The Role of the Conserved Box E Residues in the Active Site of the *Escherichia coli* Type I Signal Peptidase*

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Type I signal peptidases are integral membrane proteins that function to remove signal peptides from secreted and membrane proteins. These enzymes carry out catalysis using a serine/lysine dyad instead of the prototypical serine/histidine/aspartic acid triad found in most serine proteases. Site-directed scanning mutagenesis was used to obtain a qualitative assessment of which residues in the fifth conserved region, Box E, of the *Escherichia coli* signal peptidase I are critical for maintaining a functional enzyme. First, we find that there is no requirement for activity for a salt bridge between the invariant Asp-273 and the Arg-146 residues. In addition, we show that the conserved Ser-278 is required for optimal activity as well as conserved salt bridge partners Asp-280 and Arg-282. Finally, Gly-272 is essential for signal peptidase I activity, consistent with it being located within van der Waals proximity to Ser-278 and general base Lys-145 side-chain atoms. We propose that replacement of the hydrogen side chain of Gly-272 with a methyl group results in steric crowding, perturbation of the active site conformation, and specifically, disruption of the Ser-90/Lys-145 hydrogen bond. A refined model is proposed for the catalytic dyad mechanism of signal peptidase I in which the general base Lys-145 is positioned by Ser-278, which in turn is held in place by Asp-280.

The majority of proteins exported from a cell are made with a signal or leader peptide. These peptides are responsible for targeting proteins to the cytoplasmic membrane in prokaryotes and the endoplasmic reticulum membrane in eukaryotes (1–3). The protein is then translocated across the membrane, and the N-terminal peptide is removed from the pre-protein by a type I signal peptidase.

SPase I[1] has been isolated from Gram-negative (4–6) and Gram-positive (7, 8) bacteria as well as from several eukaryotic organisms (9–12). These endopeptidases are membrane-bound and specific for the region within the signal peptide immediately preceding the cleavage site. The substrate protein is cleaved during or after the protein is transported across the membrane bilayer. Cleavage occurs by way of nucleophilic attack by a catalytic serine Oγ on the peptide bond between the presequence and the mature region of the pre-protein.

The best-studied enzyme of this family is SPase I, or leader peptidase, of *Escherichia coli*. It has been cloned (13), sequenced (14), and purified (15, 16). Its substrate specificity has been characterized; small-uncharged residues at the P1 (-1) and P3 (-3) positions of the substrate are required for cleavage (17–19). SPase I utilizes a serine/lysine dyad mechanism to perform its enzymatic function rather than the canonical catalytic triad found in most serine proteases (20–23). To date, only a few other enzymes, such as LexA (24), UmuD (25), and the Tsp protease (26) have been identified with this unusual active site mechanism. Additional analysis is required to provide insight into other residues near this dyad in the SPase family and the roles they play, if any, in the structure and function of these enzymes.

The recent crystal structure (27) of the catalytic domain of SPase I (28–29) has revealed that most of the amino acids that are strictly conserved in Boxes B, C, D, and E of the signal peptidase family (30) are found to be located near the active site (see Fig. 1*A*). In *E. coli*, the Box B conserved region contains the catalytic serine (Ser-90), and Box D contains the catalytic lysine (Lys-145). Box E contains the conserved GDN (Gly-272, Asp-273, and Asn-274 in *E. coli*), Ser-278, Asp-280, and Arg-282 (Fig. 1*B*). In this work, we have employed a scanning mutagenesis approach to obtain a qualitative assessment of which residues in the conserved Box E are critical for maintaining a functional SPase I. We find that Ser-278, which contributes a hydrogen bond to Lys-145 and therefore helps position this residue, is critical for activity. In addition, substitution of Gly-272 with an alanine leads to a marked effect on activity. Gly-272 most likely plays a structural role, because it is located adjacent to the catalytic Ser-90 and Lys-145 dyad. Any side chain other than hydrogen at 272 results in steric crowding and perturbation of the active site. Asp-280 and Arg-282 are also important for SPase I activity. Asp-280, which forms a hydrogen bond to Ser-278, may help to position the general base Lys-145. Finally, the salt bridge between Asp-273 and Arg-146 is not required for activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli BLR(DE3) [F⁻*ompT* hsdS$_B$(r$_B^-$ mB⁻) *gal dcm* (*srl⁻rec*A) 306::Tn*10* (DE3)], BL21(DE3) [F⁻*ompT* hsdS$_B$(r$_B^-$ mB⁻) *gal dcm* (DE3)], and the pET21a and pET23b

[1] The abbreviations used are: SPase I, signal peptidase I; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis.

vectors were obtained from Novagen. *E. coli* IT41, a temperature-sensitive SPase I strain, was obtained from Dr. Yoshikazu Nakamura (31). The pING plasmid (32), which contains SPase I under the araB promoter (33), was from Dr. Gary Wilcox (Ingene, Inc.)

DNA Methods—The SPase I gene, *lepB*, was cloned into the pET23b vector by excising the *lepB* gene from the pING plasmid (33) by digestion with *Sal*I and *Sma*I. The *lepB* gene fragment was then ligated into a modified pET23b vector containing an *Sma*I site that was created upstream of the T7 termination region and containing a 72-base deletion of the T7 tag sequence. The final construct, pET23lep, contains the wild-type *lepB* gene modified with a six-histidine sequence at codons 35–40 (22). Oligonucleotide mutagenesis was performed using the Quikchange mutagenesis kit (Stratagene). Mutations were verified by plasmid isolation and sequencing (Sequenase Version 2.0). The calcium chloride method was used for DNA transformations into *E. coli* host strain BLR(DE3) (34).

Purification of 6-His-tagged Signal Peptidase Proteins—SPase I mutants containing a 6-His tag were purified using ion exchange and nickel affinity chromatography (35). Since SPase I, with a pI of 6.9, has only a weak affinity for Q-Sepharose ion exchange resin, a semi-pure dilute sample was obtained after the first chromatographic step. The nickel column served to further purify as well as concentrate the SPase I enzyme. Three liters of culture was sufficient to obtain approximately 3 mg of >95% pure protein for a majority of the mutants made.

Overnight cultures of *E. coli* BLR(DE3) cells harboring the pET23lep vector encoding the SPase I proteins were back-diluted 1:40 in 3 liters of LB media with 100 μg/ml ampicillin and 12.5 μg/ml tetracycline and grown at 37 °C to an absorbance of 0.6 at 600 nm. Expression was then induced by the addition of IPTG to a final concentration of 0.5 mM. Growth of the culture was continued for an additional 4 h, after which the cells were harvested by centrifugation and resuspended in 25 ml of lysis buffer (50 mM Tris, pH 8.0, 20% sucrose). Lysozyme (6 mg) and RNase-free DNase (Promega) (60 μl at 10 mg/ml) were added, and the solution was stirred for 10 min followed by freezing at -80 °C overnight. The lysed cells were thawed, and 200 μl of 1 M magnesium acetate was added and mixed by stirring for 10 min at room temperature. The solution was then centrifuged at 20,000 rpm for 30 min at 4 °C, and the pellet was resuspended in 25 ml of 10 mM triethanolamine, 10% glycerol, pH 7.9. After centrifugation, the pellet was resuspended by douncing in solubilization buffer (10 mM triethanolamine, 10% glycerol, 1% Triton X-100, pH 7.9) and re-centrifuged a third time. The SPase I-rich supernatant was loaded onto a 15-ml Q-Sepharose column (Amersham Pharmacia Biotech) previously equilibrated in solubilization buffer. The column was washed with 20 ml of solubilization buffer plus 5 mM magnesium sulfate, pH 7.9, and SPase I was eluted with a continuous gradient of 0–0.1 M KCl in column buffer. Two-ml fractions were collected and assayed for SPase I protein by SDS-PAGE. Fractions containing the enzyme were pooled and loaded onto a 1-ml nickel nitrilotriacetic acid-agarose column (Qiagen) equilibrated with 6-His buffer (10 mM Tris, pH 8.5, 100 mM KCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 1% detergent (either Triton X-100 or β-D-octylglucopyranoside). The column was then washed with 7 ml of 6-His buffer and 1 ml of wash buffer (6-His buffer plus 900 mM KCl). SPase I was then eluted using a 100 to 300 mM imidazole step gradient. Eluted fractions were assayed for SPase I protein by SDS-PAGE followed by GelCode Blue staining (Pierce). To remove the imidazole, pooled proteins were dialyzed against 20 mM Tris, pH 8.0, 0.5% Triton X-100 or buffer exchanged with a Centricon-10 membrane (Amicon) using 20 mM phosphate, 1% β-D-octylglucopyranoside, pH 8.0.

