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The Roles of the Prosequence of Thermolysin in Enzyme Inhibition and Folding *in Vitro**

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The zinc endopeptidase thermolysin (EC 3.4.24.27), an extracellular enzyme from *Bacillus thermoproteolyticus*, is synthesized as a preproprotein, with the prosequence (204 residues) being two-thirds the size of the mature enzyme (316 residues). This prosequence, expressed in and purified from *Escherichia coli*, inhibited thermolysin *in vitro* with an IC_{50} value of 14 nM. It also inhibited a closely related enzyme produced by *Bacillus stearothermophillus*, albeit with a 16-fold higher IC_{50} value (220 nM). The IC_{50} value for thermolysin inhibition was also increased 15-fold (210 nm) by a monoclonal antibody that recognizes an epitope close to, but not forming a part of, the active site. At a prosequence concentration of 5 μM a mammalian, thermolysin-like enzyme, neutral endopeptidase 24.11, was not inhibited. The prosequence appeared to act as a mixed, noncompetitive inhibitor of thermolysin activity, with a K_i value of 6 nM for its interaction with the enzyme alone and a K_i' value of 20 nM for its interaction with the enzyme-substrate complex. In addition, when thermolysin was denatured in 6 M guanidinium hydrochloride at acid pH and then brought to neutral pH by rapid dilution, the prosequence was found to facilitate the recovery of active enzyme in a stoichiometric manner.

The thermostable zinc metalloprotease thermolysin (EC 3.4.24.27), a 34.6-kDa neutral endoprotease secreted by *Bacillus thermoproteolyticus,* is the most well studied member of a large family of extracellular proteases secreted by both Gram-positive and Gram-negative bacteria, which have significant amino acid homologies and similar specificities in cleaving proteins at the N-terminal side of hydrophobic residues. Thermolysin has a bilobal structure, with the active site, containing one atom of zinc, situated in a cleft between the N- and C-terminal regions. X-ray crystallographic studies have shown that the proteases from *Bacillus cereus* (1) and *Pseudomonas aeruginosa* (pseudolysin, EC 3.4.24.26) (2) have virtually the same three-dimensional structures as thermolysin (reviewed in Ref. 3), and similar structures have been predicted for the enzymes from *Bacillus stearothermophillus* (NPrSte),[1] (4) *Bacillus subtilis* (5), and *B. subtilis* var. *amylosacchariticus* (6).

Like many other extracellular proteases, the thermolysin-like enzymes are synthesized as inactive preproproteins, with the prosequences, of around 200 residues, being approximately two-thirds of the sizes of their mature enzymes (7, 8, 9). Sequence homology among the prosequences is not as strong as that found between the mature enzymes, but it generally follows the same evolutionary relationship. Evidence for the autocatalytic removal of these prosequences has come from site-directed mutagenesis studies on the enzymes from *B. subtilis* (10), *B. stearothermophillus* (11), *B. Cereus* (8), and *P. aeruginosa* (12, 13).

The role of protease prosequences has been the most comprehensively studied for the bacterial serine proteases subtilisin (14–16), α-lytic protease (17–19), and carboxypeptidase Y (20–22), where they have been shown to assist the folding of their respective enzymes *in vivo* and *in vitro*. In addition, it was found that a covalent linkage between the prodomain and mature domain is not necessary for prosequence-assisted folding (15, 17, 22). With subtilisin (16) and α-lytic protease (18) the processed prosequences also act as competitive inhibitors of the mature enzymes. The noncovalently linked prosequence of the yeast vacuolar enzyme carboxypeptidase Y, however, has a low affinity for its mature enzyme *in vitro* (22).

Among the cysteine proteases, the prosequences of the papain family (23) and cathepsin B (24) inhibit their respective mature enzymes, and that of cathepsin L is essential for folding (25). For the aspartic proteases, prosequence-assisted folding has also been demonstrated for the yeast protease proteinase A (26), whereas the prosequence of the mammalian enzyme cathepsin D is important for enzyme targeting to the lysosome, although it does not appear to be necessary for folding (27).

