**Escherichia coli** Signal Peptide Peptidase A Is a Serine-Lysine Protease with a Lysine Recruited to the Nonconserved Amino-Terminal Domain in the S49 Protease Family†

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ABSTRACT: *Escherichia coli* signal peptide peptidase A (SppA) is a serine protease which cleaves signal peptides after they have been proteolytically removed from exported proteins by signal peptidase processing. We present here results of site-directed mutagenesis studies of all the conserved serines of SppA in the carboxyl-terminal domain showing that only Ser 409 is essential for enzymatic activity. Also, we show that the serine hydrolase inhibitor FP-biotin inhibits SppA and modifies the protein but does not label the S409A mutant with an alanine substituted for the essential serine. These results are consistent with Ser 409 being directly involved in the proteolytic mechanism. Remarkably, additional site-directed mutagenesis studies showed that none of the lysines or histidine residues in the carboxyl-terminal protease domain (residues 326–549) is critical for activity, suggesting this domain lacks the general base residue required for proteolysis. In contrast, we found that *E. coli* SppA has a conserved lysine (K209) in the N-terminal domain (residues 56–316) that is essential for activity and important for activation of S409 for reactivity toward the FP-biotin inhibitor and is conserved in those other bacterial SppA proteins that have an N-terminal domain. We also performed alkaline phosphatase fusion experiments that establish that SppA has only one transmembrane segment (residues 29–45) with the C-terminal domain (residues 46–618) protruding into the periplasmic space. These results support the idea that *E. coli* SppA is a Ser-Lys dyad protease, with the Lys recruited to the amino-terminal domain that is itself not present in most known SppA sequences.

Almost all proteins that are exported to the cell surface of Gram-negative and Gram-positive bacteria are synthesized in a higher-molecular weight precursor form with an amino-terminal cleavable signal peptide. During protein export, the signal peptide is cleaved from the precursor by the membrane protease signal peptidase (1). The cleaved signal peptide can be further digested by signal peptide hydrolases (2, 3).

In 1984, the protease that degrades the lipoprotein signal peptide, named signal peptide peptidase A (SppA), was purified to homogeneity (4) and shown to correspond to the previously identified inner membrane protein, protease IV (5). SppA was subsequently cloned and sequenced, and the gene-encoded protein was shown to be a tetramer (6). Sequence analysis of *Escherichia coli* SppA reveals three hydrophobic regions that are candidate membrane-spanning domains. SppA is inhibited by serine protease inhibitors such as chymostatin, leupeptin, antipain, and elastinal (4). However, unlike typical serine proteases, the SppA family of proteases does not have a conserved histidine residue, suggesting SppA is an atypical serine protease.

The *E. coli* SppA protease has been classified as part of the S49 family of proteases in the MEROPS protease database (7), specifically to the S49.001 subfamily. This group, like subfamily S49.004, contains, in addition to the carboxyl-terminal protease domain that is conserved in all the S49 family members, an amino-terminal domain. The other subfamily members (S49.002, S49.003, S49.005, and S49.006) do not contain this amino-terminal domain. Members of these groups include the sohB peptidase (8), protein C (9), protein 1510-N (10), and the archaeal signal peptide peptidase (11), respectively.

Recently, site-directed mutagenesis has been utilized (12) to identify the catalytically important residues of the archaeal SppA protease from *Thermococcus kodakaraensis* which

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contain only the C-terminal protease domain. They showed that Ser 162 and Lys 214 were critical for activity, suggesting that this protease may use a serine-lysine dyad for catalysis like signal peptidase I (13), LexA (14), UmuD (15), Tsp (16), Lon protease (17), and VP4 protease (18). However, E. coli SppA lacks a lysine at the position aligned to Lys 214.

