Cys\textsuperscript{32} and His\textsuperscript{105} Are the Critical Residues for the Calcium-dependent Cysteine Proteolytic Activity of CvaB, an ATP-binding Cassette Transporter\textsuperscript{*}

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CvaB, a member of the ATP-binding cassette transporter superfamily, is the central membrane transporter of the colicin V secretion system in \textit{Escherichia coli}. Cys\textsuperscript{32} and His\textsuperscript{105} in the N-terminal domain of CvaB were identified as critical residues for both colicin V secretion and cysteine proteolytic activity. By inhibiting degradation with \(\text{N}-\text{ethylmaleimide}\) and a mixture of protease inhibitors, a stable wild-type N-terminal domain (which showed cysteine proteolytic activity when activated) was purified. Such protease activity was Ca\textsuperscript{2+} and concentration-dependent and could be inhibited by antipain, \(\text{N}-\text{ethylmaleimide}\), EDTA, and EGTA. At low concentrations, the Ca\textsuperscript{2+} analogs Tb\textsuperscript{3+} and La\textsuperscript{3+} (but not Fe\textsuperscript{2+}) significantly enhanced proteolytic activity, suggesting that the size of the cations is important for activity. Together with comparisons of the sequences of members of the cysteine protease family, these results indicate that Cys\textsuperscript{32} and His\textsuperscript{105} are the critical residues in the CvaB N-terminal domain for the calcium-dependent cysteine proteolytic activity and secretion of colicin V.

ATP-binding cassette (ABC)\textsuperscript{1} transporters bind ATP and utilize the energy released to transport molecules across the cell membrane (1, 2). These transporters constitute one of the largest protein families found in living organisms. Members of this family transport a wide variety of compounds, including sugars, ions, antibiotics, and peptides (3, 4). Some members of this family, such as the multidrug resistance protein, \(\text{P}\)-glycoprotein (which is involved in pumping out anticancer drugs), and the cystic fibrosis transmembrane conductance regulator (which, when defective, leads to this deadly inherited disease), are clinically relevant (5, 6). Most ABC transporters consist of two hydrophobic transmembrane domains, which contain 6–12 membrane-spanning \(\alpha\)-helices and provide the specificity for the substrates that cross the membrane. They also contain two cytosolic nucleotide-binding domains, which bind and hydrolyze ATP to provide the energy for translocating substrates (1). A highly conserved region among all ABC transporters is found within the nucleotide-binding domain (~30% identity), which contains Walker A and B motifs separated by ~90–120 amino acids and a signature C motif located upstream of the Walker B site (7, 8).

Well studied examples of ABC transporters in prokaryotes are the histidine permease complex of \textit{Salmonella typhimurium} HisQMP\textsubscript{2} (9) (its ATP-binding subunit HisAP has been crystallized (10)), the maltose-binding protein MalEFGK\textsubscript{2} (11) (the crystal structure of its MalK subunit has been solved (12)), the \(\alpha\)-hemolysin transporter (13), and colicin V (14). Hemolysin B and its associated components transport the toxin hemolysin A across both membranes of \textit{Escherichia coli} (15).

The colicin V system consists of a toxin gene (\textit{cvaC}) that encodes a 103-amino acid precursor protein, the first 15 residues of which are cleaved behind two glycine residues of the leader peptide during secretion (16, 19). A second gene (\textit{cvaB}) that encodes the central ABC membrane transporter protein is predicted to have an N-terminal cytoplasmic domain that is conserved among bacterioci ABC transporters; it has been proposed to possess the protease activity that cleaves the leader peptide (14, 18–20). The N-terminal domains of the similar \textit{Lactococcus} bacteriocin transporters have been proposed to be essential for substrate recognition and processing (19).