There is currently little information regarding the roles of prosequences for the zinc metallopeptidase family. The prosequences of the mammalian enzymes carboxypeptidase A (28) and collagenase (29) inhibit their mature enzymes, and that of coccolysin from *Streptomyces cacaoi* is essential for the production of the mature enzyme *in vivo* (30). In the thermolysin family of enzymes, the proregion of the enzyme from *B. cereus* has been shown to be involved in enzyme targeting (7, 8), and intracellular fractions of *P. aeruginosa*, containing prosequence-like immunoreactivity, inhibit pseudolysin (31). More recently, it has been shown that the prosequence of pseudolysin, in either *cis* or *trans* form, is essential for the secretion of the mature, active enzyme, and it has been proposed to have a role in enzyme folding *in vivo* (32).

In many of the cases cited above it has been shown that a covalent linkage between a prosequence and its respective enzyme is not necessary for either inhibition or facilitating folding *in vivo* or *in vitro*. In the present study, the thermolysin prosequence has been expressed as an independent polypeptide

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[1] The abbreviations used are: NPrSte, neutral protease from *Bacillus stearothermophillus*; TLN, thermolysin; PRO, prosequence; mAb, monoclonal antibody.

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in *Escherichia coli*, and its potential role in the inhibition and refolding of its mature enzyme *in vitro* has been investigated.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Leu-enkephalin was purchased from Izinta (Budapest, Hungary), and Leu-enkephalin was from Bachem. The peptides P_{15} (Ala-Lys-Pro-Gly-Asp-Val-Lys-Ser-Ile-Thr-Gly-Thr-Ser-Thr-Val) and Ile-Thr-Gly-Thr-Ser-Thr-Val were prepared in the laboratory by solid phase synthesis and purified by high performance liquid chromatography, using a Vydac C_{18} column and a gradient of 10–50% acetonitrile in 0.1% trifluoroacetic acid. Leu-enkephalin, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, soybean trypsin inhibitor, and the thermolysin inhibitor phosphoramidon (*N*-(α-L-rhamnopyranosyl)-oxyhydroxy-phosphinyl)-L-leucyl-L-tryptophan) were purchased from Sigma. All culture media components were Difco products obtained from OSI (Maurepas, France). Oligonucleotides were purchased from Genosys (Cambridge, United Kingdom), and the Sequenase enzyme (version II) and [α-^{33}P]dATP were from Amersham Corp. New England Biolabs DNA modifying enzymes and the Stratagene pCR-Script cloning kit were from Ozyme (Montigny-le-Bretonneux, France); the Xpress protein expression system is an *in vitro* gene product purchased from R&D systems (Oxford, UK).

Enzymes—Thermolysin and the neutral protease NPrSte from *B. stearothermophillus* were purified from culture supernatants using a Gly-dPhe affinity column as described previously (33, 11). Alternatively, for refolding experiments, commercially available thermolysin (Boehringer Mannheim) was used. Neutral endopeptidase 24.11 (EC 3.4.24.11) was purified from rabbit kidney as described previously (34).

Bacterial Strains, Bacteriophage, Plasmids, and Growth Conditions—The bacterial strains used for subcloning were the *E. coli* strain XL1-blue (Stratagene) and, for expression work, JM109. The bacteriophage M13/T7 and the plasmid pRSET B were from the Xpress protein expression system, and the plasmid pCR-Script SK$^+$ was from the Stratagene kit. The bacteriophage was a modified M13 bacteriophage containing the gene encoding the T7 RNA polymerase. The plasmid, pTLN2, containing the gene encoding thermolysin, has been described previously (33). Routinely, *E. coli* JM109 was grown in LB broth (10 g/liter bacto-tryptone, 5 g/liter bacto-yeast, and 10 g/liter NaCl, pH 7.0) containing ampicillin (100 mg/ml), when necessary, at 37 °C. Production of bacteriophage stocks and routine recombinant DNA procedures were carried out as described (35). For protein expression, minimal medium (M9 salts, 20 mM glucose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 3 mM thiamine) was inoculated with a single colony of JM109 bearing the plasmid pTLNPRO, and the culture was incubated for 16 h at 37 °C with shaking (300 rpm). The culture was diluted (1:100, v/v) with SOB medium (20 g/liter bactotryptone, 5 g/liter bacto-yeast, 0.5 g/liter NaCl, 2.5 mM KCl, and 10 mM $MgCl_2$, pH 7.0), and incubation continued until an *A* value of 0.3 at 600 nm was obtained. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, incubation was continued for 1 h, and the absorbance was redetermined. M13/T7 bacteriophages were then added to the culture at a multiplicity of infection of 5 plaque-forming units/cell, the incubation continued for 4 h, and the cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4 °C. The pellet was washed once with Tris/EDTA buffer, and the cells were collected by centrifuging as before.

