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Use of Site-directed Chemical Modification to Study an Essential Lysine in *Escherichia coli* Leader Peptidase*

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Escherichia coli **leader peptidase, which catalyzes the cleavage of signal peptides from pre-proteins, is an essential, integral membrane serine peptidase that has its active site residing in the periplasmic space. It contains a conserved lysine residue that has been proposed to act as the general base, abstracting the proton from the side chain hydroxyl group of the nucleophilic serine 90. To help elucidate the role of the essential lysine 145 in the activity of *E. coli* leader peptidase, we have combined site-directed mutagenesis and chemical modification methods to introduce unnatural amino acid side chains at the 145-position. We show that partial activity can be restored to an inactive K145C leader peptidase mutant by reacting it with 2-bromoethylamine·HBr to produce a lysine analog (γ-thia-lysine) at the 145-position. Modification with the reagents 3-bromopropylamine·HBr and 2-mercaptoethylamine also allowed for partial restoration of activity showing that there is some flexibility in the length requirements of this essential residue. Modification with (2-bromoethyl)trimethylammonium·Br to form a positively charged, nontitratable side chain at the 145-position failed to restore activity to the inactive K145C leader peptidase mutant. This result, along with an inactive K145R mutant result, supports the claim that the lysine side chain at the 145-position is essential due to its ability to form a hydrogen bond(s) or to act as a general base rather than because of an ability to form a critical salt bridge. We find that leader peptidase processes the pre-protein substrate, pro-OmpA nuclease A, with maximum efficiency at pH 9.0, and apparent pK_a values for titratable groups at approximately 8.7 and 9.3 are revealed. We show that the lysine modifier maleic anhydride inhibits leader peptidase by reacting with lysine 145. The results of this study are consistent with the hypothesis that the lysine at the 145-position of leader peptidase functions as the active site general base. A model of the active site region of leader peptidase is presented based on the structure of the *E. coli* UmuD′, and a mechanism for bacterial leader peptidase is proposed.**

Escherichia coli leader (signal) peptidase is an integral membrane serine protease that functions to cleave off the amino-terminal leader (signal) sequence from proteins that are targeted to the cell surface of bacteria. Leader peptidase has been cloned (1), sequenced (2), overexpressed (3, 4), and purified (4, 5). The use of protease inhibitors to classify leader peptidase into a specific protease class has failed (6, 7). Site-directed mutagenesis studies have demonstrated that there is an essential serine 90 (8) and lysine 145 (9, 10) but no essential histidines or cysteines (8). The most convincing evidence for serine 90 being the nucleophile in the proteolytic reaction was provided by the work of Tschantz *et al.* (10) which showed that when serine 90 is replaced with a cysteine, leader peptidase is still active and that this thiol leader peptidase could then be inhibited by reacting it with the cysteine-specific reagent *N*-ethylmaleimide (10).

The essential serine and lysine are fully conserved within the type 1 prokaryotic and mitochondrial signal peptidases (11, 12). There are 19 lysines in the *E. coli* leader peptidase and only lysine 145 is conserved. Interestingly, the essential lysine is replaced by a histidine in the homologous yeast, chicken, and canine endoplasmic reticulum signal peptidase subunits (11, 12).

All evidence to date points toward leader peptidase utilizing a serine/lysine dyad mechanism. With this mechanism, the lysine 145 would act as the general base to abstract the proton from the hydroxyl group of the serine 90 side chain, thereby allowing for the nucleophilic attack on the scissile peptide bond of the translocated pre-protein substrate. Leader peptidase along with LexA (13), UmuD (14), and most recently, Tsp protease (15) represent the most thoroughly characterized members of the class (Clan) of serine proteases that contain an essential lysine but no essential histidine residue (16).

For lysine to act as a general base its side chain amine must be unprotonated. For this to be possible an enzyme must provide an environment for the lysine in which its pK_a would be depressed. The microenvironment near the lysine would include either a local positive charge or a hydrophobic surrounding. There are many examples of lysine residues that have significantly lower pK_a values as compared with the pK_a of 10.5 for lysine in solution (17, 18). One of the most carefully studied active site lysines is that of acetoacetate decarboxylase, which has a pK_a of 6.0 (19, 20). Moreover, there is crystallographic evidence that lysine is capable of serving as a general base for a serine residue in β-lactamase (21) and most recently in the structure of the *E. coli* UmuD′ protein (14). These two enzymes have catalytic sites that are superimposable (14).

To further investigate the role played by the essential lysine 145 in the catalysis of leader peptidase, we have combined site-directed mutagenesis and chemical modification. To simplify the interpretation of the results, we have constructed and purified an active mutant in which all native cysteine residues have been replaced with serine residues. This cysteine-less

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variant of leader peptidase will be referred to as No Cys[1] leader peptidase. We then made the K145C mutant in the No Cys leader peptidase to produce a single cysteine within the enzyme for modification. This inactive mutant, which will from now on be referred to as K145C, No Cys, regains activity after being reacted with the reagent 2-bromoethylamine·HBr to form the lysine analog γ-thia-lysine at the 145-position. We have measured with the wild-type enzyme the pH-rate profile using the pre-protein substrate pro-OmpA nuclease A. We also show that leader peptidase is inhibited by the lysine-modifying reagent maleic anhydride, and we provide evidence that the inactivation is due to its modification at lysine 145. In addition, we have modeled the active site of *E. coli* leader peptidase based on the structure of *E. coli* UmuD′ (14) and proposed a mechanism for leader peptidase. The study reported here is consistent with leader peptidase utilizing a serine/lysine dyad mechanism in its catalysis.

EXPERIMENTAL PROCEDURES

Materials—The 2-bromoethylamine·HBr (2BEA), 3-bromopropylamine·HBr (3BPA), (2-bromoethyl)trimethylammonium·Br (2BETMA), 2-mercaptoethylamine (2-MEA), and (*S*)-2-aminoethyl-L-cysteine·HCl were purchased from Sigma. The maleic anhydride was from Matheson Coleman & Bell. Oligonucleotides were synthesized at the Biochemical Instrument Center at The Ohio State University. The peptide substrate (NH_3^+-Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO-) was purchased from the macromolecular Structure Analysis Facility at the University of Kentucky.

Bacterial Strains and Plasmids—The leader peptidase proteins were expressed in MC1061 *E. coli* cells harboring the pING plasmid carrying the mutant leader peptidase gene. Six consecutive histidine residues were engineered by oligonucleotide-directed mutagenesis into the P1 (cytoplasmic) domain of leader peptidase that allowed us to purify the mutants from the chromosome-expressed wild-type leader peptidase. Briefly, amino acid residues 35–40 were substituted with histidine residues. The sequence of the oligonucleotide used is as follows: 5′-TTC GCA CCT AAA CGG CGG CGC GAA CGT CAT CAT CAT CAT CAT CAT GCT CGG GAC TCA CTG GAT AAA GCA-3′. The No Cys variant of *E. coli* leader peptidase was produced by using oligonucleotide-directed mutagenesis to replace the three cysteines at positions 21, 170, and 176 with serine residues. The oligonucleotides used to make the cysteine to serine mutations are described in Sung and Dalbey (8). The cloning of the pro-OmpA nuclease A gene into the isopropyl-β-D-thiogalactopyranoside-inducible plasmid pONF1 (22) and the overexpression of the protein were described by Chatterjee *et al.* (23).

DNA Manipulations—The DNA techniques were performed as described by Sambrook *et al.* (24). All cloning procedures used T4 kinase, T4 DNA ligase, Klenow, and restriction enzymes from Life Technologies, Inc.. Oligonucleotide-directed mutagenesis was performed as described by Zoller and Smith (25). Transformations followed the calcium chloride method of Cohen *et al.* (26).

