Dimerization Regulates the Enzymatic Activity of Escherichia coli Outer Membrane Phospholipase A*

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The outer membrane phospholipase A (OMPLA) of Escherichia coli is present in a dormant state in the cell envelope. The enzyme is activated by various processes, which have in common that they perturb the outer membrane. Kinetic experiments, chemical cross-linking, and analytical ultracentrifugation were carried out with purified, detergent-solubilized OMPLA to understand the underlying mechanism that results in activation. Under conditions in which the enzyme displayed full activity, OMPLA was dimeric. High detergent concentrations or very dilute protein concentrations resulted in low specific activity of the enzyme, and under those conditions the enzyme was monomeric. The cofactor Ca²⁺ was required for dimerization. Covalent modification of the active site serine with hexadecylsulfonylfluoride resulted in stabilization of the dimeric form and a loss of the absolute calcium requirement for dimerization. The results of these experiments provide evidence for dimerization as the molecular mechanism by which the enzymatic activity of OMPLA is regulated. This dimerization probably plays a role in vivo as well. Data from chemical cross-linking on whole cells indicate that OMPLA is present in the outer membrane as a monomer and that activation of the enzyme induces dimerization concurrent with the appearance of enzymatic activity.

Outomembrane phospholipase A (OMPLA), also known as detergent-resistant phospholipase A or PldA protein, is one of the few enzymes present in the outer membrane of Gram-negative bacteria. OMPLA hydrolyzes the acyl ester bonds in (phospho)lipids and has Ca²⁺ as an essential cofactor (1, 2). Initially, the Escherichia coli enzyme was purified and characterized (3), and its structural gene, designated pldA, was subsequently cloned and overexpressed (4–7). Recently, Brok et al. (8) reported the cloning of the pldA genes of several other Enterobacteriaceae species. Comparison of the OMPLA amino acid sequences revealed a high degree of homology within this family, but no clear homology exists with sequences of water-soluble (phospholipases). A β-barrel structure has been proposed for OMPLA (8), analogous to the outer membrane porins, of which the x-ray structures have been solved (9–11). Recently, we succeeded in the overproduction, in vitro refolding, and subsequent purification of the enzyme on a large scale (12), which allowed its crystallization (13).

Although OMPLA is embedded in its own substrate in the cell envelope, no enzymatic activity can be detected in normally growing cells (14, 15). Because OMPLA is expressed constitutively, genetic regulation cannot explain the absence of enzymatic activity. Moreover, the expressed protein is correctly transported to and inserted into the outer membrane, where it resides in a dormant state. Activity is induced by various processes that perturb the membrane, such as phage-induced lysis (16), temperature shock (4), and colicin secretion (17, 18). Similar results have been reported in vitro after reconstitution of OMPLA in lipid vesicles (19). Membrane-perturbing peptides, such as polymyxin B, melittin, or cardiotoxin, activate the enzyme. In purified, detergent-solubilized OMPLA, the enzymatic activity depends strongly on the detergent concentration (2, 5).

The present study was undertaken to determine the molecular mechanism responsible for the activation of OMPLA. The results of enzymatic activity assays, chemical cross-linking, and analytical ultracentrifugation experiments suggest that OMPLA is active as a dimer. The effects of the detergent concentration, the cofactor calcium, the covalent modification of the active site, and the enzyme concentration on dimerization were studied in detail. Data from chemical cross-linking on whole cells indicate that OMPLA is present in the outer membrane as a monomer and that activation of the enzyme induces dimer formation.

EXPERIMENTAL PROCEDURES

Chemicals—n-Dodecyl-N,N-dimethyl-1-ammonio-3-propanesulfonate (12-SB) and glutaraldehyde were purchased from Fluka and octyl-polyoxyethylene glycol (octyl-POE) from Bachem. All other chemicals were of the highest purity commercially available.

