J. Biochem. **143,** 237–242 (2008)
doi:10.1093/jb/mvm212

The Effects of Mutations in the Carboxyl-Terminal Region on the Catalytic Activity of *Escherichia coli* Signal Peptidase I

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Received October 20, 2007; accepted October 30, 2007; published online November 21, 2007

***Escherichia coli* signal peptidase I (SPase I) is a membrane-bound serine endopeptidase that catalyses the cleavage of signal peptides from the pre-forms of membrane or secretory proteins. Our previous studies using chemical modification and site-directed mutagenesis suggested that Trp^{300} and Arg^{77}, Arg^{222}, Arg^{315} and Arg^{318} are important for the proper and stable conformation of the active site of SPase I. Interestingly, many of these residues reside in the C-terminal region of the enzyme. As a continuation of these studies, we investigated in the present study the effects of mutations in the C-terminal region including amino acid residues at positions from 319 to 323 by deletions and site-directed mutagenesis. As a result, the deletion of the C-terminal His^{323} was shown to scarcely affect the enzyme activity of SPase I, whereas the deletion of Gly^{321}-His^{323} or Ile^{319}-His^{323} as well as the point mutation of Ile^{322} to alanine was shown to decrease significantly both the activity *in vitro* and *in vivo* without a big gross conformational change in the enzyme. These results suggest a significant contribution of Ile^{322} to the construction and maintenance of the proper and critical local conformation backing up the active site of SPase I.**

Key words: carboxyl-terminal residues, deletion, *Escherichia coli*, signal peptidase I, site-directed mutagenesis.

Most membrane and secretory proteins in *Escherichia coli* are synthesized *in vivo* as precursors that bear an NH_2-terminal signal (leader) peptide of 15–30 amino acid residues. This signal sequence is involved in guiding the protein into the targeting and translocating pathway by interacting with the membrane and other components of the cellular secretory machinery. The signal peptides are removed by the action of signal peptidase I (leader peptidase, SPase I) during or shortly after the protein export (*1–3*). Following the discovery of *E. coli* SPase I (*4*), the enzyme was cloned (*5*), sequenced (*2*), overexpressed (*6*, *7*), purified (*2*, *7*, *8*, *9*) and enzymatically characterized (*7*, *10*) from a wild-type strain of *E. coli*. The substrate specificity has also been investigated, indicating that small, uncharged amino acids are usually present in the substrates at the –1 and –3 positions from the site of cleavage by SPase I (*11*). This enzyme is an integral membrane endopeptidase that is typically anchored to the membrane by amino-terminal transmembrane segments (*12*, *13*). The active site and the substrate recognition site of the enzyme are in the carboxyl-terminal domain that resides on the outer surface of the cytoplasmic membrane (*2*, *14*). Further, a soluble catalytically active SPase I (Δ2–75 SPase I), which lacks the two amino-terminal transmembrane segments (residues 1–22 and 62–75) and the cytoplasmic domain (residues 23–61), was produced (*15*) and crystallized (*16*, *17*). By using site-directed mutangenesis and chemical modification methods (*10*, *18–20*), the catalytic activity of SPase I was shown to depend on the operation of a serine-lysine catalytic dyad, whereby Ser^{90} serves as the nucleophile and Lys^{145} serves as the general base in the catalytic mechanism. The Ser–Lys catalytic dyad structure of SPase I has been recently confirmed by elucidation of the three-dimensional structure of the enzyme in complex with a β-lactam inhibitor (*3*, *16*). Results of the enzymatic characterization, site-directed mutagenesis, chemical modification and three-dimensional structure study indicated that *E. coli* SPase I belongs to a novel class of serine protenases (*7*, *18–23*) that utilize a Ser–Lys catalytic dyad mechanism as opposed to the more common Ser–His–Asp catalytic triad mechanism in the cleavage reaction (*3*).

In our previous studies, we investigated the roles of the tryptophan and arginine residues in the activity of SPase I using chemical modification and site-directed mutagenesis (*7*, *22*, *24*). The results indicated that Trp^{300} and some (Arg^{77}, Arg^{222}, Arg^{315} and Arg^{318}) of the arginine residues are important for the activity. However, these residues are located fairly apart from the active site and are not directly involved in the catalytic machinery (*17*), although the P7 residue of a substrate was reported to come in close proximity to the Trp^{300} by a modelling study (*17*). They were thus assumed to be important to maintain the active conformation of the enzyme. Interestingly, many of these residues reside in the C-terminal region of the enzyme.

