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The Role of a Hydrogen Bonding Network in the Transmembrane β-Barrel OMPLA

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The hydrogen bonding of polar side-chains has emerged as an important theme for membrane protein interactions. The crystal structure of the dimeric state of the transmembrane β-barrel protein outer membrane phospholipase A (OMPLA) revealed an intermolecular hydrogen bond mediated by a highly conserved glutamine side-chain (Q94). It has been shown that the introduction of a polar residue can drive the association of model helices, and by extension it was presumed that the glutamine hydrogen bond played a key role in stabilizing the OMPLA dimer. However, a thermodynamic investigation using sedimentation equilibrium ultracentrifugation in detergent micelles reveals that the hydrogen bond plays only a very modest role in stabilizing the dimer. The Q94 side-chain is hydrogen bonded intramolecularly to residues Y92 and S96, but amino acid substitutions at these positions suggest these intramolecular interactions are not responsible for attenuating the strength of the intermolecular Q94 hydrogen bond. Other substitutions suggested that hydration of the local environment around Q94 may be responsible for the modest strength of the hydrogen bond. Heat inactivation experiments with the variants suggest that the Y92-Q94-S96 network may instead be important for thermal stability of the monomer. These results highlight the context dependence and broad range of interactions that can be mediated by polar residues in membrane proteins.

Keywords: OMPLA; hydrogen bond; membrane protein; oligomerization; analytical ultracentrifugation

Introduction

Over one-third of open reading frames are estimated to encode membrane proteins,[1] but the molecular basis of membrane protein stability is still not well understood. High resolution structural data are becoming increasingly available,[2] but in isolation, these structures tell us little about the energetics underlying the assembly process. A thermodynamic framework will be necessary in addressing questions of membrane protein folding and association and will be particularly important for dissecting the consequences of polar amino acids in these molecules. While membrane proteins are dominated by hydrophobic residues, they do contain a number of polar amino acids; these polar residues are often conserved, suggesting important structural and functional roles.[3,4] It is thermodynamically unfavorable to partition these residues into the membrane, but once partitioned, these residues have the potential to mediate energetically robust intermolecular hydrogen bonds. Understanding the energetics of side-chain hydrogen bonding will be essential to elucidating the molecular basis of membrane protein stability.

Calculations have suggested that the formation of an N-H···O=C hydrogen bond in an apolar environment could be worth as much as 4 kcal mol^{-1},[5] and indeed, model peptide studies have demonstrated

Abbreviations used: AUC, analytical ultracentrifugation; BR, bacteriorhodopsin; C14-SB, 3-(*N*,*N*-dimethylmyristyl-ammonio)propanesulfonate; C12-SB, 3-(dodecyldimethyl-ammonio)propanesulfonate; cmc, critical micelle concentration; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid); HEPC, 2-hexadecanoyl-1-ethylphosphorylcholine; HSF, hexadecylsulfonyl fluoride; OMPLA, outer membrane phospholipase A; TM, transmembrane; VDW, van der Waals; WT, wild-type.

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that the introduction of a single polar residue can be sufficient to drive the self-association of an otherwise non-interacting transmembrane (TM) helix. The peptides in these seminal studies were leucine-rich sequences with a designed interface that consisted of three valine residues and a single asparagine and were based on a water-soluble coiled-coil heptad repeat.[6,7] A combination of mutagenesis, biophysical, and biochemical methods demonstrated that the association of the peptides in both micelles and bilayers was entirely driven by intermolecular hydrogen bonding of the central asparagine residue.[6,7] When this residue was mutated to a valine, the peptide failed to associate.[6,7]

A subsequent series of host–guest experiments confirmed that other strongly polar residues were likewise capable of driving TM helix association.[4,8] These experiments found that Asp, Asn, Glu, and Gln residues were all able to drive the oligomerization of an otherwise monomeric peptide. Only one of the studies considered histidine; the His peptide did interact, but it was a weaker association than was found for the other polar residues.[4] Analytical ultracentrifugation (AUC) studies of Asp, Asn, Glu, and Gln containing helices found that the peptides assembled as trimers and that the stability conferred to the trimer by the polar residues was on the order of 3–6 kcal mol^{-1} per trimer.[8] Interestingly, each of these residues has a side-chain that can be a simultaneous hydrogen bond donor and acceptor. Neither Ser, Thr, nor Tyr, which each have only a single hydroxyl, was able to drive the association.[4,8] It is not clear whether this is a consequence of the single hydroxyl or whether the ability of serine and threonine residues to mediate interaction is affected by their ability to form intrahelical hydrogen bonds to main-chain atoms.[8,9] Nevertheless, as a consequence of these model peptide studies, it was recognized that the hydrogen bonding of polar side-chains could provide a potent driving force for membrane protein interaction, especially if the side-chain contains both a hydrogen bond donor and acceptor.

However, later work on naturally occurring sequences revealed a more complicated picture. While examples of interactions mediated by polar residues have been identified in naturally occurring TM helices including BNIP3 and TNF5,[10,11] work from the Engelman group identified several TM sequences from monotopic membrane proteins that contained a strongly polar residue but did not associate.[11] Polar residues in single pass membrane proteins had been expected to be particularly likely to mediate oligomerization, since the residue must be lipid-exposed in the monomer, but this turned out not to be the case. In yet another sequence, they found that the introduction of a polar residue only drove association when placed on the same side of the helix as a GXXXG motif (where X can be any amino acid), a sequence that is known to promote close interhelical packing.[11] These results suggested the rest of the sequence presented a steric barrier to the hydrogen bonding interaction. This work highlighted that the interaction of polar residues in membrane proteins was sensitive to the surrounding sequence context in ways not identified in the model peptide studies. It also underscored that the energetics of side-chain hydrogen bonds were not so dominated by the low dielectric of the membrane as to be able distort the helix or otherwise necessarily overcome barriers to association presented by neighboring residues.

Furthermore, recent work from the Bowie group on the stability of bacteriorhodopsin (BR) suggested that interactions are not always energetically significant even when side-chain hydrogen bonds can form.[12] Using an SDS-unfolding assay, they measured the helix–helix stability for a series of BR alanine mutants. Despite high resolution structural data indicating a number of the wild-type (WT) side-chains were involved in hydrogen bonds, the energetic contribution of these residues to stability could be largely accounted for by the extent to which they were involved in intermolecular van der Waals (VDW) packing interactions. This result led to the conclusion that the hydrogen bonds did not provide a significant additional contribution to stability.[12] Reasons for this result are unclear; the authors suggested that suboptimal geometry could in part be responsible for attenuating the strength of the hydrogen bond interactions. Alternatively, the only polar residues that can be both hydrogen bond donors and acceptors, examined in the BR study, were located at the termini of helices where they are likely water-exposed, particularly in the SDS-unfolded state.[12]

It is evident that polar residues in membrane proteins can mediate a broad range of interactions that we are only beginning to understand, and elucidating how context mediates these interactions will be important for understanding the roles of polar amino acids in membrane proteins. Insight into the interactions of polar residues will also be essential in identifying the mechanisms by which non-native polar amino acids in membrane proteins can lead to disease: in some cases, non-native hydrogen bonds have been implicated as in the cystic fibrosis transmembrane regulator (CFTR) V232D mutation[13,14] or the V664E substitution in the oncogene *neu*;[15] whereas they are not implicated in other cases such as the fibroblast growth factor recptor (FGFR) G380R achondroplasia mutation.[16] Understanding the energetics of polar residues in membrane proteins will also facilitate protein design and structure prediction. At this time, it is not possible to predict the consequence of introducing a polar residue into a membrane protein sequence.

