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Influence of hydrophobic mismatch on the catalytic activity of *Escherichia coli* GlpG rhomboid protease

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Abstract: Rhomboids comprise a broad family of intramembrane serine proteases that are found in a wide range of organisms and participate in a diverse array of biological processes. High-resolution structures of the catalytic transmembrane domain of the *Escherichia coli* GlpG rhomboid have provided numerous insights that help explain how hydrolytic cleavage can be achieved below the membrane surface. Key to this are observations that GlpG hydrophobic domain dimensions may not be sufficient to completely span the native lipid bilayer. This formed the basis for a model where hydrophobic mismatch Induces thinning of the local membrane environment to promote access to transmembrane substrates. However, hydrophobic mismatch also has the potential to alter the functional properties of the rhomboid, a possibility we explore in the current work. For this purpose, we purified the catalytic transmembrane domain of GlpG into phosphocholine or maltoside detergent micelles of varying alkyl chain lengths, and assessed proteolytic function with a model water-soluble substrate. Catalytic turnover numbers were found to depend on detergent alkyl chain length, with saturated chains containing 10–12 carbon atoms supporting maximal activity. Similar results were obtained in phospholipid bicelles, with no proteolytic activity being detected in longer-chain lipids. Although differences in thermal stability and GlpG oligomerization could not explain these activity differences, circular dichroism spectra suggest that mismatch gives rise to a small change in structure. Overall, these results demonstrate that hydrophobic mismatch can exert an inhibitory effect on rhomboid activity, with the potential for changes in local membrane environment to regulate activity *in vivo*.

Keywords: rhomboid protease; GlpG; hydrophobic mismatch; lipid–protein interactions; detergent micelles

Abbreviations: **CD**, circular dichroism; **CHAPS**, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; **CMC**, critical micelle concentration; **DLPC**, 1,2-dilauryl-sn-glycero-3-phosphocholine; **DMPC**, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; **Fos-10**, decylphosphocholine; **Fos-12**, dodecylphosphocholine; **Fos-13**, tridecylphosphocholine; **Fos-14**, tetradecylphosphocholine; **LysoFos-12**, 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine; **LysoFos-14**, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine; **Mal-8**, *n*-octyl-β-D-maltoside; **Mal-9**, *n*-nonyl-β-D-maltoside; **Mal-10**, *n*-decyl-β-D-maltoside; **Mal-12**, *n*-dodecyl-β-D-maltoside; **Mal-13**, *n*-tridecyl-β-D-maltoside; **Mal-14**, *n*-tetradecyl-β-D-maltoside; **TM**, transmembrane; **TMD**, transmembrane domain.

Additional Supporting Information may be found in the online version of this article.

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Introduction

Rhomboids represent a broad family of intramembrane serine proteases that are unusual in their ability to cleave transmembrane substrates at sites normally embedded in the lipid bilayer.[1,2] This function is mediated by an alpha-helical transmembrane domain (TMD), which forms a catalytic core that is well conserved across most species.[3] Members of the rhomboid family have been associated with a wide range of cellular processes such as quorum sensing,[4] cell growth, mitochondrial remodeling, and apoptosis.[4,5] They have also been implicated in disease states such as diabetes and cancer,[6,7] and are required for infection by apicomplexian parasites such as those that cause malaria and toxoplasmosis,[4,8] making them a potential target for drug discovery.

A detailed description of how the hydrolytic cleavage reaction is catalyzed by rhomboid proteases has been developed over the last decade, owing to a wealth of crystallographic and kinetic data obtained with the *Escherichia coli* GlpG rhomboid. Nonetheless, a number of questions remain, particularly regarding how the rhomboid can cleave sequences that are normally buried within the hydrophobic phase of the lipid bilayer, and in an α-helical conformation that must be disrupted to enter the rhomboid active site and bind in an extended conformation.[9–11] One hypothesis that has remained particularly attractive arose from observations on the location of hydrophobic and aromatic residues in x-ray crystal structures of the transmembrane domain (TMD) from *E. coli* GlpG,[12] including one determined in DMPC bicelles.[13] It was noted that the thickness of the hydrophobic belt on the surface of the GlpG structure varies from 20 Å to 25 Å, with some parts being notably shorter than the expected dimension for the hydrophobic phase of *E. coli* membranes.[12,13] This hydrophobic mismatch has been proposed to lead to thinning of the local bilayer structure, a hypothesis that is supported by molecular dynamics simulations on GlpG in lipid bilayers.[14–16] These distortions in the local membrane environment have been suggested to expose the target sequence in a substrate TM segment to the aqueous phase, destabilizing its helical structure so that it could bind the active site in the extended state required for proteolysis.[12,14,17]