In this report, we have employed site-directed mutagenesis to identify possible active site residues for E. coli SppA. We show within the carboxyl-terminal protease domain of E. coli SppA that Ser 409, which is homologous to the critical Ser 162 of T. kodakaraensis SppA (12), is essential for activity. Wild-type SppA, but not an S409 mutant where the serine is substituted for alanine, can be covalently modified with the FP-biotin serine hydrolase inhibitor. These results support the notion that Ser 409 is the active site residue in the E. coli enzyme. None of the conserved amino acid residues within the protease domain that have ionizable side chains, including all the histidine and lysine residues, are critical for activity. Strikingly, the invariant Lys 209 residue located in the amino-terminal domain is indispensable for activity and important for activation of S409. The alkaline phosphatase fusion approach suggests that E. coli SppA, which was predicted to span the membrane three times by most topology programs, spans the membrane only once with its carboxyl terminus localized to the periplasmic space. Taken together, the results provide evidence that the single-spanning E. coli SppA carries out catalysis using a serine-lysine dyad with the serine located in the conserved carboxyl-terminal protease domain and the lysine in the nonconserved amino-terminal domain.

**EXPERIMENTAL PROCEDURES**

**Materials.** Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Pfu DNA polymerase was obtained from Stratagene. Oligonucleotides were ordered from New England Biolabs.

**Bacterial Strains and Plasmids.** E. coli strains BL21(DE3), DH5α, and MC1061 [ΔlacX74, araD139, Δ ara, leu]7697, gaiU, galk, hsr, hsm, strA] were from our collection. Lin 205 [pfuA22, phoA8, ompF627(T2′), glpA24, fadL701(T2′), relAI, glpR8(glpr), glpR7 (glpR), glpD26, pit-10, spoT1] was obtained from the E. coli Genetic Stock Center. The pCR 2.1 cloning vector was purchased from Invitrogen, and the pET-28(a) expression vector was from our laboratory. The pING-1 vector with the araB promoter and the araC regulatory elements was from our collection (19).

**Cloning and Mutagenesis of E. coli SppA.** The sppA gene was cloned by the PCR method into the pET-28(a) expression vector. The E. coli chromosomal DNA from MC1061 [ΔlacX74, araD139, Δ ara, leu]7697, galU, galK, hsr, hsm, strA] was used as the template. The PCR was run for 32 cycles (95 °C for 1 min, 46 °C for 1 min, and 72 °C for 90 s) with the sense primer (5′-AAG TTT GGA GAA CAT ATG CGA ACC CTT TGG CG-3′) and the antisense primer (5′-TCA GTA CAA AAG CTT AGG GTT GGC GCA GGT C-3′) for SppA. The underlined sequences specify the restriction sites for endonucleases NdeI and HindIII, respectively. The PCR product was cloned into the cloning vector, pCR 2.1 (ampicillin resistant). SppA was then subcloned into the NdeI and HindIII sites of the pET-28(a) expression vector (kanamycin resistant). The pCR 2.1 plasmids bearing the SppA gene were digested with NdeI and HindIII, and the DNA fragment containing sppA was isolated by excision from an agarose gel. The pET-28(a) vector was prepared in the same way. The ligation product was transformed into E. coli host BL21(DE3) cells. The resulting DNA was sent to the Plant-Microbe genomics sequencing facility at The Ohio State University for confirmation.

Site-directed mutagenesis was carried out with the SppA-pET-28(a) vector as a template using the QuickChange mutagenesis PCR method (Stratagene Inc.) to incorporate different amino acid residues. Mutations were verified by DNA sequencing.

