Biochem. J. (1990) 267, 191–196 (Printed in Great Britain)

Shedding of hyaluronate synthase from streptococci

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Hyaluronate synthase was shed into the culture medium from growing streptococci (group C) together with nascent hyaluronate. The mechanism of solubilization was analysed using isolated protoplast membranes. Solubilization increased when membranes were suspended in larger volumes, but it was temperature-independent and was not inhibited by protease inhibitors. Increased hyaluronate chain length enhanced solubilization. The soluble synthase could re-integrate into Streptococcal membranes in a saturable manner. The soluble synthase behaved like an integral membrane protein, although it was not integrated into phospholipid vesicles. In sucrose velocity centrifugation the synthase had a higher sedimentation rate in detergent-free solution, indicating that it existed in an aggregated state.

INTRODUCTION

Streptococci are important causative agents of disease in man. The Lancefield groups A and C are responsible for rheumatic fever, glomerulonephritis and sub-acute endocarditis. These diseases may be autoimmune reactions elicited by bacterial antigens which cross-react with mammalian proteins [1,2]. Streptococci groups A and C are the only prokaryotes which produce hyaluronate, a ubiquitous extracellular polysaccharide on mammalian cell surfaces. Hyaluronate is synthesized in streptococci by a 52 kDa protein which cross-reacts immunologically with proteins in plasma membranes from eukaryotic cells [3,4].

Hyaluronate synthase resides in protoplast membranes of streptococci [5–8] and in plasma membranes of eukaryotic cells [9]. In streptococci, the hyaluronate capsule amplifies infectious virulence, because hyaluronate protects streptococci against immunological attacks from the infected host [10]. Hyaluronate also serves as a radical scavenger, being degraded and shed from the bacterial surface [11,12]. In addition, hyaluronate can be released by endogenous hyaluronidases from several strains [13]. It thus appears that the capsule is rapidly turned over. Growing streptococci also liberate several proteins into the culture medium, such as streptolysin, streptokinase and proteinases [14], but these proteins have not been shown to cross-react immunologically with eukaryotic proteins.

In this paper, we show that growing streptococci shed the hyaluronate synthase into the culture medium, and we analyse the mechanism of solubilization from isolated protoplast membranes.

MATERIALS AND METHODS

Materials

Radiochemicals were obtained from Amersham International, and other reagents were from Sigma Chemical Co.

General methods

Streptococci (group C, strain D181 from the Rockefeller University collection) were grown in 10 litres of Todd–Hewitt medium containing 6000 units of hyaluronidase from bovine testis. This enzyme treatment removed the bacterial capsule and facilitated the isolation of bacteria by centrifugation at 9000 *g* for 10 min by reducing the voluminous sediment. Isolation of protoplast membranes [3] and immunoblotting [15] were as described. Protoplast membranes were sedimented and purified by centrifugation at 100000 *g* for 60 min at 4 °C in an ultracentrifuge. Phospholipids were extracted with a mixture of chloroform/methanol (2:1, v/v) and determined as inorganic phosphate [16]. Antiserum was prepared by immunizing rabbits with the streptococcal hyaluronate synthase purified by preparative gel electrophoresis. Integral membrane proteins were separated from associated protein by phase separation of Triton X-114-solubilized membranes [17].

Determination of hyaluronate in culture medium

Medium from a growing culture (10 ml) was withdrawn at intervals and bacteria were sedimented at 10000 *g* for 10 min. A solution of 10% cetylpyridinium chloride (0.5 ml) was added and the mixture was incubated at 37 °C for 1 h. The precipitates were centrifuged for 10 min at 10000 *g* and dissolved in 1 ml of 1 M-NaCl. Aliquots were taken for determination of glucuronic acid by the method of Bitter & Muir [18].

