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Evidence that the Catalytic Activity of Prokaryote Leader Peptidase Depends upon the Operation of a Serine-Lysine Catalytic Dyad

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Leader peptidase (LP) is the enzyme responsible for proteolytic cleavage of the amino acid leader sequence from bacterial preproteins. Recent data indicate that LP may be an unusual serine proteinase which operates without involvement of a histidine residue (M. T. Black, J. G. R. Munn, and A. E. Allsop, Biochem. J. 282:539–543, 1992; M. Sung and R. E. Dalbey, J. Biol. Chem. 267:13154–13159, 1992) and that, therefore, one or more alternative residues must perform the function of a catalytic base. With the aid of sequence alignments, site-specific mutagenesis of the gene encoding LP (*lepB*) from *Escherichia coli* has been employed to investigate the mechanism of action of the enzyme. Various mutant forms of plasmid-borne LP were tested for their abilities to complement the temperature-sensitive activity of LP in *E. coli* IT41. Data are presented which indicate that the only conserved amino acid residue possessing a side chain with the potential to ionize, and therefore with the potential to transfer protons, which cannot be substituted with a neutral side chain is lysine at position 145. The data suggest that the catalytic activity of LP is dependent on the operation of a serine-lysine catalytic dyad.

Most proteins which are translocated across lipid bilayers are synthesized as precursor proteins (preproteins) with an N-terminal extension known as a leader or signal peptide. Leader (or signal) peptidases (LPs) are membrane-bound proteolytic enzymes present in both prokaryotes and eukaryotes which specifically catalyze the hydrolysis of the peptide bond between the N-terminal leader and the mature sequence of preproteins (38). The prokaryote enzyme responsible for this activity, LP, is known to consist of a single polypeptide chain (39), unlike the eukaryote equivalents, which consist of between two and six polypeptides (2, 15, 40). The genes encoding LP (*lepB*) of the gram-negative organisms *Escherichia coli* (39), *Salmonella typhimurium* (36), and *Pseudomonas fluorescens* (4) and that of the homologous signal peptidase 1 enzyme (*sipS*) from the gram-positive organism *Bacillus subtilis* (35) have been cloned and sequenced, and the corresponding amino acid sequences display highly significant homology.

Although the physiological role of LP is well-defined, the enzymatic mechanism is not. Proteinases in general are commonly subdivided into four groups according to the mechanism of action (serine, cysteine, metallo-, and aspartyl proteinases) (14). Since they are among the most extensively and intensively studied classes of enzymes, it might therefore be expected that the mechanism of action of LP might be partially defined by assignation to one of the four groups according to various diagnostic procedures, such as susceptibility to characteristic inhibitors or the presence of certain primary structure motifs. However, recent work in the present (4) and other (32) laboratories has unambiguously demonstrated that LP is not a member of any of these four classical groups. It has been suggested that LP may belong to an unusual class of serine proteinase which does not utilize a histidine residue as a catalytic base (4, 32), unlike the common serine proteinases, such as trypsin, which effect peptide bond hydrolysis by utilization of a catalytic triad consisting of serine, histidine, and aspartate (5). In the absence of histidine, an alternative catalytic base is clearly required for deprotonation of the serine hydroxyl group to produce an appropriate nucleophile and, presumably, for deprotonation of a water molecule to effect the deacylation reaction. Experiments involving the sensitive in vivo assay of mutated forms of LP from *E. coli* have been performed to identify the base(s) involved in catalysis.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis. Plasmid pTZlep1 (4), which carries the *lepB* structural gene, was subjected to site-directed mutagenesis to introduce a unique *Bgl*II site 265 nucleotides upstream of the initiator methionine of the *lepB* gene. This construct was used as a template for site-directed mutagenesis of the *lepB* gene. Mutant or wild-type *lepB* genes were excised as 1.3-kb *Bgl*II-*Kpn*I fragments and transferred to pNA2 (4) which had been cut with the same enzymes, thereby generating pNA9 and variants thereof. LP production is under control of the *ara* promoter, as in pRD8 (10), and is stimulated by addition of arabinose to the growth medium. Plasmid pRD9 (10) is a derivative of pRD8 and encodes a catalytically inactive variant of LP which lacks ~100 C-terminal amino acid residues. pBR322 (6) is a general-purpose cloning vector which confers resistance to ampicillin on the host cell and carries no *lepB* sequences. All plasmids were amplified in *E. coli* HB101 grown in Luria broth (25). Mutants were generated with appropriate synthetic oligonucleotides according to standard procedures (33, 34) and were identified by sequencing DNA derived from randomly selected colonies according to the dideoxynucleotide chain-termination method described by Sanger et al. (26). Mutant plasmids were used to transform *E. coli* IT41