Proteins—E. coli OMPLA was overproduced from the plasmid pP7.5 as inclusion bodies and folded in vitro and purified as described previously (12). Protein concentrations were determined spectrophotometrically using an A1%2.2cm value of 29.2 (20). OMPLA (1 mg/ml) was modified with 1 mole equivalent of hexadecylsulfonylfluoride (from a stock solution in acetonitrile) in buffer (2.5 mM 12-SB, 20 mM Tris-HCl, pH 8.3, 5 mM CaCl₂) for 16 h at room temperature (20). Subsequently, the protein solution was dialyzed twice against 100 volumes of buffer (2.5 mM 12-SB, 20 mM Tris-HCl, pH 8.3, 2 mM EDTA) and stored at −20 °C. Activity measurements revealed that 94% of the protein was modified. For analytical ultracentrifugation experiments, a sample of OMPLA was prepared in octyl-POE. To this end, 8 mg of OMPLA was loaded to a 1 ml fast-flow Q Sepharose column (Pharmacia), which was preequilibrated with buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM 12-SB). The column was washed with 25 ml of buffer (10 mM Tris-HCl, pH 8.3, 0.5% octyl-POE) and eluted with 10 ml of this buffer containing 1 M KCl. OMPLA was subsequently dialyzed twice at 4 °C against 20 volumes of 10 mM Tris-HCl, pH 8.3, containing 0.5% octyl-POE. In a similar manner, a sample of inhibited-OMPLA in octyl-POE was prepared.

Outer Membrane Phospholipase Assay—Outer membrane phospho-

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1 The abbreviations used are: 12-SB, n-dodecyl-N,N-dimethyl-1-ammonio-3-propanesulfonate; OMPLA, outer membrane phospholipase A; octyl-POE, octylpolyoxyethylene glycol; PAGE, polyacrylamide gel electrophoresis.
lipase activity was determined spectrophotometrically using 2-hexadecanoylthioethane-1-phosphocholine (21) as substrate (5). The assay buffer contained 50 mM Tris-HCl, pH 8.3, 5 mM CaCl₂, 0.1 mM dithio-bis(2-nitrobenzoic acid), 0.2 mM Triton X-100, and 0.25 mM substrate. Routinely, 50 ng of OMPLA was assayed and activities were calculated from the absorbance at 412 nm; the activities are given as μmol of substrate hydrolyzed per min and per mg protein (units/mg).

Preincubation of Enzyme in Detergent—OMPLA was diluted to a final concentration of 0.05 mg/ml in buffer (20 mM Tris-HCl, pH 8.3, 2 mM EDTA) containing various concentrations of detergent. 12-SB was used in the range of 1.25–22.75 mM, and octyl-POE was used in the range of 0.05–5% (v/v). The solutions were incubated for 16 h at room temperature, after which samples were assayed for enzymatic activity.

Chemical Cross-linking—OMPLA was incubated at 0.18 mg/ml in buffer (50 mM Hepes, pH 8.3, 100 mM KCl, containing either 20 mM CaCl₂ or 20 mM EDTA) in the presence of various concentrations of 12-SB (2–25 mM) in a total volume of 100 μl. After preincubation of the solutions for 16 h at room temperature, 5 μl of a 1% stock solution of glutaraldehyde in 2.5 mM 12-SB was added, and the reaction was allowed to proceed for 4 h at room temperature. Subsequently, 100 μl of gel loading buffer (0.1 mM Tris-HCl, pH 6.8, 3% SDS, 15.4% glycerol, 7.7% β-mercaptopethanol, and 0.008% bromphenol blue) was added, and 20 μl of this solution (corresponding to 1.8 mg OMPLA) was analyzed by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue for visualization of the protein bands. On a preparative scale, OMPLA was cross-linked at 3 mM 12-SB as described above, but after the incubation with glutaraldehyde, the cross-linking was stopped by the addition of ethanolamine to a final concentration of 50 μM, followed by extensive dialysis against buffer (20 mM Tris-HCl, pH 8.3, 2.5 mM 12-SB). Cross-linked OMPLA was purified by DEAE ion exchange chromatography similar to the purification procedure for the recombinant protein (12).

