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Biosynthesis of Peptidoglycan in *Gaffkya homari*: Reactivation of Membranes by Freeze-Thawing in the Presence and Absence of Walls†

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The reactivation of membranes from *Gaffkya homari* for the synthesis of sodium dodecyl sulfate-insoluble peptidoglycan (SDS-insoluble PG) was achieved by successive cycles of freeze-thawing (−196 versus 25°C). The presence of *G. homari* walls during this process affected the synthesis of both SDS-soluble (nascent) and SDS-insoluble PG. At two cycles the synthesis of SDS-soluble PG decreased by 70%, whereas that of SDS-insoluble PG increased sevenfold when compared with membranes reactivated in the absence of walls but assayed in the presence of walls. Moreover, at six cycles the lag time for the synthesis of SDS-insoluble PG decreased from 15 min to 5 to 7 min. Walls from *G. homari* could not be replaced with walls from *Bacillus megaterium* or cellulose. In addition to these effects, the presence of walls from *G. homari* or *B. megaterium* or of cellulose during the incubation of membranes freeze-thawed in the absence of walls increased twofold the amount of SDS-insoluble PG. Reactivated membranes showed greater sensitivities to penicillin (an inhibitor of DD-carboxypeptidase) and D-methionine (an inhibitor of LD-carboxypeptidase) than did isolated membrane-walls. The percentage of cross-linking of the SDS-insoluble PG synthesized by the reactivated system was 34%, a value similar to that observed for the polymer synthesized by isolated membrane-walls. Freeze-thawing membranes and walls together gave a complex with a density different from that of either membranes or walls. Thus, the assembly system for the synthesis and processing of PG was reconstituted in a complex of membranes and walls prepared from the isolated components. Whether this complex has the exact interrelationship between membrane and wall found in the organism has not been established.

The assembly of wall peptidoglycan (PG) requires the concerted action of a series of membrane-associated enzymes (6, 21, 31, 35). In *Gaffkya homari*, the initial product of this series is a PG which is soluble in sodium dodecyl sulfate (SDS). This glycan is processed by at least three enzymes, DD-carboxypeptidase, LD-carboxypeptidase, and transpeptidase, to form SDS-insoluble PG (8–10, 13).

The in vitro synthesis of cross-linked PG has been accomplished in a variety of systems containing membranes, membrane-walls, or permeabilized cells (6, 21, 31, 35). Many of these systems appear to retain those features which are necessary for the assembly of this wall-linked glycan. For example, membrane-walls have retained the functional spatial interrelationship between membrane and wall (22, 23, 25, 37, 38, 40). The use of membrane-walls from *G. homari* provided a system for defining some of the features of processing which are necessary for the incorporation of PG into wall (8–10, 13). It has been shown that LD-carboxypeptidase is one of the enzymes responsible for the sulfhydryl sensitivity and heat lability of the membrane-wall (26). It has been suggested that this enzyme plays a role in the relationship between membrane and wall.

In an attempt to define further this relationship, procedures were sought that would allow one to reconstitute the assembly system for PG synthesis in "membrane-walls" from purified membranes and walls. Unlike membrane-walls, membranes isolated from *G. homari* do not synthesize SDS-insoluble PG when assayed in the presence or absence of walls. The objective of these experiments was to develop a set of conditions for restoring the capacity of the membranes from this organism to synthesize SDS-insoluble PG. In the present study, restoration of activity was achieved by a process of

† We dedicate this paper to David Shemin in honor of his 70th birthday.

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successive cycles of freeze-thawing in the presence and absence of walls. By carrying out this process in the presence of walls, we observed specific effects of walls on the synthesis of both SDS-soluble and SDS-insoluble PG. Thus, it is proposed that the assembly system for the synthesis of wall PG is reconstituted in "membrane-walls" prepared from purified membranes and walls. The reactivation experiments may elucidate further the interrelationship of membrane and wall in *G. homari*.

(A preliminary report of these experiments has been presented previously [E. L. Kalomiris and F. C. Neuhaus, Fed. Proc. 39:1633, 1980].)

MATERIALS AND METHODS

Materials. We are indebted to R. K. Sinha and R. Rosenthal for authentic samples of bis-disaccharide peptide dimer and disaccharide peptide monomer isolated from *Neisseria gonorrhoeae*. We thank D. Fan for a culture of *Bacillus megaterium* 889 from which walls were prepared by the procedure described below for *G. homari*. UDP-*N*-[*U*-^{14}C]acetylglucosamine (GlcNAc) (300 mCi/mmol) was purchased from Amersham Corp. The preparation of UDP-*N*-acetylmuramyl (MurNAc)-Ala-D-Glu-Lys-D-Ala-D-Ala was described previously (11). Trypsin and the muramidase from *Chalaropsis* sp. were purchased from Sigma Chemical Co. and Miles Laboratories, respectively. Metrizamide {2-[3-acetamido-5-(*N*-methyl-acetamido)-2,4,6-triiodobenzamido]-2-deoxy-D-glucose} was purchased from Aldrich Chemical Co. The sources of other chemicals have been described previously (4, 11, 12).

