

# Characterization of a Soluble, Catalytically Active Form of *Escherichia coli* Leader Peptidase: Requirement of Detergent or Phospholipid for Optimal Activity<sup>†</sup>

William R. Tschantz,<sup>‡</sup> Mark Paetzel,<sup>‡</sup> Guoqing Cao,<sup>‡</sup> Dominic Suci,<sup>§</sup> Masayori Inouye,<sup>§</sup> and Ross E. Dalbey<sup>\*‡</sup>

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, New Jersey 08903

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**ABSTRACT:** Leader peptidase is a novel serine protease in *Escherichia coli*, which functions to cleave leader sequences from exported proteins. Its catalytic domain extends into the periplasmic space and is anchored to the membrane by two transmembrane segments located at the N-terminal end of the protein. At present, there is no information on the structure of the catalytic domain. Here, we report on the properties of a soluble form of leader peptidase ( $\Delta 2-75$ ), and we compare its properties to those of the wild-type enzyme. We find that the truncated leader peptidase has a  $k_{\text{cat}}$  of  $3.0 \text{ s}^{-1}$  and a  $K_{\text{m}}$  of  $32 \mu\text{M}$  with a pro-OmpA nuclease A substrate. In contrast to the wild-type enzyme ( $pI$  of 6.8),  $\Delta 2-75$  is water-soluble and has an acidic isoelectric point of 5.6. We also show with  $\Delta 2-75$  that the replacement of serine 90 and lysine 145 with alanine residues results in a 500-fold reduction in activity, providing further evidence that leader peptidase employs a catalytic serine/lysine dyad. Finally, we find that the catalysis of  $\Delta 2-75$  is accelerated by the presence of the detergent Triton X-100, regardless if the substrate is pro-OmpA nuclease A or a peptide substrate. Triton X-100 is required for optimal activity of  $\Delta 2-75$  at a level far below the critical micelle concentration. Moreover, we find that *E. coli* phospholipids stimulate the activity of  $\Delta 2-75$ , suggesting that phospholipids may play an important physiological role in the catalytic mechanism of leader peptidase.

In both prokaryotic and eukaryotic cells, proteins destined for the cell surface or for secretion are made with an amino-terminal leader (or signal) sequence that targets the protein into the export pathway. The leader peptide is subsequently removed by a leader (signal) peptidase during or after the exported protein is translocated across the membrane.

To date, the *Escherichia coli* leader peptidase is the most extensively studied leader peptidase. This enzyme has been purified (Zwizinski et al., 1981; Wolfe et al., 1982, 1983a; Tschantz & Dalbey, 1994), its gene cloned and sequenced (Date & Wickner, 1981), and the protein overexpressed under the control of the *araB* promoter (Dalbey & Wickner, 1985). Gene cloning and mutagenesis techniques have been employed to study the membrane biogenesis of leader peptidase (Wolfe & Wickner, 1984; Dalbey & Wickner, 1986, 1987; Dalbey et al., 1987; Zhu & Dalbey, 1989; Laws & Dalbey, 1989; von Heijne, 1989; Nilsson & von Heijne, 1990; Lee et al., 1992) and to determine its physiological role (Dalbey & Wickner, 1985). While the precise mechanism of action of leader peptidase is unknown, recent data suggest that leader peptidase is a member of a novel class of serine proteinases which utilize serine and lysine residues for catalysis (Sung & Dalbey, 1992; Black, 1993; Tschantz et al., 1993). The active site of the leader peptidase resides in the periplasmic domain (Bilgin et al., 1990), which is anchored to the membrane by two transmembrane segments

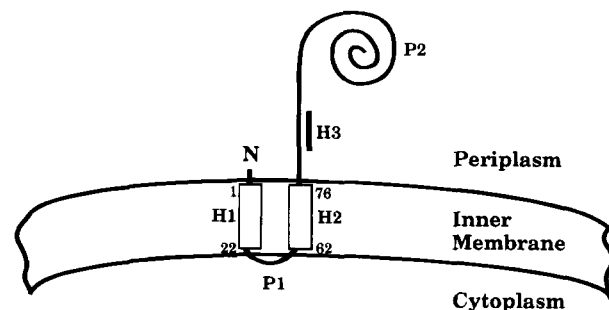


FIGURE 1: Membrane topology of *E. coli* leader peptidase. H1, H2, and H3 are hydrophobic domains, and P1 and P2 are polar domains.

(Figure 1; Wolfe et al., 1983b; Moore & Miura, 1987; San Millan et al., 1989).

Recently, there have been two important developments in the leader peptidase field. First, Kuo et al. (1993) have reported the isolation of a catalytically active, soluble form of leader peptidase, which lacks the membrane-anchoring domain. Second, a new substrate has become available that has much improved kinetic properties over previously described substrates. The  $k_{\text{cat}}/K_{\text{m}}$  with the new pro-OmpA nuclease A substrate, a hybrid secretory precursor, is 6 orders of magnitude greater than with peptide substrates (Chatterjee et al., 1995).

In this paper, we report on the characterization of the catalytic, periplasmic domain ( $\Delta 2-75$ ) of leader peptidase. We find that the truncated leader peptidase has very good catalytic parameters ( $k_{\text{cat}}$  is  $3.0 \text{ s}^{-1}$  and  $K_{\text{m}}$  of  $32 \mu\text{M}$ ) against the pro-OmpA nuclease substrate. In contrast to the wild-type enzyme,  $\Delta 2-75$  is water-soluble and has an acidic isoelectric point ( $pI$ ) of 5.6. No detectable activity is observed for  $\Delta 2-75$  proteins in which the candidate active

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\* Corresponding author.

<sup>‡</sup> The Ohio State University.

<sup>§</sup> Robert Wood Johnson Medical School.

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site residues serine 90 or lysine 145 are changed to alanine residues. Strikingly, while  $\Delta 2-75$  and the substrate are water-soluble, the detergent Triton X-100 is required for optimal activity. In addition, we find that *E. coli* phospholipids stimulate the activity of  $\Delta 2-75$ . Our results do not agree with recent studies by Kuo et al. (1993) where they find that  $\Delta 2-75$  lacks a requirement for detergent for optimal catalysis and has the same activity as the wild-type leader peptidase. Possible explanations for this discrepancy in the detergent requirement and the differences in the activity between wild-type and  $\Delta 2-75$  are discussed.

