Allosteric regulation of rhomboid intramembrane proteolysis

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Abstract

Proteolysis within the lipid bilayer is poorly understood, in particular the regulation of substrate cleavage. Rhomboids are a family of ubiquitous intramembrane serine proteases that harbour a buried active site and are known to cleave transmembrane substrates with broad specificity. In vitro gel and Förster resonance energy transfer (FRET)-based kinetic assays were developed to analyse cleavage of the transmembrane substrate psTatA (TatA from Providencia stuartii). We demonstrate significant differences in catalytic efficiency ($k_{cat}/K_{m}$) values for transmembrane substrate psTatA (TatA from Providencia stuartii) cleavage for three rhomboids: AarA from P. stuartii, ecGlpG from Escherichia coli and hiGlpG from Haemophilus influenzae demonstrating that rhomboids specifically recognize this substrate. Furthermore, binding of psTatA occurs with positive cooperativity. Competitive binding studies reveal an exosite-mediated mode of substrate binding, indicating allosteric plays a role in substrate catalysis. We reveal that exosite formation is dependent on the oligomeric state of rhomboids, and when dimers are dissociated, allosteric substrate activation is not observed. We present a novel mechanism for specific substrate cleavage involving several dynamic processes including positive cooperativity and homotropic allosteric for this interesting class of intramembrane proteases.

Keywords allostery; GlpG; intramembrane protease; kinetics; rhomboid protease

Subject Categories Membrane & Intracellular Transport; Post-translational Modifications; Proteolysis & Proteomics

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Introduction

Proteases that catalyse the hydrolysis of peptide bonds constitute the most abundant group of enzymes. They are essential in a wide variety of biological processes, including cell cycle signalling, proliferation and death, immune response and protein trafficking. Several successful strategies have been applied towards the development of currently in use protease inhibitors to treat hypertension, HIV, cancer, diabetes and coagulation disorders (Drag & Salvesen, 2010). Characterization of the role of proteases in disease and also their precise mechanism of action was essential for these drug discoveries.

Intramembrane proteases are polytopic membrane proteins that cleave transmembrane substrates and, similar to soluble proteases, are known to play roles in essential biological processes (Wolfe, 2009). Their active sites, buried within the lipid bilayer, are formed from the assembly of transmembrane helices. X-ray crystal structures for several intramembrane proteases including presenilin homologs (Hu et al, 2011; Li et al, 2013), CaaX proteases (Manolardis et al, 2013; Pryor et al, 2013), site-2-proteases (Feng et al, 2007) and several structures of rhomboid proteases (Wang et al, 2006; Wu et al, 2006; Ben-Shem et al, 2007; Lemieux et al, 2007) have provided architectural insight into this unique class of enzymes. Despite these crystal structures, our comprehension of their cleavage mechanism, substrate recognition and regulation of these lipid-embedded enzymes is in its infancy compared to soluble proteases.

Rhomboids are a well-studied family of intramembrane serine proteases (peptidases) found in all kingdoms of life. The cleavage of transmembrane substrates facilitates the release of soluble domains to the extracellular environment. They play essential roles in diverse signalling events ranging from epidermal growth factor release in eukaryotes (Lee et al, 2001; Urban et al, 2001), facilitating invasion in parasites (Sibley, 2013), to quorum sensing in bacteria (Mesak et al, 2004; Stevenson et al, 2007). Not surprisingly, rhomboid dysfunction is involved in several human diseases that include malaria, cancer and Parkinson’s disease (Bergbold & Lemberg, 2013; Chan & McQuibban, 2013; Sibley, 2013).

Rhomboids represent ‘non-classical’ serine proteases, having a catalytic dyad (Ser/His) instead of the hallmark triad, and the active site buried 10–12 Å deep from the periplasm. The hydrophilic environment of the active site is occluded from lipid bilayer and the periplasm by flexible loop 5 and transmembrane helix 5, respectively (Wang et al, 2006; Wu et al, 2006; Ben-Shem et al, 2007; Lemieux et al, 2007; Brooks et al, 2011). Both regions are proposed to act as a gate to regulate substrate entry; however, the extent of their mobility is unclear (Baker et al, 2007; Wang & Ha,
Until recently, the turnover rates for rhomboid proteases and most serine proteases is achieved by allosteric interactions. For example, with thrombin and factor Xa, an allosteric site regulates substrate gating and access to an occluded active site (Johnson et al., 2006; Di Cera et al., 2007). Oligomerization represents another regulatory mechanism for enzymes. Prokaryotic rhomboids are known to form dimers, both in their membrane domain and in the soluble cytoplasmic domain independently (Sampathkumar et al., 2012; Lazareno-Saez et al., 2013), yet it is unclear if dimerization affects function.

