The Potential Active Site of the Lipoprotein-specific (Type II) Signal Peptidase of *Bacillus subtilis*

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Harold Tjalsma, Geeske Zanen, Gerard Venema, Sierd Bron, and Jan Maarten van Dijl

From the Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, P. O. Box 14, 9750 AA Haren, The Netherlands

Type II signal peptidases (SPase II) remove signal peptides from lipid-modified preproteins of eubacteria. As the catalytic mechanism employed by type II SPases was not known, the present studies were aimed at the identification of their potential active site residues. Comparison of the deduced amino acid sequences of 19 known type II SPases revealed the presence of five conserved domains. The importance of the 15 best conserved residues in these domains was investigated using the type II SPase of *Bacillus subtilis*, which, unlike SPase II of *Escherichia coli*, is not essential for viability. The results showed that only six residues are important for SPase II activity. These are: Asp-14, Asn-99, Asp-102, Asn-126, Ala-128, and Asp-129. Only Asp-14 was required for stability of SPase II, indicating that the other five residues are required for catalysis, the active site geometry, or the specific recognition of lipid-modified preproteins. As Asp-102 and Asp-129 are the only residues involved in the known catalytic mechanisms of proteases, we hypothesize that these two residues are directly involved in SPase II-mediated catalysis. This implies that type II SPases belong to a novel family of aspartic proteases.

Signal peptidases (SPases) remove the targeting signals (i.e. signal peptides) from proteins that are translocated across the bacterial cytoplasmic membrane. This is a prerequisite for the release of the protein at the trans side of the membrane and, in some cases, the posttranslational modification of its amino terminus (for reviews, see Refs. 1–4). Although the primary structure of signal peptides is poorly conserved, three functional domains have to be present: first, a positively charged amino terminus (N-region); second, a central hydrophobic domain (H-region); and third, a polar carboxyl-terminal domain (C-region), specifying the SPase cleavage site (1). We have previously shown that five paralogous type I SPases are involved in the processing of secretory precursor proteins in *Bacillus subtilis* (5–7). Two of these, denoted SipS and SipT, are of major importance for protein secretion. In this respect, *B. subtilis* is representative for Gram-positive eubacteria and archaea, many of which contain paralogous sip gene families (8). Considerable similarities can be observed between the known type I SPases when individual amino acid sequences are compared, including strictly conserved serine and lysine residues, which form a catalytic dyad (8–11).

In contrast to the sip genes, *B. subtilis* and other eubacteria of which the genome has been sequenced completely contain only one gene for lipoprotein-specific (type II) SPases (12–14). As estimated from published genome sequences, lipoprotein precursors, which are the substrates of these enzymes, represent about 1–3.5% of the known eubacterial proteomes (14). The major difference between signal peptides of lipoproteins and those of secretory proteins is the presence of a well conserved "lipobox" of four residues in the C-region of lipoprotein signal peptides (3, 15). Invariably, the carboxyl-terminal residue of the lipobox is cysteine, which, upon lipid modification, forms the signal for the retention of the mature lipoprotein at the membrane-cell wall interface of Gram-positive eubacteria, or the inner and outer membranes of Gram-negative eubacteria (16, 17). Modification of this cysteine residue by the diacylglycerol transferase (Lgt) is a prerequisite for processing of the lipoprotein precursor by SPase II. In *Escherichia coli*, mature (apo-)lipoproteins are further modified by amino-fatty acylation of the diacylglycerol-cysteine amino group (17, 18). The latter modification is probably not conserved in all eubacteria, as *B. subtilis* and *Mycoplasma genitalium* lack an int gene for the lipoprotein aminoaoyltransferase (14).

Lipoprotein processing by SPase II is essential for cell viability of *E. coli* and other Gram-negative eubacteria (19, 20). In contrast, the SPase II of *B. subtilis* is not essential for viability, although the activity of several lipoproteins seems to be strongly impaired in the absence of SPase II (14, 21). The latter applies, for example, to the PrsA protein (14), which is required for the folding of translocated secretory proteins (22). Consequently, the secretion of α-amylase, a nonlipoprotein, was strongly impaired in cells lacking SPase II (14).

