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INCORPORATION OF PENICILLINASE INTO REAGGREGATED MYCOPLASMA MEMBRANES

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SUMMARY

Stable co-aggregates of solubilized mycoplasma membranes and extraneous proteins (bovine serum albumin and bacterial penicillinase) have been constructed. The penicillinase preparation was derived from the culture supernatant of *Bacillus cereus* strain 569/H and incorporated in a membrane-like structure formed by solubilization and reaggregation of membranes of *Acholeplasma laidlawii* (formerly *Mycoplasma laidlawaii*). In the presence of methicillin the co-aggregated enzyme showed lower thermostability and considerably higher resistance to inactivation by iodine. Antibodies to the soluble enzyme caused partial inhibition of the activity of the co-aggregated enzyme while antibodies to *A. laidlawii* membrane proteins had no effect even at concentrations sufficient to coat the co-aggregate. Partial removal of lipids from the co-aggregate restored the original characteristics of the enzyme. The properties of the co-aggregated exopenicillinase were compared with those of the native enzyme and of a membrane-bound variant of penicillinase isolated from the exopenicillinase-producing cells.

INTRODUCTION

The solubilization of *Acholeplasma laidlawii* (formerly *Mycoplasma laidlawii*) membranes by sodium dodecyl sulfate results in the separation of membrane proteins from membrane lipids [1,2]. Upon removal of the detergent in the presence of Mg^{2+}, the components reaggregate to a membrane-like structure which resembles native membranes in composition and ultrastructure[2,3]. The specificity of the reaggregation was previously investigated and recently hybrid aggregates have been described, which combine membrane protein from one species, with membrane lipids from another species of mycoplasma[4,5].

In the present communication we demonstrate the feasibility of constructing aggregates of mycoplasma membrane components and foreign, soluble proteins. Of particular interest is the enzymically active co-aggregate of solubilized membranes of *Acholeplasma laidlawii* and the exopenicillinase of *Bacillus cereus*. Such co-aggregates may prove useful in the study of enzymes immobilized in a hydrophobic biological structure.

MATERIALS AND METHODS

Acholeplasma laidlawii was grown in a modified Edward medium[6]. For the labelling of membrane lipids 50 μC of [9,10-^{3}H]oleic acid were added to each liter of the medium. The organisms were grown for 24 h at 37°. Cell membranes were isolated by osmotic lysis of the organisms and washed as described previously[2]. The exopenicillinase (β-lactamase I) and the membrane-bound penicillinase (γ-type β-lactamase)[7] of *Bacillus cereus* strain 569/H were derived and purified as before[8,9]. Bovine serum albumin was iodinated with K^{125}I (Nuclear Research center Negev, Israel) by the method of GREENWOOD *et al.*[10].

The antisera to exopenicillinase were prepared as described in a previous communication[11]. The preparation of antiserum to *A. laidlawii* and the absorption of the antiserum by the co-aggregates were performed as described by KAHANE AND RAZIN[12].

The washed *A. laidlawii* membrane suspensions (4 mg membrane protein per ml) were solubilized by 20 mM sodium dodecyl sulfate. The detergent was removed by dialysis at room temperature for 24 h against β-buffer[3] diluted 1:20 with deionized water (to be referred to as "dilute β-buffer"). After the addition of penicillinase or iodinated albumin (50 μg/ml membrane protein) to the clear dialysate, dialysis was continued against dilute β-buffer containing 20 mM $MgCl_2$. Membrane aggregates were collected after 4 days of dialysis at 4°. The aggregates were washed 5 times with a cold solution containing 0.02 M $MgCl_2$–0.01 M sodium citrate–1 M NaCl (pH 8.5) and resuspended in dilute β-buffer. Sonication of membrane aggregates was performed in an M.S.E. ultrasonic disintegrator (60 W) at 1.5A.

Penicillinase activity was determined spectrophotometrically or by the timed iodometric assay[13]. The activity was expressed in units as defined by POLLOCK AND TORRIANI[14]. Heat treatment and iodination were performed as described before[15]. Lipids were extracted from membrane aggregates by three repeated extractions with acetone–water–ammonia (90:10:0.03, by vol.) for 20 min at −5°. Protein was determined by the Folin phenol method of LOWRY *et al.*[16]. Radioactivity of tritiated materials was measured in a Packard Tri-carb liquid scintillation spectrometer using toluene–dioxane scintillation liquor[2]. ^{125}I was counted in a Packard auto γ-spectrometer.