Previous studies have shown that processing ColV-1 in membrane vesicles is dependent on the CvaA-CvaB transporter and ToIC proteins and that this activity is sensitive to inhibition by the serine/cysteine protease inhibitor antipain and the cysteine protease inhibitor \(\text{N}-\text{ethylmaleimide}\) (NEM) (21, 22). Because neither CvaA nor ColV-1 contains a cysteine residue (14, 23), CvaB is probably the target of inhibition of NEM and antipain. Sequence similarities indicate that the CvaB N-terminal domain (BntD) belongs to the cysteine protease calpain family. The catalytic residues in this family are believed to be Cys and His (24–26). In this study, we identified the residues of BntD that are critical for colicin V secretion, and we show that wild-type BntD (but not certain Cys or His BntD mutants) possesses calcium-dependent cysteine peptidase activity.

**EXPERIMENTAL PROCEDURES**

**Media and Growth Conditions**—Both liquid and solid (with 1.5% agar) TB media (10 g/liter Tryptone and 8 g/liter NaCl) were used as growth media for transformation, activity assay, and protein overproduction. Ampicillin and chloramphenicol were used at final concentrations of 100 and 30 \(\mu\)g/ml, respectively.

**Bacterial Strains, Plasmids, and Reagents**—The strains and plasmids used in this study are listed in Table I. \textit{E. coli} ColV-sensitive strain 71-18 (obtained from Dr. Roberto Kolter, Harvard Medical School, Boston, MA) was used as the lawn of cells for colicin V halo activity (14, 27, 28). Recombinant DNA manipulations were performed essentially as described by Sambrook \textit{et al.} (29). The expression vector

\* This work was supported in part by National Institutes of Health Research Grant GM34766 (to P. C. T.) and a research enhancement program grant from Georgia State University. The Georgia State University facilities were supported by the Georgia Research Alliance. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{1} The abbreviations used are: ABC, ATP-binding cassette; ColV, colicin V; NEM, \(\text{N}-\text{ethylmaleimide}\); BntD, CvaB N-terminal domain; CAPS, 3-(cyclohexylamino)propanesulfonic acid; IPTG, isopropyl-\(\beta\)-\(\text{D}\)-thiogalactopyranoside; LAPNA, \(\beta\)-arginine \(\beta\)-nitroanilide.
pTrcHis2B with a His tag at the C terminus was obtained from Invitrogen. E. coli strains DH5α and BL21 were used as the bacterial hosts. Restriction enzymes and T4 DNA ligase were obtained from Roche Applied Science or New England Biolabs, Inc. (Beverly, MA) and were used essentially as recommended by the manufacturers. Complete protease inhibitor mixture tablets were obtained from Roche Applied Science. All other chemicals were reagent-grade and were purchased from Sigma unless otherwise noted.

Site-directed Mutagenesis—All mutageneses were carried out by PCR using oligonucleotides containing the desired mutation. The standard PCR contained 20 ng of linearized plasmid template, 400 ng of each oligonucleotide primer, 200 μM each deoxynucleotide triphosphate, and 1× PCR buffer provided by the manufacturer in a total volume of 50 μl. The PCR mixture was amplified by 35 cycles of 20 s at 94°C, 20 s at 55°C, and 1 min at 72°C, followed by one cycle of 10 min at 72°C. Following the reaction, PCR amplification products were purified using QIAquick PCR purification kits (QIAGEN Inc., Valencia, CA), resolved by electrophoresis on 1% agarose gels, and purified from the gel using Qiagen gel extraction kit (QIAGEN Inc.).

To generate the desired site-directed mutagenesis constructs, a three-round asymmetric PCR strategy was used (30). For example, to generate the H105D mutation, asymmetric PCR using mutagenic primer kw71 containing 29 nucleotides of CvaB sequence and BglII-linearized pHK11 template was performed to synthesize a single-stranded DNA in the first round. This single-stranded DNA, extended linearized pHK11 template was performed to synthesize a single-stranded DNA product. The amplification products were digested with BglII and HindIII and ligated into vector pBluescript II KS(−), followed by transformation into E. coli strain DH5α (Stratagene). The resulting plasmid was used as a template for the second round of asymmetric PCR using HindIII-linearized pHK11 template, which was then purified from the gel using a Qiagen gel extraction kit (QIAGEN Inc.).

