THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 49, Issue of December 8, pp. 38813–38822, 2000
Printed in U.S.A.

The Role of the Membrane-spanning Domain of Type I Signal Peptidases in Substrate Cleavage Site Selection*

Received for publication, August 6, 2000, and in revised form, September 5, 2000
Published, JBC Papers in Press, September 11, 2000, DOI 10.1074/jbc.M007093200

Joseph L. Carlos‡§, Mark Paetzel¶‖, Greg Brubaker‡, Andrew Karla‡§, Christopher M. Ashwell, Mark O. Lively**‡‡, Guoqing Cao‡, Patrick Bullinger‡, and Ross E. Dalbey‡§§**

*From the ‡Department of Chemistry, Ohio State University, Columbus, Ohio 43210, the ¶Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and **Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157*

Type I signal peptidase (SPase I) catalyzes the cleavage of the amino-terminal signal sequences from preproteins destined for cell export. Preproteins contain a signal sequence with a positively charged n-region, a hydrophobic h-region, and a neutral but polar c-region. Despite having no distinct consensus sequence other than a commonly found c-region "Ala-*X*-Ala" motif preceding the cleavage site, signal sequences are recognized by SPase I with high fidelity. Remarkably, other potential Ala-*X*-Ala sites are not cleaved within the preprotein. One hypothesis is that the source of this fidelity is due to the anchoring of both the SPase I enzyme (by way of its transmembrane segment) and the preprotein substrate (by the h-region in the signal sequence) in the membrane. This limits the enzyme-substrate interactions such that cleavage occurs at only one site. In this work we have, for the first time, successfully isolated *Bacillus subtilis* type I signal peptidase (SipS) and a truncated version lacking the transmembrane domain (SipS-P2). With purified full-length as well as truncated constructs of both *B. subtilis* and *Escherichia coli* (Lep) SPase I, *in vitro* specificity studies indicate that the transmembrane domains of either enzyme are not important determinants of *in vitro* cleavage fidelity, since enzyme constructs lacking them reveal no alternate site processing of pro-OmpA nuclease A substrate. In addition, experiments with mutant pro-OmpA nuclease A substrate constructs indicate that the h-region of the signal peptide is also not critical for substrate specificity. In contrast, certain mutants in the c-region of the signal peptide result in alternate site cleavage by both Lep and SipS enzymes.

Proteins that are exported across the bacterial cell membrane generally contain amino-terminal signal peptides that are cleaved during translocation of the exported protein across the lipid bilayer. Cleavage of these signal peptides is accomplished by type I signal peptidases (SPase I).[1] Since these enzymes are essential for the viability of bacteria (1–4), they are currently of interest as possible targets for the design of novel antibiotics (2).

Type I signal peptidases belong to a class of serine proteases that appear to utilize a serine/lysine dyad mechanism rather than the serine/histidine/aspartic acid catalytic triad seen in the more classical serine proteases (5). The most thoroughly studied type I signal peptidase is the Gram-negative *Escherichia coli* signal peptidase (or leader peptidase (Lep)) (6). Unlike *E. coli*, which contains only one chromosomal copy of Lep, the Gram-positive *Bacillus subtilis* contains five chromosomal (SipS, SipT, SipU, SipV, and SipW) and two plasmid-encoded type I signal peptidases (SipP (pTA1015) and SipP (pTA1040) (7)). The *B. subtilis* SPases SipS, SipT, SipU, and SipV, which maintain the sequence similarity to the *E. coli* paralog, are much smaller in size, contain only one transmembrane anchor instead of two, and have a much smaller predicted β-sheet domain II than the *E. coli* enzyme (8). Five of the 30 conserved residues within signal peptidase of *B. subtilis* (SipS; 21 kDa, 184 amino acids) have been found to be critical for enzyme function (9, 10). Arg^{84} and Asp^{146} appear to be conformational determinants, while Ser^{43}, Lys^{83}, and Asp^{153} are required for activity. Ser^{43} is the putative nucleophile, while Lys^{83} is believed to act as the general base in the catalytic mechanism of SipS.

Except for residues involved in catalysis, there has been little work on the *in vitro* characteristics of purified SPases other than *E. coli* Lep. We report in this study the *in vitro* characterization of the Gram-positive *B. subtilis* type I SPase, SipS, and compare its substrate specificity with Gram-negative *E. coli* type I SPase, Lep. We find that some mutated variants of PONA are better substrates with SipS, whereas these same mutant preprotein substrates are poorer substrates for *E. coli* Lep. In addition, we find that transmembrane anchoring of the SPase I enzyme is not essential for the recognition of the correct "Ala-*X*-Ala" cleavage site in the PONA substrate. Using wild type and mutated variants of PONA, we find that truncated constructs of *E. coli* Lep or *B. subtilis* SipS are still able to maintain substrate specificity and cleave at the proper cleavage site. With respect to the signal sequence of the preprotein substrate PONA, our *in vitro* specificity studies also show that the h-region is not important for cleavage accuracy. In contrast to the effects of some mutations in the c-region of the signal peptide, no h-region changes resulted in altered cleavage site specificity for either the *E. coli* or *B. subtilis* enzymes.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Supported by National Institutes of Health (NIH) Grant GM 08512.
‖ Supported by a Procter & Gamble 1-year graduate fellowship and a Medical Research Council of Canada postdoctoral fellowship.
‡‡ Supported by NIH Grant GM32861.
§§ Supported by American Heart Association Grant-in-Aid 94011570, NIH Grant GM48805, and a gift from SmithKline Beecham Pharmaceutical. To whom all correspondence should be addressed. Tel.: 614-292-2384; Fax: 614-292-1532; E-mail: dalbey@chemistry.ohio-state.edu.

[1] The abbreviations used are: SPase I, type I signal peptidase; SipS, signal peptidases of *B. subtilis*; SipS-P2, the fragment of SipS lacking the transmembrane segment; Lep, *E. coli* leader peptidase (type I signal peptidase); WT, wild type; MBP, maltose binding protein; PONA, pro-OmpA nuclease A; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Cloning of the WT and P2 Domain of SipS—Mutagenic oligonucleotides were synthesized by the DNA core facility of the Comprehensive Cancer Center of Wake Forest University or were purchased from Integrated DNA Technologies. Isopropyl-β-D-thiogalactopyranoside, phenylmethanesulfonyl fluoride, ampicillin, and other chemical reagents were purchased from Fisher. Enzymes for recombinant DNA methods were purchased from Promega unless stated otherwise. The SipS gene from the *B. subtilis* genome was amplified by the polymerase chain reaction. The strain (8G5sipS; trpC2 try his nic ura met ada sipS) was obtained from the Ohio State University stock. Polymerase chain reaction was run for 30 cycles (94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min) in a PerkinElmer Life Sciences/Cetus thermal cycler with the sense primer (ACT GGA GGA GAT ATC ATC AAA TCA GAA AAT GTT) and an antisense primer (GGA AGC TGC TGG ATC CTA ATT TGT TTT GCG CAT for the SipS WT. The underlined sequences are the *Eco*RV and *Bam*HI restriction sites, respectively. The sense primer for the SipS-P2 construct was CTT GCT TTG GAT ATC CGC AAC TTT ATT TTT. The polymerase chain reaction fragments were cut with *Eco*RV and *Bam*HI and then ligated in frame into the *Xmn*I and *Bam*HI restriction sites of the pMAL-c2 expression vector (New England Biolabs) containing the inducible Ptac promoter. Ligations were transformed into *E. coli* host *TB1*, araΔ(lac proAB) rpsL (ϕ80 lacZΔM15) hsdR. DNA manipulations were performed as described by Maniatis *et al.* (11). The cloning procedures used *T4* DNA ligase and restriction enzymes from Life Technologies, Inc.