Although these ideas about rhomboid protease interactions with lipid have focussed on its proposed ability to modulate the local membrane environment, the possibility that the lipid bilayer might alter the behavior of rhomboid proteases has received less attention. Nonetheless, hydrophobic mismatch between the bilayer and an integral membrane protein can give rise to changes in protein structure that minimize exposure of hydrophobic amino acid side chains to the aqueous environment.[18] Studies on both model TM peptides and multispanning integral membrane proteins have shown that this often results in a change in TM helix tilt angles,[18,19] although more drastic effects have also been reported.[20] In some cases these structural changes can have a significant impact on activity, as has been demonstrated for the ion channels KcsA[21] and MscL.[22,23] Given the difference between the dimensions of the hydrophobic region of the GlpG TMD and that of a typical *E. coli* membrane, it is possible that the local lipid environment may also have an impact on rhomboid function. This idea is supported by reconstitution experiments with the *E. coli* GlpG rhomboid showing significant differences in activity against a model TM substrate that depend on the lipid used.[24] Although these results indicate that the membrane environment can modulate function, the potential influence of hydrophobic mismatch has not been directly evaluated.

To determine whether hydrophobic mismatch can influence rhomboid catalytic function, we examined the activity of an *E. coli* GlpG TMD construct in a series of detergents and lipids that vary in alkyl chain lengths. We found that maximal activity against a water soluble model substrate was obtained when the GlpG TMD was purified into detergents that contained a saturated alkyl chain with 10 or 12 carbons. Increasing the length of the alkyl chain by two carbon atoms resulted in a 2 to 3-fold reduction in activity. These trends were observed in phosphocholine, lysophosphocholine and maltoside detergents, as well as bicelles containing phosphatidylcholine-based lipids. Circular dichroism spectroscopy shows a small but reproducible difference in secondary structure content between samples reconstituted in longer-chain versus shorter-chain detergents, indicating that subtle structural differences may be enough to account for these changes in activity. Taken together, these results suggest that, even if the rhomboid's interaction with surrounding lipid is capable of membrane-thinning distortions, there is nonetheless an energetic penalty to hydrophobic mismatch that also has an impact on rhomboid activity.

Results

The GlpG rhomboid from *E. coli* was selected as the focus of our study due to the abundance of data available on its structure and activity.[2,25] This rhomboid contains an N-terminal aqueous domain (residues 1–61) on the cytoplasmic side of the membrane that is connected by a flexible linker (residues 62–90) to the catalytic TMD [Fig. 1(A)]. Although the functional role of this aqueous domain is not known, it has been shown to be important for maintaining maximum activity in some assays.[26] However, the structured cytoplasmic domain (CytD) also interacts strongly with some detergent micelles in a

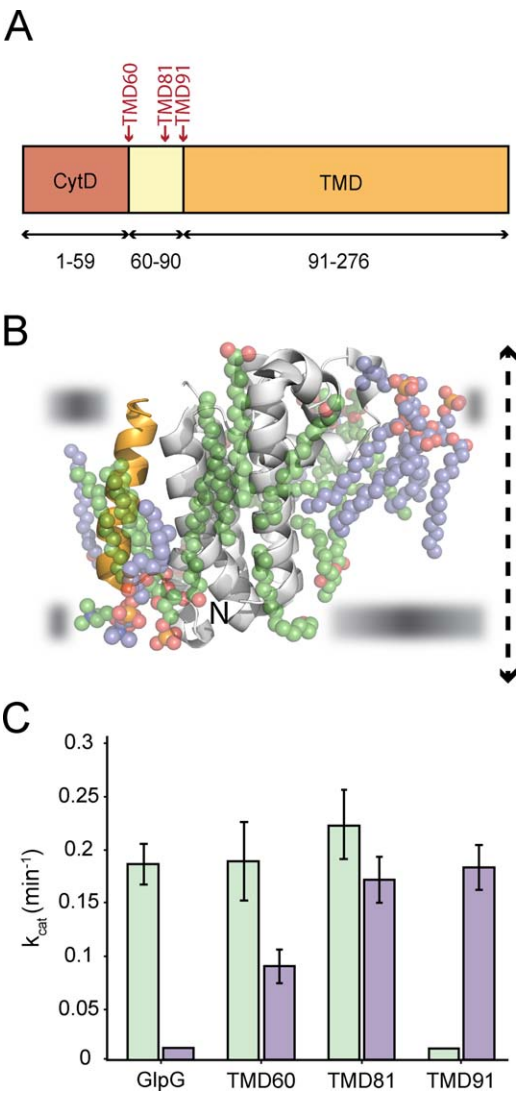

Figure 1. Activity of N-terminal truncation mutants of GlpG. A: Schematic representation of GlpG illustrating the relative size and position of the N-terminal cytoplasmic domain (CytD), linker region, and transmembrane domain (TMD). The red arrows indicate the approximate position of the truncations for TMD60, TMD81, and TMD91. B: Ribbon diagram of the x-ray structure of the TMD from *E. coli* GlpG in a DMPC phospholipid bilayer environment (PDB 2XTV), with TM5 highlighted in orange. The N-terminus is labeled (N) and the C-terminus is not visible, being on the other side in this view. The gray bars represent the approximate boundaries of the 25 Å wide hydrophobic GlpG TM region and its surrounding bilayer environment, as approximated from the position of resolved DMPC molecules from the same subunit (green) or the symmetry related subunit (blue). The 32 Å wide hydrophobic region of a native DMPC bilayer as determined by small-angle neutron scattering[41] is indicated by the dashed arrow. C: k_{cat} values for full-length GlpG, TMD60, TMD81, and TMD91 in Mal-12 (green) or Fos-12 (purple) detergent micelles. The reported error for this assay and all subsequent data represent standard deviations about average values obtained from at least three independent protein preparations. Near-zero turnover values with no error bars denote samples with no detectable activity in the casein assay.

