Role of the Cofactor Calcium in the Activation of Outer Membrane Phospholipase A†

Iban Ubarretxena-Belandia,‡ Jan-Willem P. Boots,§ Hubertus M. Verheij,‡ and Niek Dekker*,‡

Department of Enzymology and Protein Engineering and Department of Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, The Netherlands

Received June 16, 1998; Revised Manuscript Received August 7, 1998

ABSTRACT: The enzymatic activity of the outer membrane phospholipase A (OMPLA), an integral membrane protein of *Escherichia coli*, is regulated by dimerization for which the cofactor Ca^{2+} is required. In this study, the interaction of Ca^{2+} with OMPLA was characterized, with an emphasis on the role of the cofactor in the activation process and dimerization. Kinetic experiments were done in which the enzyme was solubilized in mixed micelles of substrate and different detergents. It appeared that the affinity of OMPLA for Ca^{2+} was high (12 μM) if alkylphosphocholines were used as detergent, moderate (62 μM) if sulfobetaines were used, and very low (24 mM) if alkylpolyoxyethylene glycols were used. These results show that there is a strong modulation of the calcium binding properties of OMPLA by the lipid environment. In the presence of hexadecylphosphocholine micelles, the affinity of OMPLA for Ca^{2+} was determined by three direct binding techniques. Using gel filtration, it appeared that OMPLA has one high-affinity site ($K_d \approx 36$ μM) and a second site with moderate affinity ($K_d \approx 358$ μM). Sulfonylated-OMPLA, in which the active site serine had been covalently modified with hexadecanesulfonylfluoride, was used as a mimic for the acyl-enzyme intermediate. In gel filtration experiments, this sulfonylated-OMPLA displayed binding of two Ca^{2+} per enzyme monomer both with similar high affinity ($K_d \approx 48$ μM), indicative of a strong synergistic effect of active site occupation and the affinity of the second Ca^{2+} binding site. Isothermal titration calorimetric measurements confirmed only the presence of a high-affinity Ca^{2+} binding site, whereas in fluorescence experiments only the binding of the second Ca^{2+} could be observed. Chemical cross-linking was applied to investigate which of the two Ca^{2+} sites is involved in dimerization. OMPLA was monomeric in the absence of Ca^{2+}, whereas already at low Ca^{2+} concentrations the enzyme was converted to its dimeric form. Therefore, we suggest that the first Ca^{2+} plays a role in the stabilization of the dimeric state of the enzyme. The role of the second Ca^{2+} and the observed synergy between active site occupancy and Ca^{2+} affinity are discussed.

The outer membrane phospholipase A (OMPLA; EC 3.1.1.32)[1] is an integral membrane enzyme present in the outer membrane of Gram-negative bacteria (*1*). OMPLA (31 kDa) catalyzes the hydrolysis of acylester bonds in phospholipids, and for enzymatic catalysis, the presence of the cofactor Ca^{2+} is an essential requirement (*2*, *3*). The enzyme from *Escherichia coli* has been overexpressed, purified, and partially characterized (*4*−*8*). Ser144 has been identified as the nucleophile by chemical modification (*4*) and site-directed mutagenesis (*5*). Furthermore, residues Ser152 and His142 were identified as essential residues for activity by site-directed mutagenesis (*5*, *6*). For OMPLA, a β-barrel topology similar to the porins has been proposed (*9*). Recently, this prediction has been supported by an epitope insertion study for the homologous enzyme from *Salmonella typhimurium* (*8*).

In the cell, OMPLA is constitutively expressed and is located in the outer membrane. The outer membrane is composed of phospholipids in the inner leaflet and of lipopolysaccharides in the outer leaflet (*10*). Moreover, calcium is normally abundantly present in the growth medium, and the outer membrane is permeable for calcium ions. The simultaneous presence of substrate (phospholipids), cofactor, and enzyme suggests that OMPLA would degrade the membrane lipids. However, under physiological conditions, no membrane phospholipid turnover is detected (*11*−*13*), suggesting that OMPLA normally resides in a catalytically inactive state in the outer membrane. OMPLA activity can be induced after severe perturbation of the cell envelope integrity that occurs during heat shock (*14*), phage-induced lysis (*15*), or colicin secretion (*16*, *17*). Uncontrolled breakdown of the outer membrane would have lethal

† I.U.B. was supported by a grant from the Basque government (Ikertzaileak Prestatzeko Bekak).

* To whom correspondence should be addressed at the Department of Enzymology and Protein Engineering, CBLE, Utrecht University, Padualaan 8, P.O. Box 80054, NL-3508 TB Utrecht, The Netherlands. Phone: +31-30-2532458. Fax: +31-30-2522478. E-mail: n.dekker@chem.uu.nl.

‡ Department of Enzymology and Protein Engineering.

§ Department of Biochemistry of Membranes.

[1] Abbreviations: OMPLA, outer membrane phospholipase A; C_{12}SB, dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate; C_{16}SB, hexadecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate; C_{12}PN, dodecylphosphocholine; C_{16}PN, hexadecylphosphocholine; C_8E_5, octylpentaethylene glycol ether; $C_{12}E_5$, dodecylpentaethylene glycol ether; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ITC, isothermal titration calorimetry; CMC, critical micelle concentration.

10.1021/bi9814181 CCC: $15.00
Published on Web 10/17/1998

consequences for the cell, as indeed is observed when the activity of OMPLA is strongly induced in vivo (*17*). The potential hazard of OMPLA for the integrity of the cell implies the need of a regulatory mechanism for OMPLA activity. In vitro studies with detergent-solubilized protein showed that dimerization triggers the enzymatic activity of OMPLA, and that the monomer/dimer equilibrium is critically dependent on the presence of Ca^{2+} (*18*). This observation led the authors to suggest that dimerization is the process by which the enzymatic activity of OMPLA is regulated in vivo.

In view of the crucial role of Ca^{2+} for activity and dimerization, we have studied the binding of Ca^{2+} to OMPLA with a variety of biophysical techniques. The influence of detergents on this interaction was studied as well. Our results enabled us to construct a model describing the formation of the enzyme−Ca^{2+}−substrate complex.