Expression and Purification of the MBP-SipS Fusions—E. coli (*TB1*) containing the pMAL-c2 vector with the SipS gene was grown in Luria-Bertani medium containing 100 mg/liter ampicillin. Each liter of medium was inoculated with 10 ml of the overnight cell culture. The cells were grown at 37 °C to a cell density A_{600} = 0.7, at which time isopropyl-β-D-thiogalactopyranoside was added to induce the expression of the fusion protein. The cells were allowed to grow for an additional 4 h and then harvested by centrifugation (5000 × *g* for 5 min, 4 °C). The cells were then resuspended in lysis buffer (10 mM Na_2HPO_4, 1.0 mM phenylmethanesulfonyl fluoride, 30 mM NaCl, 0.07% β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA, pH 7.0) and then lysed by passing them five times through a French pressure cell at 16,000 p.s.i. The lysate was centrifuged (27,000 × *g* for 30 min, 4 °C) and the supernatant was diluted 1:5 with 20 mM Tris-HCl, pH 7.4, and then applied to an amylose resin affinity column (New England Biolabs, 2.5 × 10 cm) at a flow rate of 1 ml/min. The column was then washed with two column volumes of 20 mM Tris-HCl, pH 7.4. The fusion protein was eluted with 20 mM Tris-HCl, pH 7.4, containing 10 mM maltose. Fractions were collected and analyzed for protein by the Bradford method (12). MBP-SipS-P2 fusion protein was further purified on a Bio-Gel HTP hydroxyapatite column (1.5 × 10 cm) using a gradient elution scheme of 10–500 mM potassium phosphate, pH 7.4. The fusion proteins were dialyzed extensively (2 × 9 liters) against 20 mM Tris-HCl, pH 7.4, and then stored frozen at −70 °C.

Cleavage of the MBP-SipS WT Fusion Protein—The MBP-SipS WT fusion protein construct contains a factor Xa cleavage site located immediately prior to the amino terminus of the SipS protein. Our preliminary studies indicated that factor Xa cleaves the MBP-SipS WT fusion very slowly (approximately only 50% cleavage in 2–3 days at room temperature). Since the factor Xa cleavage site ended with an arginine, we developed a limited trypsin (Sigma) digest procedure to cleave the MBP-SipS fusions using 0.1% weight of trypsin/weight of fusion for 1 h (13). After an optimal digestion time (~20–60 min), as determined individually from small scale experiments of each purification, the trypsin activity was inhibited using phenylmethanesulfonyl fluoride and trypsin inhibitor bound to agarose beads (Sigma).

Cleavage of the MBP-SipS-P2 Fusion Protein—The MBP-SipS-P2 fusion protein was cleaved using factor Xa. The fusion protein was incubated at room temperature for 18 h using 2.2 μg of factor Xa/mg of MBP-SipS-P2 fusion in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM $CaCl_2$. The factor Xa cleavage reaction was stopped by the addition of 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone dihydrochloride from Calbiochem.

Purification of SipS and SipS-P2 from MBP—The SipS (WT) enzyme was separated from the cleaved MBP by applying the protein to an Amersham Pharmacia Biotech FF Q Sepharose anion exchange column (1.5 × 5 cm), which was equilibrated with 20 mM Tris-HCl, pH 7.4. SipS was then eluted with a 100–700 mM NaCl gradient. Fractions containing pure SipS protein were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4, and then stored at −70 °C. The SipS-P2 protein was separated from the cleaved MBP by a Bio-Gel HTP hydroxyapatite column (1.5 × 10 cm) using a gradient of 10–500 mM potassium phosphate, pH 7.4.

Purification of WT Lep and Δ2–75 Enzymes—WT (Lep) and truncated (Δ2–75) *E. coli* type I signal peptidase enzymes were purified as described previously (14, 15).

Isoelectric Focusing of SipS—One μg of SipS WT was loaded onto an isoelectric focusing gel using the Amersham Pharmacia Biotech Phast Gel system, Phast gel 3–9 media, and Amersham Pharmacia Biotech broad range pI standards (0.2 μg of standard was applied to the gel). The gel was visualized by Phast Gel Blue R Coomassie staining.

Mass Spectral Analysis—Purified SipS, SipS-P2, and substrate PONA masses were confirmed by electrospray ionization mass spectrometry using a MicroMass VG Quattro II mass spectrometer and a Finnigan LC Q classic ion trap mass spectrometer.

Cloning, Mutagenesis, and Purification of the Pro-OmpA Nuclease A—The coding sequence for the pro-OmpA nuclease A substrate, a hybrid of the staphylococcal nuclease A fused to the signal peptide of outer membrane protein A (OmpA), was excised from pONF1 (16) by digestion with *Xba*I and *Sal*I and ligated into pET21a(+) (Novagen) that had been previously digested with *Xba*I and *Xho*I to produce the pETON2 *T7* expression vector. Mutagenesis of the pETON2 vector was performed using the "Mega-primer" method (17) and the QuikChange system (Stratagene). The complete open reading frame for each mutant was sequenced by fluorescent dye termination using a PerkinElmer Life Sciences/Applied Biosystems model 377 automated DNA sequencer. The wild type and mutant overexpressed pro-OmpA nuclease A proteins were purified as described by Chatterjee *et al.* (18). The His_6-tagged PONA mutants were also prepared as described by Chatterjee *et al.* except with the substitution of a nickel chelate affinity step for the final ion exchange chromatography step. In this final step, His_6 PONA extract was loaded onto a 1-ml bed volume Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen, Inc.) column and eluted with a stepwise 100–500 mM imidazole gradient.

Activity and Kinetic Analyses Using PONA—To determine the kinetic constants (k_{cat}, K_m) of SipS, we used the preprotein substrate pro-OmpA nuclease A. Substrate concentrations were determined by using an $E_{1\%}$ at 280 nm of 8.3 (18). The cleavage reaction (75 μl) was in 50 mM Tris, 10 mM $CaCl_2$, 1% Triton X-100, pH 8.0, with the substrate at five different concentrations (37.3, 24.9, 18.7, 12.4, and 6.2 μM). The reaction was initiated by the addition of SipS. The concentration of SipS as determined by the Pierce BCA protein assay kit was 0.33 μM. The reaction was carried out at 37 °C, and aliquots of the reaction were removed at various times so that less than 10% processing of the substrate was achieved. The reaction was stopped by the addition of 5 μl of 5× sample buffer, and the samples were frozen immediately in a dry ice/ethanol bath. The amount of pro-OmpA nuclease A that was processed by SipS was assayed by SDS-PAGE on a 17.2% gel, followed by staining with Coomassie Brilliant Blue. The precursor and mature proteins were quantified by scanning the gels on a Technology Resources, Inc. Line Tamer PCLT 300 scanning densitometer. Percentage processing was determined by dividing the area of the mature protein band by the sum of the mature and precursor band areas. The initial rates were determined by plotting the amount of product *versus* time. The V_{max}, k_{cat}, and K_m values were extracted from a $1/V_i$ *versus* 1/[S] plot where V_i is the initial velocity. We used the computer program Microcal Origins to plot the data and for linear regression analysis of the data. All values are from at least three different experiments. The comparisons of the initial rates of processing of the wild type and mutated variants of PONA by Lep or SipS enzymes were performed using a constant substrate concentration of 10 μM and a constant enzyme concentration of 10 nM (for the point mutants) or 0.33 μM (for the more extensive mutations) in 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0, 1% Triton X-100. For each 11-μl reaction volume, 11 μl of 2× dye was added after a 60-min incubation at 37 °C. Initial velocities were determined by densitometric quantitation (as above) of 10-μl aliquots taken at various time points from 84-μl reaction volumes incubated at 37 °C. All reaction samples and time points were run on 17% SDS-PAGE gels and visualized by Coomassie Blue staining.

Detergent Requirement Studies—SipS (WT) in 20 mM Tris-HCl, pH 7.4, at 19 μM (400 μg/ml) was diluted into 20 mM Tris-HCl, pH 7.4, buffer with or without 0.5% Triton X-100. The reaction was initiated by the addition of 1 μl of each dilution to 10 μl of 20 μM PONA with or without 0.5% Triton X-100. The reaction was incubated at 37 °C for 1 h and then stopped by the addition of 2 μl of 5× sample buffer. The reaction samples were then run on 17% SDS-polyacrylamide gel and visualized by Coomassie Blue staining.

Amino Acid Sequence Alignment—The amino acid sequences of Lep (Swiss-Prot number P00803) and SipS (Swiss-Prot P28628) were aligned using the program ClustalX (19). Secondary structure-based

gap penalties were used in the profile alignment of the two sequences. The secondary structure of the soluble fragment of Lep (8) (Protein Data Bank number 1B12) was calculated using the program PROMOTIF (20).