In contrast to the eubacterial type I SPases, very little is known about the mechanism that type II SPases employ for catalysis (17). In the present studies, which were aimed at the identification of potential active site residues of type II SPases, we made use of the fact that SPase II is not essential for viability of *B. subtilis*. As a first approach, all residues of the SPase II of *B. subtilis* that are conserved in the 19 known eubacterial type II SPases were mutated. The results showed that two strictly conserved aspartic acid residues are essential for the activity, but not the stability, of this enzyme, indicating that type II SPases employ an aspartic acid catalytic dyad for signal peptide cleavage of lipid-modified proteins, similar to aspartic proteases of the pepsin family. A third conserved as-
partic acid residue appears to be required for the stability of the SPase II of *B. subtilis*.

**EXPERIMENTAL PROCEDURES**

Plasmids, Bacterial Strains, and Media—Table I lists the plasmids and bacterial strains used. Tryp*®*, yeast extract medium contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). S7 media 1 and 3, used for labeling of *B. subtilis* proteins with [35S]methionine (Amersham Pharmacia Biotech), were prepared as described in Refs. 23 and 24. When required, medium for *E. coli* was supplemented with kanamycin (20 μg/ml) or ampicillin (40 μg/ml); media for *B. subtilis* were supplemented with kanamycin (10 μg/ml) or Em (1 μg/ml).

**DNA Techniques—**Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described in Ref. 29. Enzymes were from Roche Molecular Biochemicals. *B. subtilis* was transformed as described in Ref. 8. PCR was carried out with pW0 DNA polymerase (Roche Molecular Biochemicals) as described in Ref. 10. The BLAST algorithm (30) was used by a two-step PCR approach (10), using primers *lsp-myc* (Table II) was performed, using chromosomal DNA as a template. Amplified fragments were subsequently cleaved with *Sal*I and *Eco*RI and ligated into the corresponding sites of pGDL150 (26). Consequently, the wild-type or mutant *lsp* genes on pGDL150–152 were transcribed from the constitutive promoter of the *Em* gene present on pGDL48. Site-directed mutations were introduced into plasmidborne copies of *lsp-myc* by a two-step PCR approach (10), using primers *Lsp*-3 and *Lsp*-6 in combination with mutagenic oligonucleotides (Table II). Amplified fragments were cleaved with *Sal*I and *Eco*RI and ligated into the corresponding sites of pGDL48. The resulting plasmids were named pL-x, where x indicates the position and type of amino acid substitution in the corresponding mutant proteins.

**Protein Labeling, Immunoprecipitation, SDS-PAGE, and Fluorography—**Pulse-chase labeling of *B. subtilis*, immunoprecipitation, SDS-PAGE, and fluorography were performed as described previously (23, 24).

**Western Blot Analysis—**Western blotting was performed as described in Ref. 30. After separation by SDS-PAGE, proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corporation). To detect PrsA or carboxyl-terminally Myc-tagged SPase II, *B. subtilis* cells were separated from the growth medium, and samples for SDS-PAGE were prepared as described previously (23). For the separation of samples with Myc-tagged SPase II, 1 ml dithiothreitol, and 1% Triton X-100 were added to the loading buffer for SDS-PAGE as described in Ref. 31. The PrsA protein was visualized with specific antibodies and horseradish peroxidase-anti-rabbit-IgG conjugates (Amersham Pharmacia Biotech); carboxyl-terminally Myc-tagged SPase II was visualized with monoclonal anti-c-Myc antibodies (Roche Molecular Biochemicals) and horseradish peroxidase-anti-mouse-IgG conjugates.

**RESULTS**

**Conserved Domains in Type II SPases—**Thus far, the nucleotide sequences of 19 *lsp* genes for SPase II are known, allowing the detailed comparison of the deduced amino acid sequences of the corresponding proteins (Fig. 2). As demonstrated for the SPase II of *E. coli* (50), all these type II SPases have four predicted transmembrane (TM) domains (denoted TM-A to -D) (Fig. 3). Two periplasmic (Gram-negative eubacteria) or cell wall-exposed (Gram-positive eubacteria) regions are localized between the TM-A and TM-B regions and between the TM-C and TM-D domains, respectively (Fig. 3). Furthermore, five highly conserved domains (I–V) were detected in these SPases (Figs. 2 and 3): Domain I, containing no strictly conserved residues, is located in TM-A; domain II, containing the strictly conserved residues Asn-45 and Gly-47, is located in the extracytoplasmic region between TM-A and -B; domain III, containing the strictly conserved residues Asn-99 and Asp-102, is located at the junction of TM-C and the extracytoplasmic region between TM-C and -D; domain IV, containing the strictly conserved residues Val-109 and Asp-111, is located in the extracytoplasmic region between TM-C and -D; and domain V, containing the strictly conserved residues Phe-125, Asn-126, Ala-128 and Asp-129, is located at the junction of TM-D and the extracytoplasmic region between TM-C and -D (Fig. 3).