Determination of hyaluronate synthase in culture medium

Two methods were used to determine the amount of hyaluronate synthase present. 1. Synthase activity was determined as described previously by incorporation of [^{14}C]-glucuronic acid from the precursor UDP-[^{14}C]glucuronic acid into hyaluronate [19]. 2. The following Western blot technique was developed to determine the amount of the 52 kDa synthase. Culture medium (12 ml) was withdrawn at intervals and bacteria were sedimented at 10000 *g* for 10 min. Poly(ethylene glycol-6000) (3 g) was dissolved in the supernatant and the solution was shaken at 4 °C overnight. The precipitates were sedimented for 15 min at 100000 *g* in an ultracentrifuge. The pellets were dissolved in 150 μl of 20 mM-Tris/malonate buffer, pH 7.0. Aliquots (50 μl) were separated by SDS/gel electrophoresis. Definitive amounts of purified synthase were run in parallel as standards. The separated proteins were blotted on to nitrocellulose and visualized by immunoblotting with anti-(hyaluronate synthase) antibodies as described by Tsang *et al.* [15]. The stained bands were excised, the nitrocellulose was dissolved in 1 ml of ethyl acetate and the absorbance at 450 nm was measured. The amounts of hyaluronate synthase were calculated by comparison with standards.

Determination of shedding from protoplast membranes

Protoplast membranes (10 μl; 40 μg) were suspended in 150 μl of phosphate-buffered saline (20 mM-sodium phosphate/0.15 M-

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NaCl, pH 7.2) and incubated for various times at 0 °C or 37 °C. The suspensions were centrifuged in an airfuge (Beckman) for 15 min at 100000 *g*. Hyaluronate synthase activities were determined in the supernatants and pellets suspended in 20 μl of water.

Shedding during hyaluronate chain g:owth

Protoplast membranes (10 μl; 40 μg) were suspended at 37 °C in 30 μl of an unlabelled substrate solution for hyaluronate synthesis which lacked $MgCl_2$ or UDP-GlcNAc, which are required for chain elongation (160 μM-UDP-GlcNAc or 10 mM-$MgCl_2$/8 μM-UDP-glucuronic acid/1 mM-dithiothreitol/20 mM-Tris/malonate, pH 7.0). Chain elongation was initiated in one series of samples by addition of 4 μl of 0.1 M-$MgCl_2$ or 4 μl of 1.6 mM-UDP-GlcNac. To another series of samples, hyaluronidase from bovine testis (0.3 units) was added to digest nascent chains. A control series received 4 μl of water instead of $MgCl_2$. After various times at 37 °C, the solutions were diluted with 130 μl of phosphate-buffered saline and centrifuged in an airfuge as described above. Residual unlabelled substrate was removed by washing the membranes with 170 μl of phosphate-buffered saline. The pellets were suspended in 20 μl of water and the synthase activities were determined.

Negative stain preparation

The constituents of the soluble and resuspended membrane fractions were negatively stained using 1% uranyl acetate for 1 min at room temperature. The negative stain preparations were examined in a Philips EM 201 at 60 kV.

RESULTS

Shedding of hyaluronate synthase

Growing streptococci released substantial amounts of hyaluronate synthase into the culture medium (Fig. 1). This was detected by specific antibodies directed against the synthase, which had previously been identified as a 52 kDa protein in protoplast membranes [3]. A detection method had to be developed which allowed the quantification of the 52 kDa synthase without interference from other antigenic degradation products. The 52 kDa protein was precipitated from streptococcal supernatants by poly(ethylene glycol 6000), separated from other proteins by polyacrylamide-gel electrophoresis, blotted on to nitrocellulose, stained by the immunoperoxidase method and quantified by light absorbance.

Streptococci produced 0.5 μg of soluble synthase/ml at the end of the log phase. The amount of bacteria-bound synthase at this stage (A_{600} 0.6) was 1.2 μg/ml, which represented 0.55% of total protein and 13% of protein in isolated protoplast membranes. Therefore streptococci produced 1.7 μg of total synthase/ml, of which 29% had been shed into the culture medium. Simultaneously, they had released 30 μg of hyaluronate/ml at the end of the log phase. It was not possible to detect any synthase activity in the culture medium, because the enzyme was inactivated.

