Mutational Analysis of Catalytic Sites of the Cell Wall Lytic
N-Acetylmuramoyl-L-alanine Amidases CwlC and CwlV*

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The cell wall of Bacillus subtilis is ~25–30 nm thick and contains roughly 50% by weight peptidoglycan. Peptidoglycan is a heteropolymer consisting of glycan strands cross-linked by peptides. In the spore-forming Gram-positive bacterium B. subtilis, there may be 30 or more peptidoglycan hydrolases (1). Peptidoglycan hydrolases are involved in important biological processes such as cell wall turnover (2–5), cell separation (3, 6), metal cations on the activity of the CwlV1 amidase was investigated. Furthermore, the influence of divalent metal cations on the activity of the CwlV1 amidase was investigated.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Restriction endonucleases and a ligation kit were purchased from Takara Shuzo (Kyoto, Japan). Agarose S and other reagents were purchased from Nippon Gene (Toyama, Japan) and Wako Pure Chemicals (Osaka, Japan).

Plasmid Construction and Mutagenesis—DNA fragments for construction of plasmids, pKPEP31, pKPEP32, pKPEP33, and pKPEP34, were produced by PCR1 using a CwlC expression plasmid pKPEP1 as a template to express a series of C-terminal deletion mutants, D-(218–255), D-(218–255), and D-(218–255), respectively. The sense primer was an oligomer, 5’-CCGCCGAATTCTAGTTGTAAAATTTTTATTGAGTTCT-3’ (the cwlI sequence is italicized, the initiation codon is boldfaced, and the EcoRI site is underlined), and the series of mutagenized antisense primers used were 5’-GCTGCGTGGAGCT-GAGTCACATTTAAGGCAATCGAGTCT-3’, 5’-GCTGCGTGGAGCT-GAGTCACATTTAAGGCAATCGAGTCT-3’.

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† The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CHES, 2-(cyclohexylamino)-ethanesulfonic acid; CD, circular dichroism.

N-Acetylmuramoyl-L-alanine amidases are classified into one of the cell wall hydrolase groups (1, 10). The amidases specifically cleave the amido bond between the lactyl group of muramic acid and the ω-amino group of L-alanine, which is the first amino acid of the stem peptide (11). The C. subtilis CwlC amidase gene, whose product has an overall amino acid identity of 73% with the CwlM amidase from Bacillus polymyxa var. colistinus CwlV, is a catalytic domain. Site-directed mutagenesis was performed on 20 highly conserved amino acid residues within the catalytic domain of CwlC. The amidase activity was lost completely on single amino acid substitutions at two residues (Glu-24 and Glu-141). Similarly, the substitution of the two glutamic acids as catalytic residues.

The catalytic mechanism of the bacterial cell wall lytic amidases such as CwlB, CwlC, and CwlV is not well understood. In a previous study, we concluded that the CwlC amidase does not have catalytic action similar to that of serine protease as judged from the resistivity of the CwlC amidase to the serine protease inhibitor (16). The present study was undertaken to obtain new insights into the molecular mechanisms of cell wall lysis by the amidases. For this purpose, we designed a series of mutants of CwlC and CwlV amidases. First, we constructed mutants with deletions from the N-terminal or the C-terminal sides of the CwlC amidase to determine the catalytic domain. Second, to determine the catalytic amino acid residues, site-directed mutagenesis was performed on 20 amino acid residues within the N-terminal 175 amino acids (catalytic domain) of the CwlC amidase and on two amino acid residues of the truncated CwlV amidase (CwlV1). Furthermore, the influence of divalent metal cations on the activity of the CwlV1 amidase was investigated.
ATGAGCTGGAAGGTCTTTTTAAG-3', 5'-GCTGCCCTGAGCTAGTCTAAAGCTGTTGCGACCCG-3', and 5'-GCTGCCCTGACGGCTTGAATAAAAACGCTGCTGTGGGAC-3', in which the complementary sequences of the cwlC sequences are italicized, the complimentary sequence of the TAG termination codon is boldfaced, and the Pet1 site is underlined. The DNA fragment for construction of plasmid pKPEP35 to express an N-terminal deletion mutant D(1–19) was produced by PCR using the CwlC expression plasmid pKPEP1 as a template and a mutagenized oligonucleotide as a sense primer. The sense primer was 5'-CCGCGGAAT- and 5'-GCTGCCTGCAG of D-(1–320). The antisense primer was 5'-TACGTCGAGATACTCTGT-3'.