The PCR product and primer kw60 to generate a symmetric double-stranded DNA product. The amplification products were digested with HindIII and BglII and ligated into vector pBluescript II KS(−), followed by transformation into E. coli strain DH5α (Stratagene). The resulting plasmid was used as a template for the second round of asymmetric PCR using HindIII-linearized pHK11 template, which was then purified from the gel using a Qiagen gel extraction kit (QIAGEN Inc.).
Cys\textsuperscript{32} and His\textsuperscript{105} are shown in gray. Identical and similar residues are marked with asterisks and dots, respectively. The numbers on the left and right refer to amino acid positions.

\[ \text{His}100 \text{ and His}105 \text{ are conserved within the cysteine protease family.} \]

\[ \text{V secretion.} \]

\[ \text{His100 and His105 are conserved within the cysteine protease family.} \]

\[ \text{V secretion showed that Cys32 may correlate with binding buffer, followed by binding buffer at pH 6.0. The proteins described above. The supernatant containing induced soluble proteins.} \]

\[ \text{His100 with Asp, Ala, Lys, or Ser did not impair normal colicin V secretion activity, but a similar replacement of His}\textsuperscript{105} \text{ abolished it (Fig. 2A), indicating that His}\textsuperscript{105} \text{ is required for catalytic activity.} \]

\[ \text{To verify that Cys}\textsuperscript{32} \text{ and His}\textsuperscript{105} \text{ mutants are not structurally unstable, we determined CvaB proteins by immunoblotting. The FLAG epitope was introduced into CvaB, which had no effect on the colicin V activity. The results show that CvaB, wild-type CvaA, C32S CvaA, and H105D CvaA were all stable (Fig. 2C).} \]

\[ \text{H105D BntD Can Be Stably Induced—We constructed C-} \]

\[ \text{terminally His-tagged BntD under the control of the Trc promoter. Cultures containing plasmids encoding wild-type BntD or the H105D mutant were induced. Only the H105D mutant overproduced the product, which was purified by affinity chromatography (Fig. 3); the wild-type domain was not overproduced (data not shown). After the cells were lysed, the inclusion bodies containing H105D mutant proteins were harvested by centrifugation and solubilized with 6 M urea (lane 4). The supernatant was then applied to a His tag affinity column, and the nonspecific binding proteins were removed by washings. The 22-kDa H105D BntD protein was eluted with 0.3 M imidazole and used to elicit antibodies.} \]

\[ \text{To detect wild-type BntD and the H105D mutant, samples were collected before and after IPTG induction. As expected, the total cell extracts of the H105D mutant collected after IPTG induction showed a strong immunological reaction, but only weakly before induction, indicating that the H105D mutant was indeed induced (data not shown). On the other hand, wild-type extracts showed little 22-kDa protein even after induction, confirming that it is difficult to detect stable wild-type BntD under the conditions used (data not shown).} \]

\[ \text{Stabilization by Treatment with NEM and Purification of Wild-type BntD—We reasoned that because NEM is a cysteine protease inhibitor, it might stabilize wild-type BntD. Adding NEM (1 mM) to the buffer before cell lysis did indeed confer stability. The total cell extracts and inclusion bodies of wild-type BntD contained a 22-kDa protein, which could be detected immunologically.} \]

\[ \text{Accordingly, we proceeded to purify wild-type BntD in the presence of NEM. Cells were induced at low temperature for 1.5 h, yielding a soluble supernatant that contained half of the induced proteins, from which we purified wild-type BntD. Its identity (Fig. 3, lane 9) was verified by N-terminal peptide sequencing, confirming the first eight amino acids as -MDPMT-} \]

\[ \text{NRN. These preparations were used to test protease activities.} \]

\[ \text{BntD Proteolytic Activity Is Time- and Concentration-dependent—Because the sequence homology suggested that BntD is a cysteine protease, a typical substrate (LAPNA) for metallo- and cysteine aminoprotease activities was tested and found to be adequate for enzyme assay of BntD. The cleavage yielded p-nitroanilide, which could be monitored at A_{280}} \textsuperscript{nm} \text{.} \]