Purification of the pro-OmpA Nuclease A Substrate—*E. coli* strain BL21(DE3) containing pro-OmpA nuclease A encoded in the pET-21a plasmid was used for overexpression of the pre-protein substrate. The pro-OmpA nuclease A was expressed and purified as described by Chatterjee *et al.* (36).

In Vitro Activity Assay Using pro-OmpA Nuclease A—To determine the enzymatic activity of wild-type and mutant SPase I, we used pro-OmpA nuclease A as a substrate. Cleavage of this substrate was performed at different SPase I dilutions. The Pierce BCA protein assay was used to determine the concentrations of purified SPase I constructs, and an $E_{1\%}$ at 280 nm of 8.3 was used to determine the concentration pro-OmpA nuclease A (36). The starting concentration of each SPase I mutant for the dilution study was 0.1 mg/ml. Aliquots (1 μl) of enzyme (0.1, 0.01, 0.001, 0.0001, and 0.00001 mg/ml) were added to 15 μl of substrate at a final concentration of 15 μM in 50 mM Tris, pH 8.0, 1% Triton X-100. The reaction was incubated at 37 °C for 1 h then stopped by the addition of 4 μl of 5-fold sample buffer followed by quenching in a dry ice-ethanol bath. Processing of the pre-protein substrate to its mature nuclease A form was monitored by 17.2% SDS-PAGE.

In Vivo SPase I Activity Assay—The activity of SPase I *in vivo* was determined using the temperature-sensitive *E. coli* signal peptidase strain, IT41 (31). The procedure is the same as described by Sung and Dalbey (37) with the following modifications. IT41 was grown at 30 °C in M9 minimal medium (38) at pH 7.0 with 0.5% fructose and 50 μg/ml each amino acid except methionine with either wild-type SPase I or a mutated version of this enzyme in the IPTG-inducible plasmid, pET23lep. At mid-log phase, cells were grown at 42 °C for 1 h to inactivate the intrinsic temperature-sensitive SPase I. IPTG was added to a final concentration of 1 mM to induce synthesis of the mutant SPase I and incubated at 42 °C for an additional 30 min. Cell cultures (1 ml) were labeled with 200 μCi of [^{35}S]methionine for 15 s and chased with nonradioactive methionine at a final concentration of 500 μg/ml. At indicated times, aliquots (100 μl) were removed and quenched with an equal volume of ice-cold 20% trichloroacetic acid. Processing of the precursor to the outer membrane protein A (pro-OmpA) was used to determine the *in vivo* activity of the SPase I mutants. Proteins were immunoprecipitated with antibody to outer membrane protein A (15) and analyzed by SDS-PAGE and fluorography (39).

Circular Dichroism (CD) Spectroscopy—Circular dichroism measurements were conducted on a Jasco spectrapolarimeter J-500C instrument using a temperature-controlled cell at 4 °C. Protein concentrations of each SPase I construct were determined by using a molar extinction coefficient of 44,000 cm^{-1} M^{-1} at 280 nm (21).

Western Blot Analysis—BLR (DE3) (2 ml) harboring the pET23lep plasmid was grown in LB media to an absorbance at 600 nm of 0.7, then induced with IPTG (final concentration of 1 mM). After an additional 3-h incubation at 37 °C, cell cultures (100 μl) were normalized by dilution to the indicated final cell densities at A_{600} and were pelleted and resuspended in 2-fold sample buffer. Aliquots (10 μl) of dissolved cell extracts were then analyzed by 17.2% SDS-PAGE. The expression levels of the SPase I mutants were compared with the expression levels of wild-type SPase I. To do this, 10 μl of 1.1, 0.22, and 0.11 A_{600} units of cells containing overexpressed wild-type protein were assayed by 17.2% SDS-PAGE. The protein samples were electroblotted onto nitrocellulose membranes and visualized following the ECL Western blot protocol (Amersham Pharmacia Biotech).

Pulse-chase Assays—BLR(DE3) cultures (2 ml) were grown and induced the same manner as the Western blot protocol, except M9 media was used containing 0.5% fructose and all amino acids (50 μg/ml each) except methionine. After a 1-h induction, cells were labeled with 100 μCi of [^{35}S]methionine for 1 min and chased with an excess of nonradioactive methionine (500 μg/ml final concentration). Portions (100 μl) were removed at the indicated time points and added to ice-cold 20% trichloroacetic acid. Samples were then immunoprecipitated (15) and analyzed by SDS-PAGE and fluorography (39).

Structural Analysis—Hydrogen bonding contacts within the x-ray crystal structure of the *E. coli* SPase I soluble fragment (PDB 1b12) were analyzed with the program CONTACT (CCP4, 1994).

RESULTS

The x-ray crystal structure of the catalytic fragment of SPase I (27) has revealed potentially important amino acid residues located near the active site (Fig. 1*A*). This information coupled with knowledge of residues conserved in both prokaryotic SPase I and eukaryotic SPase I homologs enabled the rational design of mutants. In this work, we have focused on the conserved Box E region of the signal peptidase family (Fig. 1*B*). Box E contains the conserved tripeptide, GDN (Gly-272, Asp-273, and Asn-274 in *E. coli*), as well as the conserved Ser-278, Asp-280, and Arg-282 residues (in *E. coli*). Glycine and aspartate in the GDN consensus are strictly conserved, and the asparagine is highly conserved in all type I SPases. Ser-278 is only conserved in prokaryotic, mitochondrial SPase I (Imp1/Imp2) and the chloroplast thylakoid processing peptidase (chloroplast SPase I). We have constructed a number of mutants to test the importance of these residues and measured activity *in vitro* using the substrate pro-OmpA nuclease A. Mutants with a significant reduction in substrate processing were further analyzed *in vivo*, and their contribution to the structure and protein stability of the enzyme were also examined.