Growth of *G. homari* and preparation of membrane-walls. *G. homari*, properly named *Aerococcus viridans* subsp. *homarus* (17), was obtained from the American Type Culture Collection as *Pediococcus homari* 10400. The organism was grown as described by Hammes and Neuhaus (12) with the exception that the growth temperature was lowered to 32°C and the antifoam was omitted from the growth medium. Membrane-walls were prepared by the procedure described by Carpenter et al. (4) and modified by Neuhaus et al. (26). No antifoam was used in the disruption procedure.

Preparation of membranes and walls. For the disruption of the cells, 40-ml lots of a 15% suspension (wet weight) in 20 mM Tris-hydrochloride buffer (pH 7.8) containing 20 mM $MgCl_2$ and 1 mM dithiothreitol were mixed with 35 g of plastic beads in a Teflon bottle. Disruption was accomplished in a Bronwill mechanical cell homogenizer (Braun model MSK) at 4,000 cycles/min for three successive 5-min periods with cooling by liquid CO_2. The beads were removed by filtration through a fine-mesh cloth. Unbroken cells were removed by centrifugation at 2,500 × *g* for 10 min. To isolate crude cell walls, the supernatant fraction was centrifuged at 10,000 × *g* for 20 min. As described below, the supernatant fraction from this centrifugation was used as the source of membranes. The precipitate containing walls was suspended in buffer, and the walls were isolated by differential centrifugation between 2,500 × *g* for 10 min and 10,000 × *g* for 15 min. The walls were washed three times with 5 mM Tris-hydrochloride (pH 7.8) containing 1 M KCl (34). For the trypsin treatment of walls, the sample (3 g, wet weight) was boiled for 15 min in distilled water and transferred into 100 ml of 0.46 M Tris-hydrochloride (pH 8.1) containing 11.5 mM $CaCl_2$ and 0.1% (wt/vol) trypsin and incubated for 18 h at 37°C (43). This procedure removes adhering proteins from the walls (33, 42). After this treatment, the walls were washed three times with distilled water and then three times with 20 mM Tris-hydrochloride (pH 7.8) containing 20 mM $MgCl_2$ and 1 mM dithiothreitol. The treated walls were stored in this buffer in liquid nitrogen.

For the preparation of membrane fragments, the supernatant fraction described above was centrifuged two times at 30,000 × *g* for 10 min to remove the remaining wall fragments and cell debris. The supernatant fraction was then centrifuged at 105,000 × *g* for 2 h. The pelleted membranes were suspended in 20 mM Tris-hydrochloride buffer (pH 7.8) containing 20 mM $MgCl_2$ and 1 mM dithiothreitol. Samples (0.2 ml) of this suspension (32 to 40 μg of protein per μl) were stored at −196°C in Beem capsules (size 00; Pelco Electron Microscopy Supplies). For the experiments described in this paper, no further purification of the membrane fragments was performed.

Reactivation of membranes in the presence and absence of walls. Membranes (32 to 40 μg of protein per μl) were reactivated by successive cycles of freeze-thawing between −196 and 25°C. The first cycle of freeze-thawing was completed by thawing at room temperature membranes previously stored in a liquid nitrogen freezer. For additional cycles, the membrane suspension in a Beem capsule was placed in a canister in the freezer for at least 5 min and subsequently allowed to thaw at 25°C. The rate of freezing was 65°C/min, and the initial rate of thawing was 120°C/min. To reactivate membranes in the presence of walls, we mixed freshly prepared membranes with trypsin-treated walls (1:1, wt/wt) before storing the mixture in the freezer. Freeze-thawing of this mixture was accomplished as described for membranes.

PG assay. We assayed PG synthesis by determining the incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc in the presence of UDP-MurNAc-pentapeptide into SDS-soluble and SDS-insoluble PG. For the assay of reactivated membranes measured in the presence of walls, the reaction mixture contained the following (total volume, 135 μl): freeze-thawed membranes (120 to 150 μg of protein); walls (120 to 200 μg); 50 mM Tris-hydrochloride buffer, pH 7.8; 0.35 mM UDP-[^{14}C]GlcNAc (2.3 to 5.6 cpm/pmol); 90 μM UDP-MurNAc-Ala-D-Glu-Lys-D-Ala-D-Ala; 10 mM NH_4Cl; 70 mM magnesium acetate; and 7 mM ATP (neutralized with NH_4OH unless otherwise indicated). After incubating the mixture at 25°C for the indicated time with shaking, we terminated the synthesis by heating the mixture for 3 to 4 min at 100°C. SDS-insoluble PG was measured by the procedure described by Neuhaus et al. (26) and Carpenter et al. (4). All centrifugations were performed in a Beckman Microfuge B at 10,000 × *g* for 3 min at room temperature. For the assay of SDS-soluble PG, the reaction was terminated as described above and the SDS-insoluble material was removed by centrifugation. We assayed a sample of the supernatant fraction for SDS-soluble PG by measuring the amount of radioactivity that was immobile in solvent A.

