A Novel Thermostable Membrane Protease Forming an Operon with a Stomatin Homolog from the Hyperthermophilic Archaeabacterium Pyrococcus horikoshii*

Hideshi Yokoyama and Ikuo Matsui

From the Biological Information Research Center, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan

Membrane-bound proteases play several important roles in protein quality control and regulation. In the genome of the hyperthermophilic archaeabacterium Pyrococcus horikoshii, the open reading frames PH1510 and PH1511 are homologous to the genes nfed (nodulation formation efficiency D) and stomatin, respectively, and probably form an operon. The nfed proteins are putative membrane proteins, and the N-terminal region shows homology to ClpP-type serine proteases. Stomatin is one of the major integral membrane proteins of human erythrocytes, and its absence is associated with the hemolytic anemia known as hereditary stomatocytosis. Thus, the N-terminal region of PH1510 (1510-N, residues 16–236) was expressed and purified. From activity staining and SDS-PAGE analysis using fluorescein isothiocyanate-casein, 1510-N was identified as a thermostable endo-type protease. From site-directed mutagenesis, the conserved Ser-97 and Lys-138 are involved in proteolysis and, therefore, PH1510 is probably a serine protease with a catalytic Ser-Lys dyad. The sites of cleavage by 1510-N are rich in hydrophobic residues. The site P1 (position −1 relative to the cleavage site) is mainly leucine. P4 and P4' are mainly hydrophobic residues. Interestingly, the 1510-N protease cleaves the C-terminal hydrophobic region of PH1511. From this result and the probability of an operon, PH1510 probably functions in cooperation with PH1511. It is hypothesized that the cleavage of the stomatin-homolog PH1511 by the PH1510 protease causes an ion channel to open.

The hyperthermophilic archaeabacterium Pyrococcus horikoshii grows optimally at −100 °C, and its entire genome sequence has been determined by the National Institute of Technology and Evaluation of Japan (1). The highly thermostable gene products are expected to have industrial applications. Protein degradation plays several important roles in protein quality control and regulation (2). In mitochondrial membranes, a quality control system is present that removes non-assembled polypeptides and prevents their possibly deleterious accumulation in the membrane (3). Several membrane-bound proteases have been characterized. In Escherichia coli PtsH was identified as an ATP-dependent membrane-bound protease and investigated concisely by functional and structural analyses (2). In Archaea, the Lon protease from Thermococcus kodakaraensis KOD1 has been characterized as membrane-bound in contrast to the E. coli Lon protease, which is soluble (4). Because the archaeal FtsH homolog has not yet been identified, the Lon protease might play important roles in the archaeal membrane like the bacterial PtsH does.

Stomatin is one of the major integral membrane proteins of human erythrocytes, and its absence is associated with the hemolytic anemia known as hereditary stomatocytosis. Stomatin is thought to function as a negative regulator of univalent cation permeability (5). Its gene homologs are identified in almost all species of eukaryotes, eubacteria, and Archaea (6). Recently, it has been proposed that stomatin-like genes in prokaryotes (p-stomatin) and nfed (nodulation formation efficiency D) genes probably form an operon in at least 19 organisms (7). The nfed protein was originally identified as an ornithine cyclodeaminase (8). However, this enzyme is not related to the p-stomatin partner gene that is indicated here as "nfed", which is mislabeled in the major data bases (7). According to sequences of the p-stomatin partner gene nfed, the proteins have putative membrane-spanning segments, and the N termini of the proteins show homology to ClpP-type serine proteases (7). One member of the stomatin superfamily, prohibitin, reportedly possesses a regulatory function during membrane protein degradation by the AAA proteases of yeast mitochondria (9). Two other members, HflK and HflC in E. coli, are proposed to have a similar function in association with the bacterial membrane-bound AAA protease PtsH (10). Thus, stomatin is presumed to be some kind of partner for membrane-bound proteases. But no experimental data about the p-stomatin partner nfed has been reported. In the sequences of P. horikoshii, PH1510 and PH1511 correspond to nfed and p-stomatin, respectively. Therefore, it is speculated that PH1510 would be a protease and function in cooperation with the stomatin homolog PH1511. In this study, we characterize the N-terminal region of PH1510 as a protease with novel substrate specificity and discuss its relationship with the stomatin-like protein PH1511.