To further explore the role of polar amino acids in membrane proteins, we have investigated a highly conserved glutamine residue in a unique transmembrane β-barrel outer membrane phospholipase A (OMPLA). OMPLA is a phospholipase found in Gram-negative bacteria involved in remodeling the outer membrane under conditions of stress.[17] Dimerization of the protein is essential for enzyme activity and is stabilized by the binding of calcium and substrate.[18,19] When the crystal structure of an OMPLA dimer was solved, it revealed an intermolecular hydrogen bond at the dimer interface that was

formed by a glutamine side-chain.[20] This glutamine residue (Q94) is absolutely conserved among OMPLA sequences.[21] Furthermore, an alanine substitution at this position can disrupt glutaraldehyde cross-linking, decrease enzyme activity 4X, and reduce calcium affinity.[22] Therefore, the hydrogen bond at Q94 was generally considered to be a key protein–protein interaction that stabilized the dimer, but its thermodynamic contribution to dimer stability has never been directly addressed. Q94 is also found in an atypical polar patch of the barrel and is involved in intramolecular H-bonds with neighboring side-chains, something that is not observed in the monotopic model helices that have been used extensively to address the role of polar side-chains in membrane proteins; *a priori* it is unclear what effect this could have on the energetics of interaction. Using a combination of mutagenesis, biochemistry, and biophysics, we directly addressed the energetics of Q94 and its neighboring side-chains. We find they contribute surprisingly little to the free energy of dimerization, but rather may be important for the stability of the monomer.

Results

Q94 contributes relatively little to the dimer stability

Studies of transmembrane helices have shown that intermolecular hydrogen bonds between strongly polar side-chains can significantly stabilize helix-helix interactions.[4,6–8] The crystal structure of the OMPLA dimer suggested that a highly conserved glutamine (Q94) in the hydrophobic barrel region was mediating a similar interaction in this protein (Figure 1).[20] To directly investigate the energetic contribution of the intermolecular hydrogen bond to dimer stability, we initially substituted an alanine at position 94 (Figure 1) and used sedimentation equilibrium AUC to determine the impact of this substitution on the dimerization reaction. We have previously used sedimentation equilibrium to determine the free energy of OMPLA dimerization under four conditions: (1) in the absence of any effector molecules (monomer); (2) in the presence of calcium, a required co-factor for catalytic activity; (3) after covalent modification with a substrate analog, hexadecyl sulfonyl fluoride (HSF), but in the absence of calcium; (4) after covalent modification with HSF and in the presence of calcium.[19] We measured the free energy of dimerization for the Q94A variant under each of these four conditions and compared the results to that for WT (Figure 2; Table 1).

These effect of the alanine substitution on OMPLA dimerization was small in comparison with model helix studies in which it had been shown that the removal of a glutamine residue could shift a protein from almost completely trimeric to completely monomeric,[8] and was dependent on the condition under which we measured dimerization. The largest

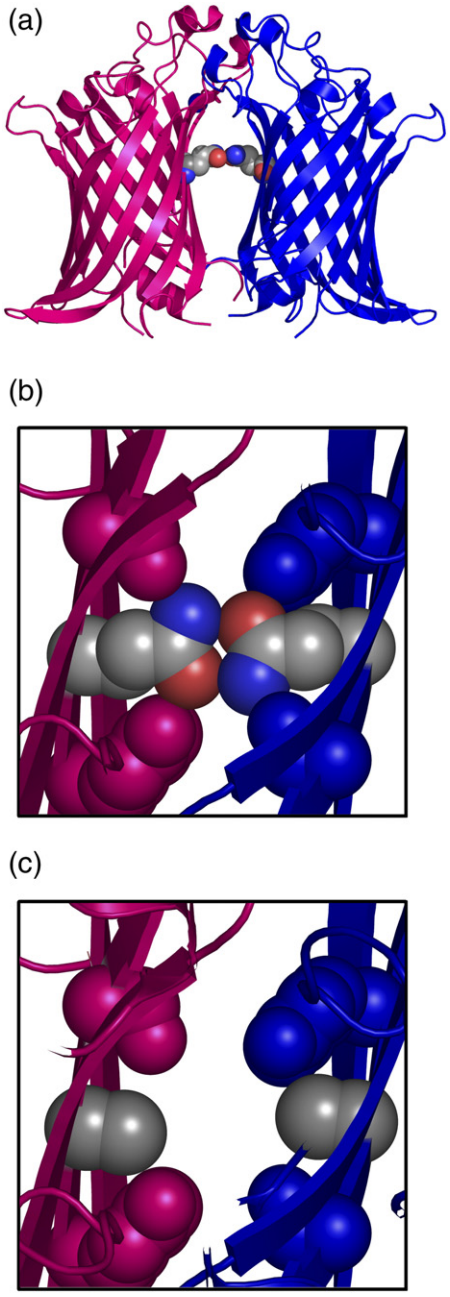

Figure 1. The intermolecular hydrogen bond at Q94. (a) The backbone of the OMPLA dimer (1QD6) is shown in ribbon form with Q94 shown in space-filling representation. Q94 is colored according to atom type with carbon in gray, nitrogen in blue, and oxygen in red. (b) A top-down view of the intermolecular hydrogen-bond at Q94. Q94 is shown in space-filling representation and colored according to atom type with carbon in gray, nitrogen in blue, and oxygen in red. The neighboring residues, tyrosine 92 and serine 96 are also shown in space-filling representation. These residues are colored according to the subunit to which they belong. (c) A view of position 94 where an Ala substitution has been made using Pymol. This model has not been energy minimized and in the actual protein the gap may be smaller. However, this model emphasizes the loss of packing interactions and represents the largest disruption the Q94A substitution could have. Figures were created using the Pymol molecular graphics system [http://www.pymol.sourceforge.net/].