Design and Amplification of a Prosequence Cassette—To obtain the thermolysin prosequence as a single, isolated polypeptide, an expression cassette was created by polymerase chain reaction amplification of the corresponding sequence in the TLN gene coupled with polymerase chain reaction-directed mutagenesis using the following primers: PRONT, 5′-GGCGTCATATGGCTTCAACGGAACAC-3′; and PROCT, 5′-CCTGTCCATGGTTACGACTTCACATCATCACC-3′. As the cleavage site between the prosequence and the mature thermolysin polypeptide is between Ser-232 and Ile-233, the last codon of the PRO cassette was that of Ser-232, followed by a stop codon (underlined), the introduction of which is directed by the polymerase chain reaction primer PROCT. Equally, this primer directs the introduction of a *Nco*I recognition sequence (double underlined). The 5′-coding extremity, defined by PRONT, consists of an artificially introduced Met codon followed by the codon for Ser-29 (underlined), the amino acid predicted to be the C-terminal residue of prothermolysin (33). Like PROCT, PRONT also directs the introduction of an endonuclease recognition sequence (*Nde*I; double underlined).

DNA amplification was performed in 100 ml of 2 mM $MgCl_2$, 200 μM dNTPs, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 20 mM Tris-HCl, pH 8.8, containing the oligonucleotides PRONT and PROCT, 2.5 units of *Taq* DNA polymerase, and 1 ng of pTLN2 DNA. The thermal cycling parameters were 94 °C for 60 s, 42 °C for 30 s, and 72 °C for 30 s during three cycles and then 94 °C for 60 s, 58 °C for 30 s, and 72 °C for 30 s (or 10 min for the 30th cycle) during 27 cycles. After amplification, the reaction mixture was extracted with an equal volume of chloroform, and the DNA was precipitated using 2.5 volumes of ethanol, recovered by centrifugation at 10,000 × *g* for 15 min at ambient temperature, and washed using 70% (v/v) ethanol. After recentrifugation, the supernatant was discarded, and the dried DNA pellet was resuspended in 10 μl of Tris/EDTA buffer and stored at −20 °C until required.

Subcloning—The blunt end, polymerase chain reaction-amplified PRO cassette was inserted into pCR-Script SK$^+$ creating pCR-Script/PRO. This plasmid was simultaneously digested with *Nde*I and *Nco*I, liberating the PRO cassette, which was than inserted into the plasmid pRSET B, thus creating pTLNPRO. After verification of this construction by DNA sequencing, pTLNPRO was used for protein expression trials.

Prosequence Purification—E. coli cells transformed with pTLNPRO were harvested by centrifugation at 10,000 × *g* for 30 min. The cells were resuspended in 50 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethanesulfonyl fluoride, 2 mM 1,10-phenanthroline, 10 μM bestatin, 100 μM leupeptin, and 2 μM pepstatin, lysed by sonification, and centrifuged at 50,000 × *g* for 40 min. Aliquots of the supernatant inhibited thermolysin activity, and this was used to assay for the presence of prosequence in the subsequent chromatographic steps. The supernatant was then passed over a column of Superdex^{TM} 75 (Pharmacia Biotech, Inc.), and proteins eluted in 50 mM Tris-HCl, pH 7.4, at 0.4 ml/min. Peak fractions containing inhibitory activity were pooled, and solid $(NH_4)_2SO_4$ was added slowly to give 40% saturation. After centrifugation at 10,000 × *g* for the 30 min, the supernatant was passed over a column of phenyl-Superose (Pharmacia), previously equilibrated in 50 mM Tris-HCl, pH 7.4, containing 40% saturated $(NH_4)_2SO_4$. Bound proteins were eluted by a gradient of 40 to 0% saturated $(NH_4)_2SO_4$ in 40 min at a flow rate of 0.5 ml/min. Thermolysin inhibitory activity eluted in a peak toward the end of the gradient, and purity was checked by SDS-polyacrylamide gel electrophoresis using 15% slab gels. Protein was measured by the method of Bradford (36).