Purification of the 6-His Tagged Leader Peptidase Proteins—The 6-His tag/nickel affinity chromatography method (27) was used to purify the overexpressed leader peptidase mutants away from the wild-type chromosome-expressed copies of leader peptidase. All leader peptidase proteins used in this study, except the wild-type, contained the 6-His tag. *E. coli* MC1061 cells containing the pING plasmid encoding the mutant leader peptidase protein were grown in M9 minimal media (1–8 liters) containing 100 μg/ml ampicillin until an absorbance of 0.5 at 600 nm was reached. Expression was induced by the addition of arabinose to a final concentration of 0.3%, and the incubation of the cultures was continued for 4 h. The cells were pelleted and then resuspended in an equal weight of 50 mM Tris, pH 7.5, 10% sucrose. The cells were frozen by dropping them into liquid nitrogen and stored at −80 °C until needed. 10 g of frozen cell nuggets were added to 25 ml of thaw buffer (50 mM Tris, pH 7.5, 20% sucrose) and thawed. Lysozyme (6 mg) and DNase (60 μl at 10 mg/ml) were added to the thawed cells and then stirred for 10 min. The mixture was then freeze/thawed in a dry ice/ethanol bath. 200 μl of 1 M magnesium acetate was added, and the solution was allowed to stir for 15 min at room temperature. The solution was then centrifuged at 18,000 rpm (4 °C, 30 min), and the pellet was resuspended in 25 ml of 10 mM triethanolamine, 10% glycerol, pH 7.5. The centrifugation step was then repeated once more. The pellet was resuspended by douncing in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol). The suspension was then centrifuged again at 18,000 rpm. The supernatant was loaded onto a 1-ml nickel column (Novagen resin) that was equilibrated with the same buffer. The column was then washed with 20 ml of binding buffer followed by a second wash with wash buffer (60 mM imidazole, 0.5 M NaCl, 10 mM Tris, pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol). The 6-His tagged leader peptidase was eluted by using an imidazole step gradient from 100 to 500 mM imidazole. Eluted fractions were assayed for protein by SDS-PAGE followed by Coomassie staining. Fractions containing 6-His leader peptidase were dialyzed against 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol and then stored at −80 °C.

Purification of the Pro-OmpA Nuclease A—The *E. coli* strain SB211 containing the plasmid pONF1 was used to overexpress the pro-OmpA nuclease A substrate, a hybrid of the signal peptide of the *E. coli* outer membrane protein A (OmpA) fused to staphylococcal nuclease A (22). The pro-OmpA nuclease A was expressed and purified as described by Chatterjee *et al.* (23).

Kinetic Assay Using Pro-OmpA Nuclease A—To determine the kinetic constants (V_{max}, k_{cat}, and K_m) of the wild-type and mutant leader peptidase proteins, we used pro-OmpA nuclease A as a substrate. Substrate concentrations were determined by using an $E_{1\%}$ at 280 nm of 8.3 (23). The cleavage reactions (75 μl) were run in TGC buffer (50 mM Tris, 50 mM glycine, 50 mM CAPS, 10 mM $CaCl_2$, 1% Triton X-100) at pH 9.0, unless indicated otherwise, containing the substrate at five different concentrations (35.2, 17.6, 13.2, 8.8, 4.4 μM). The reaction was initiated by the addition of leader peptidase (wild-type or mutant) at a final concentration of 1.37×10^{-4} μM, which was determined by the Pierce BCA protein assay kit. The reaction was carried out at 37 °C, and aliquots of the reaction were removed at various times such that less than 7% processing of the substrate was achieved. The reaction was stopped by the addition of 5 μl of 5 × sample buffer containing 10 mM $MgCl_2$, and the samples were frozen immediately in a dry ice/ethanol bath. The amount of pro-OmpA nuclease A that was processed by leader peptidase was assayed by SDS-PAGE on a 17.2% gel, followed by staining with Coomassie Brilliant Blue. The precursor and mature proteins were quantified by scanning the gels on a Technology Resources, Inc. Line Tamer PCLT 300 scanning densitometer. Percent processing was determined by dividing the area of the mature protein band by the sum of the mature and precursor band areas. The initial rates were determined by plotting the amount of product *versus* time. The V_{max}, K_m, and k_{cat} values were calculated from a $1/v_i$ versus 1/[S] plot (where v_i represents initial velocity and [S] indicates substrate concentration). We used the computer program Microcal Origins to plot the data and for linear regression analysis of the data. All values are from at least two different experiments.

pH Profile—The kinetic constants in the pH range 7 to 11 were measured using the substrate pro-OmpA nuclease A. The kinetic reactions were run as described above with the exception that the cleavage reactions were carried out in TGC buffer at the indicated pH values (7.0, 8.0, 8.5, 9.0, 9.5, 10.0, 11.0). Similar to the other kinetic assays, the amount of pro-OmpA nuclease A that was processed was quantified by SDS-PAGE and densitometry, and the kinetic parameters were extracted by using Lineweaver-Burk plots of the initial velocity values and substrate concentrations. The results plotted are the average of two separate experiments.

pH Stability Study—Leader peptidase (0.7 μg/ml) was assayed for its pH stability by dilution into TGC buffer at various pH values and incubated for 15 min (4 °C). 1 μl was then removed and added to 15 μl of pro-OmpA nuclease A (15 μM) in TGC buffer, pH 9.0, and incubated at 37 °C for 1 h. Reactions were terminated by the addition of 5 μl of 5 × sample buffer and then frozen. The amount of processing was determined by separating the precursor and the mature form by 17.2% SDS-PAGE, followed by Coomassie Brilliant Blue staining. The data were analyzed as described above using scanning densitometry. Results plotted are the average of two separate experiments.

Chemical Modification of the K145C, No Cys Leader Peptidase—The

[1] The abbreviations used are: No Cys, the cysteine-less variant of leader peptidase, all native cysteine residues were changed to serine; PAGE, polyacrylamide gel electrophoresis; OmpA, outer membrane protein A; 2BEA, 2-bromoethylamine·HBr; 3BPA, 3-bromopropylamine·HBr; 2MEA, 2-mercaptoethylamine; 2BETMA, (2-bromoethyl)trimethylammonium·Br; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CAPS, 3-(cyclohexylamino)propanesulfonic acid; TGC buffer, Tris/glycine/CAPS buffer; Me_2SO, dimethyl sulfoxide; K145C, No Cys, the leader peptidase mutant containing a unique cysteine at the 145-position; mutation nomenclature used, *e.g.* K145C, the native lysine at position 145 is changed to cysteine.

TABLE I

Kinetic parameters for the cleavage of the pre-protein substrate proOmpA nuclease A by the wild-type, mutant, and chemically modified E, coli leader peptidase

All kinetic parameters were measured at pH 9.0. All enzymes, except the wild-type (WT) contain the 6-His tag and were purified by the 6-His tag/nickel affinity chromatography method (27).