## EXPERIMENTAL PROCEDURES

**Materials.** Phenylmethanesulfonyl fluoride (PMSF)<sup>1</sup> and [<sup>35</sup>S]dATP were from Sigma and New England Nuclear, respectively. Oligonucleotides were synthesized at the Biochemical Instrument Center at The Ohio State University. The peptide substrate was purchased from the Macromolecular Structure Analysis Facility at the University of Kentucky.

**Bacterial Strains and Plasmids.** *E. coli* strains BL21(DE3) [F<sup>-</sup> ompT, r<sub>B</sub>mB], SB221 [lpp, hsdR,  $\Delta$ trpE5, leuB6, lacY, recA1/F' lacI<sup>q</sup>, lac<sup>+</sup>, pro<sup>+</sup>], and MC1061 [ $\Delta$ lacX74, araD139,  $\Delta$ (ara,leu)7697, galU, galK, hsr, hsm, strA] were from our collection. The pET3d vector (Novagen) under the control of the T7 promoter was described by Studier et al. (1990). The cloning of the pro-OmpA nuclease A gene into the IPTG-inducible plasmid pONF1 (Takahara et al., 1985) and the overexpression of the protein were described by Chatterjee et al. (1995).

**DNA Manipulations.** DNA techniques were performed as described by Maniatis et al. (1982). All cloning procedures used T4 kinase, T4 DNA ligase, Klenow, and restriction enzymes that were from Bethesda Research Laboratories. Oligonucleotide-directed mutagenesis was performed as described by Zoller and Smith (1983) with some modifications (Dalbey & Wickner, 1987). Transformations followed the calcium chloride method of Cohen et al. (1973).

**Assay for the Processing of Pro-OmpA Nuclease A.** Reaction (15  $\mu$ L) containing leader peptidase ( $1.37 \times 10^{-4}$   $\mu$ M) or  $\Delta 2-75$  ( $2.2 \times 10^{-2}$  to 2.2  $\mu$ M) was incubated at 37 °C with pro-OmpA nuclease A (5.1–41.1  $\mu$ M) in 50 mM Tris, 10 mM CaCl<sub>2</sub>, pH 8.0, in the presence or absence of Triton X-100 (1%). Typically, the reactions were terminated after 1 h by the addition of 5  $\mu$ L of 5-fold SDS sample buffer containing 10 mM MgCl<sub>2</sub> and frozen immediately. Precursor and mature nuclease proteins were separated on a 17.2% SDS–polyacrylamide gel [acrylamide:bis(acrylamide) ratio of 30:0.8], and the gel was stained by Coomassie brilliant blue. The concentration of pro-OmpA nuclease was determined using an *E*<sup>1%</sup> at 280 nm of 8.3.

**Processing of the Peptide Substrate.** The reaction was initiated by the addition of 140  $\mu$ L of wild-type (0.27  $\mu$ M) or  $\Delta 2-75$  (3.57–178  $\mu$ M) leader peptidase to 50  $\mu$ L of 1.8 mM peptide (Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO<sup>-</sup>; Dev et al., 1990) in 83 mM sodium phosphate, pH 7.7, at 37 °C. The reaction was quenched after various times by

transferring an aliquot (45  $\mu$ L) to an equal volume of 0.1% trifluoroacetic acid (TFA). The 140  $\mu$ L sample above of wild-type or  $\Delta 2-75$  leader peptidase was prepared by diluting a concentrated sample of enzyme into a volume of 95  $\mu$ L in 20 mM Tris, pH 7.4 containing either no Triton X-100 detergent or 1% Triton X-100. To this sample was added 50 mM Tris, pH 8.0, buffer with or without detergent to bring up the volume to 140  $\mu$ L. This results in a final Tris concentration of 30 mM and a pH of 8.0. After the reactions were terminated, samples were microfuged for 5 min to remove any debris or detergent present. To separate the product and reactant peptides, the samples were chromatographed on a Vydac C18 peptide column. The HPLC gradient used consisted of 97% A/3% B held for 5 min, followed by increasing the gradient linearly to 60% A/40% B over a 10 min period, which was then held constant for 5 min, and then back to 97% A/3% B over a 5 min period. In this gradient system, A = 0.1% TFA and B = acetonitrile, 0.1% TFA. Peptides were detected spectrophotometrically at 218 nm. Percent processing was determined by integration of the area under the peaks of the heptapeptide product (Phe-Ser-Ala-Ser-Ala-Leu-Ala) and the nonapeptide reactant (Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile): % processing = [(heptapeptide peptide peak)/(heptapeptide peptide peak + nonapeptide reactant peak)]  $\times$  100.

**Kinetic Assay.** The cleavage reaction contained substrate and wild-type or  $\Delta 2-75$  leader peptidase in 50 mM Tris, 10 mM CaCl<sub>2</sub>, pH 8.0, either with or without the detergent Triton X-100 (1%). The reaction was performed at 37 °C for various times with different concentrations of substrate, typically 5.1, 10.3, 15.4, 20.6, and 41.1  $\mu$ M pro-OmpA nuclease A. The reaction was initiated by the addition of wild-type leader peptidase or  $\Delta 2-75$ , and the reaction was terminated by the addition of 5-fold SDS sample buffer containing 10 mM MgCl<sub>2</sub> and frozen immediately in a dry ice/ethanol bath. The reaction with detergent contained  $1.37 \times 10^{-4}$   $\mu$ M leader peptidase or  $2.2 \times 10^{-2}$   $\mu$ M  $\Delta 2-75$ . The concentration of  $\Delta 2-75$  was 0.22  $\mu$ M when detergent was absent from the reaction. The concentration of these proteins was determined by the Pierce BCA protein assay kit. The pro-OmpA and OmpA nuclease A proteins were resolved on a 17.2% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue. The precursor and mature OmpA nuclease A bands were quantitated by scanning the gels on a Technology Resources, Inc., Liner Tamer PCLT 300 scanning densitometer. Percent processing was measured by dividing the area of the mature band by the total area of the mature and precursor nuclease A bands. The initial rates were determined by plotting the amount of product versus time. Then  $V_{\max}$ ,  $K_m$ , and  $k_{\text{cat}}$  values were extracted from a  $1/v_i$  versus  $1/s$  plot. We used Microcal Origins to plot data and to do linear regression analysis of data. The kinetic parameters for the wild-type and  $\Delta 2-75$  proteins (Table 1) are the average of three or more separate experiments.