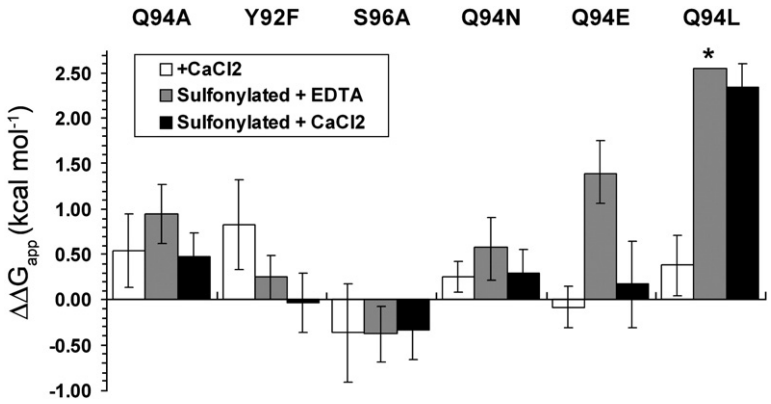

Figure 2. $\Delta\Delta G_{app}$ values for dimerization of OMPLA variants. The change in the free energy of dimerization relative to WT is reported for six single amino acid variants of OMPLA. The free energies of dimerization were measured using sedimentation equilibrium analytical ultracentrifugation under four conditions: (1) in the absence of any effector molecules (all proteins were monomeric), not shown in the graph); (2) in the presence of calcium, a required co-factor for catalytic activity (white); (3) after covalent modification with a substrate analog, hexadecyl sulfonyl fluoride (HSF), but in the absence of calcium (gray); (4) after covalent modification with HSF and in the presence of calcium (black). The $\Delta\Delta G_{app}$ values are reported in kcal mol^{-1} and represent the average of at least three independent experiments. The error bars represent the standard deviation for $\Delta\Delta G_{app}$ calculated as described in Materials and Methods. *Q94L was monomeric in the absence of calcium (gray bar); therefore, the value that represents only a lower limit to the $\Delta\Delta G_{app}$ and no error is reported.

destabilization we observed for the Q94A variant was for the sulfonylated protein in the absence of calcium (Figure 2). Under these conditions, dimerization of the alanine variant was destabilized by $\Delta\Delta G_{app} = 0.95(\pm 0.32)$ kcal mol^{-1}. However, in the presence of calcium, the cost of the alanine substitution was even smaller, with $\Delta\Delta G_{app} = 0.54(\pm 0.40)$ kcal mol^{-1} for the unmodified protein and $\Delta\Delta G_{app} = 0.48(\pm 0.26)$ kcal mol^{-1} for the sulfonylated protein. These modest energies do not result in a drastic shift in the population of dimer. Under the condition that most closely mimics active OMPLA (+HSF and +$CaCl_2$), the oligomeric distribution behaves very much like wild-type (Figure 3). These data suggest that the interaction at Q94 does not behave like the isolated side-chain hydrogen bonds in model helices and instead suggests that the local environment is influencing the energetics.

Disrupting an intramolecular hydrogen bonding network does not affect dimer stability

The strength of a hydrogen bond can be influenced by sequence context, and in the OMPLA crystal structures glutamine 94 is also hydrogen-bonded to neighboring residues on strand 3, tyrosine 92 and serine 96 (Figure 1(b)). Like Q94, Y92 and S96 are extremely well-conserved among OMPLA sequences,[21] and we hypothesized they could be important for mediating the strength of the intermolecular hydrogen bond at Q94, analogous to the way intramolecular hydrogen bonds are proposed to affect the ability of serine and threonine side-chains to mediate association. To investigate their effect, we made single amino acid substitutions (Y92F and S96A) to eliminate the intramolecular hydrogen bonding partners.

We expected it would be more energetically unfavorable to have Q94 exposed to detergent in the absence of the intramolecular hydrogen bonding partners, and that this would thereby drive the protein to bury Q94 at the protein–protein interface where its hydrogen-bonding potential could be satisfied. However, under one condition (−HSF+$CaCl_2$), the Y92F substitution was actually slightly destabilizing, and under the rest it had no effect (Figure 2). Similarly, the free energies of dimerization of the other variant, S96A, were all within error of wild-type (Figure 2). We therefore concluded that the intramolecular hydrogen bonding network plays no

Table 1. Free energies of dimerization for OMPLA variants in 2.5 mM C14-SB

	(1) Unmodified + 20 mM EDTA	(2) Unmodifed + 20 mM $CaCl_2$		(3) Sulfonylated + 20 mM EDTA		(4) Sulfonylated + 20 mM $CaCl_2$	
		ΔG_{app}	$\Delta\Delta G_{app}$	ΔG_{app}	$\Delta\Delta G_{app}$	ΔG_{app}	$\Delta\Delta G_{app}$
Q94A	Monomer	−5.13±0.38	0.54±0.40	−6.30±0.23	0.95±0.32	−7.83±0.05	0.48±0.26
Y92F	Monomer	−4.85±0.48	0.83±0.50	−7.00±0.07	0.25±0.24	−8.34±0.21	−0.04±0.33
S96A	Monomer	−6.04±0.53	−0.36±0.54	−7.63±0.21	−0.38±0.31	−8.64±0.23	−0.33±0.34
Q94E	Monomer	−5.76±0.18	−0.18±0.23	−5.86±0.22	1.39±0.36	−8.14±0.40	0.17±0.47
Q94N	Monomer	−5.46±0.09	0.22±0.17	−6.67±0.07	0.58±0.33	−8.02±0.09	0.29±0.27
Q94L	Monomer	−5.35±0.34	0.33±0.37	Monomer	≥2.5	−5.97±0.04	2.34±0.25

The apparent molar free energies of dimerization in 2.5 mM C14-SB detergent determined by analytical ultracentrifugation are reported for the OMPLA variants under each of four conditions. The values reported represent the average and standard deviation of at least three experiments. The $\Delta\Delta G_{app}$ values and errors were calculated as described in Materials and Methods.

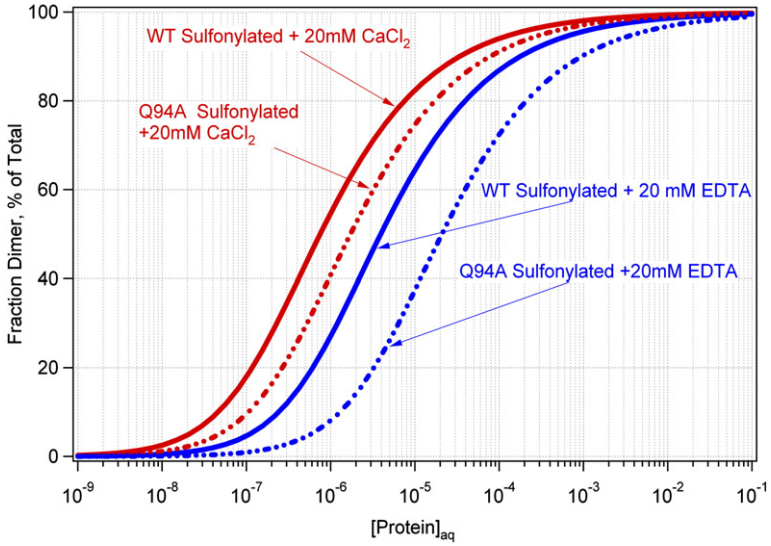

Figure 3. Dimer distribution curves for Q94A OMPLA in 2.5 mM C14-SB. From the measured ΔG_{app} values for dimerization, the fraction dimer was calculated as a function of the aqueous protein concentration. Population distributions are shown for sulfonylated WT OMPLA (continuous line) and sulfonylated Q94A (broken line) in the presence of calcium (red) and in the absence of calcium (blue). The sulfonylated protein is presumed to mimic a substrate-bound dimer. It can be seen that the Q94A substitution has little effect on the population of dimer, particularly in the presence of calcium.

role in modulating the stability of the OMPLA dimer.

