Hetero- and Autoprocessing of the Extracellular Metalloprotease (Mpr) in Bacillus subtilis

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Received 23 March 2004/Accepted 28 May 2004

Most proteases are synthesized as inactive precursors which are processed by proteolytic cleavage into a mature active form, allowing regulation of their proteolytic activity. The activation of the glutamic-acid-specific extracellular metalloprotease (Mpr) of Bacillus subtilis has been examined. Analysis of Mpr processing in defined protease-deficient mutants by activity assay and Western blotting revealed that the extracellular protease Bpr is required for Mpr processing. pro-Mpr remained a precursor form in bpr-deficient strains, and glutamic-acid-specific proteolytic activity conferred by Mpr was not activated in bpr-deficient strains. Further, purified pro-Mpr was processed to an active form by purified Bpr protease in vitro. We conclude that Mpr is activated by Bpr in vivo, and that heteroprocessing, rather than autoprocessing, is the major mechanism of Mpr processing in vivo. Exchange of glutamic acid for serine in the cleavage site of Mpr (S93E) allowed activation by Bpr in vivo, and that heteroprocessing, rather than autoprocessing, is the major mechanism of Mpr processing in vivo. Glutamic-acid-specific proteolytic activity conferred by Mpr was not activated in the S93E mutant. Furthermore, purified pro-Mpr remained a precursor form in vivo, suggesting that it is not autoprocessed. Many glutamic-acid-specific proteases such as V8 protease (5) and SPase (43) have been found in Staphylococcus aureus and some nonpathogenic bacteria (10, 13, 42); however, the processing mechanisms of glutamyl endopeptidases have not been clearly defined.

Here, we report that Mpr can be specifically activated by other extracellular proteases and that Mpr can be converted to an autoproteolytic protease by placing a glutamic acid residue in the propeptide cleavage site. The molecular evolution of protease activation mechanisms in B. subtilis is also discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cultivation was carried out in 200 ml of Luria-Bertani medium in a 2-liter flask at 37°C with shaking (200 rpm). Culture supernatants for examining protease expression and activity, as well as purification, were collected after 12 to 36 h of growth as described above. Where necessary, media were supplemented with 100 μg of ampicillin (Amp; purchased from DUCHefa)/ml for Escherichia coli and with 50 μg of kanamycin (Kan; purchased from DUCHefa)/ml and 15 μg of tetracycline (Tc; purchased from Sigma)/ml for Bacillus subtilis.

DNA manipulations and PCR. All DNA manipulations were carried out by standard protocols (25). DNA restriction digestions were performed as recommended by the supplier (New England Biolabs, Inc.). E. coli plasmids were isolated with the Qiagen spin miniprep kit (QIAGEN) and some nonpathogenic bacteria (10, 13, 42); however, the processing mechanisms of glutamyl endopeptidases have not been clearly defined.

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The gram-positive, spore-forming bacterium Bacillus subtilis secretes at least eight extracellular proteases at the end of the exponential phase of growth (21). Two major extracellular proteases are produced during the onset of sporulation, the alkaline serine protease subtilisin (AprE) (18, 38) and a neutral protease (NprE) (40). The minor extracellular proteases include Epr (2, 27), bacillopeptidase F (Bpr) (30, 31, 39), metalloprotease (Mpr) (20, 23, 28), neutral protease B (NprB) (35), wall-associated extracellular protease (WprA) (17), and Vpr (29). Expression of these proteolytic enzymes seems to be tightly regulated (33), and their major function is thought to be supplying amino acids for growth via degradation of extracellular proteins. However, some physiological roles have been discussed. For example, it has been suggested that WprA is involved in proteolysis of misfolded proteins during membrane translocation (32).