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As a continuation of these studies, we investigated in the present study the effects of mutations in the C-terminal region including amino acid residues at positions from 319 to 323, which sits behind the active site region (*17*) by deletions and site-directed mutagenesis. The results thus obtained strongly suggest that Ile^{322} is also important for maintaining the proper and critical local conformation of the structure backing up the active site of SPase I.

MATERIALS AND METHODS

Bacterial Strains, Expression Plasmids, and Other Materials—Escherichia coli strain MV1190 was used as a host for the expression vector pT7-7 as well as for the overproduction of the wild-type and mutant proteins. The pT7-7 plasmid that has the T7 promoter and the M13 phage mGP1-2 plasmid carrying the T7 polymerase gene were obtained by Dr S. Tabor (*25*). The plasmid pT7-7/*lep* was used for overproduction of SPase I in *E. coli* MV1190 as described previously (*7*). *Escherichia coli* strain IT41 encoding a chromosomal temperature-sensitive SPase I was a gift from Dr Y. Nakamura (*26*). Restriction endonucleases, DNA amplification reagents and Tag DNA polymerase were purchased from Toyobo (Tokyo, Japan) and the T4 DNA ligation kit was obtained from Takara (Kyoto, Japan). The reagents for DNA sequencing were from GE Healthcare (Foster city, CA, USA). All other reagents used were of analytical grade and obtained from Wako Pure Chem. Industries (Osaka, Japan).

Mutagenesis and Enzyme Purification—DNA manipulations were carried out as described by Sambrook *et al*. (*27*). Mutagenesis was performed according to the procedure of Kunkel (*28*). Mutations were confirmed by DNA sequencing. Each of the mutated genes was inserted back into the corresponding protein gene of the pT7-7/lep expression vector (*7*). The mutant enzymes were expressed and purified as described previously (*7*, *22*).

In Vitro and In Vivo SPase I Activity Assay—To determine the enzymatic activity of the mutant enzymes *in vitro*, we used the chemically synthesized peptide substrate (FSASALAKI) corresponding to part of the maltose-binding protein precursor containing the cleavage site (A/K) by SPase I. In the routine assay, a 10 μl aliquot of each enzyme was added to 40μl of substrate at a final concentration of 0.4 mM in 25 mM sodium phosphate buffer, pH 7.7. The reaction was allowed to proceed at 37°C for 30 min, and stopped by the addition of 50 μl of 0.1% trifluoroacetic acid, and the reaction mixture was subsequently analysed by HPLC using a C_{18} column as described previously (*7*). The activity of SPase I *in vivo* was determined using the temperature-sensitive *E. coli* SPase I strain IT41 (*26*). Measurement of SPase I activity *in vivo* was performed as described previously (*22*).

Kinetic Study—Kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) for the synthetic substrate (FSASALA/KI) were determined as described previously (*22*). The reaction was initiated by the addition of each mutant enzyme to a substrate solution containing 25 mM sodium phosphate buffer, pH 7.7. The reactions were carried out at 37°C at six or more different initial substrate concentrations (0.04–0.8 mM). The enzyme concentration used in kinetic experiments was 1.69 μM.

Determination of Thermostability—To test the thermostability, the wild-type and mutant enzymes in 10 mM potassium phosphate buffer, pH 7.0, containing 0.1% Lubrol PX, 10% glycerol and 35 mM NaCl were incubated at the indicated temperatures for 1 h. Remaining activities were measured by mixing 10 μl of each heat-treated enzyme with 40 μl of the synthetic substrate solution. The reaction was allowed to proceed at 37°C for 30 min and was stopped by the addition of 50 μl of 0.1% trifluoroacetic acid. Then the reaction mixture was analysed by HPLC as described previously (*7*).

Circular Dichroism Spectroscopy—The circular dichroism (CD) spectra of the wild-type enzyme and its mutants were recorded at a protein concentration of 2.0 μM in 25 mM sodium phosphate buffer, pH 7.7, containing 0.1% Lubrol PX and 10% glycerol with a Jasco J-720 spectropolarimeter at room temperature using the water-jacketed quartz cell with a light path of 1 mm. The sample solutions were prepared by appropriately diluting the solutions of the enzyme with 25 mM sodium phosphate buffer, pH 7.7, just before measurement. For all measurements, 1.0 nm bandwidth and 1.0 s time constant were used, and 32 scans were repeated from 200 to 250 nm at the speed of 50 nm/min with 0.1 nm/point resolution. The protein concentrations of the mutants used for CD spectroscopy were determined by amino acid analysis after acid hydrolysis.

