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DD-Carboxypeptidase and Peptidoglycan Transpeptidase from *Pseudomonas aeruginosa*

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Peptidoglycan transpeptidase and DD-carboxypeptidase have been detected in isolated membranes of *Pseudomonas aeruginosa*. Cephalosporins and penicillins fail to inhibit the transpeptidase at concentrations as high as 100 μg/ml. DD-Carboxypeptidase, on the other hand, is sensitive to inhibition by β-lactam antibiotics. The presence of dimethyl sulfoxide in the reaction mixture results in a twofold stimulation of peptidoglycan formation, whereas DD-carboxypeptidase is inhibited approximately 30%. Maximum stimulation of transpeptidase occurs in the presence of both dimethyl sulfoxide and a β-lactam antibiotic. This is in sharp contrast to the transpeptidase from *Escherichia coli*, which is sensitive to inhibition by penicillins and cephalosporins.

The final biosynthetic reaction in bacterial cell wall synthesis is the elimination of a D-alanine residue accompanied by the formation of an interpeptide bridge (see reference 13 for review and other references). This terminal reaction is catalyzed by the enzyme peptidoglycan transpeptidase. Based on a structural similarity between the substrate for the transpeptidase and penicillins, Tipper and Strominger (14) proposed that this enzyme was the site of penicillin action. Moreover, they suggested that penicillins would bind irreversibly to the transpeptidase. Subsequently, this enzyme was discovered in a particulate fraction from cell-free *Escherichia coli* extracts and found to be sensitive to penicillins (1, 4, 5). The inhibition of transpeptidation appeared to be irreversible (5).

DD-Carboxypeptidase has also been found in these membrane fractions (6). This enzyme, which is capable of hydrolyzing the terminal D-alanine residue, is also sensitive to penicillin. It has been suggested that such activity may be the result of an uncoupled transpeptidase (7, 9) or a separate entity (2).

In addition to *E. coli*, transpeptidase activity and DD-carboxypeptidase activity has been found in a number of gram-positive microorganisms (2, 8, 9, 13). To date, however, *E. coli* represents the only gram-negative bacteria from which the transpeptidase has been studied. This report concerns the successful isolation of both transpeptidase and DD-carboxypeptidase from another gram-negative organism, *Pseudomonas aeruginosa*. Evidence is presented which shows that the activity of β-lactam antibiotics on the transpeptidase from this organism is substantially different from that which has been reported for the enzymes from either *E. coli* or gram-positive organisms.

MATERIALS AND METHODS

Bacterial strain. *P. aeruginosa*-173 is a clinical isolate which has been maintained on brain-heart infusion agar, subcultured biweekly, and stored at 4 C.

Growth and harvest of cells. A seed culture that contained 400 ml of medium containing 0.8% nutrient broth, 0.8% yeast extract, 0.4% K_2HOP_4, and 0.4% glucose (autoclaved and added separately) was prepared by the addition of 5 ml of an 18-h culture and grown to mid log phase at 37 C. This culture was added to 7.6 liters of fresh, prewarmed medium in a New Brunswick fermentor. When growth reached mid to late log phase, the culture was chilled, and the cells were harvested by continuous flow centrifugation in a Sorvall RC-2 centrifuge.

Preparation of enzymes. The enzymes were prepared by a modification of the procedure of Matsuhashi et al. (11). Cells (25 g, wet weight) were suspended to a final volume of 80 ml in 0.05 M tris(hydroxymethyl)aminomethane, pH 7.5, buffer, containing 10^{-4} M $MgCl_2$ and 10^{-3} M mercaptoethanol (Buffer A), and disrupted in a French pressure cell. The debris was removed by centrifugation. The supernatant solution was centrifuged at 100,000 × *g*, and the resulting pellet was washed twice with Buffer A and finally suspended in 0.7 ml of the same buffer. Membranes prepared in this manner served as the source of the enzymes and could be stored for periods exceeding 6 months at −40 C with no appreciable loss in activity. The protein concentration was 52 mg/ml, as determined by the method of Lowry et al. (10).

Preparation of substrate. The substrate, uridine-diphosphate-*N*-acetyl muramyl-L-alanyl-D-glutamyl-*meso*-α-ε-diaminopimelyl-D-[^{14}C]alanyl-D-[^{14}C]ala-

nine, was prepared by combining the procedures of Strominger and Neuhaus (2, 5, 12). Uridine-diphosphate-*N*-acetylglucosamine was purchased from C. F. Boehringer und Soehne (Mannheim, Germany).