EXPERIMENTAL PROCEDURES

Chemicals. Restriction enzymes and DNA modifying enzymes were from New England Biolabs. Oligonucleotides were purchased from Pharmacia. Research grade dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate (C_{12}SB) was obtained from Fluka, and was purified by loading a concentrated solution of the detergent in methanol/chloroform (1:1) to an alumina column to remove acidic impurities present in the commercial preparation. The solvent was evaporated under vacuum, and the dried white powder was stored at room temperature. Hexadecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate (C_{16}SB) was purchased from Calbiochem. Dodecylphosphocholine (C_{12}PN) and hexadecylphosphocholine (C_{16}PN) were synthesized as described by van Dam-Mieras et al. (*19*). Research grade octylpentaethylene glycol ether (C_8E_5) and dodecylpentaethylene glycol ether ($C_{12}E_5$) were purchased from Fluka and used without further purification. The substrate 2-hexadecanoylthioethane-1-phosphocholine, for the OMPLA assay, was synthesized according to Aarsman et al. (*20*). Hexadecanesulfonylfluoride was synthesized as described by Horrevoets et al. (*4*). Radioactive $^{45}CaCl_2$ with a specific activity of 710 μC/mmol was obtained from Amersham. All other chemicals were of the highest purity commercially available.

Construction of the Expression Plasmid, Overproduction and Purification of OMPLA. A synthetic DNA linker consisting of two complementary oligonucleotides (5′-TATGGGAGCTCTGATCAG-3′ and 5′-AATTCTGATCAGAGCTCCCA-3′) was cloned into the *Nde*I and *Eco*RI sites of the expression vector pT7.7 (*21*). The resulting vector (pT7.71) was digested at the newly introduced unique restriction site with *Ecl*136II and *Bcl*I. The vector ppL302 (*22*) was digested with *Sph*I, and the 3′ overhang was removed by treatment with Klenow polymerase. Digestion of this DNA fragment with *Bgl*II gave the 830 bp fragment containing the *pldA* gene, coding for OMPLA. This fragment was cloned into the *Ecl*136II/*Bcl*I-digested expression vector pT7.71. The resulting construct, pN300, encodes mature OMPLA with an N-terminal extension of three amino acid residues (MGA) under control of the T7 promotor. The DNA sequence of this final construct was verified by dideoxy chain termination sequencing.

OMPLA was overproduced in strain BL21(DE3) containing the plasmid pN300 after induction with IPTG. Subsequent isolation of inclusion bodies, folding, and purification were carried out essentially as described by Dekker et al. (*22*). Unless indicated otherwise, OMPLA was diluted to the desired protein concentration in buffer (50 mM Tris/HCl, pH 8.3, 100 mM KCl) in the presence of detergent and incubated for 1 h at room temperature prior to any experiment. To exchange detergent, the protein in buffer with C_{12}SB was loaded onto a fast-flow Q-Sepharose column (8 mg of protein/mL of resin) equilibrated in buffer (20 mM Tris/HCl, pH 9.0). After washing the column with buffer containing the new detergent, the protein was eluted in the presence of the new detergent by high salt. Subsequently, the protein was loaded onto a Sephadex G-25 fine grade column (80 × 1.5 cm) in buffer (50 mM Tris/HCl, pH 8.3, 100 mM KCl) in the presence of the detergent of choice to desalt and to remove low molecular weight contaminants. Protein concentrations were determined spectrophotometrically using an $A^{1\%}_{280\ nm}$ of 29.2 (*4*). Sulfonylated enzyme was prepared by modification of OMPLA (1 mg/mL of OMPLA in 20 mM Tris/HCl, pH 8.3, 5 mM $CaCl_2$, 2.5 mM C_{12}SB) with 1 mol equiv of hexadecanesulfonylfluoride (from a stock solution in acetonitrile) in buffer for 1 h at room temperature (*4*).

CMC Measurements. The critical micelle concentration (CMC) of the detergents was measured fluorometrically using ANS (1-anilinonaphthalene-8-sulfonic acid) (*23*). In brief, a solution composed of 0.28 mM ANS in buffer (50 mM Tris/HCl, pH 8.3, 100 mM KCl) was titrated with a detergent solution. The change in ANS fluorescence intensity upon incorporation of the probe in the micelle was monitored at 480 nm under excitation at 380 nm.

OMPLA Assay. Routinely OMPLA activities were determined spectrophotometrically using 2-hexadecanoylthioethane-1-phosphocholine as a substrate. An aliquot of incubated protein sample was assayed for enzymatic activity in 1 mL of assay buffer [50 mM Tris/HCl, pH 8.3, 5 mM $CaCl_2$, 0.1 mM dithiobis(2-nitrobenzoic acid), 0.2 mM Triton X-100, 0.25 mM substrate]. This assay is referred to as the standard assay. Initial velocities were calculated from the recorded increase in absorbance at 412 nm. A unit corresponds to the conversion of 1 μmol of substrate/min. The kinetic apparent calcium dissociation constants in different detergents were determined essentially as described above with the following modifications; the assay buffer contained 10 μM EDTA, and Triton X-100 was replaced by the desired detergent. The enzymatic activities were measured at various $CaCl_2$ concentrations (added from a concentrated stock solution in water). The binding parameters were determined by nonlinear regression fitting.

Fluorescence. Fluorescence spectra were obtained with a Perkin-Elmer LS-5 spectrofluorometer at 18 °C. Excitation and emission slit widths were 5 nm. The protein sample (2.5 mL of 0.05 mg/mL) was incubated for 1 h in sample buffer (50 mM Tris/HCl, pH 8.3, 100 mM KCl) in the presence of the desired detergent. In a typical experiment, the decrease in fluorescence intensity, caused by the addition of Ca^{2+} to the protein sample, was recorded with excitation and emission wavelengths of 280 and 335 nm, respectively. The fluorescence intensity was corrected for the sample dilution, and the binding parameters were determined by nonlinear regression fitting.

Isothermal Titration Calorimetry. The thermodynamic parameters for the binding of Ca^{2+} to OMPLA were determined by isothermal titration calorimetry (ITC). ITC measurements were performed using a Microcal MCS titration calorimeter (*24*). In the calorimeter cell, 1.3 mL of protein solution was present (74 μM OMPLA, 1 mM C_{16}PN, 50 mM Tris/HCl, pH 8.3, 100 mM KCl). The titration was performed by injecting 5 μL portions of 2.5 mM $CaCl_2$ in buffer (1 mM C_{16}PN, 50 mM Tris/HCl, pH 8.3, 100 mM KCl). The data for the titration of Ca^{2+} to the protein were corrected by subtraction of the base line obtained for the titration of Ca^{2+} to buffer in the absence of protein. The binding enthalpies, the apparent dissociation constants, and the number of bound Ca^{2+} were calculated by using the computer program Origin (Microcal, Northampton, MA).

