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Mechanistic insights into intramembrane proteolysis by E. coli site-2 protease homolog RseP

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Site-2 proteases are a conserved family of intramembrane proteases that cleave transmembrane substrates to regulate signal transduction and maintain proteostasis. Here, we elucidated crystal structures of inhibitor-bound forms of bacterial site-2 proteases including Escherichia coli RseP. Structure-based chemical modification and cross-linking experiments indicated that the RseP domains surrounding the active center undergo conformational changes to expose the substrate-binding site, suggesting that RseP has a gating mechanism to regulate substrate entry. Furthermore, mutational analysis suggests that a conserved electrostatic linkage between the transmembrane and peripheral membrane-associated domains mediates the conformational changes. In vivo cleavage assays also support that the substrate transmembrane helix is unwound by strand addition to the intramembrane β sheet of RseP and is clamped by a conserved asparagine residue at the active center for efficient cleavage. This mechanism underlying the substrate binding, i.e., unwinding and clamping, appears common across distinct families of intramembrane proteases that cleave transmembrane segments.

INTRODUCTION

Intramembrane proteolysis—hydrolysis of a peptide bond within the lipid bilayer—is implicated in a variety of cellular processes throughout all three domains of life, including signal transduction and membrane protein homeostasis (1–3). In humans, deregulation of this cleavage leads to diseases such as Alzheimer’s disease (4, 5), while signal transduction through intramembrane proteolysis is associated with pathogenic infections (6, 7). The substrate cleavage is catalyzed by four distinct families of intramembrane proteases, each classified on the basis of catalytic mechanism: the zinc metalloprotease site-2 protease (S2P), the aspartic protease presenilin/signal peptide peptidase (SPP), and the serine protease Ras-converting enzyme 1 (Rce1) (8, 9), among which S2P, presenilin/SPP, and Rce1 cleave transmembrane (TM) segments of substrates. Eukaryotic S2Ps, including human S2P, have been identified in signal transduction for lipid metabolism (10), in which they perform the intramembrane proteolysis of transcription factor precursors after the extracellular region is cleaved off (11). In Rce1, two tandemly arranged periplasmic PDZ domains (PDZ tandem) were proposed to serve as a size exclusion filter to sterically hinder active site entry by any substrate having a bulky periplasmic domain (12). Rce1 also predicted to have two transmembrane β hairpins (Fig. S1), which were shown to bind the substrate near the bond that is cleaved and to contribute to substrate discrimination (24, 25). However, the absence of structural data hampers understanding of how site-1–cleaved substrates pass through the size exclusion filter to access the active center and of how the substrate TM segments exactly bind with the intramembrane β sheet of S2Ps. Because the active center of S2Ps is predicted to be located within the hydrophobic milieu of the lipid bilayer, it must both form a hydrophilic compartment around the catalytic zinc for efficient hydrolysis and accommodate the hydrophobic segments of the substrate TM domain. Concerning the S2P family, a crystal structure including the active center is available for the TM domain of the archaeon Methanothermobacter jannaschii S2P homolog (MJs2P) (26). However, MJs2P belongs to the group III subfamily of S2P and does not have PDZ domains in the extracytoplasmic region (Fig. 1C) (15). Furthermore, MJs2P was proposed to regulate substrate entry by using the TM helices flanking the catalytic core TM domain as a gate, but those helices are not present in group I S2Ps such as EcRseP and human S2P. Therefore, further structural analysis, especially on the group I S2Ps having extracytoplasmic PDZ domains, is essential for understanding the mechanism of
The PDZ tandem of RseP was proposed to perform intramembrane proteolysis of the DegS-cleaved form of RseA (site-2 cleavage). Subsequently, RseP cleaved an analog of its native substrate containing the TM segment from the K. koreensis RseA ortholog and substrate analogs for EcRseP. Mutations to the putative KkRseP active site impaired this cleavage activity (fig. S2, B and C). Furthermore, detergent-solubilized KkRseP can cleave E. coli RseA, although with reduced activity as compared with EcRseP (Fig. 2J). In this study, crystal structures of EcRseP and selenomethionine (SeMet)–substituted KkRseP were determined in complex with batimastat, an inhibitor for EcRseP (Fig. 2, Table 1, and fig. S3) (27).

**RESULTS**

**Crystallographic analysis of EcRseP and KkRseP**

For structural analysis, we purified RseP from E. coli (EcRseP) and its ortholog from marine bacterium Kangiella koreensis (KkRseP) (fig. S1). KkRseP restored the growth deficiency of E. coli rseP mutant cells (fig. S2A). In E. coli, KkRseP cleaved an analog of its native substrate containing the TM segment from the K. koreensis RseA ortholog and substrate analogs for EcRseP. Mutations to the putative KkRseP active site impaired this cleavage activity (fig. S2, B and C). Furthermore, detergent-solubilized KkRseP can cleave E. coli RseA, although with reduced activity as compared with EcRseP (Fig. 2J). In this study, crystal structures of EcRseP and selenomethionine (SeMet)–substituted KkRseP were determined in complex with batimastat, an inhibitor for EcRseP (Fig. 2, Table 1, and fig. S3) (27).

**Overall structure of EcRseP**

The final model of EcRseP (M1 to F447) is full length except for the three C-terminal residues and recombinant tag (Fig. 2, B to E). TM1 (M1 to C33) contains two zinc-coordinating His residues, H22 and H26 (fig. S4A). TM3 is divided by a loop-like bulge into two segments, TM3-N and TM3-C. TM3-C contains the third zinc-coordinating residue, D402 (fig. S4A). Besides the zinc ion in the active center, a second zinc ion was bound to H86 and H87 in the cytoplasmic region. However, its physiological role is currently unknown as H86A and/or H87A mutations did not affect the proteolytic activity (fig. S4, B to D, and table S5). EcRseP was predicted to have two intramembrane β hairpins, the C1N loop (25) and the membrane-reentrant β loop (MREβ-loop) (24), between TM1 and TM2 (fig. S1). The corresponding regions are integrated into a four-stranded β sheet (hereafter referred to as the MREβ-sheet) (fig. S5, A and B). Strand 4 corresponds to the edge strand and forms one side of the substrate-binding site where its backbone makes direct contacts with batimastat (Fig. 3). These observations are consistent with our previous findings that proteolytic activity is reduced by introducing Pro mutations into the two strands closest to the active site as observed here, either into the N-terminal region of C1N (R39 to F44 corresponding to strand 1) or into the C-terminal region of the MREβ-loop (G67 to V70 corresponding to strand 4) (25). Similarly, substrate cleavage was strongly impaired in the G43A/I61G double mutant but not by either single mutation. In the crystal structure, G43 and I61 are proximal (G43 on the C1N loop and I61G on the MREβ-loop). MJ2P also has a membrane-embedded β sheet (26), while its topology differs in strand order. However, strand 4 is still proximal to the active center (fig. S5, A and B, and d). The arrangement of the β sheet relative to the active center is the same as for the MREβ-sheet of EcRseP. Together, TM1 to TM3 and the MREβ-sheet structurally align with their equivalents in MJ2P.

A hydrophilic compartment for substrate binding and cleavage within the membrane is formed with substantial structure contributions from the MREβ-sheet (fig. S6). The edge strand of the MREβ-sheet, together with the conserved TM core region, forms a compartment that accommodates batimastat and the catalytic zinc ion. The interior of RseP during substrate accommodation and cleavage. The results provide crucial insights into the mechanism of the intramembrane proteolysis not only for S2Ps but also for the other families of intramembrane proteases that cleave the TM segments of substrates.