The PCR and expression vector pKPEP1500 (17) were digested with EcoRI and PetI, and the resultants were ligated using DNA Ligation Kit ver. 1 (Takara). The competent Escherichia coli JM109 cells were transformed using the ligation solution. The plasmids obtained from transformants were sequenced with an ABI Primo Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Life Sciences) using DNA Sequencing System 373A (Applied Biosystems).

Site-directed mutagenesis analyses of CwlC (D7N, H10Q, D14V, E26Q, E142Q) were performed by the use of QuikChange site-directed mutagenesis kit (Stratagene) using DNA Sequencing System 373A (Applied Biosystems). DNA oligomers as primer pairs were designed for the substitution of Asn-20 to Met for the first amino acid of D-(1–19). The antisense primer was 5'-GCTGCCCTGAGCTAGTCTAAAACGCTGCTGTGGGAC-3' (the complimentary sequence of the cwlC sequence is italicized, the initiation codon is boldfaced, as well as boundaries between the homologous domain and others of the amidases are shown above the thick horizontal lines). An arrow indicates the tandem repeat sequence of the amidases. The term "CwlV" represents the truncated CwlV obtained from a culture solution of B. polymyxa var. colistinus. CwlC, B. subtilis sporulation-specific amidase; CwlM, B. licheniformis amidase (12); CwlB, B. subtilis major autolysin during the vegetative growth phase (14); CwlU, B. polymyxa var. colistinus autolysin (15); CwlV, B. polymyxa var. colistinus autolysin (unpublished).

DNA oligomers as primer pairs were designed for the substitution of Glu-26 (codon: GAA) with Gln-26 (codon: CAA) and of Glu-142 (codon: GAA) with Gln-142 (codon: CAA).

PCR and DNA Sequencing—PCR was performed with a GeneAmp PCR System 9600 (Applied Biosystems). DNA sequencing was performed with double-stranded plasmid DNA as a template. Oligonucleotide primers were purchased from OligoService (Tukuba). Sequencing was performed with an Applied Biosystems Model 373A DNA sequencer using a Dye Terminator or a Dye Primer Cycle sequencing kit (Applied Biosystems).

Purification of CwlC and CwlV1 Amidases and Their Mutant Proteins—The CwlC amidase and its mutant proteins (E24A and E141Q) were overexpressed and purified as described previously (16). The CwlV1 amidase and its mutant protein (E141Q) were overproduced in E. coli KP3998 (F' hsdR299 Tn10 dcm lacI21 ara-14 proA2 lacI2 araD1389 F' [F' lacI21 proA2 lacI2 araD1389]). The fractions containing CwlV1 were collected and dialyzed against the dialysis buffer (20 mM potassium phosphate, pH 7.0). The solution was then applied onto an SP-Sepharose column (Amersham Pharmacia Biotech). The flow-through fraction containing CwlV1 amidase and its mutant protein (E141Q) was dialyzed against the dialysis buffer (20 mM potassium phosphate, pH 7.0) and purified as described above.

Preparation of Cell Wall and Peptidoglycan of B. subtilis—Cell wall from vegetative cells of B. subtilis 168S (18) was prepared essentially as described previously (19). Furthermore, for preparation of the purified peptidoglycan, the cell wall was treated in 10% trichloroacetic acid at 4 °C for 2 days to remove acid labile components such as teichoic acid and polyasaccharide (20). Removal of the teichoic acid was confirmed by the measurement of traces of inorganic phosphate. Phosphorus analysis was carried out by a combination of the methods for ashing (21) and for color development (22).

Assay of Peptidoglycan Hydrolytic Activities—For zymographic assay for amidase activities (zymography), SDS-PAGE of proteins was performed in 14% polyacrylamide gels containing a 0.1% (w/v) B. subtilis cell wall as described previously (23, 24). After electrophoresis, gels were renatured by treatment with 0.1 mM Tris-HCl (pH 8.0)
containing 1% Triton X-100 at 37 °C for 12–16 h. During the renaturation, the buffer was changed three times. Transparent bands of lysis in the translucent gel were rendered more visible by staining with 1% methylene blue (Wako, Osaka) in 0.01% KOH (24). The amount of cell wall was measured with a Shimadzu CS-9000 chromatoscanner set at 595 nm. Even in the case of an insoluble mutant protein (inclusion body), zymographic assay was effective.