\[ \text{To characterize proteolytic activity, four concentrations of BntD were tested for various times. At 2.16 \mu g/100 \mu l, BntD showed delayed activity, but reached maximal activity at 4.32 \mu g/100 \mu l; at 1.08 \mu g/100 \mu l, BntD showed low enzymatic activity; and at 0.86 \mu g/100 \mu l, BntD showed virtually no activity (Fig. 4). Low enzymatic activities were observed before a 6-h incubation with 2.16 \mu g of proteins and increased between 12 and 24 h, suggesting that cooperative aggregation or oligomerization of BntD is required for maximal activity. The higher concentration (4 \mu g) of BntD was used for all subsequent assays.} \]

\[ \text{Substrate Specificity—N\textsuperscript{\text{-}}-Benzoyl-DL-arginine p-nitroanilide, a typical serine protease substrate, was compared with LAPNA, a typical substrate for metallo- and cysteine aminoproteases. As expected, N\textsuperscript{\text{-}}-benzoyl-DL-arginine p-nitroanilide} \]
Ca²⁺-dependent CvaB Proteolytic Activity

was inactive (Fig. 5A), indicating that BntD belongs to the cysteine protease family. The effect of pH on BntD proteolytic activity was also tested, and the results show that BntD proteolysis occurred over a wide pH range, but was optimal at pH 7.5 (Fig. 5B).

To further characterize BntD, various concentrations of LAPNA were incubated with BntD in cleavage reaction buffer at 37 °C for 6 h. The activities gradually increased up to 2 mM, reaching saturation at ~3 mM (Fig. 5C). The rate of hydrolysis of LAPNA was dependent on substrate concentrations, with a $K_m$ of 1.15 mM and a $V_{max}$ of 91 pmol/min/μg BntD (Fig. 5C).

**BntD Proteolytic Activity Is Calcium-dependent**—As indicated in Fig. 1, the sequence homology suggested that BntD is a cysteine protease calpain-like protein. Calpains, which include the ubiquitously expressed μ- and m-calpains, are a family of Ca²⁺-dependent intracellular cysteine proteases from mammals, birds, and insects. The product of the sol gene in Drosophila is a calpain (36). To test whether BntD indeed requires Ca²⁺ for activity, different concentrations of CaCl₂ were tested. $p$-Nitroanilide cleavage gradually increased up to 5 mM Ca²⁺ (Fig. 6A), reaching saturation at 7.5 mM. The data demonstrate that the proteolytic activity of BntD is calcium concentration-dependent. CaCl₂ at 5 mM was used in subsequent experiments.

It has been reported that the enzymatic activity of a cysteine protease of Porphyromonas gingivalis, a black-pigmented Gram-negative anaerobe, is enhanced severalfold by divalent cations such as Ca²⁺, Mg²⁺, and Mn²⁺ (37). We tested their effect on BntD proteolytic activity. 5 mM CaCl₂, MnCl₂, and CuCl₂ each conferred moderate activity (Fig. 6B). On the other hand, KCl at 50 mM or MgCl₂ at 5 mM was less effective, and MgCl₂ at 10 mM did not compete with the CaCl₂-induced proteolytic activity of BntD (Fig. 6C). ZnSO₄ at 5 mM did not enhance any activity. Due to their similar ionic radii, Tb³⁺ and La³⁺ have been widely used to compete for calcium binding (38). We were surprised to find that TbCl₃ at 150 μM and LaCl₃ at 100 μM were as active as CaCl₂ at 5 mM and that LaCl₃ at 500 μM was 2-fold more active, but higher concentrations enhanced less activity presumably due to precipitation. At the
same concentrations, FeCl₃ was inactive. Interestingly, Havvarstein et al. (19) reported that 250 mM Na₂SO₄ increases proteolytic cleavage by LagD by 4-fold. We found that it did not enhance the proteolytic activity of BntD as effectively as Ca²⁺.

