Outer Membrane Phospholipase A Is Dimeric in Phospholipid Bilayers: A Cross-Linking and Fluorescence Resonance Energy Transfer Study†

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Received December 30, 1998; Revised Manuscript Received March 25, 1999

ABSTRACT: In the cell, the activity of outer membrane phospholipase A (OMPLA) is strictly regulated to prevent uncontrolled breakdown of the membrane lipids. Previously, it has been shown that the enzymatic activity is modulated by reversible dimerization. The current studies were carried out to define the oligomeric state of OMPLA in a membrane and to investigate the activation process. Three single-cysteine variant proteins H26C, H234C, and S144C were produced and purified to homogeneity. Using maleimido-based homo-bifunctional cross-linking reagents, H26C could be efficiently cross-linked as assessed by SDS−PAGE, whereas S144C and H234C could not be cross-linked. These data suggest that residue 26 is located close to the dimer symmetry axis. H26C was specifically labeled with 5-({[(2-iodoacetyl)amino]ethyl}-amino)naphthalene-1-sulfonic acid and *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine as the fluorescent energy donor and acceptor, respectively, and dimerization was investigated using fluorescence resonance energy transfer (FRET). Quenching of the donor in the presence of the acceptor demonstrated the dimeric nature of OMPLA, in agreement with cross-linking data. The observed FRET effect was dependent on the cofactor calcium, and the presence of substrate, indicating the specificity of the dimerization process. The labeled protein was reconstituted in phospholipid vesicles. In bilayers, OMPLA exhibited low activity and was dimeric as assessed by FRET. Addition of detergent resulted in a 70-fold increase in activity, while the protein remained dimeric. The results are discussed in terms of the activation of dimeric OMPLA due to changes in the physical state of the bilayer which occur upon perturbation of the membrane integrity.

The outer membrane phospholipase A (OMPLA,[1] EC 3.1.1.32) is an integral membrane protein present in Gram-negative bacteria (*1*−*4*). The enzyme catalyzes the hydrolysis of acyl ester bonds in phospholipids in a Ca^{2+}-dependent manner (*5*, *6*). Under physiological conditions, no membrane phospholipid turnover is detected (*7*−*9*), suggesting that OMPLA resides as a catalytically inactive species in the outer membrane. However, OMPLA activity can be triggered after severe perturbation of the cell envelope integrity which occurs during heat shock (*10*), phage-induced lysis (*11*), or colicin secretion (*12*, *13*). Uncontrolled breakdown of the outer membrane would have lethal consequences for the cell, as indeed is observed when the activity of OMPLA is strongly induced in vivo (*13*). The potential hazard of OMPLA activity for the integrity of the cell implies the need of a regulatory mechanism for activity. The regulation of OMPLA activity is not restricted to the *Escherichia coli* outer membrane, since the purified protein exhibits a similar behavior when reconstituted in lipid vesicles (*14*). In vitro experiments with OMPLA dissolved in detergent micelles have shown that the enzyme is active as a dimer, whereas the monomeric species is inactive (*15*). The monomer−dimer equilibrium was found to be modulated by the detergent and the cofactor calcium.

Here we address the question of whether a dimerization process is responsible for triggering OMPLA activity in membranes. Obviously, one would like to perform such a study with the enzyme in its natural environment, the outer membrane. However, such studies are severely hampered by the complexity of this membrane (*16*), and the practical difficulty of reconstituting its components (*17*). Alternatively, the activation of OMPLA can be studied in model membranes consisting of purified components (*14*). Assessment

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

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[1] Abbreviations: OMPLA, outer membrane phospholipase A; C_{12}-SB, dodecyl *N*,*N*′-dimethyl-1-ammonio-3-propanesulfonate; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; BMH, 1,6-bis(maleimido)hexane; IAEDANS, 5-({[(2-iodoacetyl)amino]-ethyl}amino)naphthalene-1-sulfonic acid; IANBD, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; AEDANS, *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonic acid; ANBD, *N*,*N*′-dimethyl-*N*-acetyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ethylenediamine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CMC, critical micelle concentration; R_0, Förster's distance; DOC, deoxycholate; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoic acid; PC, egg yolk phosphatidylcholine; DODAPC, dioleyldiamidophosphocholine; *E*, energy transfer efficiency.

10.1021/bi983077x CCC: $18.00
Published on Web 05/13/1999

of the dimerization of reconstituted OMPLA is complicated by the fact that the bilayer phospholipids are substrate for the enzyme, and consequently, prolonged exposure of the membrane lipids to OMPLA activity leads to extensive breakdown of the bilayer that is being studied. Therefore, a rapid method for monitoring the dimerization of the enzyme was needed. Our strategy was to use fluorescence resonance energy transfer (FRET).