**Purification of Pro-OmpA Nuclease A.** Pro-OmpA nuclease A, which is a hybrid protein of staphylococcal nuclease A fused to the signal peptide of the OmpA protein (Takahara et al., 1985), was overexpressed using *E. coli* strain SB221 bearing the plasmid pONF1. The cells were grown to the late log phase in M9-Casamino acids medium supplemented with 5  $\mu$ g/mL leucine and 5  $\mu$ g/mL tryptophan. Pro-OmpA nuclease A was induced for 4 h by the addition

<sup>1</sup> Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; OmpA, outer membrane protein A; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

of 2 mM IPTG. Pro-OmpA nuclease A was purified from these cells as described by Chatterjee et al. (1995).

## RESULTS

**Purification and Activity of Truncated Forms of Leader Peptidase.** One of the objectives of our lab is to isolate the catalytic, periplasmic domain of leader peptidase in order to solve its structure. To this end, we have constructed truncated forms of leader peptidase, namely,  $\Delta 2-75$ ,  $\Delta 2-70$ , and  $\Delta 2-60$ . These proteins were overproduced and purified according to Kuo et al. (1993), who reported the procedure for the  $\Delta 2-75$  mutant, which lacks the membrane-anchoring domain. To prepare these deletion mutants of leader peptidase, oligonucleotide-directed mutagenesis was used to create a unique *NcoI* site at various places within the leader peptidase gene (*lepB*) such that *NcoI* would cut between the codons of 60 and 61, 70 and 71, or 75 and 76. The genes encoding these truncated proteins were subcloned into the pET-3d vector, which is a T7 polymerase-dependent vector, often used in the expression of high levels of protein (Studier et al., 1990). We confirmed by Coomassie blue staining that the truncated leader peptidase proteins were expressed upon adding IPTG. Briefly, cells (200 mL) were grown, induced, and harvested, and the truncated proteins were solubilized and purified as described (Kuo et al., 1993). The cells were passed through a French Press 3 times to lyse the cells, and a Sephacryl S-100 column was used for gel filtration. Exhaustive dialysis was carried out with 0.5% Triton X-100 in 20 mM Tris, pH 7.4 (without 5 mM  $MgCl_2$ ).

After dialysis, we analyzed the samples for purity by SDS-PAGE and Coomassie blue staining. Figure 2A shows that  $\Delta 2-75$  (lane 6),  $\Delta 2-70$  (lane 5), and  $\Delta 2-60$  (lane 4) were purified to homogeneity and migrate on the gel faster than the wild-type protein (lane 1, top band). As a control, we confirmed that there is not a protein at this position if we followed the procedure exactly as described above, except we started with cells bearing the pET-3d vector without the leader peptidase insert. Two fractions were taken from the Sephacryl S-100 column at the same position where the  $\Delta 2-75$  elutes, and, after dialysis, an aliquot of this material was analyzed by SDS-PAGE and Coomassie blue staining (lanes 2 and 3).

The activity of the truncated forms of leader peptidase was assayed (Figure 2B) using pro-OmpA nuclease A, an excellent substrate for the wild-type leader peptidase (Chatterjee et al., 1995). An aliquot (2  $\mu$ L) containing wild-type (lane 1),  $\Delta 2-60$  (lane 4),  $\Delta 2-70$  (lane 5), and  $\Delta 2-75$  (lane 6) or an aliquot of the control samples (lanes 2 and 3) derived from the cells not overexpressing the truncated proteins in the pET-3d vector (see above) was incubated with the pro-OmpA nuclease A substrate for 60 min. No leader peptidase was added in lane 7. The control samples were derived from a purification preparation of cells bearing pET-3d vector without leader peptidase gene. Figure 2B shows that each of the truncated forms of leader peptidase (lanes 4, 5, and 6) have significant activity over background. The low activity of the control samples (lanes 2 and 3) shows that the measured activity of the samples containing the mutant leader peptidase proteins is due to the truncated proteins themselves and not the chromosomally-encoded leader peptidase.

We further analyzed  $\Delta 2-75$  since it had the highest activity of these truncated leader peptidases in this assay.