To increase protein expression over the original pING construct (33) the SPase I gene was cloned into the pET23b vector. The increased expression was needed for the two-part purifi-
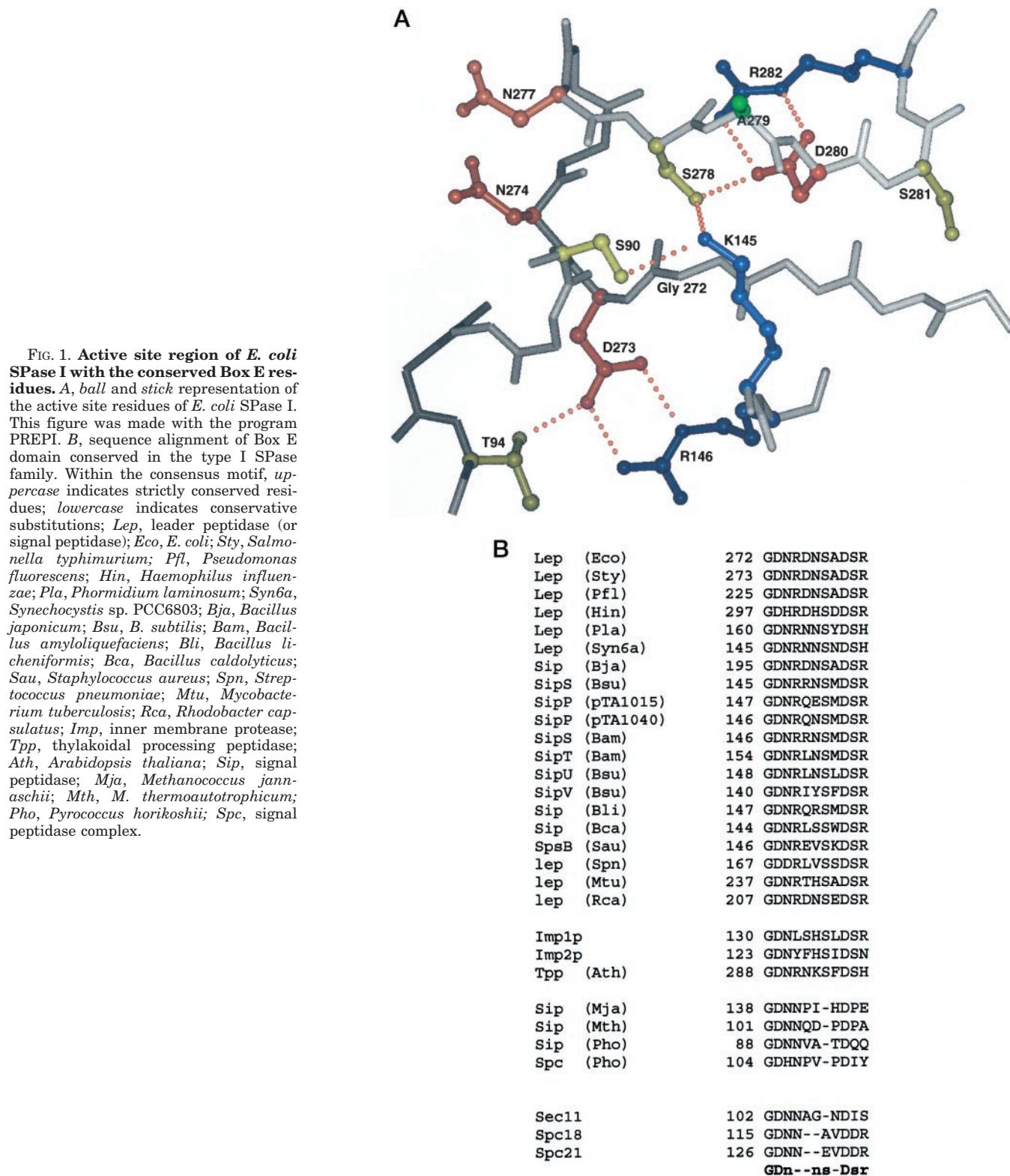
FIG. 1. **Active site region of *E. coli* SPase I with the conserved Box E residues.** *A*, *ball* and *stick* representation of the active site residues of *E. coli* SPase I. This figure was made with the program PREPI. *B*, sequence alignment of Box E domain conserved in the type I SPase family. Within the consensus motif, *uppercase* indicates strictly conserved residues; *lowercase* indicates conservative substitutions; *Lep*, leader peptidase (or signal peptidase); *Eco*, *E. coli*; *Sty*, *Salmonella typhimurium; Pfl, Pseudomonas fluorescens; Hin, Haemophilus influenzae; Pla, Phormidium laminosum; Syn6a, Synechocystis* sp. PCC6803; *Bja, Bacillus japonicum; Bsu, B. subtilis; Bam, Bacillus amyloliquefaciens; Bli, Bacillus licheniformis; Bca, Bacillus caldolyticus; Sau, Staphylococcus aureus; Spn, Streptococcus pneumoniae; Mtu, Mycobacterium tuberculosis; Rca, Rhodobacter capsulatus; Imp,* inner membrane protease; *Tpp,* thylakoidal processing peptidase; *Ath, Arabidopsis thaliana; Sip,* signal peptidase; *Mja, Methanococcus jannaschii; Mth, M. thermoautotrophicum; Pho, Pyrococcus horikoshii; Spc,* signal peptidase complex.

cation procedure employing ion exchange and nickel affinity chromatography (see "Experimental Procedures") (35) in order to obtain higher yields of pure mutant SPase I. With this new protocol the purity of each mutant (Fig. 2, *lanes 1–11* and *13–14*) is comparable with that of the wild-type enzyme (*lane 12*). For purposes of CD spectroscopic analysis, the nickel affinity column was also used to exchange the detergent Triton X-100 for the non-UV-absorbing β-octylglucopyranoside. *Lanes 10, 11,* and *12* in Fig. 2 are preparations in which this detergent

exchange method was applied.

Many Conserved Residues in the Vicinity of the Active Site Are Not Important for Enzymatic Activity or Stability—Wild-type 6-His SPase I processes pro-OmpA nuclease A to mature nuclease A even at a 10,000-fold dilution of a 0.1 mg/ml stock solution (Fig. 3, *bottom right panel*). SPase I enzymes with the single mutations T94V, R146A, R146M, G285A, N277A, and N277D all maintained activity out to 10^4-fold dilution, indicating these residues were not critical for catalysis. Since amino
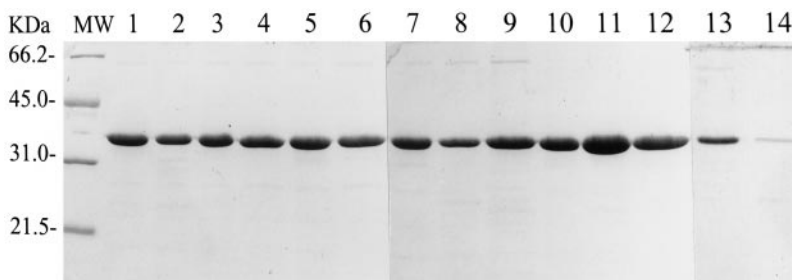
FIG. 2. **SDS-PAGE analysis of purified 6-His SPase I proteins.** Proteins were resolved on a 17.2% polyacrylamide gel followed by GelCode Blue staining (Pierce). *Lane 1*, N274A; *lane 2*, N274D; *lane 3*, T94V; *lane 4*, R146A; *lane 5*, R146M; *lane 6*, S281A; *lane 7*, G285A; *lane 8*, N277A; *lane 9*, N277D; *lane 10*, S278A; *lane 11*, G272A; *lane 12*, wild-type; *lane 13*, D273E; *lane 14*, T94V/R146A. Proteins in *lanes 1–9*, *13*, and *14* were solubilized with the detergent Triton X-100. Proteins in *lanes 10–12* were solubilized with the detergent β-D-octylglucopyranoside. *MW*, molecular mass.
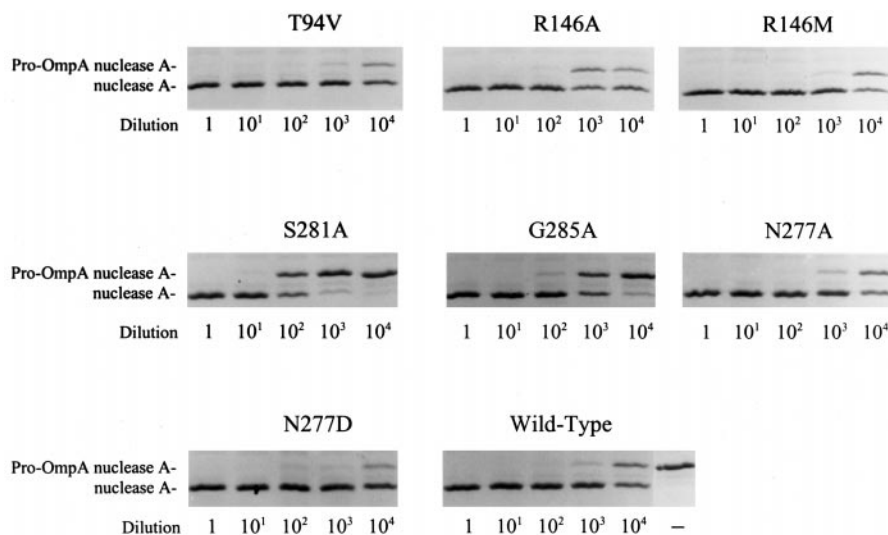
FIG. 3. **Mutations of some conserved residues near the active site do not affect *in vitro* processing.** Processing of pro-OmpA nuclease A (described under "Experimental Procedures") is initiated by the addition of 1 μl of serially diluted SPase I enzyme and incubation for 1 h at 37 °C. Wild-type *versus* mutant SPase I activities are compared by noting the differences in cleavage of pro-OmpA nuclease A into mature nuclease A. The − *lane* indicates no enzyme was added to the reaction mixture.

acid substitutions at these positions had little, if any, catalytic effect, further studies were not performed. Although the S281A enzyme showed a 100-fold decrease in activity, we also did not investigate this residue further because it is not conserved in all type I SPases and because mutation of the homologous serine residue in the *Bacillus subtilis* SipS enzyme had very little affect on activity (40).