**Carboxyl-terminally Myc-tagged SPase II Has Wild-type Activity—**Positively charged residues in the cytosolic amino- and carboxyl-terminal regions of type II SPases have been invoked in catalysis (3). Notably, the SPase II of *B. subtilis* lacks an amino-terminal cytosolic region with positively charged residues, refuting that such a region could be required for catalysis (12). Like other known type II SPases, the B.
subtilis SPase II does, however, contain positively charged carboxyl-terminal residues (four Lys residues). To determine whether the positively charged residues in the carboxyl terminus of the B. subtilis SPase II are important for catalysis, a mutant lsp gene encoding SPase II ΔC (lacking the six carboxyl-terminal residues KKKKEQ), was constructed by PCR and cloned into plasmid pGDL48 (Table III). To obtain a positive control for SPase II activity, the wild-type lsp gene was also amplified by PCR and cloned into pGDL48. The resulting plasmids, denoted pGDL150 (SPase II) and pGDL151 (SPase II ΔC), were used to transform B. subtilis 8G5 (parental strain) and 8G5-lsp (lacking the six carboxyl-terminal residues) for construction of B. subtilis 8G5-lsp, lacking the lsp sequences of the B. subtilis 8G5-lsp gene. After growth in the absence of antibiotics, cells were selected that had excised the integrated plasmid from the chromosome and lacked the 1111-base pair fragment (see under “Experimental Procedures”). The restriction sites relevant for the construction are shown (Bc, BclI; Bg, BglII; Nd, NdeI; ’ylyB, 5’ truncated ylyB gene.

**TABLE II**

| Primer  | Sequence | Mismatches with the original sequence of lsp are indicated in lowercase. |
|---------|----------|-----------------------------------------------------------------------|
| Lsp-3   | 5’-agctgtcagaCTGAACGAGGAGGACGCTTTATTG-3’ | |
| Lap-4   | 5’-ctgaattcCTGGTCTTCTGAGCGG-3’ | |
| L-dC    | 5’-atgattcctTTACCAGCGCGCGCGACCATTTGTTAATA-3’ | |
| Lsp-6   | 5’-tagattcttagttcaactctctctctctactgatcaattTTTGCTCCTTCTTCTTCTTCTTCTC-3’ | |
| L-D14A  | 5’-GTCTTTAATAGCTACGATGTCATTTGAGGAGGATATAG-3’ | |
| L-K18A  | 5’-GTTCTTTAATAGCTAGGATGTCATTTGAGGAGGATATAG-3’ | |
| L-N45A  | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-D5A   | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-W50A  | 5’-GACCCTCTAAGATCCCAGCTCGAGCCCGCTATTCGAGG-3’ | |
| L-Q5A   | 5’-GACCCTCTAAGATCCCAGCTCGAGCCCGCTATTCGAGG-3’ | |
| L-N99A  | 5’-GCCCAGCTATCGCGAGCCCGCTATTCGAGG-3’ | |
| L-D102A | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-R103A | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-V109A | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-L111A | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-P125A | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-N126A | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |
| L-A128V | 5’-GCCACATCGACGAGAGCTTATGAGGAGGATATAG-3’ | |

To monitor the activity of SPase II mutant proteins expressed by plasmids (pL-x) (Table I), processing of pre-PrsA to the mature form was studied in pulse labeling experiments with B. subtilis 8G5-lsp. As shown in Fig. 4A, processing of pre-PrsA was not affected in cells producing the SPase II mutant proteins G47A, F50A, G95A, and F125A, showing that these residues are not required for activity. Processing of pre-PrsA was mildly affected in cells producing the SPase II mutant proteins K18A, R103A, V109A, indicating that these residues are of minor importance for catalysis. Notably, pre-PrsA was not processed, or was processed very inefficiently, in cells producing...
the SPase II mutant proteins D14A, N45A, N99A, D102A, D111A, N126A, A128V, and D129A, indicating that these residues are either required for catalysis or protein stability.