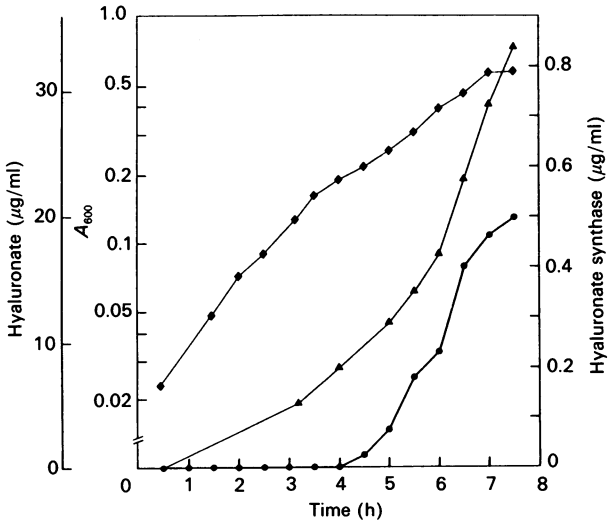

Fig. 1. Shedding of hyaluronate synthase from streptococci

Streptococci (strain D181) were grown in Hewitt–Todd medium. At the times indicated, portions were withdrawn for determination of absorbance at 600 nm (◆). Bacteria were centrifuged and the supernatants were analysed for concentrations of hyaluronate synthase (●) and hyaluronate (▲).

Characterization of shedding

A similar solubilization of hyaluronate synthase was found when isolated protoplast membranes were suspended in phosphate-buffered saline. In this solution, the synthase remained active and it was quantified in subsequent experiments by determination of enzyme activity, which was a more accurate parameter than quantitative immunoblotting. In the following experiments, the immunoblotting technique was performed only to validate enzyme determination.

The mechanism of solubilization was investigated in more detail with protoplast membranes. Membranes were prepared from streptococci harvested in the late log phase by sonic disruption and were isolated by ultracentrifugation. The synthase activity was mainly associated with the membranes, but it was also found in the supernatant after repeated ultracentrifugation and resuspension of membranes. About 20% of the synthase was solubilized by the initial washing steps. Other proteins were also shed from the membranes as detected by gel electrophoresis (results not shown), but the specific activities of the soluble synthase increased with every centrifugation step.

Shedding of the synthase from protoplast membranes might be a time- and temperature-dependent process. These parameters were determined in the following experiments. Streptococcal membranes were incubated at 0 °C or 37 °C for various times in phosphate-buffered saline. They were then sedimented by ultracentrifugation, and the remaining synthase activity was determined in the sediment. Shedding at 0 °C and 37 °C was identical and reached a plateau after 15 min (results not shown). These results suggested that solubilization was not an enzymic reaction.

Because shedding may be volume-dependent, equal amounts of membranes were serially diluted and sedimented after 15 min by ultracentrifugation. Fig. 2 shows that shedding of synthase activity indeed increased with the degree of dilution. This increase was reflected by greater amounts of soluble synthase as shown by Western blotting (Fig. 2 inset).

It is known that membrane proteins can behave as soluble proteins in ultracentrifugation, when they are inserted in small unilaminar lipid vesicles [20,21]. Therefore we determined the concentrations of phospholipids in membrane supernatants after ultracentrifugation at 100000 *g* for 30 min. However, no phospholipids were detected in the supernatants. The membrane suspension and the supernatant were also analysed by electron microscopy. The membrane suspension consisted of vesicles and the supernatant contained amorphous material. Therefore the soluble synthase was not integrated into phospholipid vesicles.

Is the soluble synthase an integral membrane protein?

It is possible that proteases could degrade and solubilize the synthase even at 0 °C to remove a membrane intercalated