The Serine at Position 278 Is Essential for Optimal Activity—In vitro and *in vivo* activity data for the S278A SPase I mutant is shown in Fig. 4. The mutation of Ser-278 to an Ala would remove the hydrogen bond from Ser-278 to the catalytic Lys-145 (Fig. 1*A*; See Table I for H-bond distances). This alteration leads to a significant loss of catalytic activity *in vitro*. Processing of the pro-OmpA nuclease A substrate by this mutant exhibited a 300-fold decrease as compared with the wild-type enzyme (Fig. 4*A*). The activity of the Ser-278 mutant was also assayed using the more sensitive *in vivo* assay, where SPase I activity was measured in its native membrane environment. This assay examines whether the plasmid-encoded S278A SPase I can restore processing of pro-OmpA at the nonpermissive temperature of 42 °C in the temperature-sensitive SPase I strain, IT41 (31). Fig. 4*B* demonstrates that the processing of pro-OmpA by *E. coli* SPase I is slowed at the nonpermissive temperature of 42 °C in IT41 bearing no plasmid, with an approximate $t_{1/2}$ of 60 s. In contrast, processing of pro-OmpA is rapid ($t_{1/2} < 10$ s) at 42 °C when it contains the pET23lep vector encoding wild-type 6-His SPase I. IT41 containing a plasmid-encoding signal peptidase S278A exhibited slow processing of pro-OmpA when compared with wild-type. The $t_{1/2}$ for the S278A enzyme is between 20 and 30 s. Immunoblot analysis indicates that the signal peptidase S278A mu-

tant is expressed within the cell in comparable amounts as wild-type (data not shown). These *in vitro* and *in vivo* results demonstrate that the Ser-278 residue is required for rapid SPase I processing.

To rule out global structural changes in SPase I due to the S278A mutation, we employed CD spectroscopy. Both S278A SPase I and the wild-type enzyme retain identical spectra between 200 and 250 nm (Fig. 5), providing evidence that the S278A protein is not grossly misfolded. Thus, the decrease in activity is consistent with and attributable to the loss of the hydrogen bonding capability of Ser-278 to Lys-145.

*Glycine 272 in the Conserved GDN Is Critical for Activity—*Since Gly-272, Asp-273, and Asn-274 are conserved and in the vicinity of the active site near the catalytic Lys-145 (27), we investigated their impact on catalysis. *In vitro* studies of G272A and N274A signal peptidases are shown in Fig. 6. The exchange of an alanine for an asparagine at position 274 had little effect on substrate processing (Fig. 6*A*, *bottom panel*). In contrast, substitution of the glycine at position 272 to alanine led to a marked effect on *in vitro* processing (Fig. 6*A*, *top panel*). Quantitation of the pro-OmpA nuclease A processing by gel band densitometry for the G272A mutant indicated that processing was impaired roughly 750-fold relative to wild-type enzyme (data not shown).

We also analyzed SPase I G272A protein for activity in intact cells. As can be seen in Fig. 6*B*, SPase I G272A has impaired *in vivo* processing of pro-OmpA in IT41 at 42 °C ($t_{1/2} \sim 50$ s), consistent with the *in vitro* results. Again, the global structural integrity of this SPase I protein appeared unperturbed as demonstrated by the CD spectrum (Fig. 5).

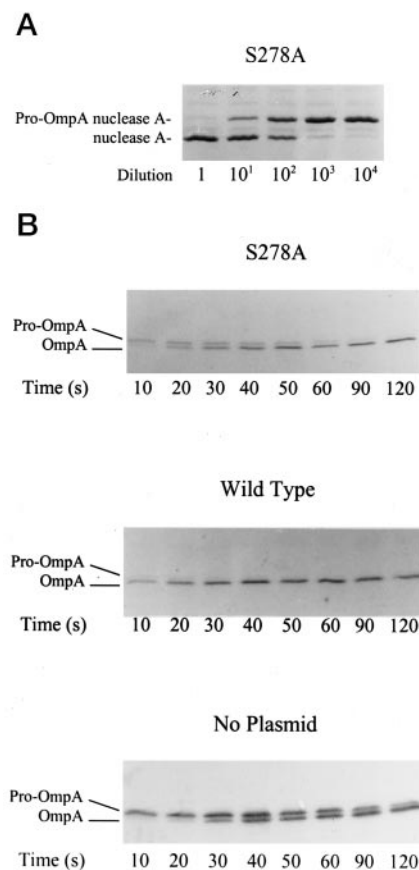*The Salt Bridge between Aspartic Acid 273 and Arginine 146*

A

S278A

Pro-OmpA nuclease A-
nuclease A-

Dilution   1 10^1 10^2 10^3 10^4

B

S278A

Pro-OmpA
OmpA

Time (s)   10   20   30   40   50   60   90   120

Wild Type

Pro-OmpA
OmpA

Time (s)   10   20   30   40   50   60   90   120

No Plasmid

Pro-OmpA
OmpA

Time (s)   10   20   30   40   50   60   90   120

FIG. 4. **Ser-278 is required for SPase I activity both *in vitro* and *in vivo*.** *A*, *in vitro* processing of pro-OmpA nuclease A (analysis as in Fig. 3). *B*, examination of *in vivo* processing in the *E. coli* temperature-sensitive SPase I strain, IT41. Mid-log phase IT41 bearing no plasmid or pET23lep encoding wild-type or mutant SPase I constructs grown at 30 °C were induced with 1 mM IPTG and incubated an additional 1 h at 42 °C. Pro-OmpA processing was then determined as described under "Experimental Procedures".

TABLE I

Hydrogen bonding distances within Box E

Atoms		Distance
		Å
Asp-273 Oδ1	Arg-146 Nε	2.9
Asp-273 Oδ2	Thr-94 Oγ	2.6
	Arg-146 Nη2	2.7
Ser-278 Oγ	Gly-272 N	3.1
	Asp-280 Oδ1	3.0
	Lys-145 Nζ	2.6
Asp-280 Oδ1	Arg-282 N	3.3
	Arg-282 Nε	2.9
Asp-280 Oδ2	Ser-278 Oγ	3.0
	Arg-282 Nη2	2.6
	Gly-272 N	3.2
Arg-282 Nε	Asp-280 Oδ1	2.9
Arg-282 Nη1	Asp-245 Oδ2	2.9
	Arg-275 O	3.1
Arg-282 Nη2	Arg-275 O	2.9
	Asp-280 Oδ2	2.6
Arg-146 Nε	Asp-273 Oδ1	2.9
Arg-146 Nη1	Gly-128 O	2.9
	Asp-273 Oδ2	2.7

Is Not Required for Activity of the E. coli SPase I—The x-ray structure has revealed that aspartic acid 273 forms a salt bridge with arginine 146 and a hydrogen bond with threonine 94 (Fig. 1*A*). To assess the significance of these interactions, we first mutated Asp-273 to alanine or asparagine. Since sufficient

amounts of the Asp-273 mutants could not be purified because of the lowered expression levels and the failure to solubilize the SPase I mutant from the membrane with detergent,[2] we measured the activity of the Asp-273 mutants *in vivo*. In Fig. 6*B* it is evident that the D273A (*middle panel*) and D273N (*bottom panel*) SPase I mutants have impaired processing *in vivo*. The processing activity at 42 °C is only slightly better with these mutants than when no plasmid is present (Fig. 4*B*). The processing $t_{1/2}$ of the D273A and D273N enzymes are close to 30 s. This demonstrates that the aspartic acid residue at position 273 is required for efficient SPase I activity when its interacting salt bridge partner, Arg-146, is also present.