**Purification of E. coli SppA.** E. coli BL21(DE3) cells harboring six-His-tagged SppA encoded by the pET-28(a) vector were used for overexpression of SppA. Wild-type SppA and its mutants were expressed and purified using cobalt affinity chromatography. A single colony was used to inoculate 100 mL of LB1 broth containing 25 µg/mL kanamycin. Twenty milliliters of this overnight cell culture was diluted into 2 L of LB broth with the same concentration of kanamycin. The cultures were grown at 37 °C until the OD600 reached 0.6, at which time they were induced with 1 mM IPTG (final concentration). After further growth for 5 h, the cells were harvested by centrifugation at 5000 rpm for 10 min with a JA-10 rotor. The cells were resuspended in lysis buffer [1 M NaCl, 25% sucrose, and 10 mM sodium phosphate (pH 7.0)] and lysed by ultrasonication. After removal of unbroken cells, the membrane fraction was spun in the centrifuge at 45000 rpm for 3 h using a Ti-70 rotor. The membrane fraction was then resuspended in extraction buffer [0.5 M NaCl, 0.5% Triton X-100, 40% glycerol, and 10 mM sodium phosphate (pH 7.0)] and stirred at 4 °C until the pellet was completely homogenized. After the centrifugation step had been repeated once more, the supernatant was isolated and applied to the affinity column. The Triton X-100 extract was loaded on the column with a 5 mL bed volume of TALON cobalt resin, which was equilibrated with the extraction buffer. The column was washed with wash buffer [300 mM NaCl, 0.5% Triton X-100, 5 mM imidazole, and 50 mM sodium phosphate (pH 7.0)]. The His-tagged protein was eluted with elution buffer [300 mM NaCl, 0.5% Triton X-100, 5 mM imidazole, and 50 mM sodium phosphate (pH 7.0)] with an increasing concentration of imidazole (50, 100, and 150 mM). Following elution, proteins were assayed for purity using a 12% SDS–PAGE gel and selected fractions were dialyzed four times with 1 L of 50 mM sodium phosphate (pH 7.0) with 1% Triton X-100 buffer to remove the imidazole.

**Assay of E. coli SppA Activity.** The activity of SppA, wild type and mutants, was measured against the chromophoric substrate Cbz-Val-ONP as follows (5). A total of 10 µg of purified enzyme was preincubated with 0.2% Triton X-100 and 10 mM sodium phosphate (pH 7.2) for 10 min at room
FIGURE 1: Alignment of the amino acid sequences of SppA protease members. (A) Amino-terminal domain of SppA proteases within the S49 family. (B) Carboxyl-terminal protease domain within the S49 family. Shown are the sequences for E. coli (Eco), Salmonella typhimurium (Sty), Vibrio cholerae (Vch), Haemophilus influenzae (Hin), Chlorobium tepidum (Cte), and Thermus aquaticus (Tko). Shown below are the consensus motifs for aligned SppA proteases. An asterisk denotes that the amino acids are identical in all sequences in the alignment. A colon means that conserved substitutions in the aligned sequences have been observed, according to the nature of the amino acid (hydrophobic, hydrophilic, acidic, or basic). A period means that semiconserved substitutions in the aligned sequences are found. The shaded residues in the carboxyl-terminal domain are invariant in all protease members in the S49 family. The shaded residue in the amino-terminal domain is absolutely conserved when the amino-terminal domain is present in the S49.001 group.

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temperature, and then 1 μL of 100 mM Cbz-Val-ONP was added. The reaction mixture (total volume of 1.0 mL) was incubated for 10 min at room temperature. The rate of p-nitrophenol release was monitored at 400 nm on a Perkin-Elmer UV–vis spectrophotometer.

FP-Biotin Reaction. A biotinylated fluorophosphonate (FP-biotin) chemical reagent was used to inactivate SppA. The reaction of wild-type, S409A, K209A, K366A, and H510A SppA with FP-biotin was performed as described by Liu et al. (20) with the following modifications. Twenty microliters of 0.1 mg/mL SppA was treated with 40 μM FP-biotin (final concentration) for 30 min at 37 °C. The reaction mixture was then divided into two aliquots. One aliquot was used for the activity assay. The other aliquot was used for the immunoblotting study after the reaction was quenched by adding 2 drops of chloroform and incubating them at 37 °C until the OD 420 was measured. The reaction was then divided into two aliquots. One aliquot was used for the alkaline phosphatase assay. For the plating assay, cells from 1 mL cultures and added directly to SDS–PAGE loading buffer. PhoA was detected by immunoblotting using the ECL Western blot detection kit (Amersham Biosciences).