Residue 145	Side chain	Lep nomenclature[a]	k_{cat}	K_m	k_{cat}/K_m
			s^{-1}	μM	$s^{-1}M^{-1}$
Lysine	$\sim CH_2CH_2CH_2CH_2NH_3+$				
(Wild type)		WT	120.0 ± 10.7	10.9 ± 2.8	$(1.1 \pm 0.2) \times 10^7$
(No Cys)		No Cys	110.2 ± 0.1	20.6 ± 6.8	$(5.4 \pm 1.0) \times 10^6$
(R146A)		R146A	36.8 ± 11.0	29.0 ± 3.0	$(1.3 \pm 0.2) \times 10^6$
Cysteine	$\sim CH_2SH$	K145C, No Cys	[b]	[b]	[b]
S-(Aminoethyl)-cysteine (γ-Thia-lysine)	$\sim CH_2SCH_2CH_2NH_3+$	K145C-EA, No Cys	1.2 ± 0.1	20.1 ± 2.1	$(6.1 \pm 1.1) \times 10^4$
S-(Aminopropyl)-cysteine (γ-Thia-homo-lysine)	$\sim CH_2SCH_2CH_2CH_2NH_3+$	K145C-PA, No Cys	0.8 ± 0.1	21.2 ± 5.1	$(4.2 \pm 0.5) \times 10^4$
S-(Aminoethylthio)-cysteine (γ-Dithio-homo-lysine)	$\sim CH_2SSCH_2CH_2NH_3+$	K145C-MEA, No Cys	0.3 ± 0.1	21.1 ± 2.4	$(1.5 \pm 0.6) \times 10^4$
S-[(Trimethylamino)ethyl] cysteine (4-Thialaminine)	$\sim CH_2SCH_2CH_2N(CH_3)_3+$	K145C-ETMA, No Cys	[b]	[b]	[b]

[a] The K145C mutant had all other cysteine residues mutated to serine (K145C, No Cys). Lep, leader peptidase.
[b] The following mutants showed no detectable activity K145C, K145C-ETMA, K145A, K145R, K145H.

reactions were carried out basically as described by Smith and Hartman (28). Briefly, leader peptidase at a concentration of 1–4 mg/ml in 50 mM Tris-HCl, pH 8.5, 1% Triton X-100 was treated with a sufficient amount of freshly prepared 2 M 2BEA (18, 28–36), 2 M 3BPA (29, 30), 2 M 2BETMA (30, 31), or 2 M 2-mercaptoethylamine (2MEA) (29) such that the final concentration of reagent was 100 mM. All reagents were titrated to pH 8.5 before adding them to the enzyme. The reaction solution was then covered with nitrogen and incubated at room temperature overnight. The reaction mixture was then dialyzed against 2 volumes of 4.5 liters of 50 mM Tris-HCl, pH 8.0, 1% Triton X-100 at 4 °C. All buffers and solutions were purged with nitrogen. The extent of reaction was assayed by quantification of cysteine residues with DTNB before and after reaction with the reagents. The extent of the 2BEA reaction was also quantified directly by amino acid composition.

Quantifying Cysteines with DTNB—A solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 100 μl at 10 mM) in 0.1 M phosphate, pH 7.28, 1 mM EDTA was added to 275 μl of 4 mg/ml leader peptidase and 625 μl of 6.4 M guanidine HCl. This mixture was incubated for 15 min at room temperature, and then its absorbance was measured at 412 nm. A molar absorbance coefficient of 13,700 M^{-1} cm^{-1} was used to calculate the moles of cysteine present in the samples (37). All solutions were made up fresh and purged with nitrogen. The concentration of the protein was determined using the Pierce BCA method. The K145C, No Cys leader peptidase was stored in reducing conditions. Before the DTNB reaction, or other modifications, the β-mercaptoethanol was dialyzed away in nitrogen-purged buffer (2 volumes of 4.5 liters of 50 mM Tris-HCl, pH 8.0, 1% Triton X-100) at 4 °C.

Amino Acid Compositional Analysis—To directly quantify the extent of aminoethylation of the cysteine 145, we measured the appearance of a γ-thia-lysine residue within the leader peptidase protein by using (*S*)-2-aminoethyl-L-cysteine·HCl (Sigma) as a standard in the amino acid compositional analysis. The amino acid analysis was performed at the W. M. Keck Foundation Biotechnology Research Laboratory in New Haven, CT, and the Biochemical Instrument Center at The Ohio State University.

Maleic Anhydride Reaction—In the time-dependent studies, maleic anhydride dissolved in Me_2SO was added to leader peptidase (0.07 mg/ml, in 10 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 2.5% Triton X-100) to a final concentration of 0.5 mM, and samples were removed at 0, 10, 30, and 60 min (38). In the concentration-dependent studies, leader peptidase (0.07 mg/ml, in 10 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 2.5% Triton X-100) was incubated at room temperature with maleic anhydride at a final concentration of 0.10, 0.25, 0.30, 0.50 mM for 60 min. After the modification, the reaction mixture was added to 12.5 μl of peptide substrate (1.5 mg/ml; NH_3^+-Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO-) (39) and incubated for 3 h at 37 °C. Reactions were terminated by the addition of an equal volume of 0.1% trifluoroacetic acid and microcentrifuged for 5 min at room temperature to pellet any particulate matter. Samples were then analyzed by high performance liquid chromatography. The elution gradient utilized to separate the cleaved from the uncleaved peptide was as follows: 97% A, 3% B held constant for 5 min, followed by a linear gradient to 60% A, 40% B over a 10-min period. This mixture was then held constant for 5 min and then brought back down to 97% A, 3% B over a 5-min period. The solvents used were A = 0.1% trifluoroacetic acid and B = 0.1% trifluoroacetic acid in acetonitrile. Peptide products were detected spectrophotometrically at 218 nm. The column used was a 25-cm Vydac C18 column. Percent processing of the peptide was determined by quantification of the 7-mer product peptide peak and the 9-mer substrate peak: % processing = (area 7-mer peak/(area 7-mer peak + area 9-mer peak)) × 100.

Combined Maleic Anhydride and 2-Bromoethylamine Reaction—A total of 0.007 μmol of the No Cys or the K145C, No Cys mutant leader peptidase was first reacted with 0.7 μmol of DTNB and then dialyzed against 50 mM Tris-HCl, 0.5% Triton X-100, pH 8.5 (buffer A). The mutants were then reacted with 2.3 μmol of maleic anhydride, incubated at room temperature for 30 min, and then dialyzed against buffer A. The cysteine in the K145C, No Cys mutant was then deprotected by reaction with β-mercaptoethanol followed by dialysis. These samples (60 μl) were then reacted overnight at room temperature with 10 μl of freshly prepared 2 M 2-bromoethylamine·HBr. This product was then reacted with maleic anhydride again. All reagents were made up fresh in nitrogen-purged buffer. After each reaction the samples were dialyzed extensively (overnight against 9 liters of nitrogen purged buffer A). The activity of the leader peptidase enzymes was assayed before and after each chemical modification step.

Alignment of Leader Peptidase with UmuD′ and Modeling the Leader Peptidase Active Site—We have aligned the amino acid sequence of the catalytic region from the solved structure of UmuD′ (residues 40–139, the total length of the UmuD protein is 218 residues) with the corresponding proposed catalytic region of leader peptidase (residues 75–202, the total length of leader peptidase is 323 amino acid residues). The alignment protocol XALIGN (40, 41) used is a derivative of the NW_ALIGN program originally developed for SEQSEE (41). The pairwise alignment module implemented in the comparison of UmuD′ and leader peptidase is based on the Needleman-Wunsch dynamic programming algorithm (42), and the sequence/structure alignment algorithms are based loosely on the protocols described by Lesk *et al.* (43). Residue anchoring and residue clustering features are analogous to the gap and extension penalties incorporated into regular dynamic programming schemes (40). The appropriate substitutions of the UmuD′ residues as well as manual manipulations were done using the program TOM-frodo (44). The coordinates for the crystal structure of UmuD′ were kindly provided by T. Peat and W. Hendrickson (14). The rendering of the modeled active site region and hydrophobic cleft adjacent to the active site of *E. coli* leader peptidase was created using the program *Raster*3D (45).