**Fig. 1. Domain organization of the S2P family members and involvement of RseP in the E. coli extracellular stress response.** The topology diagrams for (A) EcRseP and KkRseP of group I, (B) human S2P (HsS2P) of group I, and (C) MJ2P of group III, respectively. The three TM helices colored orange constitute a conserved catalytic core region. The group I S2Ps have a different number of PDZ domains. HsS2P contains a Cys-rich region inserted into the PDZ domain. The group III S2Ps (e.g., MJ2P) have a cystathionine-β-synthase (CBS) domain but no PDZ domain. Crystallographic analysis of MJ2P suggests that TM1, TM5, and TM6 (light magenta) serve as the substrate entry gate where the close proximity between TM1 and TM6 forms a gate-closed state (26). TM1 and TM6 in MV2P are less or not conserved in HsS2P and RsePs. (D) Extracellular stress causes accumulation of unfolded or denatured outer membrane proteins (OMPs) in the periplasm, together with dissociation of RseB from RseA. DegS, the E. coli ortholog of site-1 protease (S1P), is activated by interaction with the unfolded OMP and cleaves the periplasmic region of the anti–sigma factor RseA (site-1 cleavage). Subsequently, RseP performs intramembrane proteolysis of the DegS-cleaved form of RseA (site-2 cleavage), which leads to the activation of σ5. The PDZ tandem of RseP was suggested to sterically hinder the entry of the full-length RseA complexed with RseB. In this study, structure-based mutational and cross-linking analyses have been conducted to address the question of how RseP accommodates the site-1-cleaved substrates using the PDZ tandem, the PDZ C-terminal (PCT) region including H1 and H2 helices, TM4, and the MREβ-sheet.
this compartment, which is separated from the lipid bilayer by TM4, is hydrophilic because of the presence of several charged or polar residues (K71, D74, E78, R97, N108, S387, N394, H405, E412, Y428, S432, and the zinc-coordinating residues) and the exposed backbone of the edge strand (fig. S6, A and B). The previous crystallographic analysis of MjS2P had also suggested that both the exposed backbone of the edge strand and the charged residues on the TM helices coordinate water in a channel to the active site (26). The MREβ-sheet also has several charged residues on the cytoplasmic side and likely excludes lipid molecules, assisting the
hydrophilic compartment to recruit water molecules into the active center (fig. S6C). In addition, the cytoplasmic region following the edge strand may also contribute to substrate binding and cleavage. Many S2Ps, including those from both group I and group III, contain acidic residues in the region connecting the putative edge strand and the following TM region (fig. S1). Mutations of these acidic residues substantially reduced the proteolytic activity in the *Bacillus subtilis* group III S2P homolog SpoIVFB (23). In EcRseP, most of the acidic residues in the cytoplasmic region downstream of the MREβ-sheet form salt bridges with the surrounding basic residues and contribute to the formation of the hydrophilic compartment while this cytoplasmic region forms a small globular domain (fig. S6D). Our structural data also support that the acidic residues in this region are involved in binding substrate and/or recruiting water molecules to the active center.

The PDZ tandem protrudes into the periplasmic space (Fig. 2, B and C). The two PDZ domains form a pocket-like space (PDZ pocket) oriented toward the TM domain containing the active center (Fig. 2, B to D). As predicted in the previous study (21), the N-terminal residues of the PDZ C-terminal (PCT) region (P323 to T350) form an amphiphilic helix (PCT-H1) at the membrane surface (Fig. 2C). PCT-H1 leads to a loop region (the PCT-loop: G351 to G360) containing a short 3_10 helix (PCT-SH) (Fig. 2E and fig. S1). PCT-H1 and the

| Table 1. Crystallographic data and refinement. Values in parentheses are for the highest-resolution shell. PDB, Protein Data Bank. a.s.u., asymmetric unit. |
| --- |
| **Datasets** | EcRseP | KkRseP (#1) | KkRseP (#2) |
| **Data collection** | | | |
| Space group | P1 | P1 | P2_1 |
| Cell dimensions (Å) | 47.34, 56.12, 69.67 | 44.56, 49.78, 76.08 | 46.27, 40.80, 160.24 |
| α, β, γ (°) | 68.2, 74.6, 69.3 | 86.9, 79.2, 82.2 | 90, 91.6, 90 |
| No. of molecules/a.s.u. | 1 | 1 | 1 |
| X-ray source | SPring-8 BL32XU | SPring-8 BL32XU | SPring-8 BL32XU |
| Wavelength (Å) | 1.0000 | 0.9700 | 0.9700 |
| Resolution limits (Å) | 43.77–3.20 (3.31–3.20) | 49.30–3.10 (3.21–3.10) | 40.80–3.15 (3.26–3.15) |
| No. of unique reflections | 10,129 (1,028) | 11,493 (1,130) | 10,675 (1,055) |
| Completeness (%) | 99.7 (99.8) | 99.8 (99.8) | 99.6 (99.8) |
| Redundancy | 8.4 (7.8) | 135.8 (122.9) | 72.0 (66.3) |
| 〈I/σ(I)〉 | 7.5 (1.1) | 20.0 (1.4) | 13.8 (1.5) |
| R_p.i.m. | 0.098 (1.347) | 0.038 (1.601) | 0.081 (0.914) |
| CC (1/2)* | 0.995 (0.417) | 0.999 (0.821) | 0.999 (0.602) |
| **Refinement** | | | |
| Resolution limits (Å) | 43.76–3.20 (3.66–3.20) | 40.91–3.10 (3.24–3.10) | 40.55–3.15 (3.32–3.15) |
| R_work | 0.2460 (0.2952) | 0.2564 (0.4159) | 0.2654 (0.3959) |
| R_free | 0.3053 (0.3320) | 0.2987 (0.4787) | 0.2882 (0.4023) |
| No. of non-H atoms | 3,478 | 3,230 | 3,252 |
| Protein | 3,444 | 3,197 | 3,219 |
| Zn^{2+} | 2 | 1 | 1 |
| Batimastat | 32 | 32 | 32 |
| Average B factor (Å²) | 94.52 | 92.30 | 91.49 |
| Protein | 94.60 | 92.50 | 91.35 |
| Zn^{2+} | 83.44 | 64.45 | 110.74 |
| Batimastat | 87.24 | 73.57 | 105.10 |
| RMSD from ideality | | | |
| Bond length (Å) | 0.002 | 0.002 | 0.002 |
| Bond angle (°) | 0.48 | 0.50 | 0.44 |
| Ramachandran plot | | | |
| Favored (%) | 93.92 | 95.01 | 92.12 |
| Outlier (%) | 0 | 0 | 0.74 |
| PDB code | 7W6X | 7W6Y | 7W6Z |

*Correlation coefficient between intensities from random half-datasets.
actions with L435, L438, and M439 on TM4 (Fig. 3C). In addition, the phenyl group of batimastat forms van der Waals interactions with the side chain of N394 on TM3. For the side chains of batimastat, the isobutyl group is oriented toward I19 in TM1 and L390 in TM3-N in the wild type (WT), addition of 3.125 µM batimastat reduced the cleavage ratio under 3.125 µM batimastat concentration shown in (D) or (G) against the cleavage efficiencies of the model substrate [shown in (E) or (H)].

PCT-loop make direct contacts with PDZ-N. The present structural analysis also showed that the C-terminal residues of PCT (P361 to L374) also form a helix (PCT-H2), which makes a sharp turn at the C-terminal end (G375) and leads to TM3-N (Fig. 2E). PCT-H2 is accommodated into a cleft formed between TM1 and TM3-N and is located just above the batimastat bound to the active center. In EcRseP, TM4 (V423 to F447) interacts with the PCT-loop and PCT-H2 at the periplasmic side and covers the batimastat.

**Binding mode of batimastat**

The peptide-mimetic batimastat adopts an extended conformation and is flanked by the edge strand of the MREβ-sheet and TM3-N in EcRseP (Fig. 3 and fig. S7A). The main chain of batimastat forms hydrogen bonds with the main chain of L66 on the edge strand and with the side chain of N394 on TM3. For the side chains of batimastat, the isobutyl group is oriented toward I19 in TM1 and L390 in TM3-N, while the thienyl group is close to both of the residues that were reported to interact with substrates, F44 of the conserved GFG motif (25) and Y69 on the edge strand of the MREβ-sheet (24). In addition, the phenyl group of batimastat forms van der Waals interactions with L435, L438, and M439 on TM4 (Fig. 3C).