RESULTS

Elucidating the Catalytically Important Conserved Residues of E. coli SppA. The serine protease SppA has a conserved carboxyl-terminal protease domain found in all S49 family members (Figure 1B) and a nonconserved amino-terminal domain (Figure 1A) found in the S49.001 and S49.004 groups. Recently, the archeon T. kodakaraensis SppA has been classified as a serine-lysine dyad protease (12). However, while the Ser residue found in the T. kodakaraensis lines up with the Ser 409 residue in E. coli SppA, the E. coli enzyme does not have a lysine at the homologous position as the archaean protease (Figure 1B, T. kodakaraensis Lys 214). There are also no conserved histidine residues in the SppA family of proteases (Figure 1; see sequence alignment), which could function as the general base in the Ser/His/Asp triad of conventional serine proteases. However, Arg 496 and Asp 524 are absolutely conserved in the S49 family (Figure 1B) and could be possible members of a variant catalytic triad or a novel serine-arginine dyad.

To identify the serine residue that could function as the active site nucleophile in E. coli SppA, we mutated the highly conserved Ser 374, Ser 402, Ser 409, and Ser 431 residues individually to Ala residues by site-directed mutagenesis. Mutant and wild-type sequence constructs with N-terminal six-His tags were cloned into a pET-28a vector, and high
levels of expression were observed. Expressed proteins were purified to homogeneity (Figure 2) using cobalt affinity chromatography (see Experimental Procedures). Protease activities were assayed using the chromophoric substrate N-benzylxoycarbonyl-L-valine p-nitrophenyl ester (Cbz-Val-ONP) (Figure 3). With the wild-type SppA protein, hydrolysis of the Cbz-Val-ONP substrate was rapid and linear with time (data not shown). Activity was also observed with the Ser374, Ser402, and Ser431 to alanine mutants. In contrast, the Ser409 mutant had background activity, indicating the Ser 409 is critical for enzymatic activity.

To assess the role in proteolysis of the invariant Arg496 and Asp524 amino acids of SppA, we altered these residues. Figure 3 shows that R496 is not required for SppA activity; substitution with lysine (Figure 3A) or alanine (data not shown) does not lead to a marked reduction in activity. Neither is Asp 524 important for activity as the SppA D524N mutant had no reduction in activity (Figure 3A). In fact, the activity of this mutant was reproducibly higher than that of the wild-type SppA enzyme.

We also investigated the highly conserved Tyr412 for its requirement for activity since it could act as a general base. Figure 3A shows that Y412F SppA was fully active, ruling out a role of this residue as a general base. Interestingly, the Gly 410 adjacent to the catalytically important Ser 409 is critical for optimal activity. SppA with the glycine mutated to alanine severely perturbs the SppA activity (Figure 3A). Substitution of the nonconserved histidine at positions 451 and 497 had only a small effect on activity, whereas mutation of histidine at position 510 impairs activity roughly 20-fold, although it still exhibits measurable activity (Figure 3B). The data together indicate that there is no histidine in the C-terminal domain of SppA that is essential for activity.

Since T. kodakaraensis SppA has been proposed to use a serine-lysine mechanism, we assessed the catalytic role of lysine residues in E. coli SppA by mutating the C-terminal domain Lys364, Lys366, Lys397, Lys486, Lys505, Lys518, Lys535, and Lys541 residues to Ala. As one can see in Figure 3B, SppA enzymes with these single mutations all maintained activity, indicating these residues are not essential for catalysis. The K366A mutation had the greatest effect on catalysis, although it was only 5-fold. These results rule out the possibility that a lysine residue within the carboxyl-terminal domain is the general base residue.

We next tested whether the absolutely conserved Lys209 in the amino-terminal domain of E. coli SppA is crucial for activity (Figure 3B). Strikingly, mutation of Lys 209 to alanine inactivates the SppA protease. The data show that Lys 209 in the amino-terminal domain is essential for the proteolytic reaction and is consistent with the E. coli SppA employing a lysine in catalysis.

