A Soluble Enzyme Activity That Attaches Free Diaminopimelic Acid to Bdelloplast Peptidoglycan†

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ABSTRACT: An enzyme activity, responsible for the attachment of diaminopimelic acid (DAP) to bdelloplast wall peptidoglycan, was studied in an in vitro, cell-free system. Most of the activity was found in the high-speed (20000*g*) supernatant fraction of homogenates of bdelloplasts prepared from a culture of the intracellular bacterium *Bdellovibrio bacteriovorus* 109J, growing synchronously within cells of *Escherichia coli*. Peptidoglycan preparations obtained either from *E. coli* ML35 or from the walls of bdelloplasts synchronously cultured for 40 or 90 min served as the acceptors in this reaction, whereas cell wall or peptidoglycan preparations obtained from Gram-positive bacteria could not function as acceptors of DAP. The attachment activity had an apparent K_m value for DAP of 10 μM; for bdelloplast peptidoglycan, it was approximately 0.43 mg/mL, which is 13 μM with respect to peptidoglycan disaccharide peptide units. DAP attachment was partially inhibited by the structural analogues lanthionine, L-ornithine, β-aminobutyric acid, and D-serine, as well as the cell wall synthesis inhibitors penicillin G, ampicillin, and cephalexin. This enzyme activity is present only during the intracellular stage of the bdellovibrio's developmental growth cycle and may serve a stage-specific function of biochemically modifying the cell in which it grows.

The bacterial predator *Bdellovibrio bacteriovorus* 109J grows in a secure, intracellular, environment—the space between the cytoplasmic membrane and peptidoglycan of its moribund bacterial prey (Rittenberg & Thomashow, 1979; Thomashow & Rittenberg, 1980). As an adaptation to this unusual growth environment, bdellovibrios have evolved a unique dimorphic life cycle that alternates between an extracellular, nongrowing, attack-phase cell and an intracellular, growth-phase cell that can initiate growth and replication only after entering the periplasm of a Gram-negative bacterium.

Upon entry by the bdellovibrio, the attacked cell is transformed into a morphologically distinct, spherical structure termed the bdelloplast. A series of specific biochemical modifications of the prey cell peptidoglycan and outer membrane accompany this transformation. In vivo studies of the process have revealed (1) a controlled, partial degradation of the prey cell's outer envelope, which leads to the formation of a resealing pore through which the bdellovibrio enters the periplasmic space (Thomashow & Rittenberg, 1978a,b), (2) a deacetylation of the prey peptidoglycan, which precludes subsequent entry by other bdellovibrios, and (3) an attachment of fatty acids to the peptidoglycan of the bdelloplast wall in a unique reaction of as yet unknown function (Thomashow & Rittenberg, 1978c).

More recently, another type of biosynthetic modification has been described. Free diaminopimelic acid (DAP)[1] molecules, released by partial degradation of the prey cell wall during bdellovibrio attack (Thomashow & Rittenberg, 1978a), are rapidly incorporated specifically into the peptidoglycan portion of the bdelloplast wall (Ruby & Rittenberg, 1984). The incorporated DAP remains attached until it is solubilized during the lysis of the bdelloplast, just prior to the release of progeny bdellovibrios. The process of DAP attachment is not dependent upon an activity of the metabolically inactive prey bacterium and exhibits unusual characteristics, i.e., partial reversibility, and apparent independence from generation of chemical energy by the intracellular bdellovibrios.

To characterize this activity in vitro, we have described some properties of a cell-free enzyme activity that attaches DAP to bdelloplast wall peptidoglycan and compared this in vitro activity to the previously reported in vivo DAP incorporation in the intact bdelloplast (Ruby & Rittenberg, 1984). Clarification of the biochemistry of this unusual peptidoglycan-modifying activity not only is of intrinsic interest but also will help lead to a determination of the functional significance of this process in the adaptation of bdellovibrios to an intracellular growth environment.

Materials and Methods

Organisms and Crude Enzyme Preparation. Bdellovibrio bacteriovorus 109J was maintained on *Escherichia coli* ML35 as described previously (Ruby & Rittenberg, 1984). Pregrown cells of *E. coli* ML35 were used as the substrate cells for the formation of bdelloplasts. Equal volumes (200 mL each) of cell suspensions of bdellovibrios (1×10^{10} cells/mL) and *E. coli* ML35 (5×10^9 cells/mL) in HM buffer [1 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid buffer, pH 7.6, containing 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$] were mixed and incubated with vigorous shaking at 30 °C. At the desired times, 80-mL portions were removed and cooled on ice. Bdelloplasts were collected by centrifugation at 1000*g* for 15 min, washed once, and suspended in 5 mL of HM buffer. Each cell suspension was sonically disrupted at 0 °C with a Branson W200P sonicator for a total of 2 min (eight repetitions of 15-s pulses). The cell homogenate was centrifuged at 9000*g* for 10 min to remove unbroken cells and particulate material. This first, low-speed, supernatant was further centrifuged at 20000*g* for 20 min, yielding a high-speed supernatant fraction that was used as the crude enzyme preparation.