As a control, we tested whether the decreased *in vivo* activity results from lower expression levels within the cell. The amount of signal peptidase expressed was determined by Western blotting using SPase I antiserum. As can be seen in Fig. 7, both SPase I D273A and D273N are expressed at approximately one-fifth the level as pET23lep-encoded wild-type SPase I. Decreased expression levels of Asp-273 mutants may contribute slightly to the lower processing of pro-OmpA to mature OmpA in IT41 at the nonpermissive temperature.

The residues Arg-146 and Thr-94, which interact with Asp-273, were mutated to amino acids with nonpolar side chains. To our surprise, the double mutant T94V/R146A maintained almost full activity (Fig. 8*A*), demonstrating that neither the salt bridge between Asp-273 and Arg-146 nor the Thr-94/Asp-273 hydrogen bond is required (Fig. 1*A*, Table I). Moreover, SPase I is still functional when the Asp-273 residue is conservatively replaced with a glutamic acid side chain (Fig. 8*A*).

To test the requirement for an acidic side chain at position 273 even in the absence of its salt bridge partner, we mutated both the Asp-273 and Arg-146 residues simultaneously. In Fig. 8*B*, *in vivo* processing is more rapid in the mutants that contain substitutions both at Arg-146 and Asp-273 (D273A/R146A and D273A/R146A/T94V) *versus* only the single (D273A) mutant (Fig. 6*B*). Taken together, the results indicate that the Asp-273 and Arg-146 salt bridge is not required for activity, and the acidic Asp-273 is only required when the basic Arg-146 is present as well.

Aspartic Acid 280 and Arginine 282 Mutations Affect Optimal Activity—The *E. coli* SPase I crystal structure (27) indicates the carboxylate oxygen of Asp-280 is about 5.0Å from the catalytically important Nζ of Lys-145 (Fig. 1*A*). The homologous residue to Asp-280 was found to be critical for activity in *B. subtilis* (40) as well as eukaryotic yeast Sec11 (41) SPase I. The functional role of Asp-280 was examined in our *E. coli* system. D280A and D280E mutants of *E. coli* SPase I were constructed and expressed, but purification of these proteins proved rather difficult. Purification trials of the Asp-280 variants resulted in proteins exhibiting lower concentration with evidence of proteolytic degradation (Fig. 9*A*). Compared with wild-type enzyme at similar concentrations, the D280A and D280E mutants maintained more than a 1000-fold reduction in activity *in vitro* (Fig. 9*B*). With the *in vivo* assay, however, the D280A and D280E mutants of *E. coli* SPase I demonstrated much higher activity levels (Fig. 9*C*). The D280A and D280E mutants displayed slightly less *in vivo* activity than wild-type (Fig. 4*B*) but greater activity than the S278A and G272A mutants (Figs. 4*B* and 6*B*). Pulse-chase analysis indicates that the Asp-280 mutants are stable *in vivo* (Fig. 9*D*), and immunoblot analysis (data not shown) was used to verify that the lower molecular bands of purified D280A and D280E (Fig. 9*A*) were fragments of the Asp-280 mutants.

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[2] P. A. Klenotic, J. L. Carlos, J. C. Samuelson, T. A. Schuenemann, W. R. Tschantz, M. Paetzel, N. C. J. Strynadka, and R. E. Dalbey, unpublished data.
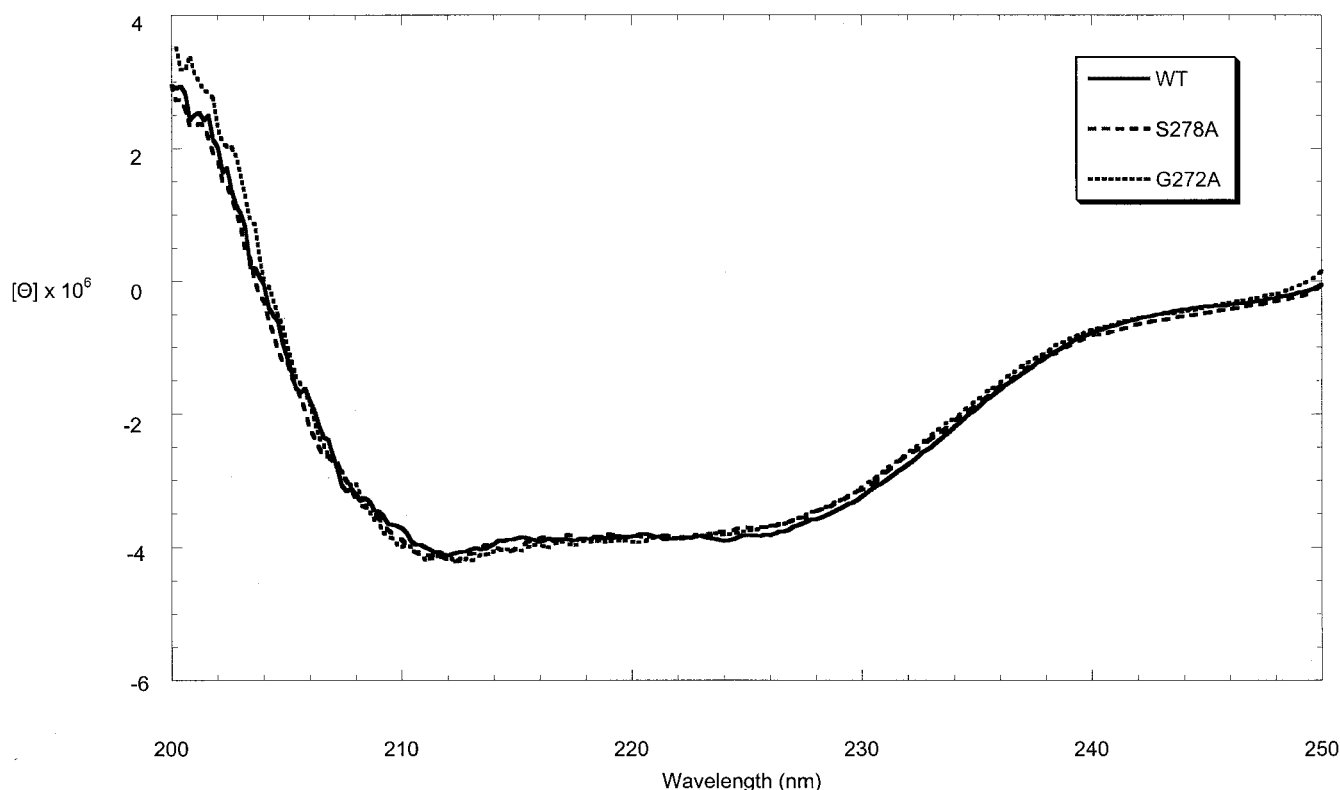
Fig. 5. **CD spectra of the wild-type (*WT*), S278A, and G272A SPase I mutants**. SPase I proteins (4 μM) were analyzed on a Jasco Spectrapolarimeter J-500C instrument in a 1-mm path-length cell at 4 °C. Spectra shown are an average of 5 scans/sample. The units of [θ] MRW are mdeg cm^2 dmol^{-1}.

To further study the role of Asp-280 in maintaining a functional SPase, its ionic interacting partner, Arg-282, was also examined via an R282M mutant. Purification of this Arg-282 variant resulted in a protein of lower concentration than wild-type enzyme but with less degradation than the Asp-280 mutants (Fig. 9*A*). Like the Asp-280 mutants, the R282M mutant displayed significantly less activity *in vitro* compared with wild-type (Fig. 9, *B* and *C*). Also, the R282M mutant displayed greater *in vivo* than *in vitro* activity but not as dramatic as the Asp-280 mutants.