Alignment of the Amino Acid Sequences of SPases I—The amino acid sequences of SPases I from various bacteria were aligned using the program ClustalW (*29*).

RESULTS AND DISCUSSION

To investigate the roles of the C-terminal region in *E. coli* SPase I, we constructed three C-terminally truncated mutants and two kinds of site-directed mutants: SPC1 (Δ323), SPC3 (Δ321–323), SPC5 (Δ319–323), I322A and *I319A*. All mutants were expressed in *E. coli* MV1190 successfully and were purified to homogeneity. Figure 1 shows that the protein expression efficiency of the mutants in *E. coli* MV1190 was similar to that of the wild-type enzyme. All the expressed mutants were purified by successive column chromatographic procedures using DEAE-cellulose, Mono P and Sephadex G-75 as described previously (*7*). All the purified mutant enzymes were homogeneous, giving a single band on SDS–PAGE at the position corresponding to an apparent molecular mass of 37 kDa, identical with that of the wild-type enzyme (Fig. 1B). In each case, the mutant enzyme was produced in the amount of 0.2–1.5 mg per 1 l of the LB broth (Table 1).

The enzymatic activities of the mutant enzymes were determined using a synthetic substrate (FSASALA/KI) (Table 1). The mutant enzyme SPC1 (Δ323) retained full catalytic activity. In contrast, SPC3 (Δ321–323) and SPC5 (Δ319–323) exhibited activities that were only about 12% and 4%, respectively, of that of the wild-type enzyme. Furthermore, the replacement of Ile^{322} or Ile^{319} with alanine also significantly decreased the specific activities. Especially, the mutation of Ile^{322} to alanine resulted in

84% loss of activity. We also measured the activity of all mutant enzymes using the *in vivo* assay (Table 1). In this assay, the three mutants, SPC3, SPC5 and I322A, were shown to have practically no activity, whereas I319A retained the activity. These results indicate that Ile^{322} is somehow important for the enzyme activity but that Ile^{319} is not so important as Ile^{322}.

The kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) of the wild-type and all mutant enzymes were determined by the *in vitro* assay using the synthetic substrate described above (Table 2). The k_{cat} and k_{cat}/K_m values decreased roughly in parallel with the relative activities by mutation, but it is notable that the K_m values of SPC3 and I322A were elevated by 1.6-fold and 2.3-fold, respectively, as compared with that of the wild-type enzyme.

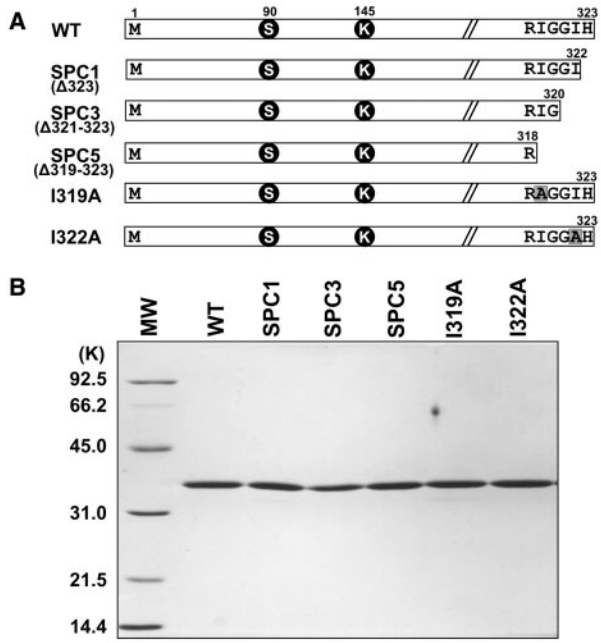

Fig. 1. **Schematic diagram and SDS–PAGE of the SPase I mutants.** (A) A schematic view of the deletion and point mutations in *E. coli* SPase I. Two active sites (S^{90} and K^{145}) are also shown. (B) SDS–PAGE of the mutant enzymes performed on a 12.5% polyacrylamide gel under reducing conditions followed by the Coomassie brilliant blue staining. MW, molecular weight markers.

