Regulated intramembrane proteolysis (RIP) plays crucial roles in both prokaryotic and eukaryotic organisms. Proteases for RIP cleave transmembrane regions of substrate membrane proteins. However, the molecular mechanisms for the proteolysis of membrane-embedded transmembrane sequences are largely unknown. Here we studied the environment surrounding the active site region of RseP, an Escherichia coli S2P ortholog involved in the σE pathway of extracytoplasmic stress responses. RseP has two presumed active site motifs, HEXXH and LDG, located in membrane-cytosol boundary regions. We examined the reactivity of cysteine residues introduced within or in the vicinity of these two active site motifs with membrane-impermeable thiol-alkylating reagents under various conditions. The active site positions were inaccessible to the reagents in the native state, but many of them became partially modifiable in the presence of a chaotrope, while requiring simultaneous addition of a chaotrope and a detergent for full modification. These results suggest that the active site of RseP is not totally embedded in the lipid phase but located within a proteinaceous structure that is partially exposed to the aqueous milieu.

Proteases contribute to functional regulation of some membrane proteins by introducing specific cleavages into them. For example, eukaryotic membrane-bound proteases such as ADAMs (a disintegrin and metalloproteinase) catalyze ectodomain shedding in which they cleave off extracellular domains of membrane proteins, including cytokines, growth factors, and adhesion molecules (1, 2). Membrane-embedded proteases are also involved in regulated intramembrane proteolysis (RIP) in which regulated cleavages are introduced into transmembrane segments of membrane proteins. An increasing number of findings have revealed that RIP plays pivotal roles in cell regulation and transmembrane signaling (3, 4). Proteases engaged in RIP include the S2P protease γ-secretase, the rhomboid protease, and the signal peptide peptidase (3, 4).

The S2P family proteases are widely distributed from bacteria to higher organisms and involved in diverse biological processes (5, 6). They possess conserved amino acid sequence motifs, a typical zinc metalloprotease motif HEXXH, and a C-terminal-located LDG motif (5, 6). Mutational alterations of the conserved residues in these motifs abolish the proteolytic activities (7–9), in agreement with the prediction that these motifs constitute a protease active site (10). The mammalian S2P protease, a founding member of RIP proteases, is an integral component of the regulatory systems for sterol metabolism and endoplasmic reticulum stress responses (11, 12). In prokaryotes, two S2P homologs of Bacillus subtilis, SpoIVFB (13) and YluC (14), control sporulation and environmental stress responses, respectively. Caulobacter crescentus MmpP participates in cell polarity determination (15). Recent studies showed that S2P homologs of Vibrio cholerae and Mycobacterium tuberculosis (YaeL and Rv2869c, respectively) are involved in the pathogenic activities of these bacteria upon infection to host cells (6, 16).

In Escherichia coli, RseP (formerly called YaeL) is the S2P ortholog and is an essential regulator of the σE pathway of extracytoplasmic stress responses (7, 8). σE is an alternative σ factor dedicated to this stress response and kept inactive by binding to the cytoplasmic domain of the membrane-bound anti-σE protein, RseA, under unstressed conditions (17, 18). Extracytoplasmic stresses such as accumulation of unassembled outer membrane proteins in the cell envelope activate DegS, another membrane protease with a periplasmic active site, which cleaves RseA on the periplasmic side (site-1 cleavage) (19). Subsequently, RseP introduces the site-2 cleavage into RseA, liberating a complex between the RseA cytoplasmic domain and σE from the membrane (7, 8). Our in vivo and in vitro studies showed that RseP cleaves RseA within its transmembrane segment (9). The RseA cytoplasmic domain thus released to the cytoplasm is degraded by cytoplasmic proteases such as ClpXP, leading to the full activation of σE (20). The site-2 cleavage of RseA by RseP is usually dependent on the prior action of DegS; in this way short-circuited cleavage of intact RseA by RseP and consequent uncontrolled elevation in the expression of the σE-controlled genes are avoided (7, 8).
Protease Active Site of RseP

Several elements, including the periplasmic PDZ domain of RseP, the Gln-rich regions in the RseA periplasmic domain, and periplasmic protein RseB, are involved in this negative regulation (21–23). RseP has unexpectedly wide substrate specificity in that it can cleave transmembrane sequences derived from lactose permease (LacY) and the signal sequence of β-lactamase that share no apparent sequence homology with the RseA transmembrane segment (9). Helix destabilizing residues in transmembrane sequences of substrates facilitate the proteolysis by RseP (9).