For spectrophotometric assay for amidase activities, the purified peptidoglycan was used. Each assay, which was performed essentially according to the published procedure (19), was carried out in duplicate. The peptidoglycan (0.33 mg/ml) was incubated with amidase (0.3 mg/ml) in 0.1 M KCl, 20 mM CHES buffer (pH 9.5) and then mixed with the purified enzyme on ice. After 1-h incubation on ice, the supernatant and the peptidoglycan were separated by centrifugation (12,000 rpm, 10 min). The proteins, which were included in the supernatant and the precipitate, were monitored by SDS-PAGE, respectively.

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were recorded on a Jasco J-600 spectropolarimeter. The CwlC amidase and its mutants (E24A, E141Q) were dissolved in 0.1 M KCl, 20 mM CHES, pH 9.5, and the CwlV1 amidase and its mutant (E142Q) were dissolved in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5. The concentrations of the proteins were ~1.0 μM for CwlC and its mutants, and ~0.6 μM for CwlV1 and its mutants. The fraction of α-helix (fractional helicity; fH) of the proteins was calculated from the formula: 

\[ fH = \left( \frac{\alpha}{\psi_{max} - \psi_{min}} \right) \times 100 \]

where \( \alpha \) is the molar concentration of protein, \( \psi_{max} \) is 222 nm, and \( \psi_{min} \) is 195 nm. The CD spectra were recorded at 25°C with a Shimadzu CD-500 spectropolarimeter. The purified CwlV1 protein sample was heated to ashes with nitric acid and sulfuric acid in a Teflon beaker. The residue was dissolved with diluted nitric acid, and the divalent metal cations in the solution were measured on a Seiko SPS4000 ICP emission spectrometer. The purified CwlV1 protein was heated to ashes with nitric acid and then excess cobalt was removed by dialysis against 20 mM KCl, 20 mM CHES buffer (pH 9.5) and then mixed with the purified enzyme on ice. After 1-h incubation on ice, the supernatant and the peptidoglycan were separated by centrifugation (12,000 rpm, 10 min). The proteins, which were included in the supernatant and the precipitate, were monitored by SDS-PAGE, respectively.

**RESULTS**

**Determination of the Catalytic Domain by Deletion Mutagenesis**—To define the boundary of the catalytic domain of the CwlC amidase, we constructed an N-terminal deletion mutant and four C-terminal deletion mutants (Fig. 2). Deletion mutants were named “D-(deleted amino acid positions)”. The expression in *E. coli* was confirmed by SDS-PAGE (data not shown), and the cell wall lytic activities of these mutant proteins were detected by zymography, that is, activity staining of enzyme. As shown in Fig. 2, the N-terminal deletion mutants D-(1–19), D-(1–42), D-(1–102), and D-(1–150) retained the cell wall lytic activities, and the mutants remained homologous domain (from Met-1 to Asn-176) in the N-acetylglucosaminyl-l-alanine amidases shown in Fig. 1. In contrast, the mutant with a further 16-amino acid deletion, D-(1–165), completely lost the activity, indicating that the C-terminal boundary of the catalytic domain was located between amino acid positions 161 and 167. This showed that the tandem repeat of the C termini of CwlC was not critical for activity. An N-terminal deletion mutant D-(1–19) in which only the first 19 amino acids were deleted lost the cell wall lytic activity completely. These results are consistent with the prediction that the homologous domain is the catalytic domain of the amidases.