Assay for Thermolysin Activity—IC_{50} values were measured at 37 °C in 100 μl of 50 mM Hepes, pH 6.8, containing 10 mM $CaCl_2$ and 0.29 nM enzyme, with 50 nM [^{3}H]Leu-enkephalin as substrate. The reactions were stopped after 30 min by the addition of 10 μl of 0.5 M HCl and the metabolite [^{3}H]Tyr-Gly-Gly separated over columns of Poropak Q (Waters) (37). When monoclonal antibodies were used, the enzyme was preincubated for 2 h at 25 °C with a 500-fold excess of antibody before activity was measured. NPrSte and neutral endopeptidase 24.11 activity were measured in the same manner. When K_m and V_{max} values for Leu-enkephalin (K_m for thermolysin, 460 μM; Ref. 33) degradation were measured, the enzyme was at 0.2 nM, and the substrate was used over a concentration range of 0.1–3.0 mM, with 50 nM tritiated peptide included as tracer. Kinetic constants were calculated by linear regression analysis using the ENZFIT program (Biosoft). In all cases substrate degradation was ≤10% total. The degradation of the pentadecapeptide P_{15} by thermolysin was quantified by measuring the appearance of one of the two metabolites formed, Ile-Thr-Gly-Thr-Ser-Thr-Tyr, by high performance liquid chromatography. The substrate was used at a concentration of 10 μM, and buffers and assay conditions were as described above. The reaction mixture was injected onto a 13-μm, 100-Å Kromasil C18 column and eluted at 1 ml/min, with a gradient of 12–27% acetonitrile and 0.7–0.6% trifluoroacetic acid in 15 min. Under these conditions, the metabolite and substrate had retention times of 7.6 and 14.7 min, respectively.

Denaturation and Renaturation—Thermolysin (0.5 mg/ml) was left to denature for 20 h at 30 °C in 100 mM glycine-H_3PO_4, pH 2.0, containing 6 M guanidinium hydrochloride. 4-μl aliquots were taken and rapidly diluted by adding a 50-fold excess of renaturation buffer, while shaking, with or without the prosequence or an equivalent quantity of bovine serum albumin. After leaving at 25 °C for 1 h, aliquots were further diluted in 50 mM Hepes, pH 7.0, containing 10 mM $CaCl_2$, to assay enzyme activity. To measure the rate of refolding, reactions were stopped by adding 10 μl of a 1 mg/ml solution of trypsin, followed 20 s later by 10 μl of a 4 mg/ml solution of soybean trypsin inhibitor. Under these conditions, the prosequence was rapidly degraded, but the activity of active thermolysin was not changed. The renaturation buffer consisted of 50 mM Hepes, pH 7.0, containing 10 mM $CaCl_2$, 5 μM $ZnCl_2$, and 4 μM phosphoramidon. When trypsin was added, the pH was at 8.0. The control enzyme, at the same concentration, was initially dissolved in 50 mM Hepes, pH 7.0 or 8.0, containing 10 mM $CaCl_2$ and treated in the same way.

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-204 S T E H V T W N Q Q F Q T P Q F I S G D L L K V N G T S P E E L V Y Q Y V E K N E N K F K F H E N A K D T L Q L K E K K N D N L G F T F M R
     K G . S I V . . E . W K . . S . V . . S . . N G G E Q A L . . . . . . . D R E N G T . R L G G R . R . R . A . I G . Q T . E . . H . V . .