Although extensive studies have characterized the biological functions of the RIP proteases, including those of the S2P family proteins, their molecular mechanisms remain largely unknown. Generally, RIP proteases have their active site residues located in predicted transmembrane segments and they are believed to catalyze proteolysis within the lipid bilayer (3, 4). This notion poses a question of how hydrolysis of a peptide bond is facilitated in the lipid environment with limited availability of water. Here, as an attempt to assess the environment around the active site motifs of RseP, we examined the ability of membrane-impermeable thiol alkylating reagents to access engineered Cys residues placed at or around the active sites. Our results suggest that the proteolytic active site is not completely embedded in the lipid phase but is in a folded structure that is either partly integrated into or stabilized by the lipid bilayer.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—E. coli K12 strains CU141 (MC4100::lacIq) (24), AD16 (Δ(pro-lac), thi/F lacP) (25), KK31 (AD16, rseP::kan, Δ(srl-recA)306::Tn10/pKK6) (26), KK211 (AD16, rseA::cat, rseP::kan) (7), and KK374 (CU141, rseA::cat, rseP::kan, degS::tet) (9) were described previously. L (27) and M9 (28) were used as complete nutrient and minimal salt media, respectively. Ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), and/or spectinomycin (50 μg/ml) were added for selection of transformants as well as for growing plasmid-bearing strains.

Plasmids—pKK6 (26) carried the rseP gene under the ara promoter control. pSTD881 (HA-MBP-RseA140) and pSTD1017 (HA-MBP-RseA(LacYTM1)140) were constructed by ligating a KpnI-HindIII fragment of pSTD797 (9) and a KpnI-HindIII fragment of pSTD835 (9), respectively, with pSTD691 (7) that had been digested with the same enzymes. pSTD892 encoding a Cys less derivative of RseP-Hisγ-Myc was constructed by site-directed mutagenesis (29) of pKK4 (9), and plasmids encoding single Cys derivatives of RseP-Hisγ-Myc were constructed by site-directed mutagenesis of pSTD892. The DNA sequences of the mutated genes were confirmed.

Complementation Assays—KK31 (a ΔrseP strain having pKK6 with ara-controlled rseP) was transformed further with a plasmid carrying the wild type or mutant forms of lac-controlled rseP-Hisγ-myc and grown in 1-ara-arabinose (0.2%) at 30 °C. The cultures were serially diluted with 0.9% saline, and portions of the diluted cultures were spotted on L agar plates containing 1 mM isopropyl-1-thio-β-D-galactopyranoside (test plates) or 0.2% arabinose (control plates) and incubated at 30 °C.

Analysis of the in Vivo Proteolytic Activities of the Single Cys Derivatives of RseP-Hisγ-Myc—Cells of KK211 (ΔrseA ΔrseP) carrying pSTD881 (HA-MBP-RseA140) and KK374 (ΔrseA ΔrseP ΔdegS) carrying pSTD1017 (HA-MBP-RseA-LacYTM1)140) were transformed further with a plasmid encoding a derivative of RseP-Hisγ-Myc to be tested. They were grown in M9 medium supplemented with 20 amino acids (20 μg/ml each), thiamine (2 μg/ml), glucose (0.4%), and isopropyl-1-thio-β-D-galactopyranoside (1 mM) at 30 °C for 3 h. Proteins were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting using anti-Myc and anti-HA antibodies as described previously (9).

Assays for Alkylation of Single Cys RseP Derivatives—Cells of KK374 (ΔrseA ΔrseP ΔdegS) carrying a plasmid encoding a RseP-Hisγ-Myc derivative were grown in L medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside and 1 mM cAMP at 30 °C for 3.5 h. They were collected by centrifugation, washed with 10 mM Tris-HCl, pH 8.1, suspended in buffer containing 50 mM Tris-HCl, pH 8.1, and 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication. After removal of unbroken cells, total membranes containing cytoplasmic and outer membranes were precipitated by ultracentrifugation and suspended in buffer containing 50 mM Tris-HCl, pH 8.1, 15% glycerol, and 1 mM Tris(2-carboxyethyl)phosphine. This membrane fraction containing inverted membrane vesicles (IMVs) (see below) was used for thiol alkylation experiments. Protease accessibility tests of three cytoplasmic membrane proteins of known orientations, HflK, HflC, and FtsH, confirmed that these membrane preparations contained cytoplasmic membrane vesicles of homogeneously inverted sidedness (supplemental Fig. S1 and data not shown).