These results suggest that the deletion or mutation to Ala of Ile^{322} significantly reduces not only the catalytic efficiency but also the substrate-binding affinity of the enzyme. As compared with the wild-type enzyme, I322A showed a lower k_{cat} value and a higher K_m value, whereas SPC5 had a lower k_{cat} value, but a similar K_m value. The reason for this difference is not clear at present; the deletion of the C-terminal five-residue peptide (IGGIH) might somehow favour the substrate binding as compared with SPC3 and I322A. It is tempting to assume that the newly formed C-terminal carboxyl group of Arg^{318} might effectively interact with some other residue in the enzyme.

Far-UV CD spectrum was measured to compare the secondary structures of the SPase I mutants with the wild-type enzyme. As shown in Fig. 2, the CD spectra of the mutant enzymes were roughly similar to that of the wild-type enzyme. These results indicated that no gross misfolding or changes in the secondary structure of the enzymes occurred through the mutations. Figure 3 shows the thermostability profiles of the mutant enzymes. After the enzymes were incubated in 25 mM sodium phosphate buffer, pH 7.7, at various temperatures for 60 min, the remaining activities were assayed. The thermostability was significantly decreased for all the mutants except SPC3. Especially notable is the decrease in thermostability of I322A; the T_m value of I322A was about 5°C lower than that of the wild-type enzyme. This suggests that Ile^{322} is important for the conformational stability of the enzyme. SPC3 was apparently more thermostable than the wild-type enzyme; the reason for this result is not certain at present.

Table 2. **Kinetic parameters for SPase I mutants.**

Enzyme	k_{cat} (h^{-1})	K_m (mM)	k_{cat}/K_m (h^{-1} mM^{-1})
Wild-type	86.4	0.52	166.2
SPC1	56.6	0.56	101.1
SPC3	20.0	0.83	24.1
SPC5	5.3	0.65	8.2
I322A	26.2	1.20	21.9
I319A	47.1	0.60	78.4

The initial rates were determined at 37°C in 25 mM sodium phosphate, pH 7.7. The concentration of the synthetic peptide (FSASALA/KI) was varied in the range of 0.04–0.8 mM. The kinetic data were analysed by double-reciprocal plots.

Table 1. **Enzymatic activities of SPase I mutants.**

Enzyme	Mutation site	Relative enzyme amount produced	*In vitro* assay		*In vivo* assay
			Specific activity (units/mg)	Relative activity (%)	Cell viability
Wild-type		1.00	12,800 ± 130	100 ± 1	Yes
SPC1	Δ323	0.95	11,263 ± 450	87 ± 4	Yes
SPC3	Δ321–323	0.15	1,574 ± 330	12 ± 3	No
SPC5	Δ319–323	0.25	550 ± 640	4 ± 5	No
I322A	Ile^{322}→Ala	0.97	2,048 ± 150	16 ± 1	No
I319A	Ile^{319}→Ala	0.90	6,244 ± 900	49 ± 7	Yes

The activities were determined by measuring the initial rates of the substrate hydrolysis. One unit is defined as the activity hydrolysing 1 pmol synthetic peptide/min. The relative activity in the *in vivo* assay was estimated from the increase in absorbance at 600 nm of *E. coli* IT41/pGP1-2 encoding each mutant enzyme relative to that encoding the wild-type enzyme. Values are means ± SD, generally based on at least five independent determinations ($n \geq 5$).

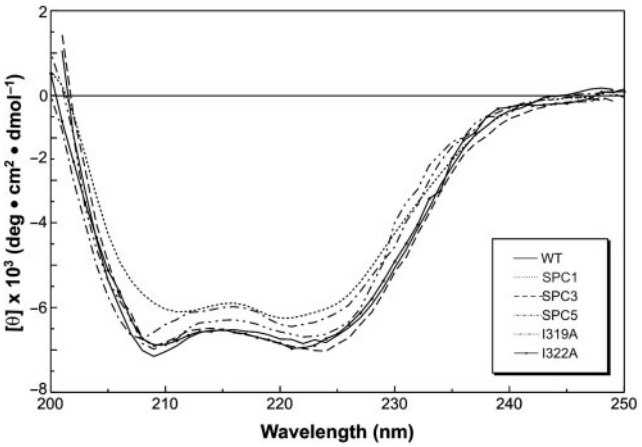

Fig. 2. **Far-UV CD spectra of the SPase I mutants.** The CD spectra of the wild-type enzyme and its mutants were recorded at a protein concentration of 2.0 μM in 25 mM sodium phosphate buffer, pH 7.7, containing 0.1% Lubral PX and 10% glycerol with a Jasco J-720 spectropolarimeter at room temperature using the water-jacketed quartz cell with a light path of 1 mm. The sample solutions were prepared by appropriately diluting the solutions of the enzyme with 25 mM sodium phosphate buffer, pH 7.7, just before measurement. For all measurements, 1.0 nm bandwidth and 1.0 s time constant were used, and 32 scans were repeated from 200 to 250 nm at the speed of 50 nm/min with 0.1 nm/point resolution. The protein concentrations of the mutants used for CD spectroscopy were determined by amino acid analysis after acid hydrolysis.