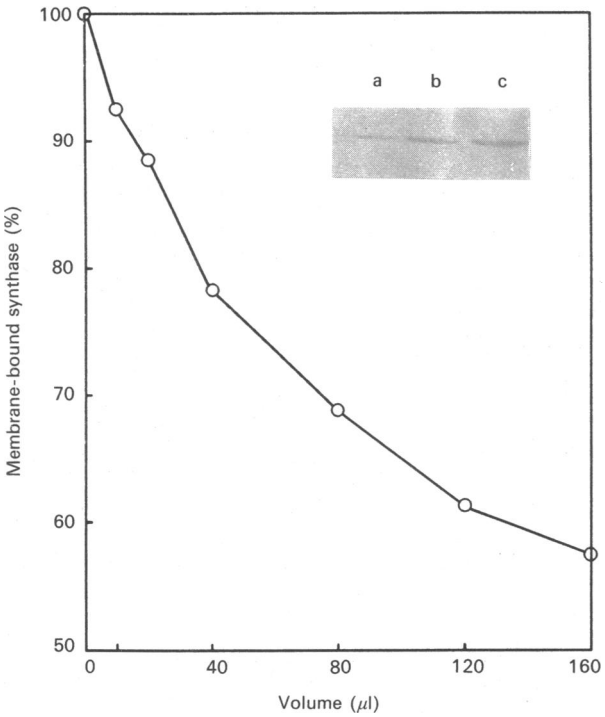

Fig. 2. Volume-dependence of shedding

Membranes (40 μg) were suspended in phosphate-buffered saline as indicated and kept at 37 °C for 15 min. They were sedimented at 100000 *g* for 15 min and their synthase activities were determined. Inset: The soluble synthase was analysed by Western blots in volumes of 30 μl (lane a), 40 μl (b) and 70 μl (c).

sequence. This possibility was analysed by comparing the extent of shedding in the presence and in the absence of protease inhibitors. Soluble and membrane-bound proteins were separated by gel electrophoresis and the synthase was visualized by Western blotting. Fig. 3 (lanes a–d) shows that protease inhibitors did not influence shedding and that the size of the soluble and membrane-bound synthases were identical, indicating that proteolysis is unlikely to have occurred.

Triton X-114 separation is a method which can distinguish integral membrane proteins and soluble proteins [17].

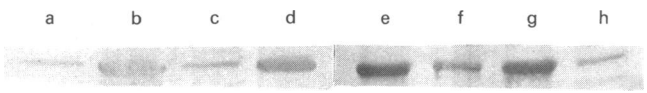

Fig. 3. Effect of protease inhibitors and Triton X-114 separation on synthase

Membranes (30 μg) were suspended in 150 μl of phosphate-buffered saline at 0 °C and sedimented in an airfuge at 100000 *g* for 20 min. The sediment was resuspended in 150 μl of phosphate-buffered saline (a,b) or phosphate-buffered saline containing 1 mM-phenylmethanesulphonyl fluoride, 10 mM benzamidine and 0.15 trypsin inhibitory units of aprotinin (c,d). After 20 min a 0 °C, the suspensions were again centrifuged in an airfuge and the synthase in the supernatants (a,c) and in the membrane pellets (b,d) was visualized by Western blotting. In another experiment the membrane pellets after the first ultracentrifugation (e,f) were resuspended in 150 μl of phosphate-buffered saline containing 1% Triton X-114. To the supernatants (g,h), Triton X-114 was added to a final concentration of 1%. The mixtures were incubated at 0 °C for 30 min and at 37 °C for 5 min. The turbidity was cleared by centrifugation at 10000 *g* for 1 min and the proteins in the organic (e,g) and aqueous (f,h) phases were analysed by Western blotting.

Streptococcal membranes were treated with hyaluronidase to remove endogenous hyaluronate and solubilized in Triton X-114. The solubilized proteins were separated at 37 °C into an organic phase containing integral membrane proteins and an aqueous phase containing the soluble proteins. The amount of synthase was compared in both phases by Western blotting. Fig. 3 (lanes e and f) shows that most of the synthase was found in the organic phase, indicating that the synthase behaved as an integral membrane protein. This separation was also performed with the soluble synthase. Fig. 3 (lanes g and h) shows that large amounts of the soluble synthase separated into the organic phase, indicating that it still contained the membrane intercalated protein sequence.

Effect of hyaluronate chain growth on shedding

If the synthase is shed from membranes as a complex with nascent hyaluronate, the chain length of the latter would influence the shedding rate, because it would make the complex more hydrophilic. In a preliminary experiment, we analysed the elongation rate of hyaluronate chains in order to obtain an appropriate time scale for measuring shedding of the synthase in the presence of hyaluronate chain growth.