Sedimentation equilibrium AUC experiments reveal dimerization can tolerate other polar residues at position 94 but not a highly hydrophobic residue

To further explore the specificity of the Y92-Q94-S96 network with regards to dimer stability, we made three more amino acid substitutions at position 94. We mutated Q94 to the closely related carboxy-amide side-chain, asparagine, to the more strongly polar and ionizable side-chain glutamic acid, and to the hydrophobic side-chain leucine to ask what features of the side-chain features were important for dimerization. Since Asn has the same hydrogen-bonding potential as Gln, one possibility was that the asparagine substitution could support association to the same extent as glutamine and possibly even enhance it because it would pay less of side-chain entropy cost upon dimerization. When purifying Q94N OMPLA, we noticed that the protein migrated as two bands on an SDS–PAGE gel, a lower band consistent with the folded monomer and a second upper band at 35 kDa (Figure 4). The upper band migrates in the same position as the folded dimer that is seen on SDS–PAGE after HSF modification or after covalent cross-linking, so initially it appeared that Q94N was capable of dimerizing in the absence of effector molecules. By pre-incubating the Q94N samples with increasing concentrations of 3-(*N*,*N*-dimethyl-myristyl-ammonio)propanesulfonate (C14-SB) detergent, we were able to collapse the two Q94N bands to a single band for folded monomer (Figure 4). Since increasing the C14-SB detergent concentration could dissociate the Q94N dimer band, these results suggested that the two bands on SDS–PAGE represented a reversible monomer–dimer equilibrium for Q94N in the absence of calcium or HSF.

However, sedimentation equilibrium experiments in pure C14-SB detergent revealed that dimerization of the Q94N variant was not stabilized in solution (Figure 2). Like the WT protein, Q94N was monomeric in solution in the absence of effector molecules, and under the other conditions, the free energies of dimerization were practically WT-like (Figure 2). The discrepancy was unexpected because typically the stability trends from SDS–PAGE typically correlate with the results from sedimentation equilibrium AUC.[23,24] However, one important difference between the SDS–PAGE assay and the sedimentation equilibrium conditions is the presence of the SDS in the PAGE assay. To explore this possibility that the Q94N dimer band was SDS induced, we added a small amount of SDS to our sedimentation equilibrium sample. With the addition of 0.3 mM SDS, there was detectable dimer for unmodified Q94N even in the absence of effector

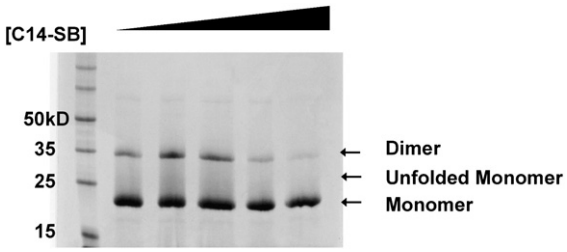

Figure 4. Association of Q94N OMPLA on SDS–PAGE. Q94N migrates as two bands on SDS–PAGE: a lower band consistent with a folded monomer and an upper band consistent with a folded dimer. The protein in the upper band can be dissociated by pre-incubating the protein with increasing concentrations of C14-SB before loading the sample on the gel. A total of 4 μl at a protein concentration of 0.94 mg ml^{-1} pre-incubated at increasing concentrations of C14 was loaded for each lane onto the 12.5% acrylamide Phast Gel. The lanes from left to right correspond to the following samples: (1) molecular mass markers; (2) 5.25 mM C14-SB; (3) 7.25 mM C14-SB; (4) 9.25 mM C14-SB; (5) 11.25 mM C14-SB; (6) 15.25 mM C14-SB.

molecules. The amount of dimer increased when the SDS concentration was increased to 0.6 mM (data not shown), confirming that SDS must be binding to and stabilizing the dimer. The fact that such a conservative substitution, such as glutamine to asparagine, could so markedly affect the SDS binding properties of a membrane protein was surprising and should be noted. It emphasizes the need to independently confirm oligomers identified on SDS–PAGE because these data suggest that SDS may not always be a passive component of the solvent.

The next substitution to glutamic acid also yielded unanticipated results. Protonated glutamic acid has a similar hydrogen-bonding pattern and similar polarity to asparagine and glutamine, but the carboxylic acid side-chain also has the potential to ionize. While Q94E did not affect the dimer stability in the presence of calcium, it was significantly destabilizing in the absence of calcium (Figure 2). As discussed further below, we postulate that these results are an indication that the Glu side-chain is incorporated in an ionized state. The results also indicate the OMPLA dimer has the potential to tolerate not just the carboxy-amides at position 94 but acidic side-chains as well.

Our final substitution of leucine to glutamine eliminated the polar character of side-chain at position 94. Substituting the roughly isosteric leucine allows us to ask if the OMPLA dimer requires something about the size of glutamine or whether the polar characteristic or hydrogen bonding character was also important. Surprisingly, the Q94L substitution strongly destabilized OMPLA dimerization, even more so than the initial alanine substitution, particularly in the +HSF background (Figure 2). Sulfonylated Q94L was completely monomeric in EDTA, and in calcium, dimerization was destabilized by $\Delta\Delta G_{app} = 2.34(\pm 0.25)$ kcal mol^{-1}. While the effect of Q94L was only $\Delta\Delta G_{app} = 0.33(\pm 0.37)$ kcal mol^{-1} for the unmodified protein in the presence of calcium, the WT interaction is already quite weak under these conditions, so it is not possible to detect a destabilization greater than 1 kcal mol^{-1}. Overall, the sedimentation equilibrium data suggest that the OMPLA dimer cannot tolerate a hydrophobic residue at position 94, and that the polar character of the side-chain is important.

Disrupting the hydrogen-bonding network at the dimer interface has little effect on enzyme activity

In light of the sedimentation equilibrium data demonstrating that a number of substitutions could be introduced at the highly conserved Y92-Q94-S96 network without destabilizing the dimer, we reconsidered the role of these amino acids in OMPLA. Dimerization is an important aspect of OMPLA function, but the sequence of this network could be conserved for its role in other aspects of function. For example, Y92 interacts with the substrate;[20] thus, substitutions in the hydrogen bonding network could affect OMPLA's affinity for substrate, enzyme kinetics, or other parameters that we cannot detect in our equilibrium centrifugation experiments. Therefore, while conservative substitutions in the Y92-Q94-S96 network could be made without affecting dimerization, it may be that these substitutions affect other aspects of the enzyme activity. To test the effect of these substitutions on OMPLA's phospholipase activity, we used a previously described colorimetric assay that monitors the cleavage of a thio-ester lysophospholipid, 2-hexadecanoylthio-1-ethylphosphorylcholine (HEPC).[18,25]

We initially tried the activity assays in 0.2 mM Triton as reported by Dekker *et al*. We measured specific activities ($U = 45$–75 μmol min^{-1} mg^{-1} for WT) that were on the order of what Dekker *et al.* reported ($U = 65$–85 μmol min^{-1} mg^{-1}). However, we found that we had large variability in our measurements in Triton. We therefore developed a protocol for the activity assay in C14-SB. Although our overall activities were lower, we found the new protocol to be more reproducible. It offers a number of other advantages for the experiment as well: (1) it reduces the heterogeneity in the assay mixture by minimizing the number of components because samples were already pre-incubated in 2.5 mM C14-SB (see Materials and Methods) and (2) performing the activity assay entirely in C14-SB allows a more direct comparison of specific activity with our thermodynamic data, which were also measured in C14-SB.