Equilibrium Gel Filtration. Measurements of Ca^{2+} binding to OMPLA were performed according to a modification of the gel filtration technique described by Hummel and Dreyer (*25*). A column (46 × 1.1 cm) of Sephadex G-25 fine grade was equilibrated with buffer (50 mM Tris/HCl, pH 8.3, 100 mM KCl, 1 mM C_{16}PN) containing $^{45}CaCl_2$. The column was loaded with a 0.5 mL aliquot of protein (1−10 mg/mL) incubated in the column buffer and was eluted with the same buffer. Fractions of 200 μL were collected, and aliquots (typically 90 μL) were transferred to scintillation vials. Subsequently, 4 mL of scintillation liquid was added to each vial, and the radioactivity was determined in a Canberra Liquid Scintillation Counter. Protein concentrations were determined spectrophotometrically. The Ca^{2+} concentration in the column buffer was determined by spectrophotometric titration with EDTA and murexide in alkaline solution (*26*). Samples (1 mL) containing at least 50 nmol of Ca^{2+} were titrated in 40 mM NaOH with a 1 mM EDTA solution in the presence of 60 μg of murexide, and the spectral change was monitored at 612 nm. The obtained data were analyzed according to Scatchard (*27*).

Chemical Cross-Linking. Protein was incubated at 0.2 mg/mL in buffer (50 mM HEPES, pH 8.3, 100 mM KCl) in the presence of detergent and in the presence of either EDTA or $CaCl_2$ at various concentrations in a total volume of 100 μL. After incubation of the solutions for 1 h at room temperature, 5 μL of a 0.2% solution of glutaraldehyde was added, and the reaction was allowed to proceed for 1 h 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% β-mercaptoethanol, and 0.008% bromphenol blue) was added. A 20 μL aliquot of this solution (corresponding to 2 μg of OMPLA) was analyzed by SDS−PAGE without heat denaturation of the protein. The gels were stained with Coomassie Brilliant Blue for visualization of the protein bands. The amount of protein in each band was determined by gel scanning with a Biorad GS-700 imaging densitometer, and the data were analyzed using the computer program Molecular Analyst software (Biorad).

RESULTS

Detergent Dependence of Enzymatic Activity. The enzymatic activity of detergent-solubilized OMPLA is strongly dependent on the detergent concentration (*3*, *18*, *28*). This dependence is related to the monomer/dimer equilibrium, and only under specific conditions is the enzyme in its active dimeric state (*18*). Since the optimum detergent concentration varies with the type of detergent, the concentration at which OMPLA displays maximum enzymatic activity had to be determined for each detergent. In Figure 1 the structures are shown of the detergents that were used in this study. The detergents can be divided into three different types according to their polar headgroup: the zwitterionic detergents C_nSB, the zwitterionic lysophospholipid analogues C_nPN, and the nonionic detergents C_nE_5. For each detergent type, we tested two compounds that differed in length of the hydrophobic chain. After incubating the protein at various detergent concentrations, an aliquot of the solution was assayed for enzymatic activity in the standard assay. In Figure 2 the activity profiles for the detergents with a short alkyl chain are shown (top panel). After incubation in C_{12}SB, the enzymatic activity showed a strong dependence on the detergent concentration, as was reported before by Dekker et al. (*18*). Similar activity profiles were obtained for C_{12}PN and C_8E_5 in which maximal activity was also displayed at detergent concentrations just above the CMC. The results obtained with long-chain detergents are shown in the bottom panel of Figure 2. For the long-chain detergents, concentrations higher than 20 times the CMC were used to ensure sufficient micelles to dissolve the enzyme. For C_{16}SB, the curve followed a similar trend as for C_{12}SB, but the optimal detergent concentration was shifted to a maximum in activity at about 50 times the CMC. Interestingly, both in C_{16}PN and in C_8E_5, the enzyme displayed maximal activity at about 40 times the CMC followed by a plateau where the enzymatic activity remained high all over the entire detergent concentration range. Apparently, in these latter two detergents, the active dimeric form of OMPLA does not dissociate at high detergent concentrations.

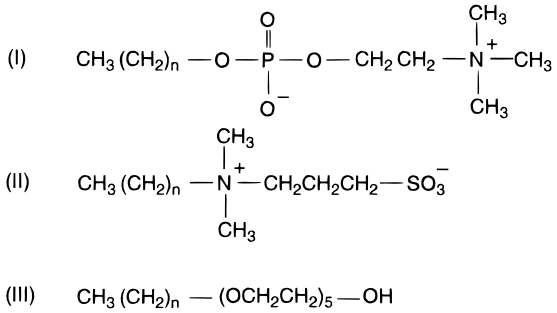

FIGURE 1: Structures of the detergents used in this study. (I) Alkylphosphocholine (C_nPN); (II) alkyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate (C_nSB); (III) alkylpentaethylene glycol ether (C_nE_5).

Detergent Dependence of the Ca^{2+} Affinity in the Kinetic Assay. In the previous experiments, the enzyme was incubated in the detergent of interest, and then the activity was determined under standard assay conditions. The fraction of active dimeric OMPLA present in the incubation mixture remained unaltered by the transfer and dilution into the assay mixture (*18*). Under kinetic conditions, no association or dissociation of OMPLA occurred as evidenced by the straight lines obtained. Given the very different behavior of OMPLA in the various detergents, it was of interest to test the activity of the enzyme toward mixed micelles of the various detergents and substrate as a function