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(18) to ampicillin resistance. All other recombinant DNA techniques were performed according to standard protocols (25). Sequence alignments were derived by using the GAP program on University of Wisconsin Genetics Computer Group software (13). pNA9 variants carrying mutant forms of the *lepB* gene are referred to as in the following example: pNA9 which expresses a *lepB* gene encoding an LP variant in which arginine 282 has been replaced with glutamine is referred to as pNA9.R282Q.

Enzyme activity. The detection of enzyme activity was in vivo in character, since the sensitivity of in vitro assays of LP is considerably lower (3). Assessment of enzyme activity depends on complementation of *E. coli* IT41, which possesses a temperature-sensitive defect in LP activity (18). Although the chromosomally encoded LP variant has significant activity at 30°C, the activity at 42°C is extremely low, and cell growth is barely perceptible over a period of several hours unless the lesion is complemented by production of plasmid-borne active LP (4, 18).

Cell growth rate. Ampicillin-resistant transformants of *E. coli* IT41 were grown in Luria broth at 42°C in the presence of ampicillin (50 $\mu g \cdot ml^{-1}$) and arabinose (0.2% [wt/vol]), and aliquots were taken at hourly intervals. Cell growth, as measured by the increase in optical density at 600 nm, was monitored.

Western blots (immunoblots). Proteins were separated by polyacrylamide gel electrophoresis (19) on 13% acrylamide (37.5:1 [acrylamide/bisacrylamide]) minigels and blotted to nitrocellulose membranes on a Bio-Rad semidry blotter according to the instructions of the manufacturer. LP was detected by probing with polyclonal antiserum to *E. coli* LP with an AuroProbe Immunogold Silver staining kit according to the instructions of the manufacturer (Amersham).

RESULTS

Although the evidence that LP is a serine proteinase is presently circumstantial, it is nonetheless of a convincing nature. It is known that of the 17 serine residues in the primary structure of *E. coli* LP, only serine 90 (*E. coli* numbering) cannot be replaced by an alanine residue and retain activity (32). It is of note that an optimal alignment of the four known LP sequences (Fig. 1) demonstrates that only three serine residues are conserved and that one of these is serine 90. Additionally, serine 90 can be replaced by a cysteine residue, resulting in a protein which retains some activity that can be abolished by incubation with *N*-ethylmaleimide (9).

Since it has been demonstrated that the histidine residues in LP from *E. coli* can all be substituted without significantly affecting activity (4) and also that none are conserved in the aligned prokaryote LP sequences (Fig. 1), histidine residues are clearly not required for catalysis, and one or more alternative residues must therefore perform this function. Candidate amino acids are obviously restricted to those which possess ionizable side chains (i.e., those susceptible to reversible protonation) and are therefore glutamate, aspartate, tyrosine, lysine, and arginine (it is known that cysteine residues in LP are not essential [4]). Examination of Fig. 1 reveals that the conserved residues of this type (numbering according to the *E. coli* sequence) are one glutamate (position 61), four aspartates (positions 129, 153, 273, and 280), two tyrosines (positions 143 and 268), one lysine (position 145), and five arginines (positions 77, 127, 146, 275, and 282). It is known that LP retains activity when glutamate 61 is replaced by valine and when arginine 77 is

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                                                                      50
Ec  MANMFALILV IATLVTGILW CVDKFFFAPK RRERQAAAQA ARDSLDKATL
St  MANMFALILV IATLVTGILW CVDKFVFAPK RRARQAAAQT ASDALDNATL
Pf  MSLNFPLLLV IAVAVCGLLA LLDLVFFAPR RRSAIASYQG SVSQPDAVVI
Bs  .......... .......... .......... .......... .......MKS

                                                                     100
Ec  KKVAPKPGWL ETGASVFPVL AIVLIVRSFI YEPFQIPSGS MMPTLLIGDF
St  NKVAPKPGWL ETGASVFPVL AIVLIVRSFL YEPFQIPSGS MMPTLLIGDF
Pf  EKLNKEPLLV EYGKSFFPVL FIVLVLRSFL VEPFQIPSGS MKPTLDVGDF
Bs  ENVSKKKSIL EWAKAIVIAV VLALLIRNFI FAPYVVDGDS MYPTLHNRER
                E              L  R F    P       S M PTL
                *                *