We measured the activity of our OMPLA variants under two conditions: (1) in an assay buffer of 2.5 mM C14-SB, the detergent concentration used in our sedimentation equilibrium experiments; and (2) in an assay buffer of 0.2 mM C14-SB, which is in the range of the critical micelle concentration (cmc) of C14-SB (0.1–0.4 mM).[26] In the activity assays by Dekker *et al.*, they measured the highest specific activities near the detergent cmc. For all OMPLA variants, we measured higher specific activities at the lower detergent concentration (Figure 5). At detergent concentrations near the cmc, we expect a maximal population of dimer, whereas the higher detergent concentration promotes dissociation of the active dimeric state of the enzyme and decreases activity.

The Q94L substitution, which had shown the greatest impact on dimerization, also showed the greatest impact on activity as expected. The Q94A variant also affected activity, but the effect was modest (Figure 5). Under the high detergent condition, the specific activity of the Q94A variant was approximately twofold lower than WT levels, which is on the order of the fourfold decrease in activity previously reported by Kingma *et al*. However, at the low detergent concentration, the Q94A substitution had no effect on activity. These results suggest that the decrease in activity seen for Q94A at higher detergent concentrations is the result of the modest destabilization of the protein dimerization and not effects on substrate binding, because lowering the detergent concentration, which promotes protein association, can rescue full activity of the variant.

Interestingly, the more conservative H-bond substitutions (Y92F, Q94N, Q94E and S96A) had no

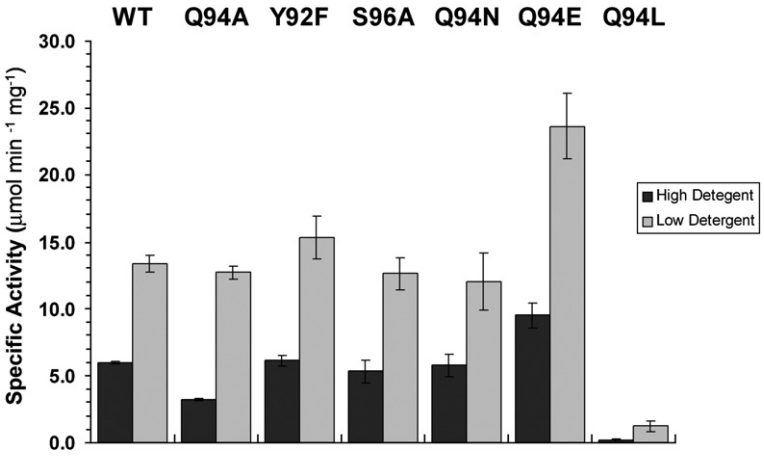

Figure 5. Specific activities of OMPLA variants. The specific activities of the OMPLA variants from this study toward the thioester-containing substrate HEPC measured using a DTNB colorimetric assay. The activity of each protein was measured under two conditions, one in which the assay buffer contained 2.5 mM C14-SB detergent (dark grey bars) and one in which the assay buffer had only 0.2 mM C14-SB detergent (light grey bars). The numbers reported represent the average of three independent measurements and the standard deviations of the measurements are given by the error bars. For all variants, the enzyme activity was increased at low detergent concentration. Consistent with our dimerization data, only Q94L significantly impaired activity.

deleterious effects on activity (Figure 5), suggesting the specificity of the Y92-Q94-S96 hydrogen bonding network may not be important for structuring the binding pocket as we had hypothesized. In fact, Q94E actually enhanced enzyme activity about twofold relative to WT levels. Q94E is positioned about 10 Å from OMPLA's active site serine, so it is hard to imagine how it can have a direct molecular influence, but perhaps Q94E indirectly affects water or calcium-binding at the active site. We also found that the addition of 0.6 mM SDS to the activity buffer did increase the activity of Q94N, consistent with a role for SDS stabilizing the dimer ($U = 28.8(\pm 3.4)$ μmol min^{-1} mg^{-1}, 0.2 mM C14-SB). This result indicates that the activity of Q94N was not impaired in the presence of SDS and suggests that SDS is not stabilizing the Q94N dimer by binding in the active site pocket.

Disrupting the Y92-Q94-S96 hydrogen bonding network destabilizes the thermal stability of OMPLA

Consistent with the earlier biochemical work showing Q94A impaired OMPLA function,[22] our investigations of OMPLA dimerization and activity suggested that alanine could not substitute for glutamine at position 94 because of relatively moderate decreases in dimerization and activity. Similarly, the Q94L substitution is clearly not found in OMPLA sequences because it significantly destabilizes dimerization and greatly reduces activity. However, the question still remained why even conservative substitutions like Y92F or Q94N, which do not impair dimerization or activity, are not found in OMPLA sequences. During the course of this work, we had noticed that the variants that disrupted the OMPLA intramolecular hydrogen-bonding network were more prone to unfold over time than WT OMPLA. This led us to hypothesize that the high evolutionary conservation of this intramolecular network might be in part due to a role in protein stability.

To test this more directly, we measured the susceptibility of the OMPLA variants to thermal denaturation. As reversible folding conditions have been identified for very few membrane proteins,[12,27,28] thermal stability is often used as a qualitative metric of membrane protein stability.[29–32] We included SDS in thermal unfolding experiments to keep the unfolded protein in solution and to lower the melting temperature, making the thermal unfolding more experimentally accessibly. The thermal unfolding experiments were done using the unmodified protein in the presence of EDTA to ensure the unfolding samples were completely monomeric, so that the effects on the monomer stability could be separated from effects on dimerization. We monitored the fraction of protein that remained folded after 10 min at 80 °C using an SDS–PAGE assay. Like many transmembrane β-barrels, folded OMPLA retains a compact structure in SDS–PAGE and migrates as a distinct band from unfolded OMPLA. This allows us to measure the relative populations of the two forms of OMPLA by comparing the relative intensities of

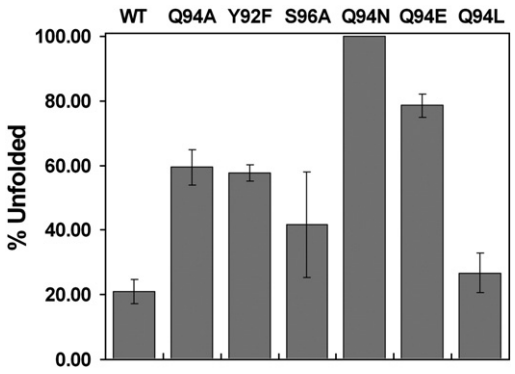

Figure 6. Thermal denaturation of OMPLA variants. The fraction of OMPLA that is unfolded after 10 min of thermal inactivation at 80 °C in the presence of SDS is reported. The population of unfolded protein was determined by measuring the relative intensities of folded and unfolded bands on SDS–PAGE after quenching the thermal inactivation. OMPLA is very thermostable, and even after 10 min at 80 °C in the presence of SDS, a large majority of the protein remains folded. However, substitutions at the Y92-Q94-S96 hydrogen bonding network increase the susceptibility of OMPLA to thermal denaturation.

the two bands. All of the substitutions at Y92-Q94-S96, with the exception of Q94L, significantly increased the fraction of unfolded protein after 10 min of thermal inactivation, consistent with a role for this region in monomer stability (Figure 6).