Catalytic parameters are crucial for understanding how substrates are cleaved and how this cleavage process is regulated. Until recently, the turnover rates for rhomboid proteases and most intramembrane proteases (presenilin, site-2-protease, signal peptidase) had not been fully assessed using steady-state methods. Presenilin has been the target of many in vitro kinetic studies both in lipid and detergent environments, as well as allosteric modulation by inhibitors (Edbauer et al., 2003; Fraering et al., 2004, 2005; Kakuda et al., 2006; Chavez-Gutierrez et al., 2008, 2012; Shelton et al., 2009). For this multi-protein complex, lipids and detergents had a drastic effect on activity (Osenkowski et al., 2008). In contrast, activity assays conducted with CaaX metalloproteases Ras-converting enzyme (Rec1p) and the yeast mating a-factor processing enzyme Ste24p, conducted in both lipid (Hollander et al., 2000; Manandhar et al., 2010) and detergent environments (Pryor et al., 2013), revealed similar catalytic parameters. A more recent paper has characterized rhomboid cleavage of substrate using reconstituted proteoliposomes (Dickey et al., 2013), demonstrating slow rate of cleavage and low specificity for substrate recognition by the active site of several prokaryotic rhomboids.

Here, we show that prokaryotic rhomboids indeed have substrate specificity using steady-state kinetic analysis of the psTatA transmembrane substrate cleavage in detergent solution assessed by both gel and real-time Förster resonance energy transfer (FRET)-based assays. Furthermore, studied rhomboids bound psTatA with positive cooperativity. Competitive binding studies with AarA and its physiological substrate psTatA show that the transmembrane substrate cleavage requires an exosite. The dimeric form of rhomboids is essential for the cleavage of the transmembrane substrate TatA, whereas the monomers are active only with a soluble substrate. A model is presented for homotropic allostery where the substrate binds to both the exosite and active site to provide a means for the regulation of specificity for this class of promiscuous intramembrane proteases.

### Results

#### Rhomboid-mediated cleavage of transmembrane substrate psTatA is specific and cooperative

The kinetics of rhomboid-mediated cleavage of psTatA was assessed using gel-based cleavage assay for hiGlpG, ecGlpG and AarA (Fig 1 and Table 1). These three classes of rhomboids represent the diverse topological arrangements found in rhomboid family (Lemberg & Freeman, 2007). Representing bacterial forms, hiGlpG has the basic core composed of six transmembrane helices (TM), while ecGlpG has the 6-TM core with a cytoplasmic domain. AarA has seven predicted transmembrane domains and reflects the topological organization found in the eukaryotic secretory class of rhomboids. Prior to kinetic calculations, all parameters for the assay were optimized, including the minimal enzyme concentration and detergent concentration to prevent non-specific aggregation of both substrates and enzymes. Time of reaction was optimized in order to ensure that the measurements were obtained during initial rate phase and a linear relationship between time and product formation was observed (Supplementary Fig S1). The plots of the reaction velocity against the corresponding substrate concentration were fitted both with Michaelis–Menten and the Hill’s equation. Based on the $R^2$ values and analysis of the residuals, the Hill’s equation was chosen as the preferred model for all three studied rhomboids (Supplementary Fig S2), suggesting cooperative substrate binding behaviour. The Hill’s coefficient ($h$) indicates the degree of cooperativity, and hiGlpG exhibited the strongest positive cooperativity with $h = 2.1 \pm 0.1$. The Hill’s coefficient for AarA was $1.70 \pm 0.18$, whereas ecGlpG was found to be an almost non-cooperative enzyme with $h = 1.2 \pm 0.1$. The catalytic parameters for the three rhomboids were determined (Table 1). The $K_{0.5}$ values for AarA and ecGlpG were in the same range with AarA being slightly higher, while a much higher $K_{0.5}$ was observed for hiGlpG. The cleavage rate of AarA for psTatA cleavage, its physiological substrate, was fastest at $1.06 \pm 0.05 \text{ min}^{-1}$. This turnover rate is similar to that calculated for the AarA-mediated psTatA cleavage in proteoliposomes (0.8 min$^{-1}$) (Dickey et al., 2013), yet in contrast, our data show that $k_{cat}$ and more importantly the cleavage efficiency ($k_{cat}/K_m$) was distinctly different for all rhomboids, revealing substrate specificity.

#### FRET-based quantitative analysis of psTatA cleavage by its native enzyme AarA

To study the physiological AarA-TatA pair in detail, a real-time FRET-based kinetic assay was developed (Fig 2). An engineered substrate CyPet-psTatA-YPet (psTatA-FRET) was used for the FRET assay. Prior to kinetic calculations, all parameters for the assay were optimized (Supplementary Fig S3). We also determined the pH optimum for all studied rhomboids, which was found to be in slightly acidic pH range (pH 5.7–6.5) (Fig 2B and Supplementary Fig S3). Most serine proteases with a catalytic Ser-His-Asp triad have a pH optimum in the basic range (pH 8–11) (Ekici et al., 2008). To analyse the FRET data, a modified method taking into account auto-fluorescence was adapted to avoid signal cross contamination, which allowed an accurate determination of the kinetic parameters (Liu et al., 2012). Consistent with the gel-based assay, the Hill equation was the preferred model for kinetic data, rendering
h = 1.8 ± 0.1 and confirming cooperative substrate binding for the AarA (Fig 2C). Our kinetic parameters obtained by FRET assay (Table 2) are in good agreement with the values from gel-based assay. ecGlpG and hiGlpG could not cleave the FRET-TatA substrate, suggesting a unique mechanism of recognition exists between the AarA-psTatA physiological pair.