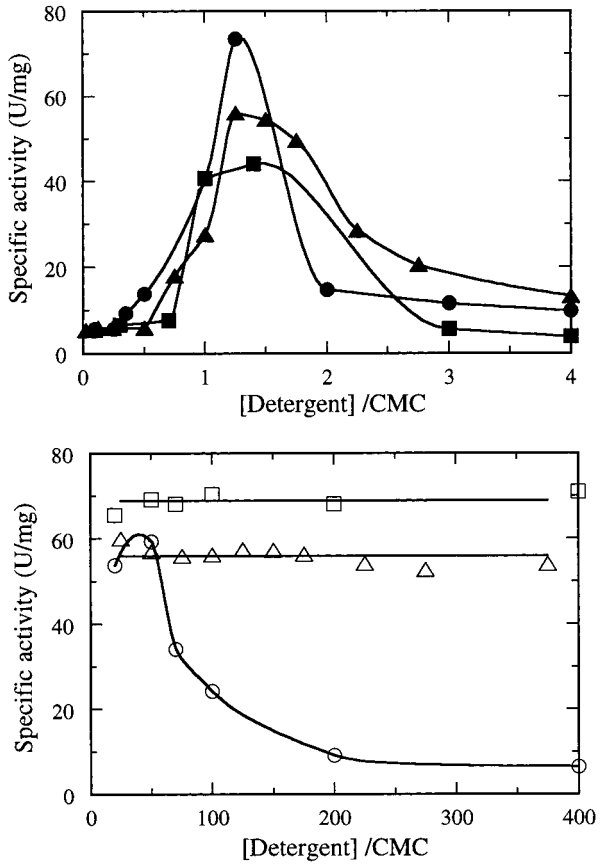

FIGURE 2: Specific activity of OMPLA as a function of the detergent concentration present in the incubation solution. OMPLA was incubated at 0.05 mg/mL in buffer containing detergent at various concentrations. After 1 h incubation, 50 ng of protein was assayed for enzymatic activity in the standard chromogenic assay. In the top panel, the data for the short alkyl chain detergents are shown: $C_{12}SB$ (●), $C_{12}PN$ (■), C_8E_5 (▲). In the bottom panel, the data obtained for the long alkyl chain detergents are shown: $C_{16}SB$ (○), $C_{16}PN$ (□), $C_{12}E_5$ (△). The detergent concentrations are expressed as the ratio of detergent concentration over its critical micelle concentration (CMC). The experimentally determined CMC values are given in Table 1.

of the Ca^{2+} concentration. OMPLA was incubated for 1 h in the detergent of interest. Then aliquots of the enzyme were added to the assay containing a fixed concentration of substrate (0.25 mM) and of detergent under study. The concentration of the detergents in the assay was equal to the concentration at which OMPLA showed optimal activity (see Figure 2), and they are listed in Table 1. Without calcium ions, OMPLA was not active in any of the detergents. When the enzymatic activity was measured as a function of the Ca^{2+} concentration, the experimental data followed a hyperbolic saturation profile. The maximum hydrolysis rate (V_{max}) and the kinetic dissociation constants (K_d) were obtained by fitting the data to a saturation curve using nonlinear regression and are summarized in Table 1. In all detergents, the enzyme is active with only little variation in V_{max}, indicating that the enzyme was in its fully active state under saturating conditions. However, depending on the detergent, large variations were observed in the affinity for Ca^{2+}. In the presence of the product analogues $C_{16}PN$ and $C_{12}PN$, the affinity for Ca^{2+} was high. In the other zwitterionic detergents $C_{16}SB$ and $C_{12}SB$ which have a "reversed" charge of the headgroup (Figure 1), the affinity

Table 1: Ca^{2+} Binding Parameters for OMPLA in Various Detergents[a]

			kinetics		
detergent	CMC[b] (mM)	[detergent][c] (mM)	V_{max} (units/mg)	$K_d(Ca^{2+})$ (mM)	fluorescence $K_d(Ca^{2+})$ (mM)
$C_{16}PN$	0.010	1.0	30	0.012	0.42
$C_{12}PN$	0.70	1.0	37	0.015	4.7
$C_{16}SB$	0.010	0.50	62	0.062	7.8
$C_{12}SB$	1.3	2.5	40	0.170	2.6
$C_{12}E_5$	0.080	2.0	55	24	5.7
C_8E_5	8.0	10.0	26	25	5.1

[a] OMPLA was incubated at 0.05 mg/mL in buffer containing detergent at a concentration where maximum enzymatic activity was displayed. After 1 h incubation the Ca^{2+} binding was determined kinetically and fluorometrically. The binding parameters have a 10% error. [b] The CMC for each detergent was determined as described under Experimental Procedures. [c] Detergent concentration at which OMPLA displayed maximum activity (see Figure 2).

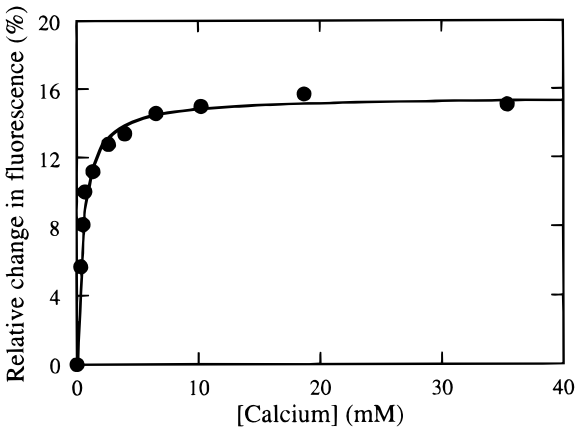

FIGURE 3: Relative change in the fluorescence of OMPLA as a function of the Ca^{2+} concentration. OMPLA was incubated in buffer at 0.05 mg/mL in 1 mM $C_{16}PN$ for 1 h. Subsequently, the change in fluorescence intensity, $(-\Delta F/F_0) \times 100$, as a function of added Ca^{2+} was monitored. The solid line represents the fitting of the data to a simple saturation model.

was about 10-fold lower. Finally, in the nonionic detergents C_8E_5 and $C_{12}E_5$, the Ca^{2+} affinity was decreased dramatically. Within the three classes of detergents, the differences observed in Ca^{2+} affinity between long- and short-chain detergents are small, indicating that only the polar head is responsible for the observed effects.