RESULTS

Purification of Mutants—The use of the 6-His tag/nickel affinity chromatography method (27) of purification has allowed us to purify the overexpressed mutants of leader peptidase away from the wild-type leader peptidase expressed by the *E. coli* chromosome. Due to leader peptidase's excellent kinetic properties, background activity from the chromosomal

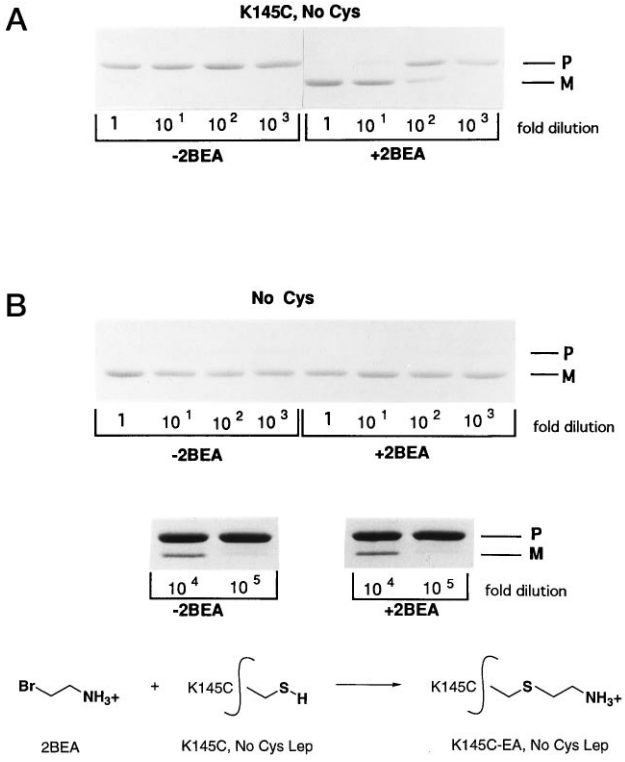

FIG. 1. **Restoring activity to the leader peptidase mutant K145C, No Cys via modification with 2BEA.** *A,* a 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 μl at 11.75 μM) (*P*) to the mature form (*M*) after a 1-h incubation with 1 μl of each dilution (starting at 2.7 μM) of the K145C, No Cys mutant of leader peptidase before and after modification with 2BEA. *B,* as a control the same experiment was run with the No Cys leader peptidase mutant containing the native lysine at the 145-position. All leader peptidase samples were purified by the 6-His tag method (see "Experimental Procedures"). The extent of the aminoethylation reaction was accessed by quantitation of free cysteines by DTNB (see Table II) and by direct amino acid compositional analysis using (*S*)-2-aminoethyl-L-cysteine as a standard. See "Experimental Procedures" for more details on the reactions and the analysis of the results. See Table I for results of kinetic reactions.

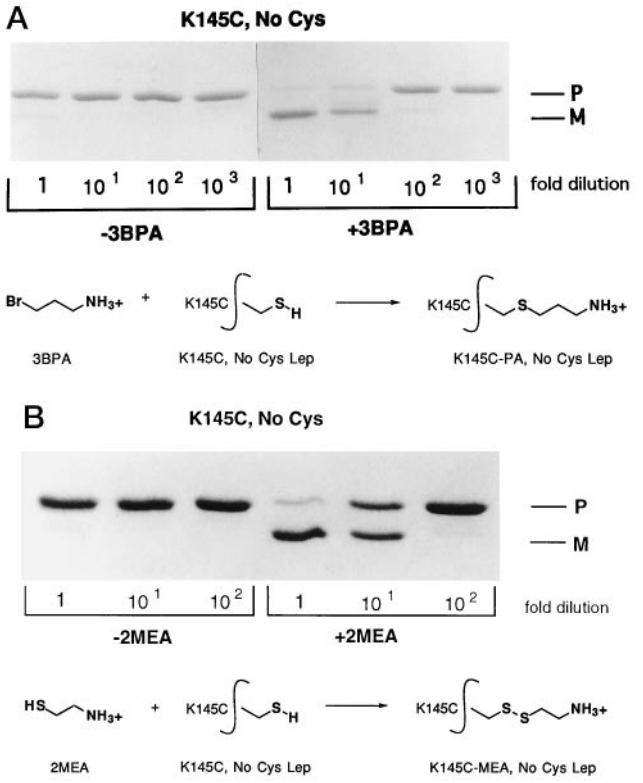

FIG. 2. **Examination of the length requirement of the side chain at the 145 position of leader peptidase.** *A,* restoring activity to the leader peptidase mutant K145C, No Cys via modification with 3BPA. A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 μl at 11.75 μM) (*P*) to the mature form (*M*) after a 1-h incubation with 1 μl of each dilution (starting at 2.7 μM) of the K145C, No Cys mutant of leader peptidase before and after modification with 3BPA. The extent of modification was assessed by quantifying the cysteine residues with DTNB before and after the reaction with 3BPA (see Table II). See "Experimental Procedures" for details of the analysis. *B*, restoring activity to the leader peptidase mutant K145C, No Cys via modification with 2MEA. A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 μl at 11.75 μM) (*P*) to the mature form (*M*) after a 1-h incubation with 1 μl of each dilution (starting at 2.7 μM) of the K145C, No Cys mutant of leader peptidase before and after modification with 2MEA. The extent of modification was assessed by quantitating the cysteine residues with DTNB before and after the reaction with 2MEA (see Table II). Other details of the modification of the K145C, No Cys mutant are described under "Experimental Procedures."

wild-type copies of leader peptidase was always a concern when assessing the activity of overexpressed mutants. A mock purification run was performed to demonstrate that there was no detectable background wild-type leader peptidase activity from this purification procedure. Cells containing the expression vector with no leader peptidase gene insert were lysed and brought through the nickel affinity column procedure. All fractions eluted from the column showed no detectable activity (data not shown). The yield of each purified mutant was approximately the same. We obtained typically 4.6 mg of purified K145C, No Cys from 10 g of frozen cell nuggets (see "Experimental Procedures").

Activity of the Wild-type and Mutant 6-His Tagged Leader Peptidases—The wild-type leader peptidase showed very impressive catalytic constants (k_{cat} = 120 s^{-1}, K_m = 10.9 μM, k_{cat}/K_m = 1.1 × 10^7 s^{-1} M^{-1}) when analyzed with the pre-protein substrate pro-OmpA nuclease A at its optimal pH of 9.0 (see pH profile below and Table I). Similarly, the No Cys variant of leader peptidase with a 6-His tag showed an almost wild-type k_{cat} value of 110 s^{-1}, yet its K_m of 20.6 μM was approximately double the wild-type value. In addition, the conserved arginine residue at the 146-position of leader peptidase does not play a critical role in catalysis. The R146A mutant showed only slightly depressed kinetic constants (k_{cat} = 36.8 s^{-1}, K_m = 29.0 μM, k_{cat}/K_m = 1.3 × 10^6 s^{-1} M^{-1}). Finally, all substitutions at the 145-position inactivated the purified protein. The mutants K145A, K145R, K145H, and K145C, No Cys purified by the 6-His tag/nickel affinity chromatography method showed no detectable activity (Table I).

Restoration of Activity to the Inactive Leader Peptidase Mutant K145C, No Cys—We were able to restore partial activity to an inactive K145C, No Cys mutant of leader peptidase by reacting it with the reagent 2BEA (Fig. 1*A*). Fig. 1*A* shows significant processing of the pro-OmpA nuclease A substrate at 1- and 10-fold dilutions of the 2BEA-modified leader peptidase. This chemically modified K145C, No Cys mutant has a k_{cat} value that is approximately 100-fold lower than that of the wild-type leader peptidase (see Table I). The control experiment in which we have used the No Cys mutant, containing the native lysine at the 145-position, showed no change in the activity upon incubation with the 2BEA (Fig. 1*B*).