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[1] Abbreviations: DAP, diaminopimelic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Peptidoglycan Preparation. Bdelloplasts were harvested from 400 mL of cultures developing synchronously for either 40 or 90 min under the conditions described for the preparation of crude enzyme. Five milliliters of 20% SDS-50 mM EDTA (pH 7.8) was added to bdelloplasts suspended in 20 mL of deionized water. The mixture was heated in a boiling water bath for 10 min, kept at room temperature overnight, and homogenized by sonication. Peptidoglycan was collected by differential centrifugation as described previously (Thomashow & Rittenberg, 1978b). The yields of bdelloplast peptidoglycan were 31.6 and 29.0 mg dry weight for 400 mL of a 40-min and 90-min synchronous culture, respectively, and contained 440 and 420 nmol of total amino sugar when analyzed by the method of Tsuji et al. (1969) after acid hydrolysis (4 M HCl, 100 °C, 2 h). On the basis of analytical data without acid hydrolysis, 10.2% and 12.4% of the amino sugar residues in the corresponding preparations were unsubstituted. *E. coli* ML35 peptidoglycan was obtained by SDS–EDTA treatment as described above. The cell wall and peptidoglycan of *Bacillus cereus* AHU1356 and *Micrococcus luteus* ATCC 4698 were obtained as described previously (Hayashi et al., 1973).

Assay of DAP Attachment Activity. The standard reaction mixture contained 12 μM [G-^{3}H]DAP (0.2 μCi), 40 mM Tris-HCl buffer, pH 8.0, 50 μg of bdelloplast peptidoglycan, and crude enzyme preparation (0.05–0.4 mg of protein) in a final volume of 0.2 mL. The mixture was incubated at 30 °C for 1 h, during which time the rate of DAP attachment was linear. The reaction was terminated by the addition of 0.2 mL of 10% (w/v) TCA. After being held at 0 °C for 2 h, the mixture was centrifuged at 14000*g* for 5 min in a Fisher microcentrifuge. The pelleted material was washed 3 times with 1 mL of 5% TCA and once with 95% ethanol. The radioactivity of the resulting insoluble material was determined in Bray's solution (Research Products International) by liquid scintillation counting. One unit of enzyme activity was defined as that incorporating 1 nmol of diaminopimelic acid residue into TCA-insoluble material per hour.

Preparation of DAP-Labeled Bdelloplast Peptidoglycan. Radioactive DAP-labeled bdelloplast peptidoglycan was prepared from bdelloplasts harvested after 50 min of labeling with [^{3}H]DAP (Ruby & Rittenberg, 1984). Two milliliters of HM buffer containing a synchronously growing suspension of bdellovibrios (2.4×10^{10} cells) in *E. coli* ML35 (1.5×10^{10} cells), to which was added 4 μCi of [^{3}H]DAP (6 nmol), was incubated at 30 °C for 50 min with vigorous shaking. At the same time, another synchronous culture containing bdellovibrios (2.8×10^{11} cells), *E. coli* ML35 (9.2×10^{10} cells), and unlabeled DAP (40 μM) in 12 mL of HM buffer was incubated. Labeled and unlabeled cultures were combined, and the bdelloplasts were harvested by centrifugation and washed once with deionized water. After resuspension of the bdelloplast pellet in 3 mL of deionized water, 1 mL of 20% SDS–50 mM EDTA was added, and the mixture was boiled for 10 min, left overnight at room temperature, and homogenized by sonication. The peptidoglycan fraction obtained by differential centrifugation yielded about 1 mg of material containing 1.2×10^5 cpm of radioactivity.

