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Properties of Cell Wall-Associated DD-Carboxypeptidase of *Enterococcus hirae* (*Streptococcus faecium*) ATCC 9790 Extracted with Alkali

REIKO KARIYAMA, ORIETTA MASSIDDA,† LOLITA DANEO-MOORE, AND GERALD D. SHOCKMAN*

Department of Microbiology and Immunology, School of Medicine, Temple University, Philadelphia, Pennsylvania 19140

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DD-Carboxypeptidase (DD-CPase) activity of *Enterococcus hirae* (*Streptococcus faecium*) ATCC 9790 was extracted from intact bacteria and from the insoluble residue (crude cell wall fraction) of mechanically disrupted bacteria by a brief treatment at pH 10.0 (10 mM glycine–NaOH) at 0°C or by extraction with any of several detergents. Extractions with high salt concentrations failed to remove DD-CPase activity from the crude wall fraction. In contrast to *N*-acetylmuramoylhydrolase (both muramidase 2 and muramidase 1) activities, DD-CPase activity failed to bind to insoluble cell walls or peptidoglycan matrices. Thus, whereas muramidase 1 and muramidase 2 activities can be considered to be cell wall proteins, the bulk of the data are consistent with the interpretation that the DD-CPase of this species is a membrane protein that is sometimes found in the cell wall fraction, presumably because of hydrophobic interactions with other proteins and cell wall polymers. The binding of [^{14}C]penicillin to penicillin-binding protein 6 (43 kilodaltons) was proportional to DD-CPase activity. Kinetic parameters were also consistent with the presence of only one DD-CPase (penicillin-binding protein 6) in *E. hirae*.

A number of years ago, we demonstrated that the autolytic peptidoglycan hydrolase activity of the bacterium that is now known as *Enterococcus hirae* (*Streptococcus faecium*) ATCC 9790 could be dissociated from its cell wall substrate by exposure of the cell wall-enzyme complex to very high salt concentrations (27). Subsequently, we showed that autolysin activity could also be dissociated from wall-enzyme complexes of *E. hirae* (3) or *Lactobacillus acidophilus* 63 AM Gasser (7) by exposure to high pH (e.g., pH 12, 0.01 N NaOH) at 0°C. The apparent high affinity for, and therefore close association of, these enzymes with their cell wall substrates seems appropriate for enzyme activities that hydrolyze bonds outside of the permeability barrier of these gram-positive bacterial species. In fact, 0.01 N NaOH extracted autolysin activity from intact cells of *E. hirae* (3).

At the time these experiments were done we did not know that *E. hirae* possessed two apparently separate and distinct muramidase activities (8, 20, 21, 29). Both muramidase 1 and muramidase 2 (21) activities appear to have a very high affinity for binding to the cell wall substrate and can be extracted from isolated, partly purified wall-enzyme complexes, as well as from intact, freeze-dried, or freshly grown bacteria, by brief exposure to pH 12 (8, 20, 29).

The presence in these neutralized pH 12 extracts of a characteristic series of polypeptides, in addition to muramidase 1 and muramidase 2, and the ability of a substantial fraction of these polypeptides to tightly bind to the acid-insoluble peptidoglycan fraction of *E. hirae* walls, plus the observation that two polypeptides, at 125 and 75 kilodaltons (kDa) after sodium dodecyl sulfate (SDS)-gel electrophoresis, not only displayed muramidase 2 activity but also bound [^{14}C]penicillin G (8), suggested that other proteins involved with cell wall assembly and/or hydrolysis might be present in the mixture extracted by alkali. This proved to be the case (8). All of the penicillin-binding proteins (PBPs) present in standard membrane preparations of this organism (5) were present in the crude cell wall fraction, and these PBPs could be dissociated from the crude wall fraction by a brief treatment at pH 12 at 0°C. Thus, it appeared that these membrane proteins, which are assumed to play one or more roles in cell wall assembly, also strongly interact with the cell wall.

By far the most abundant PBP in *E. hirae* is PBP 6 (4). PBP 6 accounts for 30 to 55% of the penicillin-binding activity of standard membrane preparations and has been shown to possess DD-carboxypeptidase (DD-CPase) activity (4, 5) and to cofractionate with muramidase 2 activity (8, 29). However, several properties of DD-CPase activity suggested that it differed from the other PBPs and muramidases in its solubility and affinity for binding to walls. Here we report attempts to selectively remove DD-CPase activity from the crude cell wall fraction and to separate it from muramidase 2 and the other PBPs present in this cell fraction.

