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Energetics of Outer Membrane Phospholipase A (OMPLA) Dimerization

Ann Marie Stanley[1], Pitak Chuawong[2], Tamara L. Hendrickson[2] and Karen G. Fleming[1]*

[1]*T.C. Jenkins Department of Biophysics, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218 USA*

[2]*Department of Chemistry Johns Hopkins University 3400 North Charles Street Baltimore, MD 21218, USA*

**Corresponding author*

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.

E-mail address of the corresponding author: karen.fleming@jhu.edu

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]