One particularly interesting result from the thermal denaturation experiment was the complete unfolding of Q94N under conditions where the wild-type protein was still approximately 80% folded. Although the unfolding conditions were chosen to ensure the proteins were monomeric, we know that SDS binds to and stabilizes the Q94N dimer. We therefore expected that Q94N might be stabilized relative to wild-type in the thermal denaturation assay because of the presence of the SDS in the assay buffer. However, Q94N was the most destabilized of our variants. Therefore, SDS can bind to and stabilize the dimer but it cannot rescue the thermal stability of the Q94N monomer.

Discussion

Hydrogen bonding at glutamine 94 plays a modest role in dimer stability

On the basis of sequence conservation and the crystal structure of the OMPLA dimer (1QD6), it was presumed that the primary role of glutamine 94 was to stabilize the dimer through the formation of an intermolecular hydrogen bond. The formation of such a hydrogen bond in an apolar environment can be quite favorable. Model compound studies and simulations of polyalanine helices suggest the formation of an N-H···O=C hydrogen bond in a hydrophobic environment can be worth as much as 2.5–4 kcal mol^{-1}.[33,34] Furthermore, the introduction of a asparagine residue can stabilize the trimerization of a transmembrane helix by as much at 6 kcal mol^{-1}.[8,35]

However, the strength of a hydrogen bond can be influenced by factors including sequence context and the local environment, and the local environment around Q94 is distinct from that of the model peptides previously used to study membrane protein hydrogen bonds. For example, in the OMPLA dimer, Q94 participates in intramolecular side-chain hydrogen bonds with Y92 and S96. Such networks are not seen in model monotopic helices. By satisfying the hydrogen-bonding potential of Q94 in the monomer, these residues could make the dissociated state more energetically favorable. Consistent with an important functional role for the hydrogen bonding network, residues Y92 and S96, like Q94, are very highly conserved among OMPLA sequences.[21] Furthermore, the crystal structure of the OMPLA dimer also suggests that Y92-Q94-S96 polar patch could be hydrated in the dimer. As illustrated in Figure 7, position 94 is actually partially surface exposed in the dimer and water molecules penetrate close to position 94 with one water molecule even hydrogen bonded to residue 96. Neutron diffraction studies of crystals of monomeric OMPLA indicate a reduced detergent density in the vicinity of Q94.[36]

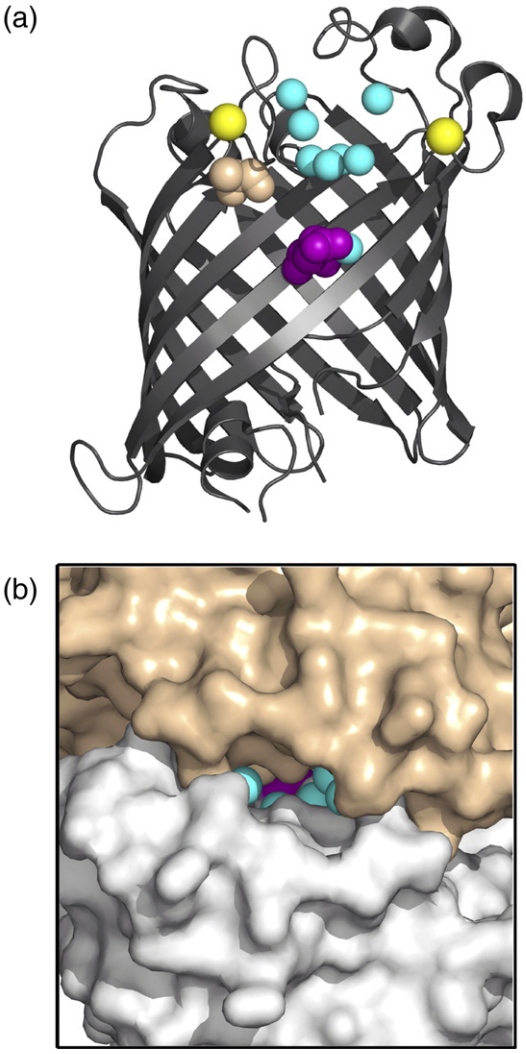

Figure 7. Water penetration in the dimer interface near Q94. (a) One of the monomers of the OMPLA dimer (1QD6) is shown in ribbon format as viewed from the dimer interface. Glutamine 94 is shown in space-filling representation and colored purple. The calcium ions are shown for both active sites in yellow space-filling representation for orientation purposes. The active site serine of the monomer that is shown is also represented in a space-filling format and is colored wheat. The crystallographic water molecules that penetrate the dimer interface near Q94 are shown as cyan spheres. (b) A top-down view of the dimer interface. The OMPLA protein surface is shown in wheat for one monomer and white for the other. Q94 remains colored purple and the crystallographic water molecules are shown as cyan spheres. This Figure illustrates the solvent exposure of Q94 and the potential hydration of the site.

In these respects, the local environment of the barrel around Q94 seems to resemble the membrane interface more than the hydrocarbon core. The interface is more chemically heterogeneous, more hydrated, and more polar than the membrane core with groups present capable of hydrogen bonding to a protein.[37] Thus, the formation of protein–protein hydrogen bonds in interfacial regions of the membrane is less energetically favorable than in the

apolar hydrocarbon core. For example, while backbone hydrogen bonds are estimated to be worth on the order of several kcal mol^{-1} in the hydrocarbon core, model peptide work demonstrates these same hydrogen bonds are worth only about 0.5 kcal mol^{-1} per residue in the more polar interfacial regions of the membrane.[38,39]

Our data indicate that although Q94 is in the barrel region of OMPLA, which is expected to be membrane embedded, the strength of the interaction at Q94 is indeed more consistent with an interfacial-like environment. The Q94A substitution destabilized the dimer about ~0.5 kcal mol^{-1} instead of the several kcal mol^{-1} that would be predicted for an isolated H-bond in an apolar environment. Our subsequent experiments indicated though that the energetics we observed were not attributable to specific intramolecular bonds to the conserved side-chains Y92 and S96, because respective phenylalanine and alanine substitutions at these positions had little effect on the free energy of dimerization. Instead, it seems the energetics may be reflecting hydration the region in Q94.