EXPERIMENTAL PROCEDURES

Chemicals and Bacterial Strains—The vector pET-21b was purchased from Novagen (Madison, WI). Ultracompetent E. coli XL2-Blue MRF' cells and E. coli strain BL21-CodonPlus (DE3)-RIL competent cells were obtained from Stratagene (La Jolla, CA). KOD-Plus polymerase was purchased from Toyobo (Osaka, Japan). Restriction enzymes and a ligation kit were obtained from Takara Shuzo (Shiga, Japan) and used according to the manufacturer’s directions. A protease inhibitor

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† To whom correspondence should be addressed. Tel.: 81-29-861-6142; Fax: 81-29-861-6151; E-mail: ik-matsui@aist.go.jp.

1 The abbreviations used are: nfed, nodulation formation efficiency D; DDM, dodecyl-β-d-maltoside; FITC, fluorescein isothiocyanate; MES, 4-morpholinolinesulfonic acid; 1510-N, residues 16–236 of PH1510; 1510-C, residues 371–441 of PH1510; 1511-N, residues 69–234 of PH1511; 1511-C, residues 189–266 of PH1511.
mixture was purchased from Roche Diagnostics. 

Construction of Expression Vectors—Using genomic DNA of P. horikoshii OT3 as a template (11), the DNA fragments encoding residues shown in Fig. 1 were amplified by PCR with two primers containing NdeI and XhoI sites. The amplified fragments were digested with NdeI and XhoI and ligated into an expression vector, pET-21b, between the NdeI and XhoI sites. Ultracompetent *E. coli* XL2-Blue MRF<sup>®</sup> cells were transformed with the ligated products. After the transformant colonies were propagated in 5 ml of 2× yeast tryptone medium containing ampicillin (100 μg/ml), the resultant plasmids were purified using a Qiagen plasmid mini kit. The DNA sequences were verified using an ABI PRISM kit and a model 310 capillary DNA sequencer (Applied Biosystems). The site-directed alanine mutants of PH1510 (residues 16–236) were prepared similarly, except that the overlap PCR method was used (12). All of the resultant proteins had hexahistidine tags at their C termini.

Expression and Purification of the Proteins—For the preparation of PH1510 N-terminal constructs shown in Fig. 1 and several alanine mutants of PH1510 (residues 16–236), *E. coli* strain BL21-CodonPlus (DE3)-RIL cells were transformed with the plasmids. The transformed cells were grown in 2 ml of the protein liquid chromatography system (Amersham Biosciences). The mixture (28 ml) was heated at 98 °C for 5 min, and 14 μl of the sample was electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS. The reaction products on the gel were visualized and quantified using a FluorImager 585 (Amersham Biosciences) with excitation and emission filters of 488 and 530 nm, respectively. The protease activity was normalized with the following formula: (percentage of cleavage) = 100 × (R<sub>X</sub> - R<sub>0</sub>)/(1 - R<sub>0</sub>), in which R<sub>X</sub> is the value of the non-degraded casein band and P is the value of all the degraded products located below the original casein band. The protease activity was visualized as clear zones resulting from casein hydrolysis. The activity was calculated with the formula P = (S + P<sub>c</sub>), in which S is the value of the non-degraded casein band and P<sub>c</sub> is the value of all the degraded products located below the original casein band. The protease activity was normalized with the following formula: (percentage of cleavage) = 100 × (R<sub>X</sub> - R<sub>0</sub>)/(1 - R<sub>0</sub>), in which R<sub>X</sub> is the value of the non-degraded casein band and P is the value of all the degraded products located below the original casein band. The protease activity was visualized as clear zones resulting from casein hydrolysis.

For the preparation of PH1510 and PH1511, the DNA fragments encoding residues 16–236 of PH1510 (both bovine; Sigma) and the purified proteins PH1510-C, 1511-N, and 1511-C were loaded on a Superose 12 gel filtration column (Amersham Biosciences) and equilibrated with 50 mM sodium phosphate (pH 7.5) containing 0.5 M NaCl and 0.1% DDM. The purified samples were analyzed by SDS-PAGE performed on a 10–15% gradient gel using the Phast system (Amersham Biosciences).