Many bacterial proteolytic enzymes are synthesized as inactive precursors, orzymogens, to prevent unwanted protein degradation and to enable spatial and temporal regulation of proteolytic activity (12). Biochemical studies of the activation mechanism of individual proteases have provided insights into their physiological functions. Every extracellular protease in B. subtilis is synthesized as a proenzyme in the cytoplasm and is processed to a mature enzyme in the extracellular milieu. The activation mechanism of B. subtilis subtilisin has been well studied. The promotor region of subtilisin is converted to an active form via intramolecular autoprocessing (19) in the extracellular protease (9). The propeptide has been shown to guide the proper folding of subtilisin in vivo and in vitro (34). Most extracellular proteases in B. subtilis seem to be activated by autoprocessing. However, the Mpr protease might be an exception in that it is not able to activate itself. Reportedly, Mpr has a high substrate specificity, recognizing a glutamic acid residue as a P1 cleavage site (20). However, a glutamic acid residue is not present in the Mpr propeptide cleavage site, suggesting that it is not autoprocessed. Many glutamic-acid-specific proteases such as V8 protease (5) and SPase (43) have been found in Staphylococcus aureus and some nonpathogenic bacteria (10, 13, 42); however, the processing mechanisms of glutamyl endopeptidases have not been clearly defined.

Here, we report that Mpr can be specifically activated by other extracellular proteases and that Mpr can be converted to an autoproteolytic protease by placing a glutamic acid residue in the propeptide cleavage site. The molecular evolution of protease activation mechanisms in B. subtilis is also discussed.
termed pSP704. The mpr gene was amplified from genomic DNA of B. subtilis 168 with primers Mpr-F (5'-AGGGAGCTCAACAATGGAATGAA) and Mpr-R (5'-ATGGATCCTTAATGATGATGATGATGACTACTTCC) was amplified with primers EPR-EF (5'-CGGATCAGGGAACTCTGTATCCC).

Construction of gene disruption vectors. bpr (4.5 kb) was amplified from chromosomal DNA of B. subtilis 168 with primers BPR-EF (5'-GGAAATTCAAAAACAGAAGTGAGATGGAAATGCAATGGGAGC) and BPR-R (5'-GGAGAGTACATTAAAATTTTCTGTTGATATAGTATTTTGGTAATTTGTTTGT). The PCR product was digested with EcoRI and cloned in EcoRI-digested pUC19 to make pUC19-Bpr.

Site-directed mutagenesis. To introduce the point mutation S93E at the propeptide cleavage site in pro-Mpr, PCR mutagenesis was carried out with pMprH6 as a template and primers S93E-F (5'-GAGCATGGAGAAACGAAAAACAGACTCAT) and S93E-R (5'-CAAAACCTTACAGCCTGAG). The resulting PCR product was digested with DpnI to remove parental plasmid and was ligated to make pMprH6(S93E). The resulting plasmid was named pMprH6(S93E), and the presence of the S93E point mutation was confirmed by DNA sequencing.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by Laemmli's method (14). Western blot analysis was performed by standard protocols (25). A His probe (Santa Cruz Biotechnology) and anti-rabbit immunoglobulin G-peroxidase conjugate (Bio-Rad) were used as primary and secondary antibodies, to detect His-tagged Mpr. The light-emitting nonradioactive ECL kit (Amersham) was used for signal detection.

Plate assay for determination of proteolytic activity. The culture supernatants from 12- to 36-h spent-culture broths of B. subtilis were plated on Luria-Bertani agar plates containing 3% (wt/vol) skim milk and Kan (25 µg/ml). Proteolytic activity was determined by observing the clear zone around colonies after 12 h of incubation at 37°C.

Determination of glutamic-acid-specific proteolytic activity. Glutamic-acid-specific protease activity was determined by a slightly modified chromogenic assay (20). Ten microcultures of culture supernatant was added to 90 µl of reaction solution (50 mM Tris·Cl [pH 7.5], 2 mM CaCl₂, 1 mM acetyl-0-p-nitroanilide [Bachem], 2% dimethylformamide). After incubation of the culture supernatant for 8 h at 37°C, the absorbance was measured at 410 nm (Bio-Rad microwell plate reader, model 550).

Protein purification. A 36-h broth culture of B. subtilis LB700 carrying either pBpr or pMpr was centrifuged (8,000 × g, 30 min at 4°C), redissolved in 20 mM Tris·HCl (pH 8.0), and dialyzed overnight against the same buffer at 4°C.
For the purification of Bpr, the dialysate was concentrated with an YM-3 membrane (Amicon Corp.) and applied to a DEAE Sepharose CL-6B (Pharmacia) column equilibrated with 20 mM Tris-HCl prior to Mono-Q (Pharmacia) chromatography. Bpr was eluted from both columns by an NaCl gradient.