On the basis of the structural data, we examined whether side chains could also affect inhibitor (and thereby substrate) accommodation in addition to the backbone interactions with the edge strand. I19N and I19F mutations to TM1 have been reported to reduce the sensitivity of EcRseP to batimastat (27). We further introduced Phe mutations to the residues interacting with batimastat on TM3 and TM4 (L390 and N394 on TM3 and L435, L438, and M439 on TM4) and examined their effects on batimastat sensitivity. To eliminate the influence of the C-terminal tags on this analysis, we introduced these mutations into a tagless EcRseP construct (Fig. 3D and fig. S7, B and C).

In the wild type (WT), addition of 3.125 µM batimastat reduced the relative cleavage ratio of the substrate to 40%. At the same batimastat concentration with N394F, the relative cleavage ratio was only reduced to 90%. I19F also showed increased resistance to batimastat as reported (27). Furthermore, batimastat resistance seems to correlate negatively with the intrinsic proteolytic activity of the mutants in the absence of batimastat, particularly with a shorter induction of EcRseP (Fig. 3, E and F). The N394F mutation with the strongest impact on batimastat resistance also reduced the proteolytic activity to a greater extent as compared to other mutations. Next, we introduced N394C, N394S, and N394D mutations to evaluate the
possibility that the reduced activity in N394F is ascribed to lower accumulation resulting from fold destabilization. We observed that all three mutants accumulated in *E. coli* but still showed substantial increase both in batimastat resistance and in reduction of proteolytic activity (Fig. 3, G to I, and fig. S7, D and E). In particular, the reduction of proteolytic activity for the isosteric mutation N394D indicates that hydrogen bonding via the amide group of the N394 side chain is critical for the cleavage. N394 may clamp a bound substrate at the active center as it interacted with the backbone of batimastat at the opposite side of the MREβ-sheet. In total, it is likely that the binding mode of batimastat partly reflects that of native RseP substrates. The substrate segments to be cleaved are thought to be extended by the strand addition, as proposed in our previous work (24), and shielded from the hydrophobic milieu of the lipid bilayer by the surrounding TM helices of RseP.

**Overall structure of KKRseP**

KKRseP produced two crystal forms with similar crystal packing (fig. S8, A to D). The two crystal structures of KKRseP are almost identical with a root mean square deviation (RMSD) of 1.34 Å for 387 Ca atoms (fig. S8, E and F), excluding some disordered loops. The structures of individual domains in KKRseP are similar to those in EcRseP, including the binding mode of batimastat via the MREβ-sheet and side chain–backbone interactions with N387 (corresponding to N394 in EcRseP) (Fig. 2, F to I, and fig. S9). However, the KKRseP structures showed substantial differences in the domain arrangement relative to those in EcRseP. For instance, the PDZ tandem is positioned further from the PCT region, and the C-terminal part of PCT (corresponds to the PCT-loop and H2 in EcRseP) is disordered (Fig. 2, F, G, and I). Thus, KKRseP adopts a PDZ-open conformation, while EcRseP is in a PDZ-closed conformation. In addition, TM4 moves away from the domains forming the active center to interact with the clef between TM1 and TM3-N in the crystal packing neighbor (Fig. 2, H and I, and fig. S10A). TM4 also interacts with the batimastat bound to the active center of the neighbor. The phenyl group of batimastat makes close contacts with V428 and L429 on TM4 (fig. S9B). In addition, residual electron density was observed close to PDZ-C. This electron density is most likely derived from the C-terminal residues of the tobacco etch virus (TEV) protease consensus sequence in the KKRseP construct (fig. S10, A and B). As the two neighboring KKRseP molecules in the crystal form an asymmetric dimer, it is probable that the accommodation of TM4 into the neighbor is an artifact of the crystal packing. However, the conformational difference between EcRseP and KKRseP raised the possibility that the PDZ tandem, the C-terminal part of PCT, and TM4 can rearrange without disrupting the TM core region. Such structural changes, if they occur, would likely affect the regulation of substrate accommodation.

**Arrangement of the PDZ tandem in EcRseP and KKRseP on the cell membrane**

To explore the possibility of domain rearrangement, we first examined the arrangement of the PDZ tandem in EcRseP and KKRseP in the spheroplast (i.e., on the cell membrane) using a methoxypolyethylene glycol 5000 maleimide (mal-PEG) accessibility assay. For EcRseP, we introduced four single-Cys mutations to the PDZ tandem in the tagless construct to eliminate the impact of the C-terminal tags on the conformation of the PDZ tandem. Consequently, we observed that the Cys residues in A136C (located outside the PDZ pocket) was efficiently modified with mal-PEG (~5 kDa). In contrast, the Cys residues in D163C, L167C, and I302C were modified only upon the addition of detergent (Fig. 4, A to C). In the crystal structure, L167 on PDZ-N makes direct contacts with PCT-H1, while D163 is deep in the PDZ pocket. I302 belongs to the carboxylate-binding loop of PDZ-C and is located inside the pocket. These results indicate that EcRseP on the membrane adopts the PDZ-closed conformation as in the crystal structure, while the PDZ pocket is sterically hindered by the bilayer and the PCT region. Next, we also examined the conformation of the PDZ tandem in KKRseP on the cell membrane. To conduct the accessibility assay on KKRseP without a C-terminal tag, we inserted an exogenous epitope, PA14 tag (28), into a β turn between K54 and H55 in the MREβ-sheet for antibody labeling. We confirmed that the resulting KKRseP(S4-PA14-55) and its four single-Cys mutants (P136C, E163C, F167C, and I302C) accumulated in *E. coli* and maintained proteolytic activity (fig. S11). If KKRseP on the membrane adopts the PDZ-open conformation as in the crystal structure, then all of the four Cys mutants should be modified to some extent in the spheroplast. Nevertheless, although E163C (proximal to PCT) underwent substantial but rather low modification, F167C (proximal to PCT) and I302C (inside the PDZ pocket) were unmodified in the absence of detergent (Fig. 4, D to F). These results suggest that the two modification sites (F167 and I302) were inaccessible because of the closer proximity between the PDZ tandem and the PCT region in KKRseP on the membrane, similar to what is observed in the crystal structure of EcRseP (PDZ-closed) rather than that of KKRseP (PDZ-open) (Fig. 4G). Thus, the PDZ-open conformation of KKRseP in the crystal may be induced by the rearrangement of the PCT region or by the accommodation of TM4 from the crystal packing neighbor.

**Importance of D446 on TM4 for substrate cleavage**

We next explored the role of TM4 in substrate cleavage. Although TM4 is less conserved compared to the other three TM regions (fig. S12) within the S2P family (15) (Fig. 5A), the binding mode of batimastat in EcRseP suggests that TM4 contributes to the formation of the hydrophilic compartment around the active site (fig. S6, A and B). Hence, we prepared two EcRseP mutants, ΔTM4 lacking the entire TM4 and C-terminal tail region (F426 to the C terminus) and ΔCTail lacking only the C-terminal tail region (D446 to the C terminus), and assessed the effect of the mutations on the cleavage. We observed that both ΔTM4 and ΔCTail did not complement the EcRseP deficiency (Fig. 5B). Furthermore, both mutants virtually lost proteolytic activity, although ΔCTail exhibited residual activity (Fig. 5C). We next mutated conserved residues on TM4 to alanine (for G431, M439, D446, and R449) or serine (for A442) (Fig. 5, A and D, and fig. S12) to examine their contributions to the cleavage. We found that only the mutation to the highly conserved D446 (fig. S1) impaired complementation and proteolytic activity. As the carboxyl side chain of D446 is positioned for interaction with the electropositive N-terminal end of the helix dipole on PCT-H2 in the crystal structure, the interaction between D446 and PCT-H2 may be critical for the maintenance of the proteolytic activity (Fig. 5E). Analysis of additional D446 mutants showed that only D446E retained proteolytic activity (Fig. 5C and fig. S13), although its complementation activity was much lower than that of the WT (Fig. 5B). Notably, despite the absence of D446 in ΔCTail, this mutant exhibited a substantial proteolytic activity. This may be due to the presence of the terminal carboxyl group of N445 in the proximity of the N-terminal
Involvement of PCT-H2 in the substrate cleavage