In AMS and EMA modification experiments, samples were subjected to the primary modification with either of these reagents and then to denaturation with SDS and the secondary modification by malPEG. We monitored the SDS-PAGE mobility retardation by the latter modification, representing the AMS- or EMA-unmodified fraction. IMVs were treated with 1 mM AMS or 0.5 mM EMA in 25 mM Tris-HCl, pH 8.1, in the presence of absence of 4 M GdnHCl and/or 1% Triton X-100 at 24 °C for the indicated time periods. Although we used 4 M GdnHCl in most experiments, results were essentially the same when the denaturant concentration was increased to 6 M in confirmatory experiments examining some crucial samples. Samples were then incubated with 62.5 mM dithiothreitol at 24 °C for 18 min to quench AMS or EMA, and proteins were precipitated with trichloroacetic acid and solubilized in buffer containing 100 mM Tris-HCl, pH 8.1, 1% SDS, and 1 mM Tris(2-carboxyethyl)phosphine. They were incubated further with malPEG (5 mM final concentration) at 37 °C for 30 min with vigorous mixing and analyzed by SDS-PAGE and immunoblotting using anti-Myc antibodies. For direct treatment of IMVs with malPEG, experiments were carried out as above except for the following two points: first, 5 mM malPEG was used instead of AMS or EMA and, second, proteins were acid precipitated and SDS solubilized for analysis by SDS-PAGE and immunoblotting without any post-denaturation treatment with malPEG. In these experiments, the treatment of samples with Cys alkylating reagents was carried out in the presence of a thiol-reducing.
forms to total RseP-His$_6$-Myc in the control sample that was modified with AMS or EMA was calculated according to the following equation: AMS or EMA modification (%) = 100 × (a−b)/a, in which a is the ratio of the malPEG-modified forms to total RseP-His$_6$-Myc in the control sample that was prepared without AMS or EMA treatment and b is the ratio of the malPEG-modified forms to total RseP-His$_6$-Myc in the AMS- or EMA-treated sample. For some unknown reasons, the RseP derivatives and other thiols and consequent interference with alkylation of the target Cys residues were not analyzed in this study.

The proportion of a single Cys derivative of RseP-His$_6$-Myc that was modified with AMS or EMA was calculated according to the following equation: AMS or EMA modification (%) = 100 × (a−b)/a, in which a is the ratio of the malPEG-modified forms to total RseP-His$_6$-Myc in the control sample that was prepared without AMS or EMA treatment and b is the ratio of the malPEG-modified forms to total RseP-His$_6$-Myc in the AMS- or EMA-treated sample. For some unknown reasons, the RseP-His$_6$-Myc derivatives with a single Cys residue at position 399, 400, or 408 showed low malPEG modification even when malPEG treatment was carried out after IMV proteins were directly acid precipitated and SDS solubilized. These proteins were not analyzed in this study.

RESULTS

Construction and Activities of Single Cys Derivatives of RseP—To investigate the environments surrounding the active site of RseP, we constructed RseP variants having single cysteines at designated positions (Fig. 1) and examined their reactivities to membrane-impermeable Cys-specific alkylating reagents. We first eliminated the 2 intrinsic Cys residues (Cys-33 and Cys-427) in RseP-His$_6$-Myc by changing them to Ala. Then selected residues in TM1 (the first transmembrane segment)-C1 (the first cytoplasmic loop) including the HEXXH motif as well as those in TM3-C2 including the LDG motif were mutated to Cys individually. The 2 His residues in the HEXXH motif are supposed to act as ligands for zinc coordination while the Glu residue is supposed to be directly involved in catalysis (5). Although the role of the LDG motif is unclear, this evolutionarily conserved and essential motif has been suggested to constitute the proteolytic active site of RseP together with the HEXXH motif (5, 10).

