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Energetics of Outer Membrane Phospholipase A (OMPLA) Dimerization

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Outer membrane phospholipase A (OMPLA) is a widely conserved transmembrane enzyme found in Gram-negative bacteria, and it is implicated in the virulence of a number of pathogenic organisms. The regulation of the protein's phospholipase activity is not well understood despite the existence of a number of high resolution structures. Previous biochemical studies have demonstrated that dimerization of OMPLA is a prerequisite for its phospholipase activity, and it has been shown *in vitro* that this dimerization is dependent on calcium and substrate binding. Therefore, to fully understand the regulation of OMPLA, it is necessary to understand the stability of the protein dimer and the extent to which it is influenced by its effector molecules. We have used sedimentation equilibrium analytical ultracentrifugation to dissect the energetics of *Escherichia coli* OMPLA dimerization in detergent micelles. We find that calcium contributes relatively little stability to the dimer, while interactions with the substrate acyl chain are the predominant force in stabilizing the dimeric conformation of the enzyme. The resulting thermodynamic cycle suggests that interactions between effector molecules are additive. These energetic measurements not only provide insight into the activation of OMPLA, but they also represent the first quantitative investigation of the association energetics of a transmembrane β-barrel. This thermodynamic study allows us to begin to address the differences between protein–protein interfaces in transmembrane proteins with a helical fold to those of a β-barrel fold and to more fully understand the forces involved in membrane protein interactions.

Keywords: thermodynamics; OMPLA; transmembrane protein–protein interactions; beta-barrel; dimerization

Introduction

Outer membrane phospholipase A (OMPLA) is a unique integral membrane enzyme found in Gram-negative bacteria.[1] The protein is widely conserved,[2] and an increasing number of studies have implicated the protein as a virulence factor in various pathogens.[3–8] OMPLA is also found in non-pathogenic strains of bacteria, suggesting a more general function for the protein.[2] While the exact biological role of OMPLA remains unclear, it has been shown that the enzyme, which is normally inactive, can be activated by processes that disrupt the integrity of the bacterial outer membrane, such as phage-induced lysis and temperature shock.[1,9–12] Under such conditions of stress, OMPLA is presumably involved in altering the composition and integrity of the bacterial outer membrane. As suggested by the toxicity of high-level overexpression of native OMPLA, uncontrolled phospholipase activity is potentially lethal to the bacterium.[10] Therefore, it is important that the activity of this protein be tightly regulated to prevent

Abbreviations used: ASA, accessible surface area; AUC, analytical ultracentrifugation; C14-SB, 3-(*N*,*N*-dimethylmyristyl-ammonio)propanesulfonate; C12-SB, 3-(dodecyldimethyl-ammonio)propanesulfonate; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid); FF, fast flow; GpA, glycophorin A; HSF, hexadecylsulfonyl fluoride; K_D, dissociation equilibrium constant; OMPLA, outer membrane phospholipase A; OS, occluded surface; S_c, shape correlation statistic; TM, transmembrane; VDW, van der Waals.

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phospholipid hydrolysis under normal physiological conditions.

Early biochemistry demonstrated that the OMPLA enzyme was active only as a dimer and that calcium binding and substrate binding modulated the dimerization and activity of the protein *in vitro*.[13] The subsequent determination of high-resolution structures of both a monomeric and dimeric state of the enzyme yielded insight into the molecular basis of these biochemical observations.[14] These crystal structures revealed that the protein had a 12 stranded β-barrel fold and that complete active sites were formed only in the dimer. The dimer contained two active sites at the interface between subunits. In each active site the calcium ion necessary for catalysis was coordinated by residues from both subunits. Substrate-binding clefts were formed at the outer-edge of the dimer interface, with the substrate acyl chain making extensive contacts with both monomers.[14] The requirement for the second subunit to complete the substrate and calcium-binding sites explained why the protein was active only as a dimer.

Despite the insight from the high-resolution structures, no clear mechanism of regulation has emerged. Control of OMPLA function, however, must involve the monomer–dimer equilibrium of the protein at a fundamental level, since dimerization is a prerequisite for activity. Although other factors such as bilayer integrity are involved in activating the protein,[11] it is clear that calcium and substrate play key roles in modulating the enzyme's monomer–dimer equilibrium.[13] Therefore, to understand the regulation of OMPLA function it is necessary to know the stability of the OMPLA dimer, as well as the extent to which the dimeric population of the enzyme is influenced by the effector molecules, calcium and substrate.

However, investigations of the thermodynamics of membrane protein interactions are scarce, and compared to soluble proteins, relatively little is known about the molecular determinants involved in these interactions. To date, thermodynamic studies of membrane protein interactions have been largely limited to helix–helix interactions,[15–22] and quantitative studies probing the association of transmembrane proteins with a β-barrel fold are absent from the literature. Therefore, even though many transmembrane β-barrels have been demonstrated to be oligomeric,[23–25] the stability of these complexes is essentially unknown. To begin to address this important question in membrane protein thermodynamics and to gain insight into the activation of OMPLA *via* its dimerization, we have used sedimentation equilibrium to determine the thermodynamics of OMPLA self-association, both in the presence and absence of its effector molecules. Our results from sedimentation equilibrium can also be directly compared to the association propensities observed for transmembrane helix dimers determined under similar conditions.[26] Such a comparison represents an important first step towards deriving general principles that define the molecular properties of membrane protein complexes.

Results

Understanding the function of a membrane protein requires knowledge of both molecular structure and molecular energetics. In the case of OMPLA, a complete understanding of the regulation of the protein requires an understanding of the stability of the active dimeric state of the enzyme and how the stability of this dimer is modulated by effector molecules. We have begun to address these questions by exploring the thermodynamics of the OMPLA self-association using sedimentation equilibrium ultracentrifugation, a technique which has proven to be very powerful in the quantitative analysis of transmembrane helix interactions.[15,18,20,27–29] Using this approach, we were able to determine the dimerization constants for unmodified and sulfonylated forms of OMPLA in the presence of EDTA, calcium, and magnesium.