RESULTS

Active Full-length (SipS) and Truncated (SipS-P2) B. subtilis Type I Signal Peptidase Were Isolated—The amino acid sequence alignment of *B. subtilis* type I signal peptidase (SipS) as it compares to *E. coli* Lep is shown in Fig. 1*A*. Whereas the 323-amino acid Lep protein has two transmembrane segments (*TM*), the 184-amino acid SipS only contains one (Fig. 1*A*). SipS-P2 is a SipS deletion mutant construct missing the transmembrane segment (amino acid residues 2–28). SipS and SipS-P2 were expressed and purified as maltose-binding protein (MBP) fusions. The amino acid sequences of the fusion linker region for MBP-SipS and MBP-SipS-P2 are shown in Fig. 1*B*. Expressing full-length and truncated *B. subtilis* type I signal peptidase as MBP fusions allowed us to purify 1-mg quantities of the enzyme. We typically obtained approximately 10 mg of pure fusion protein/liter of cell culture. Trypsin was used to cleave the WT SipS from the isolated SipS-MBP fusion protein. On the other hand, because of its higher susceptibility to trypsin digestion, factor Xa was used to cleave SipS-P2 from the SipS-P2-MBP fusion protein. Fig. 2, *A* and *C*, show the progression of the purification schemes for SipS and SipS-P2, respectively. SipS-P2 was further purified using hydroxyapatite affinity chromatography (not shown). The addition of detergent to the extraction and purification buffers (1% Triton X-100) did not increase the yield of full-length SipS with the putative transmembrane domain (not shown). The purification data in Fig. 2 reflect purification protocols without the use of any detergents.

Electrospray ionization mass spectrometry analyses of the purified protein indicate that we actually have two populations of protein differing by 2 amino acid residues. In the SipS spectrum (Fig. 2*B*), two peaks are seen corresponding to the molecular weights that would be expected if trypsin cleaved at the Arg in the factor Xa cleavage site and at the Lys^2 residue (Fig. 1*B*). There is an amino-terminal Ile resulting from the construction of the *Eco*RV restriction site. For SipS-P2, however, the molecular weights indicated by electrospray ionization mass spectrometry (Fig. 2*D*) indicate that, in addition to cleavage at the factor Xa site Arg and at Arg^{30} at the N terminus (Fig. 1*B*), cleavage occurs after the sequence -NEMR- at the C terminus of the protein. This results in truncation of SipS-P2 by 3 amino acids at its C terminus. Factor Xa is known to recognize the sequences -IEGR-, -IDGR-, and -AEGR- (21), but cleavage at other similar (secondary) sites is not uncommon (22–24). Because only two populations of proteins are evident in Fig. 2*D* for SipS-P2, cleavage at the C-terminal -NEMR- sequence appears to be better recognized than the -IEGR- or -RIMK- sequences at the N terminus of the protein.

Both SipS and SipS-P2 MBP fusion proteins exhibited enzymatic activity before and after cleavage of the MBP moiety (data not shown). The engineered linker region (10 Asn residues) probably provides sufficient flexibility to allow the preprotein substrate access to the SipS active site (Fig. 1*B*). After cleavage of the MPB fusion, purified SipS and SipS-P2 enzymes were able to process purified PONA substrate *in vitro* as shown in Fig. 3, *A* and Fig. *B*, respectively. The results indicate that the SipS-P2 variant is approximately 100-fold less active than full-length SipS. The kinetic constants of SipS were measured and are presented alongside the Lep results in Table I. Purified SipS exhibits a k_{cat} of $0.034 \pm 0.006\ s^{-1}$ and a K_m of $44 \pm 10\ \mu M$. With this substrate, the *B. subtilis* type I signal peptidase (SipS) is approximately 1300-fold less active than the *E. coli* leader peptidase. Unfortunately, the much lower activity of the truncated SipS-P2 enzyme prevented us from determining its kinetic parameters.

Interestingly, the activity of full-length SipS, an enzyme with a transmembrane segment, was only slightly activated by detergent. In a serial dilution activity profile, we observed only a slight increase (5-fold) in activity with SipS in the presence of 0.5% Triton X-100 detergent (Fig. 3*C*). In contrast, we observed approximately a 100-fold stimulation with the *E. coli* Δ2–75 (15) using the same substrate. It also is shown in Fig. 3*D* that, even without detergent, full-length SipS exhibits detectable processing of PONA down to a final reaction enzyme concentration of 7 nM (250-fold dilution lane).

Processing of PONA by *E. coli* Lep and *B. subtilis* SipS at identical cleavage sites was verified by electrospray mass spectrometry, and the results are shown in Fig. 4. The calculated mass of the full-length PONA preprotein substrate is 18,839.7 Da, while the mass of the mature nuclease A fragment after processing at the predicted cleavage site (after the sequence -FATVAQA-, discussed below) is 16,811.2 Da. Fig. 4*A* indicates complete processing of PONA in a 57 μM PONA, 0.074 μM Lep 5-min reaction (TGC buffer, pH 8.0, 37 °C), resulting in only a mature nuclease A peak of M_r 16,814.0. Conversely, a 20-min reaction of 57 μM PONA, 0.127 μM SipS results in detection of both the parent PONA substrate (M_r 18,843.0) and processed nuclease A (M_r 16,813.0) as shown in Fig. 4*B*.

Disruption of the h-region of the Signal Peptide Substrate Does Not Perturb SipS Processing—A series of point mutations in the n-, h-, and c-region of the signal peptide of PONA were constructed to determine the effects on processing by *B. subtilis* SipS. The initial velocities of SipS processing of the wild type and point mutant PONA substrates are listed in Table II. SipS processed all of the point mutant substrates within the same order of magnitude relative to the wild type substrate. The highest SipS processing occurred with the V−12A and V−4P mutants. The sequence numbering system is relative to the cleavage site (*i.e.* the residues in the signal peptide are negative, and the numbers in the mature protein are positive). The K−20D and A+1R mutants were processed with roughly twice the initial velocity of the wild type PONA substrate. The mutants at the −8 position (G−8A and G−8R) had little effect on the initial rate of processing. The L−10N mutant was processed at a slightly slower rate than wild type. As expected, the A−1R and A−3R negative control mutants displayed no detectable processing by SipS in the time periods assayed (Table II). A rather surprising result was that a double Arg mutant (V−12R/G−8R) in the h-region of the signal peptide of PONA was processed by SipS (Table II).

To look closer at the role of the h-region and the c-/h-region boundary of the signal peptide with respect to *E. coli* or *B. subtilis* SPase I processing, other more extensive amino acid mutants in the signal peptide h- or c-region of the substrate PONA were constructed. The amino acid sequences in the signal peptide region for each of these mutant variants of purified PONA are listed in Table III. A comparison of processing by purified full-length or truncated *E. coli* SPase I (Lep, Δ2–75) or *B. subtilis* SipS (SipS, SipS-P2) enzymes against these purified PONA mutant proteins are illustrated in Fig. 5. The 6HisPONA construct indicates PONA amended with a hexahistidine tag in the N-terminal region of the signal peptide. This purified variant of PONA enabled us to use a more rapid scheme for the purification of the WT form as well as some of the mutated PONA substrates. There were no differences in k_{cat}, K_m, and specificity (compared with WT with no His_6 tag) resulting from this insertion at the N terminus of PONA (data not shown). Because of the different mutations,