To examine whether the conformational rearrangement of PCT-H2 is involved in the substrate cleavage, we first performed Cys-scanning mutagenesis on PCT-H2 and the adjacent region (T350 to P381) to examine the mobility of PCT-H2 by a modified version of our accessibility assay with 4-acetamido-4′-maleimidostilbene-2,2′-disulfonate (AMS) as the probe. AMS, a soluble thiol-alkylating reagent, reacts with Cys residues exposed to the aqueous milieu. We observed that all the Cys residues on PCT-H2 were modified at comparable levels in the spheroplast (fig. S14), although some residues—such as P361, I364, A365, A368, G369, and A372—are expected to be modified less frequently if PCT-H2 is fixed in the cleft between TM1 and TM3 as in the crystal structure. Hence, PCT-H2 is thought to be mobile in EcRseP on the cell membrane. In contrast, Y379, L380, and P381 on TM3 showed low AMS modification in the spheroplast. The low AMS accessibility of these three residues is consistent with burial in the hydrophobic region of the lipid bilayer, as predicted on the basis of the crystal structure. Furthermore, we also examined the proteolytic activity of the Cys mutants and found that only G360C and G375C reduced the proteolytic activity (Figs. 5A and 6, A and C, and fig. S15). Considering the high conservation of the two Gly residues flanking both ends of PCT-H2, these results suggest that the proper positioning of PCT-H2 is important for the substrate cleavage.

Subsequently, we also tested whether PCT-H2 is involved in the regulation of the sequential cleavage. We performed a LacZ reporter assay on the Cys mutants to monitor α-deactivation resulting from deregulated cleavage of intact RseA without the prior site-1 cleavage by DegS. We found that L355C, L358C, and I362C deregulate EcRseP, although the extent of deregulation was smaller than that from L151P, a known deregulated mutant on PDZ-N (Fig. 6, B and C) (29). We also examined trypsin susceptibility of the three mutants on the cell membrane as a proxy for stability. As reported previously, L151P showed increased trypsin susceptibility (lanes 5 to 8; fig. S16) probably because this mutation disrupts the folding of PDZ-N (20). In contrast, almost no degradation was observed for the other mutants or for WT RseP (fig. S16). We also observed increased conformational flexibility in the PDZ-C domain of L358C using negative-stain electron microscopy (EM) (figs. S17 and S18), suggesting that the L358C mutation can deregulate EcRseP by altering the conformation of the PDZ tandem without destabilizing the individual PDZ domains. These results indicate that the proper positioning and mobility of PCT-H2 are also important for the regulated sequential cleavage of substrates and likely affect the positioning of TM4.

Conformational changes of the PCT region and PDZ tandem during substrate cleavage

Several lines of evidence from this study raised the possibility that the PDZ tandem and PCT region, probably in conjunction with TM4, undergo structural changes to accommodate the substrate into the active center for cleavage. Our previous in vivo photocrosslinking experiment using p-benzoyl-L-phenylalanine (BPA) indicated that buried residue T341 on PCT-H1 is accessible to RseA (21). We therefore performed an intramolecular cross-linking experiment to test whether the proteolytic activity of EcRseP is affected by immobilizing the PCT region and/or the PDZ tandem (Fig. 7, A and B). In this assay, EcRseP mutants having two Cys mutations were first expressed under isopropyl-β-D-thiogalactopyranoside (IPTG) induction, then the cells were washed, and disulfide cross-linking was
grown for 3 hours with 1 mM IPTG and 1 mM adenosine 3'-5'-cyclic monophosphate (cAMP). The cleavage efficiency was determined as in Fig. 3E. Bar plots and error bars respectively. (RseP (pYH825) or its variants were

C

Ec group of S363 (each in white) form a hydrogen bond.

basis, we could not unambiguously correlate the reduction in the substrate cleavage efficiency for the other double-Cys mutants, but we detected no substantial mobility shift for them (lanes 9 to 16; Fig. 7A). Despite the lack of band shift, we conclude that both the I173C (PDZ-N)/K347C (PCT-H1) and the D205C (PDZ-N)/S359C (PCT-loop) mutants are cross-linked because neither could be modified at the introduced Cys residues because neither could be modified at the introduced Cys residues

A representative result from three biological replicates is shown. ∆TM4 and ∆CTail indicate the F426amber and D446amber mutations, respectively. (C) In vivo cleavage assay with long induction. KA306 cells harboring one plasmid for HA-MBP-RseA148 (pK665) and one for ErSeP (pYH825) or its variants were grown for 3 hours with 1 mM IPTG and 1 mM adenosine 3’-5’-cyclic monophosphate (cAMP). The cleavage efficiency was determined as in Fig. 3E. Bar plots and error bars represent the means ± SD from three biological replicates. (D) Conserved residues on TM4. The residues mutated to alanine or serine in fig. S12 are shown as spheres. A disordered region containing R449 is indicated by a dotted line. (E) Specific interaction between D446 and PCT-H2. TM4, PCT-SH in the PCT-loop, and PCT-H2 are shown as stick models in salmon, magenta, and pink, respectively. D446 interacts with the electropositive N-terminal end of the PCT-H2 helix dipole where the side chain of D446 and the main-chain N-H group of S363 (each in white) form a hydrogen bond.

induced with an oxidizing agent, diamide. As a control, we performed the same procedure but replaced the diamide with a reducing agent, dithiothreitol (DTT). Last, a model substrate was induced with arabinose, and cleavage was monitored with an immunoblot assay. In this cross-linking experiment, the Cys-less EcRseP (indicated as WT* in Fig. 7A) cleaved the substrate efficiently under both oxidizing and reducing conditions, while the active site mutant E23Q exhibited essentially no proteolytic activity under these conditions, indicating that the diamide and DTT treatments had little impact on the proteolytic activity. We induced cross-linking between PCT-H2 and TM1 (D7C-K366C and S10C-G369C) by treatment with the oxidizing reagent diamide and then confirmed the cross-link formation by observing the oxidization-dependent mobility shift of RseP on SDS–polyacrylamide gel electrophoresis (PAGE). We actually observed that cross-linking reduced the substrate cleavage efficiency (Fig. 7A), supporting the model that PCT-H2 moves out of the cleft between TM1 and TM3-N to accommodate the substrate. We also observed a reduction in the substrate cleavage efficiency for the other double-Cys mutants, but we detected no substantial mobility shift for them (lanes 9 to 16; Fig. 7A). Despite the lack of band shift, we conclude that both the I173C (PDZ-N)/K347C (PCT-H1) and the D205C (PDZ-N)/S359C (PCT-loop) mutants are cross-linked because neither could be modified at the introduced Cys residues with a sulfhydryl-specific reagent (mal-PEG) after pretreatment with the oxidizing reagent diamide (fig. S19). For the other two mutants, we could not unambiguously correlate the reduction in the substrate
cleavage efficiency with the formation of an intramolecular disulfide bond due to the diamide-dependent destabilization (D171C/S343C) or uncertainty regarding the cross-link formation (E203C/K354C). Collectively, the results of the cross-linking experiments support a model in which the PDZ tandem and the PCT region rearrange to accommodate the substrate for cleavage. In KkRseP, the cleft between TM1 and TM3-N accommodates the TM4 from the crystal packing neighbor. We inferred that the bound TM4 might mimic the substrate and tested this prediction in an intermolecular photocross-linking experiment with EcRseP and RseA. As anticipated, pBPA introduced at the position of Y378 on the cleft (Fig. 7B) is accessible to RseA in vivo (Fig. 7C and fig. S20), which also indicates a rearrangement of PCT-H2 during substrate accommodation.