Cys-less RseP is functional as it complemented the ∆rseP mutation (data not shown). As expected, a Cys substitution for any of the conserved residues (His-22, Glu-23, His-26, and Asp-402) in the HEXXH and LDG motifs abolished the complementation activity. All the other single Cys RseP derivatives, except N389C, were active in complementation (data not shown). We examined in vivo protease activities of the single Cys RseP derivatives by co-expressing them with a model substrate, HA-MBP-RseA(LacYTM1)140, having the first transmembrane region of LacY (9) in the ∆rseA strain and examining the molecular sizes of the substrate protein accumulated in the cell by anti-HA immunoblotting (Fig. 2). All the RseP derivatives accumulated in the cell at levels comparable with the cellular abundance of the similarly expressed Cys-less and wild type RseP proteins (Fig. 2A). Whereas HA-MBP-RseA(LacYTM1)140 was detected as the full-length product (UC) in cells without RseP or in cells with the active site-mutated RseP, it was efficiently converted to the cleaved form (CL) in the presence of wild type RseP, Cys-less RseP, or a complementation-positive
single cysteine RseP variant (Fig. 2, A and B). Similar results were obtained when another model substrate, HA-MBP-RseA140, having the transmembrane segment of the physiological substrate, RseA, was used as a substrate (data not shown). Thus, most of the single Cys replacements outside the active site motifs are silent with respect to the cellular functions of RseP, suggesting that their effects on the structure of RseP are negligible. Although it is difficult to assess whether the Cys substitutions of the active site motif residues cause any gross structural alteration, we observed that the H22C active site mutations at regions predicted to be cytoplasmically exposed, apart from their kinetic effects, indicates that these residues are indeed located in the soluble cytoplasmic region of the protein.

Next, we examined AMS modifiability of cysteines introduced at position 136 and position 416 in the predicted second periplasmic region. We found that they were significantly modified when AMS modification was carried out using not only the intact spheroplasts (data not shown) but also IMVs (Fig. 4). The intactness and the sideness of IMVs were confirmed by proteinase K accessibility tests (supplemental Fig. S1). The exact reasons are unknown for the apparent access of AMS to the lumi-

**Protease Active Site of RseP**

![Image for Figure 3](Image 324x509 to 552x733)

**Figure 3. AMS modification of cytoplasmically exposed Cys residues.**

A, IMVs were prepared from cells expressing the Cys-less, A77C, or G416C derivative of RseP-His$_6$-Myc and treated with 1 mM AMS at 24 °C for 0, 5, 15, and 30 min in the presence or absence of 4 M GdnHCl (Gdn) and/or 1% Triton X-100 (TX) as indicated. Proteins were precipitated with trichloroacetic acid, solubilized in 1% SDS-5 mM malPEG, and analyzed by 7.5% SDS-PAGE and anti-Myc immunoblotting. Positions of molecular size markers are indicated in total RseP-His$_6$-Myc are graphically depicted. Average values from at least two independent experiments are shown with standard deviations.

B, the proportions of the AMS-modified form in total RseP-His$_6$-Myc are graphically depicted. Averages from at least two independent experiments are shown with standard deviations.

![Image for Figure 4](Image 60x489 to 288x733)

**Figure 4. AMS modification of periplasmically exposed Cys residues.**

A, IMVs were prepared from cells expressing the Cys-less, A136C, or A368C derivative of RseP-His$_6$-Myc and treated with 1 mM AMS at 24 °C for 0, 5, 15, and 30 min in the presence or absence of 4 M GdnHCl (Gdn) and/or 1% Triton X-100 (TX) as indicated. Proteins were analyzed as described in the legend to Fig. 2. Arrowheads indicate the malPEG-modified form of RseP-His$_6$-Myc. B, the proportions of the AMS-modified form in total RseP-His$_6$-Myc are graphically depicted. Averages from at least two independent experiments are shown with standard deviations.

The sulphydryl groups that had survived the first modification with AMS. The malPEG modification can be detected by the marked mobility retardation upon SDS-PAGE separation due to the addition of its molecular mass of ~5 kDa to the modified protein. A distinct advantage of this assay over the conventional one that uses a radio- or a ligand-labeled Cys-alkylating reagent is that both the modified and unmodified proportions are visible directly and quantifiable unequivocally. When IMVs were directly treated with AMS, the AMS modification of the Cys residues at positions 77 and 416 proceeded relatively slowly, requiring ~30 min to reach the level of 60–70%. On the other hand, these Cys residues were modified much more rapidly (within 5 min) and almost thoroughly when AMS treatment was carried out in the presence of 4 M GdnHCl, which should have denatured domains sufficiently exposed to the aqueous phase. Because the modification was also accelerated by treatment of IMVs with 1% Triton X-100, the membrane anchoring of RseP as well seems to contribute to the folding of the position 77 and position 416 regions of RseP. The fact that the modification went to completion without any denaturant or detergent, apart from their kinetic effects, indicates that these residues are indeed located in the soluble cytoplasmic region of the protein.