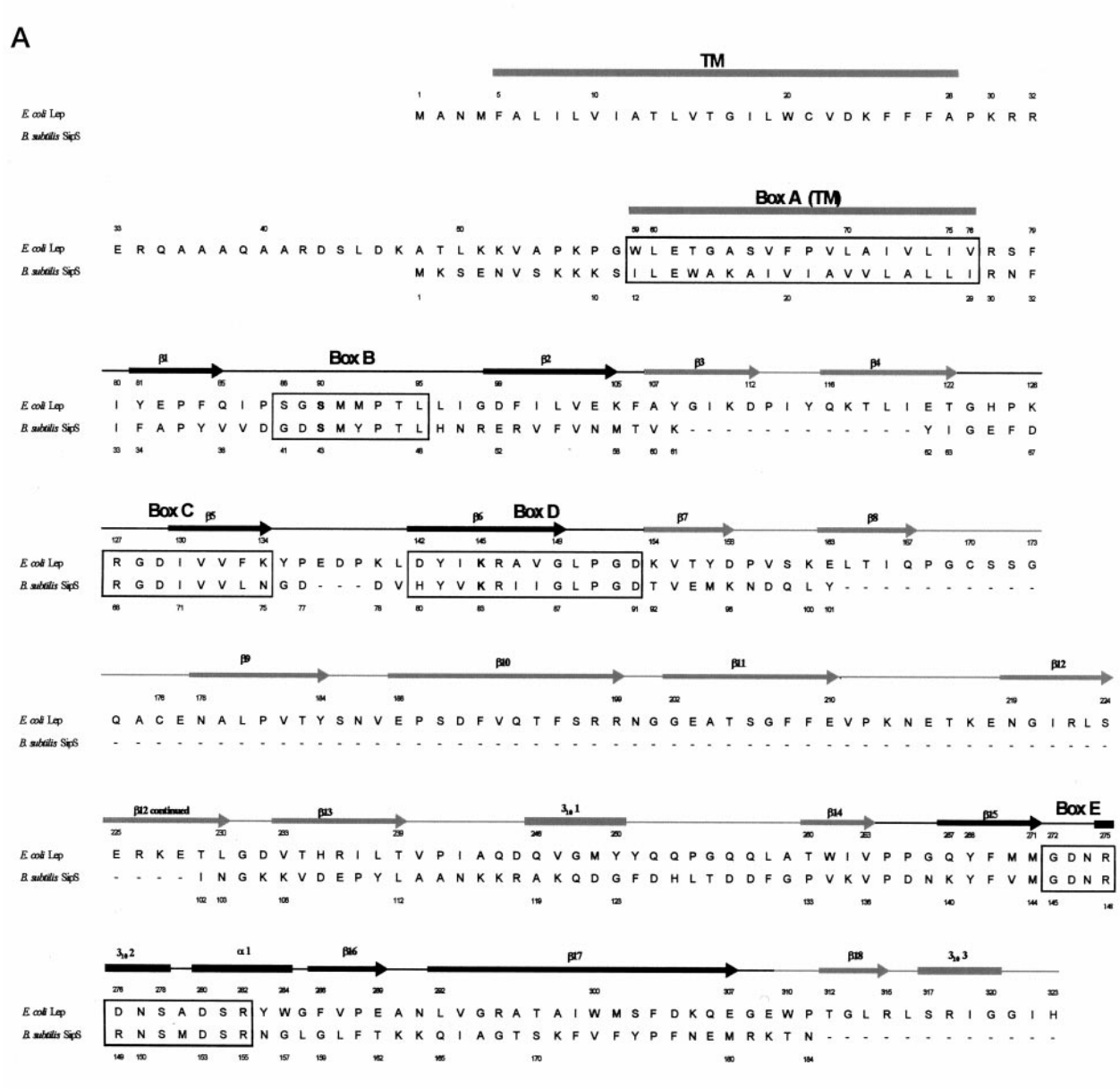

FIG. 1. **Primary amino acid sequences of *E. coli* (Lep) and *B. subtilis* (SipS) type I signal peptidases.** *A* this alignment is based on the structure of Lep (8). The secondary structure of Lep is shown *above* the *sequences*. Domain I, the catalytic domain, is indicated by *black secondary structure*, whereas domain II, the extended β-ribbon, and C terminus are indicated by *gray secondary structure*. Lep contains two transmembrane segments (*TM*), while SipS contains only one. *Boxes A–E* represent conserved regions in type I signal peptidases (6). *B*, amino acid sequence of the fusion linker region of the MBP-SipS and MBP-SipS-P2 constructs. The predicted molecular weights for MBP-SipS and MBP-SipS-P2 are approximately M_r 63,000 and 60,000, respectively. Shown in *boldface type* are amino acids for MBP and the linker region. The cleavage site for factor Xa is *underlined*.

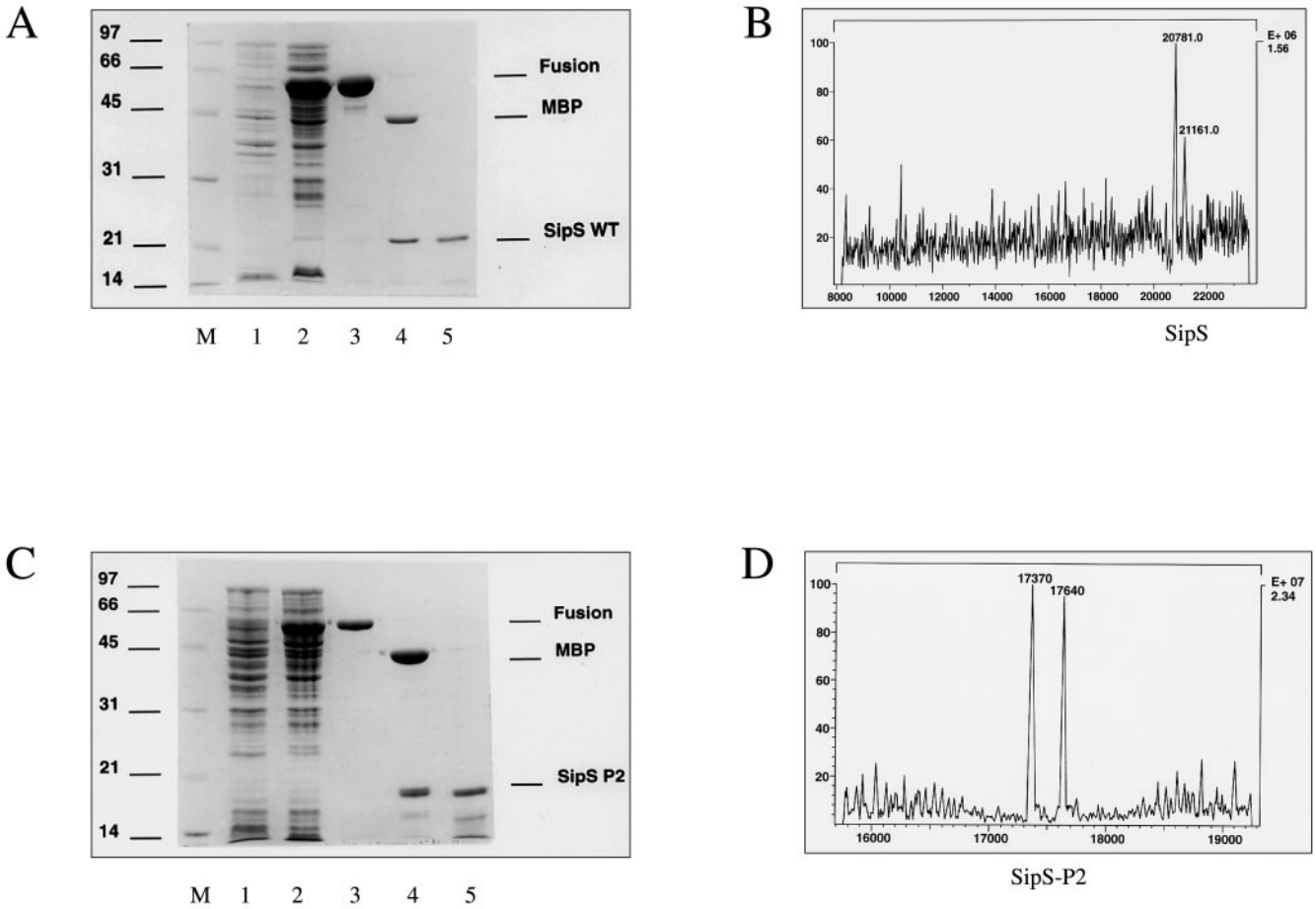

FIG. 2. **The purification of *Bacillus subtilis* type 1 signal peptidase (SipS).** A 12% SDS-polyacrylamide gel shows the purification progression of wild type SipS (*A*) and the P2 domain of SipS, SipS-P2 (*C*). Also shown are the results from the electrospray mass spectrometry analysis of purified SipS (*B*) and purified SipS-P2 (*D*). *Lane M*, molecular weight standards; *lane 1*, cell lysate before isopropyl-β-D-thiogalactopyranoside induction; *lane 2*, cell lysate after isopropyl-β-D-thiogalactopyranoside induction; *lane 3*, purified MBP-SipS (*A*) and MBP-SipS-P2 (*C*) fusion protein from the amylose affinity column; *lane 4*, MBP-SipS after trypsin cleavage (*A*) and after factor Xa cleavage of MBP-SipS-P2 (*C*); *lane 5*, purified SipS (*A*) and purified SipS-P2 after anion exchange column chromatography (*C*).

the masses of each of the starting unprocessed PONA substrates varied (see *No Enzyme* added control gel and *upper bands* in other gels), but, as shown in Fig. 5, an identical lower molecular weight band (mature nuclease A) is present in all cases where processing was detected. Cleavage of each of the purified mutated PONA substrate variants at the identical cleavage site as WTPONA (M_r ~16,800 for mature nuclease A) was also verified (as in Fig. 4) by mass spectrometry (not shown).

The Membrane-spanning Domains of B. subtilis or E. coli SPase I Are Not Required for Cleavage Site Specificity—One of the more significant results in the comparisons of *B. subtilis versus E. coli* SPase I processing of the extensive PONA signal sequence mutants is that obtained with the truncated enzymes. Although the processing by truncated enzyme constructs, Δ2–75 or SipS-P2, of wild type (or WT-His_6) PONA is slightly less efficient than with the full-length enzymes, it is shown in Fig. 5 (*lane 1*) that substrate specificity is maintained. Even without their respective transmembrane regions, these purified catalytic domains of the *E. coli* and *B. subtilis* SPase I enzymes are still able to recognize and process the substrate at the same cleavage site.