-134 F Q Q T Y K G I P V F G A V V T S H V K D G T L T A L S G T L I P N L D T K G S L K S G K K L S E K Q A R D I A E K D L V A N V T K E V P E
     . E . R H H . . . . Y . T M L A A . . . . . E . I . . . . S . . . . . . G Q P R . . K A . T V T V Q . . E A . . . Q . V T E T . . . . R . T

 -64 Y E Q G K D T E F V V Y V N G D E A S L A Y V V N L N F L T P E P G N W L Y I I D A V D G K I L N K F N Q L D A A K P G D V K S I[1] T G T S T
     T . N . E R . R L . I . P T D G T . R . . . E . . V R . . . . V . . . . V . . . . . T . . A . . . . . . . I . S R Q . . G G Q P V A . A . .

   7 V G V G R G V L G D Q K N I N T T Y S T Y Y - - - Y L Q D N T R G N G I F T Y D A K Y R T T L P G S L W A D A D N Q F F A S Y D A P A V D A
     . . . . . . . . . . . . Y . . . . . . S . . G Y Y . . . . . . . . S . . . . . . G R N . . V . . . . . . T . G . . . . T . . . . . A . . . .

  74 H Y Y A G V T Y D Y Y K N V H N R L S Y D G N N A A I R S S V H Y S Q G Y N N A F W N G S Q M V Y G D G D G Q T F I P L S G G I D V V A H E
     . . . . . . V . . . . . . . . G . . . . . . S . . . . . . T . . . G R . . . . . . . . . . . . . . . . . . . . . . . . L . F . . . . . . . G . .

 144 L T H A V T D Y T A G L I Y Q N E S G A I N E A M S D I F G T L V K F Y A N K N P D W E I G E D V Y T P G I S G D S L R S M S D P A K Y G D
     . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . E . . . . R . . . . . . . . . I . . . . V A . . A . . . . . . . . . . . .

 214 P D H Y S K R Y T G T Q D N G G V H I N S G I I N K A A Y L I S Q G G T H Y G V S V V G I G R D K L G K I F Y R A L T Q Y L T P T S N F S Q
     . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . L . . . . V . . . . . . N . . . . . M . . . . . . . . V Y . . . . . . . . . .

 284 L R A A A V Q S A T D L Y G S T S Q E V A S V K Q A F D A V G V K
     . . . . C . . A . A . . . . . . . . . . N . . . . . . N . . . . Y
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FIG. 1. **Alignment of the sequences of prothermolysin (*top*) and pro-NPrSte (*bottom*).** The numbering used is that of thermolysin, with residue 1 indicating the start of the mature enzyme. The sequence corresponding to peptide P_{15} is *underlined*, and the sequence containing the epitope recognized by $mAbT_2$ is underlined with a *dotted line*.

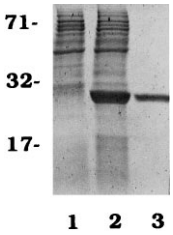

FIG. 2. **12% SDS-polyacrylamide gel electrophoresis of *E. coli* extracts and purified prosequence.** *Lane 1,* intracellular extracts from control *E. coli* cells; *lane 2,* intracellular extracts from *E. coli* cells expressing the thermolysin prosequence; *lane 3,* 5 μg of the purified thermolysin prosequence. The migration positions of molecular weight markers are shown on the *left*.

RESULTS AND DISCUSSION

Expression and Purification of the Thermolysin Prosequence—The prosequence of thermolysin, which consists of 204 residues (Fig. 1) (33), was expressed in *E. coli* (Fig. 2). Intracellular extracts of these cells inhibited thermolysin activity, unlike similar extracts from control cells, and this inhibitory activity was therefore used as an assay in subsequent chromatographic steps to purify the peptide (Fig. 2).