To determine whether the proteolytic activity of BntD requires free calcium, the divalent ion chelator EDTA and the heavy metal chelator EGTA were incubated in cleavage reaction buffer with BntD (Fig. 6C). In the presence of 5 mM CaCl₂,
FIG. 6. BntD proteolytic activity is calcium-dependent. A, BntD proteolytic activity is calcium-dependent. Increasing concentrations of CaCl₂ were added to cleavage reaction buffer and incubated for 6 h. The cleavage amount increased quickly up to 5 mM and then reached a maximum at 7.5 mM. B, cations are required for the BntD proteolytic reaction. TbCl₃ and LaCl₃ significantly increased BntD activity. MgCl₂ and Ca²⁺-dependent CvaB Proteolytic Activity.
Ca\(^{2+}\)-dependent CvaB Proteolytic Activity

**Fig. 7. Requirement of reducing agents for proteolytic activity and inhibition assay of BntD.** In the standard assay, BntD was incubated in the presence (bar 2) and absence (bar 1) of 2-mercaptoethanol. Four classes of protease have been well defined, and the specific protease inhibitors, including the substrate LAPNA, were incubated with BntD for 6 h. The cysteine protease inhibitors NEM at 5 mM (bar 3) and antipain at 2.5 mM (bar 4) and 5 mM (bar 5) showed inhibitory effects on BntD; the serine protease inhibitor phenylmethylsulfon fluoride at 5 mM (bar 6) and the aspartic protease inhibitor pepstatin A at 5 mM (bar 7) did not show any inhibition. Error bars represent the S.D. of an experiment performed in triplicate.

EDTA at 5 mM reduced BntD activity by almost 90%, whereas EGTA at 20 mM inhibited BntD activity by 75%. These data indicate that BntD proteolytic activity requires free Ca\(^{2+}\).

**BntD Proteolytic Activity Requires Reducing Agents and Effects of Inhibitors—**Our preparations contained 1 mM NEM initially in the cell extracts to stabilize the protein to allow purification of BntD, and our typical enzyme assays contained 2-mercaptoethanol and DTT. We evaluated the effects of these compounds on BntD activity. Very low activity was observed in the absence of 2-mercaptoethanol or dithiothreitol (Fig. 7, bar 1). Dithiothreitol at 5 mM or 2-mercaptoethanol at 5–10 mM (data not shown) conferred similar specific activity (32 pmol/min/µg), indicating that 2-mercaptoethanol at 5 mM is sufficient to sustain the reducing environment required for the proteolytic activity of BntD, presumably due to the sulphydryl group of the critical residue Cys\(^{32}\).

Inhibition of BntD activity by NEM was then characterized in the presence of 2-mercaptoethanol (Fig. 7). NEM at 5 mM inhibited proteolytic activity by 90% (lane 3). This observation is consistent with earlier reports that inhibition by NEM at low concentrations is reversible (39, 40) and at high concentrations is irreversible, presumably because of the inactivation by alkylation (39, 41).

Four classes of protease have been well defined: cysteine protease, serine protease, metalloprotease, and aspartic protease (42). To assign BntD more precisely to the cysteine peptidase class, its sensitivity to other groups of inhibitors was tested (Fig. 7). BntD was sensitive to the cysteine protease inhibitor antipain (in addition to NEM) in a dose-dependent manner. No inhibition was observed at 1 mM antipain (data not shown), and 2.5 and 5 mM antipain inhibited proteolytic activity by 47.5 and 73%, respectively (Fig. 7). Pepstatin A, an aspartic protease inhibitor, and phenylmethylsulfon fluoride, a serine protease inhibitor, exerted no inhibitory effect under the conditions used, indicating that BntD does not possess significant aspartic or serine protease activity.

**Lack of Proteolytic Activity of Mutants C32S and H105D—**
The 22-kDa soluble BntD proteins from CvaB mutants C32S and H105D, which lack colicin V secretion activity (Fig. 2), were purified and assayed. As expected, they lack proteolytic activity completely (Fig. 8), thus supporting the notion that Cys\(^{32}\) and His\(^{105}\) are critical residues for proteolytic activity as well as colicin V secretion.