                                                                     150
Ec  ILVEKFAYGI KDPIYQKTLI ETGHPKRGDI VVFKYPEDPK LDYIKRAVGL
St  ILVEKFAYGI KDPIYQKTLI ETGHPKRGDI VVFKYPEDPK LDYIKRAVGL
Pf  ILVNKFSYGI RLPVIDKKVI EVGDPQRGDV MVFRYPSDPN VNYIKRVVGL
Bs  VFVN...... ......MTVK YIGEFDRGDI VVL...NGDD VHYVKRIIGL
      V                      G   RGD   V           Y KR  GL
                               * *                  * **

                                                                     200
Ec  PGDKVTYDPV SKELTIQPGC SSGQACENAL PVTYSNVEPS DFVQTFSRRN
St  PGDKITYDPV AKEVTIQPGC SSGQACENAL PVTYSNVEPS DFVQTFARRN
Pf  PGDVVRYTS. DKRLFINGES ....VAEKLL GAEPNTLGSA ELYQ......
Bs  PGDTV..EMK NDQLYI.... .......... .......... .........N
    PGD
     *

                                                                     250
Ec  GGEATSGFFE VPKNETKENG IRLSERKETL GDVTHRILTV PIAQDQVGMY
St  GGEATSGFFE VPLNETKENG IRLTERKETL GDVTHRILMV PIAQDQLGMY
Pf  .......... .......... .......EKL GAVEHEI... ...RKEMSRY
Bs  GKKVDEPYLA ANKKRAKQDG ..FDHLTDDF GPV....... ..........
                                     G V

                                                                     290
Ec  YQQPGQQLAT WIVPPGQYFM MGDNRDNSAD SRYW...... ....GFVPEA
St  YQQPGQPLAT WVVPPGQYFM MGDNRDNSAD SRYW...... ....GFVPEA
Pf  RAMPDGQ... WKVPAGHYFM MGDNRDNSND SRYWDDPNIP KDLLGMVPDE
Bs  .......... .KVPDNKYFV MGDNRRNSMD SR......... .NGLGLFTKK
                  VP    YF  MGDNR NS D SR             G
                        *     * *    *  *
                                       323
Ec  NLVGRATAIW MSFDKQEGEW PTGLRLSRIG GIH.
St  NLVGKAVAIW MSFDKQEGEW PTGVRLSRIG GIH.
Pf  NIVGKAFAVW MSW..PEPKL SHLPNFSRVG LIK
Bs  QIAGTSKFVF YPFNEMRKTN .......... ....
      G
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FIG. 1. Alignment of the four known prokaryote LP amino acid sequences. Amino acid sequences were aligned with the GAP routine of the University of Wisconsin Genetics Computer Group software (13) and numbered according to the *E. coli* LP sequence. No pairwise-alignment quality score was less than 13 standard deviation units above that obtained from randomization of sequences. Ec, *E. coli*; St, *S. typhimurium*; Pf, *P. fluorescens*; Bs, *B. subtilis*. Amino acid residues which possess ionizing side chains are marked with an asterisk.

replaced by glutamate, asparagine, or leucine (3). Of the four conserved aspartates, three can be replaced by alanine (positions 129, 273, and 280) without effect, but replacement of aspartate 153 by alanine results in an inactive enzyme, thereby suggesting that the side chain of this residue may play a catalytic role (32). In the case of the mutation of aspartate 153 to alanine in LP, however, a possibly erroneous conclusion might be reached, since alanine is chemically very dissimilar to an aspartic acid and may be unlikely to successfully substitute for an aspartic acid which plays a critical structural role. Therefore, it is possible that the D153A variant of LP is inactive, for example, because of a structural disruption rather than replacement of a catalytic residue. For this reason, aspartate 153 was replaced by an asparagine residue, the side chain of which is chemically much more similar to that of an aspartic acid but which lacks the capacity to transfer protons. The possible role of the remaining amino acid residues was examined by replacement of lysine 145 by methionine, of tyrosines 143 and 268 by phenylalanine, and of arginines at positions 127, 146, 275, and 282 by glutamine. All mutants were tested for in vivo activity by their capacity to complement the lesion in the