Inhibition of Thermolysin and Related Enzymes by Prosequence—Using the pentapeptide [^{3}H]Leu-enkephalin as substrate, the purified prosequence inhibited thermolysin, with an IC_{50} value of 14 nM (Table I). The inhibition appeared relatively specific, as the peptide had a 16-fold higher IC_{50} value (220 nM) for NPrSte from *B. stearothermophillus*, which has 85 and 50% sequence identity with thermolysin in its mature region and proregion, respectively (Fig. 1). All important active site residues are conserved between the two enzymes, and several competitive inhibitors of thermolysin have been found to inhibit NPrSte with similar K_i values (33). In addition, the prosequence did not inhibit neutral endopeptidase 24.11 at a concentration of 5 μM. Although this mammalian zinc endopeptidase has little sequence identity with thermolysin, its specificity is similar, and the two enzymes are thought to have strong structural homologies in their active sites (38).

As well as inhibiting thermolysin, the prosequence was also degraded with long incubation times at 37 °C, and this was reflected in the IC_{50} values obtained, which were constant for preincubation times of the enzyme and prosequence from 0 to 60 min but increased with longer preincubation times (200 nM at 2 h).

Kinetic Constants for the Inhibition of Thermolysin by Prosequence—When K_m and V_{max} values for Leu-enkephalin degradation were measured in the presence of increasing concentrations of prosequence, K_m values were found to increase, and V_{max} values decreased, indicating mixed, noncompetitive inhibition. A Lineweaver-Burke representation of the data is shown in Fig. 3, with the intercept of the curves being above the x axis. A plot of the K_m/V_{max} values against the prosequence concentration was linear (Fig. 4*A*), giving a K_i value of 6 nM for prosequence binding to the enzyme alone. A plot of the I/V_{max} values against the prosequence concentration was also linear (Fig. 4*B*) and gave a K_i' value of 20 nM for prosequence binding to the enzyme-substrate complex. Binding of the pentapeptide substrate, therefore, appears to have only a small effect on prosequence affinity for the enzyme, implying that its major site(s) is not at the active site of the enzyme. The K_i values obtained are in the same range as those observed for the inhibition of other enzymes by their prosequences, such as 540 nM for subtilisin (16), ≥0.1 nM for α-lytic protease (18, 19), 1.89 nM for papain (23), 860 nM for papaya proteinase IV (23), 0.4 nM for cathepsin B (24), and 2.0 nM for carboxypeptidase A (28). However, the results differ from others in that, in the few cases in which the mode of inhibition of the prosequences has been determined, those of carboxypeptidase A, subtilisin, and α-lytic protease have been reported to be competitive inhibitors for their respective enzymes (28, 16, 18). A noncompetitive mode of inhibition for the thermolysin prosequence, however, could explain the results obtained with NPrSte and neutral endopeptidase 24.11 and is in keeping with a report that the noncovalent complex formed between pseudolysin and its prosequence is not disrupted in the presence of competitive inhibitors and can be purified on an inhibitor affinity column (31).

Further evidence that the prosequence interacts with thermolysin at a region distinct from the active site came from results obtained using the monoclonal antibodies $mAbT_1$ and $mAbT_2$. These antibodies recognize epitopes in thermolysin,

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TABLE I
IC_{50} values for the inhibition of thermolysin and NPrSte by the thermolysin prosequence and P_{15}

Enzyme	Substrate	Inhibitor	IC_{50}
			nM
Thermolysin	[^{3}H]YGGFL	Prosequence	14.0 ± 2.0
Thermolysin	AKPGDVKSITGTSTV (P_{15})	Prosequence	15.2 ± 1.7
Thermolysin	[^{3}H]YGGFL	AKPGDVKSITGTSTV	>100,000
Thermolysin + $mAbT_2$	[^{3}H]YGGFL	Prosequence	210 ± 15
NPrSte	[^{3}H]YGGFL	Prosequence	220 ± 16
Neutral endopeptidase 24.11	[^{3}H]YGGFL	Prosequence	>5,000

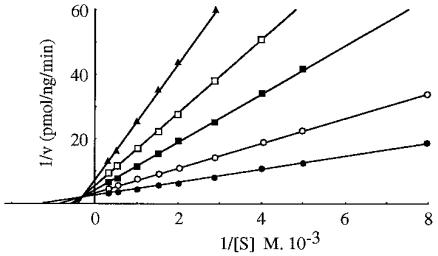

FIG. 3. **Inhibition of thermolysin by prosequence.** A Lineweaver-Burke representation of the inhibition of thermolysin by its prosequence is shown. The results shown are typical of a single experiment, and, for clarity, all the data points are not represented. The experimental conditions were as described under "Experimental Procedures," and the concentrations of prosequence (nM) were: ●, 0; ○, 10; ■, 20; □, 30; and ▲, 40.