Modification of the K145C, No Cys mutant with the reagents 3BPA and 2MEA also restored activity to this inactive mutant, although to a slightly lower extent (Fig. 2, *A–B*, and Table I). The latter recovery in activity by 2-mercaptoethylamine is most likely due to formation of a lysine analog at cysteine 145

TABLE II
Extent of reaction assayed by quantitation of cysteines with DTNB
See "Experimental Procedures" for specifics on the analysis. Lep, leader peptidase.

Reagent	Moles of Cys/mol of Lep before reaction	Moles of Cys/mol of Lep after reaction
2-Bromoethylamine · HBr	0.95 ± 0.11	0.09 ± 0.07
3-Bromopropylamine · HBr	1.13 ± 0.09	0.16 ± 0.11
2-Mercaptoethylamine	0.89 ± 0.14	0.11 ± 0.07
(2-Bromoethyl)trimethylammonium · Br	0.97 ± 0.17	0.07 ± 0.04

as reaction of the modified leader peptidase with a large excess of β-mercaptoethanol resulted in the disappearance of the restored activity (data not shown). The 2MEA reaction goes nearly to completion (see Table II). The disulfide bond formation between the reagent and the free cysteine would most likely occur during the dialysis step when the reagent would not be in great excess (see "Experimental Procedures").

Modification of the K145C, No Cys leader peptidase mutant with the reagent (2-bromoethyl)trimethylammonium·Br to form the non-titratable quaternary amine lysine analog (4-thialaminine) at the 145-position showed no detectable restoration of activity (Fig. 3). No recovery in activity of the K145C, No Cys mutant is seen even after treating the protein for 24 h with 2BETMA. In contrast, recovery is seen after 2 h of treatment with 2BEA. This is consistent with lysine 145 being involved in a critical hydrogen bond or serving as a general base in the catalysis.

To assess the extent of reaction between the K145C, No Cys mutant of leader peptidase with each of the above reagents, we used DTNB to quantitate the number of cysteines with and without the modification reaction. We have found that all of these reactions went nearly to completion (Table II). The extent of modification by 2BEA was also directly measured by amino acid compositional analysis using the standard (*S*)-2-aminoethyl-L-cysteine·HCl (Sigma). We saw the appearance of 1.1 residues of aminoethylated cysteine (γ-thia-lysine) upon reaction with 2BEA.

The pH-rate Profile of Wild-type Leader Peptidase Using a Pre-protein Substrate—The pH-rate profile of leader peptidase using the pre-protein substrate pro-OmpA nuclease A measured by plotting its k_{cat}/K_m *versus* pH, reveals a classic bell-shaped curve having a maximum activity at approximately pH 9.0 (Fig. 4*A*). The dependence of k_{cat}/K_m on pH is consistent with two ionizable catalytic residues within the free enzyme with apparent pK_a values of 8.7 and 9.3. Leader peptidase appears to be stable up until pH 11 (Fig. 4*B*).

Inhibition of Leader Peptidase with Maleic Anhydride—We have found that maleic anhydride inhibits leader peptidase in a concentration-dependent (Fig. 5*A*) and time-dependent (Fig. 5*B*) manner. The addition of 0.5 mM maleic anhydride to leader peptidase rapidly inactivated the enzyme with 80% loss in activity in 10 min (Fig. 5*B*). To investigate whether this inhibition with maleic anhydride was due to the modification of lysine 145, we reacted the K145C, No Cys mutant with maleic anhydride to modify all accessible lysines. We then attempted to react the cysteine at the 145-position with 2BEA to restore activity and also produce a single accessible amine that then could be modified by maleic anhydride with subsequent inhibition of activity. Our preliminary experiments had shown that after treatment with maleic anhydride the activity of K145C, No Cys leader peptidase was no longer recoverable (data not shown). Maleic anhydride is known to react with thiol groups as well as amino groups (46). Therefore we protected the cysteine by reacting it with DTNB to form the 2-nitro-5-thiobenzoic acid-protected cysteine before reacting it with maleic

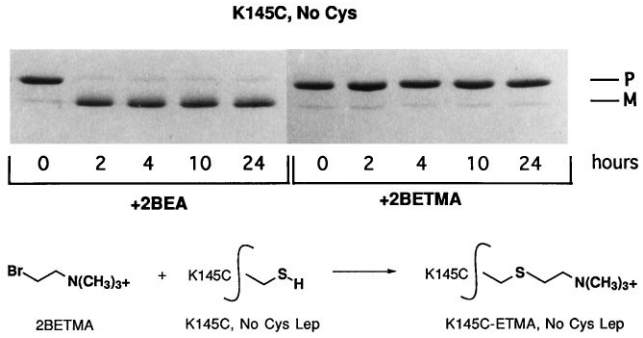

FIG. 3. **A positively charged, nontitratable lysine analog at the 145-position does not restore activity to K145C, No Cys leader peptidase.** A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 μl at 11.75 μM) (*P*) to the mature form (*M*) after a 1-h incubation of the K145C, No Cys mutant of leader peptidase (1 μl at 2.7 μM) reacted with either 2BEA or 2BETMA for various lengths of time. The extent of reaction at 24 h was assessed by quantifying the cysteine residues with DTNB before and after the reaction with 2BETMA or 2BEA (see Table II).

anhydride. We were then able, after deprotection with β-mercaptoethanol, to recover the activity of the K145C, No Cys leader peptidase by aminoethylation and then finally inhibit this recovered activity with maleic anhydride (Fig. 5*C*). The recovered activity from the DTNB/maleic anhydride/β-mercaptoethanol/2BEA reaction was approximately 33% of that seen from the 2BEA reaction alone (data not shown). Maleic anhydride is so far the only lysine-specific reagent we have found that inhibits leader peptidase to a significant extent.

Sequence Alignment of Leader Peptidase with UmuD′ and Modeling of the E. coli Leader Peptidase Active Site—As a first step toward obtaining an idea of what the active site of leader peptidase may look like, we have modeled the active site region of *E. coli* leader peptidase based on the x-ray crystal structure of *E. coli* UmuD′ (14). Previously, van Dijl and colleagues (47) have shown that these proteins are structurally and functionally related. The residue anchoring feature of the alignment program XALIGN proved to be particularly important in the alignment of UmuD′ and leader peptidase. High scoring pairwise alignments were only achieved when the two catalytic residues of the Ser/Lys dyad in each of the proteins were constrained to match. There is a 23.4% sequence identity and 37.2% sequence similarity (conservative substitutions) between the UmuD′ (residues 40–139) and leader peptidase (residues 75–202) (Fig. 6*A*). The regions of highest homology surround the putative catalytic residues (Ser-90/Lys-145 in leader peptidase and Ser-60/Lys-97 in UmuD′). There is a 31.1% identity and 53.3% sequence similarity (conservative substitutions) between UmuD′ and leader peptidase when comparing these aligned sequences between Pro-48 and Lys-98 (in UmuD′). Prior studies comparing the sequences of UmuD and *Bacillus subtilis* signal peptidase (SipS) revealed a 25% identity and 42% similarity between these proteins (47). A model of the leader peptidase active site was built by replacing the residues within the UmuD′ structure with the corresponding aligned residue from leader peptidase. The model of the leader peptidase active site reveals a shallow hydrophobic cleft just adjacent to the catalytic site. This hydrophobic cleft involves Ile-144, Leu-95, Phe-192, Ile-101, Ile-86, Val-103, Val-131, and Phe-84 (Fig. 6*C*). Similar to UmuD′, the proposed catalytic serine and lysine residues appear to be buried within a hydrophobic environment (Fig. 6, *B* and *C*).