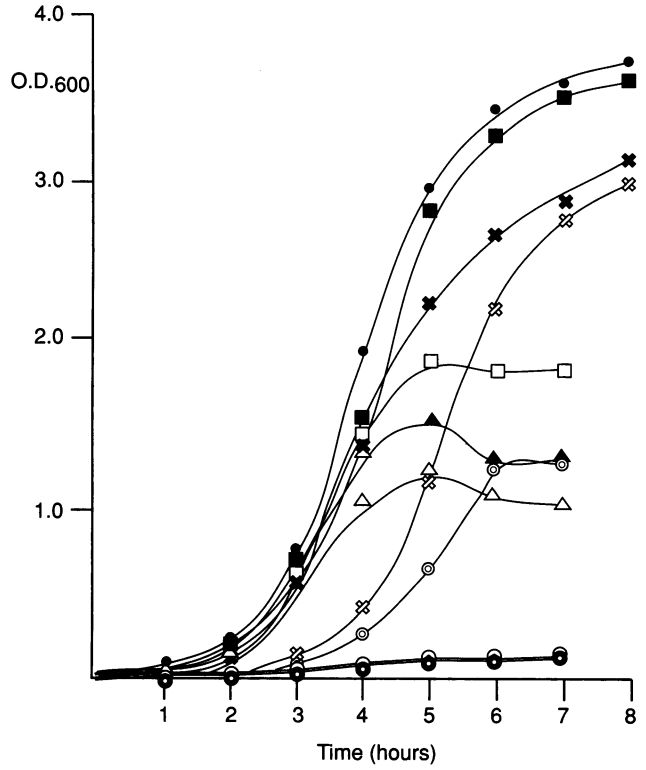

FIG. 2. Growth curve at 42°C of *E. coli* IT41 transformed with various plasmids. Cells harboring plasmids expressing various forms of *E. coli* LP were grown in Luria broth at 42°C in the presence of 0.2% (wt/vol) arabinose. The optical density of the cell suspension, measured at 600 nm (OD_{600}), was plotted as a function of time; all points are an average of two experiments. Solid circles, pNA9; open circles, pNA9.K145M; solid squares, pNA9.Y143F; open squares, pNA9.R146Q; solid crosses, pNA9.Y268F; open crosses, pNA9.R282Q; solid triangles, pNA9.R127Q; open triangles, pNA9.D153N; solid rings, pRD9; open rings, pNA9.R275Q.

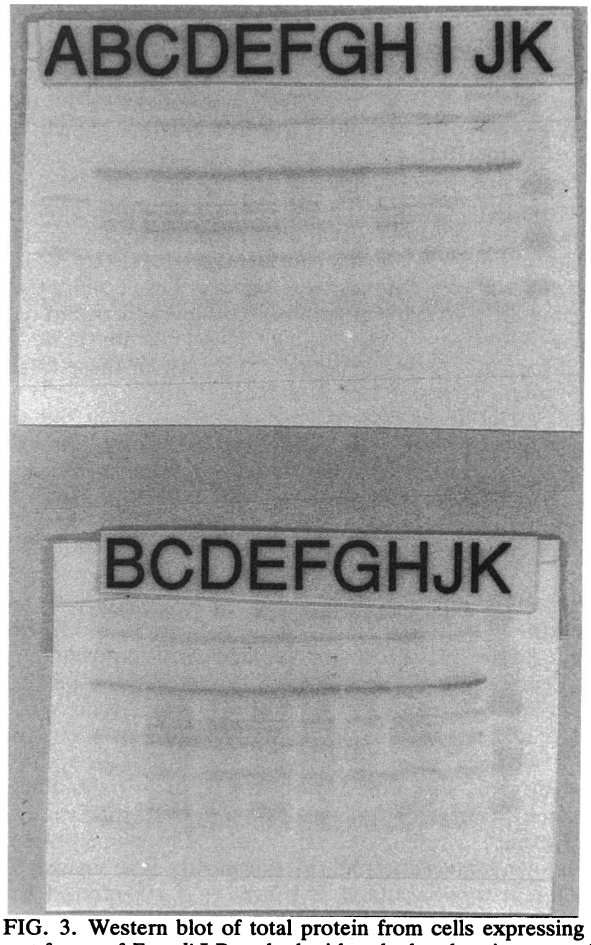

FIG. 3. Western blot of total protein from cells expressing different forms of *E. coli* LP probed with polyclonal antiserum to LP. Total protein from cells harvested at mid-log (top) phase or at stationary (bottom) phase blotted and probed with anti-LP. Lanes: A, pBR322; B, R282Q; C, R275Q; D, R146Q; E, R127Q; F, Y268F; G, Y143F; H, D153N; I, K145M; J, pNA9; K, markers (Rainbow molecular weight markers; Amersham).