DISCUSSION

Previous studies have established that the catalytic mechanism of *E. coli* SPase I requires an essential Ser-90 (37) and Lys-145 (20, 21). The Paetzel *et al.* (27) crystal structure of the inhibitor-bound soluble fragment of *E. coli* SPase I has provided the first direct evidence of the nucleophilic nature of Ser-90 and a glimpse of the neighboring residues in the active site. In the consensus sequence of the SPase I family, Ser-90 of the *E. coli* enzyme is found in the second conserved domain (Box B), whereas the catalytic Lys-145 is in the fourth conserved domain (Box D). One of the most prominent aspects of the crystal structure was the fact that the entire fifth conserved domain (Box E) of *E. coli* SPase I was found in the active site area. This Box E consensus sequence contains a cluster of three residues, GDN (beginning with Gly-272), the conserved Ser-278, the strictly conserved Asp-280, and the conserved Arg-282.

In the present study, we show that Asn-277, Gly-285, and Thr-94, are not important for *in vitro* substrate processing. This suggests that these residues are not important for enzymatic function. Although mostly conserved in the bacterial SPases, the homologous subunits (*i.e.* Sec11, Spc18, and Spc21) in the eukaryotic endoplasmic reticulum signal peptidase do not share the same conservation at these positions. The toler-

ation of these changes with respect to activity is therefore not unexpected.

In contrast, Ser-278 is necessary for efficient enzymatic action, as demonstrated by the *in vitro* and *in vivo* processing studies (Fig. 4). Serine 278 is conserved only in the bacterial, mitochondrial (Imp1/Imp2), and chloroplast type I signal peptidases (thylakoid-processing peptidases) that utilize the Ser/Lys dyad (20, 21). The Ser-278 homolog is not present in archae SPases or the endoplasmic reticulum subunit (Fig. 1*B*), which most likely uses a Ser/His dyad (41). The crystal structure of the *E. coli* SPase I-soluble fragment (27) has revealed that S278Oγ contributes a key hydrogen bond to the Lys-145 Nζ atom (Fig. 1, *A* and *B*; Table I) and is most likely essential for directing the Nζ into its proper orientation. This would explain why this Ser is conserved in type I SPases proposed to act by a Ser/Lys mechanism and its absence in Ser/His type I SPases.

Our evidence of an *in vivo* and *in vitro* stable S278A enzyme coupled with an apparently correct conformation as evidenced by similar solubility, purification, and CD spectral properties compared with wild-type enzyme points to the loss of activity of S278A as being attributable to the loss of the critical hydrogen bonding interaction that facilitates the proper orientation of the catalytic Lys-145. Sequence alignment of SPase I with SipS of *B. subtilis* shows Ser-151 aligned with the *E. coli* Ser-278. Mutation of Ser-151 to an alanine strongly reduced the *in vivo* activity of SipS, although it still had demonstrable activity compared with mutations of the catalytic Ser and Lys residues (40).

In addition to Ser-278, the Box E domain of *E. coli* SPase I contains Gly-272, Asp-273, and Asn-274, which are conserved in both prokaryotic and eukaryotic organisms. Gly-272 and Asp-273 are strictly conserved, whereas Asn-274 is a less conserved residue. Mutation of Asn-274 to an Ala had no effect on
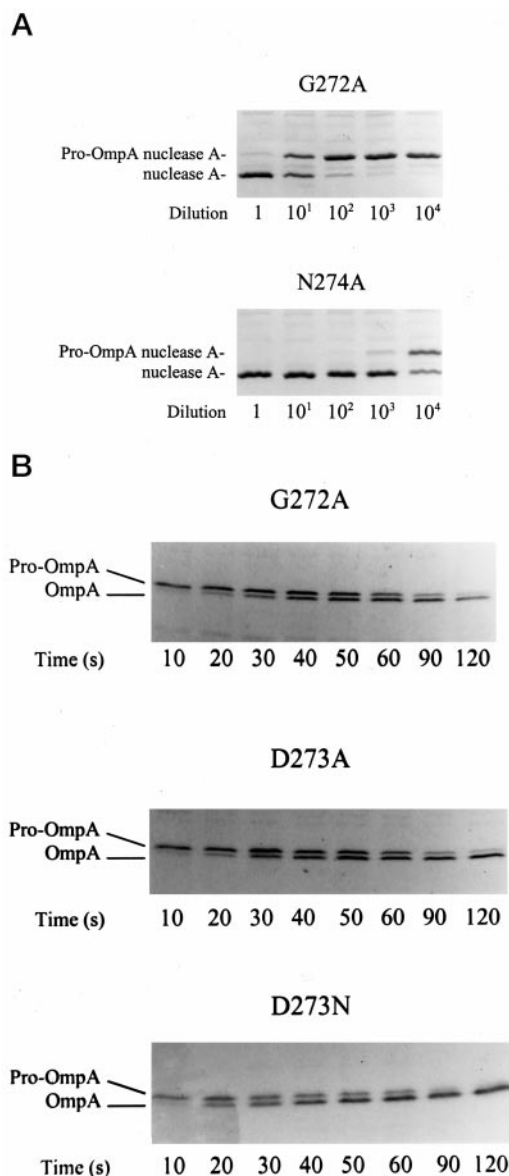
A

B

FIG. 6. **The role of Gly-272 and Asp-273 for the activity of the *E. coli* SPase I.** *A*, *in vitro* processing by serially diluted purified SPase I enzyme constructs as measured by the cleavage of pro-OmpA nuclease A (described in Fig. 3). *B*, *in vivo* processing by plasmid-encoded *E. coli* SPase I constructs (G272A, D273A, or D273N) as measured by cleavage of pro-OmpA within the IT41 *E. coli* strain. The *in vivo* assay was performed at the nonpermissive temperature of 42 °C.

FIG. 7. **Expression levels of Asp-273 SPase I mutants *in vivo*.** Western blot analysis to compare expression levels of the SPase I Asp-273 mutants within intact cells. *Lane 1*, signal peptidase control; *lane 2*, D273A; *lane 3*, D273N; *lane 4*, wild-type (*WT*) 1/10 dilution; *lane 5*, wild-type 1/5 dilution; *lane 6*, wild-type undiluted.

SPase I activity (Fig. 6*A*). This result is consistent with the studies of *B. subtilis* Type I SPase (40). The *in vitro* and *in vivo* data presented here is consistent with Gly-272 being critical for SPase I activity. Analysis of the SPase I crystal structure (Fig. 1*A*; Table I) shows that Gly-272 is located within van der Waals distance to the catalytic Lys-145 side chain. These two residues are packed such that the introduction of a side chain other than Gly at position 272 would force the Lys to shift from its optimal position and potentially cause local perturbations in the active site. Again the lack of evidence for gross structural changes based on stability, solubility, CD measurements, and ease of isolation of the G272A mutant leads us to conclude that the effects seen with G272A are solely due to the local perturbations mentioned. The mutation of the homologous Gly residue in the *B. subtilis* SipS signal peptidase, Gly-145, to an alanine residue significantly decreases *in vivo* processing of pre-β-lactamas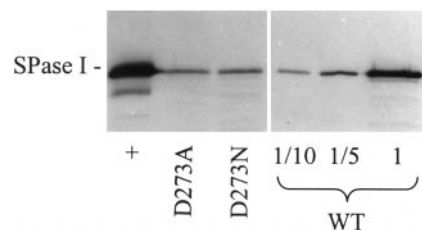e to its mature form (40). As with the *E. coli* enzyme, the Gly in the GDN consensus of *B. subtilis* SipS is more critical for activity than the Ser-151, (corresponding to *E. coli* Ser-278).

It is noteworthy that the decrease in *E. coli* SPase I activity due to mutation of the Ser-278 and Gly-272 residues was not as severe as with the substitutions of the catalytic Ser-90 and Lys-145 (37, 21, 22). No detectable *in vivo* activity was observed when Ser-90 and Lys-145 were mutated. Additionally there was no activity detected over background with the *in vitro* assay, which has a sensitivity of at least 100,000-fold. Thus mutations of the catalytic Ser-90 and Lys-145 residues cause a decrease in activity of greater than 100,000-fold.