Analytical Ultracentrifugation—Sedimentation coefficients and protein molecular weights of the detergent-protein complexes were determined by velocity and equilibrium centrifugation experiments in a Beckman Optima XL-A analytical ultracentrifuge as described by Ludwig et al. (22). OMPLA (1 mg/ml) was dialyzed at room temperature for 16 h against buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.3, containing either 0.5, 1.5, or 5% octyl-POE and either 2 mM EDTA or 20 mM CaCl₂). Samples of inhibited OMPLA were prepared in an analogous manner. After dialysis, the protein concentration was determined spectrophotometrically, and dilutions were prepared in dialysate. Velocity measurements were carried out with concentrated protein samples (1–3 mg/ml), whereas sedimentation equilibrium measurements were done with more dilute samples (0.1 mg/ml). The partial specific volume of the protein was calculated (23) from the amino acid composition to be 0.73 ml/g. Sedimentation velocity experiments yielded the sedimentation coefficient according to the Svedberg equation, using a density of 1.006 g/cm³ for the solvent. Sedimentation equilibrium data were plotted as the logarithm of the concentration vs. the logarithm of the concentration squared. Molecular weights of the complexes were calculated with a computer program similar to that of Chernyak et al. (24) using a floating baseline algorithm.

Dilution Inactivation—A stock solution of OMPLA (5 mg/ml) was diluted in buffer (20 mM Tris-HCl, pH 8.3, 2 mM EDTA, 2.5 mM 12-SB). The solutions were incubated overnight at room temperature, and subsequently the enzymatic activities were measured. The total volume of enzyme preincubation solution added to the assay was kept below 20 μl to prevent interference with the activity measurement. At the high protein concentrations, 100 ng was assayed, whereas at the lower protein concentrations, down to 0.6 ng of OMPLA was used in the enzymatic assay.

In Vivo Cross-linking—The pldA mutant E. coli strain CE1348 (8) containing pldA plasmid pPB1 (8) was grown overnight at 37 °C in 100 ml of L-broth supplemented with 100 μg/ml ampicillin. Cells were collected by centrifugation and resuspended in ice-cold buffer (100 mM NaCl, 50 mM Hepes, pH 8.3, 20 mM CaCl₂) to an A₅₄₀ of 20. Enzymatic activities of whole cells and of cell lysates, obtained by sonication at 0 °C, were measured in the chromogenic assay on a double-beam spectrophotometer. The reference cuvette did not contain dithiobis(2-nitrobenzoic acid). Samples (50 μl aliquots) were added to the sample as well as to the reference cuvette to compensate for turbidity. To 100-μl aliquots of the whole cell suspension or the cell lysates 5 μl of a 10% formaldehyde solution in water was added, and the reaction was incubated for 2 h at room temperature. Thereafter, 100 μl of SDS-PAGE loading buffer was added and the samples were heated for 10 min at 60 °C. Of the resulting solution, 25 μl were analyzed by SDS-PAGE followed by Western blot analysis using a mouse polyclonal anti-OMPLA serum as described (8).

RESULTS

Dependence of the Enzymatic Activity on Detergent Concentration—After preincubation of OMPLA in 2.5 mM 12-SB, which is slightly above the critical micelle concentration (1.3 mM), the enzyme displays full activity in the enzymatic assay (Fig. 1A). At concentrations below the critical micelle concentration, the enzyme loses its activity, presumably due to precipitation upon losing bound detergent (19). With increasing concentrations of detergent present in the preincubation, the specific activity was significantly lowered. The dependence of enzymatic activity on the concentration of detergent is not a specific feature of the zwitterionic detergent 12-SB. Similar results were obtained with several other detergents, such as β-octyglycylpropanoic acid, dodecylphosphocholine, Triton X-100, several alkylsulfobetaines, and several alkylpolyoxyethylene glycols. As an example, the results obtained with octyl-POE are shown in Fig. 1B. Previously, the dependence of the enzymatic activity on the detergent concentration has been interpreted in terms of a slow irreversible conformational transition of the enzyme (5, 19). So far, this process has not been understood at the molecular level. In this study, we have investigated whether oligomerization is responsible for the observed differences.