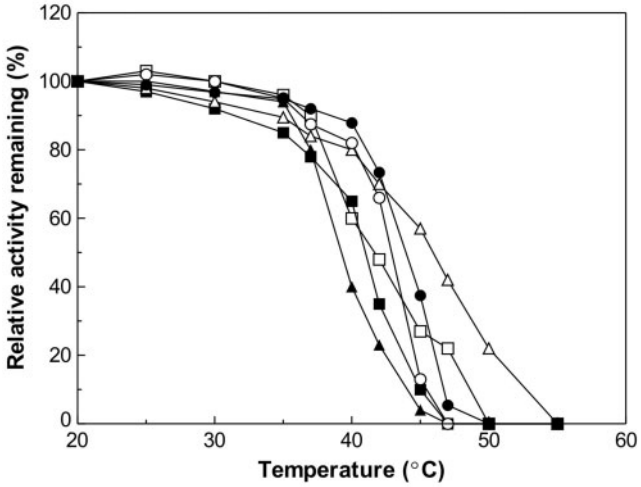

Fig. 3. **Thermostability of the SPase I mutants.** The wild-type and mutant enzymes in 10 mM potassium phosphate, pH 7.0, containing 0.1% Lubrol PX, 10% glycerol and 35 mM NaCl were incubated at the indicated temperatures for 1 h. Remaining activities were measured by mixing 10 μl of each heat-treated enzyme with 40 μl of the synthetic substrate solution. The reaction was allowed to proceed at 37°C for 30 min and was stopped by the addition of 50 μl of 0.1% trifluoroacetic acid. Then the reaction mixture was analysed by HPLC as described previously (*7*). Wild-type (solid circle); *SPC1* (open circle); *SPC3* (open triangle); *SPC5* (open square); *I319A* (solid square); *I322A* (solid triangle).

In the three-dimensional structure (Fig. 4) (*17*), Ile^{322} is suggested to interact with the side chain of Leu^{95}. The distance between the $C\gamma^2$ atom of Ile^{322} and the $C\delta^2$ atom of Leu^{95} is 3.68 Å. The side chain of Leu^{95} can also interact with that of Met^{91} neighbouring to the catalytic Ser^{90}. The distance between Cβ atoms of Leu^{95} and Met^{91} is 3.97 Å. Therefore, the lack of the interaction with Leu^{95} due to the mutation of Ile^{322} may cause a local conformational change of the catalytic Ser^{90} through the reorientation of Met^{91}. On the other hand, the side chain of Ile^{319} protrudes outside the enzyme molecule and does not interact with any group near the active site (Fig. 4). Thus, the mutation of Ile^{319} may cause less effect on the local conformation near the active site of the SPase I and hence its enzymatic activity (Table 1).

Figure 5 shows an alignment of the 14-residue amino acid sequences in the C-terminal regions of the 57 proteobacteria SPases I thus far available which correspond to the C-terminal 14-residue sequence of *E. coli* SPase I. As is clear from this figure, the residue Ile^{322} of *E. coli* SPase I is well conserved in many proteobacteria except that it is partially replaced with bulky hydrophobic amino acids such as Phe, Leu, Val and Trp. Furthermore, this homology appears to extend to other bacteria (data not shown). On the other hand, the residue Ile^{319} of *E. coli* is less conserved than Ile^{322} and even partially replaced with non-hydrophobic amino acids. These results are thus consistent with the supposition obtained above. Incidentally, it may be worthy of note that the residue Arg^{318} of *E. coli* SPase I, which was previously deduced to be one of the few important Arg residues in the