Activity Staining—For detection of the protease activity of 1510-N, casein-copolymerized SDS-PAGE was performed as described previously (13), but with a slight modification. The protein sample was kept at room temperature for 1 h in the presence of 0.8% sodium laurylsarcosine (Nacalai Tesque), 10% glycerol, and 1% β-mercaptoethanol, and electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS and 0.2% casein (Difco). After electrophoresis, the gel with duplicated runs of the left and right sides was cut in half, and one-half was washed for 1 h in 50 mM sodium phosphate (pH 7.5) containing 2.5% Triton X-100 and incubated at 80 °C for 16 h in 50 mM sodium phosphate (pH 7.5) for a proteolytic reaction. The gel was then cooled and stained with 0.1% Amido Black containing 30% methanol and 10% acetic acid. Proteolytic activity was visualized as clear zones resulting from casein hydrolysis. The other half of the gel was stained with conventional Coomassie Brilliant Blue R-250 for confirmation of the protein band.

Quantification of Proteolytic Activity—Proteolytic activity was measured by the hydrolysis of fluorescein isothiocyanate (FITC)-labeled bovine casein (Sigma). The standard reaction mixture (20 μl) consists of FITC-casein and enzyme in 20 mM sodium MES (pH 6) and was incubated at 80 °C for several minutes. To stop the reaction, the mixture was kept on ice and added with a sample buffer containing a final concentration of 0.5% SDS, 10% glycerol, and 1% β-mercaptoethanol. The mixture (28 μl) was heated at 98 °C for 5 min, and 14 μl of the sample was electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS. The reaction products on the gel were visualized and quantified using a FluorImager 585 (Amersham Biosciences) with excitation and emission filters of 488 and 530 nm, respectively. The protease activity was calculated with the formula P = (S + P<sub>c</sub>), in which S is the value of the non-degraded casein band and P<sub>c</sub> is the value of all the degraded products located below the original casein band. The protease activity was visualized as clear zones resulting from casein hydrolysis. The activity was calculated with the formula P = (S + P<sub>c</sub>), in which S is the value of the non-degraded casein band and P<sub>c</sub> is the value of all the degraded products located below the original casein band. The protease activity was visualized as clear zones resulting from casein hydrolysis.

Hydropathy Plots and Sequence Comparison—PH1510 and PH1511 are putative membrane proteins predicted by SOSUI (14) with five and two membrane-spanning regions, respectively (Fig. 1). In the peptidase data base MEROPS (15), PH1510 is classified into Clan SK according to its protein sequence. *E. coli* ClpP (2) also belongs to this group of serine proteases. Comparison of the N-terminal region of PH1510 and its homologs (Fig. 2) revealed conserved serine and lysine residues. Green et al. proposed that the nfd gene product like PH1510 could conform to Clan SF of the serine proteases, in
were initially made to express the intact PH1510 in a major band of 1510-N at 25 kDa corresponding to the pre-mature region. SDS-PAGE shows a major band at around 10 kDa, 19 kDa, and 10 kDa, respectively. The N-terminal soluble regions of PH1510 (Fig. 1) were expressed in E. coli. The protein spanning residues 16–236 (1510-N) was produced from a liter of culture.

For further experiments, 1510-C, 1511-N, and 1511-C were expressed in E. coli. The protein spanning residues 16–236 (1510-N) was produced from a liter of culture. The approximate molecular mass was quantified using gel filtration. The expected molecular mass was 1510-N was measured using gel filtration. The expected molecular mass was 1510-N was measured using gel filtration. The expected molecular mass was 1510-N was measured using gel filtration. The expected molecular mass was 1510-N was measured using gel filtration.

The catalytic site is formed by a conserved Ser-Lys dyad (7). For further experiments, 1510-C, 1511-N, and 1511-C were prepared as described under “Experimental Procedures.” SDS-PAGE shows a major band of 1510-N at ~25 kDa corresponding to the predicted mass (Fig. 3A). The constructs other than residues 16–236 of PH1510 were not fully expressed, although some expression of residues 16–197 was observed. This result indicates that the N-terminal sequences in particular would be important for protein expression. To keep the 1510-N with partly hydrophobic regions in its N terminus stable, the protein was kept in the presence of DDM throughout the purification steps, although DDM is not essential for protease activity, as indicated later.

About 1 mg of the purified protein could be produced from a liter of culture. The approximate molecular mass of 1510-N was measured using gel filtration. The expected value was ~40–50 kDa (data not shown). Thus, 1510-N would possibly form a dimer. Site-directed alanine mutants of 1510-N were prepared in the same way as the wild-type, with a similar yield (Fig. 3B).