manner that can disrupt its tertiary structure, potentially interfering with GlpG activity.[27] Since these interactions may complicate an investigation of hydrophobic mismatch on the intrinsic catalytic activity of the rhomboid TMD, we sought to work with the minimal functional unit that preserves the functional integrity of the active site. To identify this region we used a series of GlpG truncation mutants described previously,[26] that includes a construct missing only the structured CytD (starting at residue 60, called TMD60), a construct that also omits most of the flexible linker (TMD81), and a construct corresponding to the longest fragment resolved by x-ray crystallography (TMD91).

To evaluate the activity of GlpG and its truncation mutants we chose to use fluorogenic casein as a water-soluble model substrate as previously described.[26,28–31] Although rhomboids can act on transmembrane substrates, these would also require solubilisation in the detergent under investigation, a factor that can change the structure and cleavage site accessibility in these substrates.[10] In contrast, an aqueous model substrate offers the advantage that any changes in measured activity should reflect the effect of local environment on the rhomboid itself. Using this model substrate, the substrate-dependent change in initial hydrolysis rates was found to be hyperbolic (Supporting Information, Fig. S1), and fit the Michaelis-Menten equation with no requirement for cooperativity, similar to previous observations.[28,30] The reference detergent used in these studies was dodecylmaltoside (Mal-12), as this is a mild detergent that has been used for purification of active GlpG in a number of studies.[26,28,30] As shown in Figure 1(C) and Supporting Information Table S1, k_{cat} and K_m parameters for TMD60 and TMD81 were the same as those measured for the full-length protein. In contrast, TMD91 displayed no activity above background levels, even at substrate concentrations that would normally be saturating, in line with previous observations.[26]

We also repeated these studies on the same series of constructs purified into Fos-12, a detergent known to have significant propensity to bind the GlpG CytD in a partially denaturing interaction.[27] In contrast with the results in Mal-12, full-length GlpG showed no measurable activity in this detergent. Partial recovery of activity was observed in the TMD60 construct, suggesting that an interaction between CytD and Fos-12 was responsible for this loss of function. Full activity was recovered when most of the flexible linker was also omitted, with k_{cat} for TMD81 approaching that of full-length GlpG in Mal-12 Surprisingly, the same level of activity was also obtained with TMD91, suggesting that interactions with the phosphocholine headgroup were able to compensate for the loss of function observed for this construct in Mal-12 or crude membrane extracts.[26] Overall, these results suggest that

