Thermolysin, an extracellular zinc endopeptidase from *Bacillus thermoproteolyticus*, is synthesized as a pre-proenzyme and the prosequence has been shown to assist the refolding of the denatured enzyme *in vitro* and to inhibit enzyme activity (O’Donohue, M. J., and Beaumont, A. (1996) *J. Biol. Chem.* 271, 26477–26481). To determine whether prosequence cleavage from the mature enzyme is autocatalytic and if so, whether it is an intermolecular or intramolecular process, N-terminal histidine-tagged prothermolysin was expressed in *Escherichia coli*. Although partial processing to mature enzyme occurred, most of the proenzyme was recovered intact from inclusion bodies. This was then solubilized in guanidinium hydrochloride, immobilized on a cobalt-containing resin, and after dialysis against renaturation buffer, was quantitatively transformed to mature enzyme. However, when a mutation was introduced into the mature sequence to inactivate thermolysin, the proenzyme was not processed either *in vivo* or *in vitro*. In addition, mutated prothermolysin was not processed by exogenous thermolysin under a variety of experimental conditions. The results demonstrate that thermolysin maturation can proceed via an autocatalytic intramolecular pathway.

Thermolysin (EC 3.4.24.27), a 34.6 kDa extracellular neutral protease produced by the Gram-negative *Bacillus thermoproteolyticus*, is the prototype member of the M4 family of zinc endopeptidases (1). This thermostable enzyme was the first zinc endopeptidase to be crystallized, and subsequent crystallographic studies have led to a mechanism of action being proposed for the zinc metallopeptidases in general (reviewed in Ref. 2). An active site model of thermolysin has also been used as a template for the design of inhibitors for several mammalian zinc metallopeptidases, for which structural data is lacking, such as neutral endopeptidase-24.11 (EC 3.4.24.11), angiotensin-converting enzyme (EC 3.4.15.1) and endothelin-converting enzyme (reviewed in Refs. 3 and 4). The M4 enzymes are synthesized as pre-proproteins (5, 6), with thermolysin itself having a 27-residue N-terminal pre-sequence, followed by a 204-residue prosequence and the 316-residue mature enzyme (7). As compared with other enzyme classes (reviewed in Refs. 8 and 9), there have been comparatively few studies on the roles of prosequences of bacterial zinc peptides, although they have been shown to be essential for the production of active enzyme *in vivo* (10–13) probably by facilitating folding (14). The prosequences, as independent polypeptides, can also act as inhibitors of their respective mature enzymes (14, 15).

From site-directed mutagenesis studies of the M4 enzymes, it has been inferred that prosequence processing is autocatalytic, as mutations of active site residues often result in low or negligible yields of mature enzyme (16–19). In addition, certain mutations in the active site of thermolysin that change specificity lead to the production of more than one enzyme species differing by their N-terminals. For these reasons there have been few detailed studies involving the mutagenesis of residues responsible for activity and/or specificity of the thermolysin-like enzymes in contrast to the extensive research devoted to determining the factors involved in their varying degrees of thermostability (20, 21).

To investigate further thermolysin maturation and, in particular, to determine if it proceeds via an intramolecular or intramolecular autolytic mechanism, prothermolysin with an N-terminal 6-histidine tag (His$_6$-TLN)$^2$ has been expressed in *Escherichia coli*. A tagged proenzyme form with the mutation E143A in the mature sequence (His$_6$-TLN$^9$) was also expressed. This mutation was introduced to obtain a folding competent proenzyme, which would be incapable of any possible autolytic maturation. Glu-143 is an active site residue that acts as a general base, polarizing the water molecule that attacks the substrate scissile bond (2) and various mutations of this residue in other M4 proteases have been found to reduce or eliminate maturation (16–18, 22). Moreover, in one study where residual maturation and activity was obtained, crystallographic data showed that the mutation (E143S) provoked only minor localized structural changes in the mature enzyme, which has 70% sequence identity with thermolysin (22). In addition, an alanine replacement of the equivalent residue to Glu-143 in the mammalian thermolysin-like enzymes discussed above has been shown to abolish their activity (23–25).

The folding and processing of these prosequences have been studied *in vivo* and *in vitro*, in the latter case after their purification from inclusion bodies and immobilization on a Co$^{2+}$ resin via the histidine tags. The results give direct proof that thermolysin processing is autocatalytic and also show that it is likely to be an intramolecular event.