With no effector molecules, OMPLA is a monomer even at low detergent concentrations

Previous glutaraldehyde cross-linking experiments demonstrated that OMPLA would not cross-link in the absence of any effector molecules, suggesting that OMPLA has little intrinsic propensity for self-association.[13] Equilibrium sedimentation experiments with OMPLA in C14-SB micelles in the presence of 20 mM EDTA demonstrated that OMPLA was indeed monomeric. Figure 1(a) shows a representative data set for OMPLA in the presence of EDTA, which was included in the buffer to ensure no free calcium was present. In the global fit, the data were well described by a single ideal species with a molecular mass equal to that of an OMPLA monomer. The sedimentation equilibrium experiments do not eliminate the possibility that in the absence of effector molecules OMPLA forms very weak dimers that would not be detectable at the protein concentrations used in these experiments. To enable experiments at higher protein concentrations, we also carried out experiments in 0.3 cm path-length cells. These cells allowed a fourfold increase in the concentration of OMPLA to 40 μM, while still remaining in the linear range of the instrument optics. In 2.5 mM C14-SB, the protein was still monomeric. It may be that OMPLA does not dimerize in the absence of calcium, but if it does dimerize, these experiments place a lower limit on the apparent K_{Dapp} value in 2.5 mM C14-SB of $K_{Dapp} \geq 0.4$ mM ($\Delta G_{app} \geq -4.7$ kcal/mol). This lack of detectable protein–protein interaction is also consistent with the result that unmodified OMPLA crystallizes as a monomer.[14]

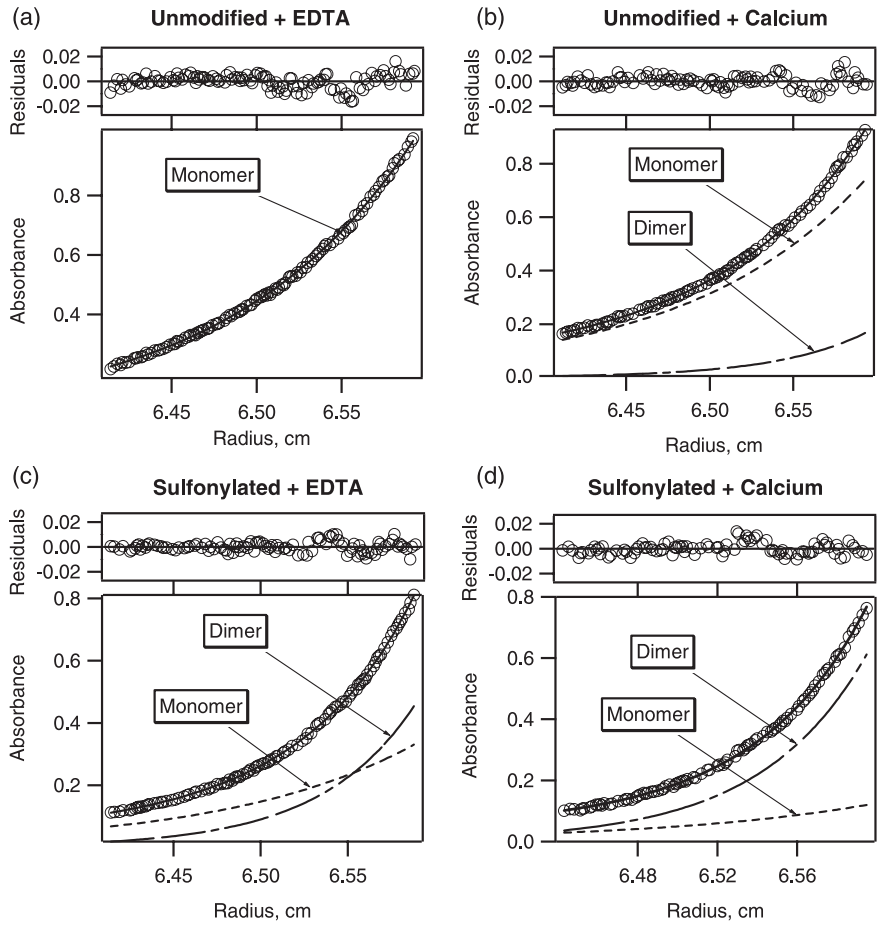

Table 1. Apparent free energies of OMPLA dimerization in 2.5mM C14

Experimental condition	ΔG_{app} (kca mol^{-1})	K_{Dapp} (μM)
(a) Unmodified	Monomer	-
(b) Unmodified with calcium	-5.68 ± 0.14	68
(c) Sulfonylated	-7.25 ± 0.23	4.9
(d) Sulfonylated with calcium	-8.31 ± 0.25	0.8

Figure 1. A sedimentation equilibrium data set (20,000 rpm, 6.6 μM) is shown for each of the four conditions of OMPLA self-association. The lower panel shows the raw data in open circles with the global fit in a continuous line. The global fits for each condition were calculated using a total of nine data sets, three concentrations (10.0 μM, 6.6 μM and 3.3 μM) and three speeds (16,300, 20,000 and 24,500 rpm). The broken lines represent the species components for a monomer–dimer fit. The upper panels illustrate the residuals for the given fit. The AUC data for the unmodified protein in the absence of calcium can be described by a single monomeric species, whereas the other conditions fit best to a monomer–dimer equilibrium. The free energies reported in Table 1 represent the average and standard deviation of at least three independent experiments.