Using the putative topology model for OMPLA (*2*), which has recently been supported by an epitope insertion study for the homologous enzyme from *Salmonella typhimurium* (*18*), three residues were selected as candidates for possible protein−protein interaction sites. Unique cysteine residues were introduced at these sites, one at a time, and sulfhydryl-directed chemical cross-linking was used to determine which of these residues are in close proximity in the homodimeric complex. The best candidate was specifically labeled with fluorescence probes, and OMPLA dimerization in detergent micelles and in lipid vesicles was investigated using FRET. The outcome of this study is discussed in terms of the regulation of OMPLA activity by the surrounding lipid phase.

EXPERIMENTAL PROCEDURES

Chemicals. Restriction enzymes and DNA-modifying enzymes were from New England Biolabs. Research grade dodecyl *N*,*N*′-dimethyl-1-ammonio-3-propanesulfonate (C_{12}-SB) was obtained from Fluka, and was purified as described previously (*19*). Phosphatidylcholine (PC) was extracted from chicken egg yolk and was purified to homogeneity according to standard procedures. Dioleyldiamidophosphocholine (DODAPC) was synthesized essentially as described by Dijkman et al. (*20*). The substrate for the OMPLA assay, 2-hexadecanoylthioethane-1-phosphocholine was synthesized according to the method of Aarsman et al. (*21*). The disulfide cross-linkers *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM) were purchased from Sigma. 1,6-Bismaleimidohexane (BMH) was purchased from Pierce. Fluorescent probes *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) and 5-({[(2-iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid (IAEDANS) were purchased from Molecular Probes. Acetylated trypsine was purchased from Sigma. All other chemicals were of the highest purity commercially available.

Overproduction and Purification of Cys Variants. The H26C, S142C, and H234C variants were constructed as described previously (*22*) and subcloned into the expression vector described by Dekker et al. (*19*). The resulting constructs encode the OMPLA variant with an amino-terminal extension of seven amino acid residues (MAR-IRAP), under control of the T7Φ10 promoter. The DNA sequence of this final construct was verified by dideoxy chain termination sequencing. The protein variants were overproduced after induction with isopropyl β-D-1-thiogalactopyranoside in *E. coli* strain BL21(DE3), transformed with the corresponding expression plasmid. Subsequent isolation of inclusion bodies, folding, and purification were carried out essentially as described previously (*19*). To avoid oxidation of the sulfhydryl group, all variants were kept under reducing conditions by the addition of 5 mM 1,4-dithiothreitol (DTT) to all buffers. To remove the DTT prior to any experiment, the protein was desalted on a G-25 Sephadex column (PD-10, Pharmacia) equilibrated and eluted with buffer A [20 mM Tris/HCl (pH 8.3) and 2.5 mM C_{12}SB]. Protein concentrations were determined spectrophotometrically using an $A_{280}^{1\%}$ of 29.2 (*23*).

Assays. Routinely, phospholipase activities were determined spectrophotometrically using 2-hexadecanoylthioethane-1-phosphocholine as a substrate. In brief, an aliquot of protein sample was assayed for enzymatic activity in 1 mL of assay buffer composed of 50 mM Tris/HCl, 5 mM $CaCl_2$, 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.2 mM Triton X-100, and 0.25 mM substrate at pH 8.3, as described previously (*6*). Phospholipid concentrations were determined from the measured P_i contents according to the method of Rouser et al. (*24*). Thin-layer chromatography (TLC) analysis was performed on HPTLC Kieselgel (Merck) plates using chloroform/methanol/water (65/25/4 by volume) as the eluent. Spots were visualized under UV light (254 nm), with I_2 vapor, with a phosphomolybdate spray, and by charring. The critical micelle concentration (CMC) of the detergent C_{12}SB was determined by the surface tension method (*25*).

Sulfhydryl Group Determination. The number of sulfhydryl groups in the protein variants was determined spectrophotometrically using DTNB (*26*). The protein was first desalted to remove DTT. Subsequently, an excess of DTNB was added (10 μL from a 10 mM stock in ethanol) to a 1 mL aliquot of the protein solution (OMPLA at ∼0.5 mg/mL). The number of sulfhydryl groups was calculated from the increase in absorbance at 412 nm using an ϵ_{412} of 13 600 cm^{-1} M^{-1} (*26*).

Site-Directed Cross-Linking. A 50 μL aliquot of disulfide cross-linker (from a 1.8 mM stock in acetonitrile) was added to 0.5 mL of a desalted protein variant. The resulting solution [16 μM OMPLA, 160 μM cross-linking reagent, 50 mM Tris/HCl (pH 8.3), 2.5 mM C_{12}SB, 9% acetonitrile, and 25 mM $CaCl_2$] was kept for 2 h at room temperature. After this time, the reaction was quenched by the addition of DTT to a final concentration of 5 mM, and the mixture was divided into two fractions. To one half was added an equal volume of gel loading buffer [0.1 M Tris/HCl (pH 6.8), 3% SDS, 15.4% glycerol, 7.7% β-mercaptoethanol, and 0.008% bromophenol blue]. Subsequently, a 20 μL aliquot of this solution (corresponding to approximately 5 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 other half was loaded onto a Q-Sepharose column equilibrated in buffer B [20 mM Tris/HCl (pH 9.0), 2.5 mM C_{12}SB, and 5 mM DTT]. After the column had been washed with buffer B to remove the free cross-linking reagent, the protein variant was eluted with 1 M KCl in buffer B. Subsequently, the protein was desalted and tested for enzymatic activity.