Gln Insertions at the h/c-region Boundary Are More Efficiently Processed by the B. subtilis Enzyme Constructs—The Q5 and Q10-PONA constructs represent 5 and 10 Gln residue insertions, respectively, to extend the c-region of the signal peptide at the h-region boundary. Gln was chosen because of its hydrophilic nature, occurrence in natural signal peptides (25), and low likelihood of alternate cleavage site introduction (26–28). The PONA signal peptide Gln amino acid insertion mutants are listed in Table III. The extension of the signal peptide c-region by 5 or 10 Gln residues resulted in the maintenance of enzyme specificity (as evidenced by a single processed band), but SipS or SipS-P2 better tolerated the changes than Lep or Δ2–75. As seen in Fig. 5, there is greater processing of Q5 or Q10PONA by either SipS or SipS-P2 (*lanes 3* and *4*). For illustrative and quantitative purposes, the initial velocities for Lep, Δ2–75, SipS, and SipS-P2 processing of the Q5PONA mutant were determined. As seen in Table IV, the initial velocity ratios indicate that the Q5PONA mutant is indeed processed more efficiently by the SipS or SipS-P2 enzymes. With an initial velocity ratio (Lep/SipS) of 0.23, the SipS initial velocity is a little over 4 times greater than the Lep initial velocity at the conditions tested. Similarly, the truncated SipS-P2 enzyme is more efficient at processing Q5PONA substrate than its *E. coli* counterpart, Δ2–75, with an initial velocity ratio of 0.39 (Δ2–75/SipS-P2).

An Extension of the h-region of the Signal Peptide Does Not Affect Cleavage Site Selection by SPase I—The disruption of the h-region did not affect processing by SipS (Table II). To further examine the role of the h-region, this domain was extended by 5 and 10 Leu residues (L5PONA and L10PONA, respectively) as listed in Table III. Leu was chosen because of its hydrophobic nature, occurrence in natural signal peptides (25), and low

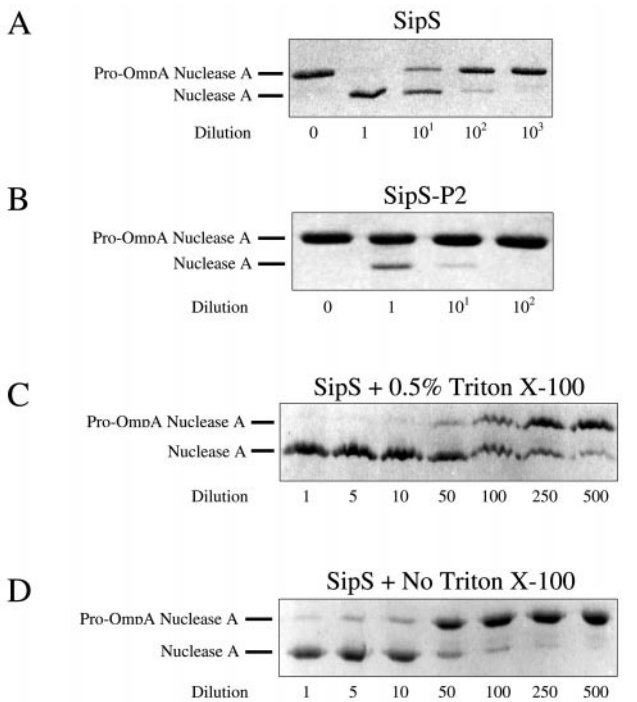

FIG. 3. **The activity and detergent requirement of purified SipS and SipS-P2 enzymes.** *A*, the processing of the pro-OmpA nuclease A substrate by serially diluted stock wild type SipS. *B*, the processing of the pro-OmpA nuclease A substrate by serially diluted stock SipS-P2. In *A* and *B*, the *0 dilution lane* indicates the no enzyme added control, and, for each reaction, 1 μl of serially diluted stock SipS or SipS-P2 enzyme, each at 18 μM (*1 dilution*), was added to 10 μl of 20 μM pro-OmpA nuclease A in 20 mM Tris-HCl, pH 7.4. *C*, serial dilution profile of SipS with 0.5% Triton X-100. *D*, serial dilution profile of SipS with no added detergent. SipS at 0.4 mg/ml was diluted into 20 mM Tris-HCl, pH 7.4, with (*C*) or without 0.5% Triton X-100 (*D*). Each reaction in *C* and *D* was initiated by the addition of 1 μl of each enzyme dilution to 10 μl of 20 μM pro-OmpA nuclease A substrate with or without 0.5% Triton X-100. After the addition of enzyme, each of the reaction samples (*A–D*) were incubated at 37 °C for 1 h and then stopped by the addition of 2 μl of 5× sample buffer and then run on 17% SDS-PAGE gels and visualized by Coomassie Blue staining.

TABLE I
Comparison of the kinetic constants of SipS with Lep using the preprotein substrate PONA

The activities were measured at pH 8.0 (see "Experimental Procedures").

	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$M^{-1}\ s^{-1}$
Lep	44 ± 9	19.2 ± 4.6	2.3×10^6
SipS	0.034 ± 0.006	44 ± 10	7.7×10^2

likelihood of alternate cleavage site introduction (26, 27). Using both the L5PONA and L10PONA substrate constructs, processing is less efficient compared with WTPONA (*lane 1*), but only one cleavage product (M_r ~16,800) is observed (Fig. 5, *lanes 5* and *6*), indicating maintenance of cleavage at the correct site. No detectable processing is observed, however, of the L10PONA substrate with the SipS-P2 truncated *B. subtilis* enzyme. No evidence of alternate site cleavage is apparent with either of these purified h-region mutants.

In contrast to h-region mutants that had no effect on cleavage site fidelity, a c-region mutant of the signal peptide of PONA results in alternate site processing. A random presequence, referred to as A13, was reported by van Dijl and colleagues (9) to enable efficient *in vivo* processing of A13-β-lactamase by both *E. coli* Lep and *B. subtilis* SipS. In our study, purified A14 and A15PONA proteins signify 6 and 10 amino sequence portions, respectively, of the reported A13 presequence inserted into the C-terminal region of the signal peptide of PONA substrate (see Table III). *In vitro*, as seen in Fig. 5 (*lane* 7), the A14PONA mutant substrate, with a six-amino acid insertion (DPLEST), gives similar results to the Q5PONA and Q10PONA mutants, whereby processing compared with WT-PONA is reduced but cleavage site specificity is maintained. Initial velocity calculations for the A14PONA substrate are shown in Table IV. The calculated initial velocity ratios of 0.72 (Lep/SipS) and 0.07 (Δ2–75/SipS-P2) confirmed that the A14PONA substrate is slightly preferred by *B. subtilis* SipS or SipS-P2. On the other hand, the 10-amino acid insertion construct (VGSGDPLEST), resulting in the mutant A15PONA, results in loss of cleavage site specificity and poor processing efficiency. *In vitro* processing of A15PONA by Lep, Δ2–75, SipS, or SipS-P2 results in two cleavage products as seen in Fig. 5, *lane 8*. As verified by mass spectrometry, the higher molecular weight product (M_r ~18,100) results from cleavage after -AIAIAVALA- in the signal sequence, while the lower molecular weight product (M_r ~16,800) results from cleavage at the "correct" site (after -DPLESTAQA-).

DISCUSSION

A soluble MBP-SipS fusion protein was purified in the total absence of detergent. The analogous *E. coli* enzyme, Lep, has two transmembrane regions and it has been found to aggregate and precipitate out of solution under identical conditions. Unlike Gram-negative *E. coli* Lep, the Gram-positive SipS protein maintained sufficient tolerance to mild trypsin digestion, enabling rapid cleavage of MBP from the purified fusion protein.

Purified *B. subtilis* SipS enzyme is able process PONA to completion (Fig. 3*A*, *1 dilution*) with a k_{cat} of 0.034 ± 0.006 s^{-1} and a K_m of 44 ± 10 μM (Table I). Although about 100-fold less active than full-length SipS, the truncated version, SipS-P2, can also process PONA substrate to completion at higher enzyme concentrations or at longer incubation periods than the 1-h end point shown in Fig. 3*B*. Purified PONA substrate thus provides a readily available *in vitro* assay of SipS or SipS-P2 activity. It is interesting that, at the conditions studied, the ratios of the initial velocities of full-length to truncated *E. coli* or *B. subtilis* enzymes were similar (Table IV). The Lep/Δ2–75 and SipS/SipS-P2 initial velocity ratios were 3.17 and 3.40, respectively, indicating similar losses in cleavage efficiency from the deletion of the transmembrane regions.