**AMS Modification Assay to Probe the Environments of the Engineered Cys Residues**—We first chose several Cys substitutions at regions predicted to be cytoplasmically exposed, periplasmically exposed, or membrane embedded based on the amino acid sequence information. Residues Ala-77 and Gly-416 are located in regions locally rich in charged residues in the C1 and the C2 loop, respectively (Fig. 1). IMVs were prepared from cells expressing either RseP(A77C)-His$_{6α}$-Myc or RseP(G416C)-His$_{6α}$-Myc and incubated with 1 mM AMS, an membrane-impermeable Cys-alkylating reagent of ~500 Da, at 24 °C for various periods (Fig. 3). After quenching AMS with dithiothreitol, proteins were acid denatured, solubilized with SDS, and subjected to the second Cys alkylation with malPEG to modify all
nal side of IMVs, an observation with precedents (30, 31). The simplest explanation may be that the small volume of the IMV lumen can be equilibrated rapidly even by slow diffusion of AMS across the membrane, although other possibilities such as translocation mediated by some channel-like membrane proteins and incomplete sealing of IMVs after the cell disruption cannot be excluded. In any case, our primary objective to distinguish between sequestered and exposed residues is not critically affected by the above observations.

We then examined RseP positions 13 and 386, expected to be lipid embedded (Fig. 5). These positions reside within clusters of hydrophobic residues in the middle of the predicted TM1 and TM3 segments, respectively (Fig. 1B). In contrast to the Cys residues in the cytoplasmic and periplasmic loop regions, those at positions 13 and 386 were not at all modified with AMS in the native membranes. Neither the presence of GdnHCl alone nor Triton X-100 alone greatly accelerated the modification. Rapid and quantitative modification of Cys residues at these positions was observed only when the membranes were treated with the denaturant and the detergent simultaneously. The synergistic effects of the chaotrope and the detergent suggest that both integration into the lipid phase and local folding of the protein contribute to the stable structure that prevents the AMS access to Val-13 and Ile-386. In any case, these residues are fully sequestered from the aqueous phase. Taken together, our model experiments show that the AMS modifiability assays can provide faithful information on the environment of a target Cys residue with respect to its exposure to the aqueous phase versus sequestration within the lipid phase and/or a protein fold.

**AMS Modifiability of Cys Residues in the Active Site Regions**—We prepared IMVs from cells expressing each of the RseP derivatives having a single Cys residue within and in the vicinity of the HEXXH motif (Fig. 1B) and treated them with AMS for 0–30 min either without any addition, in the presence of GdnHCl (Gdn), or in the presence of GdnHCl plus Triton X-100 (TX) as indicated. The HEXXH motif residues in the wild type amino acid sequence (aa in WT) are boxed. B, time course of AMS modifications are shown graphically for the H22C, E23C, F24C, and H26C derivatives of RseP-His6-Myc in the presence or absence of GdnHCl. Averages from at least two independent experiments are shown with standard deviations. Use of 6 M GdnHCl gave essentially the same results for the active site positions, suggesting that the moderate AMS modification of these residues in the presence of 4 M GdnHCl was not due to incomplete denaturation of the protein domain.
that embraces them. The residues at the C-terminal side of the active site (positions 27, 30, 33, and 37) became almost completely accessible to AMS after GdnHCl denaturation. The latter residues may be located within a proteinaceous structure exposed to the cytoplasm. The Cys residues introduced at positions 22-26 within the motif showed intermediate extents of GdnHCl enhancement in AMS modification, indicating that these residues are partially exposed but still stabilized significantly by the lipid phase integration. These results suggest that both folding and lipid integration contribute to the sequestering of the HEXXH motif residues (22-26) from the chemical modification. It is possible that they reside within a folded domain that is partially lipid phase embedded at the membrane-cytoplasm interface.