Rhomboids do not exhibit cooperativity with the soluble substrate casein

Conformational plasticity is an intrinsic property of an enzyme that enables it to exhibit cooperativity. One source of conformational change is the binding of the substrate, and conceivably, different
substrates are able to cause varied structural perturbations. Thus, it is plausible that the same enzyme can behave cooperatively with one substrate and be non-cooperative with another. To investigate this possibility, we measured the kinetics of cleavage of fluorescently labelled casein (FL-casein), a generic substrate that has been previously shown to be cleaved by ecGlpG (Wang et al., 2006; Figure 2). FRET-based AarA activity assay. A FRET-based activity assay was used to measure the catalytic parameters for AarA-mediated cleavage of psTatA-FRET.

Table 2. Kinetic parameters of AarA and active site mutant using psTatA-Förster resonance energy transfer (FRET) substrate

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_0.5$ (μM)</th>
<th>$V_{max}$ (μM min$^{-1}$)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_0.5$ (min$^{-1}$ μM$^{-1}$)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>AarA wt</td>
<td>3.9 ± 0.3</td>
<td>$2.3 \times 10^{-2}$ ± $0.1 \times 10^{-2}$</td>
<td>0.17 ± 0.07</td>
<td>$4.3 \times 10^{-2}$ ± $0.5 \times 10^{-2}$</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>AarA S150A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The purified substrate (0.13–7 μM) was incubated with AarA or mutant protein (0.135 μM) at 37°C for 2 h, and the emission intensity of CyPet at 475 nm and YPet at 530 nm was recorded with the excitation wavelength of 414 nm. The initial velocities were calculated for each substrate concentration. $K_0.5$ and $V_{max}$ were obtained from fitting the data to the Hill’s equation by plotting the initial velocities of psTatA-FRET-digestion versus the corresponding substrate concentration.
Xue & Ha, 2012; Lazarenko-Saez et al., 2013). The optimized activity assay with FL-casein for the three rhomboids, purified in 0.1% dodecyl-maltoside (DDM), was conducted as previously reported (Lazarenko-Saez et al., 2013). Plots of reaction velocity versus substrate concentration were fitted with Michaelis–Menten and Hill’s equations, revealing a clear preference for the first model for all enzymes (Fig 3). Thus, all enzymes showed non-cooperative substrate binding with casein and exhibited very similar kinetic parameters, demonstrating no specificity for this substrate (Table 3).

**Dimers are important for psTatA cleavage**

We have previously demonstrated that prokaryotic rhomboids are dimeric in detergent and lipid environments (Sampathkumar et al., 2012). Many enzymes are found as oligomers, and changes in enzymatic activity commonly accompany oligomer dissociation (Traut, 1994). To assess the effect of oligomeric state on rhomboid protease activity, the conditions for the isolation of stable monomers were identified. When purified in DDM containing buffer, all rhomboids were dimeric, whereas exchange from DDM to the chemically similar decyl-maltoside (DM) during gel filtration resulted predominantly in monomers (Fig 4A and B), with small peaks of remaining dimers for hiGlG and ecGlG. For AarA, however, upon DM exchange, a broad peak with a shoulder was observed, suggesting that a mixture of monomers and dimers was present at these conditions (Fig 4C). Monomers were obtained only when AarA was solubilized and purified in DM.

To determine whether oligomerization affects catalytic properties of rhomboids, we assessed the activity of dimers and monomers with the gel-based assay using psTatA. Rhomboids purified either in 0.1% DDM or in 0.2% DM were tested for activity using buffer containing DDM or DM (Fig 4D–F). It is important to note that for all activity measurements, the protein was diluted more than 500 times in activity buffer with detergent; thus, the amount of detergent used for purification was negligible. For all three rhomboids, we observed a loss of activity in the presence of DM, at conditions where the protein exists as a monomer. For AarA, purified in DDM, only 50% of activity was observed in DM reaction buffer, consistent with the gel filtration results, showing only partial dissociation. A full loss of activity for AarA was observed only when DM was used in both Ni-NTA purification and the activity assay. We confirmed that DM detergent does not directly influence enzymatic activity (Supplementary Fig S4). Rhomboids purified in DM regained activity when the assay was conducted in DDM, proving that the loss of activity in the presence of DM was not due to protein denaturation or to detergent itself. We were also able to demonstrate re-association of hiGlG monomers purified in DM by SEC using a buffer containing DDM (Supplementary Fig S5). Little or no aggregation was observed during gel filtration (Fig 4), verifying the mild influence of DM and DDM on rhomboid stability. Circular dichroism (CD) spectra overlap for both dimeric and monomeric forms for hiGlG, the rhomboid with the simplest enzyme topology; thus, DM did not alter structural integrity during the transition from dimer to monomer. The melting temperature ($T_m$) of dimers is slightly higher in comparison with monomers, which may be attributed to the mutual stabilization of subunits within the dimer and their dissociation as the initial step during the thermal unfolding of the enzyme.