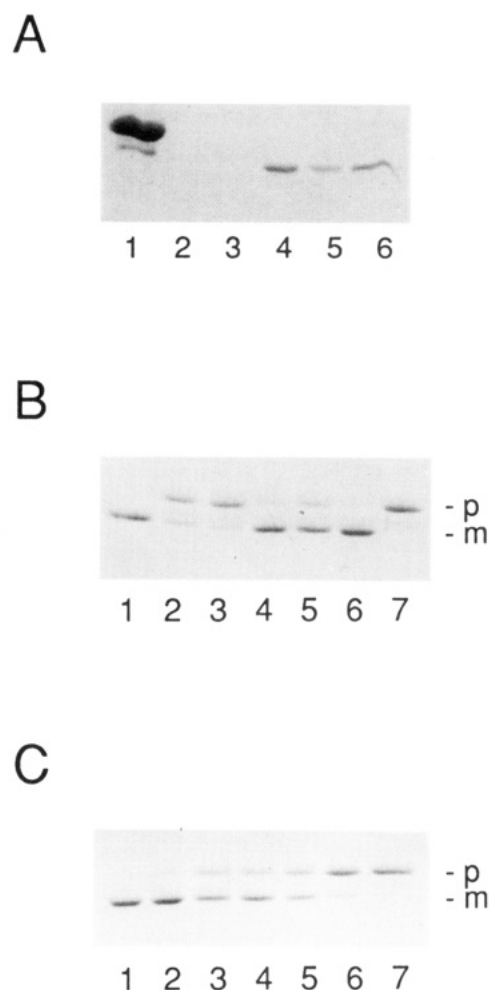


FIGURE 2: (A) Purification of the truncated forms of leader peptidase. 20  $\mu$ L of 0.7 mg/mL wild-type leader peptidase (lane 1) and 20  $\mu$ L of  $\Delta 2-60$  (lane 4),  $\Delta 2-70$  (lane 5), and  $\Delta 2-75$  (lane 6), each of 0.2 mg/mL, were applied to a SDS-PAGE (12% gel), and the gel was stained by Coomassie brilliant blue. The concentration of wild-type leader peptidase and the truncated proteins was determined by the Pierce BCA protein assay kit. As a control, 20  $\mu$ L of fractions 1 and 2 was analyzed from the Sephacryl S-100 column (where the truncated mutants elute) derived from preparation of cells bearing the pET-3d vector without a leader peptidase insert. (B) Activity of the truncated proteins. 2  $\mu$ L of 0.7 mg/mL wild-type leader peptidase (lane 1), 2  $\mu$ L of fraction 1 (lane 2) and fraction 2 (lane 3), and 2  $\mu$ L of  $\Delta 2-60$  (lane 4),  $\Delta 2-70$  (lane 5),  $\Delta 2-75$  (lane 6) (each of 0.2 mg/mL), and buffer only (lane 7) were incubated with 15  $\mu$ L of pro-OmpA nuclease A (29  $\mu$ M) in 50 mM Tris, 1% Triton X-100, pH 8.0, for 60 min at 37  $^{\circ}$ C. The reaction was then terminated by the addition of 5-fold SDS sample buffer, and the samples were analyzed by SDS-PAGE with a 17.2% polyacrylamide gel and Coomassie blue staining. (C) Concentration dependence of  $\Delta 2-75$  cleavage of pro-OmpA nuclease A. An aliquot (1  $\mu$ L) containing various concentrations of  $\Delta 2-75$  was incubated with 15  $\mu$ L of OmpA nuclease A (29 mM) in 50 mM Tris, 1% Triton X-100, pH 8.0 at 37  $^{\circ}$ C for 60 min. Lane 1, 200 ng; lane 2, 20 ng; lane 3, 4 ng; lane 4, 2 ng; lane 5, 0.8 ng; lane 6, 0.4 ng; lane 7, buffer. The reaction was terminated and analyzed by SDS-PAGE and Coomassie blue staining, as described in panel B.

Figure 2C shows that it takes 4 ng of  $\Delta 2-75$  to see 50% processing of pro-OmpA nuclease A (lane 3), compared to 0.36 ng for the wild-type leader peptidase (data not shown). Since a 500-fold dilution of  $\Delta 2-75$  gives the same processing as background in these studies, we estimate that the low background activity can account for roughly  $1/500$ th of the activity in the  $\Delta 2-75$  preparation.

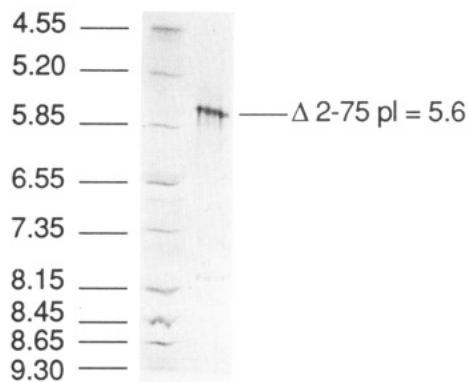


FIGURE 3: Isoelectric focusing of  $\Delta 2-75$ . 1  $\mu\text{g}$  of  $\Delta 2-75$  was loaded onto an isoelectric focusing gel using the Pharmacia Phast Gel system, Phast gel IEF 3–9 media, and Pharmacia broad-range pI standards. The gel was visualized by Phast Gel Blue R Coomassie staining. The left lane shows the pI standards (0.2  $\mu\text{g}$  of standards applied to gel).

**Determination of the pI and Water-Solubility of  $\Delta 2-75$ .** The isoelectric point of  $\Delta 2-75$  was determined by isoelectric focusing electrophoresis. We used the Pharmacia Phast gel system along with the phast gel IEF 3–9 media, which can resolve proteins with isoelectric points (pIs) between 3 and 9. All samples were run from both the anode and the cathode side of the gel to ensure that the proteins focused to the same point in the gradient. Figure 3 shows that  $\Delta 2-75$  runs as a single band on this gel system with a pI of 5.6. We also obtained the same pI for  $\Delta 2-75$  when determined by Pharmacia chromatofocusing chromatography (data not shown).

We next evaluated whether  $\Delta 2-75$  is detergent- or water-soluble by testing whether it partitions into the Triton X-114 rich phase (Bordier, 1981). Previously we found that the wild-type leader peptidase partitions into the Triton X-114 phase (Dalbey & Wickner, 1987). Triton X-114 was added to  $\Delta 2-75$  or, as a control, the wild-type leader peptidase. The samples were mixed at 4 °C, and then the temperature was raised to 37 °C, which is above the cloud point of the detergent. At this temperature, the aqueous and detergent phases can be separated by centrifugation. As can be seen in Figure 4B,  $\Delta 2-75$  is found in the aqueous fraction (lane 3), with very little protein in the detergent fractions 1 (lane 1) or 2 (lane 2). In contrast, the wild-type protein is in the detergent-rich phase (Figure 4A, lane 1). In addition to these studies, we have found that the detergent Triton-X100 can be removed from the  $\Delta 2-75$  sample by first binding the protein to Q Sepharose FF anion exchange resin and then washing the nonionic detergent away, followed by eluting the protein off the column with 0.7 M NaCl (data not shown). A very concentrated clear solution of  $\Delta 2-75$  (56 mg/mL) can be prepared in 4 mM Hepes buffer, pH 7.6, without any detergent present (data not shown), again showing that this protein is very water-soluble.