Solubilization of the DAP Attachment Reaction Product. Aliquots of the TCA-insoluble product (3000 cpm each) were treated in either deionized water (100 °C, 15 min), 5% SDS–50 mM EDTA (100 °C, 20 min), 0.02 M HCl (100 °C, 15 min), or 0.5 M NaOH (37 °C, 30 min) in a final volume of 0.2 mL. An N-acetylated product was also digested with 0.5 mg of lysozyme per 0.2 mL of 20 mM Tris-HCl buffer (pH 7.5) at 37 °C for 2 days. In each case after treatment, carrier bdelloplast peptidoglycan (0.1 mg dry weight) and an equal volume of 10% TCA were added to each mixture. The resulting precipitate and supernatant fractions were separated by centrifugation, and the radioactivity present in each was determined.

Table I: Localization of DAP Attachment Activity

	enzyme activity			
	no peptidoglycan added		250 μg/mL peptidoglycan added	
fraction	total (units)	specific (units/mg)	total (units)	specific (units/mg)
cell homogenate	25.6	0.048	105	0.20
high-speed supernatant	8.5	0.018	70.7	0.15
high-speed pellet	20.1	0.43	25.3	0.54

Materials and Analytical Methods. Protein was estimated by a modified Lowry technique (Peterson, 1979) with bovine serum albumin as the standard. N-Acetylation was carried out with acetic anhydride (final concentration 20%) in an 8% aqueous solution of triethylamine. The pentamer of *N*-acetylglucosamine was isolated from the acid hydrolysate of chitin. Monomer and dimer fractions of peptidoglycan fragments were obtained from the lysozyme digests of N-acetylated peptidoglycan from *B. cereus* AHU 1356 (Hayashi et al., 1973). Paper chromatography was carried out by the ascending method on 3MM filter paper (Whatman) in a solvent composed of butanol/pyridine/acetic acid/water (60:40:6:30). Gel filtration was performed on a 1.5 × 53 cm column of Sephadex G-50 in 50 mM ammonium carbonate, eluting with the same solvent. The elution positions of standard samples of the dimer and monomer of peptidoglycan fragments, the pentamer of *N*-acetylglucosamine, and glucose were monitored by the reducing group determination (Park & Johnson, 1949).

[G-^{3}H]DAP was obtained from Amersham Corp. DAP, ornithine, other D- and L-amino acids, penicillin G, ampicillin, cephalexin, and lysozyme were obtained from Sigma Chemical Co. Sephadex G-50 was purchased from Pharmacia Fine Chemicals. Other chemicals and solvents used were reagent grade.

Results

Localization of DAP Attachment Activity. Bdelloplasts were harvested at 50 min after mixing *E. coli* ML35 and *B. bacteriovorus* 109J, and the pelleted material was sonically disrupted. The resulting homogenate from 400 mL of bdelloplast suspension contained a considerable amount of DAP-attaching activity, even without the addition of peptidoglycan, although supplementation with isolated bdelloplast peptidoglycan (0.25 mg/mL) led to an approximately 4-fold stimulation of the DAP attachment rate (Table I). Among the cell fractions from the homogenate, the high-speed supernatant fraction contained the majority (about 75%) of the stimulatable enzyme activity; however, its specific activity was smaller than that of the high-speed pellet (Table I). Furthermore, maximal activity of the supernatant was dependent upon addition of bdelloplast peptidoglycan. On the other hand, the DAP attachment activity in the high-speed pellet showed less stimulation by exogenous peptidoglycan, probably because this fraction already contained bdelloplast wall fragments. As judged from the higher specific activity of the high-speed pellet, it may be possible that the DAP-attaching enzyme in intact bdelloplasts is bound to the membranes or walls of bdelloplasts and that during the cell disruption procedure the majority of the enzyme was released into the soluble fraction where its

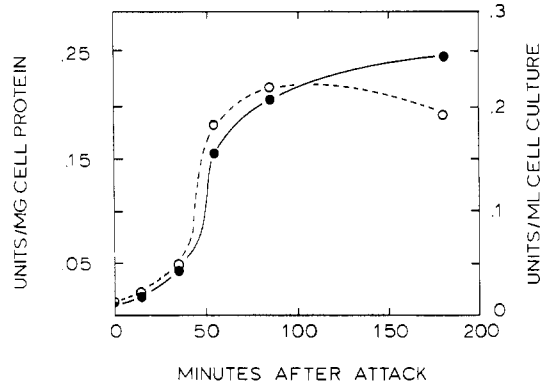

FIGURE 1: Appearance of DAP attachment activity during synchronous *Bdellovibrio* growth. Suspensions of bdellovibrios and prey cells were combined as described under Materials and Methods to produce a synchronized attack and formation of bdelloplasts. Before combining, and at times during the synchronous growth cycle, portions of the cell suspensions were removed, and the cellular material was collected by centrifugation. The DAP attachment activity present in the high-speed supernatant fraction of this material was assayed as described and plotted either as units per milliliter of cell culture (○) or as units per milligram of cell protein in the fraction (●). Data at zero time were for bdellovibrios and prey cells, both of which had levels of activity that were not significantly different from background values.