Hydration of the region around Q94 could also help to explain the relative plasticity of the Y92-Q94-S96 hydrogen bonding network in regards to dimer stability, because water molecules could compensate for and satisfy the hydrogen bonding interactions that are disrupted upon mutation. Furthermore, hydration of this region could offer an explanation for the unexpected energetics we observed for Q94E and Q94L. We found that the Q94E dimer was strongly destabilized in the absence of calcium, but when the divalent cation was included in the buffer, dimerization was restored to wild-type levels. The simplest interpretation of these results is that the glutamic acid is ionized; in the absence of calcium, the charge–charge repulsion could destabilize the dimer, but in the presence of calcium, the divalent cation could interact with and neutralize the charges, rescuing dimerization. Deprotonating a glutamic acid in an environment that resembled the hydrocarbon core of a micelle or membrane would be very energetically unfavorable. It is also expected that dehydrating a calcium ion or a charged glutamic acid would be very energetically unfavorable. However, in a hydrated environment, the scenario is more energetically feasible. Therefore, it does seem likely that residue 94 is in a microenvironment into which water can penetrate. As an aside, the unanticipated calcium-dependence of the Q94E dimerization also raises an interesting design possibility in which an acidic residue could be introduced into the interfacial region of the membrane protein to engineer an association that could be switched on by the addition of calcium.

In the case of Q94L, it might have been expected that the leucine would be well-tolerated because leucine residues are frequently found in membrane proteins,[40,41] and in fact several of the other residues at the dimer interface close to Q94 are leucine residues. Leucine is also close in size to glutamine and might pack similarly to glutamine at position 94. While the thermal denaturation results suggested the monomer still had wild-type-like stability, the leucine substitution at position 94 significantly destabilized the dimer. In fact, the leucine substitution impaired dimerization much more than alanine, so the disruption cannot just represent loss of packing or the hydrogen bond because the alanine variant would have reflected these too. It seems unlikely that the destabilization of the Q94L dimer is the result of a significant steric clash in the dimer, because leucine is actually slightly smaller than glutamine, and we find it can be accommodated in the dimer using some simple modeling in Pymol†.

Instead, we believe the energetics may be reflecting hydration around site 94, particularly in the dimer. In the OMPLA monomer, it would be possible for the leucine side-chain of the variant to interact with the hydrocarbon portion of the solvating detergent, an energetically favorable situation. However, in the dimer structure (1QD6), position 94 is clearly sequestered from detergent. If water penetrated the area as well as suggested by the crystal structure (Figure 7), we would expect it would be energetically unfavorable to transfer leucine side-chains to that environment upon dimerization. The destabilization we measure for the dimerization of Q94L+HSF (>2.34 kcal mol^{-1}) is on the order of the transfer free energy for two leucine side-chain from the membrane mimetic octanol to a membrane interface ($\Delta G = -3.8$ kcal mol^{-1}).[42,43]

The role of Q94 in a membrane environment

It is possible that the interactions at Q94 will be different in the membrane than those we have measured in detergent micelle solution, but we think it is likely that Q94 will still be in a micro-environment more like the membrane interface than the hydrocarbon core even in a bilayer. If the molecular surface of OMPLA around position 94 prefers to interact with the headgroup of detergent molecules, as suggested by the monomer crystal structure, it may also prefer to interact with the headgroup regions of lipids.[20] Furthermore, glutamine 94 is only about 5 Å below one of the rings of aromatic residues that are typically found at the edges of membrane proteins. These rings of aromatic amino acids partition into the membrane interface, and thus, in a bilayer Q94 will not be buried deeply into the hydrocarbon core. Q94 will be in close proximity to the interface and can potentially interact with lipid headgroups. Since the interior of the dimer interface, where Q94 is located, is sequestered from the lipidic solvent, we expect the region around position 94 may be quite similar in both micelles and bilayers. Therefore, we hypothesize that the local microenvironment will still attenuate the strength of the hydrogen bond in bilayers. Notably, in a molecular dynamics simulation of OMPLA in dioleoylphosphocholine (DOPC) bilayers, the intermole-cular hydrogen bond at glutamine 94 is not maintained, suggesting that it is indeed a modest interaction.[44] Hydrogen bonds

† http://www.pymol.sourceforge.net/

can be modulated by the surrounding sequence context, but are also sensitive to the local chemical environment, and together, these can modulate the ability of a polar residue to stabilize membrane protein association.

The Y92-Q94-S96 hydrogen bonding network influences monomer stability

In our investigations of the energetics of glutamine 94, we identified a number of substitutions that could be made in and around position 94 without affecting the dimerization of OMPLA. This is likely a consequence of the attenuation of the strength of hydrogen bonds in that region by the local environment. However, if other residues can be substituted in the Y92-Q94-S96 hydrogen bonding network without affecting dimerization, why is that these residues are so highly conserved in OMPLA sequences?[21] Even conservative substitutions like Y92F and Q94N are not found in OMPLA sequences. It therefore seemed likely that these substitutions were affecting some other aspect of OMPLA function that we could not detect in our sedimentation equilibrium experiments. For example, the benzyl ring of Y92 forms part of the substrate binding pocket, so it is plausible that disrupting the Y92-Q94-S96 network could impair OMPLA's phospholipase activity by interfering with substrate binding, if for example the Y92 side-chain moves into the binding pocket. Such effects on substrate binding would not be detectable in the sedimentation equilibrium experiments because the substrate analog is covalently attached. Therefore, to more completely explore the effects of disrupting the Y92-Q94-S96 hydrogen-bonding network, we measured the specific activity of each of our variants. To our surprise Q94L, which was the only variant to destabilize dimerization under all conditions, was also the only variant for which activity was significantly impaired (Figure 5). The activity of Q94A only modestly reduced under the high detergent condition, and Y92F, Q94N, Q94E, and S96A all had activity as good or better than wild-type. At first these results made the high sequence conservation of Y92-Q94-S96 even more perplexing, because we had now identified several amino acid substitutions for which both dimerization and activity were wild-type-like.

The results of the thermal denaturation experiments though provided a clue as to why these residues are not found in OMPLA. In these experiments, all of the substitutions except Q94L made OMPLA more susceptible to thermal denaturation. Surprisingly, the stability of Q94L, which had yielded the most drastic effects on dimerization and activity, was the only protein whose stability was comparable to that of wild-type. We postulate that the increase in the hydrophobicity of the protein compensated for the loss of the hydrogen bonding interactions. Nevertheless, conservative substitutions like Y92F and Q94N, which had not affected dimerization or enzyme activity, significantly increased the extent to which OMPLA was thermally unfolded. Therefore, it appears that the Y92-Q94-S96 hydrogen-bonding network may be important for the stability of the OMPLA protein. A role in stability for this region of the protein could also explain the absolute conservation of the threonine residue at position 93, whose side-chain faces into the barrel and is clearly not involved in dimerization or activity. It would be of interest to quantitatively assess the effects of disrupting the Y92-Q94-S96 hydrogen-bonding network on folding and stability and to assess them in a membrane bilayer environment. The role of Q94 in OMPLA may be more subtle and complex than suggested by the crystal structure alone, emphasizing the need to also thermodynamically explore interactions if we are to fully elucidate the role of polar amino acids in membrane proteins.