Fluorescence Labeling. The labeling reaction was started by the addition of a 100 μL aliquot of the fluorescence probe IANBD (from a 2 mM stock in acetonitrile) to the H26C variant, previously desalted to remove the DTT. The resulting mixture [3 mL of 32 μM OMPLA, 64 μM IANBD, 50 mM Tris/HCl (pH 8.3), 2.5 mM C_{12}SB, and 3% acetonitrile] was incubated in the dark at room temperature for 1 h. After this time, the reaction mixture was loaded onto a Q-Sepharose

column equilibrated in buffer C [20 mM Tris/HCl (pH 9.0) and 2.5 mM $C_{12}SB$]. After the column had been washed with buffer C to remove the free IANBD, the protein variant was eluted with 1 M KCl in buffer C. The labeling of OMPLA with IAEDANS was performed in an analogous manner. IAEDANS was added to H26C (from a 2 mM aqueous stock solution). The resulting mixture [3 mL of 32 μM OMPLA, 38 μM IAEDANS, 50 mM Tris/HCl (pH 8.3), and 2.5 mM $C_{12}SB$] was incubated in the dark at room temperature for 1 h. Subsequently, the free IAEDANS was removed by loading the reaction mixture onto a G-25 Sephadex column equilibrated and run in buffer A. All labeled proteins were dialyzed twice against buffer A. The labeling efficiency was determined spectrophotometrically and defined as the percentage of the label concentration over the protein concentration. The protein concentration was corrected for the dye absorption contribution by the relation $A_{280}^{OMPLA} = A_{280}^{obs} - f_{label}A_{label}$, where A_{label} was the sample absorbance at the wavelength of peak label absorption. The factor f_{label}, which was determined from the absorption spectrum of the free labels, has the following values: 0.071 for IANBD and 0.229 for IAEDANS. The extinction coefficients of the labels were as follows: $\epsilon_{496} = 25\,000$ cm^{-1} M^{-1} and $\epsilon_{339} = 5700$ cm^{-1} M^{-1} for IANBD and IAEDANS, respectively. The labeled protein was stored at −20 °C in the dark.

Fluorescence Resonance Energy Transfer Measurements. Unless indicated otherwise, all FRET experiments were performed by mixing the AEDANS-H26C protein (donor) in a 1/4 ratio with the native OMPLA or with the ANBD-H26C protein (acceptor) in buffer [0.05 mg/mL total protein, 50 mM Tris/HCl (pH 8.3), 100 mM KCl, and 25 mM $CaCl_2$] at a given $C_{12}SB$ concentration. A donor/acceptor ratio of 1/4 was used to promote the fraction of OMPLA dimers containing donor−acceptor pairs. Fluorescence intensity emission spectra were recorded using a Perkin-Elmer LS-5 spectrofluorometer at room temperature, in a 0.5 mL cuvette. Both excitation and emission slit widths were 5 nm. The fluorescence spectra were recorded at an excitation wavelength of 339 and with an emission wavelength range of 400−600 nm. The efficiency of the energy transfer was determined from the quenching of the fluorescence of the donor molecule by the relation $E = [1 - (F_{da}/F_{dn})] \times 100$, where E is the efficiency of the energy transfer and F_{dn} and F_{da} are the donor fluorescence intensities in the absence and in the presence of the energy acceptor, respectively.

Reconstitution of OMPLA in Phospholipid Vesicles. Purified OMPLA was incorporated into unilamellar phospholipid vesicles essentially as described previously (*14*). The solvent of an aliquot containing 20 mg of PC (stock in chloroform) was dried under reduced pressure. The dried lipid film was dissolved in 0.5 mL of buffer composed of 100 mM Tris/HCl, 4 mM EDTA, 200 mM KCl, and 100 mM deoxycholate (DOC) at pH 8.3, to which 0.5 mL of OMPLA (116 μM) in 2.5 mM $C_{12}SB$ had been added [final conditions being 28 mM PC, 58 μM OMPLA, 1.25 mM $C_{12}SB$, 50 mM Tris/HCl (pH 8.3), 2 mM EDTA, 100 mM KCl, and 50 mM DOC], resulting in an optically clear mixed micellar solution. After equilibration for 30 min, the sample was loaded onto a 70 mL G-50 Sephadex (Pharmacia) column equilibrated and run in vesicle buffer [50 mM Tris/HCl (pH 8.3), 2 mM EDTA, and 100 mM KCl]. This chromatography step was repeated once, and the fractions containing protein (based on absorbance measurements) were collected and pooled. OMPLA together with phospholipid eluted in the void volume, whereas DOC and $C_{12}SB$ were retarded, and eluted in the total volume. The resulting OMPLA vesicle suspension was translucid. The size distribution of the OMPLA vesicles was determined using light scattering by means of a Zetasizer 3000 apparatus (Malvern Instruments) using a monomodal analysis mode. The concentration of OMPLA in the proteosomes was determined by measuring the absorbance at 280 nm in the presence of 25 mM $C_{12}SB$ and 2 mM EDTA. The concentration of OMPLA was calculated using the measured absorbance corrected for the absorbance of detergent-solubilized vesicles without OMPLA. Typically, the vesicle solution contained 2 mM phospholipid and 1 μM OMPLA. The average number of OMPLA molecules per vesicle was calculated from the concentration of OMPLA, the concentration of PC, the molecular surface area occupied by a fully hydrated PC molecule (70 Å^2 according to ref *27*), and the area of the vesicles (assuming spherical geometry). The vesicle solutions could be stored at 4 °C for at least 1 week, without detectable vesicle aggregation or enzymatic breakdown of phospholipid.