We also show here there is no absolute requirement for activity for either the salt bridge between the invariant Asp-273 and Arg-146 residues or the hydrogen bond interaction between Asp-273 and Thr-94. First, substitution of Arg-146 with alanine or methionine has no effect on *in vitro* activity. Second, SPase I lacking both Arg-146 and Thr-94 was fully active *in vitro*. Third, SPase I lacking the Asp-273/Arg-146 salt bridge (D273A/R146A or D273A/R146A/T94V SPase I mutants) was active *in vivo*.

It is interesting that SPase I with the single Asp-273 mutation (either alanine or asparagine) was impaired in processing using the highly sensitive *in vivo* assay. This is in contrast to the double or triple mutant with the mutated Asp-273 residue above. It is not clear why the D273A or D273N mutants are inactive *in vivo*. It may be that these mutants are poorly expressed (Fig. 8) or may not fold or assemble into the membrane properly. Our data do not address this latter possibility. Unfortunately, the D273A and D273N mutants could not be purified and studied *in vitro* due to the poor expression as well as the difficulty in extracting these proteins from the membrane fraction with detergent. Nevertheless, the fact that the double and triple mutants (Fig. 8*B*), which remove Asp-273, are active demonstrates that there is not an absolute requirement for the Asp-273 residue.

Our results with the Arg-146 and Asp-273 substitutions in *E. coli* SPase I are in sharp contrast to those in *B. subtilis* or in yeast. Mutation of the homologous Asp-273 or Arg-146 residue in the *B. subtilis* SPase I or in the Sec11 subunit of the endoplasmic reticulum SPase I resulted in proteolytic degradation of these enzymes *in vivo* (40, 41), indicating that these residues are required for maintaining a stable, protease-resistant conformation. In our experiments the *E. coli* enzyme proved to be more stable *in vivo*, which was perhaps attributable to as yet unidentified compensating factors. Last, Bolhuis *et al.* (42) demonstrate thermal inactivation of the corresponding Arg-146/Asp-273 mutant of *B. subtilis* SipS (R84/D146), but the *E. coli* mutants (R146A or R146A/T94V) maintained identical activity as the wild-type protein at 37 °C and 42 °C in our *in vivo* experiments.[2]

Along with its salt bridge partner, Arg-282, the invariant Asp-280 residue of *E. coli* SPase I has been found to be important for enzymatic activity. The Asp-280 mutants are found to

FIG. 8. **The Salt bridge between Asp-273 and Arg-146 is not required for activity of the *E. coli* SPase I.** *A*, *in vitro* processing. Processing of pro-OmpA nuclease A using purified SPase I (at indicated dilutions) T94V/R146A or D273E. Pro-OmpA nuclease A cleavage is analyzed as in Fig. 3. *B*, *in vivo* processing. Processing of pro-OmpA in IT41 cells harboring the pET23lep vector expressing D273A/R146A or D273A/R146A/T94V mutants. The assay is as described in Fig. 4*B* and under "Experimental Procedures".
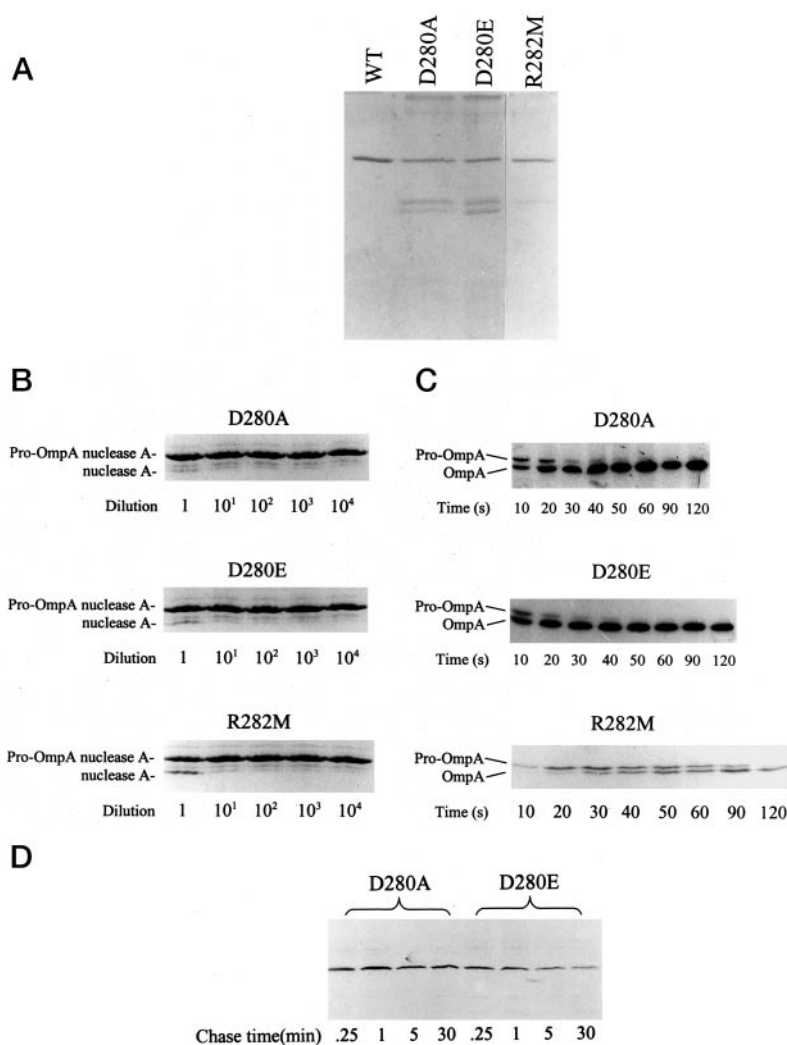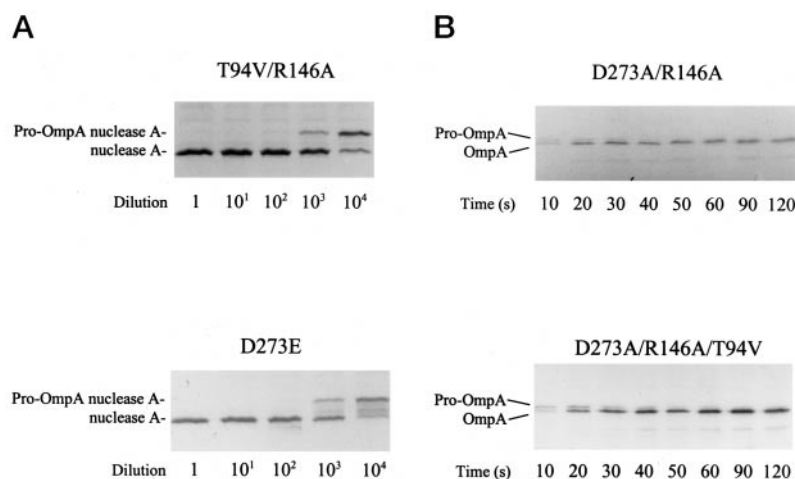

FIG. 9. **Asp-280/Arg-282 mutations affect *E. coli* SPase I activity.** *A*, purity as analyzed by SDS-PAGE of 6-His SPase wild-type (*WT*, *lane 1*), D280A (*lane 2*), D280E (*lane 3*), and R282M (*lane 4*). *B*, *in vitro* processing by serially diluted D280A (*top panel*), D280E (*middle panel*), and R282M (*bottom panel*) SPase I mutants as measured by pro-OmpA nuclease A substrate cleavage (described in Fig. 3 and under "Experimental Procedures"). *C*, *in vivo* processing of SPase I D280A (*top panel*), D280E (*middle panel*), and R282M (*bottom panel*) (described in Fig. 4*B* and under "Experimental Procedures"). *D*, *in vivo* stability of D280A and D280E mutants as analyzed by pulse chase. Two-ml cultures of BLR (DE3) cells harboring the indicated SPase I constructs were labeled with 100 μCi of [^{35}S]methionine for 15 s and then chased with 10 μl of 20 mg/ml cold methionine. Aliquots were removed at the indicated times followed by immunoprecipitation.

be stable *in vivo* (Fig. 9*D*) and display slightly lower processing than wild-type *in vivo*. R282M is also stable (not shown) but exhibits less *in vivo* activity than the Asp-280 mutants. Although mutations of Asp-280 affect activity, the *E. coli* SPase I is nevertheless quite active. Our *in vivo* Asp-280 results are in contrast to those results of van Dijl *et al.* (40) for the homologous residue in the *B. subtilis* SipS enzyme that is found to be absolutely critical for processing.