Enzyme assays. Particulate enzyme preparations were assayed for transpeptidase activity in a manner similar to that of Izaki et al. (5). The reaction mixture consisted of 0.2 M tris(hydroxymethyl)aminomethane-HCl, pH 7.5; 0.04 M $MgCl_2$; 0.4 mM uridine-diphosphate-*N*-acetylglucosamine; 0.8 mM adenosine triphosphate; the substrate (11,000 counts/min); 10 μl of enzyme preparation; dimethyl sulfoxide (Me_2SO) and/or H_2O to bring the final volume to 25 μl. After incubation for 3 h at 37 C, the reaction mixture was placed in boiling water for 1 min, followed by centrifugation for 5 min at 14,500 $\times$ *g*. The pellet was washed twice with 10-μl portions of 0.5 M tris(hydroxymethyl)aminomethane, pH 7.5, containing 0.5 M $MgCl_2$, and the washings were added to the supernatant solution. The pellet was resuspended in 10 μl of the same buffer and applied to Whatman no. 3 filter paper. The supernatant solution and the washes were also applied to the paper. After 20 h of descending chromatography in isobutyric acid-water-NH_4OH (66:33:1), the paper was scanned for radioactivity with a Nuclear-Chicago Actigraph III. The chromatogram was then cut up into a 0.5-inch origin segment and 18 additional 1-inch pieces. All chromatogram segments were added to a toluene-based scintillator, and the ^{14}C content was determined in a Nuclear-Chicago Mark I liquid scintillation counter. The peptidoglycan product remains at the origin of the chromatogram and other radioactive substances such as liberated D-alanine migrate down the chromatographic sheet. DD-Carboxypeptidase activity was determined in the same manner as the transpeptidase, except that uridine-diphosphate-*N*-acetylglucosamine was omitted from the reaction mixture. Enzyme assays performed as described above are reproducible to within 10% in a given experiment.

Chemicals and antibiotics. D-[^{14}C]alanine was purchased from Amersham/Searle, Arlington Heights, Ill. and Me_2SO was from Matheson, Coleman, and Bell, East Rutherford, N. J. Cephalexin was a kind gift from Eli Lilly, Indianapolis, Ind., and ampicillin was obtained from Bristol Laboratories, Syracuse, N.Y.

RESULTS

In the course of our studies with the transpeptidase from *E. coli*, it was discovered that transpeptidase activity was stimulated in the presence of Me_2SO (V. A. Ray, unpublished data). In extending these studies to include other gram-negative bacteria, the effect of Me_2SO on the membranes isolated from *P. aeruginosa* was examined. A twofold increase in the synthesis of cross-linked and non-cross-linked peptidoglycan was produced by the addition of Me_2SO to give a reaction concentration of 8% (Table 1). The radioactivity appearing in the insoluble peptidoglycan is low in comparison with that seen at the reference front (R_f) of D-alanine, indicating the presence of carboxypeptidase activity. It was of interest, therefore, to determine whether Me_2SO had any effect on carboxypeptidase activity. The results of such testing are shown in Table 2. It is apparent that there is less D-alanine released as the level of Me_2SO is increased from 4 to 16%, indicating a suppression of DD-carboxypeptidase activity. However, the degree of DD-carboxypeptidase inhibition at 8% Me_2SO does not appear sufficient to explain the two- to threefold increase in peptidoglycan synthesis usually observed at this concentration.

Since our membrane preparations contained larger amounts of carboxypeptidase, several compounds were examined for their ability to inhibit this enzyme. Such inhibition could conserve substrate (non-cross-linked peptidoglycan) for the synthesis of insoluble (cross-linked) peptidoglycan. Moreover, if such a compound had no effect on transpeptidase activity, much larger amounts of insoluble peptidoglycan should be observed. The results of these experiments show that all the antibiotics tested inhib-

TABLE 1. *Effect of Me_2SO on peptidoglycan formation*[a]

Me_2SO concn (%)	Peptidoglycan (counts/min)		D-Alanine released (counts/min)
	Insoluble	Soluble	
0	301	50	3,245
4	476	86	3,713
8	570	103	4,081
12	557	166	3,197
16	693	167	3,525

[a] Enzyme assays were performed as described in Materials and Methods. Substrates: uridine-diphosphate-*N*-acetyl muramyl-pentapeptide (terminal D-alanyl-D-alanine labeled with ^{14}C); and uridine-diphosphate-*N*-acetylglucosamine. Data represents the average of two determinations.

TABLE 2. *Effect of Me_2SO on DD-carboxypeptidase*[a]

Me_2SO concn (%)	Substrate remaining (counts/min)	D-Alanine released (counts/min)
0	624	3,237
4	496	2,814
8	476	2,791
12	509	2,307
16	458	2,286

[a] Enzyme assay performed as described in Materials and Methods. (Uridine-diphosphate-*N*-acetylglucosamine not present in reaction mixture.) Data represents the average of two determinations.

ited carboxypeptidase activity (Table 3). At a concentration of 10 μg/ml, cephalexin appeared to be the best inhibitor of this enzyme; however, at 100 μg/ml ampicillin and carbenicillin were the most effective inhibitors.