**EXPERIMENTAL PROCEDURES**

**Materials**—[${}^3$H]Leu-enkephalin was purchased from Izinta (Hungary) and [α-33P]dATP, [${}^3$H]lucine, the chemiluminescence kit for Western blotting, and the Sequenase enzyme (Version 2.0) were purchased from Amersham France S.A. (France). Culture media components were Difco products obtained from OSI (France). Oligonucleotides were purchased from Genosys (UK). New England Biolabs DNA-modifying enzymes and the CLONTECH Talon Co$^{2+}$-containing resin were from Genesys.

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‡ C. Marie-Claire and A. Beaumont, unpublished observations.

1 The abbreviations used are: His$_6$-proTLN, His-tagged prothermolysin; His$_6$-proTLN$,^9$, His-tagged prothermolysin with Glu-143 of the mature enzyme mutated to Ala; Gdm-HCl, guanidinium hydrochloride; [${}^3$H]Leu-enkephalin, [${}^3$H]tyro-syl-glycyl-glycyl-phenylalanyl-leucine; PAGE, polyacrylamide gel electrophoresis.

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Ozyme (France), and the Xpress protein expression system was purchased from Invitrogen (Netherlands). GenElute agarose spin columns were from Supelco and the E. coli T7 S30 extract was from Promega (France). The antipolyhistidine monoclonal antibody was from Sigma (France).

**Bacterial Strains—**E. coli DH5α was used to manipulate recombinant plasmids and E. coli JM109 (DE3), which contains the T7 RNA polymerase under the inducible control of the lacUV5 promoter, for the induction of genes under T7 promoter control. E. coli strains were transformed with plasmids as described (26) and cultured in L broth. Ampicillin was used at 100 μg/ml.

**DNA Manipulation and Polymerase Chain Reaction—**Plasmid isolation, restriction endonuclease digestions and DNA modifications were carried out as described (26). Restriction or polymerase chain reaction-amplified DNA fragments were isolated and purified after agarose gel electrophoresis. DNA sequences were determined by the chain termination technique with Sequenase 2.0 using [α-33P]dATP. Polymerase chain reaction was carried out as described (27) with slight modifications. The reaction buffer contained pfu DNA polymerase buffer (20 mM Tris, pH 8.0, 2 mM MgCl₂, 10 mM KCl, 6 mM (NH₄)₂SO₄, 0.1% (v/v) Triton X-100, 10 μg/ml nuclease-free bovine serum albumin) supplemented with 0.25 mM of each of the dNTPs and 2 units of native pfu polymerase. Amplification protocols were adapted according to the primers used. Polymerase chain reaction-based mutagenesis was carried out as described previously (28).

**Construction of the Expression Vectors—**A pvuII unique restriction site was introduced at the junction between the pre- and prosequence by site-directed mutagenesis in the previously described pTLN2 vector (7). The mutated plasmid was then digested with pvuII and EcoRI and the resulting proTLN fragment inserted in the pRSET C expression vector digested by pvuII and EcoRI to add an N-terminal His-tag to the prosequences.

**Protein Expression and Cell Fractionation—**JM109 (DE3) containing pRSET derivatives were grown in L broth, supplemented with the appropriate antibiotic at 37 °C to an A₅₉₀ of 0.6, and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Total protein and protein from the soluble and insoluble fractions were obtained as described (26). Aliquots were run on 12% SDS-PAGE and transferred to nitrocellulose membranes for Western blotting with monoclonal antibodies raised against either thermolysin (kindly provided by Dr. C. Bohuon), or polyhistidine. The relative proportions of the proteins in the different bands after Western blotting was estimated by densitometry.

**RESULTS**

**Processing of His₆-proTLN and His₆-proTLN** in Vivo and in Vitro—In extracts of E. coli cells transformed with the wild-type histidine-tagged proenzyme (His₆-proTLN), three proteins were detected after SDS-PAGE not present in control cells, which were recognized by the thermolysin antibody (Fig. 1, lanes 3 and 4) and were primarily located in the insoluble fraction. Only the protein with the highest apparent molecular weight corresponding to that of the intact His-tagged prosequence (62 kDa) was recognized by an anti-poly-His antibody (not shown). The protein with an apparent molecular mass of 58 kDa is likely to be an N-terminally truncated form of His₆-proTLN possibly due to a second origin of replication in the construction downstream from the poly-His tag, as a similar species was also obtained when [³H]His₆-proTLN was synthesized in a cell-free translation system (see below). The protein with the lowest apparent molecular mass (36 kDa) had the same mobility as a thermolysin standard, showing that some processing of the prosequence occurred intracellularly. In the case of E. coli cells transformed with His₆-proTLN’, which contains the active site mutation, no protein corresponding to mature thermolysin was detected (Fig. 1, lanes 5 and 6), supporting the proposed autocatalytic nature of thermolysin maturation. The results also show that no endogenous E. coli protease is capable of specifically processing the His-tagged proenzymes.