chromosomal *lepB* gene of *E. coli* IT41 (Fig. 2) as described in Materials and Methods. Any detectable activity of plasmid-encoded LP in cases in which an ionizing side chain has been replaced would indicate that the residue in question does not fulfill a role as a catalytic base, since it has been demonstrated that removal of active-site residues in proteinases such as trypsin or subtilisin (for example, the catalytic serine or histidine [7, 17]) results in a decrease in k_{cat} values of ~6 orders of magnitude. Since the growth rate of strain IT41 is not 0 at the nonpermissive temperature (42°C), an appropriate control plasmid (pRD9) was utilized. This plasmid encodes a catalytically inactive form of LP which lacks ~100 C-terminal amino acid residues but which is expressed to levels similar to those of authentic plasmid-encoded protein and which assembles correctly in the cytoplasmic membrane (10).

It is clear that the substitution of a methionine residue for the lysine at position 145 results in an enzyme which possesses no detectable activity, since the growth characteristics of *E. coli* IT41(pNA9.K145M) are indistinguishable from those of *E. coli* IT41(pRD9) (Fig. 2). The production of the K145M mutant does not depress the cell growth rate at 30°C (data not shown). In all other cases, the mutant LP variants possess sufficient catalytic activity to support cell growth to various degrees. Although the substitutions of phenylalanine for tyrosine at positions 143 and 268 have only a small effect on cell growth rate, all other mutations have a significant disabling effect on activity. The mutations D153N, R146Q, and R127Q all result in an enzyme which initially almost fully complements the *E. coli* IT41 temperature-sensitive phenotype. However, at a time point close to the mid-exponential growth phase of cells bearing the wild-type enzyme, there is a marked decrease in cell growth rate, and the cell cultures rapidly approach a stationary phase which is of a much lower density than that of cells bearing the authentic LP. The observation that aspartate 153 is not required for enzyme activity disproves the theory that aspartate 153 plays a crucial catalytic role (32). The mutants R275Q and R282Q both support a much-reduced initial rate of cell growth; in addition, the R275Q mutant has an effect similar to those of the D153N, R127Q, and R146Q mutants on the cell density in stationary phase.

It was considered that these effects may be due to a lower stability of the mutant protein relative to the authentic LP enzyme, the effects of which are fully realized only when the culture approaches stationary phase and protein synthesis rates become lower. Figure 3 shows the results of a Western blot of total protein from cells harboring plasmids encoding

the various mutant LP forms which has been probed with polyclonal antibodies to LP. Aliquots of cells which had been grown to mid-log phase at 30°C and then subjected for 1 h to a temperature of 42°C in the presence of 0.2% arabinose (wt/vol) were taken; in cases in which growth was supported by the plasmid concerned, they were also taken at stationary phase. Immunodetectable concentrations of LP are present in all cases, thereby demonstrating the stable accumulation of all LP variants, including the inactive K145M mutant. Chromosomal levels of LP [IT41(pBR322); Fig. 3, lane A] were not detected under these conditions.

Lysine 145 was subsequently substituted with aspartate, a residue of opposite charge, with glycine, and with serine. No detectable activity was associated with any of these further three mutant forms of LP (data not shown).

The crucial observation from these data is that the only conserved amino acid residue bearing an ionizing side chain which cannot be replaced with one which does not bear an ionizing side chain is the lysine residue at position 145.

DISCUSSION

The classical serine proteinases, such as trypsin, are among the most fully defined classes of enzyme both structurally and mechanistically. The mode of action of this type of proteinase (14, 27) involves the generation of a powerful nucleophile which attacks the carbonyl carbon of the scissile bond. The nucleophile is generated via proton abstraction from a serine (serine 195 in trypsin) hydroxyl group by an active-site base (histidine 57) whose pK_a and orientation are modulated by a specific aspartate residue (aspartate 102). These serine, histidine, and aspartate residues constitute the well-known catalytic triad of the serine proteinases (5). Subsequent to this attack, the proton is transferred from histidine 57 to the amide nitrogen, thereby generating the acyl enzyme. Deacylation is essentially a reversal of the above-described procedure. A water molecule is deprotonated by histidine 57, the resultant hydroxyl ion attacks the carbonyl carbon atom of the acyl enzyme, and deacylation is completed by transfer of the proton on histidine 57 to restore the serine hydroxyl group. Although it is clear that prokaryote LP does not belong to this class of proteinase (i.e., a serine proteinase which utilizes a Ser-His-Asp catalytic triad), evidence presented here and elsewhere (4, 32) strongly suggests that LP is a serine protease but that the proton transfers are mediated by an alternative residue. The amino acid sequence alignment shown in Fig. 1 dictates which residues are possible candidates for this role and reveals that these are limited in number. The data presented herein indicate unambiguously that the only possible candidate for the role of catalytic base in this class of enzyme is lysine 145.