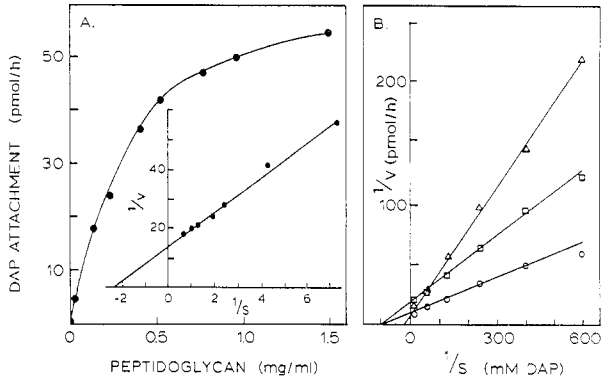

FIGURE 2: Dependence of DAP attachment rate on the concentrations of bdelloplast peptidoglycan and DAP. (A) Plot of reaction rate as a function of the concentration of bdelloplast peptidoglycan. The assay was carried out with the high-speed supernatant fraction (0.18 mg of protein/0.2 mL) under standard conditions. The inset is the double-reciprocal plot of these data. (B) Double-reciprocal plot of the DAP attachment rate as a function of DAP concentration in the absence of inhibitors (○) or in the presence of 0.5 mM lanthionine (△) or 4 mM D-alanine (□). The assay was carried out under standard conditions, using the high-speed supernatant fraction (0.28 mg of protein/0.2 mL) and bdelloplast peptidoglycan (0.35 mg/mL).

specific activity was reduced by the presence of cytoplasmic proteins.

Appearance of DAP Attachment Activity during Synchronous Growth. The high-speed supernatant fractions prepared from bdelloplasts harvested at various times during a synchronous growth cycle were assayed for DAP-attaching activity. As shown in Figure 1, the preparations from either prey cells or attack–phase bdellovibrios showed no appreciable DAP-attaching activity. Only small activities were found in the preparations obtained from cells early in the processes of attack (15 min) and bdelloplast formation (35 min). However, enzyme activity increased rapidly between 35 and 55 min into the cycle, coincident with the kinetics previously reported for DAP incorporation by intact bdelloplasts (Ruby & Rittenberg, 1984). This initial burst of total activity per milliliter of culture was followed by a leveling off that continued until lysis of the bdelloplasts. A relatively small increase in the specific enzyme activity after 85 min (Figure 1) was a consequence of a net decrease in total bdelloplast protein during intracellular bdellovibrio growth. Thus, enzyme activity appeared during the early stages of bdelloplast formation, when the attack-phase bdellovibrios were beginning their developmental transformation into growth-phase cells.

Properties of the DAP Attachment Activity. When the high-speed supernatant fraction obtained from 50-min bdelloplasts was used as a source of enzyme activity, the process of DAP attachment was linear as a function of either incubation time or amount of supernatant fraction added until about 35 pmol of DAP had been incorporated into TCA-insoluble products. In a buffer of 50 mM Tris-HCl, activity was maximal at a pH of about 8.0, and there was a 50% inhibition of activity in the presence of either 3 mM Mg^{2+} or 2.5 mM Ca^{2+}.

Preparations of either unmodified peptidoglycan obtained from *E. coli* or mixtures of modified and unmodified peptidoglycan present in both 40- and 90-min bdelloplasts were active in the DAP attachment reaction under standard conditions. On the other hand, peptidoglycan and cell wall preparations from the Gram-positive bacteria, *B. cereus* and *M. luteus*, did not serve as DAP acceptors. This finding suggested that there was a degree of specificity involving the peptidoglycan as the second substrate or acceptor of DAP. It is not known what, if any, other peptidoglycan-modifying activities are present in the crude enzyme preparation; however, peptidoglycan treated with this preparation was still solubilized by lysozyme, which suggested the absence of an N-deacetylation activity (data not shown).

When the rate of DAP incorporation was plotted against substrate (bdelloplast peptidoglycan) concentration, a typical hyperbolic curve was obtained (Figure 2A). The apparent K_m value for bdelloplast peptidoglycan determined from a linear regression of a Lineweaver–Burk plot of these data was 0.43 mg/mL (or 13 μM with respect to peptidoglycan disaccharide–tetrapeptide units). The apparent K_m for DAP was 10 μM when the concentration of bdelloplast peptidoglycan was 0.25 mg/mL (Figure 2B).