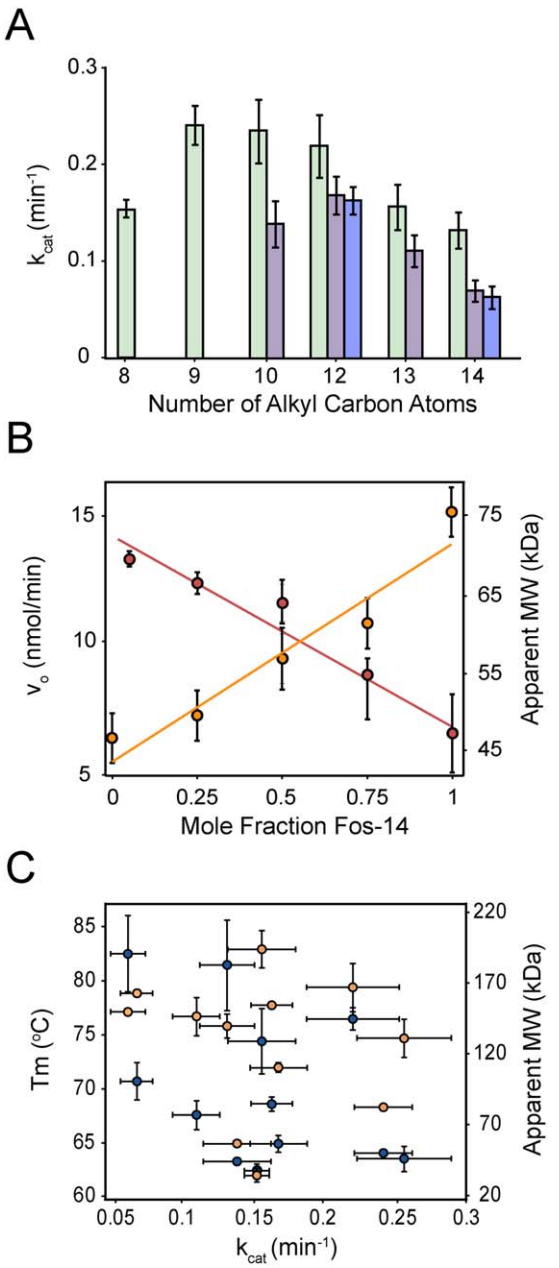

Figure 2. Effect of different detergent micelle environments on TMD81. A: Catalytic turnover numbers for TMD81 purified into maltoside (green), lysophosphocholine (blue), or phosphocholine detergents (purple). B: Proteolysis rates measured for TMD81 solubilized in 0.1% (w/v) mixed micelles comprised of Fos-12 and Fos-14 at a range of Fos-14:Fos-12 mole fractions (red). The apparent size of each protein-detergent complex (PDC) was determined using size exclusion chromatography (orange). C: k_{cat} versus the apparent molecular weight of the protein-detergent complex (blue) or the melting temperature (orange) for TMD81 in phosphocholine, lysophosphocholine and maltoside detergent micelles. Data corresponding to this graph can be found in the Supporting Information, Table S2.

TMD81 is the most robust construct for retention of proteolytic activity against this model aqueous substrate, and was therefore the focus of our investigation into hydrophobic mismatch.

To explore the effect of hydrophobic mismatch on the catalytic TM core of rhomboid protease, TMD81 was purified into phosphocholine detergents that varied in the length of the alkyl chain. As shown in Figure 2(A), maximal k_{cat} was observed in both Fos-10 and Fos-12, whereas any further increase in the alkyl chain length led to a reduction in activity. Specifically, activity in Fos-14 was threefold lower than that in Fos-12. In contrast, no difference in K_m was observed in the various phosphocholine detergents (Supporting Information Table S1), suggesting that the loss of activity is not a result of competitive inhibition of substrate binding to the active site by the longer chain detergents. Instead, the environment provided by the longer-chain detergents must have some impact on the catalytic status of the active site.

To confirm that the size of the micelle hydrophobic domain is the factor that is causing these changes in TMD81 activity, proteolysis rates were also evaluated in mixed micelles comprised of Fos12 and Fos14. It has been shown that the average size of the hydrophobic domain of a mixed micelle varies linearly with composition, allowing systematic changes to micelle size to be achieved by changing the molar ratio of long:short-chain detergents.[32] A linear relationship was observed between the Fos-12:Fos-14 molar ratio and the apparent size of the protein-detergent complex as determined by size exclusion chromatography, confirming that this relationship between size and composition was preserved in our experiment [Fig. 2(B)]. Following this trend, hydrolysis rates with a fixed amount of substrate were found to decrease linearly as the Fos-14:Fos-12 molar ratio was increased. This relationship was observed when samples were purified into Fos-12 and diluted with Fos-14, or vice versa, indicating that the changes in activity associated with each detergent are reversible.

Although the results with phosphocholine detergents suggest that the size of the micelle hydrophobic domain is responsible for the observed changes in proteolytic activity, this effect may be exaggerated due to the small size of the phosphocholine headgroup relative to more lipid-like detergents. In particular, the presence of a polar glycerol backbone spacer linking phosphocholine to the alkyl group can provide a more gradual transition between charged and hydrophobic moieties.[33,34] To determine if the headgroup size was important for this effect, kinetic parameters were measured for TMD81 in lysophosphocholine detergent micelles with saturated 12- and 14-carbon acyl chains (LysoFos-12 and LysoFos-14, respectively). As shown in Figure 2(A), the activity for TMD81 in LysoFos-12 was the same as seen in Fos-12, and also decreased by approximately 3-fold when the longer chain LysoFos-14 was used instead.

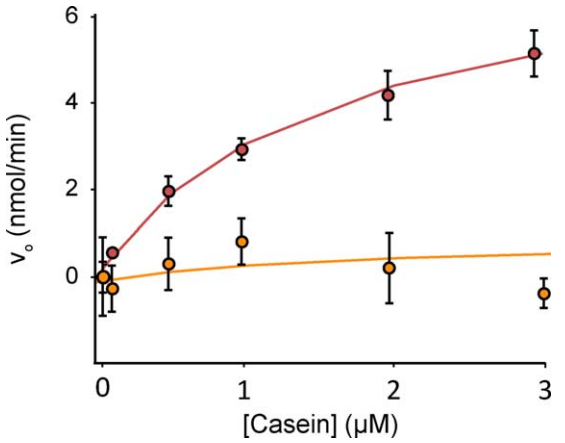

Figure 3. Michaelis-Menten plot showing the initial rate of proteolysis for TMD81 in bicelles composed of either DLPC (red) or DMPC (orange) phospholipid bicelles with CHAPS detergent.

To evaluate the role of headgroup chemistry in this phenomenon, the same series of experiments was repeated using a series of alkyl maltosides [Fig. 2(A)]. A similar trend to that seen with the Fos detergents was observed, with Mal-10 and Mal-12 supporting maximal activity, and a decrease in k_{cat} as the length of the alkyl chain increased to 14 carbons. The lower CMCs of the maltoside detergents relative to the Fos series also made it possible to purify TMD81 into shorter-chain detergents, including Mal-9 and Mal-8. Although maximal activity was retained in Mal-9, k_{cat} was found to decrease when Mal-8 was used in its place, identifying a lower limit to the optimal detergent alkyl chain length.