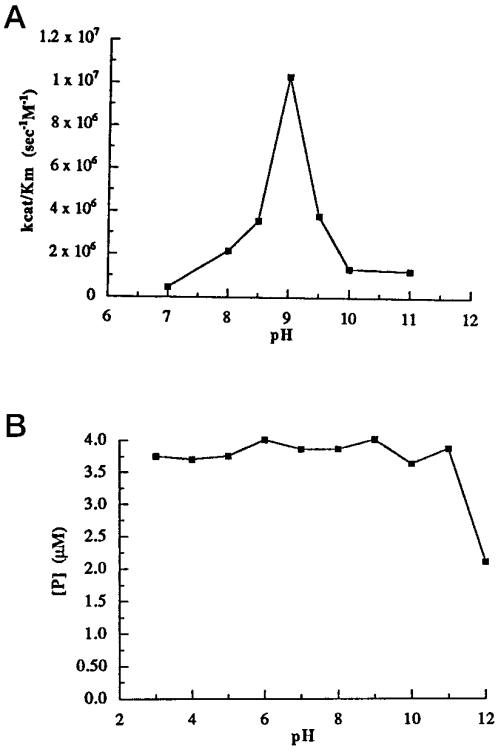

FIG. 4. *A*, pH-rate profile of leader peptidase. The kinetic constants in the pH range 7 to 11 were measured using the pre-protein substrate pro-OmpA nuclease A. The cleavage reaction was carried out in TGC buffer (50 mM Tris, 50 mM glycine, 50 mM CAPS, 10 mM $CaCl_2$, 1% Triton X-100) at the indicated pH values. The amount of pro-OmpA nuclease A that was processed was assayed by SDS-PAGE on a 17.2% gel, followed by staining with Coomassie Brilliant Blue and quantitated by densitometry (for specific reaction conditions and analysis see "Experimental Procedures"). *B*, pH stability of leader peptidase. Leader peptidase was assayed for its pH stability by dilution into TGC buffer at various pH values. Leader peptidase (0.7 μg/ml) was incubated for 15 min at 4 °C at the various pH values, and then 1 μl was removed and added to 15 μl of pro-OmpA nuclease A (15 μM) in TGC buffer, pH 9.0, and incubated at 37 °C for 1 h. The amount of pro-OmpA nuclease A that was processed was assayed by SDS-PAGE on a 17.2% gel, followed by staining with Coomassie Brilliant Blue. The concentration of the product formed [*P*] was determined from the percent processing using a scanning densitometer.

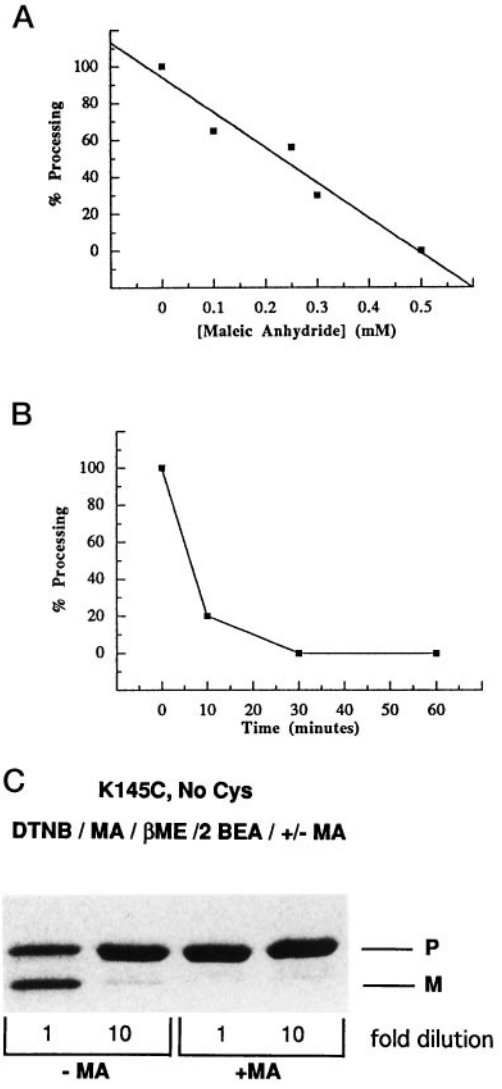

FIG. 5. *A*, the inhibition of leader peptidase by maleic anhydride with respect to maleic anhydride concentration. Leader peptidase was incubated at room temperature with maleic anhydride at a final concentration of 0.10, 0.25, 0.30, or 0.50 mM for 60 min. After the reaction, leader peptidase was assayed for activity using a peptide substrate (NH_3^+-Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO-) and high performance liquid chromatography analysis. See "Experimental Procedures" for specific reaction and analysis conditions. *B*, the inhibition of leader peptidase by maleic anhydride with respect to time. Maleic anhydride dissolved in Me_2SO was added to a final concentration of 0.5 mM, and samples were removed at 0, 10, 30, and 60 min. After the modification, leader peptidase (70 μg/ml) was assayed for activity using a peptide substrate (NH_3^+-Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO-) and high performance liquid chromatography analysis. See "Experimental Procedures" for specific reaction and analysis conditions. *C*, modification of lysine 145 with maleic anhydride inhibits leader peptidase. Protection of Cys-145 in the K145C, No Cys mutant of leader peptidase with DTNB allows for the modification of lysines with maleic anhydride (*MA*) followed by deprotection of Cys-145 with β-mercaptoethanol (*βME*) and then restoration of activity by reaction with 2BEA. This restored activity can then be inhibited by maleic anhydride. A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (*P*) to the mature form (*M*) after a 1-h incubation with dilutions of the K145C, No Cys mutant of leader peptidase before and after the final maleic anhydride modification. See "Experimental Procedures" for the details of each of the above reactions.

DISCUSSION

In prior work, we have shown that substituting lysine at the 145-position of leader peptidase with alanine, asparagine, or histidine resulted in an inactive enzyme, demonstrating that this residue was important for activity (10). To further investigate the role of lysine 145 we have purified, using the 6-His tag/nickel affinity chromatography method (27), a variety of mutants with substitutions at the 145-position and measured the activity using an assay that has a sensitivity of 100,000-fold over background. It is interesting that the mutant K145R, which maintains the positive charge at this residue, was found to show no detectable activity even when 100 μg of enzyme was added to the reaction (data not shown). Replacing the lysine 145 with a histidine also resulted in an inactive enzyme. This is consistent with our earlier results (10) and implies that if the intriguing exchange of lysine for histidine is significant, in going from prokaryotic to eukaryotic signal peptidases (11, 12), there are certainly other important structural differences as well.

It is striking that in another inactive mutant, K145C, No Cys, there is significant restoration of activity upon reaction with 2-bromoethylamine·HBr. This is a well characterized chemical modification reaction and, when combined with site-directed mutagenesis, is a convenient way to introduce an unnatural amino acid into a specific position within an enzyme (18, 28–36). Converting cysteines to aminoethylcysteine (γ-thia-lysine) residues has also been used in the past to create

A

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UmuD: LNQLLIQHPSATYFVKASGDSMIDGGISDGDLLIVDS-AITASH------     82
 Lep: IVRSFIYEP---FQIP-SG-SMMPT-LLIGDFILVEKFAYGIKDPIYQKT    118
                          *
                         90

UmuD: ---------GDIVIAAVDGE---FTVKKLQL-RPT-VQLIPMNSA--YSP    116
 Lep: LIENGHPKRGDIVVFKYPEDPKLDYIKRAVGLPGDKVTYDPVSKELTIQP    168
                                 *
                               145