A high concentration of calcium only modestly stabilizes OMPLA dimerization

It had been observed in glutaraldehyde cross-linking experiments that calcium stimulates the complete cross-linking of OMPLA as a dimer.[13] The crystal structure of the dimeric form of OMPLA (1QD6) revealed that the calcium co-factor was bound at the protein–protein interface, apparently stabilizing the dimer.[14] To investigate the extent to which calcium can stabilize the dimeric conformation of OMPLA, we carried out sedimentation equilibrium experiments in the presence of 20 mM $CaCl_2$. The data were no longer described by a single ideal species, but instead were best fit by a monomer–dimer equilibrium model. The global fitting procedure returned a value for the free energy of dimerization of $\Delta G_{app} = -5.68(\pm 0.14)$ kcal/mol in 2.5 mM C14-SB.

This value, however, is a very modest free energy. At 2.5 mM detergent, −4.7 kcal/mol is the smallest apparent free energy that can be detected in our experiments, and a significant fraction of that energy does not represent preferential interactions

and may be attributed to an energy of mixing.[30] This energy is also smaller than one might have expected from the cross-linking experiments, which suggested the protein was completely dimeric in the presence of calcium at protein concentrations equivalent to those used in the centrifuge experiments.[13] In contrast, the species plots in Figure 1(b) show that the protein is still predominantly monomeric in the presence of high concentrations of calcium when analyzed at equilibrium in solution. It seems unlikely that the choice of detergent is a significant factor in the difference between the experiments. The head group of the C14-SB detergent used in the analytical ultracentrifugation (AUC) experiments is the same as the head group for the C12-SB detergent used in the cross-linking experiments[13], and these detergents differ by only two methylene groups. Rather, covalent cross-linking does not necessarily measure equilibrium populations; instead it traps preferential oligomers, and in the case of OMPLA overestimates the effect of calcium on OMPLA dimerization.

Sulfonylation results in a significantly more stable OMPLA dimer

Hexadecylsulfonyl fluoride (HSF) is a substrate analog that specifically and covalently modifies Ser144 in the active site of OMPLA.[31] Modification of the protein by this substrate analog yielded crystals of the dimeric form of the protein. In the resulting crystal structure (1QD6), the acyl chain of HSF occupied the substrate-binding cleft situated along the periphery of the protein–protein interface.[14] Previous cross-linking studies also demonstrated the sulfonylated form of the enzyme, unlike its unmodified counterpart, could be cross-linked as a dimer both in the presence and the absence of calcium. Additionally, the modified enzyme also cross-links at higher detergent concentrations than the unmodified enzyme. Higher detergent concentrations dissociate transmembrane oligomers;[32] thus, these results suggest interactions with substrate are important for stabilizing the dimer.[13]

In the sedimentation equilibrium experiments, we find that sulfonylation has a considerable effect on the dimerization of OMPLA. Even in the absence of calcium, a significant fraction of the sulfonylated population was dimeric. Figure 1(c) illustrates the monomer–dimer fit to a representative HSF–EDTA data set, with the calculated curves for the component species shown as broken lines. In contrast to the unmodified protein, which is solely a monomer in the absence of calcium, monomer and dimer are almost equally populated for the sulfonylated protein. For HSF-modified OMPLA, the apparent free energy of dimerization was $\Delta G_{app} = -7.26(\pm 0.23)$ kcal/mol in 2.5 mM C14-SB micelles and 20 mM EDTA. The substrate analog stabilizes the OMPLA dimer more than calcium alone. The sulfonylated dimers, however, can be further stabilized by the addition of calcium. Addition of 20 mM $CaCl_2$ to the buffer results in a free energy of HSF–OMPLA dimerization of $\Delta G_{app} = -8.31(\pm 0.25)$ kcal/mol. Our results are also consistent with earlier preliminary sedimentation equilibrium analytical ultracentrifugation experiments, which determined an average molecular mass in octyl-POE detergent using single species fits.[13] In this study, Dekker *et al*. found that the protein in the absence of calcium had a molecular mass equal to that of the monomer. Additionally, they observed an increase in molecular mass in the presence of calcium and a significant increase upon sulfonylation to a molecular mass close to that of the dimer.

Magnesium can energetically substitute for calcium in the dimer

In exploring the catalytic requirements of OMPLA, it was found that magnesium did not support activity, but instead acted as a competitive inhibitor.[31,33] Magnesium's inability to support activity could result from an inability of magnesium to support the protein dimerization. To test this hypothesis, we determined the dimerization free energies of OMPLA for both the unmodified and sulfonylated versions of the protein in the presence of 20 mM $MgCl_2$ (Table 2). The unmodified protein dimerizes with a free energy of $\Delta G_{app} = -5.77(\pm 0.42)$ kcal/mol in the presence of magnesium, and HSF–OMPLA with a free energy of $\Delta G_{app} = -8.15(\pm 0.11)$ kcal/mol. These values are within error of the corresponding free energies for calcium. Although magnesium does not support activity, it can be incorporated in the dimer, likely at the catalytic calcium site, without energetic consequence.

Discussion

Interactions with substrate drive OMPLA self-association

OMPLA must dimerize to function, and we have used sedimentation equilibrium AUC to determine how biological effector molecules influence the self-association of the enzyme. The relationship

Table 2. Effect of magnesium on OMPLA dimerization in 2.5 mM C14-SB

	ΔG_{app}	$\Delta\Delta G_{app}$
Unmodified	-5.77 ± 0.42	-0.09
Sulfonylated	-8.15 ± 0.11	0.16

The free energies of OMPLA dimerization in the presence of 20 mM magnesium. The values are reported in kcal mol^{-1} and represent the average and standard deviation of four independent experiments. $\Delta\Delta G_{app}$ represents the difference between the apparent free energies $\Delta\Delta G_{app} = \Delta G_{MgCl_2} - \Delta G_{CaCl_2}$. Although magnesium does not support activity, it stabilizes dimerization to the same extent as calcium.