Analytical procedures. Protein was determined by the method of Lowry et al. (19), with bovine serum albumin as a standard. Descending paper chromatography (Whatman 3MM) was performed in isobutyric acid–concentrated NH_4OH–water (66:2:33, vol/vol/vol) (solvent A). Radioactivity on chromatograms was counted in toluene containing 0.3% 2,5-diphenyloxazole and 0.025% 1,4-bis[2-(5-phenyloxazolyl)]benzene. Radioactivity in aqueous samples was measured in a scintillation fluid described by Patterson and Greene (28). Isopycnic gradient centrifugation of freeze-thawed membranes was performed in a Beckman (model L2-65) ultracentrifuge with an SW65 rotor. The samples were applied to step gradients of Metrizamide in 20 mM Tris-hydrochloride buffer (pH 7.8) containing 20 mM $MgCl_2$ and 1 mM dithiothreitol and centrifuged in 0.8-ml cellulose nitrate tubes for 1 h at 50,000 rpm at 4°C.

Characterization of the in vitro-synthesized PG was performed by the procedures of Oka (27) and Rosenthal et al. (32). The SDS-insoluble PG was isolated from the reaction mixture and digested with the *Chalaropsis* sp. muramidase (25 U) in 90 μl of 5 mM acetate buffer (pH 5.0) at 37°C for 12 h. The digests were applied to two connected columns (1.5 by 90 cm) of Bio-Gel P6 and eluted with 0.2 M triethylamine bicarbonate (pH 7.4) (flow rate, 0.2 ml/min). The percentage of cross-linking is expressed as 0.5 of the percentage of counts per minute in the bis-disaccharide peptide. This determination does not reflect the amount of monomer that is present as trimer or higher oligomer, and, thus, it represents only the first term of the equation described by Dezélée and Shockman (5). The monomer and dimer were identified by the procedures of Hammes and Kandler (10).

RESULTS

In this study, reactivation is defined as the restoration of the ability of the membrane to synthesize SDS-insoluble PG. Restoration of this synthesis requires the formation of SDS-soluble polymer (nascent PG) and its conversion into SDS-insoluble polymer. This reactivation is accomplished by freeze-thawing membranes either in the presence or in the absence of walls.

Reactivation by freeze-thawing membranes in the absence of walls. An essential requirement for reactivating membranes was freeze-thawing. This was accomplished by freezing concentrated suspensions (32 to 40 μg of protein per μl) in a liquid nitrogen freezer (5 min) and then thawing them at room temperature (see above). Membranes that had been freeze-thawed one cycle were only 1% as effective for the synthesis of SDS-insoluble PG as membranes which had been subjected to four cycles (Table 1). The synthesis of this glycan by reactivated membranes required UDP-GlcNAc, UDP-MurNAc-pentapeptide, Mg^{2+}, ATP, and NH_4^+. These latter requirements were identical to those described for the synthesis of SDS-insoluble PG by isolated membrane-walls (4). The addition of walls to the incubation mixture facilitated the isolation of this glycan (see below).

TABLE 1. Requirements for the synthesis of SDS-insoluble PG by freeze-thawed membranes

Reaction mixture	Activity (pmol/30 min)
Complete[a]	2,170.0
− Membranes (FT[b] four cycles)	0.3
− Membranes (FT four cycles) + membranes (FT one cycle)	26.0
− ATP	1.2
− Mg^{2+}	0.8
− NH_4Cl	3.2
− UDP-MurNAc-pentapeptide	0.2

[a] The complete reaction mixture contained: 129 μg of membrane protein (freeze-thawed four cycles); 236 μg of trypsin-treated walls; 50 mM Tris-hydrochloride buffer, pH 7.8; 90 μM UDP-MurNAc-pentapeptide; 0.35 mM UDP-[^{14}C]GlcNAc (5.6 cpm/pmol); 10 mM NH_4Cl; 70 mM magnesium acetate, and 7 mM ATP (neutralized with NaOH) in a total volume of 135 μl. The amount of SDS-insoluble PG was determined as described in the text.

[b] FT, Freeze-thawed.

Without freeze-thawing, the amount of SDS-soluble PG was 16% of that observed for membranes which had undergone two cycles of freeze-thawing (Fig. 1). Although significant amounts of SDS-soluble PG were synthesized after one or two cycles, no SDS-insoluble PG was detected. The maximal amount of SDS-insoluble PG was achieved with membranes freeze-thawed four to six cycles. For example,

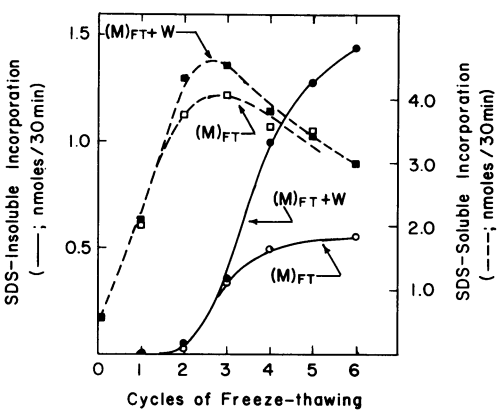

FIG. 1. Effect of number of cycles of freeze-thawing on the synthesis of SDS-insoluble (○, ●) and SDS-soluble (□, ■) PG. Membranes (120 μg) were freeze-thawed in the absence of walls for the indicated number of cycles and assayed in the presence [$(M)_{FT}$ + W] (■, ●) or absence [$(M)_{FT}$] (□, ○) of walls. The amount of walls was 160 μg. The assays for SDS-insoluble and SDS-soluble PG are described in the text.