**DISCUSSION**

In this study, we successfully determined the crystal structures of EcRseP and KkRseP as the first experimental structures for S2P with extracytoplasmic PDZ domains (group I). The structural features not only coincide with the previously proposed models but also provide new insights into the substrate accommodation mechanism. Specifically, the substrate discrimination and accommodation by EcRseP are presumed to be regulated by three processes: size exclusion, gating, and unwinding (Fig. 8).

Previous structural and mutational analyses have proposed that the PDZ tandem serves as a size exclusion filter to regulate the sequential cleavage of the substrates (20). It was also proposed that PCT-H1 is involved in the size exclusion as an adapter (21). Furthermore, most point mutations that deregulate sequential cleavage have been located in the PDZ-N domain (29). On the basis of trypsin susceptibility, those deregulated mutations are presumed to cause large structural changes or unfolding of PDZ-N (20). The present crystallographic analysis of EcRseP has shown that the PDZ-C domain protrudes in front of PCT-H2 to lie above the active center and thus appears to sterically hinder entry for substrates with a bulky periplasmic domain (Fig. 2, B to D). In addition, the PDZ-N domain makes direct contacts with the PCT-H1 in EcRseP. Thus, the unfolding of the PDZ-N domain should destabilize the interaction between PDZ-N and PCT-H1, which may also perturb the geometry of size exclusion filter to deregulate its function. The Cys-scanning mutagenesis in this study further demonstrated that the PCT-loop and PCT-H2 also regulate the sequential cleavage (Fig. 6B). The EM analysis also suggested that the mutation to the PCT-loop region (L358C) causes deregulation through fluctuations in the orientation of the PDZ-C (fig. S18). These observations indicate that the PDZ-N domain and the PCT region serve as a scaffold to place the PDZ-C domain in a position to perform the size exclusion function. A similar substrate discrimination mechanism was proposed for γ-secretase where nicastrin regulates substrate entry by steric hindrance via a bulky extracytoplasmic domain (30).

Moreover, the present study indicates that a domain rearrangement occurs during the substrate accommodation and cleavage. On the basis of the modification and cross-linking analyses, we infer that the PDZ tandem, PCT-H2, and TM4 in EcRseP serve as a gate for substrate entry. We anticipate that EcRseP on the cell membrane is

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Fig. 6. Cysteine-scanning mutagenesis analysis of the PCT-H2 and the adjacent regions. (A) Substrate cleavage with short induction. *E. coli* KK211 (∆rseA ∆rseP) cells harboring one plasmid for HA-MBP-RseA(LY1)148 (pYH20) and one for a variant of EcRseP(Cys-less)-His$_5$-Myc (pTM132) were grown at 30°C in M9-based medium for 2.5 hours and further incubated for 0.5 hours with 1 mM IPTG and 5 mM cAMP. The cleavage efficiency was determined as in Fig. 3E (see fig. S15 for the raw data). Bar plots and error bars represent the means ± SD from three biological replicates. The region of each mutation is indicated by color (magenta, pink, and gold) and label. (B) DegS-independent σ$^E$ activity of cells expressing RseP Cys mutants. Cells of ppoHP3-lacZ reporter strain AD2473 (∆degS ∆rseP) harboring pSTD343 (lacI) and a plasmid for a variant of EcRseP(Cys-less)-His$_5$-Myc (pTM101) were grown for 5 hours in L medium containing 0.1 mM IPTG and 1 mM cAMP. The measured LacZ activities are normalized as the ratio to the activity for the reporter strain expressing WT RseP (WT). The bar plot shows the means ± SD from three biological replicates. Red dashed line indicates the threshold for deregulated cleavage of intact RseA by the RseP Cys mutants. The previously isolated L151P mutant (right) shows high LacZ activity characteristic of deregulation. WT$^+$ and L151P$^+$ indicate Cys-less derivatives of WT and L151P RseP, respectively. (C) Mapping mutations on the EcRseP model. Residues where the Cys mutation impaired the proteolytic activity are indicated with red sphere models. Residues where the Cys mutations caused deregulation are indicated with cyan sphere models.
in equilibrium between the “gate-open” and “gate-closed” conformations. The AMS modification analysis indicates that PCT-H2 is mobile on the E. coli cell membrane. After the site-1 cleavage in the extracytoplasmic region, the substrate passes through the size exclusion filter to access the TM domain of RseP complexed with batimastat, indicating the importance of the conserved residue N394 in the substrate binding and translocation. The AMS analysis on EcRseP complexed with batimastat indicates the importance of the MRE structure to a “scission site” to discriminate the substrates from nonsubstrates. As the cleavage efficiency in EcRseP differs depending on the substrate, the proposed gating mechanism may also contribute to the substrate interrogation.

After the gate opening for the substrate accommodation, the substrate cleavage segments should bind with the edge strand via strand addition. We anticipate that the gate returns to a relatively closed conformation to shield the substrate TM segment from the hydrophobic milieu and to form a hydrophilic compartment around the active site for hydrolysis. Furthermore, our present structural analysis on EcRseP complexed with batimastat indicates the importance of the conserved residue N394 in the substrate binding and cleavage. We previously observed that the EcRseP(N394C) mutant in addition, it was proposed that E. coli rhomboid GlpG has an “interrogation site” for substrates in addition to a “scission site” to discriminate the substrates from nonsubstrates. As the cleavage efficiency in EcRseP differs depending on the substrate, the proposed gating mechanism may also contribute to the substrate interrogation.
showed no detectable cross-linking to Cys residues engineered into the TM segments of RseA derivatives. Thus, we inferred that N394 might not directly interact with RseA (35). However, our present crystallographic analysis demonstrated that the N394 side chain formed hydrogen bonds with the backbone of batimastat. Although the structure of the EcRseP-substrate complex remains unsolved, the present mutational analysis prompted us to propose an alternative model in which N394 directly interacts with the backbone of the substrate TM segments to stabilize a cleavage-susceptible substrate conformation. Thus, the failure to cross-link between the EcRseP(N394C) mutant and the RseA TM segment might be ascribed to the reduced affinity of this mutant to the substrate. We also observed that the N394C mutation reduced the affinity for the RseA TM segment in the common immunoprecipitation assay (35). The residue corresponding to EcRseP N394 in the B. subtilis homolog SpoIVFB (N129) has been shown to be important for substrate binding and cleavage. The SpoIVFB(N129C) mutant was disulfide cross-linked with coexpressed substrate mutants having a Cys residue in their TM segment, albeit with low efficiency (36), indicating the proximity of N129 to the bound substrate TM segment. In addition, the N129A mutation reduced the cross-linking efficiency of SpoIVFB with its substrate and reduced substrate cleavage (36–38), supporting the conclusion that N129 also stabilizes the bound substrate. Because SpoIVFB is a group III S2P, this interaction may be common across diverse S2Ps. Furthermore, clamp-like interactions with unwound substrates appear to exist across the intramembrane proteases. The cryo-EM analysis of γ-secretase classified as an aspartic protease showed that substrate binding induces the formation of a β sheet in the TM region of presenilin (Fig. 9, A and B) (33, 39). The β sheet binds with the substrate fragment via strand addition. In γ-secretase, the PAL motif is located on the opposite side of the β sheet like a clamp for the substrate. For the serine protease Rhomboid family, crystallographic analysis of E. coli GlpG in complex with substrate-derived inhibitors revealed that two loops connecting the TM helices (the L3 and L5 loops) sandwiched the peptide backbone of the inhibitor in an extended conformation (40). In the cocrytal structures, it appeared that L3 formed a parallel β sheet with the inhibitor, while L5 clamped it via backbone hydrogen bonds (Fig. 9, C and D). Furthermore, GlpG was also reported to capture a substrate peptide via the same backbone interactions with the L3 and L5 loops (31). Despite the difference in the catalytic mechanism, unwinding the TM helix into a strand conformation and stabilizing the bound substrate with a clamp structure seem to be common features in the intramembrane proteolysis of helical TM spans. Structure determination of substrate-bound and substrate-unbound forms of RseP or the group I S2Ps will deepen our understanding of the substrate
cleavage mechanism conserved across the intramembrane proteases and aid the development of strategies for regulating proteolytic activity in the membrane to prevent off-target or promiscuous cleavage.