Trypsine Treatment. A 5 μL aliquot of a trypsine solution in buffer [20 mM Tris/HCL (pH 8.3)] was added to 95 μL of a proteosome solution [final conditions being 2 mM EDTA, 50 mM Tris/HCl (pH 8.3), 100 mM KCl, 3 mM PC, 0.22 mg/mL OMPLA, and 0.22 mg/mL trypsine] in the absence and presence of 9 mM Triton X-100. The samples were incubated at room temperature for 24 h, and subsequently, a 20 μL aliquot was mixed in a 1/1 ratio with gel loading buffer. A 20 μL aliquot of this mixture was analyzed by SDS−PAGE.

RESULTS

Sulfhydryl-Directed Cross-Linking between OMPLA Monomers. Sulfhydryl-directed cross-linking has been successfully applied before in studying inter- and intramolecular interactions, for instance, in the lactose permease of *E. coli* (*28*). We now applied this technique to OMPLA. OMPLA contains no cysteine residues; therefore, unique cysteine residues were introduced by site-directed mutagenesis. As schematically depicted in Figure 1, the H26C mutation is located in the putative first loop. The S144C mutation, in which the catalytic nucleophile serine residue is replaced by a cysteine, is located on the third loop facing the extracellular media. Finally, the H234C mutation is located in a short turn at the periplasmic side. The single-Cys variants were expressed and purified to homogeneity. Both the H26C and H234C variants exhibited high specific activities comparable to that of native OMPLA (100 and 70%, respectively), indicating that the mutations did not substantially disturb the structure and function of the protein. The active site S144C variant exhibited a low activity (1% compared to that of native OMPLA) as reported previously (*22*). The number of sulfhydryl groups in each of the Cys variants was determined with DTNB. A value of 0.7−0.9 molar equiv of sulfhydryl groups in each of the variants could be determined.

The cross-linking of the single-Cys variants was investigated using three sulfhydryl-directed homo-bifunctional cross-linking reagents of various lengths, BMH, *p*-PDM, and

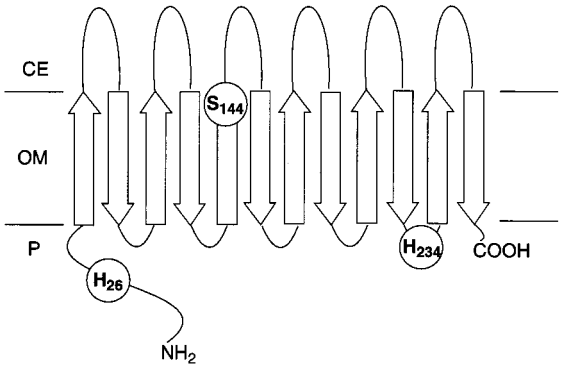

FIGURE 1: Putative topology model of OMPLA. The 12 transmembrane antiparallel amphiphatic β-strands are depicted as arrows. The extracellular loops and periplasmic turns are depicted (based on ref *2*). The positions of the single-cysteine variants are indicated: H26C in the first loop, S144C in the third extracellular loop, and H234 in the last periplasmic loop. CE represents the cell exterior, OM the outer membrane, and P the periplasm.

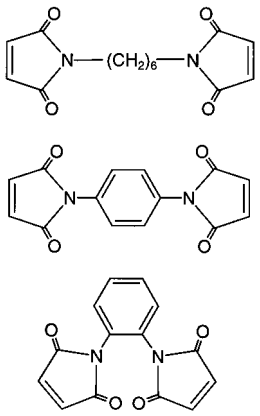

FIGURE 2: Structures of the cross-linkers. From top to bottom are shown 1,6-bis(maleimido)hexane (BMH), *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM), and *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM).