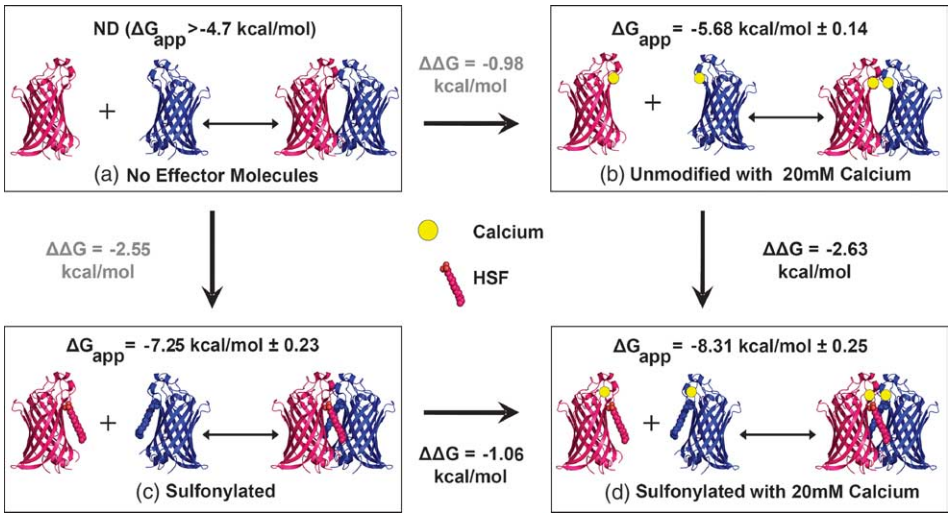

Figure 2. The thermodynamic cycle of self-association reactions for OMPLA and its effector molecules. The free energy for each of the dimerization reactions was measured in 2.5 mM C14-SB using sedimentation equilibrium AUC. The values for the free energies of association reported represent the mean and standard deviation of at least three independent measurements. The ΔΔG values represent the free energy differences between the two corresponding dimerization reactions, with the values in gray representing the smallest possible difference based on the limits for reaction (a). The association reactions are as follows: (a) Unmodified protein in the presence of 20 mM EDTA. (b) Unmodified protein in the presence of 20 mM calcium. (c) HSF modified protein in the presence of 20 mM EDTA. (d) HSF modified protein in the presence of 20 mM calcium. Modification of the protein by the substrate analog HSF favors the dimerization reaction significantly more than the addition of calcium.

between the effector molecules and dimerization can be most easily understood through the thermodynamic cycle illustrated in Figure 2. The values in gray represent upper limits based on the limit for reaction (a), which was estimated based on the concentrations at which we know the protein to be monomer. The horizontal direction represents the effects of calcium on the dimerization reaction, while the vertical direction represents the energetic effect of sulfonylation of the protein. *In vitro*, calcium stabilizes the dimer by approximately −1 kcal/mol, while occupying the acyl chain-binding site stabilizes the dimer by about −2.5 kcal/mol. These energies would suggest that substrate binding is a more significant driving force for OMPLA dimerization than is calcium binding.

In our thermodynamic cycle, calcium and HSF do not appear to interact synergistically to stabilize the dimer (Figure 2). Calcium stabilizes the unmodified protein to the same extent as the modified protein, or possibly more. The effect of calcium on dimerization of the sulfonylated protein is −1.06 kcal/mol, while −0.98 kcal/mol is the lower limit of the effect of calcium on the unmodified dimer (Figure 2). Our measurements were made using HSF, which has a small and uncharged head group that does not interact directly with the bound calcium.[14] With a large and potentially charged head group of a phospholipid substrate, the cycle may be cooperative. Evidence in the literature suggests calcium binds more tightly to the protein in the presence of phospholipid substrate.[31,34] By stabilizing the calcium in the catalytic site, interactions between the head group of a true substrate and the calcium co-factor could act cooperatively to stabilize the dimer. However, OMPLA does not require a substrate with a charged head group and shows comparable activity towards substrates with both charged and uncharged headgroups.[33]

It is instructive not just to consider the magnitude of the observed free energies in the presence of different effectors, but also to consider how these energies shift the population distribution. The consequence of the effector molecules on the oligomeric distribution of the OMPLA protein can be seen in Figure 3, where the fraction of dimer is plotted as a function of the aqueous protein concentration for both unmodified and HSF–OMPLA. Again, it can be seen, that the effects of occupying the substrate binding site stabilizes the protein dimer far more than saturating calcium. At a protein concentration of 10 μM, which is in the protein concentration range where the effector molecules have their greatest impact and in a reasonable range for biochemical experiments, calcium binding shifts only 20% of the population to the active dimeric state. At this same protein concentration, HSF-modification shifts the population to over 60% dimeric. Together, at a protein concentration of 10 μM, the effector molecules stabilize a population that is over 80% dimeric.