To examine whether the effect of hydrophobic mismatch on rhomboid protease activity extends to a more native-like lipid bilayer environment, samples of TMD81 were reconstituted into either DMPC/CHAPS or DLPC/CHAPS bicelles. The activity in DLPC bicelles was significantly lower than in detergents, with a k_{cat} of 0.01 min^{-1} (compared to 0.17 min^{-1} in Fos-12). This is in agreement with previous studies with a GlpG homologue from *Pseudomonas aeuroginosa*,[35] and can be attributed to the more ordered nature of phospholipid bilayers relative to detergents, which has been shown to restrain conformational dynamics in TM5, and correlates with a reduction in activity.[10] In line with the effect of hydrophobic mismatch observed with detergents, TMD81 activity was further reduced when DMPC was used in place of DLPC, with activity essentially being too low to detect over the range of substrate concentrations tested (Fig. 3).

One of the potential consequences of hydrophobic mismatch is a loss of structural stability, a possibility that we investigated by performing thermal denaturation experiments on all detergent-reconstituted samples. The melting temperature (T_m) of full-length GlpG in 0.1% (w/v) Mal-12 was found to be 75°C ± 1°C, in agreement with previous observations using similar methods.[36] A small increase in the melting temperature was obtained for the TMD81 truncation in Mal-12, with a value of 80°C ± 2°C, potentially reflecting a destabilizing interaction between CytD and the micelle that is eliminated by use of the truncation mutant.

When melting temperatures were measured for TMD81 samples purified into Fos-, LysoFos- and Mal-detergents, in almost all cases they were within a 15° range, showing that changes in thermal stability were not responsible for the observed differences in catalytic activity [Fig. 2(C), Supporting Information, Table S2]. The absence of a correlation between stability and activity was also demonstrated by TMD81 melting temperatures in Fos-12 and Mal-10, since these were both significantly lower than those for other samples in spite of the high activity supported in both detergents [Fig. 2(C)].

We also found no clear relationship between the apparent size of the protein-detergent complex and the catalytic activity [Fig. 2(C)]. Previous studies have shown that GlpG forms a dimer in Mal-12, but is monomeric in Mal-10,[30] observations that are in agreement with our measures of the apparent PDC size (Supporting Information Table S2). The fact that maximal activity is observed in both detergents would suggest that the oligomerization state does not alter the ability of the catalytic core to hydrolyze soluble casein substrate, in line with previous observations on full-length GlpG.[30] In addition, the apparent MW of the TMD81-Fos-10 PDC is the same as that of a TMD81 dimer with no detergent bound (47.2 kDa), suggesting that this complex contains monomeric TMD81, with the additional mass being made up of the detergent required to keep the complex in solution. There is a linear relationship between predicted MW of the protein-free phosphocholine detergent micelle and that of the PDC (Supporting Information, Fig. S2), suggesting that the oligomerization state of TMD81 in this detergent series does not change. In this case, the intercept of the line can be taken as an apparent MW of protein with no detergent bound, giving rise to a value that is closer to the expected size of a monomeric state for TMD81 (31 kDa). Therefore, although the dimeric state for GlpG appears to be necessary for cleavage of transmembrane substrates,[30] changes in oligomerization state do not account for the observed reduction in activity with the use of longer-chain detergents.

To determine if these changes in functional states could be explained by structural differences induced by solubilisation in these detergents, CD spectroscopy was performed on TMD81 purified into Fos-12 or Fos-14. As shown in Figure 4, spectra in both samples showed characteristic minima at 222

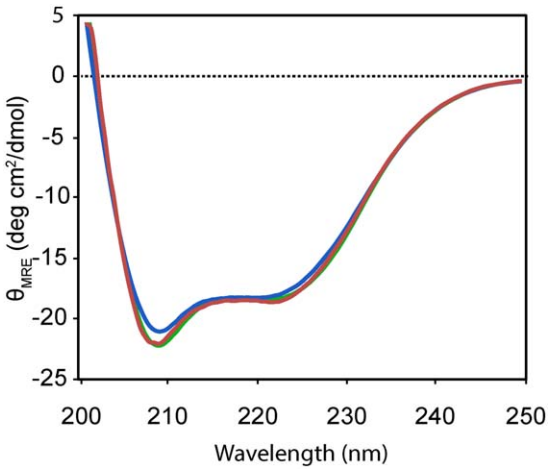

Figure 4. Effect of hydrophobic mismatch on TMD secondary structure. Circular dichroism spectra are shown for 5 μ*M* TMD81 in Fos-10 (red), Fos-12 (green), or Fos-14 (blue) at 37°C.

and 208 nm, as expected for a largely α-helical protein. Although highly similar to each other, spectra acquired in Fos-14 showed a small but reproducible difference in the relative intensities of these two minima compared to Fos-12, with the Fos-14 spectrum showing a larger relative intensity at 208 nm. Secondary structure prediction based on these spectra suggest that the Fos-12 sample had a greater proportion of α-helical structure than the less active sample in Fos-14 (91% vs. 83%). Interestingly, the Fos-10 sample, which displays comparable levels of activity relative to the Fos-12 sample, produced a CD spectrum that was virtually super-imposable with that of Fos-12. This suggests that the use of detergents with a longer alkyl chain gives rise to a change in either the structure or conformational equilibrium of the rhomboid protease, and that this change is detrimental to the catalytic integrity of the active site.