The activity of the purified *B. subtilis* enzyme, SipS, is only slightly stimulated by Triton X-100 detergent. This is in contrast to the *E. coli* Δ2–75 enzyme that is strongly stimulated by detergent (15). As shown in Fig. 1*A*, SipS is missing a second transmembrane domain and a good portion of a β-sheet domain found in Lep. This may result in a smaller hydrophobic patch surrounding the active site of SipS and lead to the lower detergent requirement observed.

In a scanning mutagenesis approach, point mutations in the signal peptide of PONA substrate were constructed to see their effect on processing by SipS enzyme. The rationale for the point mutants listed in Table II is as follows: A+1R, to see the effect of a positive charge in the first position of the mature protein; V−4P, to delineate the c- and h-region boundary, since prolines are typically found in this region (29, 30); G−8R, L−10N, and V−12R were intended to disrupt the h-region; K−20D, a negative charge here would neutralize the adjacent Lys, giving the n-region no net charge overall (a positively charged n-region is typical of signal peptides (31)); A−1R and A−3R mutants were intended as noncleavable controls following the previously reported results of −3 and −1 charged residues preventing processing (32–35).

As expected, changing the −1 or −3 residue of PONA to

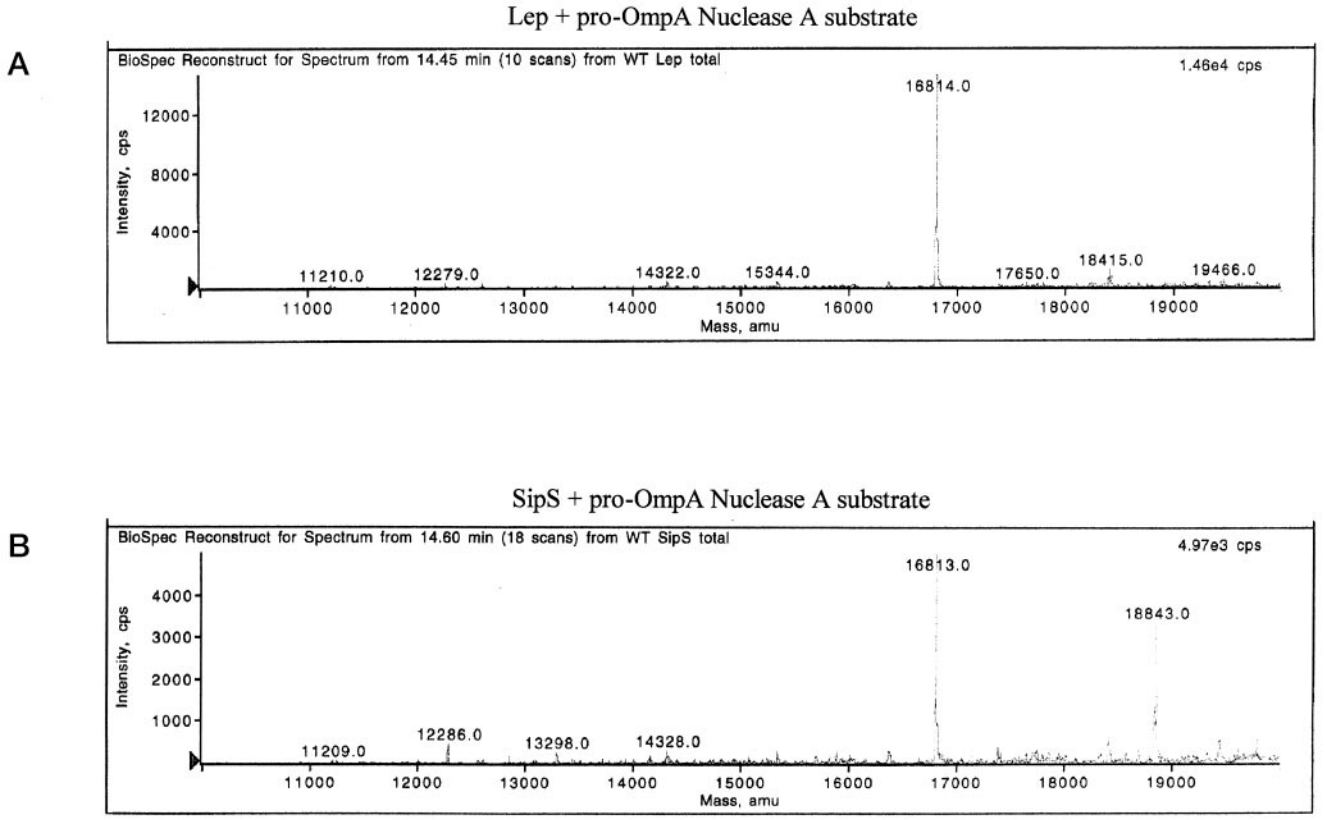

FIG. 4. **Purified Lep and SipS enzymes cleave pro-OmpA nuclease A substrate at the same cleavage site.** Electrospray mass spectrum of 0.074 μM Lep (*A*) or 0.127 μM SipS (*B*) with 57 μM pro-OmpA nuclease A substrate incubated for 90 min in 20 mM TGC, 1% Triton-100, pH 8.0, buffer.

TABLE II
The initial rates of processing of wild-type and point mutants of the preprotein substrate PONA by SipS

Substrate	Initial rate	Relative rate
	pmol/min	
WT	0.6	1.0
A + 1R	1.5	2.5
V − 4P	1.9	3.2
G − 8A	0.5	0.8
G − 8R	0.7	1.2
L − 10N	0.3	0.5
V − 12R	2.5	4.2
K − 20D	1.7	2.8
A − 1R	0.0	0.0
A − 3R	0.0	0.0
V − 12R/G − 8R	1.5	2.5

arginine prevented processing of the substrate. No processing was observed at nearby, potential alternative cleavage sites. This is in contrast to work on the precursor to the maltose-binding protein, where certain mutations at the −1 site resulted in processing at an alternative site (34). Except for G−8A and L−10N, all of these point mutants resulted in increased processing (as measured by initial velocity) *in vitro* by SipS enzyme. The mutations probably result in subtle changes to the signal peptide conformation of each individual mutant, thus affecting the interactions with SipS enzyme.

Several interesting results were obtained by studying the substrate specificity of signal peptidases using the purified full-length and truncated forms of *E. coli* Lep and *B. subtilis* SipS enzymes. First, we found that a simple tethering of the signal peptidase catalytic domain by the transmembrane segment is not a major factor involved in specificity at the −3, −1 cleavage site. This result was surprising because one compelling hypothesis in the field is that type I signal peptidase recognition of the "correct" substrate cleavage site occurs because the anchoring of the enzyme by way of its transmembrane domain limits its mobility. The −3, −1 signal peptidase cleavage site is typically an Ala-*X*-Ala motif (36, 37), and this limitation in mobility of the enzyme results in the recognition of only the correct Ala-*X*-Ala site and not other such sites in the signal peptide or mature region of the substrate. Only one such cleavage site is recognized on a substrate that is also membrane-tethered (by the h-region of the signal sequence). This hypothesis was tested by examining the specificity of truncated versions of *E. coli* Lep and *B. subtilis* SipS. Substrate cleavage at the correct site is maintained with the truncated enzymes, Δ2–75 and SipS-P2. In Fig. 5, *lane* 1, it is shown that Δ2–75 and SipS-P2 process WTPONA at the same cleavage site (after -GFATVAQA-; Table III) as full-length Lep and SipS. For both the full-length and truncated enzymes, only one (identical) mature processed band is observed. In all cases, the mature protein was determined to be M_r ~16,810 by mass spectrometry. The lack of other processed bands indicates no processing at alternative cleavage sites. Although less efficient than full-length enzyme, the truncated enzymes, Δ2–75 and SipS-P2, still cleave at the same position and do not recognize other potential cleavage sites in the presequence or the mature protein.