To investigate which of the residues that are important for SPase II activity are determinants for the stability of the enzyme, Western blotting experiments were performed. As shown in Fig. 4B, only Asp-14 was essential for the stability of SPase II-Myc, whereas all other mutant proteins were detectable.

The Conserved Domains III and V Contain Critical Residues for SPase II Activity—To verify the (in-)activity of the SPase II mutants with strongly reduced activities, pulse-chase labeling experiments were performed with \( \text{B. subtilis} \, 8G5 \) lsp. In these experiments, cells were chased with an excess of nonradioactive methionine for 10 min, as it was previously shown that within this period of time no pre-PrsA is converted to the mature form in cells lacking SPase II (14). As shown in Fig. 5, no labeled pre-PrsA was converted to the mature form in cells producing the SPase II mutant proteins D14A, D102A, A128V, and D129A, suggesting that these proteins are inactive. In contrast, low amounts of labeled mature PrsA were detectable in cells producing the N99A and N126A mutant proteins, and significant levels of pre-PrsA processing were detected in cells producing the N45A and D111A mutant proteins.

To determine the effects of the mutations in conserved residues of SPase II at steady state, the accumulation of pre-PrsA was detected by Western blotting. It has to be noted that cells lacking SPase II display alternative processing of pre-PrsA to mature-like forms that, on SDS-PAGE, migrate at a slightly reduced rate compared with mature PrsA (14). As shown in Fig. 6, only the cells producing the SPase II mutant proteins D14A, N99A, D102A, N126A, A128V, and D129A accumulated precursor and mature-like forms of PrsA.

| Conserved Domains in type II SPases. The deduced amino acid sequences of 19 known type II SPases were compared. These include type II SPases of the Gram-positive eubacteria (G\(^+\)) \( \text{B. subtilis} \) (12), \( \text{Staphylococcus aureus} \) (Sau) (33), \( \text{Staphylococcus carnosus} \) (Sca) (34), \( \text{Lactococcus lactis} \) (Lla) (GenBank\(^{TM}\) accession no. U63724), and \( \text{Mycobacterium tuberculosis} \) (Mtu) (35); the mycoplasmas (M) \( \text{M. genitalium} \) (Mge) (36) and \( \text{Mycoplasma pneumoniae} \) (Mpn) (37); and the Gram-negative eubacteria (G\(^-\)) \( \text{E. coli} \) (Eco) (38, 39), \( \text{Enterobacter aerogenes} \) (Eae) (40), \( \text{Hemophilus influenzae} \) (Hin) (41), \( \text{Serratia marcescens} \) (Sma) (GenBank\(^{TM}\) accession no. AF027768), \( \text{Pseudomonas fluorescens} \) (Pfl) (42), \( \text{Aquifex aeolicus} \) (Aae) (43), \( \text{Synechocystis} \) sp. (Syn) (44), \( \text{Helicobacter pylori} \) (Hpy) (45), \( \text{Chlamydia trachomatis} \) (Ctr) (46), \( \text{Borrelia burgdorferi} \) (Bbu) (47), \( \text{Treponema pallidum} \) (Tpa) (48), and \( \text{Rickettsia prowazekii} \) (Rpr) (49). Five conserved domains (I–V) were identified. Numbers refer to the position of the first amino acid of each conserved domain in the respective type II SPases. Residues are printed in boldface when present in at least 10 of the 19 type II SPases. Consensus sequences of each conserved domain are indicated. Uppercase letters indicate residues that are strictly conserved in all 19 type II SPases. Residues that are present in at least 10 sequences are printed in lowercase letters. Conserved residues of the SPase II of \( \text{B. subtilis} \) (SPase II (Bsu)) that are replaced by alanine or valine are indicated below the consensus sequence. Residues important for activity (\( \ast \)) or stability (\( \bullet \)) of the latter enzyme are indicated.

| Conserved Domains |
|-------------------|
| Domain I (G\(^+\)) |
| Domain II (M) |
| Domain III (M) |
| Domain IV (G\(^-\)) |
| Domain V (G\(^-\)) |

| Protein | Carboxyl-terminal amino acid sequence |
|---------|--------------------------------------|
| SPase II | DSGKKKKEQ--- --------- |
| SPase II-Myc | DSGKKEKKEQLISEEDLN |