It is also worth noting how these results might relate to the situation *in vivo*. *In vivo*, the active site of OMPLA is located in the outer leaflet of the outer membrane. This membrane leaflet is not composed of phospholipids but of lipopolysaccharides, and it has been proposed that OMPLA could serve a housekeeping function to degrade phospholipids when the bilayer asymmetry is perturbed.[10] This

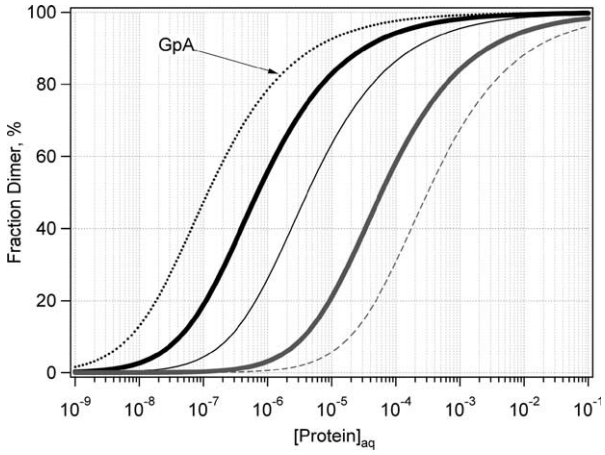

Figure 3. From the measured equilibrium constants, the fraction of dimer in 2.5 mM C14-SB can be calculated as a function of the aqueous protein concentration. The dotted line represents the upper limit to the population of OMPLA dimers in the absence of effector molecules. (---) Unmodified protein in the presence of 20 mM EDTA; (——) unmodified protein in the presence of 20 mM calcium; (——) HSF modified protein in the presence of 20 mM EDTA; (——) HSF modified protein in the presence of 20 mM calcium; (•••) glycophorin A. By comparison to the well-studied glycophorin A dimer, it can be seen that even the most stable OMPLA dimer is weaker than its helical counterpart.

proposal suggests a model where access to substrate is a key aspect of regulation, which is consistent with our thermodynamic observations that substrate is primarily responsible for promoting dimerization.

Neglecting the unusual composition of the outer membrane, one can make a crude approximation of the *in vivo* concentration of the OMPLA by roughly approximating the membrane as a spherical bilayer with a diameter of 1 μm and estimating the average surface area of lipid in the neighborhood of 60 Å^2.[35] It has been estimated that *Escherichia coli* contain about 500 copies of OMPLA,[10] so this gives a very rough estimate of the *in vivo* mole fraction of OMPLA on the order of 5×10^{-5}. Under the conditions of our experiments at 2.5 mM detergent, this mole fraction would equal a protein concentration of about 10^{-7} M. As can be seen in Figure 3, at this mole fraction OMPLA is predominantly monomeric even in the presence of both cofactors. However, it is likely that when OMPLA is restricted to the two dimensions of the bilayer the monomer–dimer equilibrium will be more favorable than what we have measured in detergent micelles, as is the case for the tetramerization of the M2 transmembrane helix.[36] A favorable shift in the equilibrium would then move things into a regime where substrate can significantly modulate the population. *In vivo* formaldehyde cross-linking suggests that OMPLA is monomeric in the *E. coli* outer membrane under normal physiological conditions, which implies that the equilibrium in bilayers is still weak overall and likely in a range where the protein dimerization can be modulated by substrate.

Magnesium does not support activity but it does support dimerization

Early biochemical studies exploring the metal requirements for OMPLA activity found that magnesium was not capable of supporting the enzyme activity and behaved as a competitive inhibitor.[31] One possible mechanism for the inhibition by magnesium is one in which magnesium can displace the catalytic calcium and also destabilizes the dimeric form of the protein. Our thermodynamic data clearly demonstrate that magnesium can be accommodated in the protein dimer without energetic consequence, and thus other factors underlie the lack of activity in magnesium. It is possible that magnesium can only be accommodated in the metal binding site when the small polar head group of HSF is present in the active site instead of the larger head group of a phospholipid substrate. This seems unlikely, however, since previous work has also shown that magnesium does not support activity towards inactivation by HSF.[31]

Alternatively, magnesium could act as an inhibitor by affecting water molecules in the active site of the protein. In the structure of the dimer (1QD6), the calcium ion in the active site appears to stabilize two water molecules that form the oxyanion hole (Figure 4). Magnesium (0.65 Å) has a much smaller ionic radius than calcium (0.99 Å)[37] and a shorter oxygen–ion (2.0–2.1 Å) distance than calcium (2.3–2.6 Å).[38] Therefore, it may be that when magnesium is bound to OMPLA, these water molecules are too far away to efficiently stabilize the negative charge on the tetrahedral transition state. Furthermore, magnesium has strict requirements for six ligands in an octahedral coordination, while calcium is less stringent, accommodating up to seven or eight ligands.[38] The coordination of calcium in the crystal structure is not octahedral (Figure 4), and therefore, when magnesium is bound to the protein it may rearrange its water ligands such that the oxyanion hole no longer has the correct geometry for catalysis.

Shape complementarity may be important for a stable membrane protein interaction

To date, the structure–energy relationships underlying transmembrane protein–protein associations have been systematically and rigorously studied in only a few helical membrane proteins, with the glycophorin A transmembrane (TM) helix dimer being the best characterized.[22,27,28] Our thermodynamic study of OMPLA self-association represents the first opportunity to compare the structure–energy relationships between a transmembrane α-helix dimer and a β-barrel dimer. Since the energetics of OMPLA and GpA dimerization have been determined experimentally in the same lipidic environment (2.5 mM C14-SB micelles), their stabilities can be directly compared. This energetic comparison shows that the glycophorin A TM