**No Detectable Activity with  $\Delta 2-75$  with Active Site Mutations.** We have used the pro-OmpA nuclease A processing assay with the  $\Delta 2-75$  protein to analyze the effects of active site mutations because it is the most sensitive assay to date. On the basis of a comparison of the activity of  $\Delta 2-75$  with background activity, we estimate that this assay system is 10-fold more sensitive than the *in vitro* system that previously was used to show that serine 90 and lysine 145 are possible active site residues (Sung & Dalbey,

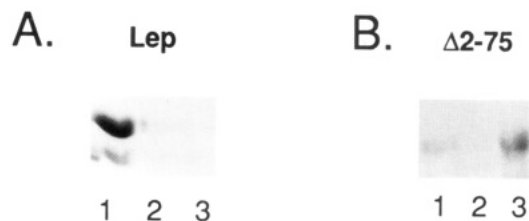


FIGURE 4:  $\Delta 2-75$  partitions into the aqueous phase in a Triton X-114 extraction study. 200  $\mu\text{L}$  of wild-type (A) or  $\Delta 2-75$  (B) leader peptidase (each 500  $\mu\text{g}/\text{mL}$ ) was solubilized in 50 mM Tris, pH 8.0, 1% Triton X-114 at 4 °C, and the sample was overlaid on a sucrose cushion (300  $\mu\text{L}$ ). After a 3 min incubation at 37 °C, the sample was microfuged for 3 min at 37 °C at 2500 rpm to separate the detergent and aqueous phases. The bottom phase is the first detergent extraction sample. The top phase was removed, and Triton X-114 was added again (to a final concentration of 0.5%) and resolubilized by incubating at 4 °C. After repeating the incubation and centrifugation step with the sucrose cushion, the aqueous phase is transferred to a new tube, and Triton X-114 is once again added to 2% final concentration and resolubilized as above. The bottom phase that remains after the second centrifugation step is the second detergent extraction sample. Finally, the centrifugation step is repeated once more after incubating the sample at 37 °C, and the top aqueous phase is saved. This is the aqueous phase sample. The two detergent and one aqueous phase samples were brought up to 800  $\mu\text{L}$  with water, and then the samples were acid-precipitated (Wolfe et al., 1982) and analyzed by SDS-PAGE with a 12% polyacrylamide gel (Tschantz et al., 1993) and stained by Coomassie brilliant blue. Samples were derived from the first detergent extraction (lane 1), the second detergent extraction (lane 2), and the aqueous phase recovered after washing 3 times with detergent (lane 3). The left panel (A) shows the wild-type leader peptidase (Lep); the right panel (B) shows  $\Delta 2-75$ .

1992; Tschantz et al., 1993). Oligonucleotide-directed mutagenesis (Zoller & Smith, 1983) was used to mutate either serine 90 or lysine 145 to alanine.  $\Delta 2-75$  bearing the S90A or K145A mutation was purified as described above and analyzed by SDS-PAGE and Coomassie brilliant blue staining. Figure 5A shows only one protein species of the predicted molecular weight is observed (lanes 2 and 3). No detectable processing of the pro-OmpA nuclease A substrate was observed even at 100 ng of  $\Delta 2-75$  with either the serine 90 to alanine mutation (Figure 5B, lane 2) or the lysine 145 to alanine mutation (Figure 5C, lane 2). These results are consistent with the serine 90 and lysine 145 being active site residues because it is expected that mutating catalytic residues would lower the activity at least 500-fold.

**Detergent Requirement for Optimal Activity.** To determine whether detergent is required for optimal activity of  $\Delta 2-75$ , we compared processing with or without the detergent Triton X-100. As described above, detergent-free  $\Delta 2-75$  was prepared by washing away the detergent after absorbing the protein onto an anion exchange resin. Figure 6A shows that when using the pro-OmpA nuclease A as a substrate that the addition of Triton X-100 (1%, final concentration) stimulates processing roughly 20–50-fold. We next asked whether a detergent micelle is required by measuring pro-OmpA nuclease A processing at different concentrations of Triton X-100 at levels far above and far below the critical micelle concentration (CMC). The CMC of Triton X-100 is 0.24 mM (Chattopadhyay & London, 1984). In the absence of detergent, there is a low level of pro-OmpA nuclease A cleavage (Figure 6B, far left panel). The addition of detergent at levels 10-fold lower than the CMC results in strong stimulation of processing (Figure 6B, middle left panel). A further increase in processing is observed when



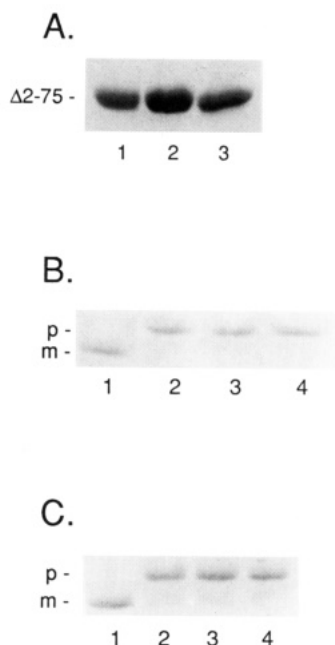


FIGURE 5: Elimination of catalytic activity by active site mutations in  $\Delta 2-75$ . (A) Purification of  $\Delta 2-75$  proteins.  $\Delta 2-75$  (lane 1),  $\Delta 2-75$  S90A (lane 2), and  $\Delta 2-75$  K145A (lane 3) were analyzed by SDS-PAGE with a 12% polyacrylamide gel and stained by Coomassie brilliant blue. 50  $\mu\text{g}$  of each protein was loaded on the gel. (B) Processing activity by S90A  $\Delta 2-75$ . Reactions (15  $\mu\text{L}$ ) contain pro-OmpA nuclease A (12  $\mu\text{M}$ ) and 1  $\mu\text{L}$  of enzyme of various concentrations and are incubated for 1 h at 37  $^{\circ}\text{C}$ . Samples were analyzed by SDS-PAGE with a 17.2% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue. Lane 1 shows the activity with wild-type leader peptidase as a positive control for cleavage. Lane 2 shows the activity of  $\Delta 2-75$  S90A (100 ng). Lane 3 shows the activity of 10 ng of S90A  $\Delta 2-75$ . Lane 4 shows the activity in the presence of buffer only. (C) Processing activity of K145A  $\Delta 2-75$ . The reactions were carried out as described in (B) with the same amounts of the K145A  $\Delta 2-75$  protein in lanes 2 and 3 as indicated for the corresponding lanes for the S90A  $\Delta 2-75$  protein shown in (B).

the detergent concentration was raised to the CMC level (Figure 6B, middle right panel), but no additional increase was seen when the detergent concentration was increased to 10-fold higher than the CMC (Figure 6B, far right panel).