Detergent Dependence of the Ca^{2+} Affinity As Determined by Fluorescence. The binding of Ca^{2+} to OMPLA could also be followed by fluorescence. Excitation at 280 nm resulted in a fluorescence spectrum with maximum intensity at 335 nm. The intrinsic fluorescence decreased when Ca^{2+} was added, and returned to the initial value when EDTA was added in excess over Ca^{2+} (data not shown). These changes in fluorescence intensity, indicative of reversible conformational changes upon Ca^{2+} binding, were used to estimate Ca^{2+} binding parameters as a function of the detergent type. The protein was incubated at the detergent concentration at which the enzyme was fully active, and the Ca^{2+} affinity was determined by measuring the fluorescence as a function of the Ca^{2+} concentration. A typical curve obtained in $C_{16}PN$ is shown in Figure 3. The relative change in fluorescence intensity ranged from 10 to 16% in the different detergent systems. The apparent calcium dissocia-

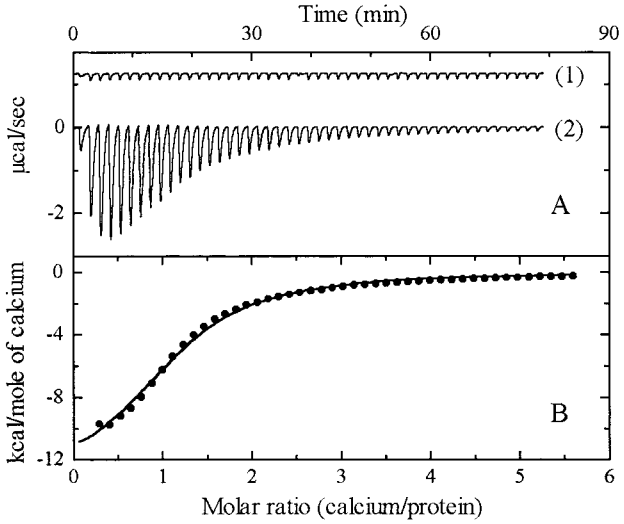

FIGURE 4: Isothermal titration calorimetry data for the binding of calcium to OMPLA. Panel A: Calorimetric response of 5 μL injections of 2.5 mM $CaCl_2$ to buffer (curve 1) and to 74 μM OMPLA (curve 2). Panel B: Integrated injection heats corrected for heats of calcium dilution. The solid line represents the fitting of the data to a simple noncooperative model.

tion constants obtained with the various detergents are summarized in Table 1. It is clear that in all the detergent systems except for C_{16}PN the affinity of OMPLA for Ca^{2+} is low, with dissociation constants in the millimolar range. It is remarkable that the Ca^{2+} affinities obtained by fluorescence are very different from the ones obtained by kinetics.

A major difference between the two systems is the presence of substrate in the kinetic assay. OMPLA, like all serine hydrolases, catalyzes ester hydrolysis via an acyl intermediate (*4*), and the Ca^{2+} binding properties of such an intermediate might well be different from those of the enzyme in the absence of substrate. Reaction of OMPLA with hexadecanesulfonylfluoride leads to the irreversible inactivation of the enzyme due to modification of the active center residue Ser144 (*4*). The modified enzyme (referred to as sulfonylated-OMPLA) can be considered as a good mimic of the acyl-enzyme. The only difference between the acyl-enzyme and the sulfonylated-OMPLA is the presence of the uncharged sulfonyl group instead of the fatty acid carbonyl group. Therefore, it is a good model to probe the Ca^{2+} binding properties of OMPLA during catalysis. Ca^{2+} binding experiments by fluorescence were performed with the sulfonylated-OMPLA under identical conditions as for the native-OMPLA. However, no significant change in the fluorescence signal could be observed upon the addition of Ca^{2+} in any of the detergents. The absence of any change in fluorescence could arise from alterations in the fluorescence properties of the enzyme, or the inability of the sulfonylated-OMPLA to bind Ca^{2+}. To investigate both possibilities, we measured Ca^{2+} binding to native- and sulfonylated-OMPLA by two other direct binding techniques.

Binding of Ca^{2+} to OMPLA As Measured by Isothermal Titration Calorimetry. We applied ITC to study the association of Ca^{2+} with native- and sulfonylated-OMPLA. To work at acceptable OMPLA concentrations, a reasonably high affinity for Ca^{2+} is needed, and, therefore, the experiments were performed in C_{16}PN. Figure 4 shows typical isothermal calorimetry titration curves obtained after addition

Table 2: Thermodynamic Parameters for the Binding of Ca^{2+} to OMPLA Determined by Isothermal Titration Calorimetry[a]

protein	K_d (μM)	n	ΔG_a (kcal·mol^{-1})	ΔH_a (kcal·mol^{-1})	ΔS_a (cal·mol^{-1}·K^{-1})
native	16	0.98	−6.6	−13.0	−21
sulfonylated	22	0.90	−6.5	−19.8	−44

[a] The experiments were performed at a temperature of 303 K with native- and sulfonylated-OMPLA in C_{16}PN. The n value represents the number of bound Ca^{2+} ions per protein molecule. The thermodynamic parameters are given for the association reaction, denoted by the subscript a.

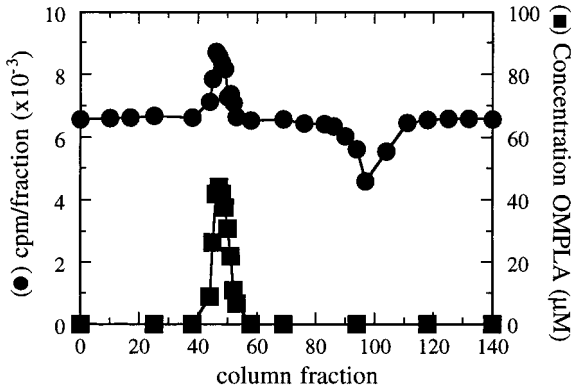

FIGURE 5: Elution profile of OMPLA in the presence of $^{45}Ca^{2+}$ on a G-25 Sephadex column. OMPLA, incubated at a concentration of 50 μM in 100 μM $^{45}Ca^{2+}$ buffer containing 1 mM C_{16}PN, was loaded onto a G-25 Sephadex fine column and was eluted in the same buffer. The counts per minute (cpm; ●) and the protein concentration (■) are plotted as a function of the fraction number.

of a $CaCl_2$ solution to buffer (panel A, curve 1), and to native-OMPLA (panel A, curve 2). Panel B shows the corrected integrated injection heats for the titration of Ca^{2+} to OMPLA. Analysis of these data yielded the dissociation constant (K_d), the number of Ca^{2+} bound per protein molecule (n), and the association enthalpy change (ΔH_a). The data for Ca^{2+} binding to native- and sulfonylated-OMPLA are listed in Table 2. ΔH_a was large and negative, whereas the entropy change upon Ca^{2+} binding is negative. Therefore, the binding of Ca^{2+} is an enthalpically driven process. The binding of Ca^{2+} to native-OMPLA is characterized by a high Ca^{2+} affinity and a stoichiometry of 1. The binding of Ca^{2+} to sulfonylated-OMPLA is indistinguishable from that to native-OMPLA.