MATERIALS AND METHODS

Bacterial growth. Fermentations (200 liters) of *E. hirae* were kindly carried out for us by E. I. DuPont de Nemours & Co., Inc. (Wilmington, Del.). The bacteria were grown in a complex medium composed of 10 g of ardamine Z, 10 g of N-Z amine B, 25.65 g of Na_2HPO_4, 16.45 g of NaH_2PO_4, and 20 g of glucose per liter at 35°C and 200 rpm and maintained at pH 6.5 to 6.8 by the addition of 10 N NaOH. When the cultures reached a concentration of 1.7 mg of bacterial dry weight per ml, the fermentation vessel was chilled, and the cells were harvested by continuous-flow centrifugation. Cells were provided to us as frozen cell paste (−70°C).

Disruption of cells and preparation of the crude cell wall fraction. All manipulations for the disruption of cells were carried out at 0 to 4°C, and the proteinase inhibitors 0.1 mM phenylmethylsulfonyl fluoride, 2 mM disodium EDTA, 5 mM nitrilotriacetic acid, 1 μg of pepstatin A per ml, and 1 μg of antipain per ml were added to all solutions. The frozen cell

* Corresponding author.
† Present address: Istituto di Microbiologia, dell' Universita di Siena, 53100 Siena, Italy.

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pastes were lyophilized, and 100-g (dry weight) portions were suspended in 600 ml of ice-cold 10 mM sodium phosphate (pH 7) containing 50 μg of DNase per ml, 50 μg of RNase per ml, and 1 mM $MgSO_4$. The cells were then disrupted in a Ribi cell fractionator (Sorvall, Inc., Norwalk, Conn.) at 40,000 lb/in^2 (5 to 10°C). The broken cell preparation was left on ice for 30 min and then centrifuged (20,000 × *g*, 30 min, 4°C). Routinely, the crude cell wall pellet was suspended in 400 ml of 10 mM sodium phosphate (pH 7), and 20-ml volumes were stored at −70°C.

Preparation of cell wall matrices. The insoluble residue of disrupted *E. hirae* or *Mirococcus luteus* bacteria (200 mg/ml) was treated with 0.5 μg of DNase per ml, 0.5 μg of RNase per ml, and 1.0 μg of pronase per ml in 10 mM sodium phosphate (pH 7) containing 1 mM $MgSO_4$ for 4 h at 37°C. The walls were pelleted (20,000 × *g*, 15 min, 4°C) and washed twice with distilled water. The washed cell walls were then suspended in 2% SDS and incubated at room temperature overnight with continuous stirring. The walls were sedimented and washed with distilled water until there was no foaming upon shaking (a minimum of 10 times). These preparations were designated SDS-washed cell walls. A portion of the *E. hirae* SDS-washed cell walls was treated with 0.1 N HCl at 60°C for 16 h (20). The suspension was neutralized with 2.5 N NaOH and centrifuged (28,000 × *g*, 15 min), and the pellet was washed five times with distilled water. This preparation was designated *E. hirae* peptidoglycan (EHPG). A portion of the EHPG was then N-acetylated with acetic anhydride (15).

Alkaline extraction of DD-CPase and muramidase 2. All manipulations were carried out at 0 to 4°C, and all solutions contained the proteinase inhibitors mentioned above, unless otherwise stated. pH 10 extractions were performed by the addition of 10 mM glycine–NaOH (pH 10) to the sedimented crude cell wall fraction or intact cells. The pH was adjusted to 10 with 2.5 N NaOH after the addition of the proteinase inhibitors. After rapid resuspension, the samples were centrifuged (16,000 × *g* or 28,000 × *g*, 15 min, 4°C), and the supernatant fraction was decanted and rapidly adjusted to pH 7 by the addition of 0.5 M sodium acetate (pH 5.4). pH 12 extractions were carried out by suspending the sedimented crude cell wall fraction or intact cells in ice-cold distilled water containing the proteinase inhibitors and then, immediately before centrifugation (16,000 × *g* or 28,000 × *g*, 15 min, 4°C), bringing the suspension to pH 12 by adding an appropriate volume of 2.5 N NaOH. Immediately after centrifugation, these supernatants were also neutralized to pH 7 by the addition of 0.5 M sodium acetate (pH 5.4).