Purification of pro-Mpr was carried out as follows. The dialysate was applied to a DEAE Sepharose CL-6B (Pharmacia) column equilibrated with 20 mM Tris-HCl (pH 8.0). pro-Mpr was eluted from the column by an NaCl gradient. The active fractions were dialyzed against 20 mM Tris-HCl (pH 8.0), concentrated with an YM-3 membrane, loaded onto a Sephacryl S-100 (Pharmacia) column, and fractionated.

The purification of His6-tagged pro-Mpr(S93E) was performed as follows. A 36-h broth culture of B. subtilis LB700 carrying pMprH6(S93E) was centrifuged (6,000 × g; 30 min), and the supernatant was applied to a Ni-nitrilotriacetic acid superfowl (QIAGEN) column. pro-Mpr(S93E) was eluted with imidazole buffer.

**RESULTS**

Propeptide cleavage of metalloprotease (Mpr) by heteroprocessing in B. subtilis. The signal peptide and propeptide sequences of Mpr are reported to be composed of 34 and 59 amino acids, respectively, (28), and a previous study showed that the propeptide cleavage site should be between Ser93-Ser94 in pro-Mpr (see Fig. 6). It was reported that proteolytic activity of Mpr is highly specific to glutamic acid (20). Therefore, we thought that Mpr was unlikely to be processed by a self-cleaving mechanism like that of subtilisin. To investigate Mpr processing, we overexpressed Mpr in multiple-protease-deficient strains of B. subtilis and examined the secreted proteins in supernatants of 36-h cultures by SDS-PAGE for patterns of Mpr processing. We noted a remarkable difference in the molecular masses of Mpr proteins (overexpressed from pMpr) between strain DB104, which lacks the NprE and AprE extracellular proteases, and strain DB428, which lacks the Epr and Bpr extracellular proteases as well as NprE and AprE. While a 33-kDa protein was overexpressed in DB428, a 28-kDa protein was overexpressed in DB104 (see Fig. 2A), N-terminal amino acid sequences of the 33- and 28-kDa proteins indicated (see Fig. 6) were determined to be AENPO and SIIGT, respectively. The molecular masses of these proteins correspond well to those of pro-Mpr and mature Mpr, suggesting that Bpr and/or Apr is a strong candidate(s) in the processing of pro-Mpr and further, that pro-Mpr was activated not by itself (autoprocessing), but by other proteolytic activities (heteroprocessing).

Activation and processing of pro-Mpr in vivo by Bpr and not by Epr. To investigate whether Epr, Bpr, or both was required for the processing of pro-Mpr, we expressed Mpr and MprH6 in different multiple-protease-deficient B. subtilis strains including DB104 (aprE nprE), SB300 (aprE nprE epr), PB300 (aprE nprE bpr), and DB428 (aprE nprE epr) and detected the proteolytic activity on skim milk-agar plates. We observed a prominent clear zone around colonies of DB104 and epr-deficient SB300 strains but not around colonies of bpr-deficient PB300, DB428, and LB700 (aprE nprE epr bpr nprB wprA) strains (Fig. 1) at 36 h of growth. We examined pro-Mpr secretion in DB104 and SB300 strains by SDS-PAGE (Fig. 2A) and immunoblot (Fig. 2B) analyses and found that pro-Mpr was completely processed into the mature form. However, in bpr-deficient strains pro-Mpr protein remained throughout the time course.

To confirm that Mpr was processed into an active form, we tested whether the culture supernatants of different strains were able to hydrolyze a synthetic chromogenic substrate of Mpr, acetyl-Glu-nitroanilide. The glutamic acid-specific proteolytic activity of Mpr was observed in the culture supernatants of SB300 and DB104 strains harboring pMpr (Fig. 2C).
However, in the culture supernatants of \textit{bpr}-deficient PB300, DB428, and LB700 strains carrying pMpr, no glutamic acid-specific proteolytic activity was observed (Fig. 2C).

\textbf{Activation of pro-Mpr by Bpr in vitro.} We purified both Bpr and pro-Mpr from culture supernatants of overexpressing strains to examine pro-Mpr processing in vitro. We observed that purified Bpr activated purified pro-Mpr in vitro as detected by the production of the mature form of Mpr by SDS-PAGE analysis (Fig. 3A) and by the activation of Mpr in the colorimetric assay for glutamic-acid-specific proteolytic activity (Fig. 3B). From these results, we conclude that Bpr specifically converts inactive pro-Mpr to active Mpr through proteolytic processing of the Mpr propeptide.