Protoplast membranes were isolated from streptococcal cultures which contain hyaluronidase to remove the capsule and thus facilitate isolation of bacteria. Therefore these membranes synthesized hyaluronate from short chains upon incubation with substrate. Chain elongation could be monitored by gel filtration on Sephacryl S-1000. Fig. 4 shows the elution profiles after

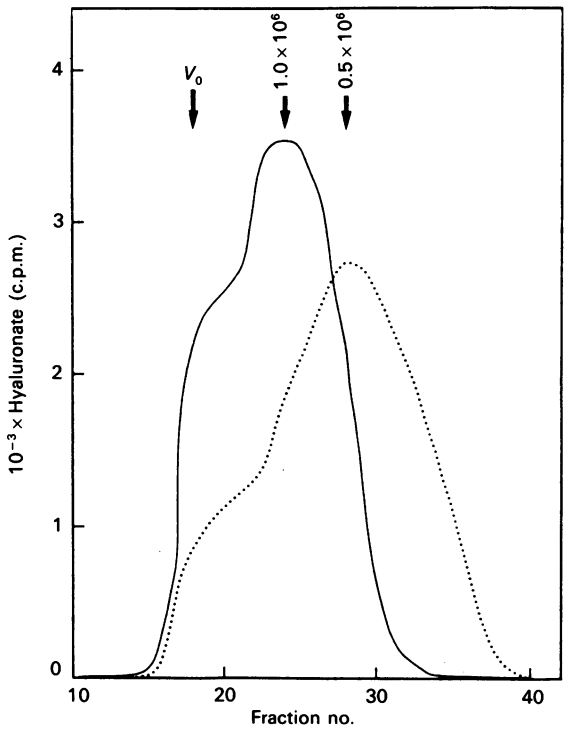

Fig. 4. Growth of hyaluronate chains

Streptococcal membranes (6 μg) were incubated in 120 μl of buffer containing radioactive substrate (160 μM-UDP-GlcNAc/8 μM-UDP-[^{14}C]glucuronic acid/1 mM-dithiothreitol/20 mM-Tris/malonate, pH 7.0) for 60 min (····) or 120 min (——). Chain elongation was stopped by addition of 10 μl of 10% SDS, the mixture was dialysed against water and the size of hyaluronate was determined by gel filtration on a Sephacryl S-1000 column (0.6 cm × 48 cm) with phosphate-buffered saline/0.1% SDS as eluant. Fractions of 0.35 ml were collected and their radioactivity was determined. Molecular mass (Da) is indicated by the arrows.

incubation at 37 °C for 60 and 120 min. The molecular masses were calculated from $K_{av.}$ values for hyaluronate obtained from the manufacturer. The $K_{av.}$ values of the growing chain population was 0.45 after 60 min and 0.32 after 120 min, corresponding to average molecular masses of 5.15×10^5 and 1.04×10^6 Da respectively. These results show that the chains were started *de novo* or from small nascent fragments. The elongation rate was equivalent to about 22 repeating units/min.

The experiment just described showed that hyaluronate grew to a large size within 2 h. Shedding of the synthase was measured during chain elongation. Membranes were suspended in buffers containing substrates for hyaluronate synthesis but lacking $MgCl_2$ and chain elongation was initiated by addition of $MgCl_2$ after various times. A control series did not receive $MgCl_2$. Membranes were sedimented and the residual synthase activity was determined. Fig. 5(*a*) shows that hyaluronate chain growth enhanced solubilization of the synthase. Similar results were obtained when membranes were incubated in a solution containing $MgCl_2$ but lacking UDP-GlcNAc and in which chain growth was initiated by addition of UDP-GlcNAc (results not shown). The amounts of soluble synthase were again visualized by Western blotting. Fig. 5(*b*) verifies that increased amounts of the synthase were shed during chain growth of hyaluronate. Shedding was not enhanced when chain elongation was impossible due to the lack of one constituent such as $MgCl_2$ or UDP-GlcNAc. When hyaluronidase was added during active synthesis, shedding was not increased. This indicated that shedding was dependent on hyaluronate chain length.