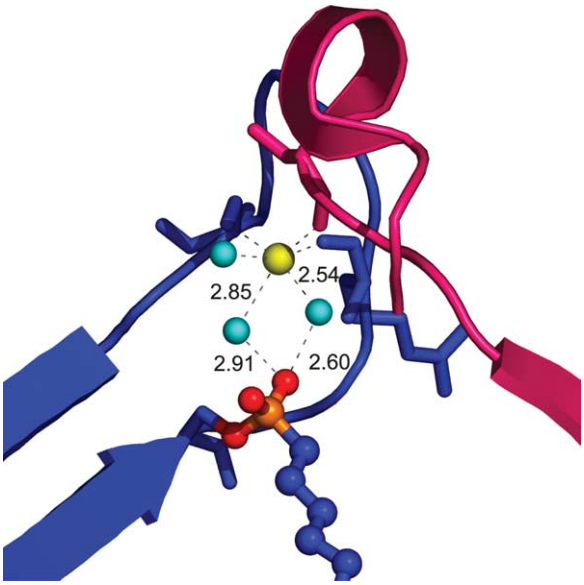

Figure 4. An illustration of the catalytic calcium-binding site in OMPLA (1QD6). The covalent inhibitor HSF is shown in ball and stick representation. Residues from the subunit to which the HSF is attached are shown in blue and the residues from the opposing subunit are shown in pink. The calcium ion is represented as a yellow sphere, and the coordinated water molecules that can be seen in the crystal structure are shown as cyan spheres. The calcium appears to coordinate two water molecules that could form the oxyanion hole that would stabilize the negative charge that develops on the tetrahedral intermediate during catalysis. This Figure was generated using Pymol Molecular Graphics System (http://www.pymol.org) and the distances are given in angstrom units.

dimer ($\Delta G_{app} = -9.30$ kcal/mol)[26] is at least 4.6 kcal/mol more stable than the OMPLA dimer (in the absence of effector molecules) and is still 1.1 kcal/mol more stable than even the strongest OMPLA dimer (the sulfonylated protein with 20 mM calcium).

Ultimately, we would like to understand how the stabilities of these dimeric complexes are determined by the accompanying changes in protein–protein interactions, protein–lipid interactions, and lipid–lipid interactions. Of these, particularly with the aid of high-resolution structures, protein–protein interactions are the easiest to characterize. High-resolution structures have been determined for both OMPLA and GpA, and therefore allowed us to explore the physicochemical and geometric properties of the protein–protein interface that may be correlated with and possibly predictive of stability. Such parameters are frequently used to characterize proteins in the soluble protein interaction field, and such characterizations have lead to empirical correlations that are useful, for example, in distinguishing physiological interfaces from interfaces that are artifacts of crystal contacts.[39,40]

It is likely that the relationships between such parameters and stability will be different in membrane proteins as compared to soluble proteins, especially since the hydrophobic effect, an important force in soluble protein interactions, will be lacking in membrane–protein interactions. In characterizing the interactions in the two classes of proteins, different parameters may turn out to be important. Given that packing interactions are known to be a key determinant of stability in membrane protein interactions,[27,28,41] it might be expected that the geometric parameters of the interface will be particularly relevant in characterizing transmembrane protein interactions. Therefore, we began our consideration of the structure–energy relationships of glycophorin A and OMPLA dimerization by first examining the extent of surface area buried at the interface and the shape complementarity of the protein surfaces.

Accessible surface area (ASA) is a frequently used parameter to characterize the size of the interacting surfaces of soluble protein interactions.[39–43] In soluble proteins, there is a weak correlation between the amount of accessible surface area buried at the interface and dimer stability, with more stable dimers burying more surface area.[40] Additionally, in membrane proteins, it has been demonstrated that the energetic cost of point mutations in helix–helix interactions is strongly correlated with changes in the buried accessible surface area.[41] Data from bacteriorhodopsin and GpA suggest that the burial of 38 Å^2 of accessible surface area provides about 1 kcal/mol of stability,[41] which is surprisingly close to the value from soluble protein folding of 40 Å^2 buried per 1 kcal/mol of stability.[44] The correlation between buried ASA and changes in energetics in membrane proteins can be interpreted as reflecting changes in packing and the accompanying van der Waals (VDW) interactions that result from the mutations.[41] A potential corollary to these results is that the extent of the transmembrane protein surface buried at the interface may be an indication of the stability of the interaction.

We have chosen to use an occluded surface (OS) algorithm to calculate the extent of molecular contact area at the interface of the OMPLA and GpA dimers.[27,28,45] Occluded surface is related to, but distinct from, the buried accessible surface area. While the radius of a water molecule is a logical probe size for ASA calculations in soluble proteins, it is not clear what this probe should be in membrane proteins where the protein is embedded in a heterogeneous lipidic environment. The OS algorithm is based on a molecular surface area not a solvent accessible surface area and therefore may be more sensitive to packing interactions in protein–protein complexes. Occluded surface area can be interpreted as representing the extent of surface area involved in VDW packing interactions. As with accessible surface area and consistent with the idea that membrane–protein interactions are stabilized largely by VDW interactions, the energetic cost

of point mutations has also been shown to be well-correlated with changes in occluded surface area, with mutational studies of GpA suggesting that 26 Å^2 of occluded surface area provides 1 kcal/mol of stability.[27,28] The crystal structures of monomeric (1QD5) and dimeric (1QD6) OMPLA suggest that there are no large structural rearrangements upon dimerization. Therefore, we used the crystal structure of the sulfonylated dimer to explore the protein–protein interface of both the unmodified and sulfonylated dimers. The OS for the dimers are as follows: 502 Å^2 for GpA, 1041 Å^2 for unmodified OMPLA, and 1506 Å^2 for sulfonylated OMPLA. Molecular dynamics simulations suggest that the binding cleft collapses in the absence of substrate, so the OS we calculate may represent a lower limit for the extent of the contact surface for the unmodified dimer.[46] Although the large net contact areas for the OMPLA dimers suggest extensive packing and favorable VDW interactions, the self-association constants of OMPLA are substantially weaker than that of GpA, suggesting that net contact area alone is not the sole determinant of dimer stability.

In comparing the different OMPLA dimers, the more stable sulfonylated dimer does occlude more surface area than the unmodified dimer. The stabilization is, however, far less than the −18 kcal/mol predicted by the mutagenesis studies.[27,28,41] The much smaller observed stabilization of −2.5 kcal/mol may reflect the entropic costs of restricting the otherwise freely rotating chain of the HSF moiety. Changes in the extent of contact area may still prove to be an important parameter in estimating the cost of point mutations, but total occluded surface area may not be as relevant in predicting absolute interaction stability.

