA was freed from dithiothreitol after application onto a Q-Sepharose column, washing with buffer A (2.5 mM C_{12}SB, 20 mM Tris/HCl, pH 3.8, 20 mM $CaCl_2$) and subsequent elution with 1 M KCl in buffer A. The protein was incubated overnight for optimal disulfide bond formation. The dimeric species of OMPLA was further purified by application onto a Superdex G-200 column (Pharmacia).

OMPLA activity assay

OMPLA activities were determined spectrophotometrically using hexadecanoylthioethane-1-phosphocholine (C_{16}thioglycol*P*Cho) as a substrate. OMPLA was incubated overnight at a concentration of 0.05 mg·mL^{-1} in buffer (20 mM Tris/HCl, pH 8.3, 2 mM EDTA, 2.5 mM C_{12}SB). Routinely, 50 ng of protein was assayed for enzymatic activity in 1 mL of assay buffer (50 mM Tris/HCl, pH 8.3, 5 mM $CaCl_2$, 0.2 mM Triton X-100, 0.1 mM dithiobis(2-nitro-benzoic acid), 0.25 mM substrate). Initial velocities were calculated from the increase in A_{412}. One unit corresponds with the conversion of 1 μmol of substrate per minute.

Calcium binding measured in the kinetic assay

Kinetic Ca^{2+} binding constants were determined using the aforementioned assay with minor modifications. Instead of 5 mM $CaCl_2$, 10 μM of EDTA was added to the assay buffer. Calcium was titrated to the assay buffer after which activity measurements were performed. Upon graphical representation of the specific activity vs. the concentration of calcium a hyperbolic saturation curve is obtained which is fitted according to Michaelis–Menten kinetics. Thus the parameters K_{Ca} and the V_{max} were obtained.

To study the effect of assay parameters on calcium affinity, the following parameters were varied: the concentration of Triton X-100 (200 or 500 μM), the concentration of substrate (10, 20 or 33 mol%), the type of substrate (C_{16}thioglycol*P*Cho, $C_{18:1}$dithio*P*Cho, $C_{18:1}$dithio*P*Etn or $C_{18:1}$dithio*P*Gro) and type of detergent (C_{16}*P*Cho, C_{12}SB, $C_{12}E_5$).

Chemical cross-linking

OMPLA was incubated at 0.2 mg·mL^{-1} in buffer (50 mM Hepes, pH 8.3, 100 mM KCl and 2.5 mM C_{12}SB and either 20 mM $CaCl_2$ or 2 mM EDTA) in a total volume of 100 μL. After 1 h, 10 μL of 1% glutaraldehyde in 2.5 mM C_{12}SB was added. The reaction was allowed to continue for 15 min at room temperature. Subsequently, 100 μL of gel loading buffer (0.1 M Tris/HCl, pH 6.8, 3% SDS, 15.4% glycerol, 7.7% 2-mercaptoethanol and 0.008% bromphenol blue) was added and 20 μL of this solution (corresponding to 2 μg of OMPLA) was analysed by SDS/PAGE. Visualization of the bands was achieved by staining with Coomassie Brilliant Blue.

Reconstitution of OMPLA in phospholipid vesicles

Phospholipids were solubilized in chloroform/methanol (1 : 1, v/v) and the organic solvent was removed under

reduced pressure. The dried lipid film was hydrated with buffer composed of 50 mM Tris/HCl, pH 8.3, 2 mM EDTA, 100 mM KCl to a final phospholipid concentration of 6 mM. To this suspension 2-octylglucopyranoside was added to yield an optically clear mixed micellar solution. OMPLA was added to a final concentration of 7 μM. Bio-Beads® were washed with buffer and added to the phospholipid solution at a concentration of 80 $mg{\cdot}mL^{-1}$. This mixture was incubated for 1 h under constant slow rotation. The Bio-Beads procedure was repeated three times each with a fresh batch of beads.