**MATERIALS AND METHODS**

**Construction of expression plasmids for structural analysis**

A pUC118-based plasmid was constructed to produce EcRseP for structural analysis. The resulting plasmid, designated as pNY1452, carried a gene encoding the WT full-length EcRseP fused with a C-terminal tag containing the TEV consensus sequence and a His\(_8\) tag: -LESSG-ENLYFQG-QFTS-HHHHIC. For negative-stain EM, an expression plasmid for the L385C mutant, designated as pNY1550, was constructed from pNY1452 by the inverse polymerase chain reaction (PCR) method.

In parallel, we also searched for RseP orthologs suitable for structural analysis. Genes of 14 orthologs were amplified using genomic DNA obtained from the Japan Collection of Microorganisms (RIKEN, Microbe Division) and subcloned into Nde I and Xho I sites of a modified pET-21b vector. Using the constructed plasmids, the orthologs were produced with a C-terminal tag containing the TEV consensus sequence and a His\(_8\) tag: -LESSG-ENLYFQG-QFTS-HHHHIC. To examine the production level and dispersity, the orthologs produced in small-scale culture were subjected to detergent screening with fluorescence-detection size exclusion chromatography (FSEC) after the C-terminal His\(_8\) tag was labeled using a peptide-based multivalent nitroltriamic acid (NTA) fluorescent probe, P3NTA. From the screen, an ortholog Kkor_1905 from _K. koreensis_ str. DSM 16069 (KkRseP; UniProtKB: C7R5Z1), a Gram-negative bacterium isolated from seawater of the tidal flat (42), was selected as a promising candidate for structural analysis. The expression plasmid carrying the KkRseP gene without codon optimization was designated as pNY1432. The highest yield of monodisperse KkRseP sample was obtained with an optimized preparation using n-dodecyl-β-D-maltoside (DDM) supplemented with cholesteryl hemisuccinate (CHS). The strains and plasmids used in the structural analysis including the procedures described below are summarized in tables S1 and S2.

**Production and purification of full-length EcRseP and KkRseP**

EcRseP was overproduced in _E. coli_ C43(DE3) (Lucigen). _E. coli_ C43(DE3) cells transformed with the expression plasmid pNY1452 were grown at 30°C to an optical density at 600 nm (OD\(_{600}\)) of 0.7 to 0.8 in LB medium [Bacto Tryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (10 g/liter); without pH adjustment] supplemented with ampicillin (50 µg/ml), followed by induction of overexpression with 0.1 mM IPTG and incubation at 30°C for an additional 4 hours. Cells were harvested by centrifugation and lysed by sonication in 10 mM tris-HCl (pH 7.4) with 150 mM NaCl. The cell lysates were centrifuged at 40,000 g for 45 min at 4°C. Subsequently, the supernatant was separated by ultracentrifugation at 200,000 g for 90 min at 4°C. The membrane fraction collected as precipitate was suspended in 10 mM tris-HCl (pH 7.4) with 150 mM NaCl and was ultracentrifuged again under the same conditions. Last, the precipitated membrane fraction was suspended in 10 mM tris-Cl (pH 7.4) with 150 mM NaCl and NaCl (10 g/liter); without pH adjustment supplemented with ampicillin (50 µg/ml), followed by induction of overexpression with 0.1 mM IPTG and incubation at 30°C for an additional 4 hours. Cells were harvested by centrifugation and lysed by sonication in 10 mM tris-HCl (pH 7.4) with 150 mM NaCl and was ultracentrifuged again under the same conditions. Last, the precipitated membrane fraction was suspended in 10 mM tris-Cl (pH 7.4) with 150 mM NaCl, and the total protein was quantified using the bicinchoninic acid assay. The resuspended membrane fraction was diluted to adjust the protein concentration to 10 mg/ml using bovine serum albumin as a standard.

EcRseP was solubilized by adding an equal volume of a solubilization buffer containing 40 mM tris-HCl (pH 8.5), 150 mM NaCl, and 2% sucrose monododecanoate (SM) to the suspension of the membrane fraction. After incubation at 4°C for 1 hour, the mixture was ultracentrifuged at 210,000 g for 90 min at 4°C. The supernatant was applied to NZ:1 antibody–conjugated Sepharose resin (anti-PA tag), and the unbound fraction was washed out with a buffer containing 10 mM tris-HCl (pH 8.5), 150 mM NaCl, and 0.05% SM. EcRseP was eluted from the resin with a buffer containing 10 mM tris-HCl (pH 8.5), 150 mM NaCl, 0.05% SM, and PA14 peptide (0.1 mg/ml; EGGVAMPGAEDDVV). The C-terminal tag was cleaved off by adding TEV protease to the elution fraction, followed by incubation at 20°C overnight. The reaction mixture was then applied to a Superdex 200 10/300 GL SEC column (Cytiva). The peak fraction containing putative monomeric EcRseP was collected and again applied to the same SEC column to remove oligomeric EcRseP and aggregated TEV protease. After the second round of SEC, monodisperse EcRseP sample was obtained with high purity (~95%).
To produce KkRseP, the plasmid pNY1432 was transformed into E. coli C43(DE3) harboring pRARE2. For the native KkRseP, E. coli cells were grown at 30°C to an OD$_{600}$ of 0.6 to 0.7 in a LB medium supplemented with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml), followed by induction of overexpression with 0.1 mM IPTG and incubation at 16°C for an additional 18 hours. To produce the SeMet-substituted KkRseP, E. coli cells were first cultured in an LB medium supplemented with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) grown at 30°C overnight. The cells were further inoculated into M9 medium supplemented with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) and grown at 30°C. At an OD$_{600}$ of 0.3, a mixture of amino acids was added to the medium where the final concentrations were as follows: lysine (100 mg/ml), phenylalanine (100 mg/ml), threonine (100 mg/ml), valine (50 mg/ml), leucine (50 mg/ml), isoleucine (50 mg/ml), and SeMet (60 mg/ml). At an OD$_{600}$ of 0.7 to 0.8, 0.1 mM IPTG was added to induce the overproduction, followed by incubation at 16°C overnight. The cells were lyzed by sonication in 10 mM potassium persulfate (K$_2$S$_2$O$_8$, 1.0000 Å) with 150 mM NaCl, followed by centrifugation at 20,000 rpm for 30 min at 4°C. Subsequently, the supernatant was further separated by ultracentrifugation at 200,000g for 60 min at 4°C. The collected membrane fraction was lastly suspended in 20 mM tris-HCl (pH 8.0), 300 mM NaCl, 15% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and the total protein concentration was adjusted to 10 mg/ml.

KkRseP was solubilized by adding an equal volume of a solubilization buffer containing 40 mM tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 10% glycerol, 2% DDM, and 0.4% CHS to the suspension of the membrane fraction. After incubation at 4°C for 1 hour, the mixture was ultracentrifuged at 210,000 rpm for 30 min at 4°C. The supernatant was applied to Ni-NTA agarose resin, and the tag-cleaved KkRseP was collected in the unbound fraction. KkRseP was further purified with a Superdex 200 10/30 GL SEC column (Cytiva).

Crystallographic analysis of full-length RsePs

Batimastat (Toronto Research Chemicals) was added to the SEC elution fractions of EcRseP and KkRseP, respectively, at a final concentration of 300 µM. Subsequently, EcRseP and KkRseP were concentrated up to 7 to 12 mg/ml by ultrafiltration and incorporated into lipidic cubic phase (LCP) by mixing the protein solution and monoolein (NU-CHECK-PREP Inc.) with a volume ratio of 5:8 using two syringes attached with a coupler. Crystallization conditions were searched and optimized by microbatch crystallization method using Laminex sandwich plates and the MemMeso screening Kit (Molecular Dimensions). Aliquots (50 or 100 nl) of the protein–monoolein mixture were dispensed onto 96-well glass plates and overlaid with 800 nl of crystallization solution using a mosquito LCP (TTP LabTech) or Crystal Gryphon LCP (Art Robbins Instruments). The crystals of EcRseP used for data collection were obtained in a crystallization solution containing 30% (v/v) PEG 500 dimethyl ether, 100 mM NaCl, 100 mM MgCl$_2$, and 100 mM Hepes-Na (pH 7.0), while those of KkRseP were generated from crystallization solutions containing 28 to 30% (v/v) PEG 500 monomethyl ether, 100 mM NaCl, 100 mM CaCl$_2$, and 100 mM Hepes-Na (pH 7.0). Crystals were harvested using MicroMounts (MiTeGen) or LithoLoops (Molecular Dimensions) and flash-frozen in liquid nitrogen.