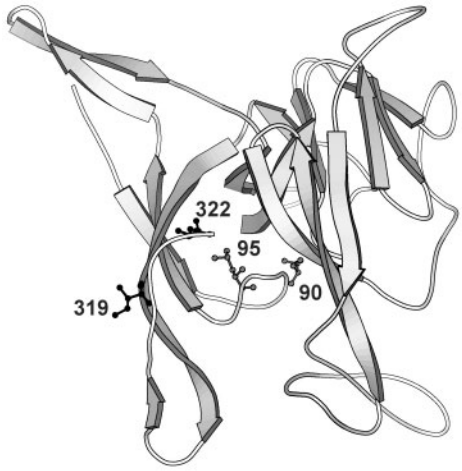

Fig. 4. **Schematic representation of the backbone conformation of SPase I.** The side chains of Ile^{319} and Ile^{322} (black), and Ser^{90} and Leu^{95} (grey) are drawn by the ball-and-stick model. The structure was presented from the PDB atomic coordinates 1B12 (*16*) with a slight modification using the program MolScript (*30*).

enzyme (*24*), is also highly conserved in the SPases I of other bacteria (Fig. 5).

Ile^{322} (this study), Trp^{300} (*22*) and some Arg residues including Arg^{318} (*24*) are located fairly apart from the active site of SPase I and are not directly involved in the catalytic machinery. Their mutations, however, resulted in a great loss of enzymatic activity, indicating that

	*		*
Escherichia coli (NP_417063)	WPTGLRLSRIGGIH	*Helicobacter pylori* (NP_223241)	RYLVRWERMFKSVE
Shigella flexneri (NP_708420)	WPTGVRLSRIGGIH	*Helicobacter pylori* (NP_207371)	RYLVRWERMFKSVG
Salmonella enterica (NP_457111)	WPTGVRLSRIGGIH	*Wolinella succinogenes* (NP_907517)	DYKIRWNRIGKSIE
Yersinia pestis (NP_406234)	WPTGVRLSRIGGIH	*Campylobacter jejuni* (NP_282017)	DKNVRWERIGRFVD
Yersinia enterocolitica (AAQ11415)	WPTGVRLSRIGGIH	*Rickettsia rickettsii* (NP_00153218)	LKPWVESVRLNRIF
Erwinia carotovora (YP_051368)	WPTGVRLSRIGGIH	*Rickettsia conorii* (NP_359793)	LKPWIESVRLNRIF
Photorhabdus luminescens (NP_930561)	WPTGVRFSRIGGIN	*Rickettsia sibirica* (NP_00142370)	LKPWIESVRLNRIF
Vibrio cholerae (NP_232091)	IPTGVRFNRVGGIH	*Rickettsia prowazekii* (NP_220508)	LKPWIESVRLSRIF
Vibrio parahaemolyticus (NP_798952)	IPTGVRFNRIGGIH	*Bradyrhizobium japonicum* (NP_771702)	FWRWPWAVRWNRFF
Vibrio vulnificus (NP_760461)	IPTGVRFNRIGGIH	*Rhodopseudomonas palustris* (NP_948038)	IWRWPTAVRWGRIF
Buchnera aphidicola (NP_240083)	WPTGIRINRIGSIH	*Magnetospirillum magnetotac* (ZP_00048501)	LWRWPTDVRWNRIF
Buchnera aphidicola (NP_660598)	WPTGIRIKRIGNIY	*Magnetospirillum magnetotac* (ZP_00051050)	VWRWPWAIRYARLL
Buchnera-aphidicola (NP_777862)	WPTGIQFDRIGNIY	*Brucella suis* (NP_697674)	WPTDVRFNRLFTWV
Candidatus Blochmannia florida (NP_878820)	WPTGIKLDRIGMLK	*Mesorhizobium loti* (NP_108011)	IWKWPSLWRVSRLF
Haemophilus influenzae (ZP_00157522)	PTGFRFERFFTAIK	*Bartonella henselae* (NP_033344)	IWRWPFDVRWNRLF
Haemophilus influenzae (NP_438188)	PTGFRFERFFTAIK	*Bartonella quintana* (NP_032107)	IWRWPFDVRWNRLF
Haemophilus influenzae (ZP_00154746)	PTGFRFDRFFTAIK	*Sinorhizobium meliloti* (NP_385177)	IWKWPANLRYDRLF
Haemophilus somnus (ZP_00133139)	PKGIRFSRMFTSIK	*Bradyrhizobium japonicum* (NP_767807)	WTCAATGFRLARFF
Actinobacillus pleuropneumo (ZP_00134799)	PSGLRFDRMFTSIN	*Bradyrhizobium* (NP_767807)	VWTWLSGFRLARFF
Pseudomonas aeruginosa (NP_249459)	MSNLPNFSRVGVIH	*Rhodopseudomona palustris* (NP_946177)	VTDWFSGFRVARFF
Pseudomonas fluorescens (P26844)	LSHLPNFSRVGLIK	*Serratia marcescens* (NP_941103)	TDHSWDSRFWGTVK
Burkholderia pseudomallei (YP_109022)	WMNFSDLKRIGSFN	*Salmonella typhi* (NP_058222)	TDHSWDSRFWGPVK
Ralstonia solanacearum (NP_519182)	WMNLGDWKRIGSFH	*Bradyrhizobium japonicum* (NP_772268)	HLVGKVTRIFWSLD
Azoarcus (YP_160177)	WFNFSDMKRIGGFH	*Desulfotalea psychrophila* (YP_063843)	RWTSIKWARIGRLV
Bordetella bronchiseptica (NP_890282)	WMNFSDLSRIGRFH	*Neisseria meningitidis* (NP_283755)	MNFGDFGRAGTAIR
Bordetella parapertussis (NP_885463)	WMNFSDLSRIGRFH	*Ralstonia solanacearum* (NP_519837)	WYLPRLARIGRPLD
Bordetella pertussis (NP_881060)	WMNFSDLSRIGRFH	*Magnetospirillum magnetotac* (ZP_00051050)	GLLVPPAPRRVPVH
Nitrosomonas europaea (NP_842323)	WNFNDLSRIGTLIK	*Proteus vulgaris* (NP_640175)	GHSDKELTRTGIIP
Acinetobacter (YP_047167)	GFKIPSFNRNGTID		