Characterization of vesicles

TLC was used to check the purity of the components and to follow detergent removal during vesicle preparation. TLC was performed on HPTLC Kiesegel (Merck) plates using dichloromethane/methanol/water (85 : 20 : 3, v/v/v) as eluents. Spots were visualized with I_2 vapor followed by charring with phosphomolybdate reagent. Vesicle size was determined by light scattering in the Zetasizer 3000 (Malvern Instruments). Phospholipid concentrations were determined by measurement of the inorganic phosphate content [18]. The OMPLA content was determined by estimation from SDS/PAGE analysis using purified OMPLA as a reference.

The average number of OMPLA molecules per vesicle was calculated from (a) the concentration of OMPLA; (b) the concentration of phospholipid, (c) the molecular surface area occupied by a fully hydrated phosphatidylcholine molecule being 70 $Å^2$ according to [19], (d) the molecular dimensions of OMPLA calculated from the crystal structure (600 $Å^2$) and (e) the surface area of the vesicles (assuming spherical geometry).

The orientation of OMPLA in proteoliposomes was determined by limited proteolysis. Chymotrypsin was added to both the intact liposome preparation and solubilized liposomes to a final concentration of 0.2 $mg{\cdot}mL^{-1}$ and incubated at room temperature for 16 h. Subsequently, the products were analysed on SDS/PAGE. Visualization of protein was achieved by staining with Coomassie Brilliant Blue.

Complete solubilization of liposomes was achieved by the addition of 5 molar equivalents of Triton X-100. Both intact and solubilized liposomes were incubated at a concentration of 2, 20 or 200 mM $CaCl_2$. Samples were taken after 1, 5, 15, 60 min or 24 h of incubation. The reaction was stopped by the addition of 250 mM EDTA. The extent of phospholipid hydrolysis was analysed by TLC.

RESULTS

Construction of a covalent OMPLA dimer

The absence of cysteines in wild-type OMPLA allows for the introduction of a unique intermolecular disulfide bond covalently linking two OMPLA monomers. For the construction of a well-defined OMPLA dimer, a His26 → Cys OMPLA variant was employed. In the structure of dimeric OMPLA, His26 in one monomer is located in a highly flexible N-terminal region in close proximity to its counterpart in the other monomer (Fig. 1A) at a distance of more than 30 Å from the active site Ser144 and the catalytic calcium site.

His26 → Cys OMPLA was expressed without a signal sequence and accumulated in inclusion bodies. These were folded and purified mainly as described by Dekker *et al.* [14] in the presence of dithiothreitol. To obtain covalent dimers, dithiothreitol was removed using anion-exchange chromatography and the His26 → Cys variant OMPLA was eluted in the presence of calcium at the optimal detergent concentration for dimer formation. SDS/PAGE analysis showed that about 50% of the protein migrated with an apparent molecular mass of 42 kDa corresponding with the dimeric species of OMPLA [12], whereas 50% migrated at 27 kDa, corresponding with wild-type OMPLA (Fig. 2, lane 2). The dimeric species of OMPLA was purified by gel filtration, yielding covalent OMPLA dimer with a purity of over 90% (Fig. 2, lane 3).

Dependence of enzymatic activity on detergent in preincubation

It has been shown that OMPLA activity strongly depends on the concentration of detergent used in the preincubation.