120 μg of membrane protein incorporated 500 pmol of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc into SDS-insoluble polymer in the absence of walls during the assay. In our experiments, membranes (freeze-thawed six cycles) from different batches of cells, which were assayed in the absence of walls, yielded approximately 50% of the activity which was observed with membranes assayed in the presence of saturating concentrations of walls. However, for 120 μg of membrane protein from different batches, this activity varied from 1.5 to 3.0 nmol of [^{14}C]GlcNAc incorporated into SDS-insoluble PG in 30 min. Essentially, no effect of walls was observed on the synthesis of SDS-soluble PG. The sums of SDS-soluble and SDS-insoluble polymers in the presence and absence of walls with membranes freeze-thawed four to six cycles were not equal (Fig. 1). This observation reflected the progressive solubilization of the SDS-insoluble polymer in the absence of walls during the isolation procedure. Thus, the addition of walls to freeze-thawed membranes during the incubation facilitated the precipitation and subsequent isolation of the SDS-insoluble PG.

Comparison of SDS-insoluble PG synthesized by membranes reactivated in the absence of walls with that synthesized by isolated membrane-walls. Three types of experiments were used to compare SDS-insoluble PG synthesized by reactivated membranes and by isolated membrane-walls. These experiments compared the time courses of SDS-insoluble PG synthesis, the sensitivities of its synthesis to benzylpenicillin and D-methionine, and the percentages of cross-linking of the in vitro-synthesized SDS-insoluble PG. Each of these experiments probed a specific feature of the reactivated system.

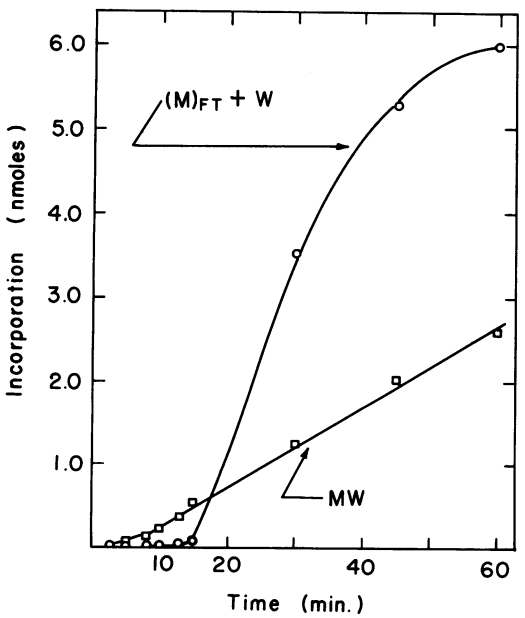

FIG. 2. Time courses of SDS-insoluble PG synthesized by isolated membrane-walls (MW) (□) and reactivated membranes assayed in the presence of walls [$(M)_{FT}$ + W] (○). ○, 129 μg of membrane protein (freeze-thawed six cycles in the absence of walls) and 295 μg of walls were used. □, 130 μg of isolated membrane-walls (freeze-thawed six cycles) was used.

A comparison of the time courses of synthesis of SDS-insoluble PG catalyzed by reactivated membranes and isolated membrane-walls revealed a difference between the two systems (Fig. 2). Reactivated membranes assayed in the presence of walls synthesized no SDS-insoluble PG during the first 15 min, whereas isolated membrane-walls synthesized significant amounts of this polymer during this time interval. The lag time of 15 min was a characteristic feature of membranes reactivated in the absence of walls.

The sensitivities of the systems for the synthesis of SDS-insoluble PG to benzylpenicillin and D-methionine reflected the requirement for the actions of DD-carboxypeptidase and LD-carboxypeptidase, respectively. In Fig. 3, the sensitivities of the reactivated membranes are compared with those of the isolated membrane-walls. Both systems were sensitive to the action of benzylpenicillin, a specific inhibitor of the DD-carboxypeptidase in *G. homari* (8). However, at concentrations greater than 2 μM, the system containing reactivated membranes was more sensitive to the action of the antibiotic than the system containing isolated membrane-walls (Fig. 3A). For example, 5 μM benzylpenicillin inhibited isolated membrane-walls by 60%, whereas reactivated membranes assayed in the presence of walls were completely inhibited by this concentration. The reactivated membranes were also more sensitive to the action of 6 mM D-methionine, a specific inhibitor of the LD-carboxypeptidase (9) (Fig. 3B).