RESULTS

In the course of dialysis of solubilized *A. laidlawii* membranes against dilute β-buffer containing 20 mM Mg^{2+} most of the lipids and proteins reaggregate to membrane-like structures[2]. When reaggregation was carried out in the presence of soluble penicillinase or bovine serum albumin an aggregate was formed which contained membrane material and an appreciable amount of the soluble protein. There was no association of extraneous soluble proteins with membraneous structures when such proteins were added to native membranes or to preformed membrane reaggregates. Fig. 1 shows that the amount of albumin bound to the aggregate was only slightly affected by repeated washing of the aggregate with a cold eluent solution (1.0 M NaCl in 0.1 M sodium citrate, pH 8.5)[8] to which 0.02 M $MgCl_2$ was added to stabilize the aggregate. In contrast, a large part of the initially bound penicillinase was removed

by washing. The remaining enzyme (8–10 μg penicillinase per mg membrane protein) which was not removed even after ten additional washings will be refered to as co-aggregated enzyme. Subsequent sonication caused progressive solubilization of the co-aggregates, but the ratio of penicillinase activity to the total protein remained constant in the supernatant fluid and in the sedimentable material (Fig. 2).

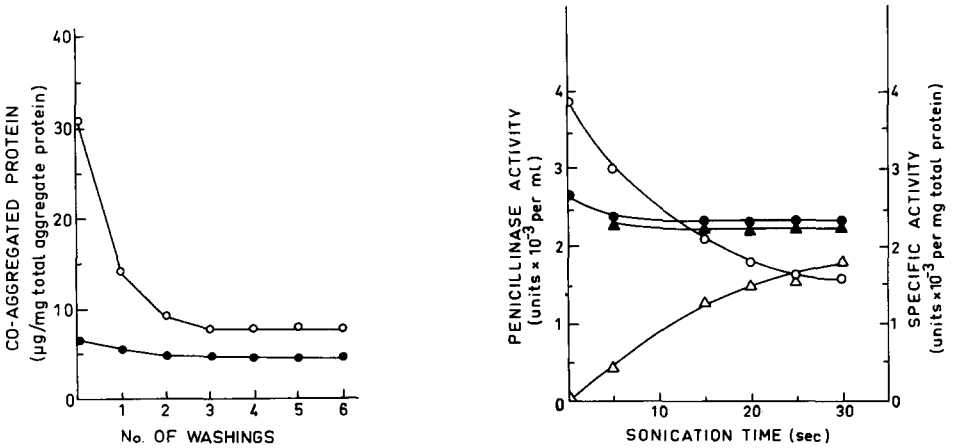

Fig. 1. Release of penicillinase and bovine serum albumin from co-aggregates. The co-aggregates formed from solubilized membrane components and the soluble proteins, were washed repeatedly with a solution of 1 M NaCl in 0.05 M sodium citrate (pH 8.5). The release of soluble proteins was monitored as described in MATERIALS AND METHODS. ○. penicillinase; ●, bovine serum albumin.

Fig. 2. Solubilization of co-aggregated proteins by sonication. 1-ml volumes of the co-aggregate suspensions were sonicated for various time periods. The samples were centrifuged at 34000 × g for 45 min and penicillinase activity was determined in both sediment and supernatant. Open symbols, penicillinase activity; closed symbols, specific activity. Circles, Activity in sediment; triangles, activity in supernatant.

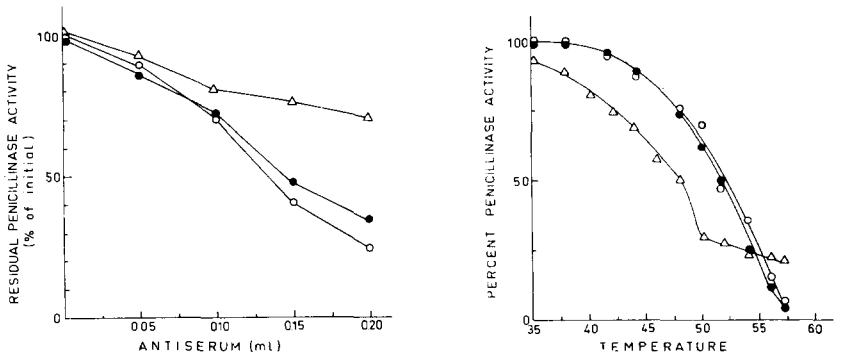

Fig. 3. Inhibition of free and co-aggregated enzyme by antiserum to exopenicillinase. ○, free enzyme; △, co-aggregated enzyme; ●, co-aggregated enzyme sonicated for 3 min.