Second, a longer signal peptide h-domain attached to the substrate does not necessarily make it a better substrate. One reason to expect this is that the average Gram-positive signal peptide is 32.0, while the average Gram-negative signal peptide is 25.1 amino acids in length (38, 39). This greater average length in the Gram-positive species is due in part to the longer h-regions of the signal peptide. In our studies, however, the extension of the h-region in the presequence of PONA by 5 and 10 Leu residues did not improve its cleavability by the Gram-

TABLE III
Representative signal sequences of the pro-OmpA nuclease A substrate mutants used in this study

Construct	Signal sequence[a]
	−20 −12 −8 −3−1+1
WTPONA	MKKTAIAIAVALAGFATVAQA-ATSTKKLHKE
6HisPONA	M**HHHHHH**KKTAIAIAVALAGFATVAQA-ATSTKKLHKE
Q5PONA	MKKTAIAIAVALAGFA**QQQQQ**TVAQA-ATSTKKLHKE
Q10PONA	MKKTAIAIAVALAGFA**QQQQQQQQQQ**TVAQA-ATSTKKLHKE
L5PONA	MKKTAIAIAVALAGFA**LLLLL**TVAQA-ATSTKKLHKE
L10PONA	MKKTAIAIAVALAGFA**LLLLLLLLLL**TVAQA-ATSTKKLHKE
V-12R/G-8R	M**HHHHHH**KKTAIAIA**R**ALA**R**FATVAQA-ATSTKKLHKE
A14PONA	M**HHHHHH**KKTAIAIAVALAGFATV**DPLEST**AQA-ATSTKKLHKE
A15PONA	M**HHHHHH**KKTAIAIAVALAG**VGSGDPLEST**AQA-ATSTKKLHKE
A13[b]	MLKKVILAAFILVG**VGSGDPLEST**AQA-β-lactamase

[a] The h-region is shown underlined as a point of reference, the mutant amino acid insertions are shown in bold, and the cleavage site (scissile bond) is shown as a dash. The n-region precedes the h-region, and the sequence between the h-region and the scissile bond is the c-region.
[b] The A13 van Dijl presequence (9) is illustrated to show the amino acid sequence used for the A14PONA and A15PONA substitutions.

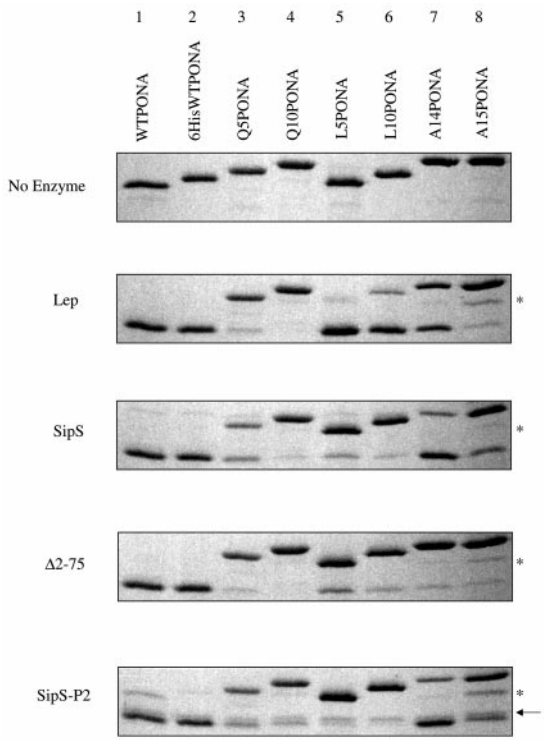

FIG. 5. **Processing of WT and mutant PONA by Lep, Δ2–75, SipS, and SipS-P2.** Shown are 17% SDS-PAGE analyses comparing cleavage of the indicated PONA substrate constructs by each of the purified enzymes. For each lane, 1 μl of enzyme was added to 10 μl of the indicated PONA substrate (10 μM substrate and 0.33 μM final enzyme concentration) and incubated for 1 h at 37 °C in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0, 1% Triton X-100. See "Experimental Procedures" for additional details. The *arrow* (SipS-P2 gel) indicates SipS-P2 enzyme (M_r ~17,400). The *asterisk* indicates alternate cleavage of A15PONA, resulting in the additional higher molecular weight band (M_r 18,080 as verified by mass spectrometry).

positive *B. subtilis* enzyme. The insertion of 5 Leu residues (L5PONA) resulted in overall slower processing by all of the enzyme constructs studied (Lep, Δ2–75, SipS and SipS-P2; Fig. 5, *lanes* 5 and 6). The insertion of 10 Leu residues (L10PONA) resulted in a much more severe decrease in processing than L5PONA for each of the enzymes used. In fact, no processing was detected at all for processing of L10PONA by SipS-P2 even after an extended overnight incubation (not shown) at the conditions used for Fig. 5 (1-h incubation). There were no observed changes in "enzyme preferences" for these two PONA h-region mutants. Like with WTPONA, there was greater processing by Lep and Δ2–75 of L5PONA or L10PONA substrate than by SipS (or SipS-P2) enzymes (Fig. 5). With respect to specificity, the signal peptide h-region insertion mutants did not exhibit any evidence of alternate site cleavage. Both L5PONA and L10PONA were processed at the same cleavage site as WTPONA substrate.

Third, disruption of the length of the h-domain in the signal peptide of PONA by Arg substitutions at either the −12 or −8 position (Tables II and III) did not affect processing. Even the introduction of both positive charges to obtain the double Arg mutant (Table III), resulted in a substrate displaying a measurable initial velocity with SipS (Table II). These results clearly show that the length of the hydrophobic domain can be shortened without a marked effect on signal peptide processing by the *B. subtilis* signal peptidase. This is surprising in light of recent results by Stein *et al.* (40), who showed that the insertion of 10 consecutive leucine residues into a short fluorogenic peptide substrate (41) resulted in a dramatic 10^4 increase in k_{cat}/K_m by *E. coli* Lep.

Fourth, although the c-regions of Gram-positive bacterial signal peptides are also typically longer than the c-region of Gram-negative bacterial signal peptides (38, 39), the lengthening of the c-region of PONA did not improve its efficiency as a substrate for Gram-positive SipS. In our study, the Q5PONA and Q10PONA constructs were created to extend the c-region of the signal sequence of PONA by 5 and 10 Gln residues, respectively (see Table III). No change in cleavage site specificity was observed, but processing trials of these substrates indicated that SipS or SipS-P2 more efficiently cleaves them than the *E. coli* enzymes. In Fig. 5, *lanes 3* and *4*, greater processing is observed for the *B. subtilis* enzyme(s) than the *E. coli* signal peptidase for Q5PONA and Q10PONA. Since the results for Q5PONA were more dramatic than with Q10PONA, the initial velocities were determined for the Q5PONA substrate mutant (see Table IV). For Q5PONA, the SipS initial velocity is over four times greater and the SipS-P2 velocity is about 2.5 times greater than the calculated initial velocities for Lep and Δ2–75, respectively. An extension of the c-region was made to mimic a portion of the van Dijl sequence reported to be processed by both Lep and SipS (9). This 6-amino acid insertion of the sequence DPLEST into the c-region of the presequence of PONA produced a mutant (A14PONA) with similar cleavage characteristics to the Gln_5 insertion mutant, Q5PONA. Like Q5PONA, the initial velocities of A14PONA processing are greater for the *B. subtilis* enzyme constructs than the *E. coli* enzyme constructs (Fig. 5, *lane* 7 and Table IV). A further extension of this A14PONA mutant by an additional 4 residues (to produce A15PONA, which includes more of the van Dijl A13 presequence) again resulted in a substrate that was better processed by SipS and SipS-P2, but cleavage at another cleavage site was now apparent with all of the enzymes (Fig. 5, *lane* 8, *asterisk*).

TABLE IV
Initial velocities (V_i) of purified E. coli (Lep, Δ2–75) and B. subtilis (SipS, SipS-P2) signal peptidases using WTPONA, Q5PONA, and A14PONA substrates

Substrate	Enzyme	V_i	Initial velocity ratio			
			Lep/Δ2–75	Lep/SipS	SipS/SipS-P2	Δ2–75/SipS-P2
		μmol/min				
WTPONA	Lep	24.4 ± 0.3	3.17	34.3	3.40	36.8
	Δ2–75	7.7 ± 0.2				
	SipS	0.71 ± 0.19				
	SipS-P2	0.21 ± 0.05				
Q5PONA	Lep	0.011 ± 0.006	1.06	0.23	1.84	0.39
	Δ2–75	0.010 ± 0.003				
	SipS	0.049 ± 0.003				
	SipS-P2	0.026 ± 0.002				
A14PONA	Lep	0.098 ± 0.024	10.5	0.72	1.01	0.07
	Δ2–75	0.009 ± 0.001				
	SipS	0.136 ± 0.034				
	SipS-P2	0.135 ± 0.029				