The digestion of the SDS-insoluble PG by the *Chalaropsis* sp. muramidase provides a sensitive method for establishing the cross-linkage of the polymer. We compared the elution profile of the degraded SDS-insoluble PG synthesized by isolated membrane-walls with that of the degraded SDS-insoluble PG synthesized by reactivated membranes assayed in the presence of walls (Fig. 4). Only the monomers and dimers were identified and quantitated. The percentage of cross-linking was 34% for SDS-insoluble PG synthesized by reactivated membranes assayed in the presence of walls and 34% for the polymer synthesized by isolated membrane-walls. It would appear that the SDS-insoluble PG synthesized by reactivated membranes has the same

degree of cross-linking as that formed by isolated membrane-walls. The percentages of cross-linking are lower than those reported previously (10) because only monomers and dimers were considered in our calculations.

Reactivation by freeze-thawing membranes in the presence of walls. A comparison between reactivated membranes freeze-thawed in the absence of walls and isolated membrane-walls revealed significant differences in the time courses of formation of SDS-insoluble PG and in the sensitivities of this synthesis to penicillin and D-methionine. On the basis of these differences, reactivation by freeze-thawing membranes and walls together was considered as a procedure for reconstituting the assembly system for the synthesis of SDS-insoluble PG.

Specific effects of walls on the synthesis of both SDS-soluble and SDS-insoluble PG were observed when membranes were freeze-thawed in the presence of walls (Fig. 5 and 6). Figure 5 illustrates the syntheses of both classes of glycans by membranes subjected to an increasing number of cycles in the presence of walls. In contrast to membranes freeze-thawed for two cycles in the absence of walls, the synthesis of SDS-soluble PG decreased by 70% whereas that of SDS-insoluble PG increased sevenfold. Membranes freeze-thawed for six cycles in the absence of walls attained maximal reactivation for the synthesis of SDS-insoluble PG. Therefore, the presence of walls during six cycles of freeze-thawing would not be expected to have a major effect on the amount of this polymer synthesized in 30 min.

We also compared the time courses of synthesis of SDS-insoluble PG catalyzed by membranes freeze-thawed in the absence and presence of walls (Fig. 6). As indicated in the previous section, membranes reactivated in the absence of walls synthesized SDS-insoluble PG with a distinct lag time of 15 min (Fig. 2 and 6). In contrast, the lag time for isolated membrane-walls was 3 to 5 min (Fig. 2). When membranes were freeze-thawed in the presence of walls (Fig. 6), the lag time decreased from 15 min to 5 to 7 min. Thus, one of the differences between isolated membrane-walls and reactivated membranes was minimized by freeze-thawing membranes in the presence of walls.

The systems containing membranes reactivated in the presence or absence of walls were more sensitive to the actions of penicillin and D-methionine than was the system containing isolated membrane-walls (Fig. 3). Freeze-thawing membranes in the presence of walls decreased the inhibitory response of these compounds to a small extent as compared with membranes reactivated in the absence of walls. These responses indicated differences between isolated membrane-walls and membranes reactivated in the presence of walls.

Membranes reactivated in the absence of walls synthesized SDS-insoluble PG which was cross-linked 34% (Fig. 4). The SDS-insoluble PG synthesized by membranes reactivated in the presence of walls was also partially characterized by digestion with the *Chalaropsis* sp. muramidase. The cross-linking of this PG was 32%. These results indicated that membranes reacti-

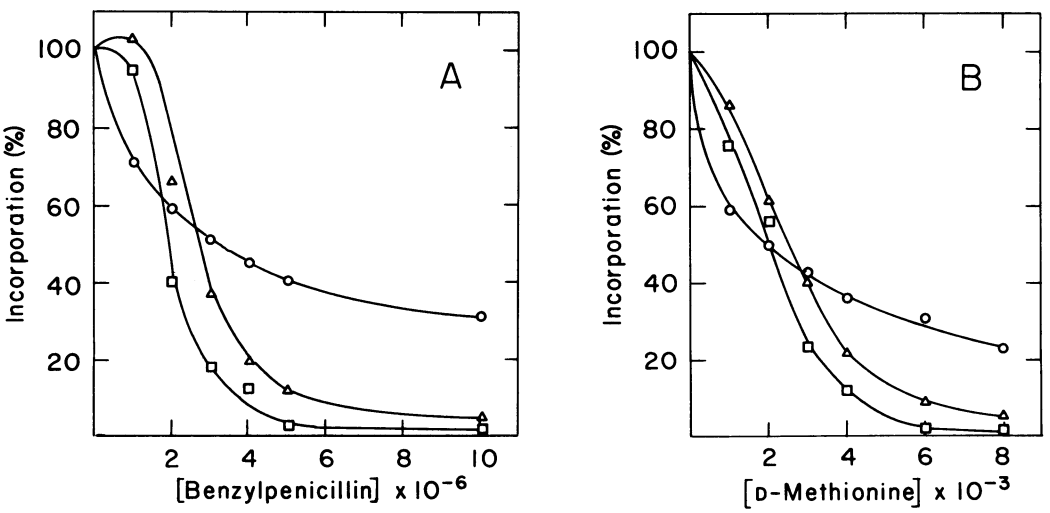

FIG. 3. Effect of benzylpenicillin (A) and D-methionine (B) on the synthesis of SDS-insoluble PG by isolated membrane-walls (○) and membranes freeze-thawed six times in the presence (△) and absence (□) of walls. For reactivated membranes, 150 μg of membrane protein was used with 170 μg of walls. For isolated membrane-walls, 150 μg of membrane protein was used. The assays for SDS-insoluble PG are described in the text.