Diffraction data from the crystals obtained from the LCP crystallization were collected at SPRing-8 beamline BL32XU (43) using an EIGER X9M detector (Dectris). Microfocused x-rays with a beam size of 15 µm by 10 µm at wavelengths of 1.0000 Å (EcRseP native data), 1.2800 Å (Zn-anomalous diffraction data), and 0.9700 Å (Se-anomalous diffraction data), respectively, were used for both raster scan and data collection. A dataset with a total oscillation range of 10° and 0.1° oscillations per frame was collected from each crystal under an absorbed dose of 10 megayrays. The partial datasets collected with the automated data collection system ZO (44) were merged, integrated, and scaled using the KAMO system (45), which integrates BLEND (46), XDS, and XSCE (47, 48). Diffraction intensities were converted to structure factors using the CCP4 suite where 5% of the unique reflections were randomly selected as a test set for the calculation of free R factor (49).

Initial phases of the SeMet-substituted KkRseP crystal were determined by a combination of molecular replacement and single-wavelength anomalous diffraction methods (MR-SAD). After MR using the structure of KkPDZ-C domain as a search model, the MR phases were manually modified and fit into the electron density map using the program COOT (51). The updated models were refined with phenix.refine (52) iteratively while monitoring the stereochemistry with MolProbity (53). The structure of EcRseP was solved by MR using the partial model of KkRseP in addition to the individual EcPDZ-N [Protein Data Bank (PDB) code: 2ZPL] and EcPDZ-C (PDB code: 2ZPM) models (29). The PCT domain and TM4, where the conformations were largely different from those of KkRseP, were modeled manually. Model modification, structure refinement, and validation were performed similarly to those for KkRseP. Statistics for data collection and refinement are summarized in Table 1.

Structural superposition and RMSD calculation were performed by a pairwise alignment protocol using LSQKAB (54). Figures for protein structures were prepared with PyMOL (The PyMOL Molecular Graphics System, version 2.3, Schrödinger LLC).

**Media for biochemical analysis**

L medium [Bacto Tryptone (10 g/liter), yeast extract (5 g/liter), NaCl (5 g/liter); pH adjusted to 7.2 by using NaOH] and M9 medium [without CaCl$_2$ (55)] supplemented with thiamine (2 µg/ml) and 0.4% glucose were used to culture *E. coli* cells. Ampicillin (50 µg/ml), chloramphenicol (20 µg/ml), and spectinomycin (50 µg/ml) were added for selecting transformants and for growing plasmid-harboring cells.

**In vivo proteolytic activity assay of EcRseP and KkRseP and their derivatives**

The in vivo proteolytic activities of EcRseP and KkRseP were analyzed essentially as described previously (24, 56). Precultured cells were inoculated into an M9 medium supplemented with 20 µg/ml of each of the 20 amino acids, thiamine (2 µg/ml), 0.4% glucose, 1 mM IPTG, and 1 mM adenosine 3′,5′-cyclic monophosphate (cAMP) and grown at 30°C for 3 hours. The proteins were precipitated by
trichloroacetic acid (TCA) treatment, washed with acetone, sus-
pended in SDS sample buffer with 2-mercaptoethanol (2ME), and
analyzed by Laemmli SDS-PAGE and immunoblotting using an
Immobilon membrane filter (MilliporeSigma) and appropriate
antibodies. Proteins were visualized by a Lumino image analyzer
LAS-4000 mini (Cytiva) using ECL (enhanced chemiluminescence)
or ECL Prime Western Blotting Detection Reagents (Cytiva). Rabbit
polyclonal anti–hemagglutinin (HA) [HA-probe (Y-11), Santa Cruz
Biotechnology], anti–maltose-binding protein (MBP) (57), anti-Ec-RseP
(19), and anti-SecB (21) antibodies and rat monoclonal anti-PA anti-
bodies (58) were used for immunoblotting. Also, for detection of His-
tagged proteins, anti–His antibodies from the Penta–His HRP
Conjugate Kit (Qiagen) were used. In the short induction method,
precultured cells were inoculated into an L medium with 0.4% glu-
cose and grown at 30°C for 2.5 hours. Collected cells were sus-
pended in 100 μl of L medium and preincubated at 30°C for 10 min
in an Eppendorf Thermomixer comfort (600 rpm). After the
addition of 1 mM IPTG, the cells were incubated at 30°C for 15 min with
shaking. Last, the proteins were precipitated by the TCA treatment
described above. Cleavage efficiencies of the substrates were calculated
according to the following equation: cleavage efficiency (% = 100 ×
(cleaved) / (total cleaved) + (full length)).

In vitro substrate cleavage assay with purified EcRseP or KkRseP
Model substrate HA-RseA148 was synthesized using the PUREfrex
1.0 reconstituted cell-free protein synthesis kit [Gene Frontier Co.,
Japan; (59, 60)], using a template DNA (PCR-amplified from pYH18)
and standard reagents including 0.02% DDM and 35S-labeled me-
thionine (35S-Met). EcRseP or KkRseP was purified according to the
same procedures as those for the samples for crystallization. Syn-
thetized 35S-labeled substrate was mixed with purified EcRseP [final
concentration, 2.5 ng/μl (50 μM)], KkRseP [final concentration, 100 ng/μl (2.0 μM)], or each of the respective protein buffers (mock)
and incubated at 37°C in buffer containing 50 mM tris-HCl (pH 8.1),
2.5% glycerol, 5 μM zinc acetate, 0.05% DDM, 100 mM NaCl, 10 mM
DTT, and 5 mM zinc chelator 1,10-phenanthroline (PT+) or 5% dimethyl
sulfoxide (DMSO; PT−) with shaking in an Eppendorf Thermomixer
comfort (500 rpm) for the indicated periods. Samples were then mixed
with an equal volume of 2× SDS sample buffer plus 2ME and
boiled for 5 min. The proteins were separated by SDS-PAGE using
a 15% bis-tris gel and MES SDS running buffer (61) and visualized
using a PhosphorImager BAS5000 (Cytiva).

In vivo batimastat sensitivity assay
Batimastat inhibition in vivo of proteolytic activity of RseP deriva-
tives was evaluated using a mutant strain that lacks the accA gene
coding a component of a multidrug efflux pump to increase the sen-
sitivity to batimastat as described previously (27) with several modifica-
tions. YH2902 (ΔacrA ΔrseP ΔacrA) cells harboring two plasmids
encoding an EcRseP-derived (pYH825 based) and a model sub-
strate HA-MBP-RseA(LY1)148 (pYH124) were inoculated into L
medium with 0.4% glucose and grown at 30°C for 2.5 hours.
Collected cells were suspended in L medium and divided into three
portions. Each portion received 12.5, 3.125, or 0 μl (final concen-
tration) batimastat (MilliporeSigma), the stock solution of batimastat
was dissolved in DMSO and preincubated at 30°C for 10 min in an
Eppendorf Thermomixer comfort (600 rpm). Samples were mixed
with IPTG (final concentration, 1 mM) to induce protein expres-
sion and incubated for 1 hour with shaking. Proteins were TCA-
precipitated and analyzed by SDS-PAGE and immunoblotting.