To investigate the predominant oligomeric state in the lipid bilayer, we took advantage of the exposed cytoplasmic domain of ecGlG, which is known to form domain-swapped dimers (Lazarenko-Saez et al., 2013). We examined the oligomeric state of the cytoplasmic domain of ecGlG upon shedding from the membrane, demonstrating the prevalent oligomeric state in its native lipid environment is dimeric (Fig 4G and H). A crucial aspect of this study was that neither the proteins were exposed to detergent nor the samples were concentrated, providing an assessment of the protein in its native environment.

**Table 3. Kinetic parameters of AarA, ecGlG and hiGlG using FL-casein as a substrate**

<table>
<thead>
<tr>
<th>Rhomboid</th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µM min$^{-1}$)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_M$ (µM$^{-1}$ min$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>AarA</td>
<td>1.8 ± 0.47</td>
<td>0.066 ± 0.012</td>
<td>0.36 ± 0.068</td>
<td>0.2 ± 0.092</td>
</tr>
<tr>
<td>ecGlG</td>
<td>2.5 ± 0.37</td>
<td>0.067 ± 0.022</td>
<td>0.37 ± 0.122</td>
<td>0.148 ± 0.068</td>
</tr>
<tr>
<td>hiGlG</td>
<td>65 ± 2.1</td>
<td>0.070 ± 0.069</td>
<td>0.38 ± 0.037</td>
<td>0.05 ± 0.020</td>
</tr>
</tbody>
</table>

The reaction mixture contained 0.179–8.95 µM of BODIPY FL-casein, activity buffer (50 mM MES, pH 6.0, 150 mM NaCl, 20% glycerol, 0.1% DDM) and 0.179 µM of rhomboid enzyme. The substrate was mixed with the activity buffer and incubated at 37°C for 1 h in the dark. The reaction was started with the protease. Fluorescence emission at 513 nm was measured at 37°C every 5 min over 2 h in a Fluostar fluorescence microplate reader with an excitation wavelength of 503 nm.
Interestingly, monomers of all three rhomboids were able to cleave casein (Supplementary Fig S6), revealing that the monomeric form of the enzyme is functional with the soluble substrate but lacks the elements needed for the psTatA transmembrane substrate cleavage. These results lead us to question whether the catalysis of rhomboid’s native substrates is governed by the active site alone or whether another mechanism, such as allosteric interactions, is involved in the process.

**The cleavage of psTatA is governed by allosteric interactions**

To determine whether the cleavage of psTatA is dependent on allosteric interactions, we performed competition binding assays using casein as a main substrate and psTatA as a competing substrate for the three rhomboids: hiGlpG, ecGlpG and AarA (Fig 5). Rhomboid protease was reacted with FL-casein in the presence of different concentrations of psTatA and casein. The cleavage of psTatA was monitored by SDS-PAGE and the absorbance of the bands was measured. The results showed that the cleavage of psTatA is dependent on the presence of casein, indicating that the cleavage of psTatA is governed by allosteric interactions.
concentrations of psTatA. Initial velocities were measured at each substrate concentration and plotted against the corresponding casein concentration. The obtained data sets were fitted simultaneously and globally to competitive, non-competitive and mixed inhibition. For AarA, the best model was estimated to be non-competitive inhibition with a global $R^2$ of 0.973 (Fig 5A). Global fits of the AarA data set for competitive and mixed inhibition revealed clear systematic deviations from best-fit lines. The determined $K_i$ for psTatA is $9.2 \pm 0.7 \, \mu M$, represented by $K_i$ for the AarA-psTatA complex (Fersht, 2002). For ecGlpG, the global fit revealed competitive inhibition as best-fit model with global $R^2$ of 0.967 (Fig 5B) with $K_i$ for ecGlpG-psTatA being $4.29 \pm 0.60 \, \mu M$, which is significantly lower than that previously observed with detergent solubilized ecGlpG-TatA, 119 $\mu M$ (Dickey et al., 2013). The derived kinetic constants are shown on the plots. For hiGlpG, no effect on activity was observed with increasing psTatA concentration (Supplementary Fig S6). This can be explained by the high $K_{i,5}$ of hiGlpG for psTatA (119 $\mu M$) that exceeds the possible concentration range to retain a soluble substrate. Competition binding assays were also performed with monomeric form of the AarA (Supplementary Fig S7). As performed for the dimers, FL-casein cleavage was monitored with increasing amounts of psTatA. No difference in cleavage was observed in the presence of TatA, further confirming the monomeric form of AarA does not bind psTatA.