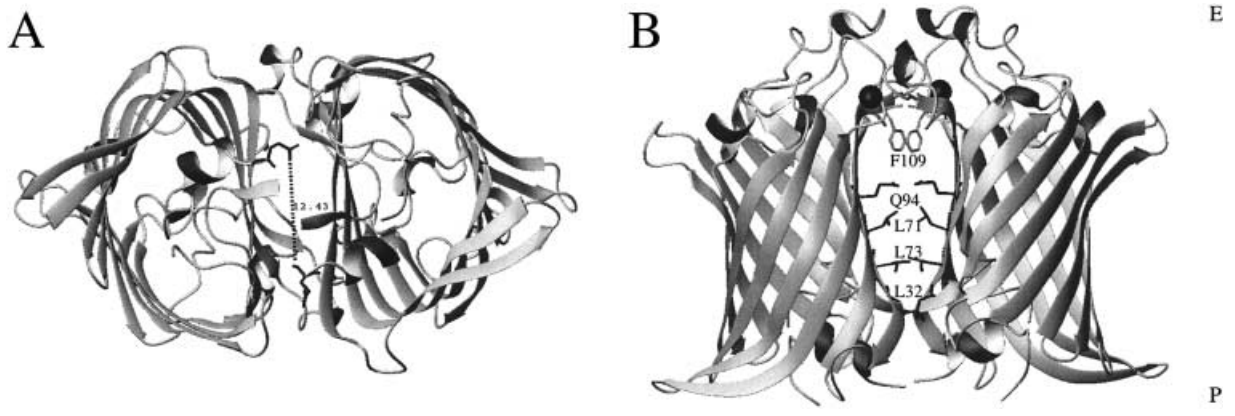

Fig. 1. Structure of OMPLA. (A) Bottom view of OMPLA highlighting the distance between Glu25 of both monomers. In the structure, the 13 N-terminal residues and residues 26–31 could not be resolved due to high crystallographic B-factors. (B) Side view of OMPLA highlighting the residues involved in dimerization. The catalytic calcium ion is represented as a black sphere. E represents the extracellular side, and P represents the periplasmic side of the structure.

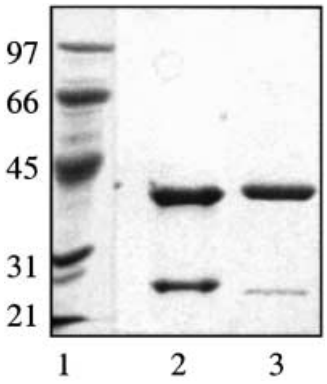

Fig. 2. SDS/PAGE analysis of OMPLA dimerization. Lane 1, molecular mass marker; lane 2, OMPLA after overnight preincubation under dimerization favouring conditions; lane 3, OMPLA after purification by gel filtration.

The effect of the detergent concentration on enzymatic activity of wild-type OMPLA and the covalent dimer is shown in Fig. 3. Whereas the enzymatic activity of wild-type OMPLA decreased with increasing concentration of C_{12}SB, the activity of the OMPLA dimer was not affected. The decrease in activity of native OMPLA was not due to impaired calcium affinity, as OMPLA preincubated at 1.5, 2.5 or 5 mM C_{12}SB displayed similar calcium affinity of around 10 ± 3 μM.

Because OMPLA activity not only depends on the concentration but also on the type of the detergent used in the preincubation, the activities of wild-type and dimeric OMPLA were assessed for several detergents, among which the zwitterionic lysophospholipid analogue C_{16}*P*Cho and the reverse zwitterionic detergent C_{12}SB, a nonionic detergent $C_{12}E_5$, and the anionic detergents DOC and SDS. The results are summarized in Fig. 4. Whereas wild-type OMPLA activity displayed high sensitivity towards the type and concentration of detergent, enzymatic activity of the covalent OMPLA dimer was insensitive towards any detergent used during preincubation.

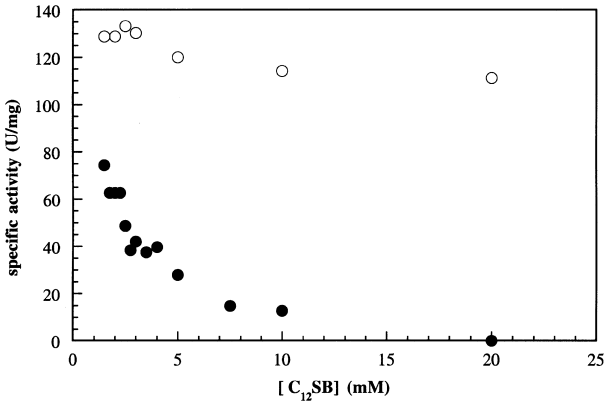

Fig. 3. Activities of the OMPLA dimer and wild-type OMPLA as a function of the concentration of detergent present during preincubation. Covalent OMPLA dimer (○) or wild-type OMPLA (●) was incubated at various concentrations C_{12}SB and the activity was tested in the standard assay.