We next examined the AMS reactivity of Cys residues introduced into the TM3-C2 region, which contains the LDG motif (Fig. 7). The Cys residues in this region of the IMV-integrated RseP reacted very poorly with AMS whereas they were readily modified under the solubilized and denatured conditions. In the presence of GdnHCl alone, the Cys residues at position 402 in the LDG motif as well as at the neighboring residues (positions 392, 398, and 404) showed intermediate levels of AMS modification, whereas those at positions 405, 406, and 409 were fully modified. Thus, the LDG motif residues behaved similarly to the HEXXH residues, consistent with the notion that these residues constitute a single active site domain of RseP, probably at the membrane-cytoplasm interface.

**Accessibility of the RseP Active Site to malPEG, a Reagent Having Larger Molecular Size**—In the above experiments, AMS modifiability of several Cys residues in the active site motif regions was only negligibly or moderately enhanced by treatment with GdnHCl. Similar results were obtained when EMA, a Cys-specific alkylating reagent of size comparable with AMS, was used instead of AMS (data not shown). To obtain additional information on the nature of the protein fold in the active site region, we then examined the accessibility of the active site positions to a bulkier thiol modifier, malPEG (Fig. 8). In this experiment, IMVs were treated directly with malPEG in the presence or absence of GdnHCl or the GdnHCl-Triton X-100 combination. Upon SDS-PAGE, the modified proteins migrated as an upshifted band. None of the cysteines introduced into positions 22, 23, and 26 in the HEXXH motif as well as Cys at position 402 in the LDG motif received significant malPEG modification in intact IMVs. In the presence of GdnHCl, they exhibited much lower levels of modification with malPEG than with AMS. All of these Cys residues were efficiently modified with malPEG in the simultaneous presence of GdnHCl and Triton X-100. Also, Cys at position 77 (in the cytosolic loop) was modified efficiently even without any denaturant or detergent. Therefore, the relatively low efficiency of modification of Cys at the active site regions in the presence of GdnHCl was not due to the intrinsically low reactivity of malPEG as compared with AMS. Thus, the active site positions are less accessible to malPEG of molecular mass ~5 kDa than to AMS or EMA of molecular mass ~500–700 Da in the presence of the denaturant alone, suggesting that the denaturant makes an opening of a limited size to the structure containing the active site.

**DISCUSSION**

Elucidation of the molecular mechanisms responsible for intramembrane proteolysis is important for our understanding of physiological and pathological processes in which intramembrane proteolysis plays key roles, providing the basis for our possible control over such events. However, the membrane-embedded nature of this class of proteases makes their mechanistic analysis difficult. One standing question about the RIP proteases is the environment in which peptide bond hydrolysis is catalyzed. Does it occur within the plane of the lipid bilayer? If so, how are water molecules for the hydrolytic reaction provided to the membrane-embedded protease active site?

The recently determined three-dimensional structures of the *E. coli* rhomboid protease GlpG have provided the first structural information on the spatial arrangement of the active site in RIP proteases (32, 33). The GlpG active site residues are located within the hydrophilic cavity that opens to the aqueous milieu. Our cysteine modification experiments similar to those reported in this study supported the occurrence of the structure
scanning mutagenesis in combination with specific alkylation of the introduced cysteines has been used to study local topology and membrane integration of membrane proteins. In many of the previous studies, labeling with a radio- or a ligand-labeled Cys-alkylating reagent was used to indicate Cys modification (e.g. Refs. 34–37). However, these assays only report the modified fraction of the target protein, making it difficult to estimate quantitatively the modification efficiencies. In contrast, our gel shift assays allow visualization of both the modified and unmodified species of the target protein, thus directly reporting the modification extents and allowing quantitative and kinetic analyses of the modification.

None of the residues around the active site motifs of RseP was accessible to AMS in the native membrane-integrated state. These results suggest that the active site region of RseP is not significantly exposed to the aqueous environments on either side of the membrane. Therefore, the active site residues are all buried within a structure, either proteinaceous folding, lipid phase anchoring, or both. Cys at positions 22, 23, 24, and 26 in the H22EXXH motif and Cys at position 402 in the LD402G motif, which were virtually unmodifiable in the intact IMVs, became AMS modifiable to significant extents when treated with GdnHCl. These observations indicate that the active site of RseP exists in a folded proteinaceous structure that is not completely embedded in the lipid phase of the membrane. If this structure has sufficiently large openings to the aqueous milieu, water molecules required for proteolysis would be easily available, as suggested for GlpG. Alternatively, if it is segregated from the aqueous milieu by a proteinaceous gate, some signal such as substrate binding might induce a conformational change in RseP that enables water molecules to gain access to the active site.