Detection of Protease Activity—Using the purified 1510-N, protease activity was detected with staining (Fig. 4). The protein sample was mildly denatured with sodium lauroylsarcosine instead of SDS and not heated; it was then electrophoresed on a casein-copolymerized polyacrylamide gel. After incubation at 80 °C for 16 h, the gel was stained with Amido Black. As a result of Coomassie Brilliant Blue R-250 staining, a major protein band at ~45 kDa was observed (Fig. 4). The corresponding band was cleared from the background by casein hydrolysis. The result indicates that 1510-N shows protease activity. The gel filtration analysis indicated that 1510-N possibly forms a dimer. Further analyses are needed to make clear whether 1510-N functions in a dimeric form.

Measurement of Protease Activity Using FITC-casein—To quantify the protease activity, FITC-casein and 1510-N were mixed and incubated at 80 °C for several minutes. The reaction mixture separated with SDS-PAGE was visualized using a FluorImager (Fig. 5A). The FITC-casein consists of FITC-a-casein (appeared ~30 kDa) and FITC-b-casein (~23 kDa). The a- and b-caseins are phosphorylated (16), and their differences in phosphorylation probably contribute to the different rates of migration on SDS-PAGE despite their similar molecular masses. As the reaction proceeded, the 20-kDa degraded band, some minor bands, and the FITC-peptides located at the bottom of the gel increased. Assuming that the substrate shows the total fluorescence intensity of the bands of FITC-a- and b-caseins and that the product shows the total intensity of the bands below the substrate, the protease activity was measured and the normalized activity was calculated as indicated under “Experimental Procedures.” β-Casein is not cleaved as effectively as α-casein, as indicated later. Thus, the cleavage of α-casein is mainly measured in the analysis. The proteolytic activity is shown as the percentage of cleavage versus time (Fig. 5B).

Proteolytic activity was measured as described above in several buffer solutions at various pH values. The optimal pH was ~5–6 (Fig. 6A). The temperature dependence was measured similarly, and elevated activity was observed with temperatures from 50 to 98 °C (Fig. 6B). This observation indicates that...
Fig. 5. Measurement of the protease activity with SDS-PAGE using FITC-casein. FITC-casein (0.5 µg) and 1510-N (0.25 µg) were incubated at 80 °C for each minute in 10 mM Tris-HCl (pH 7.8), and the reaction mixtures were electrophoresed on a 10% polyacrylamide gel. A, FITC-casein and degraded products were detected using a FluorImager. The numbers shown on the left of the gel indicate the corresponding molecular mass. The FITC-casein consists of FITC-α-casein (~30 kDa) and FITC-β-casein (~23 kDa). As the reaction proceeded, the 20-kDa band, some minor bands, and the FITC-peptides located at the bottom of the gel increased. B, the result from panel A was calculated as indicated under “Experimental Procedures” and plotted as the percentage of cleavage values versus time.

Fig. 6. Characteristics of the 1510-N protease measured using FITC-casein. The activity is indicated as the mean of the percentage of cleavage versus time for three different time points. A, pH dependence of the protease activity. The proteolytic reaction was performed using FITC-casein (0.5 µg) and 1510-N (0.25 µg) at 80 °C in 50 mM buffer. ○, sodium acetate; △, sodium phosphate; +, sodium MES; [ ], Tris-HCl; and ×, glycine-NaOH. The error bars were omitted for clarity. B, temperature dependence of the protease activity. The proteolytic reaction was performed at each temperature using FITC-casein (0.5 µg) and 1510-N (0.1 µg) in 20 mM sodium MES (pH 6).

1510-N is a hyperthermostable protease. As the salt concentration increases, the activity decreases; the level of activity in 0.1 M NaCl was about one-tenth of that in 0 M NaCl (data not shown). Divalent cations generally have inhibitory effects on the activity: MgCl₂, 14% residual activity; MnCl₂, 4% activity; CaCl₂, 11% activity; and ZnCl₂, 9% activity (in 10 and 20 mM sodium MES buffer (pH 6) in comparison with the control lacking a divalent cation in the MES buffer). Detergents also inhibit the activity: 0.1% DDM, 38% activity; 0.01% SDS, 53% activity; and 1% SDS, 1.2% activity (in comparison with the control).