Chemical Cross-linking of OMPLA in Micelles—OMPLA was preincubated with various concentrations of detergent (2–25 mM 12-SB) and subsequently incubated with glutaraldehyde. The products of the cross-linking reaction were analyzed by SDS-PAGE. After preincubation in 2 mM 12-SB and subsequent cross-linking, only high molecular weight aggregates were vis-

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2 N. Dekker, unpublished observations.
Dimerization of OMPLA

Inhibition of OMPLA with hexadecylsulfonylfluoride leads to the irreversible inactivation of the enzyme due to modification of the active center residue Ser-144 (20). The modified enzyme can be considered a mimic of the acyl enzyme that is thought to occur during catalysis. Therefore, it was of interest to investigate how this modification would influence the dimerization process. Interestingly, even without cross-linking, a considerable amount of the dimer was visible on the gel (Fig. 2A, lane 3). OMPLA that was preincubated at 2.5 or 3 mM 12-SB, was efficiently cross-linked into the dimer (Fig. 2B, lanes 4–9). After preincubation in 2.5 or 3.0 mM 12-SB, inhibited OMPLA was completely cross-linked into the dimer (Fig. 2B, lanes 4 and 5). At higher concentrations of 12-SB, inhibited OMPLA could be cross-linked as well (Fig. 2B, lanes 6–9). This finding is in sharp contrast to the results obtained for the unmodified enzyme (Fig. 2A). Apparently, the modification of the active site leads to stabilization of the dimer. The amounts of dimer formed after cross-linking at high detergent concentrations were considerably higher when compared to the amount of dimer already present in the sample of inhibited OMPLA before cross-linking.

Because Ca$^{2+}$ is an essential cofactor for the enzymatic hydrolysis of phospholipids by OMPLA (1, 2), we studied the role of this cofactor in dimerization. OMPLA was preincubated in 3 mM 12-SB in the presence of Ca$^{2+}$. After reaction with glutaraldehyde, the protein appeared to be cross-linked efficiently (Fig. 2C, lane 2). In the presence of EDTA, little cross-linking was observed (lane 3). In contrast, the inhibited OMPLA was efficiently cross-linked, independent of the presence of Ca$^{2+}$ or EDTA (Fig. 2C, lanes 4 and 5).

OMPLA (4 mg) was cross-linked on a preparative scale in 3 mM 12-SB, which resulted in the formation of over 80% dimer. The enzyme was purified from the reagents by DEAE cellulose ion exchange chromatography (yield: 3 mg). In this step, the residual amount of monomeric OMPLA present after cross-linking could not be completely separated from the covalent dimer, resulting in a 10% contamination of monomer in the dimer preparation (data not shown). After purification, the cross-linked OMPLA was still enzymatically active, albeit at a reduced level (50%). This observation opened the possibility of studying the enzymatic activity of this covalent dimer under conditions in which native OMPLA loses activity. The cross-linked dimer was preincubated at various concentrations of 12-SB. In contrast to native protein, the specific activity of cross-linked OMPLA was independent of the detergent concentration present during preincubation (Fig. 3).

**Dilution Inactivation**—The results of the chemical cross-linking experiments described above suggest that the dimeric state of OMPLA is the active form of the enzyme. To test the
stability of the dimer, dilutions of OMPLA to very low protein concentrations were prepared. These dilutions were preincubated to reach equilibrium, and subsequently the enzymatic activities were measured. At high protein concentrations in the preincubation, the specific activity remained constant, but below 1 μg/ml the specific activity was strongly reduced (Fig. 4), underscoring the assumption that the active state is an oligomeric form. The reduction in specific activity is 8-fold for the lowest enzyme concentration tested, indicating that the monomer of OMPLA has only low activity or no activity at all. Experiments at lower protein concentrations were not feasible due to limitations in sensitivity of the kinetic assay. The dilution inactivation data were fitted to a saturation curve. A dissociation constant of 3.4 nM was derived, assuming a simple equilibrium between an active dimer and an inactive monomer. The dilution inactivation experiment described above for the native enzyme was also carried out with the purified, cross-linked dimer (Fig. 4). Below 1 μg/ml, only a slight decrease in activity was observed, presumably due to the 10% monomer contamination in the dimer preparation. The fact that the dimeric enzyme was active over this whole protein concentration range demonstrates that the reduction in activity for the native enzyme is due to dissociation and not to nonspecific losses.