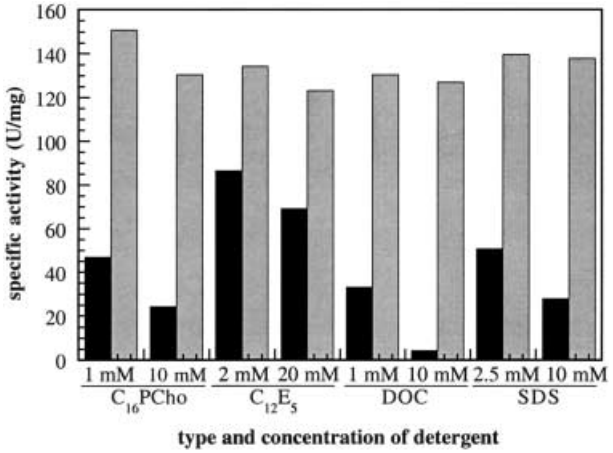

Fig. 4. Activities of the OMPLA dimer and wild-type OMPLA after preincubation in different detergents. The activity of wild-type OMPLA is depicted by black bars whereas the activity of the OMPLA dimer is indicated by the grey bars.

Calcium binding studies

Calcium binding in both wild-type OMPLA and the covalent OMPLA dimer was studied using kinetic assays in which the mole fraction of substrate was varied as well as the level of the detergent Triton X-100. Table 1 reveals that at any Triton X-100 concentration for wild-type OMPLA calcium affinities improved with increasing mole fractions of substrate in the detergent. An increased level of Triton X-100 in the assay adversely affected calcium affinity. In contrast, the calcium affinity of covalent OMPLA dimer remained around 4 μM regardless of the concentration of substrate or Triton X-100 used in the assay.

OMPLA displays broad substrate specificity and is active on both monoacyl and diacyl ester substrates with any polar head group [1]. The dependence of calcium affinity on the substrate used in kinetic assays was investigated. The results of experiments with several substrates are summarized in Table 2. Whereas for wild-type OMPLA calcium affinity depended both on the presence of one or two acyl chains in the substrate and the type of polar head group, in the covalent OMPLA dimer calcium affinity was high and relatively invariant for all substrates used. This strongly suggests that the type of substrate influences dimerization thereby indirectly influencing binding of the catalytic calcium for wild-type OMPLA.

Subsequently, it was investigated whether the type of detergent in the kinetic assays had any effect on calcium affinity of covalent OMPLA dimers. The results are compared with previous results obtained for wild-type OMPLA [20] and are shown in Table 3. Whereas the type of detergent strongly influenced calcium affinities of wild-type OMPLA, the covalent OMPLA dimer was virtually insensitive towards the detergent and displayed high affinity for calcium under all experimental conditions.

Activation of OMPLA reconstituted in $C_{18:1}$*P*Cho vesicles

Wild-type OMPLA and its covalent dimer were reconstituted in $C_{18:1}$*P*Cho vesicles using the Bio-Beads method. This resulted in the formation of vesicles with an average size of

Table 1. Calcium affinities determined in kinetic assays. Catalytic calcium binding was determined in both wild-type OMPLA and the covalent OMPLA dimer in the kinetic assay, in which the mole fraction of substrate in the assay as well as the concentration of Triton X-100 (TX-100) was varied. Substrate affinities (K_m*) have been determined at both Triton X-100 levels.

	[TX-100] (μM)	K_m* (mol%)	K_{Ca} (μM)		
			10 mol% S	20 mol% S	33 mol% S
Wild-type OMPLA	200	11	46	16	9
	500	7.9	508	212	35
OMPLA dimer	200	7	7.5		7.2
	500	5	1.1		2.4

Table 2. Maximum activities and calcium affinities determined in the kinetic assay using a variety of substrates. The calcium binding parameters were measured in the activity assay containing 500 μM Triton X-100 and 10 mol% of substrate.