While these studies show that the detergent Triton X-100 promotes  $\Delta 2-75$ -catalyzed preprotein cleavage, it is not clear as to how the cleavage is affected. To measure more precisely the effect of detergent on cleavage, we determined the kinetic parameters in the presence or absence of detergent by measuring the initial rates of cleavage at various pro-OmpA nuclease A concentrations. Table 1 summarizes the kinetic constants that were determined from the initial velocity plots. In the presence of detergent, the calculated values of  $k_{\text{cat}}$  is  $3.0 \pm 0.4 \text{ s}^{-1}$ , and  $K_m$  is  $32 \pm 3 \mu\text{M}$ . In the absence of detergent, the kinetic constants are much poorer. The  $k_{\text{cat}}$  is  $0.14 \pm .04 \text{ s}^{-1}$ , and the apparent  $K_m$  is  $199 \pm 50 \mu\text{M}$ . Therefore, with no detergent present, the  $k_{\text{cat}}$  has decreased roughly 20-fold, and the apparent  $K_m$  has increased 6-fold. We also measured the kinetic constants for the wild-type enzyme with the pro-OmpA nuclease A substrate in the presence of detergent to see how comparable  $\Delta 2-75$  is at catalysis; with the wild-type enzyme, the  $k_{\text{cat}}$  is  $44 \pm 9 \text{ s}^{-1}$ , and the  $K_m$  is  $19.2 \pm 4.6 \mu\text{M}$ , indicating that indeed  $\Delta 2-75$  is very active with a  $k_{\text{cat}}$  that is only 15-fold lower than that of the wild-type protein. The  $K_m$  values are similar.

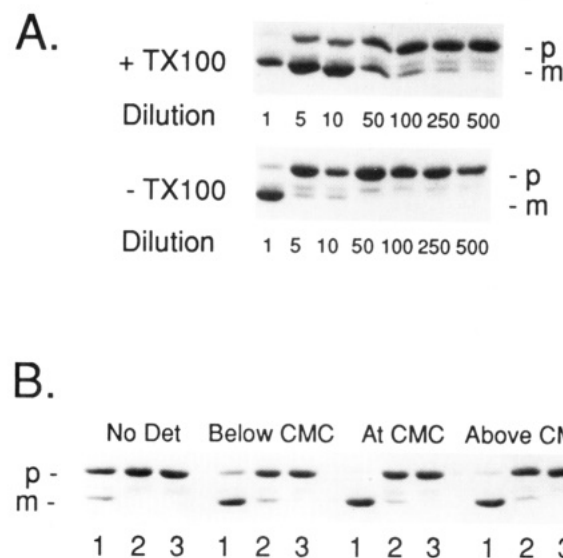


FIGURE 6: (A)  $\Delta 2-75$  requires the detergent Triton X-100 for optimal activity. Processing of pro-OmpA nuclease A by  $\Delta 2-75$  at various concentrations was tested by diluting a  $\Delta 2-75$  stock solution (100  $\mu\text{g}/\text{mL}$ ) into 50 mM Tris, pH 8.0, with or without Triton X-100. The reaction contained 15  $\mu\text{L}$  of pro-OmpA nuclease A (23  $\mu\text{M}$ ) in 50 mM Tris, pH 8.0, with or without 1% Triton X-100. The reaction was initiated by the addition of  $\Delta 2-75$  at various dilutions, and incubations were continued for 1 h at 37  $^{\circ}\text{C}$ . The reaction was terminated by the addition of 5-fold SDS-PAGE sample buffer, and the samples were subjected to SDS-PAGE gel and Coomassie brilliant blue staining. (B) Dependence of the  $\Delta 2-75$  activity on the Triton X-100 critical micelle concentration (CMC).  $\Delta 2-75$  was incubated at various concentrations at 37  $^{\circ}\text{C}$  for 1 h in the presence of pro-OmpA nuclease A and buffer (50 mM Tris, pH 8.0) or Triton X-100 (see below for the concentrations). Briefly, reactions contained 15  $\mu\text{L}$  of pro-OmpA nuclease A (21 mM) and 1  $\mu\text{L}$  of  $\Delta 2-75$  at a concentration of 0.1 (lane 1), 0.01 (lane 2), or 0.001 mg/mL (lane 3) and 1  $\mu\text{L}$  of buffer (50 mM Tris, pH 8.0) or the detergent at different concentrations. The detergent levels (final concentrations) were 10-fold below the CMC (0.024 mM Triton X-100), at the CMC (0.24 mM Triton X-100), and 10-fold above the CMC (2.4 mM Triton X-100). The processing of pro-OmpA nuclease A (p) to nuclease A (m) was analyzed by SDS-PAGE using a 17.2% polyacrylamide gel and stained by Coomassie blue staining.

Table 1: Kinetic Constants of Lep Constructs<sup>a</sup>

	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )
WT lep	$44 \pm 9$	$19.2 \pm 4.6$
$\Delta 2-75$		
+TX-100	$3.0 \pm 0.4$	$32 \pm 3$
-TX-100	$0.14 \pm 0.04$	$199 \pm 50$

<sup>a</sup> See Experimental Procedures for how constants were determined.

We also examined the requirement for detergent with a peptide substrate, Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO<sup>-</sup>, which corresponds to the -7 to +2 region of the maltose binding protein precursor (Dev et al., 1990). This peptide is cleaved by leader peptidase between the alanyl and lysyl residues. We incubated  $\Delta 2-75$  at various enzyme concentrations with the peptide substrate at 37  $^{\circ}\text{C}$  in the presence or absence of 1% Triton X-100. At the indicated times (Figure 7), the percent processing was determined by quantitation of the reactant and product peptides which were separated using a reversed-phase analytical C18 column utilizing a H<sub>2</sub>O/acetonitrile gradient. Figure 7 shows the percent processing at various times after the addition of leader peptidase or  $\Delta 2-75$ . As observed with the pro-OmpA nuclease A substrate, the addition of detergent leads to a