Assay of enzymatic activities. DD-CPase activity was determined by the enzymatic estimation of the amount of D-alanine liberated (13). Routinely, enzyme preparation and 5 mM diacetyl-L-Lys-D-Ala-D-Ala were incubated in 30 μl (final volume) of 10 mM sodium phosphate (pH 7) for 2 h at 37°C. Muramidase 2 activity was assayed by the ability to hydrolyze either EHPG or SDS-washed *M. luteus* cell walls (21). The standard assay consisted of 0.17 mg of either substrate per ml and the enzyme preparation in a final volume of 1.5 ml of 10 mM sodium phosphate (pH 7). Dissolution of substrate was monitored (A_{450}) on a Gilford 300 spectrophotometer. One enzymatic unit is defined as the amount of enzyme that results in a decrease of 0.001 *A*/min.

Protein assay. Protein was determined by the bicinchoninic acid method (30; Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard.

Preparation of standard protoplast membranes. *E. hirae* was grown in 200 ml of Todd-Hewitt broth containing 2% glucose, the exponential-phase cells at a concentration of 0.23 mg of cell dry weight per ml were chilled on ice, and the bacteria were harvested by centrifugation and washed once with ice-cold water. Cellular lysis was carried out at a concentration of 12 mg of cell dry weight per ml in 10 mM sodium phosphate (pH 7) containing hen egg white lysozyme (10 μg/ml), DNase (5 μg/ml), and RNase (2.5 μg/ml). Lysis was complete after 45 min at 37°C. The lysates were centrifuged (80,000 × *g*, 30 min, 4°C), the membrane pellets were suspended in 3 ml of 10 mM sodium phosphate (pH 7), and 200-μl samples were stored at −70°C.

Penicillin binding assay. Penicillin-binding activity was determined at a concentration of [^{14}C]penicillin G of 40 μg/ml (5). The assay mixture contained 50 μl of extract or membranes (150 to 300 μg of protein) and 20 μl of 10 mM sodium phosphate (pH 7) containing 2.8 μg of [^{14}C]penicillin G (56 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). After 15 min at 37°C, the reaction was quenched with 500 μg of cold penicillin G, followed by the addition of 75 μl of 2× SDS sample buffer (125 mM Tris hydrochloride [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% glycerol) and incubation at 100°C for 2 min.

SDS-polyacrylamide gel electrophoresis. The electrophoresis method used was similar to that described by Laemmli (22). The stacking and separating gels were 4 and 10% polyacrylamide at pH 6.8 and 8.8, respectively. Gels were subjected to electrophoresis at 10 mA of constant current for approximately 16 h under a continuous SDS-Tris-glycine buffering system (pH 8.3). Gels were stained with Coomassie blue R-250 (0.275% Coomassie blue R-250, 50% methanol, 10% acetic acid). PBPs were visualized by standard fluorographic techniques (5) with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.) as the fluor. X-ray film (X-Omat; Eastman Kodak Co., Rochester, N.Y.) was exposed for 15 to 60 days at −70°C to visualize the prominent PBP 6.

Densitometric analysis of radioautograms. Density of bands in radioautograms was determined by using a Dumas image analysis system (Drexel University, Philadelphia, Pa.) and a program designed to calculate relative integrated densities.

RESULTS

Extraction of DD-CPase activity from the crude cell wall fraction. Initial studies showed that the insoluble residue of disrupted *E. hirae* retained about one-half of the total DD-CPase recovered in all fractions. After two washes with 10 mM sodium phosphate (pH 7), the crude cell wall pellet retained about one-third of the total recovered DD-CPase activity. The DD-CPase activity present in the pH 7 washes and the DD-CPase activity present in the original supernatant fraction sedimented at 100,000 × *g* and thus were considered to be membrane-bound. Further washes of the crude wall fraction with 150 mM sodium phosphate (pH 7.5) or with up to 5 M sodium or lithium chloride failed to extract significant amounts of DD-CPase activity. However, extraction of the crude walls at pH 12 (0.01 to 0.02 N NaOH) at 0°C removed DD-CPase activity along with muramidase 1 and muramidase 2 activities (3, 8, 20, 29) plus a broad but characteristic assortment of proteins as detected by Coomassie blue staining of SDS gels (8). At least seven of these polypeptides bound [^{14}C]penicillin G (D. L. Dolinger, I. Said, G. D. Shockman, and L. Daneo-Moore, unpublished data), including one at 43 kDa, which has been called PBP 6 and has been shown to have DD-CPase activity (4, 5).