**DISCUSSION**

In this study, Cys\(^{32}\) and His\(^{105}\) were identified as critical catalytic residues involved in BntD proteolytic activity. Purification of wild-type BntD was difficult previously because of its proteolytic activity, which is presumably capable of self-cleaving.\(^2\) The stability and purification of the wild-type domain were accomplished in the presence of the cysteine protease inhibitor NEM in cell extracts (~7.2 mg/ml proteins), which presumably interacts with the sulphydryl group of the cysteine residue. Inhibition by 1 mM NEM in the cell extracts during BntD purification was apparently reversible, as has been reported previously (39, 40). In vitro cleavage showed that the proteolytic activity of BntD was indeed inhibited by NEM; addition of dithiothreitol restored inhibition at low concentrations of NEM, indicating that NEM inhibition is reversible at low concentrations, whereas at high concentrations of NEM (>2 mM), inhibition is not reversible.\(^3\) Although NEM is traditionally regarded as a cysteine protease inhibitor, the inhibition of BntD by NEM in this work does not demonstrate that inhibition by NEM is mediated through covalent modification of Cys\(^{32}\) because alkylation at a site other than a cysteine residue has been shown (41). The nature of the reversible and nonreversible inhibition by NEM at different concentrations is under investigation.

It has been reported that the enzymatic activity of cysteine proteases is enhanced severalfold by divalent cations such as Ca\(^{2+}\) (37, 43) and Mg\(^{2+}\) and Mn\(^{2+}\) (37). Our study shows that Ca\(^{2+}\) stimulated the BntD reaction in a time-dependent (Fig. 4) and concentration-dependent (Fig. 6A) manner. Low concentrations of calcium showed a moderate effect, but high concentrations exerted progressively accelerated BntD activity. Pal et al. (44) reported that the active triad of calpain in the absence of Ca\(^{2+}\) is not assembled into a catalytically active conformation, as evidenced by crystallization in the absence of calcium. Our data support this finding because little proteolytic activity was observed in the absence of Ca\(^{2+}\). Other divalent cations (Mn\(^{2+}\) and Cu\(^{2+}\)) exhibited a moderate effect. Tb\(^{3+}\) and La\(^{3+}\), calcium analogs because of their similar ionic radii and coordination

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\(^2\) K.-H. Wu and P. Tai, unpublished data.

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KCl showed moderate LAPNA cleavage; MnCl\(_2\) and CuCl\(_2\) showed half-activity of the CaCl\(_2\) addition. No activity was detected in the absence of CaCl\(_2\) (negative control), ZnSO\(_4\) and FeCl\(_2\) addition, suggesting that Ca\(^{2+}\) is required for BntD proteolytic activity. Ca\(^{2+}\) is required for BntD proteolytic activity. 10 mM MgCl\(_2\) did not compete with 5 mM CaCl\(_2\)-induced cysteine proteolytic activity. BntD-specific activity was reduced by addition of 5 mM EDTA and 20 mM EGTA by chelating Ca\(^{2+}\) divalent cations. Error bars represent the S.D. of an experiment performed in triplicate.
properties, have long been used to probe presumed calcium binding (38). Interestingly, low concentrations of Tb³⁺ and La³⁺ significantly enhanced BntD proteolytic activity, whereas FeCl₃ did not, which is consistent with the physiological role of CvaB in secretion of colicin V under conditions of iron depletion (45). Based on the above results, the size of the cation appears to be important, but trivalent charges ion can be ruled out. It has been reported that Zn²⁺ inhibits caspase-6 (46) and caspase-3 (47) activities due to the inherent tendency of Zn²⁺ to react with thiols (46). The present study shows that ZnSO₄ did not enhance BntD proteolytic activity, indicating that this effect was probably due to the interaction of Zn²⁺ with the thiol group. EDTA and EGTA, chelators of metal ions, abolished the stimulating effect of calcium, indicating that free calcium is required for the proteolytic activity of BntD. A typical calpain protease has approximately four or five EF-hands, which include the calcium-binding domain (48), but a computer search did not reveal an EF-hand motif in BntD. On the other hand, the EF-hand structure is also absent in several other calpain proteins, including human calpain-7, and despite the absence of the EF-hand or Ca²⁺-binding motifs, the recombimant human calpain protease still shows catalytic activity in the presence of calcium (49). Moreover, non-EF-hand Ca²⁺-binding sites have been revealed in the cysteine protease region of calpain, and Ca²⁺-binding aligns the active site of calpain (50). Taken together, these results indicate that BntD is a member of the calpain-like cysteine peptidase family and is a Ca²⁺-concentration dependent cysteine protease.