Discussion

One of the most unique aspects of rhomboid protease function is its ability to cleave polypeptide sequences that are normally embedded in the lipid bilayer in an α-helical conformation. It has been proposed that hydrophobic mismatch between the rhomboid and its lipid environment gives rise to localized membrane thinning, forcing the target sequence out of the lipid membrane to facilitate unfolding and binding.[12,14,37–39] However, hydrophobic mismatch also has the potential to alter the activity of the rhomboid itself, a possibility we have investigated in this work. We found that the isolated catalytic domain from *E. coli* GlpG encompassed by TMD81 does show different activities that depend on the length of the alkyl chain of the detergent or lipid used to maintain it in solution. For all the systems tested, it appears that saturated alkyl chains comprised of more than 12 carbon atoms reduced activity of the catalytic core, even when reconstituted into bicelles. Since this difference was observed for detergents with different headgroups, this likely reflects the impact of changing the dimensions of the hydrocarbon phase.

Although it is possible that the observed differences are a consequence of hydrophobic mismatch, detergent molecules also have the potential to interfere with substrate binding through competitive inhibition. In fact, a crystal structure of the TMD of *E. coli* GlpG in β-octyl glucoside shows a detergent molecule bound to a groove formed by TM helices 2 and 5, and the headgroup extending into the active site.[13] However, this occupation of the active site by detergent was only observed for the inactive mutant S201T, with the WT structure showing β-octyl glucoside bound to the same groove, but without headgroup interactions with the active site. Also, a GlpG TMD crystal structure in DMPC bicelles has been determined that resolved a number of well-ordered lipids, none of which enter the active site.[13] Our data is consistent with these latter observations, since a classic competitive inhibitor would be expected to increase K_m, but was found to be the same in our experiments within a detergent series. Therefore competitive inhibition by detergent does not explain the trends we observed.

The fact that TMD81 appears to be adversely affected by the use of detergents and lipids with alkyl/acyl chains that exceed 12 carbon atoms is highly suggestive of a change in activity being caused by hydrophobic mismatch. Structural data on micelles and bilayers confirms the correlation between alkyl/acyl chain length and the size of the hydrophobic phase. According to small angle x-ray scattering studies, the micelles used in our study are spheroidal in shape, with the short axis of the hydrocarbon phase being similar for detergents with the same alkyl chain length.[32,40] For Mal-12 and Fos-12, these values were found to be 33 and 31 Å, respectively,[32,40] which are already larger than the average hydrophobic thickness of 25 Å for GlpG, confirming that rhomboids have the ability to compensate for moderate degrees of hydrophobic mismatch. The approximately 5 Å increase in thickness of the hydrocarbon phase that is estimated to result from the use of tetradecyl chains appears to exceed this ability, as manifested by the decrease in activity. Meanwhile, the 22 Å hydrophobic phase of Mal-8 detergent micelles[40] is shorter than the average hydrophobic thickness of GlpG by ~3 Å, indicating reduced flexibility for the accommodation of detergents with shorter alkyl chain lengths. Interestingly, the dimensions of the hydrophobic phase for DMPC bicelles is similar to that of Fos-12 and Mal-12 detergents, with a length of 32 Å as determined by small

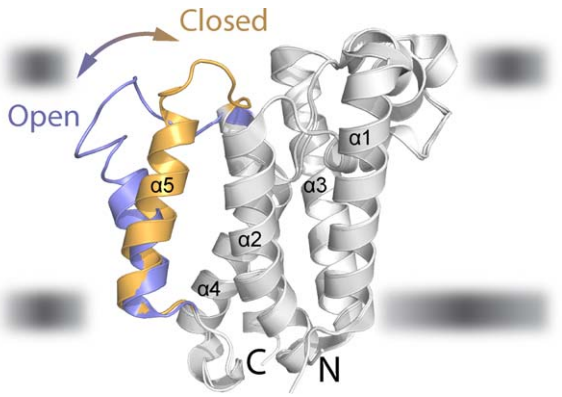

Figure 5. Superposition of two crystal structures representing the proposed model for GlpG substrate gating in which TM5 and the adjacent L5 region transitions from a closed conformation (orange, PDB 2XTU) to an open conformation (blue, PDB 2NRF) to allow substrate access into the active site.

angle neutron scattering.[41] Since TMD81 was not active in this lipid, this suggests that the more ordered bilayer environment provided by phospholipid bicelles reduces the ability of this rhomboid to accommodate hydrophobic mismatch.