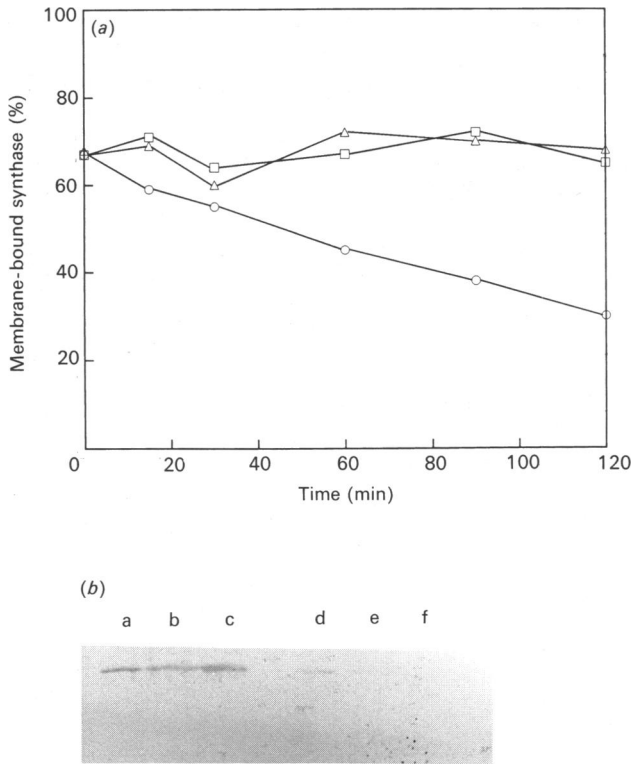

Fig. 5. Effect of hyaluronate on shedding

(*a*) Membranes were incubated with unlabelled substrate without $MgCl_2$. One series received $MgCl_2$ for chain elongation for the times indicated (○) and the other samples served as controls (△). Hyaluronidase was added to one series of samples to digest nascent chains (□). Membranes were separated from the substrate by ultracentrifugation and their hyaluronate synthase activity was determined. (*b*) Membranes (0.5 mg) were incubated in 2 ml of substrate for hyaluronate chain growth for 15 min (a), 60 min (b) or 120 min (c) and in substrate lacking UDP-GlcNAc for 15 min (d), 60 min (e) or 120 min (f). The soluble fractions were separated from membranes and analysed by polyacrylamide-gel electrophoresis and Western blotting.

Re-integration of the synthase into the particulate fraction

Is solubilization of the synthase a reversible process? This question was studied by re-integration of the synthase into the particular fraction. For these experiments, synthase-deficient membranes were required to eliminate endogenous synthase activity in recipient membranes which could mask the newly incorporated activity. These membranes were isolated from a synthase-deficient mutant [3]. Increasing amounts of the inactive