The non-competitive inhibition with the dimeric AarA indicates that psTatA binds to the enzyme in a region other than the active site, suggesting the presence of a remote binding site (exosite) for substrate recognition. Furthermore, this clearly demonstrates the initial binding of psTatA to the exosite drives a conformational change in the active site of AarA. In the presence of psTatA, AarA still binds casein with the same affinity, but its active site is no longer in the optimal arrangement to cleave casein. In contrast to AarA, psTatA binds to dimeric ecGlpG competitively, suggesting no conformational changes in the active site are induced upon psTatA binding to ecGlpG exosite, if any binding occurs at all. Competition studies with monomeric AarA show that dimerization is important for allosteric crosstalk between active site and exosite. Taken together, these results clearly indicate that the exosite interaction is the initial step in specific substrate binding of psTatA to AarA.

**Discussion**

In this paper, we report the steady-state kinetic analysis of rhomboid intramembrane protease family members. Kinetic experiments with transmembrane substrate revealed deviations from Michaelis–Menten enzyme kinetics. Sigmoidal saturation curves and Hill’s coefficients indicate positive cooperativity of TatA cleavage, suggesting that conformational changes occur upon substrate binding to these rhomboids. Our competitive binding studies provide strong evidence that the studied rhomboid proteases also have allosteric properties. Allosteric regulation is an efficient mechanism for modulation and regulation of protein activity to prevent non-specific substrate cleavage by altering the substrate accessibility and affinity. The classical model for enzyme allosteric behaviour assumes the presence of two non-equivalent binding sites, where one of the sites is a regulatory exosite and the other is the catalytic site. If substrate binds to both sites, it is an example of homotropic allosterism. The involvement of an exosite in rhomboid substrate binding has been proposed in a previous report (Strisovsky et al., 2009). Our competition experiments confirm the presence of an exosite and validate the model for allosteric regulation of AarA activity. This is the first evidence of homotropic allosteric activation for any intramembrane protease family member by its natural substrate.
Based on our kinetic evidence, we propose the following model for psTatA cleavage by AarA (Fig 6). Since psTatA is the only identified substrate for any prokaryotic rhomboid, AarA-psTatA represents the only known physiological enzyme–substrate pair in propkaryotes. The process of substrate binding to rhomboids is achieved through a multi-step dynamic process. In the lipid bilayer, AarA exists as dimer, containing both an active site and an exosite. Our in vitro studies revealed that dimer assembly is required for activity with the transmembrane substrate psTatA, and when dimers reversibly dissociate into monomers, the function is lost. In contrast, monomeric form is capable of cleaving FL-casein, suggesting that the active site is not affected upon dissociation. Crystal structures further support the integrity of the active site of the monomeric form (Wang et al., 2006; Lemieux et al., 2007; Vinothkumar et al., 2011). Taken together, these results suggest that rhomboids may have two different mechanisms for substrate recognition; the transmembrane substrate is recognized on the hydrophobic belt of the enzyme by the exosite, which facilitates the substrate entry laterally into the active site. We propose soluble substrates, such as FL-casein, do not require initial exosite binding and approach the active site from the soluble face of the enzyme via the opening of loop 5. Our model also may explain the discrepancy observed from mutations in the proposed gating regions. Mutations disrupting the interaction between helices 2 and 5 are known to enhance TM cleavage of FL-casein, do not require initial exosite binding and approach the active site in a non-competing pathway further suggestive of different sites of substrate recognition (Sampathkumar et al., 2012). The prevalent oligomeric state for ecGlpG, expressed in E. coli membranes, was dimeric state as well. These results validate the physiological relevance of above model and lead us to speculate that allosteric may represent a regulatory mechanism for TM protein recognition for the rhomboid family. In support of this hypothesis, the mitochondrial PARL protein is known to recognize several substrates in a non-competing pathway further suggestive of different sites of substrate recognition (Sekine et al., 2012; Chan & McQuibban, 2013). Furthermore, the eukaryotic rhomboid RHBDD2 has been shown to oligomerize (Ahmedi et al., 2013), and while this is an inactive iRhom, it is known to bind TM substrates (Lemberg & Freeman, 2007). It is possible that dimerization is not essential for TM substrate cleavage but could enhance cleavage. An alternative interpretation to our observations is that DM not only disrupts dimerization but could also bind to the exosite and prevents substrate binding at high concentrations above the DM critical micelle concentration (CMC).

The broad specificity of rhomboids and its physiological relevance was discussed in the other reports (Urban et al., 2002; Strisovsky et al., 2009; Dickey et al., 2013). In the present study, we have determined the turnover rates and \( K_{\text{cat}} \) values for three rhomboid family members in detergent solution (Table 1). These two parameters are interrelated, and an enzyme may achieve an ideal

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**Figure 6.** A schematic illustration of rhomboid exosite-mediated substrate recognition and allosteric regulation of cleavage.