Provided that the treatment with the denaturant alone can completely denature the extramembrane regions of RseP, the relatively inefficient AMS modification at the several active site residues under the denaturation condition would suggest that the RseP active site is positioned, at least partly, inside the plane of the membrane. It is also possible that complete denaturation of the extramembrane regions requires the simultaneous treatment with the denaturant and the detergent. In the latter case, the active site-containing domain may well be in a cytosolically exposed protein fold. Whereas AMS and malPEG were similar in their ability to modify the active site cysteines in the presence of both GdnHCl and Triton X-100, malPEG of larger molecular size than AMS exhibited much less efficient modification in the presence of GdnHCl alone. This can be also interpreted in the context of the above two possibilities, either the membrane cavity (in the former case) or the partially denatured proteinaceous domain (in the latter case) has an opening of limited size under that condition. Although it is difficult to discriminate between these two possibilities at this time, the former appears to explain more easily the cleavage by RseP of the substrate membrane proteins within their transmembrane segments.

Recently three-dimensional structural models of γ-secretase based on electron microscopy and single particle analyses have been reported (38, 39). Lazarov et al. (39) reported that γ-secretase has a chamber-like structure of ~20–40 Å within the interior of the complex, although its details are determined from the crystal in the native and membrane-integrated GlpG. The GlpG structure suggests that water molecules are readily accessible to the protease active site. However, structural information on other RIP proteases, including the S2P family proteins, is not yet available to an extent that would illuminate the reaction mechanisms. The biochemical analysis that we have undertaken in this study should be useful as a complement to any structure that is expected to be solved for a S2P protease in the future.

We examined accessibility of membrane-impermeable and thiol-specific alkylating reagents to Cys residues introduced into the protease active site regions. Similar approaches of Cys-

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4 S. Maegawa, K. Koide, K. Ito, and Y. Akiyama, unpublished results.
still unclear because of the low resolution of the reported structure. It was suggested that this chamber, which is segregated from the surrounding lipids, contains the proteolytic active site and that substrate transmembrane segments are translocated into it to receive proteolysis. Recent biochemical evidence based on the modifiability of introduced Cys residues with reagents that react with free sulfhydryl groups accessible to water supports a hydrophilic active site pore in γ-secretase that opens to the external aqueous milieu (40, 41). Our results suggest that the protease active site of RseP resides in a folded domain positioned at the interface between the lipid and aqueous phases, which are largely sequestered from the cytoplasm. It is conceivable that the RseP active site is located within a chamber-like structure. Given the similar architecture of the active site region of a rhomboid protease, GlpG, RIP proteases may generally possess active site residues that are segregated from the lipid environment of the membrane.

Our previous study showed that the presence of helix-destabilizing residues in transmembrane segments of substrate proteins promotes their cleavage by RseP (9). Destabilization of a substrate helix might facilitate its entrance into the hypothetical active site chamber of RseP, providing a feature that helps discrimination of substrate and non-substrate transmembrane segments by RseP. γ-Secretase is a multiprotein complex, and the above-mentioned chamber could be formed at interfaces of the subunit assembly (39). Although the oligomeric nature of RseP is unknown, the possibility must be considered that it oligomerizes to form an active site chamber that is appropriately sequestered from the aqueous and lipidic environments but still kept competent in recruiting water molecules and a substrate transmembrane segment. Structural studies of RseP will greatly advance the elucidation of the molecular mechanism that allows regulated proteolysis of membrane-integrated regions of specific membrane proteins.