Influence of Cell Wall Synthesis Inhibitors and Amino Acids on the DAP Attachment Reaction. In a synchronous-cycle suspension of bdelloplasts, DAP incorporation was previously shown to be independent of continued protein synthesis and metabolic energy generation (Ruby & Rittenberg, 1984). While the in vitro DAP attachment reaction described herein proceeded without any apparent exogenous energy source, the crude enzyme preparation could have contained ATP or other energy currency. Partially purifying the enzyme activity by ammonium sulfate precipitation (45–60% saturation) and dialysis did not decrease the total activity, suggesting that small molecules like ATP are not required in the reaction.

Addition of penicillin G or other cell wall synthesis inhibitors has been previously reported not to inhibit DAP incorporation in bdelloplasts (Ruby & Rittenberg, 1984). However, because of the possibility that DAP attachment in vitro might occur through a transpeptidation mechanism, we determined the effect of penicillin on in vitro activity, without the possible in vivo influences of a membrane barrier or, perhaps, a naturally occurring penicillinase. As shown in Table II, addition of 100 μg of penicillin G, ampicillin, or cephalexin per milliliter of standard assay mixture inhibited the DAP attachment reaction to similar extents (31–49%). The inhibitory concentrations of these compounds were 20–100 times higher than those required for inhibition of other known transpeptidation reaction systems (Strominger et al., 1971; Wickus & Strominger, 1972b.)

Table II: Effect of Cell Wall Synthesis Inhibitors on the DAP Attachment Reaction

addition	concn (μg/mL)	DAP (pmol) attached	activity (%)
none		32.8	100
penicillin G	10	26.6	81
	100	16.7	51
ampicillin	10	23.9	73
	100	18.0	55
cephalexin	10	30.1	92
	100	22.6	69

Table III: Effect of Amino Acids and Analogues on the DAP Attachment Reaction

addition	DAP attached (pmol)	activity (%)
none	32.4	100
D-glutamic acid	33.0	102
L-glutamic acid	32.5	100
L-lysine	31.5	97
glycine	21.4	66
L-alanine	21.5	66
D-alanine	18.5	57
D-serine	12.0	37
β-aminobutyric acid	9.7	30
L-ornithine	7.8	24
DL-lanthionine	4.8	15

It has been previously reported that the DD-DAP attachment enzyme system of *Bacillus megaterium* is specifically inhibited by D-series amino acids (Wickus & Strominger, 1972a). As shown in Table III, the DAP attachment enzyme activity described herein was strongly inhibited by a 330-fold excess (4 mM) of lanthionine, L-ornithine, β-aminobutyric acid, and D-serine and moderately by D-alanine, L-alanine, and glycine. Lanthionine, a DAP analogue that has a sulfur atom replacing the central C-4 methylene of DAP, and that has been found as a natural component of the cell wall of *Fusobacterium nucleatum* (Kato et al., 1979), also competes effectively with DAP for incorporation into peptidoglycan in bdelloplast suspensions (unpublished data). As shown in Figure 2B, lanthionine and D-alanine were competitive and noncompetitive inhibitors of the DAP attachment reaction, respectively.

Characterization of the Reaction Product. Strong acid hydrolysis (4 M HCl, 100 °C, 4 h) of the TCA-insoluble enzyme reaction product, followed by paper chromatography, gave a single radioactive spot with a migration coefficient the same as that of authentic DAP, and distinguishable from other amino acids or analogues (Figure 3). The same results were obtained with the acid hydrolysate of in vivo DAP-labeled bdelloplast peptidoglycan. Thus, in both cases, labeled DAP residues are incorporated in an unmodified form by an amide-like bond.

The chemical properties of the linkage of the DAP molecule with the TCA-insoluble peptidoglycan product of the reaction were examined as follows. Treatment of radioactive DAP-labeled material with either hot SDS–EDTA, weak acid, or weak alkali solubilized only between 3% and 9% of the label, suggesting a strong chemical bonding. Nevertheless, about 95% of the radioactivity was recovered as soluble material if the DAP-labeled product was digested with lysozyme after N-acetylation with acetic anhydride. When this solubilized material was subjected to gel chromatography on a Sephadex G-50 column, the majority of the radioactivity was recovered in the fraction that eluted together with monomeric fragments of peptidoglycan [*N*-acetylglucosaminyl(β1→4)-*N*-acetylmuramic acid (tripeptide)] (Figure 4). A small amount of radioactivity was also found as the dimer of the peptidoglycan fragment. Taken together, these results strongly suggested that labeled DAP residues were incorporated into the peptidoglycan moiety, probably in its peptide portion. Interestingly, the lysozyme digestion of bdelloplasts whose peptidoglycan had been DAP labeled (Ruby & Rittenberg, 1984) produced approximately equal amounts of monomeric and dimeric peptidoglycan fragments.