Further experiments showed that DD-CPase activity could

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TABLE 1. Extraction of DD-CPase and muramidase 2 activities from the crude cell wall fraction[a]

Fraction	Protein		DD-CPase activity		Muramidase 2 activity	
	mg/ml	Total (mg)	Total (nmol/min)	Sp act (nmol/min per mg of protein)	Total (U)	Sp act (U/mg of protein)
1. pH 7, 10 mM sodium phosphate wash	10.1	242	367	1.5	312	1.3
2. pH 7.5, 150 mM sodium phosphate wash	2.3	55	230	4.2	168	3.1
3. pH 10, 10 mM glycine-NaOH extract	4.3	118	503	4.3	220	1.9
4. First pH 12, NaOH extract	9.3	316	1,401	4.4	7,888	25.0
5. Second pH 12, NaOH extract	1.1	34	233	6.9	2,263	66.6

[a] The crude cell wall fraction from 5 g of dried bacteria was successively extracted with 25 ml of each solution. However, the volumes of fractions 3 to 5 were larger because of the volume needed to neutralize each of these fractions. Centrifugation was at 28,000 × g for 15 min at 4°C.

be removed from the washed crude wall fraction by extractions at pH 9.5 and higher. For example, two to three extractions with 10 mM glycine-NaOH at pH 10 removed essentially all of the DD-CPase activity, as did extractions with any of several detergents such as 1% Triton X-100, octylglucoside, or 3-[(3-cholaminopropyl)-dimethylammonio]-1-propanesulfonate. In contrast to DD-CPase activity, extractions at pH 10 removed little to no muramidase 2 activity, but the latter activity was extracted when the pH was increased to 12 (Table 1) (8). Thus it proved possible to partially separate these two activities.

Correlation between DD-CPase activity and PBP 6. The crude cell wall fraction was successively extracted with 10 mM sodium phosphate (pH 8), 10 mM glycine-NaOH (pH 10), and twice with 0.02 N NaOH. All of the fractions were examined for DD-CPase activity and the presence of PBP 6. The resulting fluorogram (Fig. 1) showed the presence of one prominent radioactive band at about 43 kDa, the same mobility as PBP 6 in standard protoplast membrane preparations of *E. hirae* (Fig. 1, lane 5). Since PBP 6 is by far the most abundant PBP in *E. hirae* (4, 5), this fluorogram was incubated for a relatively short time, so that the amount of [^{14}C]penicillin bound could be quantified by densitometric analysis of the PBP bands at 43 kDa. Thus, the other, less prominent PBPs that can be detected in these and similar preparations upon longer exposure intervals are not seen in this fluorogram. The regression line (Fig. 2) showed a correlation between the amount of [^{14}C]penicillin bound (pixel area per 100 μg of protein) and specific activity of DD-CPase (picomoles per minute per 100 μg of protein) in each fraction.

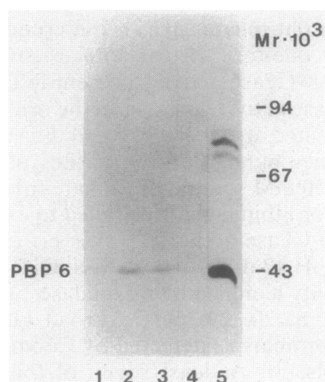

FIG. 1. PBP 6 as detected by fluorography. Samples of 50 μl of extract were used for the binding of [^{14}C]penicillin G. Lanes: 1, pH 8, 10 mM sodium phosphate extract; 2, pH 10, 10 mM glycine-NaOH extract; 3, first pH 12 NaOH extract; 4, second pH 12 NaOH extract; 5, standard protoplast membrane preparation.