One clue as to the weak stability of the OMPLA dimer, despite the extensive area of interacting protein surface, may come from the geometry of the protein interface. In Figure 5, the molecular surface is shown as a dot surface colored by ray length, and it illustrates the discontinuous nature of the OMPLA interface. It can also be seen that much of the protein surface does not contact the other subunit and that the interface contains a number of voids. One convenient method for quantifying the geometric complementarity of a protein–protein interface is to calculate a shape correlation statistic S_c.[47] An average protein–protein interaction has a value of $S_c = 0.70–0.76$,[47] and the non-physiological interfaces in crystal lattices are typically observed to have an $S_c = 0.55–0.60$ (D. J. Leahy, personal communication). The unmodified OMPLA dimer has a very low shape complementarity factor of only $S_c = 0.635$. This number is reflective of the apparent voids at the interface, which are likely costly to bury. The net energetic contributions of voids will depend in part on the nature of the interactions of those surfaces with lipids in the monomeric form. However, it has been shown that cavity-forming mutations in lysozyme can be well-described by two energy terms, one reflecting the difference in the free energy of transfer of the two

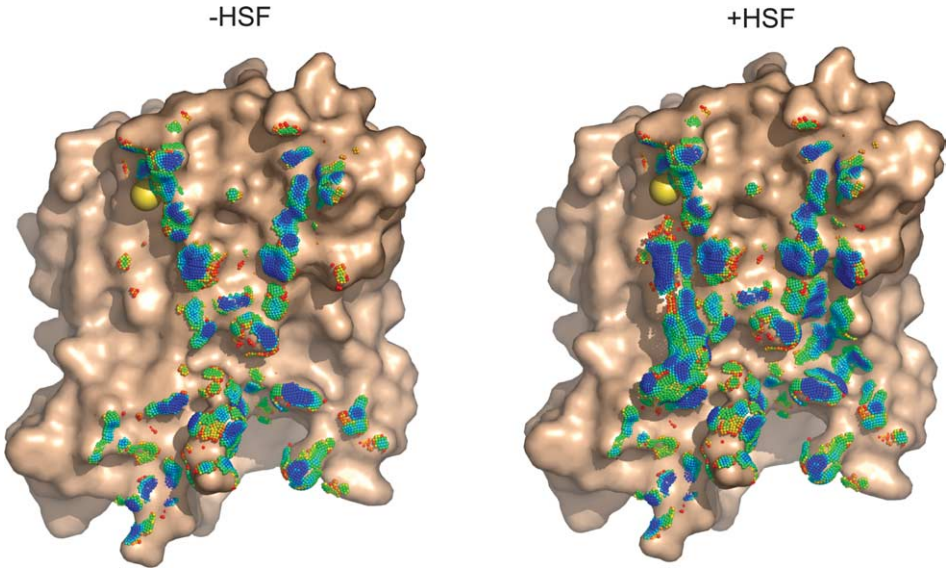

Figure 5. A single subunit of the HSF-modified OMPLA dimer (1QD6) showing a dot surface representation of the inter-monomer occluded surface. The dot surface is colored by ray length, with blue representing the closest molecular contacts and red representing the most distant contacts. (a) The dot surface on the left represents just the protein–protein contacts of the unmodified dimer. In the absence of substrate, the protein interaction surface is discontinuous, there are few close contacts, and much of the protein surface is not buried. The dot surface on the right represents the interface in the sulfonylated dimer (1QD6) and represents both protein–protein contacts and protein–substrate contacts. The additional surface represents the surface of the HSF molecule covalently attached to the subunit shown and the pocket into which the HSF from the opposing subunit would pack. Consistent with a more stable dimer, the interacting surface in the presence of the substrate analog is larger, more continuous, and includes more close contacts. These Figures were generated using Pymol Molecular Graphics System (http://www.pymol.org).

residues and a second term that depends on the size of the cavity created. This second term presumably reflects the loss of favorable VDW interactions.[48] Therefore, we hypothesize that this second term will still contribute to membrane protein interactions, and the effects of large to small mutations in helical membrane proteins are consistent with this idea.[27,41] Thus, we expect the interface of OMPLA to be destabilized relative to an interface with a higher shape complementarity and fewer cavities as compared to an interface in which a void is filled, for example by mutation to a larger residue. The low S_c value for OMPLA is in stark contrast to the optimized interface of GpA, which yields a relatively high S_c value, $S_c = 0.728$.

Figure 5 shows that the dimer interface in the presence of substrate analog is larger and more continuous. Although the value is still relatively low, the additional protein–acyl chain interaction surface from HSF-modification leads to a significant increase in the S_c value to $S_c = 0.667$. This improvement in the interface complementarity is consistent with the considerable stabilization of the dimer observed upon sulfonylation. The gap index, another measure of complementarity used in characterizing soluble protein interactions,[49] also reflects the trend seen in S_c. While all three proteins score well as compared to soluble protein interactions (average gap index = 2.0 Å),[40] the sulfonylated protein (1.03 Å) does better than unmodified OMPLA (1.3 Å) and GpA (0.3425 Å) does better than both OMPLA dimers. The intrinsic protein–protein interface in OMPLA is apparently not optimized for interaction, but instead seems to be optimized for interaction only in the presence of substrate. The dimer can nicely accommodate the acyl chain of a substrate molecule, and it is the interactions with the bound substrate that are primarily responsible for promoting the protein dimerization.