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REFERENCES

1. Huovila, A. P., Turner, A. J., Pelto-Huikko, M., Karkkainen, I., and Ortiz, R. M. (2005) Trends Biochem. Sci. 30, 413–422
2.Blobel,C.P.(2000)Carr.Opin.CellBiol.12,606–612
3. Weihofen, A., and Martoglio, B. (2003) Trends Cell Biol. 13, 71–78
4. Wolfe, M. S., and Kopan, R. (2004) Science 305, 1119–1123
5. Kinch, L. N., Ginalska, K., and Grishin, N. V. (2006) Protein Sci. 15, 84–93
6. Makino, H., and Gleckman, M. S. (2006) Microbes Infect. 8, 1882–1888
7. Kanekura, K., Ito, K., and Akiyama, Y. (2002) Genes Dev. 16, 2147–2155
8. Alba, B. M., Leeds, J. A., Ounuyrky, C., Lu, C. Z., and Gross, C. A. (2002) Genes Dev. 16, 2156–2168
9. Akiyama, Y., Kanekura, K., and Ito, K. (2004) EMBO J. 23, 4434–4442
10. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) Cell 100, 391–398
11. Rawson, R. B., Zelenski, N. G., Nijhawan, D., Ye, J., Sakai, J., Hasan, M. T., Chang, T. Y., Brown, M. S., and Goldstein, J. L. (1997) Mol. Cell 1, 47–57
12. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prys, R., Brown, M. S., and Goldstein, J. L. (2000) Mol. Cell 6, 1355–1364
13. Rudner, D. Z., Fawcett, P., and Losick, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14765–14770
14. Schobels, Z., Zellmeier, S., Schumann, W., and Wiegert, T. (2004) Mol. Microbiol. 52, 1091–1105
15. Chen, J. C., Viollier, P. H., and Shaparo, L. (2005) Mol. Microbiol. 55, 1085–1103
16. Matson, J. S., and DiRita, V. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 16403–16408
17. Missiakas, D., Mayer, M. P., Lemaire, M., Georgopoulos, C., and Raina, S. (1997) Mol. Microbiol. 24, 355–371
18. De Las Peñas, A., Connolly, L., and Gross, C. A. (1997) Mol. Microbiol. 24, 373–385
19. Walsh, N. P., Alba, B. M., Bose, B., Gross, C. A., and Sauer, R. T. (2003) Cell 113, 61–71
20. Flynn, J. M., Levenklen, I., Sauer, R. T., and Baker, T. A. (2004) Genes Dev. 18, 2292–2301
21. Kanekura, K., Ito, K., and Akiyama, Y. (2003) EMBO J. 22, 6389–6398
22. Grigorovs, I. L., Chaca, R., Zhong, H. J., Alba, B. M., Rhodius, V., Herman, C., and Gross, C. A. (2004) Genes Dev. 18, 2686–2697
23. Bohn, C., Collier, J., and Bouloc, P. (2004) Mol. Microbiol. 52, 427–435
24. Akiyama, Y., Ogura, T., and Ito, K. (1994) J. Biol. Chem. 269, 5218–5224
25. Bohn, C., Collier, J., and Bouloc, P. (2004) Mol. Microbiol. 52, 427–435
26. Akiyama, Y., and Ito, K. (1985) EMBO J. 4, 3351–3356
27. Kanekura, K., Akiyama, Y., and Ito, K. (2001) Gene 281, 71–79
28. Davis, R. W., Botstein, D., and Roth, J. R. (1980) Advanced Bacterial Genetics: A Manual for Genetic Engineering. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Sawano, A., and Miyawaki, A. (2000) Nucleic Acids Res. 28, E78
31. Kimura, T., Ohnuma, M., Sawai, T., and Yamaguchi, A. (1997) J. Biol. Chem. 272, 580–585
32. Kimura, T., Ohnuma, M., Sawai, T., and Yamaguchi, A. (1997) J. Biol. Chem. 272, 580–585
33. Sawano, A., and Miyawaki, A. (2000) Nucleic Acids Res. 28, E78
34. Kimura-Someya, T., Iwaki, S., and Yamaguchi, A. (1998) J. Biol. Chem. 273, 32806–32811
35. Wada, T., Long, J. C., Zhang, D., and Vik, S. (1999) J. Biol. Chem. 274, 17353–17357
36. Hu, Y. K., and Kaplan, J. H. (2000) J. Biol. Chem. 275, 19185–19191
37. Ermolova, N., Madhvan, R. V., and Kack, R. H. (2006) Biochemistry 45, 4182–4189
38. Ogura, T., Mio, K., Hayashi, I., Misyashta, H., Fukuda, R., Kopan, R., Kodama, T., Hamakubo, T., Iwatsubo, T., Tomita, T., and Sato, C. (2006) Biochem. Biophys. Res. Commun. 343, 525–534
39. Lazarov, V. K., Fraering, P. C., Ye, W., Wolfe, M. S., Selkoe, D. J., and Li, H. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6889–6894
40. Tolia, A., Chavez-Gutierrez, L., and De Strooper, B. (2006) J. Biol. Chem. 281, 27633–27642
41. Sato, C., Morohashi, Y., Tomita, T., and Iwatsubo, T. (2006) J. Neurosci. 26, 12081–12088