o-PDM (distance between reactive groups of 16, 10, and 6 Å, respectively). Their structures are depicted in Figure 2. Each Cys variant was cross-linked for 2 h with each of the three reagents, and the products were analyzed by SDS−PAGE (Figure 3). When the H26C variant was reacted with the cross-linkers, the protein migrated with an apparent molecular mass of 45 kDa (Figure 3, lanes 2−4), which corresponds to the dimeric species of OMPLA (*15*). These data suggest a close proximity between residues of both monomers at position 26 under dimeric conditions. The cross-linked protein exhibited full activity, indicating that the cross-linking did not substantially alter the native structure of the protein. The S144C and H234C variants migrated with an apparent molecular mass of 27 kDa (Figure 3, lanes 5−10), which corresponds to the monomeric protein (*19*), suggesting that these residues are widely spaced in the dimer of OMPLA. The absence of cross-linking for S144C and H234C could be explained in terms of the sulfydryl group being inaccessible for the cross-linker. However, this possibility is not very likely, since the sulfydryl group of both variants exhibited good reactivity toward DTNB. Moreover, the enzymatic activity of the variant S144C could be abolished by the addition of *n*-ethylmalemide. Therefore, the most likely possibility is that these residues are widely spaced in the dimer of OMPLA.

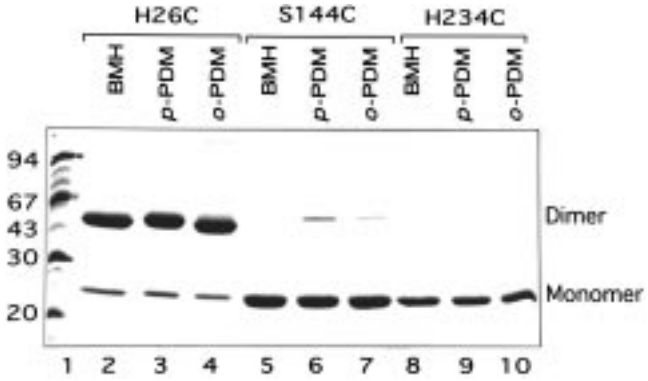

FIGURE 3: SDS−PAGE analysis of the chemical cross-linking of single-Cys variants. The protein variants were cross-linked with homo-bifunctional sulfhydryl specific reagents with linker lengths of 16 (BMH), 10 (*p*-PDM), and 6 Å (*o*-PDM): lane 1, molecular mass markers; lanes 2−4, H26C; lanes 5−7, S144C; and lanes 8−10, H234C. The cross-linker used in each individual experiment and the single-Cys variants are denoted on the top of the gel. The migration positions of monomeric OMPLA and of dimeric OMPLA are indicated on the right. The mass (in kilodaltons) of the molecular mass marker proteins is indicated on the left.

Dimerization of OMPLA in Detergent Micelles As Monitored by FRET. Residue 26 in one monomer is located in close proximity to its counterpart in the adjacent monomer. To be able to monitor dimerization (i) without perturbation of the system, (ii) in a sensitive way, and (iii) in real time, we exploited FRET. IAEDANS (energy donor) and IANBD (energy acceptor) were used as fluorescence labels. We selected this FRET couple because of its good spectral overlap (*29*), and the fact that the R_0 (27 Å according to ref *30*) is considerably larger than the distance between the sulfhydryl groups at position 26 ($\leq$6 Å as determined by cross-linking). In addition, these labels react selectively with sulfhydryl groups via their iodoacetamide groups. The fluorescence probes were incorporated into the H26C variant with stoichiometries of 0.9 and 0.7 for IAEDANS and IANBD, respectively. The reaction with the sulfhydryl group was highly selective as judged from the low levels of incorporation of the fluorescent probes into the native protein ($<$5% modification under identical labeling conditions). The fluorescence-labeled proteins exhibited specific activities identical to those of the nonlabeled ones. Moreover, the cofactor and detergent dependence of dimerization were unaffected, indicating that the labeling did not perturb the native structure of the protein. The fluorescence excitation and emission spectra of AEDANS-H26C and ANBD-H26C in C_{12}SB were collected (not shown). For AEDANS-H26C, peak fluorescence excitation and emission intensities were observed at 339 and 462 nm, respectively. For ANBD-H26C, the values were 492 nm for the peak excitation intensity and 520 nm for the peak emission intensity, indicating good spectral overlap between the labeled proteins. However, the emission intensity of the acceptor was considerably lower than that of the donor, in agreement with the reported lower quantum yield of the acceptor compared to the donor, and as a result, the donor fluorescence lifetime and intensity decrease (*31*). Therefore, for practical reasons, the *E* between the donor and acceptor was measured by the donor quenching method (*32*). This method is based on the fact that the presence of an energy acceptor in the vicinity of an excited energy donor provides an additional relaxation pathway for

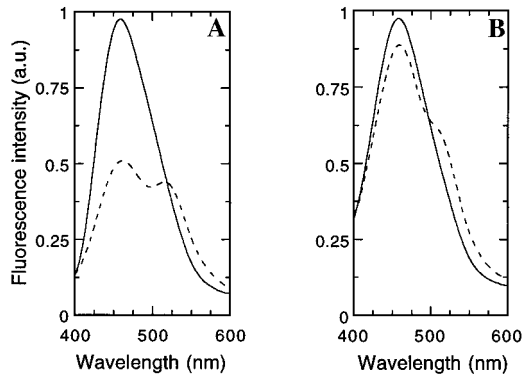

FIGURE 4: Fluorescence intensity emission spectrum of the labeled H26C variant in detergent micelles. (A) The spectrum of a 1/4 mixture of AEDANS-H26C and native OMPLA (—) and the emission spectrum in which native OMPLA was replaced by ANBD-H26C (- - -). The spectra were recorded in buffer containing 2.5 mM C_{12}SB and 25 mM calcium. (B) Spectra from an analogous experiment carried out in 2 mM EDTA. The excitation wavelength was 339 nm. The F_{dn} and F_{da} were 462 and 459 nm, respectively.