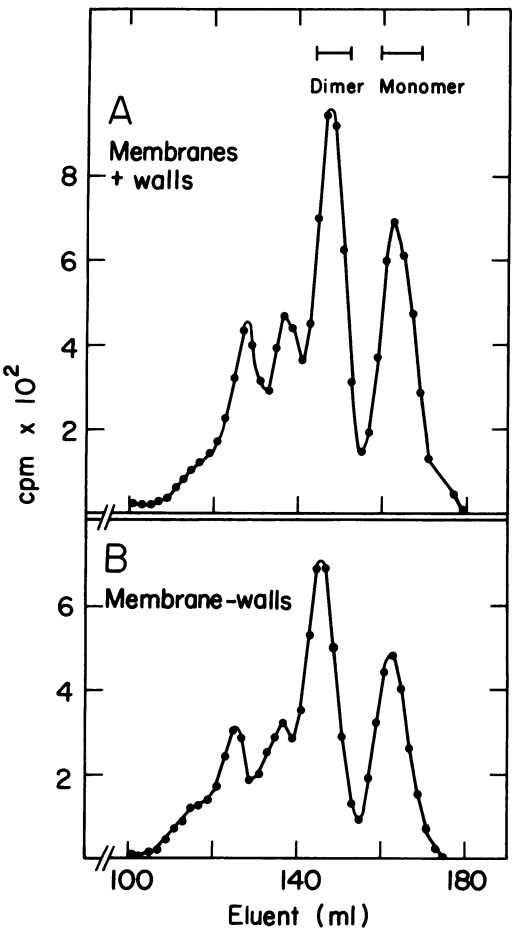

FIG. 4. Elution profiles of [^{14}C]GlcNAc-labeled degradation products derived from PG synthesized by membranes freeze-thawed for six cycles (A) and by isolated membrane-walls (B). For (A), SDS-insoluble PG was synthesized by freeze-thawed membranes to which walls were added in the reaction mixture. SDS-insoluble PG was isolated from reaction mixtures for PG synthesis, degraded with the *Chalaropsis* sp. muramidase, and filtered on Bio-Gel P6 as described in the text.

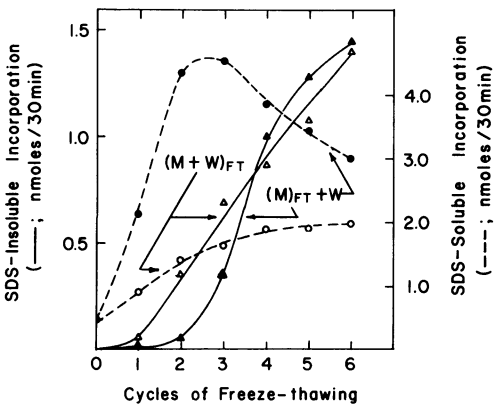

FIG. 5. Effect of number of cycles of freeze-thawing in the presence of walls on the synthesis of SDS-insoluble and SDS-soluble PG. Membranes (120 μg of protein) were freeze-thawed for the indicated number of cycles in the presence of walls [(M + W)$_{FT}$; △, ○] and in the absence of walls. The membranes reactivated in the absence of walls were then assayed in the presence of walls [(M)$_{FT}$ + W; ▲, ●]. The amount of walls was 160 μg. The assays for the synthesis of SDS-soluble and SDS-insoluble PG are described in the text.

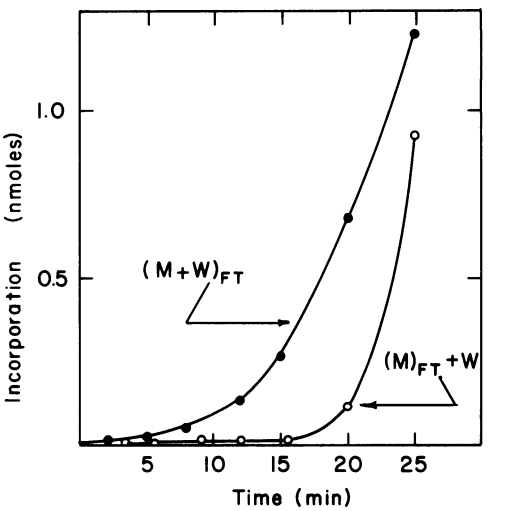

FIG. 6. Time courses of SDS-insoluble PG synthesized by membranes freeze-thawed for six cycles in the presence [(M + W)$_{FT}$] (●) and in the absence [(M)$_{FT}$ + W] (○) of walls. Reaction mixtures for each time point contained 150 μg of membrane protein and 170 μg of walls (dry weight). Membranes reactivated in the presence of walls [(M + W)$_{FT}$] were assayed as described in the legend to Fig. 5. Membranes reactivated in the absence of walls [(M)$_{FT}$ + W] were assayed in the presence of walls as described in the legend to Fig. 1.

vated in the presence and absence of walls catalyzed the same degree of cross-linking as that catalyzed by isolated membrane-walls.