The substrate specificity results reported here support a hypothesis of enzyme-substrate interactions provided by the c-region of the signal peptide playing an important role in specificity. A simple Gln_5 insertion (Q5PONA) into the c-region appeared to be even better than a more deliberate 6-residue insertion (A14PONA) in yielding a substrate more preferred by *B. subtilis* SipS or SipS-P2 than by either *E. coli* signal peptidase construct. With the −3 and −1 residues providing the primary interactions with the enzyme, secondary and perhaps transient interactions during the course of catalysis are provided by the c-region of the signal peptide. While we did not have sufficient quantities of Q5PONA substrate to perform kinetic analyses on it, we were able to determine kinetic parameters for Lep and SipS processing of the A14PONA mutant. The k_{cat}, K_m, and k_{cat}/K_m values obtained are, respectively, $0.043 \pm 0.003\ s^{-1}$, $42.5 \pm 1.0\ \mu M$, and $1.0 \times 10^3\ M^{-1}\ s^{-1}$ for SipS and $0.014 \pm 0.003\ s^{-1}$, $16.6 \pm 3.1\ \mu M$, and $8.4 \times 10^2\ M^{-1}\ s^{-1}$ for Lep. For both SipS and Lep, the K_m values for A14PONA are similar to WTPONA (see Table I). Thus, assuming that $K_m \approx K_d$, the purified signal peptidases from *B. subtilis* and *E. coli* bind the mutant A14PONA substrate equally as WTPONA. The 6-amino acid sequence insertion into the c-region of the signal peptide of PONA resulting in A14PONA results only in a large decrease in k_{cat} for Lep (no change in K_m within error), while the SipS kinetic parameters are essentially unchanged. The Lep enzyme experiences a severe drop in catalytic efficiency by at least 3 orders of magnitude (compare Table I values). These results are consistent with enzyme-substrate interactions effected by the A14PONA mutations that are not present in the Michaelis complex. This suggests transient enzyme-substrate interactions, provided by the c-region of the signal peptide, that are not present in the ground state enzyme-substrate complex.

In summary, we have purified enzymatically active *B. subtilis* SPase I, which exhibits activity with the preprotein substrate, PONA. Mass spectrometry has directly shown that identical *in vitro* processing of PONA occurs by two signal peptidases from different organisms, Gram-positive *B. subtilis* (SipS) and Gram-negative *E. coli* (Lep). Disproving the hypothesis that the hydrophobic membrane segment(s) of the SPase I enzyme plays a direct role in determination of the substrate cleavage site, our experiments show that the Lep and Sips enzymes, lacking their respective transmembrane domains (Δ2–75 and SipS-P2, respectively), retain the same substrate specificity as the parent native enzymes. In addition, the h-region signal transmembrane anchor of the signal peptide substrate also is not a factor in cleavage site recognition. For either enzyme, the disruption or even lengthening of the h-region of the preprotein substrate only altered the enzyme-substrate interaction, as evidenced by changes in the rate of cleavage, but the correct bond was still cleaved. Lengthening the h- or c-region of the signal peptide of PONA substrate did not make this preprotein a better substrate despite the statistical evidence of signal peptides in Gram-positive eubacteria having longer h- and c-regions than Gram-negative organisms. Based on more experimental evidence, a good longer term objective is to eventually determine, more precisely, some of the determinants of specificity for both Gram-positive and Gram-negative bacteria. Other than the −1 and −3 residues, what are the specific enzyme-substrate interactions that occur during cleavage of the substrate? With the isolation of *B. subtilis* SipS in sufficient quantities to do physical characterizations, we are now in a position to attempt to answer questions such as these.

Acknowledgments—Special thanks goes to Don Ordaz at (Ohio State University Department of Microbiology Bio-fermentation facilities) and to Mark Schiller for help with the His_6 PONA construct.

REFERENCES

1. Date, T. (1983) *J. Bacteriol.* **154,** 76–83
2. Cregg, K. M., Wilding, I., and Black, M. T. (1996) *J. Bacteriol.* **178,** 5712–5718
3. Zhang, Y. B., Greenberg, B., and Lacks, S. A. (1997) *Gene (Amst.)* **194,** 249–255
4. Klug, G., Jager, A., Heck, C., and Rauhut, R. (1997) *Mol. Gen. Genet.* **253,** 666–673
5. Paetzel, M., and Dalbey, R. E. (1997) *Trends Biochem. Sci.* **22,** 28–31
6. Dalbey, R. E., Lively, M. O., Bron, S., and van Dijl, J. M. (1997) *Protein Sci.* **6,** 1129–1138
7. Tjalsma, H., Bolhuis, A., van Roosmalen, M. L., Wiegert, T., Schumann, W., Broekhuizen, C. P., Quax, W. J., Venema, G., Bron, S., and van Dijl, J. M. (1998) *Genes Dev.* **12,** 2318–2331
8. Paetzel, M., Dalbey, R. E., and Strynadka, N. C. (1998) *Nature* **396,** 186–190
9. van Dijl, J. M., de Jong, A., Vehmaanpera, J., Venema, G., and Bron, S. (1992) *EMBO J.* **11,** 2819–2828
10. van Dijl, J. M., de Jong, A., Venema, G., and Bron, S. (1995) *J. Biol. Chem.* **270,** 3611–3618
11. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
13. Patterson, W. R., and Poulos, T. L. (1994) *J. Biol. Chem.* **269,** 17020–17024
14. Klenotic, P. A., Carlos, J. L., Samuelson, J. C., Schuenemann, T. A., Tschantz, W. R., Paetzel, M., Strynadka, N. C., and Dalbey, R. E. (2000) *J. Biol. Chem.* **275,** 6490–6498
15. Tschantz, W. R., Paetzel, M., Cao, G., Suciu, D., Inouye, M., and Dalbey, R. E. (1995) *Biochemistry* **34,** 3935–3941
16. Takahara, M., Hibler, D. W., Barr, P. J., Gerlt, J. A., and Inouye, M. (1985) *J. Biol. Chem.* **260,** 2670–2674
17. Sarkar, G., and Sommer, S. S. (1990) *BioTechniques* **8,** 404–407
18. Chatterjee, S., Suciu, D., Dalbey, R. E., Kahn, P. C., and Inouye, M. (1995) *J. Mol. Biol.* **245,** 311–314
19. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25,** 4876–4882
20. Hutchinson, E. G., and Thornton, J. M. (1996) *Protein Sci.* **5,** 212–220
21. Nagai, K., and Thogersen, H. C. (1984) *Nature* **309,** 810–812
22. Nagai, K., Perutz, M. F., and Poyart, C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7252–7255
23. Eaton, D., Rodriguez, H., and Vehar, G. A. (1986) *Biochemistry* **25,** 505–512
24. Wearne, S. J. (1990) *FEBS Lett.* **263,** 23–26
25. von Heijne, G. (1986) *J. Mol. Biol.* **192,** 287–290
26. Laforet, G. A., and Kendall, D. A. (1991) *J. Biol. Chem.* **266,** 1326–1334
27. Jain, R. G., Rusch, S. L., and Kendall, D. A. (1994) *J. Biol. Chem.* **269,**

16305–16310
28. Nilsson, I., and von Heijne, G. (1991) *J. Biol. Chem.* **266,** 3408–3410
29. Perlman, D., and Halvorson, H. O. (1983) *J. Mol. Biol.* **167,** 391–409
30. Rosenblatt, M., Beaudette, N. V., and Fasman, G. D. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 3983–3987
31. von Heijne, G., and Abrahmsen, L. (1989) *FEBS Lett.* **244,** 439–446
32. Kuhn, A., and Wickner, W. (1985) *J. Biol. Chem.* **260,** 15914–15918
33. Karamyshev, A. L., Karamysheva, Z. N., Kajava, A. V., Ksenzenko, V. N., and Nesmeyanova, M. A. (1998) *J. Mol. Biol.* **277,** 859–870
34. Fikes, J. D., Barkocy-Gallagher, G. A., Klapper, D. G., and Bassford, P. J., Jr. (1990) *J. Biol. Chem.* **265,** 3417–3423
35. Shen, L. M., Lee, J. I., Cheng, S. Y., Jutte, H., Kuhn, A., and Dalbey, R. E. (1991) *Biochemistry* **30,** 11775–11781
36. von Heijne, G. (1983) *Eur. J. Biochem.* **133,** 17–21
37. von Heijne, G. (1985) *J. Mol. Biol.* **184,** 99–105
38. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Int. J. Neural. Syst.* **8,** 581–599
39. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng.* **10,** 1–6
40. Stein, R. L., Barbosa, M. D. F. S., and Bruckner, R. (2000) *Biochemistry* **39,** 7973–7983
41. Zhong, W., and Benkovic, S. J. (1998) *Anal. Biochem.* **255,** 66–73