Alkaline extraction of DD-CPase activity (PBP 6) from fresh intact bacteria. To examine the stability of DD-CPase activity and PBP 6, a pH 12 extract of fresh, intact *E. hirae* cells was prepared, in the absence of added proteinase inhibitors, from an exponential-phase culture grown in S broth (3) to a bacterial concentration of 0.39 mg/ml. The culture was chilled to 0°C, and the bacteria were pelleted and washed with ice-cold water. The cells (400 mg) were then suspended in 3 ml of ice-cold water and, immediately before centrifugation, brought to pH 12 by the addition of 2.5 N NaOH. Immediately after centrifugation (28,000 × g, 15 min, 4 °C), the supernatant was decanted and neutralized to pH 7 with 0.5 M sodium acetate (pH 5.4). This fresh extract was incubated at 37°C, and at intervals samples were taken for assay of [^{14}C]penicillin-binding activity and DD-CPase activity. After 18 h at 37°C the intensity of PBP 6 at 43 kDa was the same as that of the zero time control, and lower-molecular-weight radioactive degradation products, such as the 30-kDa polypeptide observed by others (5, 9), were not seen (Fig. 3). Also, DD-CPase activity remained unchanged after 18 h at 37°C (data not shown).

Inability of DD-CPase activity to bind to cell wall matrices. Previous observations concerning the purification of muramidase 2 activity (8) indicated that both PBP 6 (and DD-CPase) and muramidase 2 activities were enriched in the same fractions. A major step in the purification of muramidase 2 involves its ability to bind to walls or the peptidoglycan fraction of walls of *E. hirae* (8). The fraction of pH 12 extracts bound to these affinity matrices also contained significant amounts of PBP 6 (8) and DD-CPase activity (unpublished data). However, only 15 to 25% of the PBP 6 and DD-CPase activities present in neutralized pH 10 or 12 extracts appeared to bind to these matrices, raising the possibility that two different enzymes might be present. On

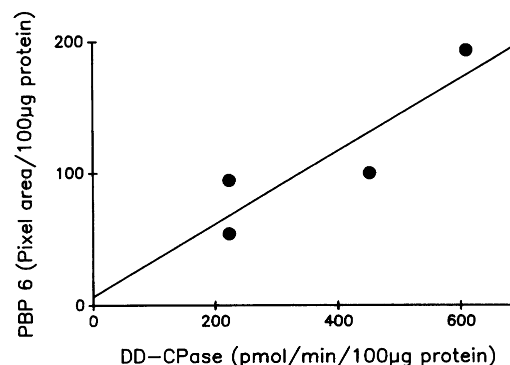

FIG. 2. Regression curve for DD-CPase specific activity versus penicillin-binding activity obtained from Fig. 1.

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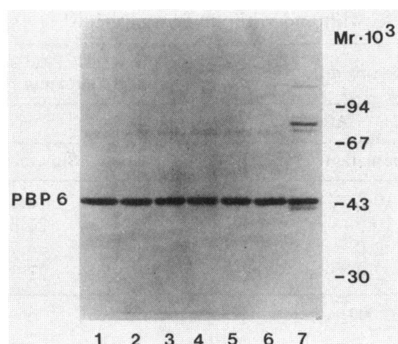

FIG. 3. Stability of PBP 6. Alkaline extract (pH 12) from fresh intact cells without proteinase inhibitors was incubated at 37°C. At various times, 50 μl of the extract was used for the binding of [^{14}C]penicillin G. Lanes: 1, 0 h; 2, 1 h; 3, 2 h; 4, 4 h; 5, 6 h; 6, 18 h; 7, standard protoplast membrane preparation.