Ca^{2+} Binding to OMPLA by Gel Filtration. Direct binding experiments were performed by gel filtration using $^{45}Ca^{2+}$ under equilibrium conditions, to obtain both the stoichiometry and the Ca^{2+} affinity of the OMPLA−Ca^{2+} complex for native- and sulfonylated-OMPLA in an independent way. For reasons of comparison, the experiments were carried out in the presence of 1 mM C_{16}PN. In Figure 5 a typical gel filtration experiment with native-OMPLA is shown. OMPLA eluted in the void volume, and at the same position, an increase in the Ca^{2+} concentration is observed indicative of complex formation. The experiment was repeated at various Ca^{2+} concentrations. From the "peak", areas the corresponding saturation levels (υCa) at each Ca^{2+} concentration were calculated, and the obtained data were analyzed according to Scatchard (*27*). As shown in Figure 6 the data for native OMPLA fit as two straight lines, suggesting the presence of two classes of binding sites with

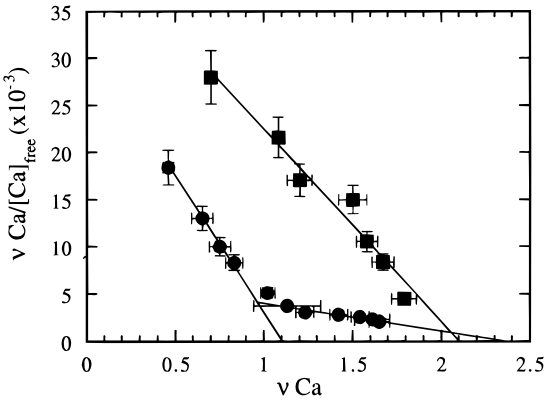

FIGURE 6: Scatchard plot for native- and sulfonylated-OMPLA. The ratio of the saturation (υCa) over free Ca^{2+} concentration is plotted over υCa for native- (●) and sulfonylated-OMPLA (■). The standard deviation of each value is indicated with error bars.

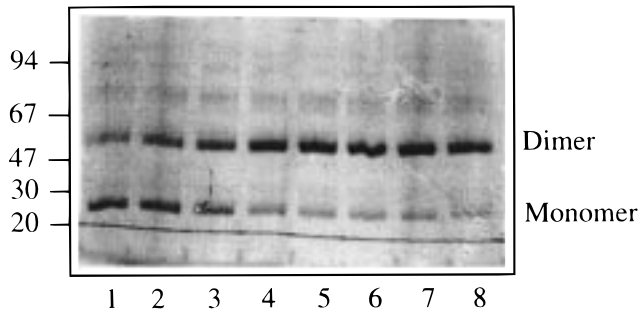

FIGURE 7: SDS−PAGE of OMPLA after cross-linking. The results for the cross-linking of OMPLA in 1 mM C_{16}PN at various Ca^{2+} concentrations are shown. Lane 1, 5 mM EDTA; and lanes 2−8, 25, 50, 100, 200, 400, 1000, and 5000 μM Ca^{2+}, respectively. The migration positions of monomeric and dimeric OMPLA are indicated.

different affinities. The extrapolation of the first linear part of the curve to the abscissa gives a value of 1.1 ± 0.05. It can be concluded that a high-affinity site exists with a corresponding dissociation constant for the OMPLA−Ca^{2+} complex of 36 ± 1 μM. Extrapolation of the second part of the curve to the abscissa gives a value of 2.4 ± 0.4, suggesting the presence of a second site with a dissociation constant of 358 ± 45 μM.

Similar experiments were performed with sulfonylated-OMPLA. As shown in Figure 6, the data for sulfonylated-OMPLA fit to a single straight line. Extrapolation to the abscissa gives a value of 2.1 ± 0.2 for the stoichiometry of the complex and a dissociation constant of 48 ± 4 μM. This observation shows that sulfonylated-OMPLA can bind two Ca^{2+} with comparable, high affinity. These results suggest that upon sulfonylation the affinity of the first Ca^{2+} site did not change, whereas the affinity for the second Ca^{2+} site is increased.

Cross-Linking. Previous studies have shown that Ca^{2+} is required for enzymatic activity and for dimerization of OMPLA (*3*, *18*). We now show that OMPLA binds two Ca^{2+} per protein molecule. To find out which site is involved in dimerization, the protein was incubated at various Ca^{2+} concentrations, and subsequently cross-linking reagent was added. The cross-linking was assessed by SDS−PAGE. After cross-linking in the presence of EDTA, the protein mainly runs as a monomer (Figure 7, lane 1). When Ca^{2+} was present in increasing concentrations, increasing amounts

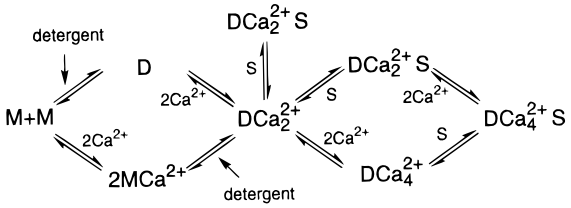

FIGURE 8: Model for the formation of the substrate−OMPLA−Ca^{2+} complex. The possible pathways leading to the formation of the active species are depicted. The OMPLA monomer is represented as M and the dimer as D. S represents the substrate, and the exact stoichiometry of the dimer/substrate complex is not known. Calcium binding also involves a dimerization step of OMPLA, which itself is strongly dependent on the applied conditions. The effect of detergent on Ca^{2+} binding is indicated in the model and explains the large variations in apparent K_d's.

of the protein could be cross-linked into the dimeric form of OMPLA (Figure 7, lanes 2−8). The amount of dimer reached its maximum already at about 100 μM Ca^{2+} (lane 4), suggesting that the Ca^{2+} dissociation constant associated with the dimerization process is well below 100 μM. Gel filtration showed that the binding of the two Ca^{2+} to native-OMPLA occurred with dissociation constants of 36 and 358 μM. It can be concluded that dimerization occurs in the Ca^{2+} concentration range where only the first Ca^{2+} site is saturated. The sulfonylated-OMPLA was efficiently cross-linked already in the absence of Ca^{2+} (data not shown), suggesting that sulfonylation stabilizes the dimeric form.