\textbf{Activation of pro-Mpr(S93E) mutant in vivo by autoprocessing.} Because Mpr was reported to have glutamic acid-specific endopeptidase activity, the propeptide cleavage site of Mpr was mutated to include a glutamic acid residue (mutation S93E) to determine whether Mpr was capable of autoprocessing. Prominent clear zones around colonies of all strains expressing this mutant protein were observed in the skim milk-agar plate assay even after only 12 h of growth (Fig. 1). SDS-PAGE (Fig. 4A) and immunoblot (Fig. 4B) analyses showed that the mutant pro-Mpr was largely processed into an active form from 12 h of growth, even in \textit{bpr}-deficient strains. The Mpr(S93E) mutant displayed proteolytic activity similar to or greater than that of wild-type Mpr in the colorimetric assay.
suggesting that S93E mutant may be activated more efficiently by an autoprocessing mechanism (Fig. 4C).

**Activation of pro-Mpr(S93E) mutant in vitro by autoprocessing.** We tried to purify pro-Mpr(S93E) from culture supernatants of overexpressing strains to examine pro-Mpr(S93E) processing in vitro. We could obtain a complex consisting of pro-Mpr(S93E) and mature Mpr(S93E) because secreted pro-Mpr(S93E) is immediately converted into the mature form during the purification process. However, we could observe that purified pro-Mpr (S93E) was activated by autoprocessing in vitro throughout the time course as detected by the increase of the mature form of Mpr(S93E) in immunoblot analysis (Fig. 5). Thus, we conclude that the S93E mutation allows efficient autoprocessing of Mpr.

**DISCUSSION**

The activation mechanism of many extracellular proteases in *B. subtilis* has been investigated. Subtilisin is well characterized and known to be activated by autoprocessing (34). Most extracellular proteases in *B. subtilis* like subtilisin, have been thought to be autoprocessed. It was reported that Bpr might be susceptible to autolysis (22). We observed that Epr and NprB were each fully processed when expressed from a plasmid in the seven-protease-deficient LB700 strain, indicating that they might be activated by autoprocessing (data not shown). In the case of Mpr, however, the recent report that Mpr is a glutamic-acid-specific endopeptidase (20) and that glutamic acid is absent in the propeptide cleavage site of pro-Mpr led us to consider whether Mpr was activated by another protease.

Proteolytic activity was observed on skim milk-agar plates in an aprE nprE mutant carrying pMpr but not in an aprE nprE epr bpr mutant carrying pMpr, suggesting that Mpr might require Epr or Bpr for activation rather than being autoprocessed. To identify which was required for Mpr activation, we investigated pro-Mpr processing in bpr-deficient and epr-deficient derivatives of DB104 (PB300 and SB300, respectively) (Table 1). The skim milk plate assay indicated that bpr, but not epr, was required for Mpr processing (Fig. 1).

This finding was confirmed by SDS-PAGE analysis with Coomassie blue staining, demonstrating that pro-Mpr (33...
kDa) was accumulated and not converted into the mature form (28 kDa) in culture supernatants of bpr-deficient strains carrying pMprH6 (Fig. 2A). However, a 28-kDa form could be detected in bpr-deficient strains PB300 and DB428 by immunoblotting (Fig. 2B). To determine whether pro-Mpr was properly processed in the bpr-deficient strains or nonspecifically processed by other proteolytic activities to an apparently mature size, we examined the glutamic-acid-specific protease activity in various B. subtilis strains carrying pMprH6. As shown in Fig. 2C, no glutamyl proteolytic activity was observed in the culture supernatants of bpr-deficient strains carrying pMprH6. This suggests that the 28-kDa protein detected by Western blotting in the bpr-deficient strains is an inactive form of MprH6, although it is processed at the N terminus because the C-terminal His6 tag is being detected.

These in vivo findings are supported by in vitro studies demonstrating that purified Bpr processes purified pro-Mpr to a proteolytically active form of the expected size in vitro (Fig. 3). We conclude from these in vivo and in vitro results that Bpr specifically processes pro-Mpr to mature Mpr in vivo. This heteroprocessing activity represents a novel function of bacil- lopeptidase F in B. subtilis.