**His₆-proTLN and His₆-proTLN’**, which represented around 40% of the total protein present in the inclusion bodies, were solubilized in 6 M Gdm-HCl and independently immobilized on a Co²⁺-containing resin by their N-terminal tags. Around 200–300 μg of the proenzymes were attached/ml of resin, which was then extensively washed with a high salt buffer to eliminate contaminating protein (Fig. 2). Dialysis of immobilized His₆-proTLN against renaturation buffer led to the total disappearance of the proenzyme and the concomitant appearance of a protein corresponding in molecular weight to mature thermolysin. The majority of this protein was found in the supernatant of the dialysate and was fully active against the thermolysin substrate [³H]Leu-enkephalin (Fig. 3, lanes 1–3). No protein was detected on the dialyzed resin by Coomassie Blue staining; most of the prosequence probably had been digested by thermolysin during the course of the dialysis (Fig. 3, lane 4), whereas a Western blot showed the presence of some mature enzyme, which represented ≤5% of the total. The resin-associated enzyme could be eluted with 500 mM imidazole, which removes His-tagged proteins, and was therefore presumably noncovalently attached to the remaining prosequence or C-terminally truncated derivatives. With the active site mutant, His₆-proTLN’, no evidence for processing was observed in line with the in vivo results, the proprotein being recovered intact and still attached to the resin after dialysis (Fig. 3, lanes 5–7). The E143A mutation therefore either eliminated maturation or reduced it to undetectable levels under the conditions used.

Dialysis of the Gdm-HCl solubilized extracts without prior attachment of the proenzymes to the Co²⁺-resin resulted in aggregate formation. Some active mature thermolysin was formed in extracts containing His₆-proTLN, but the recovery was <1.0% of that obtained with a similar quantity of
immobilized proenzyme.

Intermolecular Versus Intramolecular Processing—The preceding results not only demonstrate the autocatalytic nature of thermolysin maturation but also suggest that it is an intramolecular event. For further confirmation of this, His$_6$-proTLN and His$_6$-proTLN' were co-immobilized on the resin in a 1:1 protein ratio. Renaturation by dialysis in this case resulted in both the formation of active mature thermolysin and the retention of a proenzyme form on the resin (Fig. 3, lanes 8–10). Similar results were obtained when the dialysis time was increased from 48 to 96 h. As the amount of proenzyme retained on the resin was approximately 50% of that originally bound, it seemed likely that this corresponded to His$_6$-proTLN'. To verify this, $[^3]$HHis$_6$-proTLN' was produced in a cell free system (Fig. 4), which led as in vivo (Fig. 1) to the synthesis of two proteins, one of 62 kDa corresponding to the entire His-tagged prosequence, which was recognized by both the antithermolysin and antipolyhistidine antibodies and the second of 58 kDa, which was only recognized by the antithermolysin antibody (not shown) implying as discussed above a second origin of replication downstream from the poly-His tag. When the wild-type prosequence was synthesized under the same conditions a third protein corresponding to mature thermolysin was also obtained, which was used as a standard for other fluorographic experiments.

When $[^3]$HHis$_6$-proTLN' was also co-immobilized as a tracer with His$_6$-proTLN and His$_6$-proTLN', >99.8% of the radioactivity remained associated with the resin after 48 h dialysis, confirming that the active thermolysin produced from the wild-type proenzyme is unable to process the mutated proenzyme. The resistance of His$_6$-proTLN' to proteolysis by thermolysin also demonstrates that this protein has assumed a folded conformation. To see whether thermolysin might be able to process the mutated enzyme if the latter were not attached to a support, $[^3]$HHis$_6$-proTLN' and its N-terminally truncated form were incubated in solution with different concentrations of thermolysin for 1 h at 37 °C. Degradation of both proteins was observed only at the highest enzyme, proenzyme ratio used (1:1), and this was nonspecific in nature with no detectable radiolabeled mature thermolysin being formed (Fig. 4).