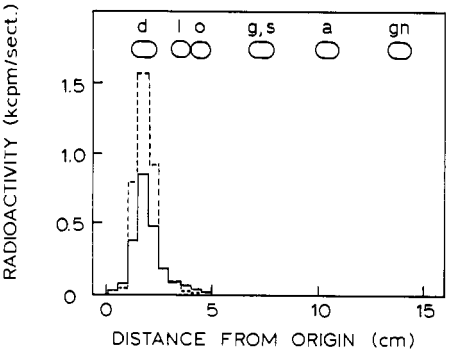

FIGURE 3: Paper chromatography of acid hydrolysates of the in vitro DAP attachment reaction product and in vivo DAP-labeled bdelloplast peptidoglycan. The TCA-insoluble labeled reaction product (4200 cpm) and labeled bdelloplast peptidoglycan (2500 cpm) were hydrolyzed in 0.4 mL of 4 M HCl at 100 °C for 4 h in sealed tubes. The hydrolysates were subjected to paper chromatography, and the paper was cut into 5-mm sections, which were measured for radioactivity by liquid scintillation counting. Radioactivity profiles of the hydrolysate of the in vitro reaction product (solid line) and DAP-labeled bdelloplast peptidoglycan (dashed line) are indicated. The migration positions of DAP (d), lysine (l), ornithine (o), glycine (g), serine (s), alanine (a), and glucosamine (gn) are outlined by the designated ovals.

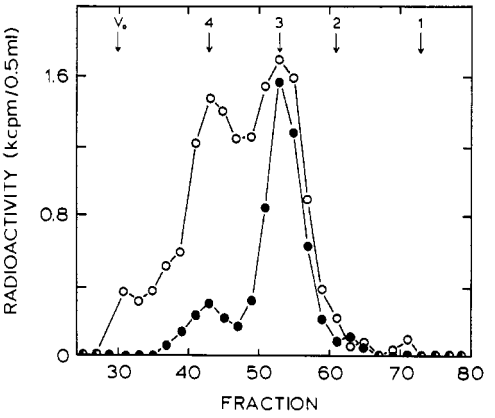

FIGURE 4: Sephadex G-50 chromatography of the in vitro DAP attachment reaction product and in vivo DAP-labeled bdelloplast peptidoglycan after N-acetylation and lysozyme digestion. The TCA-insoluble labeled reaction product (13 000 cpm) and labeled bdelloplast peptidoglycan (33 000 cpm) were N-acetylated with acetic anhydride in 8% triethylamine. After recovery by centrifugation, the products were digested with 0.5 mg of lysozyme/mL 20 mM Tris-HCl buffer, pH 7.4, at 37 °C for 48 h. The chromatograms of the soluble fractions of the treated, in vitro reaction product (●) and the treated, in vivo bdelloplast peptidoglycan (○) were produced as described under Materials and Methods. Arrows indicate the elution positions of glucose (1), a pentamer of *N*-acetylglucosamine (2), and the monomer (3) and dimer (4) fragments of authentic peptidoglycan. V_0 indicates the void volume of the column.

The labeled DAP residues in the in vitro reaction product seemed to be covalently bound to peptidoglycan through a linkage stable against weak acid and weak alkali treatment. These results suggested an amide linkage and are consistent with the conclusion that DAP residues are incorporated into the hot SDS–EDTA-insoluble fraction presumed to be bdelloplast peptidoglycan (Ruby & Rittenberg, 1984). However, because peptidoglycan preparations obtained from Gram-negative bacteria by hot SDS–EDTA treatment contain considerable amounts of non-peptidoglycan components (Schleifer

& Kandler, 1972), further characterization will be required to confirm the above conclusion.