Fig. 4. Effect of heating on penicillinase activity. Reaction mixtures consisted of 0.2 ml of 0.5% gelatin containing about 100 units of enzyme and 0.2 ml of 0.1 M phosphate buffer (pH 7.3). The tubes were incubated at the indicated temperatures for 2 min and transferred to an ice bath. The residual penicillinase activity was determined by the spectrophotometric and by the timed iodometric assay[13]. ○, free exoenzyme; △, membrane-bound enzyme (γ-type β-lactamase); ●, co-aggregated enzyme.

The penicillinase activity of the co-aggregate was not affected by antiserum to *A. laidlawii* membranes and even a prolonged incubation (3 days) of the aggregate with excess antibodies did not reduce the enzymic activity. On the other hand, antiserum to penicillinase had a slight inhibitory effect which could be increased by prolonged sonication of the co-aggregate. Typical results are shown in Fig. 3.

The relative rates of hydrolysis of benzylpenicillin and methicillin by penicillinase remained unchanged after the incorporation of the enzyme into the aggregate. Similarly, there was no change in the susceptibility to inactivation by iodine[17] or in thermostability. Indeed a detailed comparison of the temperature-dependent inactivation of the free and incorporated enzyme shows no difference between the two preparations throughout the range of temperatures tested (Fig. 4). All this is in contrast to the behaviour of the naturally occurring membrane-bound variant (γ-type β-lactamase) which hydrolyses methicillin at a higher relative rate[18], is extremely sensitive to iodine[19] and has a distinctive thermostability pattern (Fig. 4). However when tested in the presence of methicillin, the co-aggregated enzyme appeared to be much more resistant to iodination but more sensitive to thermal inactivation than the soluble enzyme. Susceptibility to iodination in the presence of methicillin has been found to provide the most sensitive tool for distinguishing between closely related exopenicillinases[7]. The results presented in Fig. 5 show a striking decrease in the susceptibility to iodination when the exoenzyme is incorporated in the aggregate. The original response of the exoenzyme to increasing concentrations of meticillin was partly restored when 62% of the co-aggregated lipids were removed (Fig. 5).

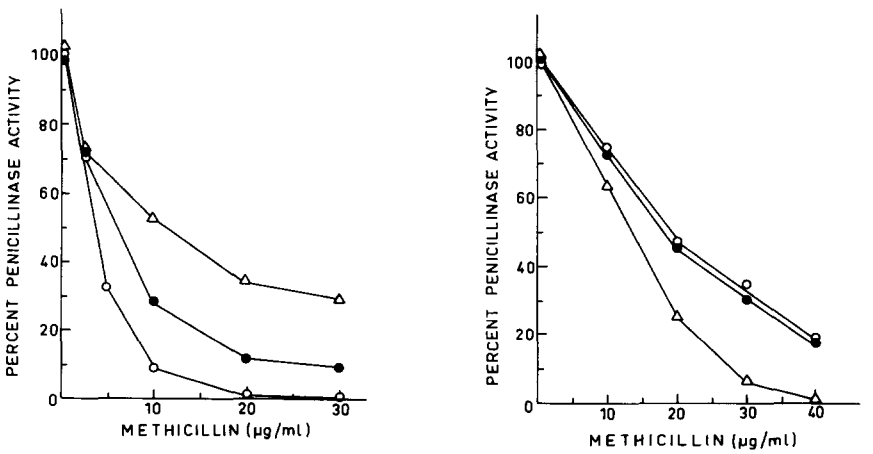

Fig. 5. Inhibition of penicillinase activity by iodine in the presence of methicillin. Reaction mixtures consisted of 0.2 ml of 0.5% gelatin containing 180 units of enzyme, 0.2 ml of iodine solution (0.025 M I_2 in 0.125 M KI) various concentrations of meticillin and deionized water added to a total volume of 1 ml. The tubes were incubated at 0° for 5 min and the residual activity was measured by the timed iodometric assay[13]. ○, free exoenzyme; △, co-aggregated exoenzyme; ●, lipid-depleted co-aggregated exoenzyme.

Fig. 6. Thermal inactivation of penicillinase in the presence of methicillin. Reaction mixtures consisted of 0.2 ml of 0.5% gelatin containing 180 units of enzyme and 0.2 ml of 0.1 M phosphate buffer (pH 7.3). The tubes were incubated at 48° for 2 min and transferred to an ice bath. The residual penicillinase activity was determined by the timed iodometric assay[13]. ○, free exoenzyme; △, co-aggregated exoenzyme; ●, lipid-depleted co-aggregated exoenzyme.