Inhibition of SppA by FP-Biotin. To provide further evidence that Ser 409 is the active site serine residue in the E. coli SppA enzyme, we tested whether wild-type SppA, unlike the Ser409Ala mutant, is modified with the biotinylated fluorophosphonate inhibitor (FP-biotin), which has been shown to inhibit serine hydrolases (20). First, we tested whether the reagent FP-biotin inhibited the SppA enzyme. Figure 4A shows that the addition of FP-biotin significantly decreased the activity of the wild-type enzyme compared
with the mock treated enzyme. Next, we examined whether the FP-biotin treated enzyme is modified with the inhibitor but remains unchanged when the candidate active site Ser 409 residue is mutated. As one can see in Figure 4B, a streptavidin reactive band is detected on the immunoblot with wild-type SppA, but not with the S409A SppA mutant. These results support the notion that serine 409 is indeed the active site residue. Likewise, we examined whether Lys 209 is critical for activation of the Ser such that it can react with the FP-biotin inhibitor. Figure 4D shows that the K209A
SppA mutant only poorly reacts with the inhibitor in comparison to wild-type SppA. Since K366A and H510A exhibited low activity in Figure 3, we also included these mutants in the FP-biotin reaction assay. Both SppA mutants reacted with the FP-biotin inhibitor, although poorly, compared to the wild-type protein (Figure 4F). Note only one-third of the WT SppA sample was loaded onto the gel for immunoblot analysis compared to K366A and H510A samples (Figure 4F).

Topology of E. coli SppA. Hydropathy analysis and topology programs do not provide a clear indication of the membrane orientation of E. coli SppA. The topology program TMHMM predicts one transmembrane segment from residue 21 to 43 (23), while TopPred2 predicts residues 29–45, 398–414, and 421–441 are transmembrane segments (24). Both programs predict the amino terminus of SppA is localized to the cytoplasm and the carboxyl terminus protrudes into the periplasm.

The membrane topology of SppA was experimentally probed using the well-established PhoA fusion method (25). PhoA is enzymatically active only when it is exported to the periplasm; cytoplasmic PhoA is inactive. Therefore, fusion of PhoA to periplasmic domains of SppA would result in high PhoA activity, whereas fusion to cytoplasmic loops of SppA would result in low PhoA activity. To determine whether the C-terminal domain transmembrane segments predicted by TopPred2 were real, the PhoA mature sequence was fused to SppA after residues 377, 419, and 501. As a negative control, we used a construct in which PhoA was fused after the first hydrophobic domain of the leader peptidase. Previous studies with the H1Lep–PhoA fusion (H1) showed that PhoA is localized primarily to the cytoplasm, although the first apolar domain (without the carboxyl-terminal positively charged residues present) can export a small percentage of PhoA to the periplasmic space (27). All the PhoA fusion constructs were expressed in the PhoA− strain to determine the expression level and the PhoA activity of the PhoA fusion proteins. Figure 5 confirms by immunoblotting that the various PhoA constructs are expressed in the cell. We measured the activity of PhoA in liquid using the chromophoric substrate p-nitrophenyl phosphate. Table 1 summarizes the PhoA activities of the SppA fusion proteins. All the SppA fusion proteins gave high enzymatic activities of alkaline phosphatase; the normalized AP activities are 21.5, 28.5, 16.9, and 23.3 units for the 618 (full-length), 501, 419, and 377 SppA–PhoA constructs, respectively. The negative control (H1) gave a normalized AP activity of 3.7. The results are consistent with all regions of the C-terminal domain of SppA being periplasmic. Because there are no other candidates for TM segments other than the one at the beginning of the protein, the data support a model in which SppA spans the membrane one time with its large carboxyl-terminal domain and most of its N-terminal domain protruding into the periplasmic space.
DISCUSSION

In this report, we present two pieces of data that suggest that Ser 409 is directly involved in catalysis of *E. coli* SppA. The first datum is the fact that Ser 409 of *E. coli* SppA, which is homologous to the proposed active site serine residue in *T. kodakaraensis* SppA (12), is critical for enzymatic activity. Second, the FP-biotin serine hydrolase inhibitor modifies wild-type SppA but does not label the protein when Ser 409 is mutated, suggesting that Ser 409 is modified by the inhibitor (Figure 4).