the donor. The fluorescence emission spectrum of AEDANS-H26C mixed with native OMPLA and the emission spectra in which the native protein was replaced by ANBD-H26 are shown in Figure 4. In the presence of the cofactor calcium (essential for dimerization), a large quenching of the donor emission was observed (left panel), as evidenced by the decrease in fluorescence intensity at 462 nm, which resulted in an *E* of 50%. In the presence of EDTA, however, a small *E* of 9% was observed (right panel). The difference in *E* is indicative of Ca^{2+}-induced dimerization.

Dimerization as a Function of Detergent and Substrate. Using FRET, we investigated the effect of the detergent concentration on dimerization. Both activity and *E* at various detergent concentrations were determined in the presence of calcium. At a C_{12}SB concentration of 2.5 mM [approximately twice the CMC (1.3 mM) of C_{12}SB determined under our experimental conditions], the protein was fully active, as reported previously (*6*), and a large *E* was observed, indicating that the protein was present in the dimeric state, in agreement with previous results (*15*). When the C_{12}SB concentration was increased to 10 mM, the activity sharply decreased, and so did the energy transfer, indicating that at high detergent concentrations the dimer dissociated into monomers. It can be calculated that the ratios of detergent micelles to protein molecules at 2.5 and 10 mM C_{12}SB are 14 and 100, respectively. These values illustrate that (i) at 2.5 mM C_{12}SB dimerization is not the result of a limiting concentration of micelles and (ii) the increased number of micelles drives dimer dissociation by a competition effect.

Next, the effect of the phospholipid substrate on dimerization was investigated. The fluorescence emission spectrum of a 1/4 mixture of AEDANS-H26C and ANBD-H26C in 2.5 mM C_{12}SB and EDTA was recorded. Under these conditions, OMPLA is monomeric, and no FRET could be detected (see Figure 4, right panel). The addition of PC to a final concentration of 0.1 mM resulted in an *E* of 45%, indicative of substrate-induced OMPLA dimerization in mixed micelles in the absence of calcium ions.

Assessment of Dimerization in Phospholipid Vesicles. The fluorescence-labeled H26C variant was reconstituted in PC vesicles. The gel filtration method used for the reconstitution

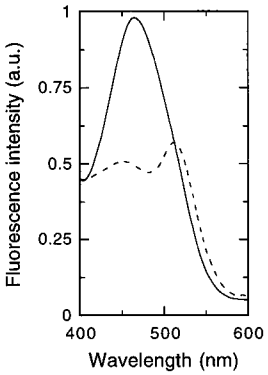

FIGURE 5: FRET of the labeled H26C variant in vesicles. The fluorescence intensity emission spectrum of a 1/4 mixture of AEDANS-H26C and native OMPLA (—) after reconstitution in lipid vesicles is shown. Donor quenching due to FRET is apparent in the emission spectrum in which native OMPLA was replaced by ANBD-H26C (- - -). The spectra were recorded in buffer containing EDTA with an emission wavelength range of 400−600 nm and at an excitation wavelength of 339 nm. The fluorescence intensity on both spectra was corrected for differences in protein concentration. The F_{dn} and F_{da} were 458 and 453 nm, respectively.

Table 1: FRET and Enzymatic Activity of OMPLA[a]

conditions	addition	activity (h^{-1})	*E* (%)
C_{12}SB micelles	EDTA	−	9 ± 2
C_{12}SB micelles	Ca^{2+}	−	50 ± 4
vesicles	EDTA	nd[d]	41 ± 6
vesicles	Ca^{2+}	0.1	48 ± 5
mixed micelles[b]	EDTA	nd	40 ± 5
mixed micelles[b]	Ca^{2+}	7.1	38 ± 5
DODAPC vesicles	EDTA	−	58 ± 1
DODAPC vesicles	Ca^{2+}	−	55 ± 1
mixed micelles[c]	EDTA	−	53 ± 2
mixed micelles[c]	Ca^{2+}	−	54 ± 3

[a] The *E* between the labeled H26C OMPLA variant is given under various conditions. OMPLA activity was measured using TLC analysis. The activity is defined as the reciprocal of the time in hours necessary to hydrolyze 50% of the original PC. The experimental conditions are described in the legend of Figure 6. The values represent the mean value of a duplicate experiment, and the standard deviation is also given. [b] C_{12}SB/PC molar ratio of 3. [c] C_{12}SB/DODAPC molar ratio of 3. [d] nd means no activity could be detected.