Conclusions

OMPLA has proven to be an amenable system for the biophysical study of the self-association of transmembrane β-barrels. We have successfully used sedimentation equilibrium to rigorously determine the association constants for this protein under a variety of conditions. We have determined that sulfonylation with a covalent substrate analog stabilizes the OMPLA dimer by −2.5 kcal/mol, a significantly larger energy than from the addition of the calcium co-factor (−1 kcal/mol). Our studies also suggest that the extent of buried surface area is not a sufficient descriptor of a stable transmembrane interaction, but that the geometric complementarity of the interface is also important. Quantitative measurements of self-association should prove valuable in elucidating the mechanism of OMPLA regulation, as well as begin to shed light on the association of TM β-barrels in general. Having a system that is well understood in detergent micelles will also aid in the development of methods for studying the association of OMPLA in a more native bilayer environment.

Materials and Methods

Expression and purification of OMPLA

The open reading frame for the mature OMPLA polypeptide, lacking its signal sequence, was PCR amplified from *E. coli* strain MG1655 and cloned into a pET-11a vector. The construct was verified by DNA sequencing and then transformed into HMS174(DE3) cells. The protein was expressed and purified as described before, with the exception that the cells were lysed by French press and C14-SB was substituted for C12-SB during the purification.[50] SigmaUltra grade C14-SB was purchased from Sigma.

HSF-modification of OMPLA

HSF was synthesized as described and the identity of the purified compound was confirmed by NMR.[31] SigmaUltra grade C12-SB was purchased from Sigma. 1.5–2 mg of purified refolded OMPLA was exchanged by ion-exchange chromatography using DEAE Sepharose FF into 3 ml of a buffer containing 20 mM Tris (pH 8.3), 400 mM KCl, and 5 mM C12-SB for labeling with HSF. The protein elution was diluted into a final reaction buffer of 20 mM Tris (pH 8.3), 200 mM KCl, 2.5 mM C12-SB, and 20 mM $CaCl_2$. A 12× molar excess of HSF from a 50 mg/ml solution in $CHCl_3$ was added to the reaction, which was then incubated and rotated overnight at room temperature.

The colorimetric substrate arachidonoyl-thio-PC (Cayman Chemicals), coupled with Ellman's reagent, was used to monitor OMPLA phospholipase activity. On average, 5–10 μg OMPLA was assayed in 200 μl of 250 mM Tris (pH 8.3), 20 mM $CaCl_2$, 2.5 mM C12, 1 mM arachidonoyl-thio-PC, and 1 mM DTNB. A loss of activity toward this substrate was used to confirm complete (>98%) modification of the protein with HSF (data not shown). To test the stability of the protein modification and to confirm that HSF was not significantly hydrolyzed during the time course of the sedimentation equilibrium experiments, sulfonylated OMPLA samples were prepared in conditions identical to that of the sedimentation equilibrium experiments. These samples were then monitored over several days for the reappearance of activity. Both in the presence and absence of calcium, the activities of the sulfonylated samples remained well below 2% of that for an equivalent unmodified protein sample for more than five days. This is consistent with a previous report that HSF moiety of the modified OMPLA was stable to hydrolysis for several weeks.[31] As with the unmodified protein, the HSF-modified protein was exchanged by ion-exchange chromatography into the buffer for the sedimentation equilibrium experiments (see below).

Sedimentation equilibrium analytical ultracentrifugation

Purified protein was exchanged into 20 mM Tris (pH 8.3) 7.5 mM C14-SB, 600 mM KCl by ion-exchange chromatography with DEAE Sepharose FF. Samples were then diluted to the appropriate concentration and a final

buffer composition of 20 mM Tris (pH 8.3), 2.5 mM C14-SB, 13% ^2H_2O, and either 20 mM $CaCl_2$, 20 mM $MgCl_2$, or 20 mM EDTA. ^2H_2O (13%) was included to make the density of the buffer equal to that of C14-SB, so that the contribution of the detergent to the buoyant molecular mass of the protein was negligible. Sedimentation equilibrium experiments were performed in a Beckman XL-A analytical ultracentrifuge using six-sector cells and sample volumes of 110 μl. Data were collected at 280 nm for three initial protein concentrations (10.0 μM, 6.6 μM, and 3.3 μM) and three rotor speeds (16,300 rpm, 20,000 rpm, and 24,500 rpm). Data were acquired at 0.001 cm intervals with ten replicates. Samples were centrifuged until equilibrium was reached, as determined by WINMATCH†. The density of the buffer, the partial specific volume of the protein, and the buoyant molecular mass of the protein were calculated using the software SEDNTERP.[51] The radial distribution profiles for the nine data sets for a particular condition were globally analyzed using the non-linear least-squares curve-fitting procedure of the program NONLIN[52] and equilibrium constants were converted to molar units using the molar extinction coefficient $\epsilon = 90{,}444\ \text{mol}^{-1}\ \text{cm}^{-1}$.[31]

Interface calculations

GpA calculations were done using model 7 from the GpA NMR structure (1AFO). OMPLA calculations were done using the 1QD6 PDB file, either including or deleting the HSF atoms for the sulfonylated or unmodified protein, respectively. The inter-chain occluded surface area was calculated using the OS 7.2.2 algorithm[45] using a maximum ray length of 2.8 Å. The surface shape complementarity of the interfaces were calculated using the ccp4 implementation of the S_c algorithm using default values.[49] The default atom radii for protein residues were used, and the carbon radius for the HSF molecule was set to 1.95. Figures were generated using PYMOL Molecular Graphics System‡. The gap index (Å) = gap volume (Å^3)/ΔASA of dimerization (Å^2) was calculated as described[49] using the program SURFNET[53] to calculate the gap volume and MSROLL[54] to calculate the ASA.

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† www.bbri.org/rasmb.

‡ DeLano, W. L. (2002). The Pymol Molecular Graphics System. http://www.pymol.org. DeLano Scientific, San Carlos, CA, USA.

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