**Determination of Critical Amino Acids by Site-directed Mutagenesis**—Site-directed mutagenesis of the N-terminal amino acid residues in the catalytic domain was performed to identify the amino acids involved in the cell wall lytic activity. Fig. 3 shows the amino acid sequence of the homologous catalytic domains of several amidases in the CwlB family. The uppermost line indicates the N-terminal 176 amino acids of the CwlC amidase corresponding to the catalytic domain. We selected 20 amino acids for site-directed mutagenesis on CwlC amidase according to the following criteria. The amino acids critical for the cell
The amidase activities of the CwlC mutants detected by zymography. The amidase activities of the mutants are shown with the percent activity relative to wild-type. Because of experimental error, activities of less than 1% are ignored. The site-directed mutants were divided into three groups on the basis of their cell wall lytic activity: none (less than 1% activity of the wild-type; E24A, E24S, E24Q, D55V, H79L, N81I, E141V, and E141Q), partial (1–10% activity of wild-type; K25I and R52L), reduced and unchanged (10–100% activity of wild-type; D7N, H10Q, D14V, S51G, D55S, D55N, R63Q, D73A, S77G, H79N, N81S, R120L, K123Q, S133A, T147I, D150V, and Q161L) (Fig. 4A). Although the mutants D55V, H79L, and N81I did not show lytic activity at all, the related mutants D55S, D55N, H79N, and N81S had more than 20% of the activity of the wild-type. In contrast, all of the mutations at E24 and E141 resulted in a loss of cell wall lytic activity. The mutants E24A and E141Q were overproduced in E. coli and purified as described previously. Fig. 4B shows results of SDS-PAGE (left-hand side) and zymography (right-hand side) of the CwlC mutants. Zymography is the detection method of enzymatic activity in situ after SDS-PAGE as described under "Experimental Procedures." Neither E24A nor E141Q mutants showed cell wall lytic activity. From the measurement of the turbidity decrease of the purified peptidoglycan as a substrate, it was concluded that the E24A and E141Q mutants did not decompose the peptidoglycan at all (Fig. 4C).

CD spectra of the native CwlC amidase and the mutant amidases (E24A and E141Q) were measured in 20 mM CHES buffer (pH 9.5) containing 100 mM KCl at 10 °C. Fig. 5A, CD spectra of CwlC and its mutants (E24A and E141Q) in 20 mM CHES buffer (pH 9.5) containing 100 mM KCl at 10 °C. Fig. 5B, CD spectra of CwlV and its mutant (E142Q) in 20 mM HEPES buffer (pH 7.0) containing 100 mM KCl at 10 °C.

Influence of the substitution of the conserved glutamic acid with aspartic acid on the enzymatic activity of the CwlC amidase. The amidase activities of the mutants were detected by zymography and are shown with the percent activity relative to wild-type. Because of experimental error, activities of less than 1% are ignored. E24D/E141D is a double mutant at Glu-24 and Glu-141. The E24Q and E141Q mutants shown in Fig. 4 are displayed again.
terminal domain (321–499) of the CwIV amidase is the homologous catalytic domain (Fig. 1). In our laboratory, the truncated CwIV (CwIVt: 317–499) was purified from the supernatant of the culture. The truncated CwIVt retained considerable cell wall lytic activity. We also constructed a plasmid, which expresses the truncated CwIV D-(1–320), named CwIV1, in which Met was used as the substitute for the first amino acid Lys-321. Zymography and the decrease in turbidity of the peptidoglycan (Fig. 7) monitored the cell wall (peptidoglycan) lytic activity of the purified CwIV1. In performing these analyses, it was found that the CwIV1 amidase consisting of only the catalytic domain had cell wall lytic activity. Glu-26 and Glu-142 of the CwIV amidase correspond to Glu-24 and Glu-141 of the CwIC amidase as judged by an alignment of the catalytic domains of CwIC homologous amidases, respectively (Fig. 3). Therefore, site-directed mutagenesis at Glu-26 and Glu-142 in the CwIV1 amidase was performed to confirm that the glutamic acids were critical to the cell wall lytic activity and the catalytic amino acid residues of the CwIB family N-acetylmuramoyl-l-alanine amidases. The mutants E26Q and E142Q of the CwIV1 amidase were overproduced in E. coli. The mutant E142Q was purified in a similar manner as the wild-type. On the other hand, the addition of Zn2+ to the reaction mixture stimulated the degradation of the peptidoglycan. On the other hand, the addition of Zn2+, Mn2+, and Co2+ to the reaction mixture stimulated the degradation of the peptidoglycan in that order. The specific activity of the CwIV1 amidase in the presence of cobalt ion was five times larger than that in the presence of zinc ion. The specific activity of the intact CwIV1 amidase (the purified amidase that was not treated with EDTA) was ~5700 units/mg. The addition of manganese and calcium ion hardly influenced the degradation of the peptidoglycan. The removal of superfluous divalent metal cations from the CwIV1 amidase solution did not result in a significant change in the reactivity of the amidase (data not shown). In this study, we did not attempt to examine the influence of the divalent metal cation on the CwIC amidase activity because of its poor solubility.