We propose a preliminary model of transmembrane substrate cleavage by rhomboid protease. Dimerization allows the formation of an exosite, either via conformational changes upon dimerization, or the exosite is located at the interface (not depicted in this illustration). The initial binding of the substrate’s transmembrane segment by its exosite recognition motif (red) to the rhomboid intramembrane-located exosite will allow the cleavage at a low rate \( k_{\text{cat}-\alpha} \) and induce subtle conformational changes in the active site (A) resulting in the optimal active site arrangement and increased rate of catalysis \( k_{\text{cat}-\beta} \). The overall rate measured is therefore an apparent \( k_{\text{cat}} \) \( (k_{\text{cat-app}}) \).
balance by optimizing the ratio of $k_{\text{cat}}/K_M$, which describes enzyme’s efficiency for any substrate. The determined values for ecGlpG and hiGlpG were lower by a factor of 10 and 100, respectively, in comparison with AarA. These values permit a meaningful quantitative comparison for rhomboids ability to react with psTatA and allow us to conclude that rhomboid-mediated cleavage is more efficient towards the natural substrate and hence specific. Our kinetic data allowed us to evaluate the efficiency of the rhomboid protease and demonstrate that this enzyme was slow. The slow rate of enzyme has already been anticipated due to non-canonically arranged of the active site catalytic dyad instead of triad and the lipidic environment surrounding the enzyme. When catalytic triad of trypsin was converted to Ser–His dyad, a 10-fold decrease in the activity was observed (Craik et al., 1987). However, the turnover rate of rhomboids was found to be comparable to some soluble serine proteases (http://www.brenda-enzymes.org/index.php4) (Tschantz & Dalbey, 1994; Gallagher et al., 2001) having similar catalytic architecture. For example, with the E. coli signal peptide I (SPase), the $k_{\text{cat}}$ values range from 0.016 to 660 min$^{-1}$ for different substrates (also see BRaunschweig ENzyme Database, http://www.brenda-enzymes.org for more information). The E. coli SPase is a membrane-tethered enzyme having a large soluble protease domain with a Ser–Lys dyad that removes amino-terminal signal sequence from proteins translocated from cytoplasm (Auclair et al., 2012). Similar to rhomboids, SPases are known to cleave substrates with small side-chain residues in the P1 position (Nielsen et al., 1998).

A recent paper has reported kinetic parameters of rhomboid proteases using TatA as a substrate in reconstituted proteoliposomes (Dickey et al., 2013). The cleavage of psTatA by ecGlpG rhomboid in these conditions obeyed Michaelis–Menten kinetics and showed no cooperativity. These data are consistent with our observations, revealing that ecGlpG behaviour with psTatA is almost non-cooperative (n = 1.2) and demonstrates non-specific or no psTatA binding to rhomboid ecGlpG exosite. Furthermore, the above paper also reported that detergent solubilization increased the rate of substrate cleavage (Dickey et al., 2013). However, the turnover rate of AarA enzyme with psTatA in proteoliposomes, 0.8 min$^{-1}$, is comparable to our values, 1.06 min$^{-1}$ (Table 1), suggesting detergent does not have a drastic effect on catalysis. Analogously, the CaaX protease has similar activity in both membrane and detergent solubilized state; this study was conducted with C$_{12}$E$_7$ detergent, also having a very low CMC similar to DDM. Simple membrane proteins such as rhomboids, purified in a single step with mild detergents such as DDM and DM, tend to retain native protein structure and function. Endogenous lipids can be co-purified at lipid protein ratios as high as 36:1, as observed for a polytopic 12-transmembrane membrane protein after purification in DDM (Wang et al., 2003). In our system, the substrate and enzyme molecules likely are surrounded by mixed detergent:lipid micelles, explaining why we observe similar kinetics to that measured in the lipid bilayer. Lastly, our competition studies reveal the affinity of the psTatA substrate for ecGlpG. In detergent solution, we observe a significantly higher affinity, $K_d$, 4.29 ± 0.60 μM, compared to that observed with reconstituted enzyme (119 μM; Dickey et al., 2013). At present, the reason behind this discrepancy is unclear and probably can be explained by the differences in utilized methods.

The location of the enzyme’s exosite remains to be determined. Based on a crystal structure of ecGlpG in lipid environment, a plausible exosite at the interface of TM2/TM5 has been suggested (Vinothkumar, 2011). Our current data do not allow us to make any predictions about the conformational changes that occur during dimerization and allosteric communication between exosite and active site. Rhomboid-inhibitor structures revealed only minor structural changes; however, inhibitor binding does not truly mimic the multi-step substrate binding process and thus may not induce structural changes similar to that with substrates (Vinothkumar et al., 2011; Xue & Ha, 2013). An enzyme–substrate complex crystal structure will provide information on the conformational changes associated with substrate binding. We propose that allosteric interactions facilitate movements required for substrate entry into the buried active site via loop 5 and helix 5. Substrate–exosite interactions may also play a role in substrate gating by tethering the substrate in a canonical conformation proximal to catalytic machinery, which increases the probability of substrate access into the active site. Understanding the structural changes that occur upon dimerization and allosteric activation will furthermore resolve the longstanding controversy over substrate gating into the membrane-buried active site. It also remains to be seen whether eukaryotic rhomboids are also dimeric and allosterically regulated. This would have significant implications for therapeutic design, since there are a number of advantages in using allosteric modulators over classic orthosteric ligands. Our work describes a unique example of allosteric interactions for an intramembrane protease and will set the stage to explore the role of allostery for other intramembrane proteases.