DISCUSSION

Previously, it has been shown that Ca^{2+} is essential for the enzymatic activity (*3*) and required for the dimerization of OMPLA (*18*). In the present paper, we have focused on the characterization of Ca^{2+} binding to OMPLA, with an emphasis on the role of Ca^{2+} in the activation and dimerization processes of the enzyme. Our kinetic experiments using zwitterionic detergents showed micromolar affinity for Ca^{2+} by OMPLA (Table 1), as observed by Horrevoets et al. in the standard kinetic assay containing Triton X-100 and substrate (*3*). Direct binding studies using ITC confirmed the high affinity of the enzyme for Ca^{2+} (Table 2). Under similar conditions, however, fluorescence showed low affinity for Ca^{2+} (Table 1). The observation that both with ITC and with fluorescence very different Ca^{2+} affinities were obtained, suggests the presence of two Ca^{2+} binding sites per OMPLA molecule. Gel filtration experiments confirmed this hypothesis (Figure 6). It is remarkable that both ITC and fluorescence alone were not sufficient to completely characterize the Ca^{2+} binding to OMPLA. Apparently, binding of the first Ca^{2+} does not affect the fluorescent properties of the protein. For ITC, it should be emphasized that besides the interaction of Ca^{2+} with its ligands in the binding site, many other processes are concurrent, for example, dimerization of the protein, possible conformational changes of the subunit, and redistribution of the surrounding detergents, and each of these processes may contribute to the net heat effect that is measured by ITC upon addition of Ca^{2+} to the protein.

The experimental data for Ca^{2+} binding to OMPLA are summarized in the model shown in Figure 8. The first step of this model is the formation of the calcium complex of the dimer (DCa_2^{2+}). Two possible routes leading to DCa_2^{2+}

can be envisaged; one in which dimerization precedes Ca^{2+} binding and the other in which the binding of Ca^{2+} occurs prior to dimerization. Although experimentally it has been shown that OMPLA is mainly monomeric in the absence of Ca^{2+}, still low amounts are present as dimer (*18*). Calcium could then function as a sink by which monomeric OMPLA is converted to the Ca^{2+}-loaded dimeric form. Alternatively, in the other route Ca^{2+} binds to monomeric OMPLA, after which the conformation of the subunit changes resulting in a dimerization-competent state. So far, we have not been able to experimentally attack the outlined possibilities. It is noteworthy that in either pathway dimerization and Ca^{2+} binding are interdependent processes. Therefore, any parameter affecting the monomer/dimer equilibrium will influence Ca^{2+} binding. This interdependence might explain the large variation in apparent calcium dissociation constants in different detergents obtained in the kinetic assay.

The second part of the model outlines the formation of the catalytic OMPLA−Ca^{2+}−substrate complex. The kinetic experiments suggest that saturation of the high-affinity site is sufficient for activity. If this is the case, the OMPLA−Ca^{2+}−substrate complex will be composed of the protein dimer with two Ca^{2+} plus substrate ($DCa_2^{2+}S$). However, the Ca^{2+} binding data for the sulfonylated-OMPLA, a mimic of the acyl-enzyme intermediate, obtained by gel filtration strongly indicate that both Ca^{2+} binding sites are occupied during catalysis. This observation favors an OMPLA−Ca^{2+}−substrate complex composed of the dimer protein with four Ca^{2+} plus substrate ($DCa_4^{2+}S$) as the most probable active species. The order of Ca^{2+} and substrate binding cannot be elucidated at this stage. In line with the observed effect of the active site occupancy on Ca^{2+} binding, the positive influence of the detergent $C_{16}PN$ on the binding affinity for the second calcium (Table 1) is noteworthy. This detergent can be considered as a substrate analogue, and, therefore, could have affinity for the active site. The interaction of $C_{16}PN$ with the active site might induce a synergistic effect on calcium binding analogous to that observed upon sulfonylation.

To address the question of the role of each Ca^{2+} in OMPLA, it is relevant to compare OMPLA with several other β-barrel outer membrane proteins that also bind calcium. Biochemically it has been shown that Ca^{2+} plays an essential role in the stabilization of the quaternary structure of the porins of *Rhodobacter sphaeroides* and *Vibrio cholerae* (*29*, *30*). In the absence of Ca^{2+}, these trimeric proteins dissociate into their subunits. In the X-ray structure of *Rhodobacter capsulatus* porin, Ca^{2+} is bound at the interface between subunits, providing a structural basis for the trimer stabilization by calcium (*31*). In view of the similarities in secondary and oligomeric structure between OMPLA and the porins, and our finding that the binding of the first Ca^{2+} by the enzyme is concurrent with dimerization, we propose that the first bound Ca^{2+} has a structural role in OMPLA acting as a molecular glue stabilizing the dimer.

The role of the second Ca^{2+} is discussed in terms of the model shown in Figure 9. Ca^{2+} could directly interact with the substrate (Figure 9: I, II) in a manner that is analogous to how Ca^{2+} interacts with the substrate in water-soluble phospholipases A_2. Several crystal structures of these phospholipases in complex with a transition-state analogue

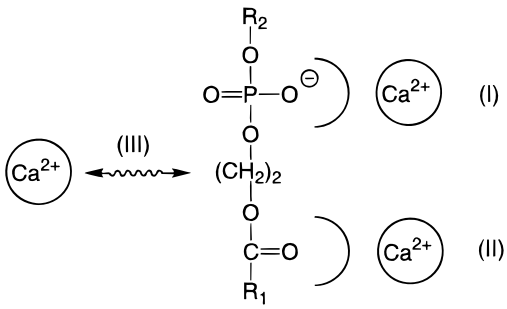

FIGURE 9: Schematic representation of the interaction of the second Ca^{2+} with OMPLA. (I) Direct interaction between Ca^{2+} and the phosphate group; (II) direct interaction between Ca^{2+} and carbonyl; (III) indirect interaction via conformational change between Ca^{2+} and OMPLA.

have been reported (*32*, *33*). Based on these structures, it has been suggested that the transition state is stabilized via direct interaction of Ca^{2+} with the phosphate group and the oxyanion. A possible direct interaction in OMPLA between Ca^{2+} and the phosphate group of the substrate (possibility I) was tested by comparison of two substrates: 2-hexadecanoylthioethane-1-phosphocholine and the related compound lacking the phosphocholine headgroup, hexadecanoylthioglycol. We argued that if the substrate interacts via the negatively charged phosphate with Ca^{2+}, then removal of this interaction would lead to a considerable change in the Ca^{2+} affinity. As it turned out, the Ca^{2+} dissociation constants obtained for both substrates were almost identical, suggesting that no interaction takes place with the phosphate (unpublished data). Calcium could also act as a ligand for the oxyanion that is formed during catalysis (possibility II). Alternatively, the Ca^{2+} binding site could be separated from the catalytic site, and still both sites could interact allosterically (possibility III). At this stage, we can rule out the first possibility, and we are left with two possible modes of interaction for the second Ca^{2+}.