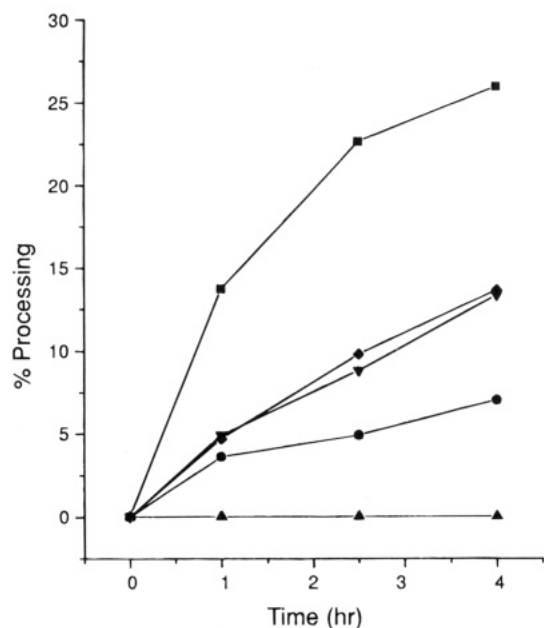


FIGURE 7: Detergent Triton X-100 promotes the activity of  $\Delta 2-75$  with a peptide substrate. Percent processing of the peptide substrate (Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO<sup>-</sup>) was assayed with Triton X-100 at 3.57  $\mu\text{M}$  ( $\blacktriangledown$ )  $\Delta 2-75$  or at various concentrations ( $\blacktriangle$ , 3.57  $\mu\text{M}$ ;  $\bullet$ , 35.7  $\mu\text{M}$ ;  $\blacksquare$ , 178  $\mu\text{M}$ ) in the absence of Triton X-100. As a control, the percent processing was determined with the wild-type leader peptidase ( $\blacklozenge$ , 0.27  $\mu\text{M}$ ) in the presence of Triton X-100. Reactions were initiated by the addition of 50  $\mu\text{L}$  of peptide (1.8 mM stock) to 140  $\mu\text{L}$  of the enzyme sample at the indicated concentrations. At the indicated times, aliquots were removed, and the percent processing was determined (see Experimental Procedures).

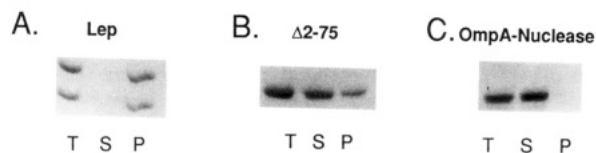


FIGURE 8: Majority of  $\Delta 2-75$  and the pro-OmpA nuclease A substrate do not aggregate in the absence of detergent. 50  $\mu\text{g}$  each of wild-type leader peptidase (A),  $\Delta 2-75$  (B), and OmpA nuclease A (C) in 2 mL of 20 mM Tris, pH 8.0, buffer was centrifuged 5 h at 100000g at 4  $^{\circ}\text{C}$ . After centrifugation, the supernatant was transferred to a new tube, and the pellet was resuspended in a volume equal to the starting volume. Samples were then analyzed on a 17.2% SDS-polyacrylamide gel, and the gel was stained with Coomassie brilliant blue. Samples were derived from uncentrifuged samples (T), the pellet fraction (P), and the supernatant fraction (S).

marked stimulation of processing of the peptide. For example, Figure 7 shows that 3.5  $\mu\text{M}$   $\Delta 2-75$  leads to more peptide processing in the presence of detergent than with 35  $\mu\text{M}$   $\Delta 2-75$  without detergent. Moreover, we find that the wild-type leader peptidase is able to process the peptide substrate approximately 20-fold better than the  $\Delta 2-75$ .

One possibility is that the detergent is required to prevent  $\Delta 2-75$  or the pro-OmpA nuclease A substrate from denaturing and aggregating. To test this idea, we centrifuged the samples containing either  $\Delta 2-75$  or pro-OmpA nuclease A at 100000g for 5 h at 4  $^{\circ}\text{C}$  and examined whether the protein is found in the pellet. As can be seen in Figure 8, the majority of the total  $\Delta 2-75$  protein (T) was located in supernatant fraction (S) with very little in the pellet fraction (P) after centrifugation. In addition, the pro-OmpA nuclease substrate was located exclusively in the supernatant fraction

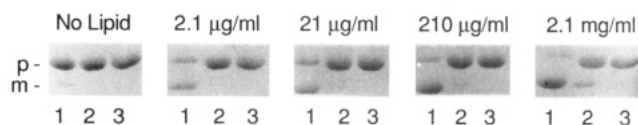


FIGURE 9: *E. coli* phospholipids stimulate the activity of  $\Delta 2-75$ .  $\Delta 2-75$  was incubated at various concentrations at 37  $^{\circ}\text{C}$  for 1 h in the presence of pro-OmpA nuclease A. Briefly, reactions contained 10  $\mu\text{L}$  of pro-OmpA nuclease A (31  $\mu\text{M}$ ), 1  $\mu\text{L}$  of  $\Delta 2-75$  at concentrations of 0.1 mg/mL (lane 1), 0.01 mg/mL (lane 2), or 0.001 mg/mL (lane 3), and 1  $\mu\text{L}$  of phospholipid mixture or buffer (50 mM Tris, pH 8.0). The lipid concentrations were 2.1  $\mu\text{g}/\text{mL}$ , 21  $\mu\text{g}/\text{mL}$ , 210  $\mu\text{g}/\text{mL}$ , or 2.1 mg/mL. Pro-OmpA nuclease A and OmpA nuclease A were resolved on a 17.2% polyacrylamide gel and stained by Coomassie brilliant blue. *E. coli* total lipid extract was purchased from Avanti Polar Lipids, Inc. 100  $\mu\text{L}$  of the lipids (25 mg/mL) was transferred to a glass vial and dried under nitrogen. 100  $\mu\text{L}$  of 50 mM Tris-HCl, pH 8.0, was added to the lipid and vortexed to make the phospholipid mixture (Tanford, 1973).

(S), while the wild-type leader peptidase and its proteolytic fragment (bottom band) were found exclusively in the pellet fraction (compare total and pellet lanes); the wild-type protein denatures in the absence of detergent because the detergent stabilizes its two transmembrane segments.