the other hand, other observations such as quantitative variations in the distribution of DD-CPase activity between the supernatant and pellet fractions and the effects of detergents on this distribution (see below) suggested that the presence of DD-CPase activity in the cell wall pellet fraction, and apparently bound to peptidoglycan, could be due to its sedimentation during the 28,000 × *g* centrifugation procedure used; the latter seems to be the case (Table 2). The exposure of the pH 12 extract to the peptidoglycan matrix before centrifugation (16,000 × *g*), either in the absence or presence of 0.5% Triton X-100, resulted in the loss of two-thirds to over four-fifths of the muramidase 2 activity but not in a decrease in DD-CPase activity (Table 2, samples 2 and 3). In addition, centrifugation of the pH 12 extract at 100,000 × *g* for 1 h resulted in the loss of about three-fourths of the DD-CPase activity from the supernatant (Table 2, sample 4), but more than one-half of the muramidase 2 activity remained in the supernatant. Furthermore, muramidase 2 activity in the 100,000 × *g* supernatant efficiently bound to either EHPG or N-acetylated EHPG, whereas the DD-CPase activity failed to bind to any of the matrices

TABLE 2. Lack of affinity of DD-CPase activity[a] for several cell wall-derived matrices

Sample	Protein (mg/ml)	DD-CPase activity (nmol/min per ml)	Muramidase 2 activity (U/ml)
1. pH 12 extract of crude walls (Table 1, fraction 4)	9.3	41	232
2. 16,000 × *g* (15 min) supernatant of sample 1, plus EHPG	7.2	41	77
3. 16,000 × *g* (15 min) supernatant of sample 1, plus EHPG in 0.5% Triton X-100	7.7	45	39
4. 100,000 × *g* (1 h) supernatant of sample 1	4.2	11	118
5. 16,000 × *g* (15 min) supernatant of sample 4 after binding to and centrifugation in the presence of:			
EHPG	3.6	12	14
N-acetylated EHPG	3.7	12	7

[a] EHPG or N-acetylated EHPG (0.5 mg) was added to 1 ml of pH 12 extract (Table 1, fraction 4). After 30 min at 0°C with occasional mixing, the samples were centrifuged and supernatants were assayed for DD-CPase and muramidase 2 activities.

tested, which included *E. hirae* cell walls and *M. luteus* walls in addition to the two shown in Table 2, sample 5. In other experiments, DD-CPase activity also failed to bind to any of a variety of wall-derived matrices, even though the amounts of matrices used (2.5 mg/ml) were about fivefold higher than those used in the experiment summarized in Table 2. Similarly, DD-CPase activity present in pH 10 extracts failed to bind to EHPG or the other wall matrices (data not shown).

Is the DD-CPase activity present in the neutralized pH 10 and 12 extracts of the crude wall fraction truly in solution? Data presented in Table 2 that only about one-fourth of the DD-CPase present in pH 12 extracts remained in the supernatant after centrifugation at 100,000 × *g* for 1 h suggested that, in contrast to muramidase 2 activity, DD-CPase activity might be micellar or membrane bound and not truly in solution. To examine this further, 1-ml portions of fractions 1 and 3 of the experiment shown in Table 1 were centrifuged at 100,000 × *g* in the absence and presence of 0.5% Triton X-100. The pellets were suspended in 1 ml of 10 mM sodium phosphate (pH 7), and both fractions were assayed for protein and DD-CPase activity (Table 3). The sum of DD-CPase activities recovered in the two fractions was frequently somewhat higher than the value observed before centrifugation, especially in the absence of Triton X-100, and was thought to be due to removal of inhibitors. Although the amounts of protein sedimented were similar in the presence or absence of Triton X-100, in each case only a small fraction of the DD-CPase activity (3 to 9% of the total activity recovered) was sedimented in the presence of the detergent, whereas about 80% of the total DD-CPase activity recovered was sedimented in the absence of Triton X-100. Longer centrifugations (3 h) at 100,000 × *g* in the absence of Triton X-100 sedimented additional DD-CPase activity (data not shown), and similar results were obtained with fraction 4 of the experiment shown in Table 1 (pH 12 extract; data not shown). We interpret these data as indicating that most of the DD-CPase activity present in the alkaline extracts was particulate or micelle associated. Examination of these fractions for [^{14}C]penicillin-binding activity (data not shown) showed a good correlation between the intensity of the PBP 6 band (43 kDa) and the amount of DD-CPase activity present in that fraction.