**Analytical Ultracentrifugation—**Analytical ultracentrifugation was applied to study the monomer-dimer equilibrium in further detail. For membrane proteins, the determination of the molecular weight by this technique is complicated by the presence of detergents and/or lipids, which are needed for solubilization of the protein. The determined molecular weight of the complex is the sum of the molecular weight of the protein and of the adhering detergent/lipid molecules. However, when a detergent is chosen with the same density as the buffer, the contribution of the detergent is canceled out. Octyl-POE has a density of near unity and has been successfully applied in the molecular weight determination of various membrane proteins (22, 26). Analytical ultracentrifugation experiments were carried out with OMPLA in the presence of octyl-POE at concentrations of 0.5, 1.5, and 5%, at which OMPLA has optimal, intermediate, or strongly reduced enzymatic activity, respectively (compare Fig. 1B). In sedimentation velocity experiments, OMPLA behaved as a homogenous complex at all octyl-POE concentrations. Sedimentation equilibrium experiments were carried out to determine the molecular weight of OMPLA in the protein-detergent complex, and the results are summarized in Table I. A molecular weight of 55,000 was derived at optimal detergent concentration in the presence of the cofactor Ca$^{2+}$. This value is intermediate between the calculated masses of the monomer (31,500 Da) and the dimer (63,000 Da) and indicates the existence of an equilibrium, with 86% of the protein in the dimeric form. At higher octyl-POE concentrations, the molecular weight decreased to values close to that of the monomer. In the presence of EDTA the observed molecular weight agrees within experimental error with that of the monomeric form, showing that Ca$^{2+}$ is essential for stabilization of the dimer of native OMPLA. The effect of the cova lent modification of the active site on dimerization was also studied in analytical ultracentrifugation experiments. The molecular weight of inhibited OMPLA corresponds to the dimeric state, irrespective of the detergent concentration (Table I). Whereas for native OMPLA the dimerization was absolutely dependent on calcium, the monomer-dimer equilibrium was only partly shifted toward the monomer in the absence of calcium in the case of the inhibited enzyme.

**Chemical Cross-linking of OMPLA in Vivo—**In normally growing cells, OMPLA is present in the outer membrane, but enzymatic activity is only detectable after perturbation of the membrane. We investigated whether or not activation in vivo can be correlated with dimerization. For these experiments, we used the expression plasmid pRB1, which carries the *pldA* gene encoding OMPLA under control of its own promoter (8). CE1348 cells with or without plasmid pRB1 were grown overnight and the enzymatic activity of OMPLA present in intact cells was measured. OMPLA activities of cells carrying the plasmid (5 milliunits/ml of cell culture) did not deviate significantly from that of CE1348 cells without plasmid. Possible explanations for this lack of activity are: (i) the substrate cannot reach the enzyme, (ii) the enzyme is not expressed, or (iii) the enzyme is in an inactive state. The first possibility is unlikely because the active site of the enzyme is supposedly present at the cell surface (8) and because the substrate is small enough to diffuse into the periplasmic space. The second option was tested by Western blotting, which showed that in cells carrying the plasmid, OMPLA was produced in a heat-modifiable, correctly folded form (data not shown). We conclude that the enzyme apparently resides in the outer membrane in a dormant state. After activation of OMPLA by sonication an activity of 110 milliunits/ml of the plasmid-containing cell culture was measured, whereas the activity of the CE1348 cells without plasmid remained low (5 milliunits/ml). After chemical cross-linking of OMPLA with formaldehyde in the nonactivated cells, the enzyme was detected on Western blots as the folded monomeric form at 27,000 and also partially as unfolded protein at 31,500 (Fig. 5, lane 1). The appearance of the unfolded form is presumably due to the 10-min incubation at 60 °C in loading buffer before SDS-PAGE needed to obtain clear gels.

**Table I**

| Protein | Octyl-POE MW (Ca$^{2+}$) | Octyl-POE MW (EDTA) |
|---------|--------------------------|---------------------|
| OMPLA   | 0.5                      | 55,000$^a$          |
|          | 1.5                      | 35,400$^a$          |
| OMPLA   | 5.0                      | 31,800$^a$          |
| Inhibited OMPLA | 0.5           | 61,300$^b$          | 47,000$^b$                  |
| Inhibited OMPLA | 1.5           | 69,600$^b$          | ND                           |
| Inhibited OMPLA | 5.0           | 69,000$^b$          | ND                           |

$^a$ 18,000 rpm.