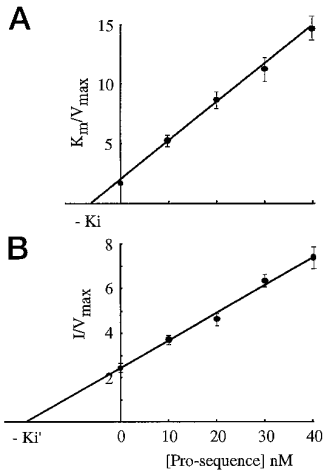

FIG. 4. **Inhibition of thermolysin by prosequence.** *A,* I/V_{max} values for the inhibition of thermolysin by its prosequence, plotted against prosequence concentration. The intercept on the *x* axis gives $-K_i$. *B,* K_m/V_{max} values for the inhibition of thermolysin by its prosequence, plotted against prosequence concentration. The intercept on the *x* axis gives $-K_i'$.

contained within residues 206–254 and 255–299, respectively, in the C-terminal domain of the enzyme, without affecting enzyme activity (data not shown), and $MAbT_1$, but not $mAbT_2$, also recognizes NPrSte (11). Preincubation of thermolysin or NPrSte with $mAbT_1$ had no effect on the inhibition of either enzyme by the thermolysin prosequence. In contrast, $mAbT_2$ increased the IC_{50} value of the prosequence for thermolysin 15-fold (Table I) and, as expected, had no effect on the inhibition of NPrSte. The exact epitope recognized by $mAbT_2$ is unknown, but residues 255–299 form two surface α-helices, linked by an exposed loop, which lie below the active site cleft. NPrSte has 7 amino acids different in this sequence, which might partially explain the differences in affinity of the prosequence for the two enzymes.

The major binding site in the active site of thermolysin is the S_1' subsite (3, 39), which ensures enzyme specificity by accepting large hydrophobic residues. Peptide substrates can additionally interact with the S_1, S_2, and S_2' subsites. These substrates, however, generally have high μM–mM affinities, and peptide-based active site inhibitors of thermolysin require a strong zinc-chelating group, such as a phosphate, hydroxamate, carboxylate, or sulfydryl, to achieve K_i values in the nM to low μM range (3).

Therefore, even if the N-terminal residues of the prosequence were to occupy the active site after processing, this alone would not be expected to give a nanomolar inhibition. This was confirmed by the poor affinity for the enzyme of the pentadecapeptide P_{15}, (IC_{50}, >100 μM; Table I), which constitutes the −8 to +7 sequence of prothermolysin (Fig. 1). High performance liquid chromatography analysis showed that P_{15} was cleaved by thermolysin between Ser-7 and Ile-8, which corresponds to the maturation site *in vivo* (data not shown), and this peptide would therefore not only occupy the S_1 and S_2 but also the S_1' and S_2' subsites.

P_{15} was also used as a substrate in inhibition assays to see whether prosequence binding might hinder its access to the active site to a greater extent than the pentapeptide substrate. The IC_{50} value obtained, however (15 nM), was virtually identical to that observed with [^{3}H]Leu-enkephalin (Table I).

It would seem, therefore, that *in vitro* at least, the strong inhibition of thermolysin by its prosequence is primarily due to interactions away from the active site of the enzyme, some of which may occur at, or close to, the two α helices formed by residues 255–299. This may resemble the situation with subtilisin, even though inhibition by its prosequence is competitive (16), as x-ray crystallographic studies of the noncovalently linked complex of the two proteins have shown that, although the N-terminal tetrapetide of the prosequence occupies the S_1–S_4 subsites of the active site, there are additional interactions between the 77-residue peptide and two surface α helices of the enzyme (40).