To test the specificity of the wall effects, the walls from *G. homari* were replaced with either walls from *B. megaterium* or cellulose (Table 2). When the membranes from *G. homari* were freeze-thawed two and six cycles in the presence of either *B. megaterium* walls or cellulose, there was an inhibition of SDS-insoluble PG synthesis. However, when the membranes were reactivated in the absence of walls, the addition of walls from either *G. homari* or *B. megaterium*

TABLE 2. Specificity of wall effects on SDS-insoluble PG synthesis by freeze-thawed membranes from *G. homari*

Addition	SDS-insoluble PG (pmol/30 min)[a]			
	FT[b] two cycles		FT six cycles	
	$(M)_{FT}$ + W	$(M + W)_{FT}$	$(M)_{FT}$ + W	$(M + W)_{FT}$
None	170		1,600	
Walls (*G. homari*)	190	400	2,840	2,220
Walls (*B. megaterium*)	210	114	2,560	1,380
Cellulose	70	34	2,110	470

[a] $[(M)_{FT} + W]$, Membranes were freeze-thawed in the absence of walls or cellulose and assayed in the presence of walls or cellulose. $[(M + W)_{FT}]$, Membranes were freeze-thawed in the presence of walls or cellulose. Membranes (125 μg of membrane protein) were freeze-thawed in the presence or absence of walls (90 μg) for either two or six cycles of freeze-thawing. For the addition of cellulose, 80 μg was used in place of the walls. The amount of SDS-insoluble PG was measured in the assay described in the text.

[b] FT, Freeze-thawed.

during the assay facilitated the isolation of SDS-insoluble PG. Thus, from the results shown in Table 2, it was concluded that the reactivation of membranes in the presence of walls shows specificity for the walls from *G. homari*.

Formation of a membrane-wall complex from membranes and walls. The results in Fig. 5 and 6 suggested that freeze-thawing membranes and walls together may promote the formation of a membrane-wall complex. This complex was demonstrated by isopycnic centrifugation with a step gradient of Metrizamide. This substance is chemically inert and nonionic and has a relatively low viscosity (7, 30); it thus allows separation of particles of relatively similar densities.

When membranes and walls were freeze-thawed together for six cycles, a complex was formed which had a density different from that of either freeze-thawed (six cycles) membranes or walls (Fig. 7, lane 3). In contrast, membranes freeze-thawed for six cycles in the absence of walls did not form a complex with added walls under the conditions of the isopycnic centrifugation (Fig. 7, lane 2). Walls centrifuged in the absence of membranes had the same density as those centrifuged in the presence of membranes. Membranes subjected to two cycles of freeze-thawing had a lower density than those subjected to six cycles. When membranes were freeze-thawed for two cycles in the presence of walls, a complex was also formed, but its density was similar to that of walls (data not shown). These observations indicated that freeze-thawing membranes in the presence of walls formed a complex of membranes and walls that was stable to the conditions of isopycnic centrifugation. Furthermore, this complex did not dissociate in the reaction mixture for the synthesis of SDS-insoluble PG (data not shown).

DISCUSSION

The reactivation of membranes from *G. homari* for the synthesis of SDS-insoluble PG was achieved by successive cycles of freeze-thawing. It is proposed that these cycles induce modifications in the membrane, allowing for the synthesis of the SDS-insoluble polymer. We have described two types of effects that walls have on PG synthesis. The first showed specificity for walls from *G. homari* and required that membranes be freeze-thawed in the presence of these walls. It is suggested that the addition of walls from *G. homari* during the freeze-thaw

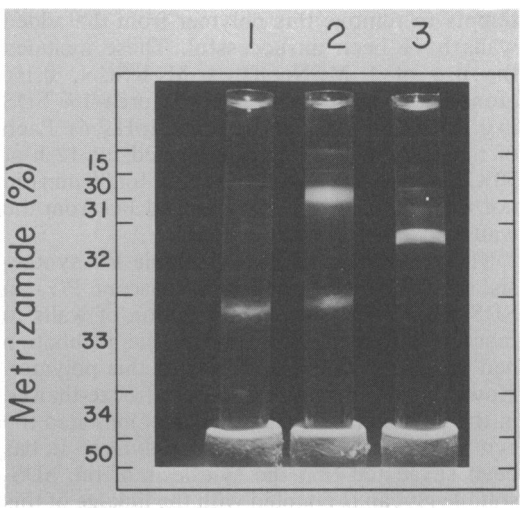

FIG. 7. Isopycnic centrifugation of membranes freeze-thawed for six cycles in the presence and absence of walls. The samples in 20 mM Tris-hydrochloride buffer (pH 7.8) containing 20 mM $MgCl_2$ and 1 mM dithiothreitol were applied to step gradients of Metrizamide and centrifuged to equilibrium as described in the text. The samples were as follows: Lane 1, membranes (260 μg of protein) freeze-thawed for six cycles; lane 2, membranes (260 μg of protein) freeze-thawed for six cycles and mixed with 400 μg of walls; lane 3, membranes (260 μg of protein) and walls (400 μg) freeze-thawed together for six cycles.

process facilitates these membrane modifications such that fewer cycles of freeze-thawing are required to achieve synthesis of the polymer. Neither walls from *B. megaterium* nor cellulose can replace walls from *G. homari* for this type of effect. Thus, reactivation of membranes for the synthesis of SDS-insoluble PG may be accomplished with fewer cycles of freeze-thawing when membranes and walls are freeze-thawed together.