**Phospholipids Stimulate the Activity of  $\Delta 2-75$ .** Since the detergent Triton X-100 enhanced the activity of  $\Delta 2-75$ , we asked whether phospholipids play a role in catalysis. *E. coli* lipid extract was purchased from Avanti Polar lipids, Inc., and used to prepare a mixed phospholipid bilayer system by vortexing following established techniques (Tanford, 1973; see legend to Figure 9). We then tested the ability of  $\Delta 2-75$  to cleave pro-OmpA nuclease A in the presence of no phospholipid, or 2.1  $\mu\text{g}/\text{mL}$ , 21  $\mu\text{g}/\text{mL}$ , 210  $\mu\text{g}/\text{mL}$ , or 2.1 mg/mL phospholipid. Figure 9 shows that the addition of phospholipid stimulates the activity of  $\Delta 2-75$ . There is an increase in the amount of pro-OmpA nuclease A converted to the mature nuclease A as the concentration of micelles is increased from 2.1  $\mu\text{g}/\text{mL}$  to 2.1 mg/mL.

## DISCUSSION

We report here the isolation of the soluble, catalytically active form of *E. coli* leader peptidase and compare its properties to the native membrane-bound enzyme. We find that  $\Delta 2-75$ , the periplasmic catalytic domain, retains excellent activity. It has a  $k_{\text{cat}}$  of 3.0  $\text{s}^{-1}$  and a  $K_{\text{m}}$  of 32  $\mu\text{M}$  with the pro-OmpA nuclease A substrate whereas the wild-type protein has a  $k_{\text{cat}}$  of 44  $\text{s}^{-1}$  and a  $K_{\text{m}}$  of 19.2  $\mu\text{M}$  (Table 1). In contrast to the wild-type protein ( $pI$  of 6.8), the periplasmic domain is water-soluble and has a more acidic isoelectric point ( $pI$  of 5.6). Additionally, we provide further evidence that serine 90 and lysine 145 are active site residues since their replacement with the nonnucleophilic alanine residue results in at least a 500-fold reduction in the activity.

Strikingly, we demonstrate that the detergent Triton X-100 promotes  $\Delta 2-75$ -catalyzed cleavage of pre-protein and peptide substrates. For example, we find in the presence of Triton X-100 the  $k_{\text{cat}}$  is 20-fold higher and has an apparent  $K_{\text{m}}$  that is 6-fold lower than without detergent. We also find *E. coli* phospholipids, comprised of mainly phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Raetz & Dowhan, 1990), stimulate the activity of  $\Delta 2-75$ . Presently, it is not clear whether the requirement for detergent or phospholipid is a substrate or an enzyme effect. For example, detergent may be required to stabilize the leader peptidase hydrophobic stretch (83–98) within  $\Delta 2-75$ , which

contains the catalytic serine 90 residue at its center, or it may be needed to stabilize the OmpA leader peptide of the pro-OmpA nuclease A substrate. However, we favor the idea that the detergent (or phospholipid) requirement is an enzyme effect. First, much larger  $\Delta 2-75$  crystals are formed when detergent is present in the crystallization buffer (M. Paetzel, M. Tchernaiia, N. Strynadka, R. Dalbey, and M. N. G. James, unpublished data), and we find that detergent stimulates the activity of  $\Delta 2-75$  with two different substrates. It is intriguing that detergent is required for optimal activity at concentrations far below the critical micelle concentration of Triton X-100, where the equilibrium between monomers and micelles strongly favors the monomer form of the detergent (Helenius & Simons, 1975). This suggests that single detergent molecules, themselves, might be sufficient to enhance the catalytic activity of leader peptidase.

We ruled out that detergent is required to keep the  $\Delta 2-75$  enzyme, the pro-OmpA nuclease A, and Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile substrates soluble. Both  $\Delta 2-75$  (Figure 4) and pro-OmpA nuclease A (data not shown) partition into the aqueous phase in a Triton X-114 extraction study, and the peptide substrate is quite soluble in aqueous buffer.

Kuo et al (1993) have reported that  $\Delta 2-75$  has the same catalytic activity in the presence or absence of detergent and also that  $\Delta 2-75$  has the same activity as wild-type leader peptidase. The reason for these discrepancies reported here and Kuo and colleagues is not clear. Some differences in the experimental protocols should be mentioned. Kuo et al. (1993) used the peptide substrate Ac-Trp-Leu-Val-Pro-NLeu-Leu-Ser-Phe-Ala-Ala-Glu-Gly-Asp-Asp-Pro-Ala-NH<sub>2</sub>, corresponding to -8 to +7 positions of the procoat protein. This peptide is a very poor substrate for wild-type leader peptidase; the  $k_{\text{cat}}$  is 0.0027 s<sup>-1</sup> (Kuo et al., 1993). In the present paper, the  $k_{\text{cat}}$  for the wild-type enzyme is 44 and 0.027 s<sup>-1</sup> with the pro-OmpA and peptide (Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO<sup>-</sup>) substrates, respectively. The peptide substrate and the more physiological pre-protein substrate used in this work may be better at discriminating small changes in leader peptidase activity than the substrate used by Kuo et al. (1993). In addition, Kuo et al. (1993) removed the detergent using an Extracti-Gel D column. Although this procedure is useful, it is well-known that residual detergent usually remains even after great efforts have been made to remove the detergent (Allen et al., 1980).

In conclusion, we have determined that the periplasmic domain of leader peptidase retains good catalytic properties only 15-fold lower than the wild-type enzyme. However, we find that the presence of detergent or phospholipid is still required for optimal catalysis, suggesting that phospholipids may play an important role in vivo in the catalytic mechanism. This is easy to envision since within the cell the active site serine 90 residue is spatially close to the membrane surface where it could cleave preproteins as they are translocated across the membrane. Finally, very recently, we have been able to purify large amounts of homogeneous and concentrated  $\Delta 2-75$  using a new purification procedure which has allowed us to obtain crystals of  $\Delta 2-75$  that are suitable for X-ray crystallography (M. Paetzel, M. Tchernaiia, N. Strynadka, R. Dalbey, and M. N. G. James, unpublished data). Work is now underway to solve the structure of leader

peptidase, a member of a new protease family, and this should help in the elucidation of the novel catalytic mechanism.

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