$^b$ 14,000 rpm.
enzyme was transferred to a solution containing the detergent could account for this discrepancy. First, the enzyme was preincubated at low detergent concentrations, the results of the preincubation and chemical cross-linking identified the dimer of OMPLA.

Analytical ultracentrifugation experiments indicate that OMPLA is present in an inactive monomeric form in the outer membrane of resting cells and that the enzyme dimerizes upon activation.

**DISCUSSION**

In the present study, we have investigated whether the activity of OMPLA is regulated by oligomerization. The results of kinetic experiments, chemical cross-linking and analytical ultracentrifugation provide experimental support for a dimerization model in which the enzyme is in equilibrium between an inactive monomeric and an active dimeric state. The dimer of OMPLA was present at low detergent concentrations, whereas at high detergent concentrations the enzyme was monomeric. In the kinetic experiments, up to a 15-fold reduction in activity upon dimer dissociation was observed. This figure represents an upper limit for the activity of the monomeric species, and a more precise determination was hampered by sensitivity limitations of the assay at extremely low protein concentrations. Based on our observations, we cannot exclude the possibility that the monomer has some low enzymatic activity. However, the absence of enzymatic activity of OMPLA in both lipid vesicles (2) and in the outer membrane (14, 15) suggests that the monomer is inactive.

Analytical ultracentrifugation has the advantage that the ratio of dimers and monomers can be determined under equilibrium conditions. The dimeric form was present at low detergent concentrations, whereas at intermediate or high detergent concentrations, the monomer was predominant. The dimerization equilibrium was also studied by chemical cross-linking. The cross-linking proved to be very efficient, and the results were in full agreement with the data obtained in the analytical ultracentrifugation experiments. Analytical ultracentrifugation and chemical cross-linking identified the dimer of OMPLA only at low detergent concentrations, and preincubation of OMPLA under these conditions resulted in full activity. At intermediate detergent concentrations, the results of the preincubation experiments deviate from those obtained by the other techniques. Under those conditions, the preincubation experiments still gave considerable activities, suggesting that significant amounts of dimer were present. Several factors could account for this discrepancy. First, the enzyme was preincubated in 12-SB, but for the activity measurement, the enzyme was transferred to a solution containing the detergent Triton X-100. Second, the presence of substrate in the assay results in the formation of the acyl enzyme intermediate. Our results obtained with the inhibited OMPLA indicate a strong stabilization of the dimeric state for the acyl enzyme analog, suggesting that under assay conditions the monomer-dimer equilibrium could be shifted toward the dimer. Third, the presence of the cofactor calcium could also influence the monomer-dimer equilibrium. All of these possibilities could contribute to the overestimation of the amount of dimer.

Dilution inactivation of oligomeric enzymes is a process in which the protein concentration is decreased below the dissociation constant for oligomerization. OMPLA, being a membrane protein, is present in the micellar phase. We accomplished dilution inactivation by varying the protein/detergent ratio. This ratio varied in two ways. In the preincubation experiment, the protein concentration was kept constant, and increasing the detergent concentration resulted in dimer dissociation. In the dilution inactivation experiment, decreasing the protein concentration at a fixed detergent concentration resulted in dimer dissociation. Final proof for the dimer model came from our kinetic measurements with cross-linked OMPLA, which retained enzymatic activity. Changing the protein/detergent ratio in this case did not affect the enzymatic activity, confirming our dimerization model.

Oligomeric regulation of enzyme activity has been reported for many water-soluble enzymes (reviewed in Ref. 27). Dilution inactivation has been described for several of these enzymes (28–30). There is to our knowledge only one membrane enzyme for which oligomeric regulation has been described before, i.e., Ca$^{2+}$-ATPase (31, 32). This enzyme is regulated by dimerization, and its activity is dependent on both the detergent concentration and the protein concentration (31). Moreover, the dimerization was Ca$^{2+}$-dependent (32). OMPLA is the second example of a membrane protein for which dimerization is the mechanism of activity regulation.