Intermolecular processing however, might only occur if preceded by intermolecular folding. Free mature thermolysin previously denatured in 6 M Gdm-HCl was therefore incubated in the dialysis in a 1:1 molar ratio with immobilized His$_6$-proTLN' and tracer $[^3]$HHis$_6$-proTLN. Under these conditions no evidence for the conversion of $[^3]$HHis$_6$-proTLN' to mature enzyme was observed (Fig. 5). In addition only <0.5% of the original thermolysin activity was recovered from the dialysate, similar to that found when the same quantity of denatured mature enzyme was dialyzed in the presence of the Co$_{2+}$ resin alone.

**DISCUSSION**

To determine whether prothermolysin processing can proceed through either intermolecular or intramolecular autolysis, N-terminally His-tagged wild-type and mutated proenzymes

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**Fig. 2.** Purification of His$_6$-ProTLN and His$_6$-ProTLN' from inclusion bodies. Inclusion bodies from E. coli expressing His$_6$-proTLN or His$_6$-proTLN' were solubilized in 6 M Gdm-HCl, and the proenzymes were purified on a Co$_{2+}$ resin as described under “Experimental Procedures.” Aliquots of both preparations were applied to 12% SDS-PAGE, and the gels were stained with Coomassie Blue. Lanes 1 and 3, inclusion bodies from cells expressing His$_6$-proTLN and His$_6$-proTLN', respectively. Lanes 2 and 4, His$_6$-proTLN and His$_6$-proTLN', respectively, after purification. The running positions of molecular weight markers (kDa) are shown on the right side.

**Fig. 3.** Western blot of the immobilized denatured prosequences before and after dialysis. Lanes 1–4, immobilized His$_6$-proTLN; resin before dialysis against renaturation buffer (lane 1), resin after dialysis (lane 2), dialysate supernatant (lane 3), and resin after dialysis stained by Coomassie Blue (lane 4). Lanes 5–7, immobilized His$_6$-proTLN'; resin before dialysis (lane 5), resin after dialysis (lane 6), and dialysate supernatant (lane 7). Lanes 8–10, co-immobilized His$_6$-proTLN and His$_6$-proTLN'; resin before dialysis (lane 8), resin after dialysis (lane 9), and dialysate supernatant (lane 10). Samples were run on 12% SDS-PAGE and transferred to nitrocellulose sheets for Western blotting with an antithermolysin monoclonal antibody. The running positions of molecular weight markers (kDa) are shown on the right side, and the arrow on the left side indicates the running position of the thermolysin standard.

**Fig. 4.** Autoradiogram of $[^3]$HHis$_6$-ProTLN' before and after incubation with thermolysin. 40 ng of $[^3]$HHis$_6$-proTLN' were incubated for 1 h at 37 °C with 0 (lane 1), 0.04 (lane 2), 0.4 (lane 3), 4.0 (lane 4), and 40 (lane 5) ng of thermolysin. The reactions were stopped by heating at 100 °C for 5 min in SDS-PAGE sample buffer, and the samples were run on 12% SDS-PAGE before fluorography as described under “Experimental Procedures.” The running positions of molecular weight markers (kDa) are shown on the right side. The arrow on the left side indicates the running position of the thermolysin standard.

**Fig. 5.** Autoradiogram of immobilized $[^3]$HHis$_6$-ProTLN' after dialysis in the presence or absence of mature thermolysin. His$_6$-ProTLN' solubilized from inclusion bodies and $[^3]$HHis$_6$-proTLN' were co-immobilized on the Co$_{2+}$ resin and dialysed against renaturation buffer in the presence or absence of an equivalent molar ratio of denatured thermolysin. Samples were run on 12% SDS-PAGE before fluorography as described under “Experimental Procedures.” Lane 1, resin after dialysis in the absence of thermolysin; lane 2, resin after dialysis in the presence of thermolysin; lane 3, dialysate supernatant after dialysis in the presence of thermolysin. The running positions of molecular weight markers (kDa) are shown on the right side, and the arrow on the left side indicates the running position of the thermolysin standard.
were expressed in E. coli. The presence of the His-tag allowed the proenzymes, extracted from inclusion bodies, to be attached to a solid support, thus avoiding aggregation during renaturation and giving conditions where intramolecular processing could be differentiated from intermolecular processing.