### Materials and Methods

#### Expression and purification of ecGlpG, hiGlpG and AarA

Rhomboid genes were cloned into pBAD-Myc/HisA plasmid (Invitrogen, Canada), having C-terminal Tobacco Etch Virus (TEV) protease cleavage site, Myc-epitope and His$_6$-tag. The vector was transformed into TOP10 chemically competent E. coli cells. The protein was induced with 0.002% arabinose and expressed at 24°C for 8 h in LB media. The cells were harvested, resuspended in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, Tris-buffered saline (TBS) and lysed under high pressure (EmulsiFlex-C3). The membranes were isolated by ultracentrifugation at 95,800 g for 2 h, solubilized in 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol, 1% (w/v) DDM or 2% (w/v) DM and applied onto a Ni-NTA column chromatography (Qiagen, Ontario, Canada). The proteins were eluted with 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol, 1% (w/v) DDM or 2% (w/v) DM and applied onto a Ni-NTA column chromatography (Qiagen, Ontario, Canada). The proteins were eluted with 50–500 mM of imidazole, 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 20% glycerol, 0.1% DM or 0.2% DM. The His-tag was removed by TEV protease (1 mg per 100 mg of protein, overnight, 16°C), and a subsequent Ni-NTA column was performed to remove uncleaved protein and TEV protease. The flow-through was collected and concentrated using 30,000 MWCO concentrators (Millipore, USA). The protein samples were flash-frozen and stored at −80°C.

#### Rhomboid proteolytic activity assay with psTatA

The cleavage reaction mixture consisted of 0.5–15 μM of psTatA substrate mixed with 0.13 (for AarA) and 0.33 μM (for ecGlpG and hiGlpG) enzyme in 50 mM 2-(n-Morpholino)ethanesulfonic acid
Rhomboid proteolytic activity assay with FL-casein as substrates

The ecGlpG, hiGlpG and AarA cleavage assays using FL-casein as substrates were performed as previously described (Lazareno-Saez et al., 2013).

SDS-Tricine and SDS-PAGE gels

SDS-Tricine gels were used for gel-based activity assays for high resolution of proteins smaller than 30 kDa. SDS-PAGE gels were used for purity control. Standard protocols of both systems were employed.

Expression and purification of psTatA-FRET

We measured kinetic parameters of psTatA cleavage by AarA with a Förster resonance energy transfer (FRET)-based protease assay using an engineered FRET pair, CyPet and YPet (derived from cyan fluorescence protein and yellow fluorescence protein). Rhomboid substrate psTatA was cloned between CyPet and YPet FRET pair in pBad HisB vector. This fluorescent protein pair exhibits 20-fold energy transfer efficiency when compared to the parental pair (Nguyen & Daugherty, 2005). The vector containing CyPet-psTatA-YPet substrate (psTatA-FRET) was transformed into GlpG knockout cells from the Keio library (glpG::Kn; Baba et al., 2006). Expression in GlpG knockout cells prevented premature cleavage of the substrate. The cells were grown in LB media with ampicillin (100 μg L\(^{-1}\)) and kanamycin (30 μg L\(^{-1}\)) until OD\(_{600}\) reached 0.6 followed by induction with 0.02% of arabinose. The protein was expressed for 18 h at 24°C, cells were lysed, cell debris was removed with centrifugation (22,320 g), and the supernatant was loaded on the Ni-NTA column equilibrated with 50 mM Tris–HCl pH 8.0, 200 mM NaCl, 20% glycerol and 0.1% DDM. The protein was eluted with 200–350 mM of imidazole, concentrated and, loaded on SEC Superdex 200(16/60) (GE, Canada) equilibrated with the same buffer. The fractions were assessed with SDS-PAGE, and the protein concentration was determined by the BCA method. Pure protein (> 95%) was pooled, concentrated and flash-frozen prior to storage at −80°C.

Providencia stuartii TatA purification

Providencia stuartii TatA (psTatA) was purified using anti-flag resin as described previously (Lazareno-Saez et al., 2013).

FRET-based protease kinetic assay

CyPet-psTatA-YPet was incubated with AarA at 37°C in buffer at pH 6.0 containing 50 mM MES pH 6.0, 150 mM NaCl, 20% glycerol and 0.1% DDM for 20 min. The final concentration of AarA was fixed at 0.135 μM, and the final concentration of CyPet-psTatA-YPet was varied from 0.13 to 7 μM. FRET-based AarA cleavage assay was conducted by measuring the emission intensity of CyPet at 475 nm and YPet at 530 nm with the excitation wavelength of 414 nm in a fluorescence multi-well plate reader (SynergyMx, BioTek). To negate the effect of signal cross contamination, we determined the CyPet and YPet direct emissions and total emissions at 530 nm. The emission of the recombinant protein CyPet-psTatA-YPet was measured at 475 nm when excited at 414 nm to determine the CyPet direct emission; the emission was measured at 530 nm when excited at 475 nm to determine the YPet direct emission. The fluorescence emission was measured every 5 min for 2 h. We then obtained digested concentration of substrate using the following equation, developed by Liu and co-workers (Liu et al., 2012):