Comparison of the kinetic parameters of DD-CPase activity in alkaline extracts of the crude cell wall fraction with activity in membrane fractions. As mentioned above (Table 3), about one-fifth of DD-CPase activity extracted at pH 10 remained in the supernatant after centrifugation (100,000 × *g*, 1 or 3 h) in the absence of Triton X-100. To further compare DD-CPase activity in these fractions, kinetic parameters were examined. K_m (millimolar) and V_{max} (nanomoles per minute per milligram of protein) values of tripeptide hydrolyzed in 10 mM sodium phosphate (pH 7) were calculated from Lineweaver-Burk plots (Table 4). Significant differences were not observed between DD-CPase activity present in the pH 7 wash of the crude cell wall fraction (which primarily contains membrane material), pH 10 or 12 extracts of crude walls, and the supernatants (100,000 × *g*, 1 h) of neutralized pH 10 and 12 extracts. K_m and V_{max} values obtained in 50 mM carbonate buffer (pH 10) for membrane-bound enzyme reported by Coyette et al. (4) were comparable to our results. The concentration of benzylpenicillin that inhibited DD-CPase activity by 50% was also determined. Again, significant differences were not observed. The concentration of benzylpenicillin that inhibited 50% of the binding of [^{14}C]penicillin to PBP 6 was previously reported (5) also to be 1.2 to 1.6 μM.

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TABLE 3. Sedimentation of DD-CPase activity at 100,000 × g (1 h) with and without Triton X-100[a]

Sample	Triton X-100 (0.5%)	Protein (mg/ml)			DD-CPase activity (nmol/min per ml)		
		Before	After		Before	After	
			Supernatant	Pellet		Supernatant	Pellet
Fraction 1, Table 1 (pH 7 wash of crude walls)	Absent	10.1	10.2	1.6	15.3	8.5	28.5
	Present	11.2	10.5	1.5	20.0	21.3	1.8
Fraction 3, Table 1 (pH 10 extract of crude walls)	Absent	4.3	2.0	1.1	18.3	4.7	19.8
	Present	4.2	2.8	0.3	22.3	22.3	0.6

[a] Protein concentrations and DD-CPase activities are given before and after centrifugation at 100,000 × g.

DISCUSSION

Our previous observations that all of the six PBPs that are found in membrane preparations of *E. hirae* are also present in the crude cell wall fraction, along with the two muramidases of this organism (8, 20, 29), led us to further investigate these activities to gain an insight into the possible relationship of their cellular location to their activity or activities. Since PBP 6, which accounts for 30 to 55% of the penicillin-binding activity of standard membrane preparations (4, 5), has been shown to possess DD-CPase activity (4), we examined its cellular localization and properties. Because of the presence of PBP 6 in the crude cell wall fraction, it did not surprise us to find that about one-third of the sum of the recovered DD-CPase activity was present in the crude wall fraction. Although the presence of both DD-CPase activity and PBP 6 activity in the crude wall fraction was consistent with the presence of the same activity as that present in membranes, the presence of more than one DD-CPase in other bacterial species (32) and the idea that perhaps the wall-associated DD-CPase could be relatively easily obtained in a water-soluble form led us to further investigate and attempt to purify it from the crude wall fraction.

Initial results indicated that, indeed, DD-CPase activity could be released to the supernatant from the crude wall fraction by brief exposure to high pH or by extraction with any one of several detergents but that it was not released to the supernatant by exposure to high salt concentrations. Since the presence of a detergent frequently leads to further complexities, such as the formation of mixed micelles (14, 16), which make it difficult if not impossible to distinguish between water-soluble and water-insoluble substances, we chose to try to extract DD-CPase activity at elevated pH values.

TABLE 4. Comparison of kinetic parameters and benzylpenicillin inhibitions of DD-CPase in various fractions

Sample	K_m (mM)	V_{max} (nmol/min per mg of protein)	ID_{50}[a] (μM)
Membrane-bound enzyme	11[b]	33[b]	1.2[c]
1. pH 7 wash of crude walls	22	1.2	1.3
2. pH 10 extract of crude walls	20	8.5	1.4
3. pH 12 extract of crude walls	20	8.7	1.2
4. 100,000 × g (1 h) supernatant of sample 2	20	3.4	0.9
5. 100,000 × g (1 h) supernatant of sample 3	20	6.6	1.1

[a] ID_{50}, Concentration of benzylpenicillin that inhibited DD-CPase activity by 50%.
[b] In 50 mM carbonate buffer (pH 10) (4).
[c] In 50 mM sodium cacodylate buffer (pH 6) (6).