The lipid extraction procedure carried out at $-5°$ was mild and only 8 % of the penicillinase activity was destroyed after three successive extractions. The enzyme retained after partial extraction of the lipids could now be eluted by washing with the NaCl– citrate solution (see MATERIALS AND METHODS). After five successive washings 82 % of the co-aggregated enzyme was eluted and found to be indistinguishable from the native enzyme. The effect of co-aggregation on the thermostability of the exoenzyme in the presence of methicillin is shown in Fig. 6. Labilization of exopenicillinases to heat by methicillin has been previously described[7] and is further discussed below. The increased thermolability of the co-aggregated enzyme, shown in Fig. 6, is significant if not dramatic, and has been repeatedly confirmed. Moreover, the partial removal of the co-aggregate lipids, which has been described above, resulted in the complete restoration of the heat-inactivation pattern of the free exoenzyme, as shown by the overlapping curves obtained for the two preparations (Fig. 6).

DISCUSSION

The nonspecificity of the reaggregation phenomenon previously described[4,5] is further demonstrated in this work by showing that extraneous soluble proteins can co-aggreaate with membrane components. Unlike the hydrophobic membrane proteins which are almost completely incorporated in the presence of 20 mM $MgCl_2$ (ref. 2), the soluble proteins were found to co-aggregate to a limited extent.

The association of penicillinase with the membrane reaggregate appears to be due in part to electrostatic bonds, since a large part of the enzyme is easily eluted with a concentrated electrolyte solution. The remaining enzyme is not released by repeated washings and is probably bound to the aggregate by other, mainly hydrophobic bonds.

The possibility that the enzyme is trapped in closed vesicles seems to be eliminated by the observation that repeated sonication, which would readily disrupt such vesicles, did not result in selective solubilization of penicillinase. The constant ratio of penicillinase to total protein in the solubilized and precipitable fractions throughout that treatment could in fact be taken as a *prima facie* indication that the enzyme is incorporated in the same way as the reaggregated membrane proteins. However, selective elution of penicillinase can be obtained after partial removal of the co-aggregate lipids. Thus, although the precise location of the enzyme in the co-aggregate is not clear, the close association with the lipid components indicates that the enzyme has been embedded in a largely hydrophobic environment.

It was expected that the environmental constraint imposed by the embedding structure will modify the conformation of penicillinase, especially in view of the remarkable flexibility of this enzyme[20]. This was essentially corroborated in subsequent experiments, although the extent of modification was surprisingly small. Indeed, there was no change in the main characteristics which serve to distinguish between the extracellular and membrane-bound variants of penicillinase formed by *B. cereus*. These include thermostability, susceptibility to inactivation by iodine, and relative rates of hydrolysis of benzylpenicillin and methicillin.

A further criterion which is relevant to such comparison is the complete lack of inhibition of the catalytic activity of the natural membrane-bound enzyme by antibodies to the extracellular variant[19]. The embedded enzyme is clearly susceptible

to inhibition by such antibodies, although considerably less so than the soluble exoenzyme (Fig. 3). This difference could indicate a change in conformation involving antigenic determinants[20], although decreased accessibility of the embedded enzyme which would readily account for the difference, has not been ruled out.

A comparison involving more subtle and functionally significant differences was made at the level of enzyme-substrate interactions. The basic observation here is that the substrates have free access to the active site of the embedded enzyme, even when the co-aggregate is coated with antibodies to the original membrane. Yet, while the catalytic activity of the enzyme appears to be unmodified, the interaction of the enzyme with substrates is evident from the comparison of the conformative responses (*i.e.* the substrate-induced changes in conformation) of the free and bound enzymes. It has been shown[21] that the conformative response of the exopenicillinase to certain substrates (including methicillin) is characterized by concomitant appearanc to certain substrates (including methicillin) is characterized by concomitant appearance of sensitivity to heat and to iodination. The conformative response can be constrained (*e.g.* by homologous antibodies[22]) and a similar constraint is found in the natural membrane-bound penicillinase, which is thus considerably less responsive to the labilizing effect of such substrates[9]. The conformative response of the embedded exoenzyme does indeed reflect the constraining effect of the membrane-like structure and it differs from that of the free exoenzyme[21]. Of particular interest is the observation that it also differs qualitatively from the conformative response of the antibody-bound exopenicillinase in reaggregated membranes of *A. laidlawii* leads to the formation of a firmly embedded derivative, which is fully active.

The general properties of the new derivative are similar to those of the exoenzyme and unlike those of the natural membrane-bound variant of penicillinase. However, the conformative response of the co-aggregated enzyme to methicillin reflects the effect of the new environment, and sets it apart from the naturally occurring penicillinases.

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