Detection of Catalytic Residues—Using the 1510-N mutants (Fig. 3B), the protease activity was measured with FITC-casein. For each mutant and wild-type sample, the percentage of cleavage with time as shown in Fig. 7A is plotted for four different enzyme quantities, and an average value of percentage of cleavage versus time to the amount of the enzyme (percentage/min/µg) relative to the wild-type is indicated with the S.D. (Fig. 7B). S97A shows 0.08% activity compared with the wild-type. The 0.08% activity is not significantly above background. Thus, Ser-97 is considered to be a catalytic residue. T62A, K138A, and D168A have relatively low levels of activity of 5.5, 1.2, and 3.2%, respectively. From activity staining, D168A shows a 25-kDa band instead of 45-kDa band (data not shown). Although Asp-168 may be involved in proteolysis, a different oligomeric form of D168A possibly contributes to the weaker activity. Other mutants show levels of activity relatively similar to that of the wild-type protease. These results indicate that 1510-N is a serine protease with a catalytic residue, Ser-97.

Using protease inhibitors, the protease activity of 1510-N was measured, and values relative to the control with no inhibitor were calculated. The relative activity was 83% in 5 mM iodoacetic acid (cysteine protease inhibitor), 33% in 5 mM EDTA (metalloprotease inhibitor), and 79% in 5 µM pepstatin (aspartic protease inhibitor), indicating that these inhibitors do not remarkably inhibit the protease activity of 1510-N. In the case of typical serine protease inhibitors, the relative activity was 79% in 5 mM phenylmethylsulfonyl fluoride and 51% in 1 mM 3,4-dichloroisoumarin. These serine protease inhibitors do not remarkably affect the activity.

Sequence Specificity of Cleavage—To elucidate the sequence specificity of the cleavage site, certain proteins were used as substrates for the 1510-N protease. Among the α- and β-caseins 1510-C, 1511-N, and 1511-C, relatively good substrates are α-casein and 1511-C. Specifically degraded products are shown in the SDS-PAGE pattern (Fig. 8). N-terminal sequences of the degraded products were determined (Table I). The α-13k (13 kDa) product is from αs1-casein, and the α-14k (14 kDa) product is mainly from αs2-casein (Table I). Considering that α-casein contains αs1- and αs2-caseins at a ratio of 6:1 (17), the minor product, α-14k (14 kDa), is a relatively major one among αs2-casein-degraded products (Table I). The α-20k (20 kDa) product mainly shows the N terminus of αs1-casein on N-terminal sequencing and partly contains the minor product (~10%) of αs1-casein (α-20k-2 in Table I). The 1511–10k (10 kDa) product is a major degraded product from 1511-C (Table I). β-casein was not degraded effectively, but minor 12k (12 kDa) products were produced (Table I). From other substrates like 1510-C and 1511-N, no cleaved products were observed.
1510-N probably cleaves the substrate specifically. The cleavage sites contain many hydrophobic and aromatic residues. Some features of sequence specificity are observed for the upper three major products in Table I. The site P1 (position -1 relative to the cleavage site) is leucine, whereas P4 is valine or leucine. P4’ (position +4 relative to the cleavage site) contains hydrophobic residues such as methionine or proline. On the other hand, the lower four minor products in Table I indicate that leucine is located at P2, P1’, or P2’. The 1510-N protease possibly recognizes leucine at the cleavage site.

**DISCUSSION**

In this report, the N-terminal region of PH1510 was identified as a novel hyperthermostable serine protease. Following sequence alignment of the nfed homologs, relatively conserved aspartic acids were mutated and the protease activity of their alanine mutants was measured, but no suitable candidates for a catalytic residue were found (Fig. 7B). D168A shows relatively little activity as indicated, but Asp-168 is not highly conserved. His-107, the only histidine of 1510-N, is not a catalytic residue (Fig. 7B). Thus, 1510-N is not a typical serine protease with a catalytic Ser-Lys dyad. Lys-138 might act as a catalytic residue were found (Fig. 7B).