Such extractions proved to be successful (Table 1) and, in fact, suitable for the partial separation of DD-CPase activity from muramidase 2 activity (as well as from muramidase 1 activity; data not shown). However, despite the observation that most of the DD-CPase activity present in the crude wall fraction could be extracted by successive treatments at pH 10, the presence of sometimes substantial amounts of DD-CPase in more than one fraction (e.g., Table 1), the inability of substantial amounts of DD-CPase to bind again to cell walls, or the insoluble peptidoglycan fraction of walls (Table 2) led us to investigate the possibility that DD-CPase (and PBP 6) was, in reality, in membrane fragments or micelles and merely cosedimented with the walls or peptidoglycans. The series of experiments summarized in Table 2 clearly demonstrates that DD-CPase activity (and PBP 6) is not bound directly to the cell wall or peptidoglycan matrix but is instead hydrophobic protein that is present in the crude wall fraction, probably because of its ability to associate with other hydrophobic molecules, including those that appear to have a more specific affinity for the insoluble wall. Thus, for example, DD-CPase activity remained in the supernatant even after centrifugation at 100,000 × g for 1 h in the absence of Triton X-100 (Tables 2 and 3) and failed to cosediment with EHPG, re-N-acetylated EHPG (Table 2), or walls of *E. hirae* or *M. luteus*. In view of these results, it is not surprising that the wall-associated DD-CPase activity had kinetic properties indistinguishable from those of membrane-bound DD-CPase activity and that we observed a good correlation between DD-CPase activity and the amount of PBP 6 present in various fractions.

DD-CPase as a bacterial membrane-bound protein has been studied extensively in bacilli, *Escherichia coli*, and *E. hirae* (12, 37). *E. coli* possess both membrane-bound and water-soluble DD-CPase activities (32). Three DD-CPases were purified from *E. coli* and were called DD-CPases IA, IB, and IC. DD-CPase IA was extracted from the membrane fraction with 2% Triton X-100, purified, and identified as PBPs 5 and 6 (31). DD-CPase IB was obtained from the membrane fraction after 0.5 M LiCl extraction, and periplasmic DD-CPase IC was obtained from the supernatant after ultracentrifugation of disrupted cells. DD-CPases IB and IC were indistinguishable and were identified as PBP 4 (17, 23). Our results are consistent with the presence of only one DD-CPase (PBP 6) in *E. hirae*. DD-CPases of bacilli (34, 35) and *E. hirae* (5, 9) were subjected to proteolysis to yield active, water-soluble fragments. In *E. hirae*, trypsin was used to generate 30-kDa water-soluble fragments from the 43-kDa membrane-bound PBP 6 (5, 9). It is thought that proteolytic fragments from the membrane-bound forms might have an important physiological role in natural environments. Proteolysis does not seem to be a factor in our observations (Fig. 3), although the possibility of an alkaline-

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labile protease cannot be eliminated. Recently, membrane topology of low-molecular-weight PBPs (DD-CPase) has been established at the molecular level. DD-CPase is anchored in the membrane by a small C-terminal peptide segment in *E. coli* (11, 18). *E. hirae* (10), *Bacillus subtilis* (36), and *Bacillus stearothermophilus* (36), and the active-site serine residue is located close to the N-terminal end of the protein of *E. hirae* (10), *E. coli* (1) and *B. subtilis* (33). This type of membrane topology suggests that DD-CPase is membrane bound via short C-terminal segments with its catalytic domain exposed with an extracytoplasmic orientation toward the peptidoglycan.

Although the bulk of the data presented indicate that the DD-CPase (PBP 6) of *E. hirae* is a membrane protein, they also suggest that DD-CPase (PBP 6) strongly associates with the cell wall of this organism. There is convincing evidence in two gram-positive bacteria, *Staphylococcus aureus* (24, 25) and *Gaffkya homari* (2, 19, 26), that the fraction of the membrane that closely associates with the cell wall in the form of wall-membrane complexes relatively efficiently carries out peptidoglycan synthesis in vitro. The fraction of DD-CPase activity that is closely associated with the wall could be involved with cellular processes that lead to modifications of previously assembled wall such as the formation of, or regulation of the formation of, additional peptide cross-bridges or repair reactions (28).

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