	Wild-type OMPLA		Covalent OMPLA dimer	
Substrate	V_{max} (U·mg^{-1})	$K_{Ca^{2+}}$ (μM)	V_{max} (U·mg^{-1})	$K_{Ca^{2+}}$ (μM)
C_{16}thioglycol*P*Cho	46	508	90	4
$C_{18:1}$dithio*P*Cho	16.2	3161	27	17.5
$C_{18:1}$dithio*P*Gro	7.1	134	9	3.5
$C_{18:1}$dithio*P*Etn	18	1170	45	15

Table 3. Calcium binding parameters for the covalent OMPLA dimer in various detergents. To facilitate comparison between wild-type OMPLA and the covalent dimer, wild-type OMPLA calcium binding parameters are copied from [21]. OMPLA displayed maximum enzymatic activity at the detergent concentrations used. The binding parameters have a 20% error.

		Covalent dimer		Wild-type		
Detergent	CMC (mM)	[Detergent] (mM)	V_{max} (U·mg^{-1})	$K_{Ca^{2+}}$ (μM)	V_{max} (U/mg)	$K_{Ca^{2+}}$ (μM)
C_{16}*P*Cho	0.010	1	59	3.7	30	12
C_{12}SB	1.3	2.5	83	8.7	40	170
$C_{12}E_5$	0.080	2	116	48	55	24 000

150 nm. Rough calculations showed that for wild-type OMPLA approximately 740 OMPLA monomers were incorporated per vesicle, resulting in a surface density of 3.2%, whereas approximately 1500 dimers (i.e. 3000 monomers) were incorporated (yielding a surface density of 15%). Chymotrypsin has been shown to cleave after Tyr56 in the extracellular loop 1 (A. Busquets, University of Barcelona, Dept. Fisicoquímica, Spain, personal communication). Hence, chymotrypsin cleavage provides information about the surface-exposure of the extracellular loops of OMPLA. For wild-type OMPLA reconstituted in vesicles, approximately 50% of the loops were cleaved by chymotrypsin and hence surface-exposed, whereas only about 10% of the loops in the covalent OMPLA dimer vesicles were surface-exposed.

The activation of OMPLA reconstituted in phospholipid vesicles was assessed by incubation at several calcium concentrations. Degradation of phospholipids was followed on TLC and the time necessary to degrade 50% of the phospholipids at different calcium concentrations was estimated. Surprisingly, wild-type OMPLA and the covalent OMPLA dimer behaved identically after reconstitution in phospholipid vesicles. The degradation half-lives of the phospholipids at different calcium concentrations are shown in Table 4. High calcium concentrations were required to activate the protein when present in an intact bilayer. Solubilization of the vesicles with a fivefold molar excess of Triton X-100 resulted in 100-fold faster degradation of the phospholipids.

To study the role of dimerization *in vivo*, a plasmid was constructed encoding tandem OMPLA connected by a SGSGS-linker under control of the *pldA* promoter. Using Western blotting on cell lysates, we could demonstrate the presence of a 62-kDa OMPLA-construct. However, this

Table 4. Time necessary to degrade 50% of the phospholipids in DOPC vesicles at various calcium concentrations. The experiments were performed at room temperature in a buffer containing 50 mM Tris/HCl, pH 8.3, 2 mM EDTA, 100 mM KCl. The vesicles were solubilized using fivefold molar excess of Triton X-100.

	50% degradation after	
[Calcium] (mM)	Intact vesicles	Solubilized vesicles
2	≫ 24 h	15 min
20	≈ 10 h	5 min
200	≈ 1.5 h	1 min

62-kDa protein corresponded with unfolded OMPLA dimer. Subsequently, the construct was expressed under control of a T7 promoter. The dimer accumulated in inclusion bodies that could not be folded *in vitro* to yield active OMPLA dimer.