A common mechanism used by proteins to resolve the energetic cost of hydrophobic mismatch is to induce deformations in membrane structure in order to accommodate the dimensions of the protein hydrophobic belt.[18,22] This outcome reflects the relative incompressibility of proteins compared to that of lipid bilayers. Detergent micelles require even less energy to be deformed, providing an explanation for the ability of TMD81 to be maximally active in detergent micelles with hydrophobic regions larger than the protein hydrophobic belt. Nonetheless, when the energetic cost of inducing membrane deformations becomes more significant, structural changes in proteins can also be induced, for example, by altering helix tilt angles (eg: KcsA ion channel),[21] or changing packing interactions between transmembrane helices (e.g. Ca^{2+} ATPase).[42] In the case of GlpG, hydrophobic mismatch is most pronounced in the vicinity of TM helix 5 [Fig. 1(B)],[13] a region thought to play a role in substrate gating.[10,43,44] Most structures show this helix to be closely associated with the core TMD structure, however, it has also been captured in a state where it is bent away from the active site (Fig. 5).[43] It has been suggested that this structure represents an open state that facilitates entry of substrate into the active site. Since hydrolysis of the acyl-enzyme intermediate does not appear to be rate-determining,[45] conformational change involving TM helix 5 has been proposed to be the slow step in the catalytic cycle.[10,45] This hypothesis is supported by increased catalytic turnover numbers for GlpG mutants designed to decrease the stability of packing interactions between TM helices 5 and 2.[44] Enhanced hydrolysis rates for GlpG in detergents relative to those in lipid bilayers[10,24] also correlate with an increase in the dynamics of this TM helix.[10] These findings suggest that TM helix 5 represents a control point for GlpG activity, with greater mobility being associated with faster rates of proteolysis. Given the severity of hydrophobic mismatch in the vicinity of this helix, TM helix 5 may be acting as a sensor of the local lipid environment. Interestingly, this helix also contains a lipid-facing residue at its N-terminus (Arg227) that is required for full activity in Mal-12 and crude membrane extracts.[26]

On the basis of the model of TM5 (along with L5) acting as a substrate gate, it is possible that hydrophobic mismatch with the lipid environment could increase the energy required to access the open state, thereby slowing the rate of substrate hydrolysis. Although it might be expected that a shift in the conformational dynamics to favour the closed state would manifest as an increase in secondary structure content, predictions from the CD spectra suggest a small decrease in α-helix content upon reconstitution into micelles that reduce TMD81 activity. However, despite its reproducibility, the change in the CD spectrum is subtle, and the difference between the two states is smaller than the uncertainty associated with the prediction method.[46–48] It is also possible that the difference between the two states is not localized to just one part of the structure, but instead reflects subtle changes dispersed through a wider network of interactions linking environmental changes to active site structure. We also cannot rule out the possibility that the longer chain systems create a steric barrier that physically impedes substrate entry, a phenomenon that could result in the decreased turnover numbers we have observed.

The accumulation of data on protein-lipid interactions is making it increasingly apparent that the local membrane environment can play an important role in influencing both the structure and function of integral membrane proteins. Through our studies we were able to demonstrate that changes in the dimensions of the hydrophobic phase can alter the activity of the rhomboid protease, providing further insight into the complex relationship between rhomboids and the local lipid environment. This could have consequences for rhomboid function *in vivo*, since the composition of bacterial membranes can vary in response to nutrient depletion, growth rate and environmental stress,[49–51] and also contain microdomains enriched in lipids of different headgroup and alkyl chain identities.[52,53] Similarly, in eukaryotes, lipid rafts and localized membrane distortions play a key role in protein sorting, signal transduction, and cleavage of transmembrane substrates by α- and γ-secretases.[54–56] The resulting

differences in membrane properties and extent of hydrophobic mismatch could be used to regulate the activity of rhomboid proteases, with longer and more ordered lipid bilayers being inhibitory to rhomboid function. Although not examined in this study, the effect of longer hydrocarbon phases would be expected to have an even larger effect on rhomboid activity against transmembrane substrates since the thicker membrane would make it more difficult to access buried cleavage sites. Overall, the ability of the hydrophobic phase of the lipid environment to modulate GlpG activity provides an additional mechanism for the regulation of intramembrane protease activity.

Materials and Methods

Protein expression and purification into detergent micelles

Full length *E. coli* GlpG rhomboid protease, along with truncation mutants TMD60 (residues 60–278), TMD81 (residues 81–276) and TMD91 (residues 91–276) were expressed and purified as previously described.[26] In all purifications, the membrane fraction was extracted from the pellet using 1% (w/v) Mal-12 (Anatrace), with nickel affinity and size exclusion chromatography steps being done in the presence of 0.1% (w/v) of the desired detergent. In some cases it was necessary to use higher detergent concentrations to exceed the critical micelle concentration for these steps. Consequently, 0.2%, 0.35%, and 1.3% (w/v) concentrations were used for purifications into Mal-10, Mal-9, and Mal-8, respectively, whereas 0.3% (w/v) was used for purification into Fos-10. In addition to the detergent of interest, purified samples contained 50 m*M* Tris-HCl pH 7.4, 150 m*M* NaCl and 100 μ*M* EDTA (GlpG buffer). Concentrations of purified samples were determined using a BCA assay (Pierce).