The second type of wall effect on the synthesis of SDS-insoluble PG did not show specificity for walls from *G. homari* and did not require that membranes and walls be freeze-thawed together. The addition of walls during the assay increased twofold the apparent synthesis of SDS-insoluble PG by reactivated membranes. Either walls from *B. megaterium* or cellulose could replace walls from *G. homari*. This type of wall effect may result from an enhanced precipitation of the glycan polymer by providing a supporting matrix during the incubation. In the case of walls from *G. homari*, both types of wall effects were observed. Thus, it is suggested that these walls not only can facilitate the necessary membrane modifications required for PG formation, but they may also facilitate the precipitation of the synthesized glycan. It is not known whether the newly synthesized PG is covalently linked to the preexisting glycan of the added walls. All attempts to remove this polymer from the added walls have been unsuccessful. These included the use of 1 M NaOH, 1 M KSCN, 0.1% monochloroacetic acid, and 9 M urea–1% SDS in 0.1 M sodium phosphate buffer (pH 7.6). Each of these treatments was performed for 12 h at 50°C. Moreover, 2% SDS at 100°C for 30 min did not release the newly synthesized PG from the walls.

The restoration of SDS-insoluble PG synthesis requires the formation of nascent PG, an SDS-soluble polymer. The addition of walls to reactivated membranes during the incubation had no effect on the synthesis of this polymer. However, when membranes were freeze-thawed in the presence of walls, the walls inhibited the synthesis of the SDS-soluble polymer. It has been suggested that the synthesis of the SDS-soluble glycan is coupled with the linkage of this nascent polymer to preexisting PG (24, 35). Uncoupling of the system could lead to an enhanced rate of SDS-soluble PG formation. It is proposed that the presence of walls during the reactivation of membranes facilitates the restoration of the coupling process involved in the synthesis of the SDS-insoluble glycan from nascent polymer. This proposal was supported by the observation that membranes freeze-thawed in the presence of walls from *G. homari* gave a shorter lag time for the time course of SDS-insoluble PG synthesis. In addition, the amount of SDS-soluble PG formed decreased when membranes were freeze-thawed in the presence of walls. Thus, as the coupling process is effected, the amount of SDS-soluble polymer decreases while the amount of SDS-insoluble polymer increases.

The molecular mechanisms by which freeze-thawing modifies or stresses the membrane structure are not clear (1–3, 14, 16, 18, 20, 36, 39, 41). The resulting modifications might include: permeability changes in the membrane; changes in the optimal topology of the enzymes which are involved in synthesizing and processing PG; changes in the sidedness of vesicles; changes in the size of the vesicles as a result of fusion; "solubilization" and reassembly of PG-synthesizing enzymes. We propose that freeze-thawing membranes effects a specific modification in the membrane organization required for the synthesis of SDS-insoluble PG. This process provides a convenient method for reactivating membranes from *G. homari* for PG synthesis without introducing external agents.

Membranes which are isolated from ruptured cells of *G. homari* have low activity for the synthesis of SDS-soluble PG and no activity for the synthesis of SDS-insoluble PG. On the other hand, membranes which are isolated as membrane-walls are capable of synthesizing SDS-insoluble glycan. In the isolation of membrane-walls, it is proposed that walls stabilize an active state of the membrane for PG synthesis. This active state is lost during the course of isolating membranes free of walls. Weston et al. (40) suggested that membranes isolated as membrane-walls from *Micrococcus luteus* synthesize wall-linked PG more efficiently when the interface (membrane-wall junction) between the membrane and wall remains intact. In studying PG synthesis in *B. megaterium* KM, Reynolds (29) observed that protoplast membrane preparations are relatively inactive unless the protoplasts from which the membranes are derived are first incubated in a growth medium. This process, which has been termed reconditioning, has been proposed to correct damage in the protein-lipid structure of the membrane that is required for PG synthesis. Kanegasaki and Wright (15) observed that freeze-thawing mixtures of cell envelope and lipid intermediates is required for the efficient polymerization of the *Salmonella* O-antigen. They suggested that this process facilitates the entry of the lipid into the cell-membrane matrix, permitting the lipid intermediate to interact with the polymerase. These observations bear certain phenomenological resemblances to those described for membranes from *G. homari*.

In summary, our results indicated that it is

possible to reactivate membranes by freeze-thawing and that, if the process is carried out in the presence of walls, specific effects of walls can be observed. Under these conditions, a complex of membranes and walls was demonstrated whose density was different from that of either membranes or walls. It is suggested that a specific interrelationship between membrane and wall may have been effected. Whether these reactivated membranes have the exact interrelationship of the membrane and wall found in the organism has not been established.

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