produced large unilamellar phospholipid vesicles (≈700 Å in diameter), which were stable upon storage at 4 °C for several days. TLC analysis showed that the detergents C_{12}-SB and DOC were efficiently removed to less than 1% of the original amounts. According to a rough calculation (see Experimental Procedures), the vesicles contained approximately 15 OMPLA molecules. To determine if reconstitution had a denaturing effect on OMPLA, the reconstituted protein was analyzed by SDS−PAGE. The protein migrated at exactly the same position as the detergent-solubilized OMPLA (not shown), and exhibited the characteristic heat modifiability (*19*), indicative of the presence of the β-barrel structure. In the presence of EDTA, no PC hydrolysis could be observed, as judged from TLC analysis, for as long as 24 h of incubation of the vesicles at room temperature. Under these conditions, FRET experiments were performed (Figure 5). Efficient energy transfer was observed, as evidenced by the quenching of the donor emission at 462 nm. The obtained *E* (Table 1) was large and comparable to the value obtained for the detergent-solubilized protein under conditions where the protein was mainly dimeric. These results suggest that

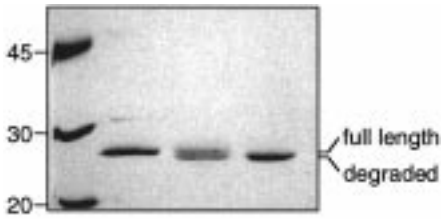

FIGURE 6: Orientation of OMPLA in proteosomes as determined by proteolysis. SDS−PAGE analysis of trypsin digestion of reconstituted OMPLA under various conditions: lane 1, molecular mass markers with masses (in kilodaltons) indicated on the left; lane 2, sample of proteosomes; lane 3, sample of proteosomes incubated overnight with trypsin; and lane 4, sample of proteosomes incubated overnight with trypsin in the presence of Triton X-100.

in phospholipid vesicles OMPLA is dimeric, even in the absence of cofactor calcium. It should be noticed that the large *E* could also result from the formation of higher-order protein complexes, but with the low amounts of OMPLA per proteosome (15 protein molecules per vesicle), this possibility is not very likely. The addition of the cofactor Ca^{2+} (final concentration of 25 mM) to the vesicles led to a low but measurable OMPLA activity. Under these conditions, a large *E* was also observed, indicating that the majority of the protein was dimeric. The low activity of the reconstituted protein could be due to a preferred orientation of the protein in the bilayer, by which, for example, the calcium binding sites could be present at the lumen of the vesicle, thereby offering a simple explanation for the absence of activity. To determine the orientation of OMPLA in the vesicle, the proteosomes were incubated with trypsine in the presence or absence of Triton X-100 (Figure 6, lanes 3 and 4, respectively). With intact vesicles, only partial proteolytic degradation of OMPLA was observed, whereas in the presence of detergent, all the OMPLA was degraded. Our results showed that OMPLA was present in the vesicles in both orientations, in line with recent electron microscopy data on the reconstituted enzyme (*33*).

Next, the effect of the addition of detergent to the vesicles was investigated. To trigger full activity, the detergent C_{12}-SB was added to the vesicles. The addition of C_{12}SB above the CMC in a C_{12}SB/PC molar ratio of 3 resulted in the complete solubilization of the vesicles, as evidenced by absorbance measurements at 600 nm. The *E* and activity of the enzyme at various conditions are given in Table 1. In these mixed micelles of PC and C_{12}SB, a 70-fold increase in OMPLA activity was observed when calcium was added, as compared to the conditions in the absence of detergent. It should be emphasized that during a complete FRET measurement approximately 1% of the proteosome phospholipids were hydrolyzed, indicating that significant phospholipid hydrolysis is not a determining factor in our measurements. To exclude the interference of hydrolysis products in our measurements, the experiments were repeated in vesicles of the nonhydrolyzable lipid DODAPC. As shown in Table 1, the results are very similar to the ones obtained in PC vesicles. Our data suggest that OMPLA is dimeric and poorly active in the bilayer system, whereas in mixed micelles, the activity of the enzyme is enhanced considerably.

DISCUSSION

The experiments described here have shown that the dimeric H26C variant could be efficiently cross-linked with full retention of activity even with the shortest linker, suggesting a close proximity between the amino termini of both monomers in the dimeric complex (Figure 1). Such an interpretation is supported by the observation that for this variant, spontaneous disulfide formation between monomers was detected by SDS−PAGE in the absence of cross-linker under nonreducing conditions (not shown). The question then arises as to the role of the amino terminus. In the porins of Gram-negative bacteria, of which the X-ray structure is known (*34*−*36*), the trimeric structure is stabilized by interactions between segments of the monomers in the aqueous phase. In view of the structural similarities between OMPLA and the porins (*2*), we propose that monomer−monomer interactions in the amino terminus region contribute to the stabilization of dimeric OMPLA. The absence of cross-linking for S144C and H234C most likely reflects the fact that these cross-link target sites are outside the range of the cross-linkers. Interestingly, this result suggests that the active sites of the two monomers are not in close proximity in the dimeric complex.