The number of the divalent metal cations, which bind to the native CwIV1, was determined by ICP emission spectrometer as summarized in Table I. The binding numbers of Zn2+, Mn2+, and Fe2+ per native CwIV1 were ~0.5, ~0.2, and ~0.1, respectively. With respect to Co2+-substituted CwIV1 and Co2+-substituted E142Q, one Co2+ bound one molecule of CwIV1 and one molecule of E142Q. These results indicate that one divalent metal cation, mostly Zn2+, binds one molecule of the native CwIV1 and the substitution of the Glu-142 of the CwIV1 amidase to Gln does not influence the binding of the divalent metal cation to the amidase.

FIG. 7. The amidase activities of the CwIV1 mutants. A, SDS-PAGE (left) and zymography (right) of the CwIV1 mutants. E142Q was a purified soluble protein. The inclusion body of the insoluble E26Q protein was also used as a sample for SDS-PAGE. The purified proteins (~2 μg) were applied from lane 1 to lane 6. B, the amidase activities of the CwIV1 mutants detected by zymography. The amidase activities of the mutants are shown with the percent activity relative to wild-type. Because of experimental error, activities of less than 1% are ignored. C, the amidase activities of the wild-type and the mutant detected by the relative turbidity decrease of the peptidoglycan. Reaction conditions are described under “Experimental Procedures.” Neither E26Q nor E142Q mutants had cell wall lytic activity (Fig. 7, A and B). From the measurement of the turbidity of the purified peptidoglycan as a substrate, it was concluded that the E142Q mutant did not decompose the peptidoglycan (Fig. 7C).

FIG. 8. Influence of the divalent metal cations on the peptidoglycan lytic activity of the CwIV1 amidase. Preparation of the divalent metal cation-free CwIV1 and the reaction conditions are described under “Experimental Procedures.” Results are expressed as the relative turbidity (absorbance at 540 nm) decrease of the peptidoglycan. The term “intact” represents the purified CwIV1 without any treatment.

CD spectra of the native CwIV1 amidase and the mutant amidase (E142Q) were measured in 20 mM HEPES buffer at pH 7.5 (Fig. 5B). The spectrum of the E142Q was almost the same as that of the native CwIV1. This indicates that the replacement of Glu-142 by glutamine does not significantly influence the secondary structure of the mutant.

Influence of Divalent Metal Cations on the Peptidoglycan Lytic Activity of the CwIV1 Amidase—Because of the low solubility of the CwIC and its mutant proteins, the influence of divalent metal cations on amidase activity was studied solely on the CwIV1 and its mutant proteins. The CwIV1 amidase was finally dialyzed against the buffer containing 5 mM EDTA and 20 mM Tris-HCl (pH 7.5). We measured the influence of the divalent metal cations (1 mM) on the decrease in turbidity of the peptidoglycan as a substrate. The divalent metal cation-free CwIV1 solution (0.3 μg/15.4 pmol) was added to the solution containing the B. subtilis peptidoglycan (final 0.33 mg/ml, 0.3 A540), divalent metal cation (final 1 mM), 0.1 M KCl, and 20 mM HEPES (pH 7.0). The reaction mixture (1 ml) was incubated at 37 °C, and the time course of the turbidity changes at 540 nm was measured (Fig. 8). The peptidoglycan was scarcely digested by the EDTA-treated CwIV1 amidase. The addition of magnesium and calcium ion hardly influenced the degradation of the peptidoglycan. On the other hand, the addition of Zn2+, Mn2+, and Co2+ to the reaction mixture stimulated the degradation of the peptidoglycan in that order. The specific activity of the CwIV1 amidase in the presence of cobalt ion was five times larger than that in the presence of zinc ion. The specific activity of the intact CwIV1 amidase (the purified amidase that was not treated with EDTA) was ~5700 units/mg. The removal of superfluous divalent metal cations from the CwIV1 amidase solution did not result in a significant change in the reactivity of the amidase (data not shown). In this study, we did not attempt to examine the influence of the divalent metal cation on the CwIC amidase activity because of its poor solubility.
Catalytic Site of Cell Wall Lytic Amidases CwlC and CwlV