The catalytic residues of cysteine protease are believed to be Cys and His (24, 25). It has been suggested that a histidine residue located in the vicinity of the active-site cysteine residue is required to form a thiolate-imidazole ion pair (51). The amide oxygen of the Asn side chain is believed to form a hydrogen bond with the nitrogen atom of His (24, 25), creating a Cys-His-Asn triad that is often considered analogous to the Ser-His-Asp arrangement of serine proteases. The conserved region around Cys is QGQD/GW/A. Cys²⁵ of CvaB is conserved among the cysteine proteases. We have demonstrated here that it is also involved in secretion activity. The conserved regions around His and Asn of cysteine proteases are -HF- and -NPWGW, respectively (Fig. 1). His¹⁰⁵ of CvaB is in this region, but Asn is absent. Mutants in which His¹⁰⁵ was replaced by other amino acids exhibited no protease activity. The presence of the imidazole ring is essential for proteolysis presumably because no other amino acid side chain can substitute for the aromatic property of a protonated imidazole ring contribute to the tautomeric property of imidazole. It has been suggested that a histidine pair. However, the active Asn residue was not identified in BntD.² As predicted by three-dimensional modeling, Trp¹⁷, Asp¹⁰², and Val¹⁰⁸ of BntD may interact with the imidazole ring of His¹⁰⁵. The possible involvement of these residues is under investigation. Gln²⁶ of BntD is also conserved among cysteine proteases. It has been reported that Gln in papain forms an “oxyanion hole” with Cys, which in turn stabilizes the Cys and His ion pair (57). Replacements of Gln²⁶ with Lys significantly affected CoV secretion activity.³ The role of Gln²⁶ is under investigation.

Alignment in different data base systems shows that BntD has high identities to the peptidase C39 family,³ which includes lantibiotic and non-lantibiotic bacteriocins. They are synthesized as precursor peptides containing N-terminal leader peptides, which are cleaved off during maturation. These bacteriocins have leader peptides of the so-called double-glycine type: the processing site with two conserved glycine residues in positions 1 and 2. Peptide bacteriocins, including colicin V (18, 23), are exported across the cytoplasmic membrane by the ABC transporter, in which the proteolytic domain resides in its N-terminal part. Havarstein et al. (19) showed that the N-terminal domain of LagD, a transporter of lactococcin G, possesses proteolytic activity against lactococcin G derivatives containing the double-glycine peptides. Our study indicates that BntD indeed possesses protease activity, which has been shown to be a novel calcium-dependent cysteine protease.

BntD in low amounts reacted slowly with the substrate LAPNA, requiring 6 h to show significant cleavage. Even at high protein concentrations, the Vₘₐₓ was still relatively low; a cooperative effect of aggregation or oligomerization of BntD is probably required at low protein concentrations. This low enzymatic activity may be a characteristic of energy-dependent proteases. It is generally believed that the nucleotide-binding domain of the ABC transporter binds and hydrolyzes NTPs, which provides energy for ABC transporters to secrete (53). It is not certain whether the proteolytic activity of BntD can be enhanced by its C-terminal domain ATPase. Zhong et al. (21) showed that processing CoIV-1 requires the CvaA-CvaB transporter, the TolC protein, membrane integrity, and energy. In addition, the processing of CoIV-1 is rapid (21). The slow enzymatic activity of BntD suggests that the cooperative effects with other transporter components and energy might be required for higher proteolytic activity in vitro for cleavage and secretion of colicin V (21).

Acknowledgments—We are grateful to John Ingraham and Sandra Adams for commenting on the manuscript, Wen-Pin Tzeng for excellent technical support and helpful discussions on mutant constructions, and Jenny Yang and Giovanni Gadda for discussions and suggestions. We thank XiaoTian Zhong for involvement in the early phase of mutant construction and discussions, Roberto Kolter for strains and plasmids, Malcolm Johns for protease assay advice, Carolyn Carter for peptide sequencing, and Ping Jiang for DNA sequencing.

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