Discussion

Biochemical and structural modifications of the outer envelope of Gram-negative bacteria occur during the early phases of entry and intracellular growth of *B. bacteriovorus* (Thomashow & Rittenberg, 1980; Ruby & Rittenberg, 1984). *Bdellovibrio*-specific degradative enzymes, including a protease, a glycanase, and a peptidase, are believed to be responsible for producing a discrete pore in the prey cell outer membrane and peptidoglycan through which the bdellovibrio enters (Thomashow & Rittenberg, 1978a). Simultaneously, N-deacetylation of the amino sugar residues functions to halt and contain these hydrolytic activities (Thomashow & Rittenberg, 1978b). In addition, attachment to the bdelloplast peptidoglycan of fatty acid and DAP molecules, released during envelope degradation (Thomashow & Rittenberg, 1978a,c), has been demonstrated during the formation of the secure, modified structure in which the bdellovibrio grows (Ruby & Rittenberg, 1984). For none of these degradative or biosynthetic activities has the enzyme system been characterized in any detail.

In the present study, a cell-free, in vitro assay was developed to clarify the biochemistry of DAP attachment to bdelloplast peptidoglycan. When a crude, soluble extract of bdelloplasts was combined with bdellovibrio-modified (i.e., deacetylated) bdelloplast peptidoglycan, or unmodified *E. coli* peptidoglycan, radioactively labeled DAP was readily incorporated into a TCA-insoluble product that behaved chemically like peptidoglycan. The TCA-insoluble reaction product could not be solubilized by treatment with hot SDS–EDTA, weak acid, or weak alkali. However, after N-acetylation, the product was sensitive to lysozyme digestion, resulting in the formation of labeled disaccharide peptide units (Figure 4). The only radioactivity released by acid hydrolysis of this labeled material was present as a single spot that comigrated with DAP on paper chromatography (Figure 3). We conclude, therefore, that free DAP is incorporated into peptidoglycan in an unmodified form.

A biochemically similar reaction has been previously reported for a cell-free enzyme activity obtained from cells of *Bacillus megaterium* (Wickus & Strominger, 1972a). This activity also was reported to incorporate free DAP intact into monomeric and dimeric disaccharide units of peptidoglycan in a reaction that is partially reversible by the addition of a high concentration of excess free DAP. In this system, however, the activity is not soluble but instead is associated with the membrane fraction, and the incorporation of free DAP is dependent upon the simultaneous synthesis of peptidoglycan from uridine nucleotide precursors. Furthermore, our studies using amino acid analogues of DAP suggested that the DAP-attaching enzyme reactions of the bdelloplast system and the *B. megaterium* system differ in their substrate specificity. In the latter system, D-amino acids specifically inhibit the DAP attachment, while in the bdelloplast system, some L-amino acids were equally potent inhibitors (Table III). The two sets of results may be reconciled by assuming a difference in the linkage mode of each product. Structural characteristics of both substrates, the DAP and the bdelloplast peptidoglycan, may be related to these dissimilar results.

Native peptidoglycans of *E. coli* and *B. cereus* have similar structural characteristics and belong to the same peptidoglycan class: the directly cross-linked, meso-DAP type (A1γ), according to the classification proposed by Schleifer and Kandler (1972). However, the peptidoglycan and cell wall fractions prepared from *B. cereus* were unable to serve as the acceptor molecules in the *Bdellovibrio* DAP-attaching reaction. Although the reason for this specificity is not clear, it may be related to differences in the concentration of potential DAP-accepting peptide units (i.e., un-cross-linked penta- and tetrapeptide residues) in the two peptidoglycan preparations. The molar ratio of alanine to glutamate was between 2.0 and 2.2 for the *E. coli* peptidoglycan and between 1.5 and 1.9 for that of *B. cereus* (data not shown). While this difference is numerically small, it indicates that, compared to the *B. cereus* peptidoglycan, the *E. coli* peptidoglycan used in this study contained a relatively higher concentration of potential DAP-accepting peptide units. To fully explain these data, it will be necessary to determine (1) the molecular position of DAP attachment, (2) the distribution of tri-, tetra-, and pentapeptide units in the peptidoglycan preparations used, and (3) the effect of removal of the terminal D-alanine residues from active acceptor peptidoglycan by treatment with D,D-carboxypeptidase.

Both the membrane-bound *Bacillus* and the soluble *Bdellovibrio* enzyme systems are independent of any added energy donor, and so are distinct from the ATP-dependent (Katz et al., 1967) or tRNA-dependent (Roberts et al., 1968) systems previously described. Because of its sensitivity to penicillins (Table II), albeit only at rather high concentrations (Strominger et al., 1971; Wickus & Strominger, 1972b), the DAP-attaching reaction in the bdelloplast system may utilize a transpeptidation reaction that is energetically similar to that in typical peptidoglycan synthesis, that is, the isoenergetic formation of cross-linked peptide units (Rogers, 1980). At the present time, we have no evidence that DAP attachment is accompanied by the liberation of terminal amino acid residues from an acceptor peptide unit.