A catalytic Ser-Lys dyad (21, 22), were classified into Clan SK in the MEROP data base (15). According to the data base, 1510-N belongs to Clan SF (20). Recently, tail-specific protease and C-terminal endoprotease, which have a catalytic Ser-Lys dyad and belong to Clan SF, were classified into Clan SK in the MEROP data base (15). According to the data base, 1510-N belongs to Clan SK. These results also support the 1510-N protease with a catalytic Ser-Lys dyad. Lys-138 might act as a general base to increase the nucleophilicity of the active site Ser-97 as indicated in the Ser-Lys dyad mechanism (19). According to a structural modeling study of CtpC protease, a threonine residue is hydrogen-bonded to the catalytic lysine, and the interaction might be important for activity (22). The Thr-62 of 1510-N might function similarly.

Considering that the SDS-PAGE pattern of degraded products shows clear bands (Fig. 8), 1510-N is an endo-type protease. Also, it only cleaves specific proteins. Clearly, it specifically recognizes the substrate. The sequences around the cleavage sites are rich in hydrophobic residues, and substrates are mainly cleaved after leucine. Sites P4 and P4’ are mainly hydrophobic residues. No other definitive sequence specificity was found. The tail-specific protease, one of the Clan SK protease members, selectively degrades proteins bearing a nonpolar C terminus. In particular, a protein with the C-terminal sequence WVAAA is a good substrate for the protease (23). The signal peptide peptidase A (SppA, also known as protease IV) belongs to Clan SK and is a typical membrane protein (24). Signal peptides and their cleavage sequences have been investigated extensively, and sites P1 and P3 are, for the most part, alanines in bacteria (25). In the case of 1510-N, P1 is mainly leucine. It seems that 1510-N may have some features in common with SppA in other respects. 1510-N might possibly recognize secondary or tertiary structures, in particular specific structures of membrane-bound proteins. One of the substrates, 1511-10k (10 kDa) (Table I), has all hydrophobic residues from P4 to P4’. The cleavage site is between residues 238 and 239 of PH1511. The region from residue 235 to 242, corresponding to P4 to P4’, shows high hydrophobicity (PH1511 in Fig. 1) and thus is considered to be anchored to the membrane. The 1510-N protease possibly recognizes and cleaves specific proteins that are located at the membrane surface and are in a particular structure.

An analysis with an operon-predicting algorithm (26) indicated that PH1510 (nfed homolog) and PH1511 (stomatin homolog) are involved in proteolysis. Typical serine protease inhibitors such as phenylmethylsulfonyl fluoride and 3,4-dichloroisocoumarin do not remarkably inhibit the activity of 1510-N. Inhibitors of cysteine protease, metalloprotease, and aspartic protease do not remarkably affect the activity either. These results support the possibility that 1510-N is a serine protease with a catalytic Ser-Lys dyad, because serine proteases with catalytic Ser-Lys often show little or no reactivity with typical serine protease inhibitors (18, 19). Bacterial leader peptidase 1 and the repressor LexA are known as serine proteases with a catalytic Ser-Lys dyad and belong to Clan SF (20). Recently, tail-specific protease and C-terminal endoprotease, which have a catalytic Ser-Lys dyad (21, 22), were classified into Clan SK (15). According to the data base, 1510-N belongs to Clan SK. These results also support the 1510-N protease with a catalytic Ser-Lys dyad. Lys-138 might act as a general base to increase the nucleophilicity of the active site Ser-97 as indicated in the Ser-Lys dyad mechanism (19). According to a structural modeling study of CtpC protease, a threonine residue is hydrogen-bonded to the catalytic lysine, and the interaction might be important for activity (22). The Thr-62 of 1510-N might function similarly.

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The cleavage sites were determined with N-terminal sequences of the products degraded by 1510-N. Under the column marked “Product name,” the top three items are major products, and bottom four are minor products. The percentage value in parentheses shows the ratio of the proteolytic products in the sequenced protein band. Each ratio was calculated with the peak intensity of PTH-amino acids derived from seven cycles of Edman degradation. The bold type indicates hydrophobic or aromatic residues. 10k, 12k, 13k, 14k, and 20k refer to 10, 12, 13, 14, and 20 kDa, respectively.