Fig. 5. **Alignment of the C-terminal 14-residue sequence of *E. coli* SPase I with the corresponding sequences of SPases I from other proteobacteria.** The accession number is given in parenthesis. Asterisk denotes the residues corresponding to Ile^{322} in *E. coli* SPase I.

certain amino acid residues away from the active site may contribute significantly to the active conformation, hence the activity of the enzyme. These results thus constitute an example of the impact of non-active site mutation in enzyme. Similar results may be obtained with other enzymes. Indeed, non-active site amino acid substitutions were reported to be the major factors leading to the decreases in inhibitor binding to the HIV-1 protease (*31*).

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology to Y.K. (11760077).

REFERENCES

1. Wickner, W., Driessen, A.J.M., and Hartl, F.-U. (1991) The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Annu. Rev. Biochem.* **60**, 101–124
2. Wolfe, P.B., Wickner, W., and Goodman, J.M. (1983) Sequence of the leader peptidase gene of *Escherichia coli* and the orientation of leader peptidase in the bacterial envelope. *J. Biol. Chem.* **258**, 12073–12080
3. Paetzel, M., Dalbey, R.E., and Strynadka, N.C.J. (2000) The structure and mechanism of bacterial type I signal peptidases, A novel antibiotic target. *Pharmacol. Ther.* **87**, 27–49
4. Chang, C.N., Blobel, G., and Model, P. (1978) Detection of prokaryotic signal peptidase in an *Escherichia coli* membrane fraction: endoproteolytic cleavage of nascent f1 pre-coat protein. *Proc. Natl. Acad. Sci. USA* **75**, 361–365
5. Date, T. and Wickner, W. (1981) Isolation of the *Escherichia coli* leader peptidase gene and effects of leader peptidase overproduction *in vivo*. *Proc. Natl. Acad. Sci. USA* **78**, 6106–6110
6. Dalbey, R.E. and Wickner, W. (1985) Leader peptidase catalyzes the release of exported proteins from the outer surface of the *Escherichia coli* plasma membrane. *J. Biol. Chem.* **260**, 15925–15931
7. Kim, Y.-T., Muramatsu, T., and Takahashi, K. (1995) Leader peptidase from *Escherichia coli*: overexpression, characterization, and inactivation by modification of tryptophan residues 300 and 310 with *N*-bromosuccinimide. *J. Biochem.* **117**, 535–544
8. Zwizinski, C. and Wickner, W. (1980) Purification and characterization of leader (signal) peptidase from *Escherichia coli*. *J. Biol. Chem.* **255**, 7973–7977
9. Wolfe, P.B., Silver, P., and Wickner, W. (1982) The isolation of homogeneous leader peptidase from a strain of *Escherichia coli* which overproduces the enzyme. *J. Biol. Chem.* **257**, 7898–7902
10. Tschantz, W.R., Sung, M., Delgodo-Partin, V.M., and Dalbey, R.E. (1993) A serine and a lysine residue implicated in the catalytic mechanism of the *Escherichia coli* leader peptidase. *J. Biol. Chem.* **268**, 27349–27354
11. van Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* **133**, 17–21
12. Moore, K.E. and Miura, S. (1987) A small hydrophobic domain anchors leader peptidase to the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.* **262**, 8806–8813
13. Bilgin, N., Lee, J.I., Zhu, H., Dalbey, R.E., and von Heijne, G. (1990) Mapping of catalytically important domains in *Escherichia coli* leader peptidase. *EMBO J.* **9**, 2717–2722
14. Zimmermann, R., Watts, C., and Wickner, W. (1982) The biosynthesis of membrane-bound M13 coat protein. *J. Biol. Chem.* **257**, 6529–6536
15. Kuo, D.W., Chan, H.K., Wilson, C.J., Griffin, P.R., Williams, H., and Knight, W.B. (1993) *Escherichia coli*