Calcium binding in dimer interface variants

To study the conditions for dimerization and thus the importance of proper positioning of the monomers with respect to each other, the contribution of the dimerization interface to efficient catalysis was assessed by site-directed mutagenesis. In the dimeric enzyme, most interactions between OMPLA monomers occur in the hydrophobic membrane-embedded area. The hydrophobic side chains of Leu32, Leu71, Leu73 and Leu265 exhibit a knob-and-hole interaction, the aromatic residues Tyr114 and Phe109 display stacking interactions, and the side chain of Gln94 is hydrogen bonded with its counterpart of the other molecule within the dimer [21]. All these residues, except Leu32, Leu73 and Gln94 are also involved in substrate binding. Three variants were constructed to determine the importance of the diverse interactions for dimerization, i.e. Leu32 → Ala/Leu71 → Ala/Leu73 → Ala (Leu variant), Phe109 → Met and Gln94 → Ala. All variants were expressed and folded *in vitro* with efficiencies similar to wild-type OMPLA. None of the variants were affected in affinity for the standard assay substrate C_{16}thioglycol*P*Cho (data not shown). The results of our covalent OMPLA dimer demonstrated that a properly positioned dimer always displays high calcium affinity. Hence, calcium affinity can be used as a sensitive probe for proper dimerization. It is noteworthy that all mutations at the dimer interface are located at more than 15 Å from the catalytic calcium ion (Fig. 1B). In the standard assay, wild-type OMPLA has a calcium affinity of 12 μM with a maximum activity of around 80 U·mg^{-1}. For the Phe109 → Met variant and the Leu variant maximum activities were similar to wild-type OMPLA, whereas the Gln94 → Ala variant retained 25% of wild-type activity. For the Phe109 → Met variant, also calcium affinity was similar to wild-type OMPLA. A large decrease in calcium affinity was observed for both the Leu variant (7.3 mM ± 0.4) and the Gln94 → Ala variant (0.9 mM ± 0.15), emphasizing the importance of these residues for dimerization and formation of a proper catalytic calcium site in OMPLA. These results were confirmed by glutaraldehyde cross-linking experiments (Fig. 5) that revealed that only the Phe109 → Met variant is able to form a dimer in the presence of calcium.

To correct for the impaired dimerization of the Leu variant, we constructed a covalently bound Leu variant dimer containing an additional His26 → Cys mutation. This Leu variant dimer was resistant against dissociating forces such as detergent concentration similar to the covalent wild-type dimer. The calcium affinity of this Leu variant dimer was only modestly improved, being 1.2 mM (± 0.4), whereas maximum activity was comparable to wild-type OMPLA. This 300-fold lower calcium affinity compared to the wild-type OMPLA dimer demonstrates that for this impaired Leu variant a physical link is not sufficient for proper positioning of the OMPLA monomers to bind calcium in an optimal fashion.

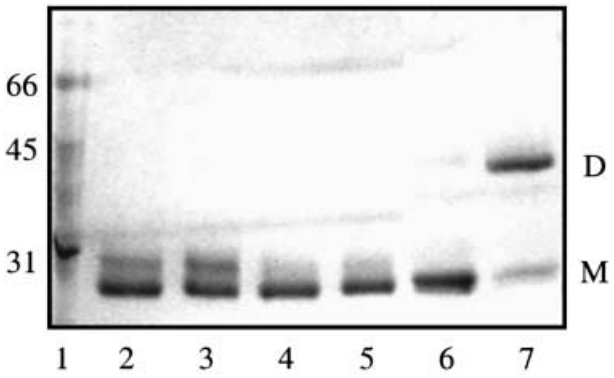

Fig. 5. Calcium-dependent glutaraldehyde cross-linking of dimer interface variants. Lane 1, molecular mass marker; lane 2 and 3, Leu32 → Ala/Leu71Ala/Leu73Ala variant; lane 4 and 5, Gln94Ala variant; lane 6 and 7, Phe109 → Met variant. The samples in even lanes were incubated at 2 mM EDTA before cross-linking, whereas the samples in odd lanes were incubated at 20 mM calcium before cross-linking.