On the basis of the proximity between the cysteine residues in the H26C dimer, this position was selected for the introduction of the fluorescence labels. In the dimer, a large energy transfer ($E = 50\%$) was observed between labeled monomers in detergent micelles (Table 1). A rough calculation[2] estimates that the maximal *E* that one can theoretically obtain in our system is approximately 56%. A higher *E* is not possible, since the labeling was not complete, and therefore, a considerable fraction of donor−OMPLA molecules was not complexed with acceptor−OMPLA molecules. Comparison of the calculated and measured *E* values suggests that in the dimeric complex the distance between probes is much smaller than R_0, in agreement with the cross-linking data.

In detergent micelles, the dimerization was dependent on calcium, as previously reported (*15*, *37*), indicating the specific nature of the association process. Interestingly, the substrate promoted the dimerization of OMPLA, a finding that has been suggested previously on the basis of results showing that the dimeric state of the enzyme was stabilized by covalent modification of the active site (*15*). The ability of substrates to influence protein oligomerization has only been documented for a few enzymes, notably, glyceraldehyde-3-phosphate dehydrogenase (*38*) and nitric oxide synthase (*39*). An attractive possibility is that in OMPLA the substrate is an effector, and as such contributes to the modulation of activity.

A remarkable outcome of our experiments is the observation of very low enzymatic activity, even when OMPLA is in the dimeric state and surrounded by substrate in the presence of calcium. This conclusion is supported by experiments in which a reconstituted covalent dimer of the

[2] This calculation involved the following steps. Taking into account the labeling efficiency (90 and 70% for the donor- and acceptor-labeled OMPLA, respectively), we calculated the amount of donor-labeled OMPLA (D), acceptor-labeled OMPLA (A), and nonlabeled OMPLA (N), and then, given the D/A ratio of 1/4 used in each FRET experiment, the fraction of DD, DA, and DN dimers was calculated; finally, if it is assumed that maximal energy transfer occurs between DA pairs [i.e., $R \ll R_0$, where *R* is the distance between the fluorescence probes and R_0 (Förster's distance) is the distance at which 50% energy transfer occurs], the maximal *E* can be calculated.

H26C variant exhibited an analogous behavior (unpublished results). Only when the bilayer structure is perturbed, the phospholipase activity becomes apparent. This result is in agreement with previous studies by Horrevoets et al. (*14*), who showed that the activation of OMPLA was dependent on changes in the physical state of the bilayer. One could envisage two possible models for rationalizing the activation of OMPLA in bilayers. (i) Although OMPLA resides as a catalytically competent dimer, in an intact bilayer the substrate presentation to the enzyme is not optimal, or (ii) the changes in the physical properties of the surrounding lipid phase give rise to distinct dimeric species, thereby, tuning enzyme activity. With regard to the first option, it should be mentioned that the packing of the lipid molecules in the bilayer might result in an unfavorable substrate conformation and orientation, resulting in an increased energy barrier for substrate binding, and, hence, a decrease in the catalytic rate. Another possibility is that the products of the OMPLA-catalyzed reaction somehow inactivate the enzyme, resulting in the so-called product inhibition. In line with both rationales, it is noteworthy that the collapse of the bilayer into mixed micelles correlates with changes in the packing of the lipid molecules (*40*), and this fact might result in a favorable presentation of the substrate or in an enhanced product removal. Alternatively, with regard to the second option, it has long been recognized that the lipid environment can influence parameters such as the conformational flexibility (*41*), the lateral diffusion (*42*), or simply the orientation of proteins. For OMPLA, these parameters might restrict the enzyme within the bilayer, resulting in the stabilization of a poorly active conformation of the dimer. This so-called "conformational trapping" has been observed for large peptides, and has been suggested as a regulatory mechanism for membrane protein activity (*43*). One can envisage that the changes in the physical properties of the surrounding lipids, as a result of the transition to micellar structures, facilitate structural rearrangements in the OMPLA dimers with the ensuing enhancement in enzymatic activity. At this moment, we cannot discriminate between either substrate or enzyme models. It should be emphasized, however, that both models are not mutually exclusive, particularly, since in our case the bilayer is at the same time the solvent and the substrate for the enzyme.

Several examples can be found in the literature in which a correlation has been observed between the lipid surroundings and the function of a membrane enzyme such as the plasma membrane Ca^{2+}-ATPase (*44*), diglucosyldiacylglycerol synthase from *Acholeplasma laidlawii* (*45*), the Na^+/K^+-ATPase from the plasma membrane of animal cells (*46*), the cardiolipin synthase from rat (*47*), and the lactose permease of *E. coli* (*48*). However, the understanding of the mechanism underlying these observations is scanty. The data presented here on the regulation of OMPLA activity provide a basis for further investigating and for rationalizing regulatory mechanisms in and at biological membranes.

ACKNOWLEDGMENT

We thank Mr. Ruud Cox for the synthesis and purification of the detergent C_{12}SB.

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BI983077X