We cannot exclude the possibility that subtilisin (AprE) and neutral protease (NprE) also play roles in Mpr processing. We could not determine whether Mpr was activated by AprE and NprE by the skim milk plate assay, because strains that are aprE+ nprE+ (strains 168 and PB100) form clear zones regardless of whether they are overexpressing Mpr. However, we found significant glutamyl proteolytic activities and fully processed (28-kDa) Mpr bands in the culture supernatants of wild-type strain 168 and also in bpr-deficient PB100, suggesting that Apr and/or Npr could be involved in Mpr processing (data not shown). Multiple proteases may allow pro-Mpr to be more rapidly and fully converted into an active form in the extracel- lular milieu. Epr had little effect on Mpr processing (Fig. 2A and B). However, we observed that Mpr had slightly lower levels of glutamyl proteolytic activity in the absence of Epr than in the presence of Epr (Fig. 2C). This result implies that Epr contributes to Mpr processing.

In pathogenic bacteria, extracellular proteases can enhance bacterial virulence through degradation of critical host pro- teins and by mimicking the activity of host regulatory proteases that control important zymogen systems (6). In the staphylo- coccal extracellular proteolytic system, zymogen activation by other extracellular proteases was recently reported (26). In nonpathogenic bacteria such as B. subtilis, extracellular proteases may have other functions. As mentioned, all extracel- lular proteases of B. subtilis are known to have no effect on either exponential growth in complex medium or sporulation (29). However, production and secretion of extracellular proteases increase during postexponential growth (7). During that pe- riod, protein degradation by extracellular proteases supplies nutrients for the cells. Moreover, the extracellular proteases play a role in the activation of other proteases. For example, WprA, Apr, and Vpr are reported to activate an antimicrobial subtilin (3). The present study shows a novel function of extracellular proteases, which is to activate other extracellular proteases in B. subtilis.

Because Mpr is a glutamic-acid-specific endopeptidase that specifically forms a peptide bond after the P1 glutamic acid site (20) but does not have a P1 glutamic acid at its own processing site, we constructed the His6-tagged Mpr(S93E) mutant to determine its effect on processing. Interestingly, the S93E mu- tant was fully processed into an active form in all the B. subtilis strains tested, regardless of protease deficiencies (Fig. 4), indicating that pro-Mpr(S93E) was activated by autoprocessing. The in vitro studies demonstrated that purified pro-Mpr(S93E) was processed to a proteolytically active form of the expected size in vitro by the time course (Fig. 5). From these in vivo and in vitro results, we conclude that the S93E mutant allows auto- processing. Moreover, we confirmed that processing of Mpr(S93E) occurs at the proper site between amino acids 93 and 94 of the mature enzyme (Mature; amino acids 94 to 313). Lined and dashed arrows indicate heteroprocessing by other extracellular proteases, and autoprocessing, respectively.
and 94 (Fig. 6) by N-terminal sequence analysis of the mature form from LB700. As in the results shown in Fig. 4C, there was an approximately twofold decrease in activity in the LB700 strain that lacks seven exopeptidases. The decrease in Mpr activity in LB700 carrying pMpr(S93E) may be due to the absence of the wprA gene product (LB700 is the only strain examined here which lacks wprA) which is associated with the protein secretion apparatus in B. subtilis (1). Absence of WprA might influence the secretion of Mpr and so affect Mpr processing. Alternatively, the decrease might be due to effects of the multiple mutations on growth rate or other factors that affect the colorimetric assay. Regardless, the processing of pro-Mpr(S93E) in the absence of Bpr and other exopeptidases suggests strongly that S93E allows autoprocessing.

With regards to the autoprocessing mechanism, the alteration of serine into glutamic acid residue in pro-Mpr might help its propeptide fit properly into the P1 pocket for processing by self cleavage. This indicates that the propeptide sequence determines whether Mpr undergoes heteroprocessing or autoprocessing. This alteration of the processing mechanism could be explained in the viewpoint of the molecular evolution or autoprocessing. This alteration of the processing mechanism is associated with the propeptide of Bacillus licheniformis ATCC 14580. J. Biol. Chem. 267:23782–23788.