Refolding of Thermolysin by Its Prosequence in Vitro—When thermolysin was denatured at acid pH in 6 M guanidinium hydrochloride and then rapidly diluted in renaturation buffer, a small percentage of enzyme activity (≤2%) was recovered, compared with a nondenatured control. However, when different concentrations of the prosequence were included in the renaturation buffer, the percentage of enzyme activity recovered rose, reaching a maximum of around 20% of control levels, with a stoichiometric ratio of prosequence:enzyme (Fig. 5). Maximal enzyme activity was recovered within 2 min (Fig. 5, *inset*), and this activity was stable for up to at least 72 h (not shown). No difference in recovery was observed when the renaturation was carried out at pH 7.0 or 8.0. The competitive inhibitor of thermolysin, phosphoramidon, was included in the renaturation buffer at a concentration 50 times higher than its K_i value, as, in its absence, the yield of active enzyme was reduced by 65%. The role of the inhibitor is not clear, but it may act to prevent autolysis during the refolding process (18) and/or stabilize the refolded state. High salt concentrations inhibited refolding (50% inhibition at 0.5 M NaCl), and the optimal level of zinc ions was between 2.5 and 10 μM, the activity recovered

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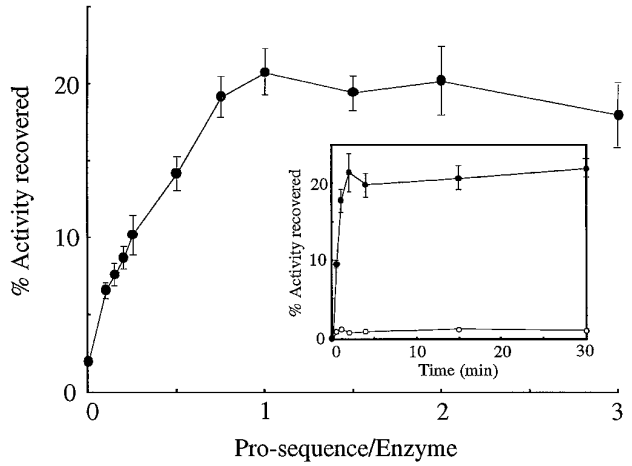

FIG. 5. **Effect of the thermolysin prosequence on the refolding of denatured enzyme *in vitro*.** Thermolysin was denatured in 6 M guanidinium hydrochloride under acid conditions and rapidly diluted in a 50-fold excess of 50 mM Hepes, pH 7.0, 10 mM $CaCl_2$, 5 μM $ZnCl_2$, and 4 μM phosphoramidon containing different concentrations of prosequence, as described under "Experimental Procedures." Activity is expressed as a percentage of control enzyme. *Inset,* activity recovered from denatured thermolysin after renaturation in the presence and absence of a molar equivalent of prosequence, as a function of time. The reaction was stopped by adding trypsin as described under "Experimental Procedures." Activity is expressed as a percentage of control enzyme.

being reduced at either higher or lower concentrations. The replacement of the prosequence by equivalent quantities of bovine serum albumin did not facilitate enzyme folding.

Two mechanisms have been proposed for the facilitation of protease folding by their prosequences. For α-lytic protease (19) and subtilisin (41, 42) they are thought to lower the energy of a rate-limiting transition state at a late stage of folding (reviewed in Ref. 43), whereas for carboxypeptidase Y, it has been suggested that the prosequence functions as a chaperone and reduces the rate of nonproductive folding or aggregation (22). Further studies will be required to elucidate the exact mechanism of action of the thermolysin prosequence.

Concluding Remarks—The *in vitro* results presented here for thermolysin, from the Gram-positive *B. thermoproteolyticus*, together with those of recent *in vivo* and *in vitro* studies on pseudolysin, from the Gram-negative *P. aeruginosa* (31, 32), have now established the role of the prosequences of the thermolysin family of bacterial enzymes in the folding of their mature enzymes. In addition, the processed prosequences, by remaining associated with the folded enzyme, could prevent unwanted proteolysis. The prosequences of these zinc peptidases therefore act in a manner similar to that of many of their equivalents in the serine, cysteine, and aspartate protease families.

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