These studies are consistent with Asp-280 and Arg-282 being important for maintaining a fully functional *E. coli* SPase I

enzyme. Unfortunately, we were not able to determine whether these residues are important for maintaining the structural integrity of the enzyme or play a direct catalytic role, since we were not able to purify the protein in sufficient amounts for structural and catalytic characterization. However, we favor the idea that it plays a structural role, because the x-ray crystal structure revealed that not only is Asp-280 held firmly in place by a strong salt bridge to Arg-282, but that it hydrogen bonds to Ser-278 and interacts with the main chain of Gly-272 and Arg-282 as well (Fig. 1*A*, Table I). The hydrogen bonding of

Asp-280 to Ser-278 also likely aids the Ser-278 H-bonding interaction with the catalytic Lys-145.

The results of these studies have allowed us to refine our model of the catalytic details of the Ser/Lys mechanism of SPase I as well as explain the conserved nature of many of the Box E residues. Residues Ser-278 and Asp-280 help position the general base Lys-145 residue. Ser-278 contributes a hydrogen bond directly to the epsilon amino group of the catalytic Lys-145 and, therefore, helps orient it with respect to the catalytic Ser-90. The Gly-272 residue is located very near the catalytic dyad and has been maintained through evolution. The studies of Gly-272 presented here are consistent with the crystal structure of SPase I, which reveals that any residue with a Cβ atom at the 272 position would sterically interfere with the Lys-145 side chain.

REFERENCES

1. Wickner, W., Dreissen, A. J., and Hartl, F.-U. (1991) *Annu. Rev. Biochem.* **60,** 101–124
2. Pugsley, A. P. (1993) *Microbiol. Rev.* **57,** 50–108
3. Dalbey, R. E., and Robinson, C. (1999) *Trends Biochem. Sci.* **24,** 17–22
4. Zwizinski, C., and Wickner, W. (1980) *J. Biol. Chem.* **255,** 7973–7977
5. van Dijl, J. M., van den Bergh, R., Revers, T., Smith H., Bron, S., and Venema, G. (1990) *Mol. Gen. Genet.* **223,** 233–240
6. Black, M. T., Munn, J. G. R., and Allsop, A. E. (1992) *Biochem. J.* **282,** 539–543
7. van Dijl, J. M., de Jong, A., Vehmaanpera, J., Venema, G., and Bron, S. (1992) *EMBO J.* **11,** 2819–2828
8. Cregg, K. M., Wilding, I., and Black, M. T. (1996) *J. Bacteriol.* **178,** 5712–5718
9. YaDeau, J. T., and Blobel, G. (1989) *J. Biol. Chem.* **264,** 2928–2934
10. Baker, R. K., and Lively, M. O. (1987) *Biochemistry* **26,** 8561–8567
11. Greenburg, G., Shelness, G. S., and Blobel, G. (1989) *J. Biol. Chem.* **264,** 15762–15765
12. Schneider, A., Behrens, M., Scherer, P., Pratje, E., Michaelis, G., and Schatz, G. (1991) *EMBO J.* **10,** 247–254
13. Date, T., and Wickner, W. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6106–6110
14. Wolfe, P. B., Wickner, W., and Goodman, J. M. (1983) *J. Biol. Chem.* **258,** 12073–12080
15. Wolfe, P. B., Silver, P., and Wickner, W. (1982) *J. Biol. Chem.* **257,** 7898–7902
16. Tschantz, W. R., and Dalbey, R. E., (1994) *Methods Enzymol.* **244,** 285–301
17. Kuhn, A., and Wickner, W. (1985) *J. Biol. Chem.* **260,** 15914–15918
18. Fikes, J. D., Barkocy-Gallagher, G. A., Klapper, D. G., and Bassford, P. J., Jr. (1990) *J. Biol. Chem.* **265,** 3417–3423
19. Shen, L. M., Lee, J.-I., Cheng, S., Jutte, H., Kuhn, A., and Dalbey, R. E. (1991) *Biochemistry* **30,** 11775–11781
20. Black, M. T. (1993) *J. Bacteriol.* **175,** 4957–4961
21. Tschantz, W. R., Sung, M., Delgado-Partin, V. M., and Dalbey, R. E. (1993) *J. Biol. Chem.* **268,** 27349–27354
22. Paetzel, M., Strynadka, N. C. J., Tschantz, W. R., Casareno, R., Bullinger, P., and Dalbey, R. E. (1997) *J. Biol. Chem.* **272,** 9994–10003
23. Paetzel, M., and Dalbey, R. E. (1997) *Trends Biochem. Sci* **22,** 28–31
24. Roland, K. L., and Little, J. W. (1990) *J. Biol. Chem.* **265,** 12828–12835
25. Peat, T. S., Frank, E. G., McDonald, J. P., Levine, A. S., Woodgate, R., and Hendrickson, W. A. (1996) *Nature* **380,** 727–730
26. Keiler, K. C., and Sauer, R. T. (1995) *J. Biol. Chem.* **270,** 28864–28868
27. Paetzel, M., Dalbey, R. E., and Strynadka, N. C. J. (1998) *Nature* **396,** 186–190
28. Kuo, D. W., Chan, H. K., Wilson, C. J., Griffin, P. R., Williams, H., and Knight, W. B. (1993) *Arch. Biochem. Biophys.* **303,** 274–280
29. Tschantz W. R., Paetzel, M., Cao, G., Suciu, D., Inouye, M., and Dalbey, R. E. (1995) *Biochemistry* **35,** 3935–3941
30. Dalbey, R. E., Lively, M. O., Bron, S., and van Dijl, J. M. (1997) *Protein Sci.* **6,** 1129–1138
31. Inada, T., Court, D. L., Ito, K., and Nakamura, Y. (1989) *J. Bacteriol.* **171,** 585–587
32. Johnson, S., Lee, J. H., and Ray, D. S. (1985) *Gene (Amst.)* **34,** 137–145
33. Dalbey, R. E., and Wickner, W. (1985) *J. Biol. Chem.* **260,** 15925–15931
34. Cohen, S. N., Chang, A. C. Y., and Han, L., (1973) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 2110–2114
35. Smith, M. C., Furman, T. C., Ingolia, T. D., and Pidgeon, C. (1988) *J. Biol. Chem.* **263,** 7211–7215
36. Chatterjee, S., Suciu, D., Dalbey, R. E., Kahn, P. C., and Inouye, M. (1995) *J. Mol. Biol.* **245,** 311–314
37. Sung, M., and Dalbey, R. E. (1992) *J. Biol. Chem.* **267,** 13154–13159
38. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
39. Ito, K., Date, T., and Wickner, W. (1980) *J. Biol. Chem.* **255,** 2123–2130
40. van Dijl, J. M., de Jong, A., Venema, G., and Bron, S. (1995) *J. Biol. Chem.* **270,** 3611–3618
41. van Valkenburg, C., Chen, X., Mullins, C., Fang, H., and Green, N. (1999) *J. Biol. Chem.* **274,** 11519–11525
42. Bolhuis, A., Tjalsma, H., Stephenson, K., Harwood, C. R., Venema, G., Bron, S., and van Dijl, J. M. (1999) *J. Biol. Chem.* **274,** 15865–15868