UmuD: ITIS-S--EDTL--DVFGV----VIHVV--KAMR                    139
 Lep: GCSSGQACENALPATYSNVEPSDFVQTFSRRNGG                    202
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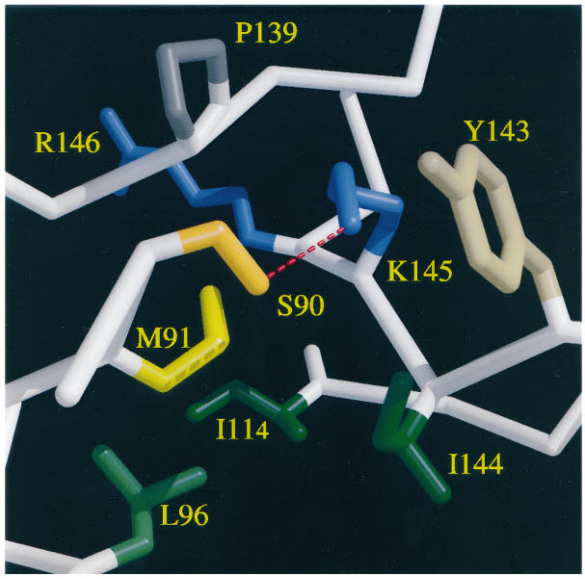
B



C

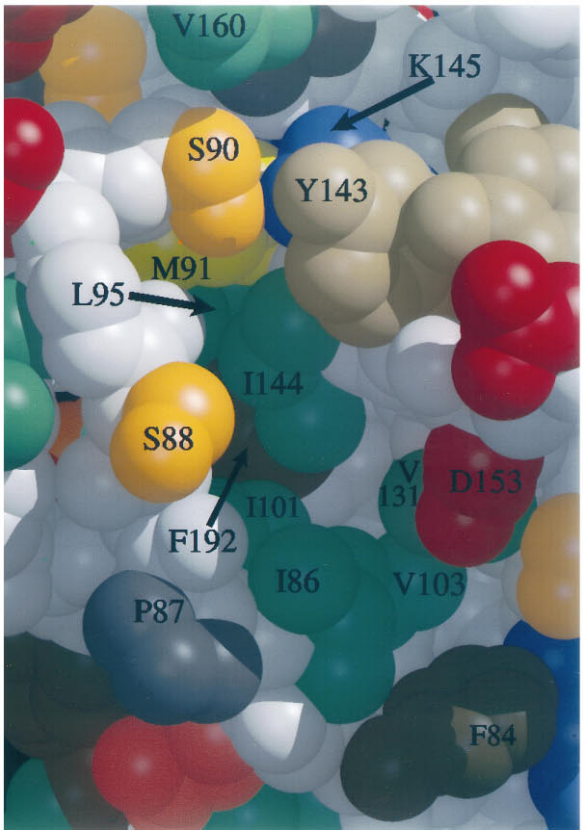


FIG. 6. **Model of the *E. coli* leader peptidase active site based on the *E. coli* UmuD′ crystal structure.** *A*, the sequence alignment of *E. coli* leader peptidase (residues 75–202) and *E. coli* UmuD′ (residues 40–139). The protocol XALIGN (40, 41) was used to perform this alignment. The proposed active site residues of leader peptidase are marked by *asterisks*. *B*, ball-and-stick rendering (45) of the modeled active site region of *E. coli* leader peptidase based on the structure of *E. coli* UmuD′ (14). *C*, space-filling representation (45) of the hydrophobic cleft adjacent to the active site of *E. coli* leader peptidase, modeled from the structure of *E. coli* UmuD′ (14). See "Experimental Procedures" and results for details of the alignment and modeling programs and procedures.

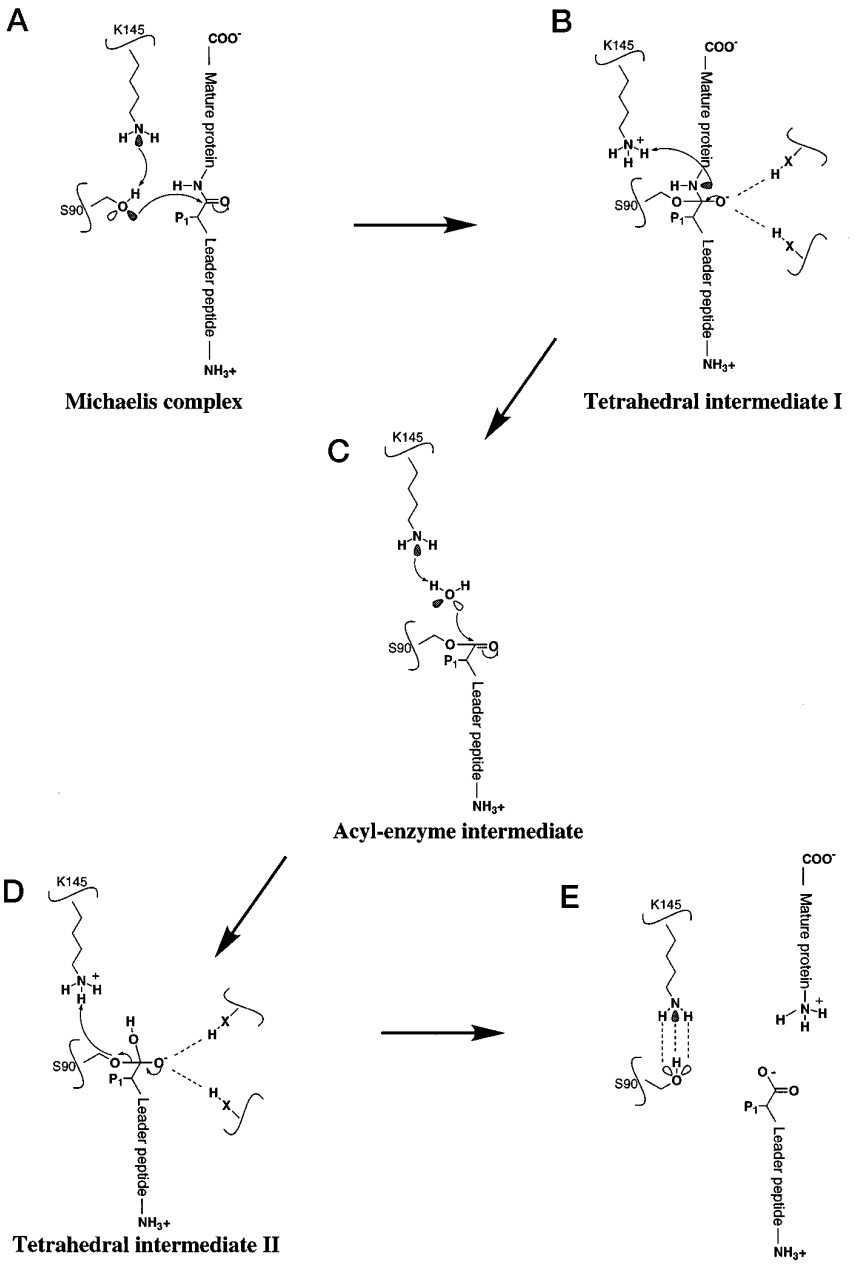


FIG. 7. **The proposed mechanism of *E. coli* leader peptidase based on site-directed mutagenesis and chemical modification studies.**

new trypsin-susceptible sites in proteins (35, 36). The fact that we did not observe total restoration of activity (approximately 1% of the wild-type activity) is somewhat surprising since the side chain of the γ-thia-lysine is only subtly different from that of lysine. The only difference in the proteins would be the substitution of one thioether group for a methylene group. The bond length of C-C is 1.54 Å, whereas that of C-S is 1.82 Å, and the bond angle of C-C-C is 109°, whereas that of C-S-C is 105° (18). Model building studies indicate that the primary amine group in γ-thia-lysine and lysine can be superimposed to within 0.1 Å (30). To date, there has not been a reported crystal structure of a protein containing a γ-thia-lysine. Although there has been a report of a small molecular structure of the amino acid itself, the authors (48) state that due to unexpected bond length and torsion angles in this structure it would be difficult to predict the C2 to C5 separation distance of a γ-thia-lysine in a macromolecule from this structure.