TABLE I

| Protein                | Divalent metal cation per protein | Specific activity |
|------------------------|-----------------------------------|------------------|
|                        | Mn²⁺ | Fe²⁺ | Co²⁺ | Ni²⁺ | Cu²⁺ | Zn²⁺ | Cd²⁺ |
| CwlV1 (native)         | 0.21 | 0.07 | <0.02 | <0.04 | <0.02 | 0.53 | <0.01 |
| CwlV1 (Co²⁺-substituted) | 0.72 | 1.18 |        |        |        |      |      |
| E142Q (Co²⁺-substituted mutant) | 0.53 |      |      |      |      |      |      |

a Divalent metal cations were measured on an ICP emission spectrometer.

b One unit of amidase activity is defined as the amount of amidase necessary to decrease the A₄₅₀ of a cell wall suspension by 0.001 min⁻¹, measuring the maximum initial rate.

c The substitution method of cobalt ion was described under "Experimental Procedures."

d N.D., not detected by both turbidity measurement of the cell wall in vitro and zymography.

DISCUSSION

A wide variety of lytic enzymes of bacterial cell wall have been isolated from bacteria and bacteriophages as well as the animal and plant kingdoms. Lysozymes (endo-N-acetylmuramidase) have long served as model systems for the study of protein structure and function (27, 28). Hen egg white lysozyme is one of the enzymes in which the catalytic mechanism has been extensively investigated (29–31). In contrast, although the N-acetylmuramoyl-t-alanine amidases, which lyse the cell wall of bacteria and are widely found in living organisms, play important roles in cell cycles, their enzymatic characterizations have been little studied. To consider reaction mechanism of the amidases in the CwlB family, we performed mutational analyses at first with respect to the CwlC and the truncated CwlV (CwlV1) amidases to elucidate their catalytic residues. Next we examined the influence of divalent metal cations on amidase activity.

Catalytic Amino Acid Residues of the Cell Wall Lytic N-Acetylmuramoyl-t-alanine Amidases—The results of the mutagenesis in the catalytic domains of the CwlC and CwlV1 amidases indicated that Glu-24 and Glu-141 in the CwlC amidase and Glu-26 and Glu-142 in the CwlV1 amidase are catalytically essential. These two glutamic acids are strictly conserved through N-acetylmuramoyl-t-alanine amidases and the proteins with the homologous amino acid sequence listed in Fig. 9. The mutant E141Q of the CwlC amidase and the mutant E142Q of the CwlV1 amidase were overproduced in E. coli and purified. The changes from glutamic acid to glutamine were isosteric. The CD spectra of these mutants were identical with those of the wild-type amidases. This suggests that the loss of amidase activity of these mutants is not attributable to destruction of the structure of the amidases. Similar purification of the mutant amidases (E24Q of CwlC and E26Q of CwlV1) was attempted, but both mutants formed inclusion bodies in E. coli. Because the E24A mutant of CwlC did not form inclusion bodies, it was overproduced in E. coli and purified. The CD spectrum of the E24A mutant (Fig. 5a) showed that it was almost identical with the wild-type in structure. Furthermore, to reaffirm the indispensability of the two glutamic acids, CwlC mutants, in which aspartic acid was substituted for Glu-24 and/or Glu-141, were constructed and their amidase activities were examined. The E24D and E141D mutations reduced appreciably the amidase activities, compared with the wild-type CwlC. The double-substituted E24/141D mutant lost all amidase activity. The decrease in both side-chain lengths with the change of the glutamic acid residues to aspartic acid residues is attributable to the shortening of the distance between the glutamic acid residues at the catalytic site. From these results, we propose that Glu-24 and Glu-141 of CwlC and Glu-26 and Glu-142 of CwlV1 are the most likely candidates for the essential catalytic residues.