Surprisingly, we found that Lys 209 is the best candidate for a general base residue for the *E. coli* SppA peptidase. This Lys 209 residue is localized to the nonconserved amino-terminal domain of *E. coli* SppA peptidase. All other candidates for a general base residue were found not to be essential for catalytic activity (Figure 3). Also we found that the mutagenesis of Lys 209 to an alanine markedly weakened the ability of the SppA to be modified with the FP-biotin inhibitor (Figure 4). This is consistent with Lys 209 being important for activation of the Ser 409 nucleophile such that it can be reactive toward substrates and inhibitors.

A serine and lysine residue is also implicated in the catalytic mechanism from the X-ray structure of *E. coli* SppA that was determined in the course of this work (Figure 6; 26). The 2.4 Å structure revealed that Ser 409 and Lys 209 are indeed active site residues for the *E. coli* SppA peptidase. The structure reveals that the *Oγ* atom of Ser 409 is within hydrogen bonding distance of the *Nζ* atom of Lys 209 and that there are no other titratable functional groups within the vicinity of the *Oγ* atom of Ser 409 other than the *Nζ* atom of Lys 209. In fact, given that there is no sequence similarity and no similarity in protein fold, the active site architecture of *E. coli* SppA is strikingly similar to that of the *E. coli* signal peptidase. It appears that bacteria have converged on the same catalytic mechanism (Ser/Lys) to both cleave off the signal peptide (13) and hydrolyze the remaining signal peptide. In eukaryotic organisms, these same processes are catalyzed by a Ser/His/Asp protease [within the signal peptidase complex (27)] and an aspartic protease mechanism [within the signal peptide peptidase (28)].

These data, along with recent studies (12, 29), suggest that all SppA proteases are serine-lysine dyad peptidases. Members of this family of proteases all contain an active site Ser in the protease domain. They differ only in the location of the critical lysine residue. Members of two of the subfamilies (*S49.001* and *S49.004*) have a highly conserved lysine in the amino-terminal domain (Figure 1A) that we showed (Figure 3) is crucial for the activity of *E. coli* SppA. Members of the other subfamilies (*S49.002*, *S49.003*, and *S49.006*) lack most of this domain and contain an absolutely conserved Lys residue in the protease domain located 52 residues (except for the *S49.005* subfamily which has Lys 41 residues downstream from the Ser) downstream from the invariant Ser [see the MEROPS protease database (7)]. Both the lysine and serine residues were shown to be critical for the activity of *T. kodakaraensis* SppA (*S49.006*) (12), and the structure of protein 1510-N from *Pyrococcus horikoshii* (*S49.005*) revealed a Ser and a Lys residue at the active site region (29).

In our mutagenesis work on *E. coli* SppA, we also discovered that mutation of G410, S402, H510, and K366 impairs activity (Figure 3). The structure of *E. coli* SppA shows that the G410 main chain nitrogen contributes to the oxyanion hole (26), which explains its importance in activity. If G410 is changed to any other residue, it would sterically compete for space with the carbonyl oxygen of the scissile bond. The structure of the SppA active site reveals that K209 may achieve the depressed p*Kₐ* that is required for it to function as a general base at biological pH by providing a hydrophobic microenvironment that shields the lysine from water. His 510 is located close to the K209 residue and has its polar side chain pointing away from the catalytic dyad. Changing H510 to a smaller residue may weaken the ability of the residues to shield the dyad from water, thus impairing activity. It is not clear from the structure why mutation of S402 impaired activity. Nor is it clear why a single mutation of K366 located on the charged patch at the large opening of SppA (26) would drastically affect the activity of SppA.

In addition to this work on the catalytic mechanism, we investigated the membrane topology of SppA. In contrast to what is predicted by several topology programs (23, 24), our results using alkaline phosphatase fusion analysis indicate that the *E. coli* enzyme spans the membrane only once and has its carboxyl-terminal domain, as well as the portion of the N-terminal domain containing the essential base, Lys 209, in the periplasmic space. Moreover, the recently determined X-ray structure of SppA, lacking the amino-terminal transmembrane segment, shows that previously predicted transmembrane segment 2 with the active site Ser 409 and transmembrane segment 3 are buried within the globular domain of SppA (26). The combined data indicate that SppA spans the membrane with an amino-terminal transmembrane segment and its globular domain located in the periplasmic space (Figure 6B).

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