Conclusions

The crystal structure of the OMPLA dimer revealed an intermolecular hydrogen bond mediated by a highly conserved glutamine side-chain. It was presumed that this interaction played a key role in mediating dimerization, but our direct thermodynamic investigations have revealed that Q94 only contributes modestly to dimer stability. Single amino acid substitutions at this and neighboring positions revealed that it may be hydration of the local environment around Q94 that attenuates the strength of the hydrogen bond. Q94 is hydrogen bonded intramolecularly to Y92 and S96, which are also very highly conserved residues in OMPLA sequences, but this H-bond network does not seem to be important for dimerization. Interestingly, it may be important for stability of the OMPLA monomer. Overall, the Y92-Q94-S96 sequence seems to be so well conserved in OMPLA because it simultaneously optimizes dimerization, activity, and protein stability. Our results highlight the context dependence of polar interactions in membrane proteins and emphasize the need to combine thermodynamic data with structural information if we are to understand the molecular determinants of membrane protein stability.

Materials and Methods

Purification of OMPLA variants

Wild-type OMPLA and single amino acid variants were expressed and refolded as described, with the exception that the cells were lysed by French press and 10 mM C12-SB was substituted for Triton X-100 in the refolding.[19,45] We found 3-(dodecyldimethyl-ammonio)propanesulfonate (C12-SB) improved the refolding efficiency for our variants, as well as for wild-type OMPLA. Refolded protein from a 500 ml growth was exchanged into C14-SB detergent by anion exchange on a 25 ml diethylaminoethyl (DEAE) Fast Flow Sepharose column. The column was equilibrated in 20 mM Tris (pH 8.3), 5 mM C14-SB, and 50 mM KCl, and the protein was eluted 20 mM Tris (pH 8.3), 5 mM C14-SB, and 400 mM KCl. The protein was dialyzed overnight at 4 °C to a final concentration of 20 mM Tris (pH 8.3), 5 mM C14-SB, and 50 mM KCl.

Folded protein was separated from unfolded protein by FPLC on a Q6 column from Bio-Rad in 20 mM Tris (pH 8.3), 5 mM C14-SB, and 2 mM EDTA using a gradient 0–1 M KCl gradient. The gradient was adjusted for optimal separation. Most of the folded variants eluted between 260 mM and 310 mM KCl. Fluka Brand C12-SB and 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.[25] SigmaUltra grade C12-SB was purchased from Sigma. Purified protein was reacted with HSF as described.[19] For the variants, a 12–50× molar excess of HSF from a 50 mg ml^{-1} in $CHCl_3$ was added to the reaction, which was then incubated and rotated overnight at room temperature. Q94A was incubated with HSF overnight at 37 °C and Q94L was incubated with HSF for 48–72 h at 37 °C.

The colorimetric substrate 2-hexadecanoylthio-1-ethylphosphorylcholine (HEPC; Cayman Chemicals), coupled with 5′-dithiobis(2-nitrobenzoic acid) (DTNB), was used to monitor OMPLA phospholipase activity. On average, 1–5 μg OMPLA was assayed in 200 μl of 250 mM Tris (pH8.3), 20 mM $CaCl_2$, 2.5 mM C12, 1 mM HEPC, and 1 mM DTNB. A loss of activity toward this substrate was used to confirm complete (>98%) modification of the protein with HSF. Up to 10 μg of Q94L was used to ensure that this less active mutant was completely labeled.

Sedimentation equilibrium analytical ultracentrifugation

Purified protein was prepared for sedimentation equilibrium as described.[19] As before, 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 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). Samples were centrifuged until equilibrium was reached, as determined by WINMATCH‡. 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.[46] The density of the buffer, the partial specific volume of the protein, and the buoyant molecular weight of the protein were calculated using the software SEDNTERP,[47] and equilibrium constants were converted to molar units using the molar extinction coefficient $\varepsilon = 90{,}444$ mol l^{-1} cm^{-1}.[25] The free energies reported represent the average and standard deviation of at least three independent experiments. $\Delta\Delta G_{app}$ and the corresponding error were calculated as follows:

$$\Delta\Delta G_{app} = \Delta\Delta G_{mut} - \Delta\Delta G_{WT} \quad (1)$$

$$\sigma^2_{\Delta\Delta} = \sigma^2_{mut} + \sigma^2_{WT} \quad (2)$$

Specific activity measurements

The colorimetric substrate HEPC coupled with DTNB was used to measure the specific activity of OMPLA. Similar to previously reported activity assays, OMPLA was pre-incubated overnight at 0.2 mg ml^{-1} in 20 mM Tris (pH8.3), 2.5 mM C14-SB, 200 mM KCl, and 2 mM EDTA. The specific activity of OMPLA is sensitive to the protein and detergent concentration at which it is incubated prior to its addition to the assay buffer.[18] A total of 0.2 μg of OMPLA was added to an assay buffer to give a final assay volume of 500 μl. The assay buffer contained 50 mM Tris (pH8.3), 20 mM $CaCl_2$, 1 mM HEPC, 0.8 mM DTNB, and either 0.2 mM or 2.5 mM C14-SB. The absorbance at 412 nm was recorded every 5 s using the kinetic mode in Beckman DU 520 UV/Vis spectrophotometer. The rate tool in kinetic mode was used to calculate the slope of absorbance *versus* time, and this was converted into specific activity using an extinction coefficient for 5-thio-2-nitrobenzoic acid of $\varepsilon = 13{,}600$ mol l^{-1} cm^{-1}.

Thermal denaturation measurements

As in the activity assays, OMPLA was pre-incubated overnight at 0.2 mg ml^{-1} in 20 mM Tris (pH8.3), 2.5 mM C14-SB, 200 mM KCl, and 2 mM EDTA. EDTA was included to ensure the proteins were monomeric. Five μl of SDS-loading buffer was added to 45 μl of protein, such that the final concentration of SDS-loading buffer of 0.5X (25 mM Tris (pH6.8), 1% (w/v) SDS, 0.05% (w/v) bromophenol blue, 5% (v/v) glycerol); it was found that the bands on the gel were better defined when a final concentration of 0.5X was used instead of 1X buffer. The samples were then heated at 80 °C in an MJ Research PTC-200 Peltier Thermal Cycler for 10 min. The samples were then quenched at 4 °C for 3 min and analyzed by SDS–PAGE on a 12.5% acrylamide gel on a Phamacia PhastSystem. The gels were scanned at 600 dpi on an Epson Perfection 1240U scanner and analyzed using the gel analysis tool in the NIH's ImageJ program.[48]

Hydrogen bond identification

Hydrogen bonds were identified using the program HBPLUS[49] and the PDB files for the OMPLA monomer (1QD5) and the OMPLA dimer (1QD6). PDB files for OMPLA simulated in DOPC bilayers were kindly provided by Dr Marc Baaden.

‡ http://www.bbri.org/rasmb

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.05.009

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