The wild-type proenzyme was transformed to mature enzyme both in vivo and in vitro in the latter case after purification and therefore in the absence of other proteolytic enzymes. In addition, no processing was observed for the proenzyme containing the active site mutant, E143A, either in vivo or in vitro. These results demonstrate the autocatalytic nature of thermolysin processing and are also in favor of an intramolecular mechanism, as His6-proTLN was quantitatively transformed to mature enzyme when sequestered using only 4–6% of the theoretical resin capacity to limit the chance of intermolecular reactions. However, as it could be argued that even under these conditions only two favorably positioned molecules are required to start a chain reaction, further studies were undertaken to see whether thermolysin could process the mutated proenzyme, His6-proTLN'. No evidence for specific intermolecular processing was found when His6-proTLN and His6-proTLN' were co-immobilized on the resin prior to renaturation by dialysis or when free [3H]His6-proTLN' was incubated with exogenous thermolysin. In addition the prosequence of immobilized His6-proTLN' was unable to facilitate the refolding of mature denatured thermolysin. It can therefore be concluded that prothermolysin processing can take place via an intramo

termolecular mechanism.

However, it should be pointed out that the results obtained from these mainly in vitro studies do not entirely rule out the existence of an intermolecular mechanism in vivo. This is illustrated by the data available for the serine bacterial protease, subtilisin. Whereas the results of several studies are in favor of an intramolecular mechanism for prosubtilisin folding and processing (30–34), an intermolecular pathway has also been suggested with the consequence of one molecule acting as the folding template for the mature sequence of the second (33, 35, 36). It has been proposed that both mechanisms might function in vivo with the intermolecular route occurring during periods of maximum synthesis (33, 36). Although some of the results obtained in this study argue against a similar bimolecular mechanism for thermolysin, i.e. the prosequence region of immobilized His6-proTLN' did not appear to act as a template for the refolding of the mature sequence, further studies will be required to determine whether the two pathways may function for thermolysin processing in vivo.

In addition, to facilitate folding, the thermolysin prosequence as an independent polypeptide is a specific noncompetitive inhibitor of the mature enzyme with a nanomolar affinity (14). An inhibitory role has also been found for the pro sequences of other enzymes (15, 37–39), and this is generally thought to prevent unwanted intracellular proteolysis. In the case of thermolysin (14) and subtilisin (40) it has also been shown that inhibition is temporary as the prosequence is eventually degraded by their respective mature enzymes. Given that intramolecular folding and processing of prothermolysin have now been shown to be feasible, another role for the inhibitory properties of the prosequence can be envisaged.

Thermolysin is a bicalloidal enzyme with the active site in a cleft between its N- and C-terminal domains, and in addition to the single catalytic zinc atom in this active site, the enzyme also binds four calcium atoms. Thermal denaturation of thermolysin is rapid and irreversible due to the local unfolding of exposed sequences, followed by autolysis (20, 21, 41). The calcium atoms, by protecting surface-exposed loops against unfolding, stabilize the enzyme, and calcium-3, which binds to the N-terminal domain, has been shown to be particularly important in this respect (20, 21, 42). In addition the N-terminal domain of thermolysin, which is predominantly formed of β-sheets, is less stable than the predominantly α-helical C-terminal domain (41). Prothermolysin maturation occurs between a serine and an isoleucine residue (7), in line with the preference of the enzyme for cleaving substrates at the N-terminal side of hydrophobic residues (2). Therefore for intramolecular processing to occur, the proenzyme must not only have a functional active site but Ile-1 of the mature sequence should also be positioned in the S1′ subsite prior to hydrolysis. However, in the crystallographic structure of the enzyme, Ile-1 is 29 Å away from the active site cleft. It therefore follows that a repositioning of the N-terminal residues of the mature enzyme occurs after processing, and it is likely that the calcium-3 binding site would not be formed until this stage. Again, parallels can be drawn with the serine protease, subtilisin, where intramolecular folding also necessitates an N-terminal rearrangement and the formation of a Ca2+-binding site after processing (31, 32). The temporary inhibition of thermolysin by its prosequence after autolytic processing could therefore help to stabilize the enzyme against irreversible autolysis before calcium-3 binding.

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Thermolysin Maturation