When transpeptidase activity was measured under similar conditions, it became evident that all of the antibiotics which inhibited the DD-carboxypeptidase not only failed to inhibit the transpeptidase, but also increased the formation of insoluble peptidoglycan, the product of the transpeptidase reaction (Table 4). This increase in transpeptidase activity was not observed in the absence of Me_2SO. The liberated D-alanine in these experiments is the result of residual DD-carboxypeptidase activity (Table 3) and transpeptidase activity.

DISCUSSION

These experiments have demonstrated the presence of peptidoglycan transpeptidase in membranes of *P. aeruginosa*. The transpeptidase from this organism appears not to be similar to the transpeptidase of *E. coli;* the *E. coli* enzyme is inhibited by penicillins and cephalosporins (13), whereas the *P. aeruginosa* enzyme is not. The membrane isolates from *Pseudomonas* also contain large amounts of DD-carboxypeptidase activity. We believe that initially we were unable to detect transpeptidase activity because the carboxypeptidase rapidly degraded the uridine-diphosphate-*N*-acetyl-muramyl-pentapeptide precursor. But, since the carboxypeptidase is highly susceptible to inhibition by both penicillins and cephalosporins, we were able to detect transpeptidase activity in the presence of these antibiotics.

TABLE 3. *Effect of some β-lactam antibiotics on DD-carboxypeptidase*[a]

Antibiotic	Substrate remaining (counts/min)	D-Alanine released (counts/min)
None	787	3,916
Ampicillin (10)[b]	9,377	870
Ampicillin (100)	10,296	287
Penicillin G (10)	6,755	2,355
Penicillin G (100)	8,324	973
Carbenicillin (10)	9,218	923
Carbenicillin (100)	10,427	300
Cephalexin (10)	9,548	687
Cephalexin (100)	9,499	689

[a] Enzyme assays performed in the presence of 8% Me_2SO as described in Materials and Methods (uridine-diphosphate-*N*-acetylglucosamine not present in reaction mixture). Data represents the average of four determinations.

[b] Concentration in micrograms per milliliter.

TABLE 4. *Effect of some β-lactam antibiotics on transpeptidase*[a]

Antibiotic	Peptidoglycan (counts/min)		D-Alanine released (counts/min)
	Insoluble	Soluble	
None[b]	590	121	2,178
None (no Me_2SO)	187	46	4,069
Ampicillin (10)[c]	912	272	1,751
Ampicillin (100)[c]	1,002	453	1,374
Penicillin G (10)[c]	945	226	2,404
Penicillin G (100)[c]	1,058	461	1,817
Carbenicillin (10)[c]	860	261	1,884
Carbenicillin (100)[c]	998	507	1,170
Cephalexin (10)[c]	1,218	257	1,676
Cephalexin (no Me_2SO) (10)[c]	252	62	1,426
Cephalexin and ampicillin (10 and 10)	842	315	1,564
Cephalexin and pencillin G (10 and 10)	892	299	1,550
Cephalexin and carbenicillin (10 and 10)	837	311	1,571

[a] Enzyme assays performed as described in Materials and Methods in the presence of 8% Me_2SO except where indicated (uridine-diphosphate-*N*-acetylglucosamine and uridine-diphosphate-*N*-muramyl-pentapeptide [terminal D-alanyl-D-alanine labeled with ^{14}C] present in reaction mixture).

[b] Concentration in micrograms per milliliter; represents the average of two determinations.

[c] Concentration in micrograms per milliliter; represents the average of six determinations.

When the carboxypeptidase was inhibited by these antibiotics in the presence of Me_2SO, we observed an increase in the formation of insoluble peptidoglycan. Clearly, *Pseudomonas* transpeptidase activity is not inhibited by β-lactam antibiotics. A possible explanation for the lack of transpeptidase inhibition could be provided by assuming that all of the antibiotic is bound by the carboxypeptidase. However, maximum binding of cephalexin to carboxypeptidase was observed at 10 μg of cephalexin per ml (Table 3). When transpeptidase activity was assayed in the presence of both cephalexin (10 μg/ml and another antibiotic (either 10 or 100 μg/ml), the maximum inhibition observed was only 20% (Table 4; unpublished data).

The addition of Me_2SO to the reaction mixture provided a method by which it was possible to separate the transpeptidase and carboxypeptidase reactions while slightly suppressing DD-carboxypeptidase activity. Although the exact function of Me_2SO remains unknown, it may be postulated that it makes the particular enzyme more available to the substrate.

Transpeptidase has been described as the site of cephalosporin and penicillin intrinsic activity

by Strominger and others (13). Our studies indicate that this is not the case for *P. aeruginosa;* none of the antibiotics herein tested had a significant effect on the transpeptidase from *Pseudomonas*. Even carbenicillin, a penicillin known to have cidal effects on *Pseudomonas*, was essentially inactive as an inhibitor of transpeptidase. Thus, the precise mechanism of action of penicillins against *Pseudomonas* remains unknown.

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