Other studies that have utilized aminoethylation to restore activity to an inactive lysine to cysteine mutant include ribonuclease A (30), aspartate aminotransferase (18), and ribulosebisphosphate carboxylase/oxygenase (28). These studies have also reported incomplete restoration of activity by aminoethylation (8, 7, and 60%, respectively). The fact that the functionally restored leader peptidase K145C-EA, No Cys (lysine 145 changed to aminoethyl cysteine) shows a reduced k_{cat} value, and yet a normal K_m value (as compared with the No Cys mutant, Table I) is consistent with the lysine 145 being directly involved in catalysis. In these studies we have removed the other cysteines in leader peptidase to simplify the interpretation of the aminoethylation results. The cysteine-less (No Cys) leader peptidase had almost wild-type activity (Table I).

As a control, we confirmed that the 2BEA reagent had no effect on the activity of the No Cys mutant (Fig. 1*B*.) showing that the restoration of the activity upon the reaction with 2BEA is from the aminoethylation of the cysteine 145 residue. If the restoration of the activity in the K145C, No Cys mutant were due to modification of some other residue other than the cysteine 145, the addition of 2BEA to the No Cys mutant would have increased the activity in this enzyme as well. The inactive K145H mutant also showed no recovery of activity when

treated with 2BEA (data not shown).

To assess length requirements of the side chain at the the 145-position, we reacted the K145C, No Cys mutant with 3-bromopropylamine·HBr and 2-mercaptoethylamine that generates a side chain amine that is 5 atoms from the C_α instead of 4 atoms like lysine. These modifications also restored activity to the K145C, No Cys mutant, although slightly less than that seen with 2-bromoethylamine (Fig. 2, *A* and *B*, and Table I), revealing that there may be significant flexibility in the active site. It is not clear why the conservative substitution of γ-thia-lysine for lysine only gives approximately 1% the activity of the wild type, yet the longer residues γ-thia-homo-lysine and γ-dithio-homo-lysine give almost the same activity as γ-thia-lysine. One possible reason why leader peptidase with γ-thia-lysine is not fully active is that γ-thia-lysine prefers a gauche (C-S-C) side chain torsion angle, whereas lysine prefers an anti-(C-C-C) side chain torsion angle (30, 49).

Reacting the K145C, No Cys mutant with the reagent 2BETMA, which puts a positively charged, nontitratable lysine analog (4-thialaminine) at the 145-position, resulted in no restoration of activity (Fig. 3). This is consistent with the K145R result and also with the hypothesis that lysine 145 is essential due to a critical hydrogen bond or its role as a general base and not because of its charge. It is possible that the additional bulkiness of the methyl groups on the ϵ-amino group within the lysine analog (4-thialaminine) is responsible for the lack of activity.

The pH-rate profile that shows a bell-shaped curve when the k_{cat}/K_m is plotted *versus* pH is consistent with two ionizable groups in the free enzyme with apparent pK_a values of approximately 8.7 and 9.3 (Fig. 4*A*). It is likely that the apparent pK_a value of 8.7 from the ascending limb of the plot corresponds to the pK_a of lysine 145 that would be required to be deprotonated to function as a general base. Lysine 145 would have a pK_a value 1.8 units lower than a free lysine in solution (pK_a = 10.5). Leader peptidase is not irreversibly affected by pH changes until pH 11 (Fig. 4*B*); therefore, denaturation of the protein at pH values higher than about pH 9 would not be an explanation for the descending arm of the pH profile (pK_a = 9.3). It could be that a protonated residue with a pK_a of 9.3 is essential for leader peptidase activity, such as a residue involved in an oxyanion hole might be deprotonated at this pH. It is also possible that this pK_a reflects a deprotonation of a residue involved in a salt bridge important for active site geometry. The descending arm of the pH-rate profile might correspond to the deprotonation of a tyrosine residue functioning as a critical hydrogen bond donor for binding or for catalysis. There is a conserved tyrosine 143 (Fig. 6, *B* and *C*), just two residues from the critical lysine, but this residue has been changed to a phenylalanine by Black *et al.* (9) and found to be nonessential using a very sensitive *in vivo* assay. van Dijl *et al.* (47) also found this tyrosine to be nonessential in *B. subtilis* SipS.

There is a strictly conserved arginine directly carboxyl-terminal to the proposed catalytic lysine. It is well known that spatially proximal positive charges to a lysine can decrease its pK_a. Westheimer's hypothesis (50) concerning such an effect has recently been confirmed by Highbarger *et al.* (19). To investigate the possible importance of the immediately proximal positively charged arginine at position 146, this residue was mutated to an alanine. The less than dramatic decrease in activity resulting from the R146A mutation indicates that it is not the positive charge from Arg-146 that is responsible for the decreased pKa of Lys-145 (see Table I). Moreover, when we look at the modeled active site of leader peptidase (Fig. 6*B*), we see that the charged guanidinium group of Arg-146 is pointed completely away from the active site region and therefore would be predicted to have a very small electrostatic effect on Lys-145. Therefore, it is more likely that the decreased pK_a of Lys-145 comes from its hydrophobic environment (Fig. 6, *B* and *C*). It is important that in future studies the apparent pK_a values acquired from the pH-rate profile be assigned to specific residues by other methods such as NMR spectroscopy (51).

We have found that leader peptidase is inhibited by the lysine modifying reagent maleic anhydride (Fig. 5). Kim *et al.* (52) found only low level inhibition of leader peptidase upon the addition of the lysine modifiers succinic anhydride and trinitrobenzene sulfonic acid. It is not yet clear why only maleic anhydride was successful in modifying lysine 145. Other lysine-modifying reagents we have tried without any effect on activity include potassium cyanate, trinitrobenzene sulfonic acid, succinic anhydride, acetic anhydride, and pyridoxyl 5-phosphate. It may be that these other reagents were not able to gain access to the active site region due to the active site's proposed hydrophobic environment. Another possibility is that maleic anhydride is the only reagent tried so far that can bind and induce a conformation within leader peptidase that allows lysine 145 to be reactive (lowered lysine pK_a). Finally, lysine 145 may be less reactive to modification by lysine-specific reagents because of its possible hydrogen bond to serine 90 and the potentially buried nature of Lys-145.

Taken together, the results from this study as well as others (9, 47) are consistent with the hypothesis that leader peptidase utilizes a lysine as its general base. We have proposed a mechanism for leader peptidase whereby the deprotonated ϵ-amine of lysine 145 abstracts the proton from the Oγ of serine 90 making it nucleophilic enough to attack the scissile bond of the pre-protein substrate (Fig. 7*A*). The tetrahedral intermediate I could be stabilized by a yet unidentified oxyanion hole, and the breakdown of the tetrahedral intermediate I to form the acyl-enzyme intermediate would be accelerated by the protonation of the leaving amine (mature protein) via lysine 145 (Fig. 7*B*). It is possible that the lysine 145 could also act as the general base in the formation of tetrahedral intermediate II, whereby lysine 145 would activate a water that would attack the ester carbonyl of the acyl-enzyme intermediate (Fig. 7*C*).

There are many unanswered questions regarding the mechanism of leader (signal) peptidase. From site-directed mutagenesis and chemical modification studies, it is clear that serine 90 and lysine 145 are critical residues, but it is not yet clear whether there exists an oxyanion hole similar to the classical serine proteases. Another unanswered question is whether lysine 145 serves as the general base in both activation steps of the reaction. The forthcoming x-ray crystal structure of the soluble fragment of *E. coli* leader peptidase (53) may help to answer these questions regarding the mechanism of this very unique serine protease.

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