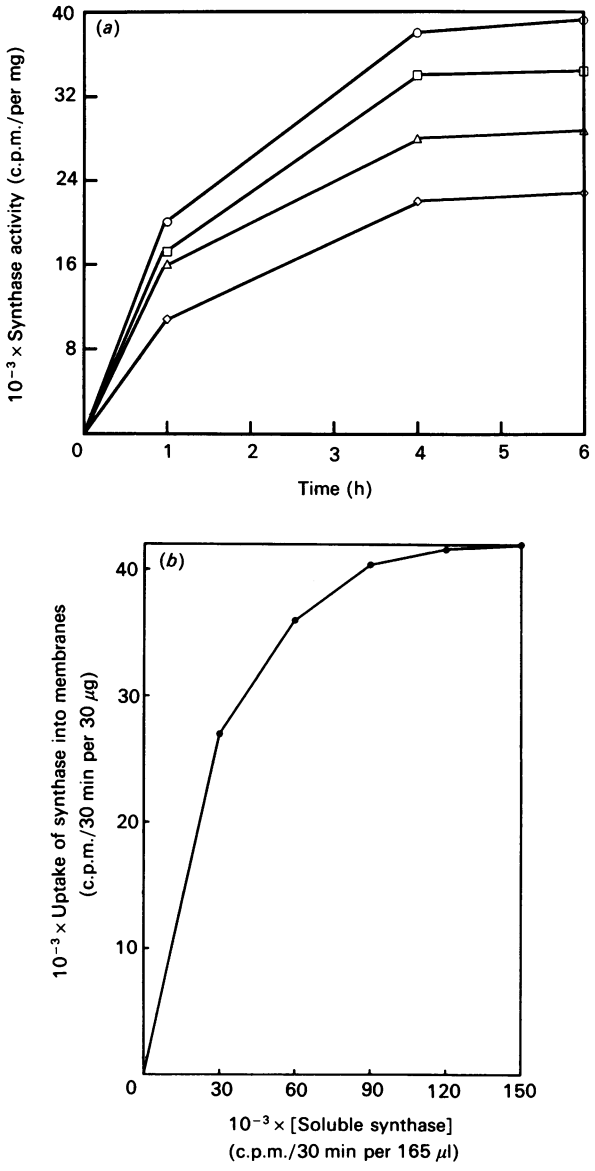

Fig. 6. Re-integration of soluble synthase

(*a*) Increasing amounts of inactive membranes (◇, 250 μg; △, 500 μg; □, 750 μg; ○, 1000 μg) were incubated with 4 ml of active soluble synthase for 1, 4 or 6 h, sedimented by centrifugation and the incorporated synthase activity was determined. (*b*) Inactive membranes (30 μg) were incubated with increasing concentrations of the 100000 *g* supernatant from active membranes in 165 μl of phosphate-buffered saline at 0 °C for 4 h. The membranes were sedimented at 100000 *g* for 30 min and their synthase activity was determined.

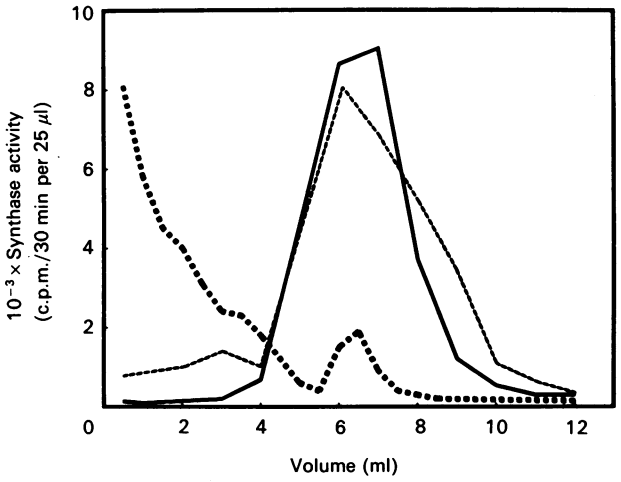

Fig. 7. Sucrose velocity centrifugation of synthase

Membranes (160 μl, 4.8 mg) were sedimented from a phosphate-buffered saline suspension for 30 min at 100000 *g*. The supernatant was loaded on to a 12 ml gradient of 5–20 % sucrose in phosphate-buffered saline and centrifuged for 20 h at 160000 $g_{av.}$ at 4 °C. The gradient was fractionated from the bottom into 0.5 ml fractions and the synthase activity was determined from portions of 25 μl (· · · ·). The supernatant was supplemented with digitonin to a final concentration of 1 %, loaded on to a 5–20 % sucrose gradient containing 0.5 % digitonin and centrifuged as above (----). The membrane pellet was solubilized in 5 ml of phosphate-buffered saline/0.5 % digitonin by pipetting and stirring at 0 °C for 30 min. Undissolved material was removed by centrifugation at 100000 *g* for 30 min. The supernatant was loaded on to a 12 ml gradient of 5–20 % sucrose in phosphate-buffered saline/0.5 % digitonin. Centrifugation and determination of synthase activity was performed as above (——).

membranes were incubated for 1–6 h with constant amounts of active soluble synthase; they were then sedimented and the synthase activity was determined. Fig. 6(*a*) shows that the soluble synthase was incorporated into the particulate fraction in a time- and concentration-dependent manner. Altogether, 90 % of the soluble synthase was incorporated into 1 mg of protoplast membranes after 6 h.

In another experiment, increasing amounts of soluble synthase were added to constant amounts of inactive membranes. The incorporation of the synthase into membranes was found be hyperbolic and reached a saturation maximum (Fig. 6*b*).