DISCUSSION

Previous studies have shown that *in vitro*, OMPLA activity is controlled by reversible dimerization. Only in dimeric OMPLA high affinity calcium sites are formed that are essential for catalysis [22,23]. It has been shown that the affinity for calcium depends largely on the type of detergent used in the kinetic assay [20]. However, the relations between detergent, calcium binding and dimerization were poorly understood. Therefore, we have constructed a covalent OMPLA dimer using His26 → Cys OMPLA. In the structure of dimeric OMPLA His26 is located in a flexible region of the N-terminus. Thus a disulfide bond can be formed at more than 30 Å from the active center and catalytic calcium site without apparent distortion of the dimer interface. The covalent dimer displayed even higher activity than wild-type OMPLA. In contrast to wild-type OMPLA, activity of the covalent dimer in a micellar system was not affected by experimental conditions such as type and concentration of detergent used for solubilization of OMPLA. Interestingly, unlike wild-type OMPLA, the covalent OMPLA dimer displayed a high affinity for calcium (4 μM) regardless of detergent or substrate used in the kinetic assay, demonstrating that high affinity calcium binding is strictly correlated with correct dimerization. The absence of high affinity calcium binding of wild-type OMPLA in certain detergents can therefore be explained by dissociation of OMPLA dimers, because the catalytic calcium site is formed by residues from both monomers within the dimer. It was wondered whether this observed behaviour of OMPLA in micellar systems would be identical in a membrane environment.

To mimic the membrane environment, the dimer was reconstituted in a phospholipid bilayer. Because the OMPLA dimer *in vitro* invariantly has a high affinity for calcium, rapid degradation of the phospholipids was anticipated upon exposure of the vesicles to calcium. However, for both native OMPLA and the covalent OMPLA dimer only degradation of the phospholipid vesicles was observed after addition of detergent yielding a micellar system. This indicates that dimerization *per se* is not

sufficient for OMPLA activation in a bilayer environment but that perturbation of the bilayer is also essentially required to activate OMPLA. We propose that tight lipid packing in a bilayer may not allow proper formation of OMPLA dimers. These results agree well with the *in vivo* situation as *E. coli* cells always try to maintain a physical state of their lipids that is close to a bilayer–nonbilayer phase transition [24] in which OMPLA resides in an inactive state. OMPLA gets activated when this system becomes perturbed. Alternatively, the dimer is present in the vesicle with an optimal interface, but the protein cannot undergo dynamic transitions allowing access of substrate and/or calcium ions to the interface. This hypothesis, however, seems rather unlikely as in the crystal structure both the catalytic calcium site and the substrate binding pocket already seem easily accessible.

Our studies revealed that a proper OMPLA dimer displays high affinity for calcium. Hence, calcium binding can be used to monitor the capacity of OMPLA variants to dimerize properly. This is illustrated by the poor calcium affinity of OMPLA variants with an impaired dimeric interface, e.g. the Leu32 → Ala/Leu71 → Ala/Leu73 → Ala and Gln94 → Ala variants. These changes are introduced at a distance of at least 15 Å from the active centre and catalytic calcium site. Most likely, the reduced hydrophobicity of the triple Leu to Ala variant or the lack of an intermolecular hydrogen bond between residues 94 destabilize OMPLA dimers such that no high affinity calcium site can be formed.

Interestingly, the poor dimeric interface in the Leu variant can only partially be restored by introduction of a covalent linkage identical to the covalent wild-type dimer. These results emphasize that an intact dimeric interface is required for the formation of a high affinity calcium site.

While in this study the importance for proper dimerization of OMPLA has been clearly indicated, it is yet unclear why OMPLA dimers are not formed correctly in a phospholipid bilayer. Further studies will be needed on this aspect to fully understand activation of OMPLA *in vivo*.

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