### Table 1: Cleavage site sequences

| Product name | P6 | P5 | P4 | P3 | P2 | P1 | P1’ | P2’ | P3’ | P4’ | P5’ | P6’ |
|--------------|----|----|----|----|----|----|----|----|----|----|----|----|
| 1511-10k (90%) | S  | N  | V  | I  | V  | L  | M  | L  | P  | M  | E  | M  |
| α-13k-1 (100%) | Y  | K  | Y  | P  | Q  | L  | E  | I  | V  | P  | N  | S  |
| α-14k-1 (70%) | Q  | Y  | L  | Q  | Y  | L  | Y  | Q  | G  | P  | I  | V  |
| α-20k-2 (10%) | P  | Q  | E  | V  | L  | N  | E  | N  | L  | L  | R  | F  |
| β-12k-1 (40%) | P  | G  | P  | I  | H  | N  | S  | L  | P  | Q  | N  | I  |
| β-12k-2 (40%) | F  | A  | Q  | Q  | S  | Q  | L  | V  | Y  | T  | F  | P  |
| β-12k-3 (10%) | N  | I  | P  | P  | L  | T  | Q  | T  | P  | V  | V  | V  |

Fig. 9. Schematic representation of the hypothetical model of PH1510, PH1511, and an ion channel. The PH1510 and PH1511 models are based on hydrophathy plots and the putative membrane-spanning regions shown in Fig. 1. For simplicity, PH1510 and PH1511 are shown in a monomeric state, although the protease domain of PH1510 is probably in a dimeric form as indicated, and the stomatin homologous to PH1511 is reported to be oligomeric (27). Stomatin in humans is thought to function as a cation channel regulator. The soluble region of stomatin plugs into a cation channel with the stomatin N-terminal region embedded in the cell membrane (5). PH1511 (p-stomatin) has two membrane-spanning regions in its N terminus (Fig. 1) and a putative membrane-anchored region in its C terminus (residues 235 to 242 as indicated). When the PH1510 protease cleaves the C-terminal membrane-anchored region of PH1511, the residual soluble region of PH1511 is freed from the C-terminal anchored membrane, and then a cation channel opens.

Fig. 10. Sequence alignment of C-terminal amino acids for PH1511 and its homologs. The figure is prepared similarly as in Fig. 2 with PH1511 (from P. horikoshii; NCBI accession number NP_143371); PAB1933 (P. abyssi; NP_126340); PA0452 (P. aeruginosa; NP_249143); SMB20989 (S. meliloti; NP_437654); MEC-2 (C. elegans; NP_741797); and stomatin (human; NP_004090). Filled arrowhead shows proteolytic cleavage site of 1511-C by the 1510-N protease. The underlined region is rich in hydrophobic residues.

Prolate Forming an Operon with a Stomatin Homolog

This hypothesis shown in Fig. 9 is consistent with the result that the epithelial sodium channel in Xenopus is activated by proteolysis (28). Direct evidence relating to the function of stomatin is not reported in this article, but a relationship between the epithelial sodium channel and the stomatin-like protein has been established (7, 29). One member of the stomatin superfamily, prohibitin, is reported to have a role in the regulation of mitochondrial AAA proteases in yeast (9), and this report shows a relationship between prohibitin and proteases. In bacteria and Archaea, on the other hand, stomatin and nfed homologs are found, but to our knowledge there are no reports about the relationship between p-stomatin and ion channels. However, stomatin homologs are present in almost all species of eukaryotes, bacteria, and Archaea. Assuming that stomatin homologs in eukaryotes and p-stomatin in prokaryotes have common features, it seems possible, as suggested in Fig. 9, that stomatin homologs have a common function in cooperation with proteases and ion channels in almost all species.

Another hypothesis is also proposed. The precursor PH1511 would become an active form with its C-terminal region degraded by the PH1510 protease and would then regulate an ion channel. On the other hand, it seems possible that PH1511 regulates the PH1510 protease activity as the stomatin-like protein regulates AAA protease (9, 10). Further study is needed to elucidate the molecular mechanism behind the relationship among stomatins, ion channels, and proteases.

Green et al. (7) pointed out further that the central membrane-spanning region of the nfed protein is partly homologous to a sterol-sensing domain found in some eukaryotic proteins. One of the sterol-sensing domains, the sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP), plays essential roles in regulating cholesterol homeostasis (30). Cholesterol acts as an effector molecule, and proteolysis is linked to the cholesterol concentration in eukaryotic cells. It seems possible that the central membrane-spanning region of PH1510 acts like a SCAP and that the protease
activity of the N-terminal domain is regulated with a cholesterol-like molecule binding to the central region of PH1510. Some studies about membrane-based proteolysis by the SCAP/SREBP system have been reported (30, 31). PH1510 and PH1511 might be good targets for study.

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