What is the nature of the Ca^{2+} binding sites in OMPLA? Generally, to achieve micromolar affinity for Ca^{2+}, the binding site would contain at least one, but more likely two or even three negatively charged carboxylates. The primary structure of OMPLA has 15 glutamic acid and 16 aspartic acid residues. Based on sequence comparison of OMPLAs from *E. coli*, *Salmonella typhimurium*, *Klebsiela pneumonia*, *Proteus vulgaris* (*9*), *Pantoea agglomerans* (*7*), and *Campilobacter coli* (*34*), only five of these residues are absolutely conserved. These residues (Glu66, Glu111, Asp149, Asp184, and Asp251) are the likely candidates for being calcium ligands, and future structural and mutational work is needed to reveal their exact role.

ACKNOWLEDGMENT

We are greatly indebted to Mr. Ruud Cox for synthesizing the substrate and the hexadecanesulfonylfluoride inhibitor, and to Dr. Ton Aarsman for his help with the gel filtration experiments.

REFERENCES

1. Scandella, C. J., and Kornberg, A. (1971) *Biochemistry 10*, 4447−4456.
2. Tamori, Y., Nishijima, M., and Nojima, S. (1979) *J. Biochem. 86*, 1129−1138.

3. Horrevoets, A. J. G., Hackeng, T. M., Verheij, H. M., Dijkman, R., and de Haas, G. H. (1989) *Biochemistry 28*, 1139−1147.
4. Horrevoets, A. J. G., Verheij, H. M., and de Haas, G. H. (1991) *Eur. J. Biochem. 198*, 247−253.
5. Brok, R. G. P. M., Ubarretxena-Belandia, I., Dekker, N., Tommassen, J., and Verheij, H. M. (1996) *Biochemistry 35*, 7787−7793.
6. Brok, R. G. P. M., Dekker, N., Gerrits, N., Verheij, H. M., and Tommassen, J. (1995) *Eur. J. Biochem. 234*, 934−938.
7. Brok, R. (1995) Ph.D. Thesis, Utrecht University.
8. Merck, K. B., de Cock, H., Verheij, H. M., and Tommassen, J. (1997) *J. Bacteriol. 179*, 3443−3450.
9. Brok, R. G. P. M., Brinkman, E., van Boxtel, R., Bekkers, A. C. A. P. A., Verheij, H. M., and Tommassen, J. (1994) *J. Bacteriol. 176*, 861−870.
10. Lugtenberg, B., and van Alphen, L. (1983) *Biochim. Biophys. Acta 737*, 51−115.
11. Weiss, J., Beckerdite-Quagliata, S., and Elsbach, P. (1979) *J. Biol. Chem. 254*, 11010−11014.
12. Patriarca, P., Beckerdite, S., and Elsbach, P. (1972) *Biochim. Biophys. Acta 260*, 593−600.
13. Audet, A., Nantel, G., and Proulx, P. (1974) *Biochim. Biophys. Acta 348*, 334−343.
14. de Geus, P., van Die, I., Bergmans, H., Tommassen, J., and de Haas, G. H. (1983) *Mol. Gen. Genet. 190*, 150−155.
15. Cronan, J. E., and Wulff, D. L. (1969) *Virology 34*, 241−246.
16. Pugsley, A. P., and Schwartz, M. (1984) *EMBO J. 3*, 2393−2397.
17. Luirink, J., van der Sande, C., Tommassen, J., Veltkamp, E., de Graaf, F. K., and Oudega, B. (1986) *J. Gen. Microbiol. 132*, 825−834.
18. Dekker, N., Tommassen, J., Lustig, A., Rosenbusch, J. P., and Verheij, H. M. (1997) *J. Biol. Chem. 272*, 3179−3184.
19. van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., and de Haas, G. H. (1975) *Biochemistry 14*, 5387−5394.
20. Aarsman, A. J., van Deemen, L. L. M., and van den Bosch, H. (1976) *Bioorg. Chem. 5*, 241−253.
21. Tabor, S. (1990) *Expression Using the T7 RNA Polymerase/Promoter System. Current Protocols in Molecular Biology* (Ausuble, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.) Greene Publishing and Willey-Interscience, New York.
22. Dekker, N., Merck, K., Tommassen, J., and Verheij, H. M. (1995) *Eur. J. Biochem. 232*, 214−219.
23. Vendittis, E., de Palumbo, G., Parlato, G., and Bocchini, V. (1981) *Anal. Biochem. 115*, 278−286.
24. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem. 179*, 131−137.
25. Hummel, J. P., and Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530−532.
26. Scarpa, A. (1972) *Methods Enzynol. 24*, 343−351.
27. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
28. de Geus, P., Riegman, N. H., Horrevoets, A. J. G., Verheij, H. M., and de Haas, G. H. (1986) *Eur. J. Biochem. 161*, 163−169.
29. Weckesser, J., Zalman, L. S., and Nikaido, H. (1990) *J. Bacteriol. 159*, 199−205.
30. Chakrabarti, S. R., Chaudhuri, K., Sen, K., and Das, J. (1996) *J. Bacteriol. 178*, 524−530.
31. Weiss, M. S., and Schulz, G. E. (1992) *J. Mol. Biol. 227*, 493−509.
32. Scott, D. L., Otwinowski, Z., Gelb, M. H., and Sigler, P. B. (1990) *Science 250*, 1563−1566.
33. Scott, D. L., White, S. P., Browning, J. L., Rosa, J. J., Gelb, M. H., and Sigler, P. B. (1991) *Science 254*, 1007−1010.
34. Grant, K. A., Ubarretxena-Belandia, I., Dekker, N., Richardson, P. T., and Park, S. F. (1997) *Infect. Immun. 65*, 1172−1180.

BI9814181