Shedding as an aggregate

We tested the possibility that the soluble and membrane-bound synthases existed in different aggregation states by using velocity sedimentation in sucrose gradients. Membranes were solubilized by digitonin and proteins were separated in a 5–20 % sucrose gradient containing 0.5 % digitonin. Fig. 7 shows that the synthase migrated to the middle of the gradient. The soluble synthase was centrifuged on a similar gradient lacking 0.5 % digitonin and most of it migrated to the bottom of the gradient. When digitonin was added to the soluble synthase and centrifuged on the sucrose gradient containing 0.5 % digitonin, the synthase again activity migrated to the middle of the gradient. These results indicated that the synthase was shed from membranes as aggregates which could be disrupted by detergents.

DISCUSSION

Large amounts of hyaluronate synthase were shed from growing streptococci together with hyaluronate. Protoplast membranes also released a soluble synthase in an enzymically active form which could not be sedimented at 100000 *g*. The process of solubilization was studied with purified membranes, because the culture medium inhibited the synthase activity which was a more convenient parameter for the amount of enzyme than Western blotting. In all experiments, the soluble enzyme mass corresponded to soluble enzyme activity.

Solubilization of the synthase was not accompanied by release of lipids, eliminating the possibility that small unilaminar vesicles are formed. This was corroborated by electron microscopy. Protease inhibitors did not inhibit shedding and the soluble and membrane-bound synthases had identical molecular masses, suggesting that solubilization was a non-enzymic reaction. Triton X-114 phase separation indicated that the soluble synthase had retained the membrane intercalated protein sequence, since most of it partitioned into the detergent phase.

Hyaluronate chain growth increased the amount of synthase liberated from membranes. This effect was dependent on chain length, because increased shedding was not observed in the presence of hyaluronidase. This result suggested that the synthase was solubilized together with hyaluronate. Large nascent hyaluronate chains attached to the synthase probably increased the hydrophilicity of the complex. It is not known under which conditions the synthase is dissociated from nascent hyaluronate chains, but it seems likely that dissociation rate of the complex is slower than the shedding from membranes.

The soluble synthase re-integrated into inactive membranes in a time- and concentration-dependent manner. Thus solubilization and re-integration could be reversible reactions. An equilibrium between the soluble and the membrane-bound form could also explain why dilution of membranes led to increased solubilization, and why solubilization was complete after 15 min.

How can an integral membrane protein be solubilized without proteolytic degradation or loss of a hydrophobic protein sequence? The hydrophobic segment could be modified or masked. Self-aggregation of hydrophobic segments could be responsible for increased solubility. Aggregation of the soluble synthase could indeed be demonstrated by sucrose velocity centrifugation. The aggregates of the soluble synthase could be dispersed by detergents.

Solubilization of hyaluronate synthase from growing streptococci and from protoplast membranes led us to assume that both processes were mediated by the same mechanism. However, we cannot exclude the possibility that additional reactions participate in solubilization from bacteria, such as enzyme modification.

Is it an advantage for streptococci to release the synthase together with nascent hyaluronate? Because they are attacked by the immune system of the infected host, they try to liberate their surfaces from adsorbed material. Under aerobic conditions the capsule is degraded by radicals to release hyaluronate together with adsorbed material. Under non-aerobic conditions, however, radical-induced degradation could be reduced and long hyaluronate chains may be complexed to the synthase. In this case, two shedding mechanisms are feasible: dissociation of hyaluronate from the membrane-bound synthase which then replaces hyaluronate, or release as a complex of synthase and hyaluronate. We have shown here that the latter process is likely in streptococci, but we cannot exclude the first possibility.

The bacterial hyaluronate synthase cross-reacted with its eukaryotic counterpart [4]. Since streptococci are unique in eliciting rheumatic fever which could be caused by cross-reacting antibodies [1], the soluble synthase liberated into an infected host could be responsible for an autoimmune reaction.

We thank Mrs. D. Thiele, Mrs. A. Blanke and Mrs. G. Reinhold for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 310, B3 and B8). H. R. was a Heisenberg fellow of the Deutsche Forschungsgemeinschaft.

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Received 29 March 1989/14 November 1989; accepted 12 December 1989