Reconstitution into phospholipid bicelles

Samples of TMD81 were first reconstituted into phospholipid vesicles which were then converted into bicelles by solubilisation in detergent, based on protocols developed for the integration of functional membrane proteins into bicelles.[57,58] Thin films of DLPC or DMPC phospholipids (Avanti Polar Lipids) were prepared as previously described,[24] and hydrated in detergent-free GlpG buffer to produce a 20 mg/mL suspension. This was then extruded through a 1 μm filter using an Avanti Mini-Extruder, and the resulting liposomes incubated with 0.12 m*M* 12-Mal in GlpG buffer for 30 min. Purified TMD81 in 0.1% (w/v) Mal-12 was added to produce a final TMD81 concentration of 0.1 mg/mL in a 1 mg/mL lipid solution. After a 60-min incubation at room temperature, Mal-12 was removed by overnight dialysis. Residual detergent was removed by adding 2 aliquots of 30 mg Amberlite beads (Sigma-Aldrich) per mg Mal-12, with an incubation time of 1–2 h per aliquot. Proteoliposomes were collected via ultracentrifugation, re-suspended in FPLC buffer, and re-extruded through a 1 μm filter. Successful and complete reconstitution into liposomes was confirmed by density gradient centrifugation in a 0-50% sucrose gradient (200,000*g* for 90 min), followed by SDS-PAGE analysis of liposome and pellet fractions. CHAPS detergent (Bioshop) was added to the proteoliposomes to achieve a molar phospholipid:bicellar detergent ratio (q_{eff}) of 1, with a total bicelle concentration of 1.5% (w/v) to yield small isotropic bicelles similar to those employed in previous rhomboid studies.[35] The mixture was then subjected to multiple cycles of heating to 42°C[57] and cooling to room temperature.

Kinetics measurements

Detergent-solubilized rhomboid protease (0.25 μ*M*) was incubated at 37°C with varying concentrations of BODIPY-labeled casein (Invitrogen) in GlpG buffer containing the detergent of interest. Initial hydrolysis rates were determined as described previously[26] using a Spectramax Gemini XS microplate reader, plotted as a function of substrate concentration, and fit to the Michaelis-Menten equation to determine K_{m} and V_{max}. For the Fos-12/Fos-14 mixed-micelle experiments, purified TMD81 in 0.1% (w/v) Fos-12 was diluted with GlpG buffer containing 0.1% (w/v) Fos-14 to produce the desired Fos-12:Fos-14 ratio. Similar experiments were also done starting with TMD81 in Fos-14 and diluting with Fos-12. The activity of TMD81 in these mixed micelle systems was assessed using 0.25 μ*M* TMD81 with 1.5 μ*M* substrate. SDS-PAGE analysis of fractions taken at several time points during a reaction was used to convert fluorescence intensities into molar concentrations of product as previously described,[28] and gave rise to k_{cat} values that were comparable to previously determined values for full length GlpG in Mal-12.[28] The reported uncertainty for this assay, and all experimental data presented in tables and associated graphs, typically represent standard deviations about average values obtained from three independent protein preparations.

Circular dichroism and thermostability

CD spectra were collected on 5 μ*M* TMD81 in GlpG buffer containing the detergent of interest at 37°C using a Jasco J-815 CD spectropolarimeter. Each spectrum was acquired with eight accumulations, a data-pitch of 0.2 nm and at a scan rate of 20 nm/min. To ensure that accurate relative concentrations of TMD81 were used in spectral comparisons, band intensities of CD samples run on SDS-PAGE were used to determine concentrations for mean residue molar ellipiticity calculations. Each spectrum was

acquired on at least two independent samples, and a high level of reproducibility was observed between individual trials. Secondary structure analysis on the resulting spectra was carried out with the CDPro software using the CONTIN method and the SMP56 reference set.[46] CD spectroscopy was also used to measure TMD81 thermostability in detergent micelles. Samples were heated from 35°C to 91°C at a rate of 0.3°C/min., and the ellipticity at 222 nm was monitored. The data was fit using a two-state Boltzmann curve to determine the melting temperature (T_m).[36]

Protein-detergent complex size estimations

Molecular weight standards (Amersham, Sigma-Aldrich) comprised of albumin (67 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa), and lysozyme (14.3 kDa) were loaded onto a Superdex 200 10/30 size exclusion chromatography column (GE Lifesciences) in GlpG buffer, and used to generate a calibration curve, with the elution volume of blue dextran (~2000 kDa) being used to determine column void volume.[59] Elution volumes from TMD81 samples run under the same conditions with the appropriate detergent were used with the calibration curve to calculate an apparent molecular weight for the protein detergent complex.

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