To determine the effects of the mutations in conserved residues of SPase II at steady state, the accumulation of pre-PrsA in \( \text{B. subtilis} \, 8G5 \) lsp was analyzed by Western blotting. It has to be noted that cells lacking SPase II display alternative processing of pre-PrsA to mature-like forms that, on SDS-PAGE, migrate at a slightly reduced rate compared with mature PrsA (14). As shown in Fig. 6, only the cells producing the SPase II mutant proteins D14A, N99A, D102A, N126A, A128V, and D129A accumulated precursor and mature-like forms of PrsA.
analyze pre-PrsA processing by mutant SPase II proteins, B. subtilis 8G5 lsp (mutant) SPase II proteins (SPase II-Myc) is indicated. Yeast extract medium. The position of carboxyl-terminally Myc-tagged or pL-x were withdrawn after overnight growth at 37 °C in tryptone/ (SPase II; negative control), pGDL152 (SPase II-Myc), or pL-x were labeled with [35S]methionine for 90 s prior to immunoprecipitation, SDS-PAGE, and fluorography. The conserved domains (I–V) in which the respective mutations are located and the positions of precursor and mature forms of PrsA are indicated. Variations in the amount of label in different lanes relate only to the incorporation of precursor and mature forms of PrsA are indicated. The conserved domains (I–V) in which the respective mutations are located and the positions of precursor and mature forms of PrsA are indicated. Variations in the amount of label in different lanes relate only to the incorporation of core and mature-like forms of PrsA in cells of the parental strain B. subtilis 8G5 (wild-type), B. subtilis 8G5 lsp (no SPase II; negative control), or B. subtilis 8G5 lsp harboring plasmids pGDL150 (SPase II; negative control), pGDL152 (SPase II-Myc), or pL-x, were withdrawn after overnight growth at 37 °C in tryptone/yeast extract medium. The position of carboxyl-terminally Myc-tagged (mutant) SPase II proteins (SPase II-Myc) is indicated.

Taken together, our findings show that residues Asn-99, Asp-102, Asn-126, Ala-128, and Asp-129 are critical for SPase II activity and that Asp-14 is critical for SPase II stability.

DISCUSSION

In the present paper, we document the mapping of six functionally important residues of SPase II of B. subtilis. These are Asp-14, Asn-99, Asp-102, Asn-126, Ala-128, and Asp-129. All of these residues are predicted to be localized close to the external surface of the cytoplasmic membrane. Only one residue, Asp-14, was required for the stability of the enzyme, showing that it is an important structural determinant. This view is supported by the fact that the replacement of the equivalent Asp residue in the SPase II of E. coli (Asp-23) by glycine merely resulted in temperature sensitivity of the enzyme (3). In addition, Asp-14 of the B. subtilis SPase II is not conserved in the type II SPases of M. genitalium and M. pneumoniae, which have been shown to contain active type II SPases (52, 53). In contrast, mutation of the other five residues required for activity of the B. subtilis SPase II did not significantly affect the stability of this enzyme, showing that these residues are directly or indirectly required for catalysis. Interestingly, unlike the SPase II of E. coli (38), the B. subtilis SPase II did not require positively charged residues at the carboxyl terminus for activity, which implies that these residues are structural, rather than catalytic, determinants for the E. coli SPase II.

The observation that SPase II lacks conserved serine residues and that the only conserved lysine residue is not required for activity rules out the possibility that type I and type II SPases make use of similar catalytic mechanisms. Furthermore, the lack of conserved cysteine and histidine residues and the previous finding that purified SPase II of E. coli was active in the absence of metal ions (32) demonstrate that SPase II does not employ the well defined catalytic mechanisms of thiol- or metalloproteases. Consequently, the present observation that two strictly conserved aspartic acid residues are essential for SPase II activity indicates that this enzyme belongs to the aspartic proteases. This hypothesis is supported by the obser-
vation that the SPase II of *E. coli* could be inhibited by pepstastin, a known inhibitor of aspartic proteases (32).