While there are a number of biochemical similarities and differences between the cell-free *Bdellovibrio* and *Bacillus* DAP-attaching enzyme preparations, it is the distinctions at the cellular level that provide both our best information about and a particular source of interest in the possible biological function(s) of the *Bdellovibrio* activity. The *Bdellovibrio* DAP-attaching activity appears during the early stages of bdelloplast formation, yet no significant activity is detectable in cell extracts of either prey cells or free-swimming attack-phase bdellovibrios (Figure 1). Thus, the in vitro evidence suggests that the source of the DAP incorporating activity is the attached attack-phase cell as it begins its differentiation into an intracellular growth-phase cell. Furthermore, it can be concluded that, because the bdelloplast peptidoglycan substrate is external to the bdellovibrio cell, this activity must be produced as an excreted, extracellular enzyme. These results are all in agreement with the characteristics reported for in vivo DAP incorporation by developing bdelloplasts (Ruby & Rittenberg, 1984) and suggest that the activity is specifically expressed during bdelloplast formation and may function in this necessary stage in the development of the bdellovibrio life cycle.

Registry No. DAP, 583-93-7.

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Yeast Redoxyendonuclease, a DNA Repair Enzyme Similar to *Escherichia coli* Endonuclease III†

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ABSTRACT: A DNA repair endonuclease (redoxyendonuclease) was isolated from bakers' yeast (*Saccharomyces cerevisiae*). The enzyme has been purified by a series of column chromatography steps and cleaves OsO_4-damaged, double-stranded DNA at sites of thymine glycol and heavily UV-irradiated DNA at sites of cytosine, thymine, and guanine photoproducts. The base specificity and mechanism of phosphodiester bond cleavage for the yeast redoxyendonuclease appear to be identical with those of *Escherichia coli* endonuclease III when thymine glycol containing, end-labeled DNA fragments of defined sequence are employed as substrates. Yeast redoxyendonuclease has an apparent molecular size of 38 000–42 000 daltons and is active in the absence of divalent metal cations. The identification of such an enzyme in yeast may be of value in the elucidation of the biochemical basis for radiation sensitivity in certain yeast mutants.

The genetics of the DNA repair pathways of *Saccharomyces cerevisiae* have been studied extensively. A number of loci have been identified that, when mutated, result in an increased sensitivity to chemical and physical DNA damaging agents. Of the nearly 100 different mutants so far characterized, 35 of these are radiation sensitive and have been grouped into 3 epistasis groups termed RAD3, RAD6, and RAD52 and are thought to constitute different cellular responses to ultraviolet and/or ionizing radiation-induced DNA damage [for a review, see Game (1983)]. Although a wealth of information exists concerning the genetic aspects of yeast DNA repair, there are comparatively few studies that address the biochemical characteristics of this system. Such existing investigations have focused primarily on the identification of various apurinic/apyrimidinic (AP)[1] endonuclease activities (Pinon, 1970; Chlebowicz & Jachymczyk, 1977; Armel & Wallace, 1978, 1984; Bryant & Haynes, 1978a; Futcher & Morgan, 1979; Akhmedov et al., 1982), although there are reports identifying other enzymes including uracil–DNA glycosylase (Crosby et al., 1981) and UV-specific endonucleases (Bryant & Haynes, 1978b; Bekker et al., 1980).

In consideration of the types of DNA repair enzymes that should be present in yeast, it is noteworthy that mutants in several RAD loci (*rad6*, *rad9*, *rad18*) are sensitive to both UV light and ionizing radiation (Game, 1983). Previous studies by our laboratory (Doetsch et al., 1986, 1987; Helland et al., 1986) and others (Bachetti & Benne, 1975; Gates & Linn 1977; Katcher & Wallace, 1983; Brent, 1983) have indicated that *Escherichia coli* endonuclease III and a similar mammalian enzyme, redoxyendonuclease, recognize oxidative DNA damage induced by both UV light and X-rays. Thymine glycol, a major product of ionizing radiation damage (Teoule & Cadet, 1978), and other ring-saturated, ring-cleaved, and ring-contracted base damage products appear to be substrates

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[1] Abbreviations: bp, base pair(s); Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethyl sulfoxide; AP, apurinic/apyrimidinic; SDS, sodium dodecyl sulfate.

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