Calcium is an essential cofactor of OMPLA. One can envisage two possible roles for calcium: a structural role and a functional one. For the water-soluble phospholipase A$_2$, the Ca$^{2+}$ ion plays a functional role in polarizing the carbonyl moiety of the scissile ester bond and in binding the phosphate group of the substrate (33, 34). Although OMPLA does not share sequence homology with water-soluble phospholipase A$_2$, calcium could play a similar role in the catalytic machinery of OMPLA. However, a clue for the role of calcium in OMPLA might come from the structure of several porins in which it has been reported that Ca$^{2+}$ is required to maintain the quaternary structure. In the crystal structure of the trimeric porin from Rhodobacter capsulatus, a shared Ca$^{2+}$-binding site is located at the subunit interface (10, 11), and Ca$^{2+}$ is also essential to maintain the trimeric structure of the R. sphaeroides porin (35). OMPLA has been proposed to be structurally related to the outer membrane porins, having a β-barrel topology in common (8). Therefore, we suggest that Ca$^{2+}$ plays a structural role in the stabilization of the dimeric state.

Previously, it has been suggested that conformational changes were responsible for the activation of OMPLA (2, 5, 19, 20). The reactivity of the active site and the number of active sites per molecule have been determined as a function of the detergent concentration by the covalent modification of the active site serine with hexadecylsulfonylfluoride (20). The number of active sites was one per molecule of OMPLA, and this number was constant, irrespective of detergent concentration. The reactivity with the inhibitor was the highest near the critical micelle concentration, and the reactivity was strongly diminished at high detergent concentrations. The authors of that study concluded that the enzyme could adopt different
conformations and that there exists a slow reversible equilibrium between these states. In view of our current dimerization model, the reduced reactivity can be explained in terms of decreasing concentrations of dimer upon increasing the detergent concentration. The fact that even at high detergent concentrations all of the available enzyme can still be modified, albeit with reduced reactivity, suggests that the dimer that has reacted with the inhibitor is withdrawn from the monomer-dimer equilibrium. Apparently the association of monomers into the active dimer is slow compared to the chemical reaction with the inhibitor. Our results show that dimerization is an important factor in the activation process. Dimerization of the enzyme could induce conformational changes of the protein due to the formation of the subunit interface. Such a conformational change might then lead to the formation and/or optimization of the active site. Besides conformational changes, dimerization could induce activation by the formation of shared active sites by complementarity, or as the case for several other oligomeric enzymes, such as citrate synthetase (36), glutamine synthetase (37), aspartate aminotransferase (38), triose phosphate isomerase (39), and human immunodeficiency virus protease (40). At this moment we do not have experimental support in favor of shared active sites. It is hoped that the x-ray analysis will provide insight into the mechanism by which the activity in the dimer is induced. In this respect, it is noteworthy that OMPLA crystallizes in space group P3\(_2\)1 (13) with one molecule per asymmetric unit. The presence of a crystallographic two-fold symmetry axis in this space group would be consistent with a dimer being the building block of the crystal.\(^3\)

\(^3\) B. W. Dijkstra, personal communication.

In vivo, OMPLA is present in the outer membrane in a dormant state. The results of the cross-linking experiments with nonactivated cells showed that OMPLA is monomeric under these conditions. The absence of efficient cross-linking is a negative result, but it is probably not an artifact of the technique that was used. Cross-linking with formaldehyde has been applied successfully for protein complexes in the outer membrane and in the periplasm of whole cells (41, 42). OMPLA can be activated by various treatments that have in common the fact that they perturb the membrane and finally lead to cell lysis. OMPLA was activated by sonication, which is a crude method resulting in the breakage of the cells. After this step, the enzymatic activity was easily detectable, and the dimeric enzymatic activity in the dimer is induced. In this respect, it is noteworthy that OMPLA crystallizes in space group P3\(_2\)1 (13) with one molecule per asymmetric unit. The presence of a crystallographic two-fold symmetry axis in this space group would be consistent with a dimer being the building block of the crystal.\(^3\)

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