Substituted cysteine accessibility analysis of single-cysteine
EcRseP or KkRseP mutants
A mal-PEG accessibility assay was performed with single-Cys mutants of
EcRseP or KkRseP using essentially the same method as described
previously (20, 56). KK374 cells (57) carrying a plasmid encoding a
tagless EcRseP Cys mutant or a KkRseP Cys mutant with an internal
PA14 tag were cultured in L medium containing 0.4% glucose, 1 mM
IPTG, and 1 mM CAMP at 30°C for 2.5 hours and converted to
spheroplasts by lysozyme/EDTA treatment as described previously
(29). Spheroplast samples were treated with water or 2% Triton
X-100 at 0°C for 3 min in the presence of 5 mM MgCl2, 1 mM PMSF,
and 1 mM tris(2-carboxyethyl)phosphine (TCEP; MilliporeSigma).
Samples were mixed with an equal volume of 2× reaction buffer
[60 mM tris-HCl (pH 8.1) and 2 mM mal-PEG (MilliporeSigma)]
and incubated at 4°C for the indicated periods. The Cys modifica-
tion reaction was stopped by adding 10% 2ME. The proteins were TCA-
precipitated and analyzed by SDS-PAGE and immunoblotting.

Single-Cys EcRseP mutants were modified in two steps with AMS
and mal-PEG using essentially the same method as described previ-
ously (20, 56). Spheroplast samples of KK374 cells expressing a
single-Cys mutant of RseP-PA-His-Myr (RseP-HM) were prepared as
above and then treated with water or 1% Triton X-100 at 0°C for
30 min in the presence of 10 mM MgCl2, 1 mM PMSF, and 1 mM
TCEP. After prewarming at 24°C for 5 min, samples were treated
with 1 mM AMS (Thermo Fisher Scientific) in the presence or ab-
ence of 1% Triton X-100 at 24°C for 5 min. Following quenching of
AMS by incubation with 62.5 mM DTT at 24°C for 18 min, the pro-
teins were precipitated and washed with 5% TCA. Samples were
solubilized in 100 mM tris-HCl (pH 8.1) containing 1% SDS and
1 mM TCEP and treated with 5 mM mal-PEG at 37°C for 1 hour
with vigorous shaking to modify free thiols. AMS/mal-PEG-modified
proteins were analyzed by SDS-PAGE and immunoblotting.

The proportion of RseP-HM Cys mutants modified with AMS was
calculated according to the following equation: AMS modification
(%) = 100 × (a − b)/a, in which a is the ratio of the mal-PEG-
modified forms to total RseP-HM in the control sample that is
prepared without AMS treatment, and b is the ratio of the mal-
PEG-modified forms to total RseP-HM in the AMS-treated sample.
For immunoblotting analysis of mal-PEG–labeled proteins, an
Immobilon-P SQ membrane filter (MilliporeSigma) and transfer
buffer containing 24 mM tris-base, 192 mM glycine, 10% methanol,
and 0.05% SDS were used.

Complementation assay
E. coli KK31 [ΔrseP/pKK6 (PBAD-rseP)] cells harboring a plasmid
encoding EcRseP or its derivatives under the lac promoter were grown at
30°C in an L medium containing 0.02% l-arabinose for 2 hours.
The cultures were serially diluted with saline, and 3 μl of the diluted cul-
tures was spotted on L agar plates containing 1 mM IPTG to test com-
plementation or 0.02% l-arabinose as a control for monitoring total cell
counts. The plates were incubated at 30°C for the indicated period.

β-Galactosidase activity assay
The δ6 activity was assayed by monitoring β-galactosidase (LacZ)
activity expressed from a chromosomal δ6-dependent lacZ reporter
Site-directed in vivo photocrosslinking

Site-directed in vivo photocrosslinking was carried out using essentially the same method as previously described (21). Cells harboring pEVOL-pBpF and a plasmid encoding an RseP(E23Q)-HM derivative were grown at 30°C for 4 hours in an M9 medium containing 0.5 mM PBPA (Bachem AG). After adding spectinomycin (100 μg/ml) to stop further protein synthesis, a portion of the culture was withdrawn and ultraviolet (UV)–irradiated for 10 min at 4°C using a B-100 AP UV lamp (365 nm; UVP LLC). Proteins were TCA-precipitated and dissolved in SDS sample buffer with 2ME.

Disulfide cross-linking–mediated domain immobilization experiment

The in vivo proteolytic activity of EcRseP after domain tethering by EcRseP was examined as follows. AD2544 cells harboring two independently inducible plasmids, each encoding an RseP derivative with a pair of Cys mutations under the P_1 lac promoter (pYH835 based) or the model substrate HA-MBP-RseA(LY1)148 (pTM949) under the P_Bad promoter, were used. Precultured cells were inoculated into L medium with 0.4% glucose, grown at 30°C for 1.5 hours, and divided into two portions. Collected cells were washed with and resuspended in 150 μl of L medium with 5 mM IPTG and incubated at 30°C for 30 min in an Eppendorf Thermomixer comfort (600 rpm) to induce expression of the RseP derivatives. After incubation, each of the two samples was washed with IPTG-free L medium, resuspended in L medium with 5 μM di- amide (for oxidation) or 10 μM DTT (for reduction), and further incubated at 30°C for 30 min. The cells were washed again with L medium and resuspended in L medium containing 0.02% l-arabinose with (for the +DTT sample) or without (for the +diamide sample) 1 mM TCEP and incubated at 30°C for 30 min to induce substrate expression. Last, proteins were precipitated with TCA, resuspended in SDS sample buffer without 2ME, and analyzed by SDS-PAGE and immunoblotting.

To evaluate the efficiency of intramolecular disulfide cross-linking for double-Cys RseP, all TCA-precipitated postreaction samples were dissolved in 100 mM tris-HCl (pH 8.1) with 1% SDS and vigorously shaken for 30 min at room temperature. Then, the samples were divided into two portions, treated with 0 or 1 mM mal-PEG at 37°C for 1 hour with vigorous shaking to modify free thiols, mixed with 2× SDS sample buffer containing 2ME, and lastly analyzed by SDS-PAGE and immunoblotting.

Multiple alignment analysis of bacterial RseP homologs

Selection and amino acid sequence alignment of RseP homologs were performed as follows. BLAST search (blastp) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed, using the amino acid sequence of K_rseP[K. koreensis str. DSM 16069; Kkor_1905 (UniProtKB: C7R52I)] as the query, against the nonredundant UniProtKB/SwissProt sequence database. From the 66 S2P homologs found by this search, duplicates, nontemplar RseP homologs, and homologs with obviously different molecular sizes were manually excluded, leaving 39 bacterial homologs. Multiple sequence alignment was performed with these 39 species by the Clustal W ver.2.1 program (www.clustal.org/clustal2) using the genetic information processing software Genetyx (GENETYX Corporation, Japan).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://doi.org/10.1126/sciadv.abbp0911

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Involvement of FtsH in protein assembly into and through the membrane. I. Mutations that reduce retention efficiency of a cytoplasmic PsrA promoter. *J. Bacteriol.* 177, 4121–4130 (1995).

The atomic coordinates were deposited in the PDB with the accession codes as follows: Ec RseP complexed with the 12C7 Fab (EMD-33409) and Ec PDZ-C domain complexed with the 12C7 Fab (7W6Z), Kk RseP in the PDZ-C domain (7W70), and Ec PDZ-C domain complexed with the 12C7 Fab (7W8N). The authors declare that they have no competing interests. Materials and availability:

The atomic coordinates were deposited in the PDB with the accession codes as follows: full-length EcRseP (7W6X), full-length KkRseP in the P1 crystal (7W6Y), full-length KkRseP in the P2 crystal (7W6Z), KPDZ-C domain (7W70), and ECPDZ-C domain complexed with the 12C7 Fab (7W71). The negative-stain EM maps were deposited in the Electron Microscopy Data Bank with accession codes as follows: WT EcRseP complexed with the 12C7 Fab (EMD-33409) and EcRseP(L358C) mutant complexed with the 12C7 Fab (EMD-33410). All other data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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Mechanistic insights into intramembrane proteolysis by E. coli site-2 protease homolog RseP

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