\[
\text{FL}_{475} = \frac{M - x}{M} \left( \text{FL}_{475}^0 - \alpha \text{FL}_{414}^0 \frac{\text{CyPet}}{\text{CyPet} + \text{YPet}} - \beta \text{FL}_{475}^0 \frac{\text{YPet}}{\text{CyPet} + \text{YPet}} \right) + \alpha \left( \frac{M - x}{M} \right) + \beta \text{FL}_{475}^0 \frac{\text{YPet}}{\text{CyPet} + \text{YPet}}
\]

\[\text{FL}_{475}^0\] and \[\text{FL}_{414}^0\] are total fluorescence emission at 530 nm when excited at 414 nm before and after digestion, respectively, \(M\) is the total amount of CyPet-psTatA-YPet, and \(x\) is the amount of digested CyPet-psTatA-YPet. \(\alpha\) is ratio of fluorescence emission by CyPet at 530–475 nm under excitation at 414 nm, whereas \(\alpha \text{FL}_{414} \frac{\text{CyPet}}{\text{CyPet} + \text{YPet}}\) is CyPet direct emission at 475 nm when excited at 414 nm. Similarly, \(\beta\) is ratio of fluorescence emission by YPet at 530–475 nm under excitation at 475 nm. Using this ratio, YPet direct emission \(\left( \beta \text{FL}_{475}^0 \frac{\text{YPet}}{\text{CyPet} + \text{YPet}} \right)\) at 530 nm when excited at 475 nm was calculated.

Here, 30/68 gives the molecular mass ratio of CyPet-psTatA to YPet. The plot of emission of CyPet-psTatA-YPet at 475 nm under excitation at 414 nm versus amount yields a straight line, and the constant \(k\) determines the slope of this line. Similarly, a linear relationship between the emission of CyPet-psTatA + YPet (1:1 molar ratio) under excitation at 414 nm and the protein amount is found. In this case, \(j\) describes the slope of the plot. Both parameters, \(j\) and \(k\), were calculated from standard plots of fluorescence emission versus amount of protein and used in the equation.

Before kinetic calculations, all the parameters for the assay were optimized. It was verified that the proportionality between the
fluorescence emitted and the amount of the substrate used in the assay was linear. The minimal concentration of the enzyme that gave a linear dependence of amount of generated product with time was chosen, as well as the minimal time of reaction within the linear part of the curve.

For pH optimization, enzymatic activity was measured with psTata-FRET spanning the interval of pH 2–9. Tris-buffer was chosen deliberatively to maintain the correct pH range to avoid effects of buffering compounds on the activity. For ecGlpG and hiGlpG, the pH optima was measured in the same manner, using FL-casein as a substrate (Lazarenko-Saez et al., 2013).

Circular dichroism

Ni-NTA purified hiGlpG in 0.1 % DDM was further purified by size-exclusion chromatography in a buffer containing 25 mM Tris pH 8, 150 mM NaCl, 5% glycerol and either 0.1% DDM or 0.2% DM to harvest protein in either dimeric or monomeric forms, respectively. CD measurements were performed using JASCO-720 spectrophotometer. All spectra were recorded using 0.1 mm path length cell at a protein concentration of 0.2 mg ml⁻¹ and 0.14 mg ml⁻¹ for monomers and dimers, respectively. Melting curves of the samples were measured at 222 nm between 25 and 90°C with a gradient resolution of 0.2°C. The rate of temperature change was 0.5°C min⁻¹. The bandwidth of the equipment was 1 nm with a response time of 1 s.

Inside-out vesicle preparation

ecGlpG was cloned with a TEV protease site inserted between residues 91 and 92, separating N-terminal cytoplasmic and C-terminal membrane domains. In a 2:l culture, ecGlpG was expressed and the membrane fraction was isolated as described above. Membranes were resuspended in 15 volumes of TBS and pelleted by ultracentrifugation at 95,800 g for 1 h. From the supernatant, the liberated cytoplasmic domain was assessed by SDS-PAGE. Prior to gel filtration, membranes were resuspended in 15 volumes of TBS and subjected to three freeze-thaw cycles to create inverted membrane fraction. As a negative control, the remaining membranes were incubated in a similar manner without TEV protease. The membrane fractions were pelleted at 95,800 g for 1 h. From the supernatant, the liberated cytoplasmic domain was assessed by SDS-PAGE. Prior to gel filtration, TEV protease was removed using Ni-NTA column; the flow through, containing the cytoplasmic domain, was loaded on a Superdex 75 (10/30) column (GE, Canada).

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Author contributions

EA, NG, PMS, and MWM conducted kinetic experiments. EA, PP and MJL analysed data and wrote the paper. All authors edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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