leader peptidase: production of an active form lacking a requirement for detergent and development of peptide substrates. *Arch. Biochem. Biophys.* **303**, 274–280
16. Paetzel, M., Dalbey, R.E., and Strynadka, N.C.J. (1998) Crystal structure of a bacterial signal peptidase in complex with a β-lactam inhibitor. *Nature* **396**, 186–190
17. Paetzel, M., Dalbey, R.E., and Strynadka, N.C.J. (2002) Crystal structure of a bacterial signal peptidase apoenzyme: implications for signal peptide binding and the Ser-Lys dyad mechanism. *J. Biol. Chem.* **277**, 9512–9519
18. Sung, M. and Dalbey, R.E. (1992) Identification of potential active-site residues in the *Escherichia coli* leader peptidase. *J. Biol. Chem.* **267**, 13154–13159
19. Black, M.T. (1993) Evidence that the catalytic activity of prokaryote leader peptidase depends upon the operation of a serine-lysine catalytic dyad. *J. Bacteriol.* **175**, 4957–4961
20. Paetzel, M., Strynadka, N.C., Tschantz, W.R., Casareno, R., Bullinger, P.R., and Dalbey, R.E. (1997) Use of site-directed chemical modification to study an essential lysine in *Escherichia coli* leader peptidase. *J. Biol. Chem.* **272**, 9994–10003
21. Black, M.T., Munn, J.G.R., and Allsop, A.E. (1992) On the catalytic mechanism of prokaryotic leader peptidase I. *Biochem. J.* **282**, 539–543
22. Kim, Y.-T., Muramatsu, T., and Takahashi, K. (1995) Identification of Trp300 as an important residue for *Escherichia coli* leader peptidase activity. *Eur. J. Biochem.* **234**, 358–362
23. Kuo, D., Weidner, J., Griffin, P., Shah, S.K., and Knight, W.B. (1994) Determination of the kinetic parameters of *Escherichia coli* leader peptidase activity using a continuous assay: the pH dependence and time-dependent inhibition by β-lactams are consistent with a novel serine protease mechanism. *Biochemistry* **33**, 8347–8354
24. Kim, Y.-T., Kurita, R., Kojima, M., Nishii, W., Tanokura, M., Muramatsu, T., Ito, H., and Takahashi, K. (2004) Identification of arginine residues important for the activity of *Escherichia coli* signal peptidase I. *Biol. Chem.* **385**, 381–388
25. Tabor, S. (1990) Expression using the T7 RNA polymerase/promoter system in *Current Protocols in Molecular Biology* (Ausubel, FM., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) pp. 16. 2. 1–16. 2. 11 Greene Publishing and Wiley Interscience, New York
26. Inada, T., Court, D.L., Ito, K., and Nakamura, Y. (1989) Conditionally lethal amber mutations in the leader peptidase gene of *Escherichia coli*. *J. Bacteriol.* **171**, 585–587
27. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488–492
29. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
30. Kraulis, P.J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950
31. Olsen, D.B., Stahlhut, M.W., Rutkowsky, C.A., Schock, H.B., vanOlden, A.L., and Kuo, L.C. (1999) Non-active site changes elicit broad-based cross-resistance of the HIV-1 protease to inhibitors. *J. Biol. Chem.* **274**, 23699–23701