Influence of Divalent Metal Cations on Amidase Activity—Our analysis shows that Zn²⁺ can serve as a cofactor for the activity of the CwlV amidase, but it is less effective than Mn²⁺ and Co²⁺. On the other hand, Mg²⁺ and Ca²⁺ are not entirely essential cofactors of the amidase. Comparisons of amidase activities in mixtures of Zn²⁺ and either Mg²⁺ or Ca²⁺ suggested that Zn²⁺ bound to the amidase with higher affinity than Mg²⁺ and Ca²⁺ (data not shown). We also observed a peak for the optimal concentration of Zn²⁺ at ~0.2 mM and that of Co²⁺ at ~1.0 mM. But higher concentrations of these cations were inhibitory (data not shown). The reason for the decreased activity at higher concentrations is not clear. However, the most striking result from these experiments was the observation that the heavy metal cations Zn²⁺, Mn²⁺, and Co²⁺ supported the cell wall lytic activity of the CwlV amidase, whereas Mg²⁺ and Ca²⁺, the presumed physiologically relevant cations, did not. This observation prompted us to examine whether Zn²⁺ or any of the other metal cations could serve as cofactors for cleavage of the amide bond of the peptidoglycan.

Catalytic Mechanism of the N-Acetylmuramoyl-t-alanine Amidase—T7 lysozyme (amidase) cuts the amido bond between the lactyl group of the muramic acid residue and the δ-amino group of the t-alanine residue in the peptidoglycan similar to B. subtilis CwlC amidase or B. polymyxa var. colistinus CwlV amidase. But T7 lysozyme is very different from the CwlC amidase family in amino acid sequence. With respect to the...
catalytic residues of T7 lysozyme, no acidic amino acid residue is found inside of the cleft, in which Zn$^{2+}$, Mn$^{2+}$, or Co$^{2+}$ is chelated as a cofactor (32). Consequently, we can say that the reaction mechanisms of the CwlC and CwlV amidases differ from that of T7 lysozyme, because the critical catalytic residues of the bacterial amidases are two glutamic acids.

Comparison of the structures of matrilysin, thermolysin, and carboxypeptidase A, which are zinc metalloendopeptidases, reveals both similarities and differences in their active sites (33). The metallocenzyms have a common catalytic zinc site in which the zinc atom is coordinated by three protein ligands and a Glu residue is considered to act as a nucleophile or a general base catalyst in the subsequent reaction of the amide bond of the peptidoglycan. The remaining polarization of the carbonyl bond might accelerate the cleavage can might coordinate with the zinc ion, and then the resulting critical Glu residues (Glu24-CwlC, Glu26-CwlV1) and the two protein molecule (Table I) suggesting Glu-26 not Glu-142 of CwlV1 is essential for binding of the divalent metal cation. Consequently, it would be possible to argue that one of the Glu-141-CwlC amidase were reduced to 23% and 15%, respectively (Fig. 9, His-10 and His-79 of CwlC are conserved residues that correspond to His-10 and His-80 of CwlV1. The amidase activities of the H10Q and H79N mutants of the CwlC amidase were reduced to 23% and 15%, respectively (Fig. 4A). On the other hand, the H79L mutant did not show detectable activity. The E142Q of CwlV1 contained one cobalt ion per protein molecule (Table I) suggesting Glu-26 not Glu-142 of CwlV1 acts as a cofactor for the activity of the amidases. To elucidate the reaction mechanism and the peptidoglycan recognition mechanism of the amidases, further studies, including crystallization experiments and/or deliberated site-directed mutagenesis, are necessary.