Although the pK_a value of the side chain of lysine is ~10.8 when in solution (30), it is known that the functional pK_a value of amino acid side chains can be radically altered by the protein environment (11). The class A and class C β-lactamases are worthy of consideration in this respect since, although these enzymes are not proteinases, they are nonetheless amide hydrolases. It has been known for some time that the class A and class C β-lactamases hydrolyze the cyclic amide ring of β-lactam antibiotics by attacking the carbonyl carbon of the amide bond and that the relevant nucleophilic group is furnished by the side chain of a serine residue; the side chain is rendered nucleophilic because of proton abstraction by a nonhistidine residue, which in the case of the class A enzyme has been proposed to be a lysine and/or a glutamate residue (16, 20, 31); in the case of the class C enzyme, a tyrosine residue has been proposed (22). Evidence suggests that the class A β-lactamase mechanism may involve the sequential operation of two bases with different chemical identities, in that a lysine residue catalyzes deprotonation of the catalytic serine hydroxyl group and a glutamate, via an intervening water molecule, catalyzes deprotonation of the hydrolytic water molecule (1, 31). It seems unlikely that the mechanism of action of LP will prove to be similar in fine detail, since neither glutamate nor aspartate residues are required for activity.

Perhaps the most significant comparison of LP can be made with LexA, a protein which represses ~20 genes which are involved in the SOS response and DNA repair in *E. coli* (37). It has been demonstrated that the cleavage of the LexA protein is an autocatalytic intramolecular event that is catalyzed by a serine-lysine catalytic dyad (28). Mutations of serine 119 and lysine 156 inactivate the enzyme (28), and the majority of partially disabled enzyme variants resulting from random mutagenesis have been shown to be due to substitutions in the regions of primary structure close to the catalytic serine and lysine residues (21). In addition, LP and LexA have several other similarities: both enzymes are resistant (as indeed are the β-lactamases [8, 23]) to inhibition by diisopropyl fluorophosphate, a diagnostic inhibitor of serine proteinases, at concentrations (1 mM) which totally inhibit the classical serine proteinases (24); both enzymes appear to display relatively low activities compared with, e.g., trypsin; and the proposed catalytic serine and lysine residues of both enzymes are immediately N terminal to a methionine and an arginine residue, respectively (24) (Fig. 1). This last observation may be of a wider significance, since sequence similarity with certain eukaryotic signal peptidase subunits has been proposed previously (35). The similarities are clustered into five regions, one of which includes serine 90 and one of which includes lysine 145 (*E. coli* numbering). It is interesting that both the yeast Sec11 and canine Spc18/21 subunits have a histidine residue in place of the lysine 145, raising the intriguing possibility that the eukaryotic catalytic subunits operate by a more-classical serine proteinase mechanism. However, a note of caution should be applied, since alignments of any of the prokaryote sequences with those of the yeast Sec11 or canine Spc18/21 subunits are of very low statistical significance (<3 standard deviation units above random alignments; data not shown).

Conclusion. The data presented here reveal that of all conserved amino acid residues in prokaryote LP variants, lysine 145 is the only amino acid possessing an essential ionizable side chain. Lysine 145 is therefore the sole candidate for a potential catalytic base in the mechanism of action of LP. This observation, together with the published evidence that serine 90 is catalytic, strongly suggests that the enzymological activity of prokaryote LP is dependent on the operation of a serine-lysine catalytic dyad. It is interesting to speculate on the possibility that the serine-lysine dyad is, unlike the Ser-His-Asp triad, innately inefficient and that it exists in some enzymes because of the lack of selective pressure to increase rates beyond those displayed by enzymes such as LP (k_{cat}, $300 \cdot h^{-1}$ [12]) and LexA (k_{max}, $\sim 7 \cdot h^{-1}$ [29]).

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