Aspartic proteases are a group of proteolytic enzymes of the pepsin family that share the same catalytic mechanism and usually function in acidic environments (54–56). The known aspartic proteases of eukaryotes are monomeric enzymes, which consist of two subdomains, both containing the conserved sequence Asp-Thr-Gly. All three residues contribute to the active site (57, 58). Furthermore, conserved hydrogen bonds between the catalytic Asp residues and conserved Ser or Thr residues that are located at the +3 position relative to the active site Asp residue of retroviral aspartic proteases. Consequently, the active site Asp residues of retroviral aspartic proteases lack the conserved hydrogen bonding, which explains why these enzymes have a much higher optimum pH than pepsin-like proteases (63). Notably, type II SPases lack the conserved Asp-Thr/Ser-Gly motif of previously described (eukaryotic and viral) aspartic proteases. Moreover, like in the viral aspartic proteases, conserved Ser or Thr residues are absent from the +3 position relative to the putative active site Asp residues. Instead, type II SPases contain strictly conserved Asn residues at the +3 position (Fig. 2), which are very important for activity. These observations imply that the type II SPases belong to a novel class of aspartic proteases. As no type II SPases or otherwise related proteins have been identified in archaea or eukaryotes, it seems that this novel class of aspartic proteases has evolved exclusively in eubacteria.

The present observations suggest that SPase II of *B. subtilis* employs Asp-102 and Asp-129 for catalysis. By analogy to the known catalytic mechanism of aspartic proteases, this implies that Asp-102 and Asp-129, and their equivalents in other type II SPases, form a catalytic dyad. It remains unclear whether the pK_a of these residues is reduced by hydrogen bonding, as described for eukaryotic aspartic proteases. If such hydrogen bonds do not exist in type II SPases, this would explain the high optimum pH (7.9) of the SPase II of *E. coli* (39). The absence of conserved Ser/Thr residues does not, however, exclude the possibility that the pK_a of active site aspartic acid residues is modulated by other residues. In fact, this could be one possible role of other residues (i.e., Asn-45, Asn-99, Asp-111, Asn-126, and Ala-128) required for the activity of SPase II of *B. subtilis*. Alternatively, the latter residues could be required for the geometry of the active site of type II SPases, or the specific recognition of the diacyl-glyceryl-modified cysteine residues in the lipobox of preproteins.

**FIG. 7.** Model for signal peptide cleavage by SPase II. At the start of the catalytic cycle of SPase II, only one of the two active site Asp residues is protonated, and the active site contains a lytic water molecule (A). Upon binding of the signal peptide (SP) of a lipid-modified preprotein, the carbonyl carbon of the scissile peptide bond is hydrated by the lytic water molecule. This is accompanied by the deprotonation of one active site Asp residue and the protonation of the other Asp residue (B). Next, one hydroxyl group of the tetrahedral reaction intermediate donates a proton to the charged Asp residue, and simultaneously, the peptidic nitrogen accepts a proton from the other Asp residue (C). The latter event results in cleavage of the scissile peptide bond and regeneration of the initial protonation state of the Asp residues. Finally, the signal peptide (SP) and the mature lipoprotein (mLP) are released and replaced by a new lytic water molecule (D). The cytoplasmic (in), and extracytoplasmic (out) sides of the membrane and the amino termini of the signal peptide and the mature lipoprotein are indicated.
Based on the catalytic mechanism of the asparagine protease of the human immunodeficiency virus 1 (64, 65), which is required for viral replication (66), we propose the following mechanism for type II SPases. At the start of catalysis, the active site contains a so-called “lytic” water molecule, and only one of the active site asparagine acid residues is protonated (Fig. 7A). Upon binding of a lipid-modified precursor, the carboxyl carbon of the scissile peptide bond is hydrated (Fig. 7B), resulting in a tetrahedral intermediate (Fig. 7C). During this event, a proton is transferred (via the lytic water molecule) from one active site asparagine acid residue to the other (Fig. 7, C and D). Next, one hydroxyl group of the tetrahedral intermediate donates a proton to the charged asparagine acid residue, and simultaneously, the nitrogen atom at the scissile peptide bond accepts a proton from the other catalytic asparagine acid residue. The latter event results in peptide bond cleavage (processing), regeneration of the catalytic site of SPase II, and release of the mature lipoprotein and the cleaved signal peptide from the enzyme (Fig. 7D).

This model is particularly attractive, because both essential asparagine acid residues are predicted to be located in close proximity to the extracytoplasmic surface of the membrane, similar to the active site serine residue of type I SPases (9, 10). This is the place where C-regions of exported precursors are likely to be cleaved.

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