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REFERENCES

1. Smith, T. J., Blackman, S. A., and Foster, S. J. (2000) Microbiology 146, 249–262
2. Holý, J.-V., and Tuomanen, E. I. (1991) J. Gen. Microbiol. 137, 441–454
3. Doyle, R. K., and Koch, A. L. (1987) Crit. Rev. Microbiol. 15, 169–222
4. Archibald, A. R. (1989) in Bio/Technology Handbooks Vol. 2 (Harwood, C. R., ed) pp. 217–254, Plenum Press, New York
5. Wu, H. C., and Tokunaga, M. (1986) Curr. Top. Microbiol. Immunol. 125, 127–157
6. Fein, J. E., and Rogers, H. J. (1976) J. Bacteriol. 127, 1427–1442
7. Smith, T. J., and Foster, S. J. (1995) J. Bacteriol. 177, 3855–3862
8. Moriyama, R., Hattori, A., Miyata, S., Kudoh, S., and Makino, S. (1996) J. Bacteriol. 178, 6059–6063
9. Ishikawa, S., Yamane, K., and Sekiguchi, J. (1998) J. Bacteriol. 180, 1375–1380
10. Nogroho, F. A., Yamamoto, H., Kobayashi, Y., and Sekiguchi, J. (1999) J. Bacteriol. 181, 6230–6237
11. Harz, H., Burgdorf, K., and Holzig, J.-V. (1990) Mol. Biochem. 190, 120–1280
12. Kuroda, A., and Sekiguchi, J. (1991) J. Bacteriol. 173, 7304–7312
13. Ishikawa, S., Kawahara, S., and Sekiguchi, J. (1999) Mol. Gen. Genet. 262, 738–748
14. Shida, T., Hattori, I., and Sekiguchi, J. (2000) Biosci. Biotechnol. Biochem. 64, 1522–1525
15. Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T., and Horiiuchi, T. (1987) Protein Eng. 1, 327–332
16. Aoki, T., and Sekiguchi, J. (1987) Agric. Biol. Chem. 51, 2901–2909
17. Kuroda, A., and Sekiguchi, J. (1990) J. Gen. Microbiol. 136, 2209–2216
18. Dehart, H. P., Heath, H. E., Heath, L. S., LeBlanc, P. A., and Sloan, G. L. (1995) Appl. Environ. Microbiol. 61, 1475–1479
19. Baginski, E. S., Fou, P. P., and Zak, B. (1967) Clin. Chem. 13, 326–332
20. Chen, P. S., Jr., Torribara, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756–1758
21. Amaxini, U. K. (1970) Nature 227, 680–685
22. Rashid, M. H., Sato, N., and Sekiguchi, J. (1995) FEMS Microbiol. Lett. 132, 131–137
23. Kaneo, T., Sato, S., and Kotsani, H., et al. (1996) DNA Res. 3, 109–136
24. Chen, Y.-H., Yang, J. T., and Martinez, H. M. (1972) Biochemistry 11, 4120–4131
25. Blake, C. C. F. (1965) Nature 206, 757–761
26. Kelly, J. A., Siedek, A. R., Sykes, B. D., James, M. N. G., and Phillips, D. C. (1979) Nature 282, 875–8788
27. Phillips, D. C. (1966) Sci. Am. 215, 78–80
28. Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Bupley, J. A. (1972) in The Enzymes (Boyer, P., ed) Vol. 7, 3rd Ed., pp. 663–868, Academic Press, New York
29. Ueki, K., Ho, Y., Kato, Y., and Imoto, T. (1997) J. Biol. Chem. 272, 19767–19781
30. Cheng, X., Zhang, X., Pihlgrath, J. W., and Studier, F. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4034–4038
31. Auld, D. S. (1997) Structure Bonding 89, 29–50
32. Markenir, L. P., Brooks, G. S., Cutfield, J. F., Sullivan, P. A., and Withers, S. G. (1997) J. Biol. Chem. 272, 3161–3167
33. Vaseux, G., Wang, J., Corvoll, P., and Llores-Cortes, C. (1996) J. Biol. Chem. 271, 9069–9074
34. Sekiguchi, J., Akeo, K., Yamamoto, H., Khasanov, F. K., Alonso, J. C., and Sekiguchi, A. (1990) J. Bacteriol. 177, 5582–5589
35. Troup, B., Jahn, M., Hungerer, C., and Jahn, D. (1994) J. Bacteriol. 176, 673–680
36. Tsui, H.-C. T., Zhao, G., Feng, G., Leung, H.-C. E., and Winkler, M. E. (1994) Mol. Microbiol. 11, 189–202
37. Blatner, F. R., Plunkett, G., 3rd, Bloche, C. A., Perri, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Bode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) Science 277, 1453–1474
38. Xu, X., and Elliott, T. (1995) J. Bacteriol. 175, 4990–4999
39. Tomb, J.-F., Dougherty, B. A., and Merrick, J. M., et al. (1998) Science 280, 496–512
40. Tomb, J.-F., White, O., and Kerlavage, A. R., et al. (1997) Nature 388, 539–547
41. Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., Overbeek, R., Snead, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olsen, G. J., and Swanson, R. V. (1998) Nature 392, 353–358
