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Isolation of streptococcal hyaluronate synthase

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Hyaluronate synthase was isolated from protoblast membranes of streptococci by Triton X-114 extraction and cetylpyridinium chloride precipitation. It was identified as a 52000-M_r protein, which bound to nascent hyaluronate and was affinity-labelled by periodate-oxidized UDP-glucuronic acid and UDP-*N*-acetylglucosamine. Antibodies directed against the 52000-M_r protein inhibited hyaluronate synthesis. Mutants defective in hyaluronate synthase activity lacked the 52000-M_r protein in membrane extracts. Synthase activity was solubilized from membranes by cholate in active form and purified by ion-exchange chromatography.

INTRODUCTION

Hyaluronate is synthesized at the inner side of plasma membranes by alternate transfer of UDP-hyaluronate to the substrates UDP-*N*-acetylglucosamine and UDP-glucuronic acid (Prehm, 1983*a*,*b*). The nascent chains extrude through the membranes into the extracellular matrix (Prehm, 1984). This synthesis can be inhibited by periodate-oxidized UDP-glucuronic acid or UDP-*N*-acetylglucosamine, which can also be used as affinity labels for the synthase (Prehm, 1985). We have used these inhibitors to identify the synthase from streptococcal protoblast membranes, which have a 100-fold-higher specific activity than mammalian cells (Markovitz & Dorfman, 1962). Since the synthase binds nascent hyaluronate, we took advantage of this affinity to purify the enzyme in active form.

MATERIALS AND METHODS

Materials

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

General methods

The streptococcal C strain D181 was obtained from the Rockefeller University collection. Established procedures were used for mutagenization (Adelberg *et al.*, 1965), determination of the hyaluronate synthase (Prehm, 1980), isolation of streptococcal protoblast membranes (Sugahara *et al.*, 1979) and Triton X-114 extraction (Bourdier, 1981).

Isolation of the hyaluronate synthase

The streptococcal strain D181 was grown in Hewitt–Todd medium at 37 °C with agitation to $A_{600} = 1.0$ (Holmström & Ricina, 1967). To maintain the pH at 7.0, 10 M-NaOH was added during cultivation. The culture was chilled to 4 °C, and the bacteria were sedimented at 9000 *g* for 20 min. After being washed with phosphate-buffered saline (150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.2), the bacterial suspension in 200 ml of phosphate-buffered saline was sonified at 120 W in a cooling rosette at 4 °C four times for 3 min at 5 min intervals. The disrupted bacteria were centrifuged at 9000 *g* for 20 min, and the supernatant was centrifuged at 120000 *g* for 60 min. The sediment contained crude protoblast membranes, which were suspended by sonication in the appropriate buffer at 4 °C and again sedimented at 120000 *g* for 60 min. The membranes were suspended in 40 mM-sodium phosphate buffer, pH 6.8, by sonication at a protein concentration of 5 mg/ml. To load the active synthase with hyaluronate, the suspension was incubated for 30 min at 37 °C with 1 mM-UDP-*N*-acetylglucosamine, 1 mM-UDP-glucuronic acid, 4 mM-dithiothreitol and 10 mM-$MgCl_2$. The membranes were separated from the substrate buffer by ultracentrifugation at 120000 *g* for 30 min. The membranes were suspended in 40 mM-sodium phosphate buffer, pH 6.8. Triton X-114 was added to a final concentration of 2% (v/v) at 4 °C. The sample was warmed at 37 °C until the solution became turbid. It was sedimented in an Eppendorf centrifuge at 10000 *g* for 1 min. The upper, aqueous, phase was withdrawn and again subjected to the same Triton X-114 extraction. Cetylpyridinium chloride (10%, w/v) was added to the aqueous phase of the second phase separation to a final concentration of 1% (w/v) and the mixture was kept at 37 °C for 30 min. A white precipitate formed, which was centrifuged at 10000 *g* for 5 min. The precipitate was suspended in 10% (w/v) sodium acetate in methanol by sonication at room temperature and again centrifuged at 10000 *g* for 3 min. The precipitate was dissolved in the appropriate buffer containing detergent and used for further analysis. It contains highly enriched hyaluronate synthase.

Purification of the enzymically active synthase

A 1 mg portion of streptococcal membranes in 0.5 ml of 40 mM-sodium phosphate buffer, pH 6.8, was solubilized by addition of 50 μl of 10% (w/v) sodium cholate at 0 °C. The solution was applied to a 2 ml column of DEAE-Sephacel at 4 °C and eluted with 10 ml of a linear gradient of 0–0.6 M-NaCl in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1% sodium cholate. Fractions (1 ml) were collected, and 50 μl portions were tested for hyaluronate synthase (Prehm, 1980).

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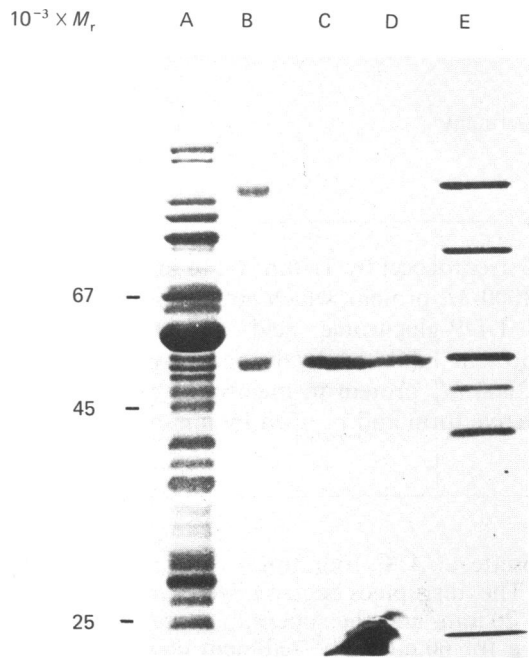

Fig. 1. Identification of the hyaluronate synthase

Lane A shows the Coomassie Blue-stained gel of proteins from streptococcal protoblast membranes. Lane B shows membrane proteins extracted with hyaluronate as described in the Materials and methods section and stained with Coomassie Blue. Lanes C and D show fluorography of the affinity-labelled proteins. Streptococcal membranes (200 μg) were incubated with 1 mCi of periodate-oxidized substrates [^{3}H]UDP-glucuronic acid (lane C) or [^{3}H]UDP-*N*-acetylglucosamine (lane D) (specific radioactivity 17.6 Ci/mmol) for 60 min at 37 °C in 100 μl of 40 mM-sodium phosphate buffer, pH 6.8. The mixture was diluted with 10 ml of 40 mM-sodium phosphate buffer, pH 6.8, and the membranes were sedimented at 100000 *g* for 30 min. The membrane pellets were dissolved in 100 μl of loading buffer and subjected to SDS/polyacrylamide-gel electrophoresis. The gel was stained with Coomassie Blue, impregnated with 2,5-diphenyloxazole and exposed to a Kodak X-ray film for 40 h. Lane E shows the silver-stained proteins eluted from the DEAE-Sephacel column that had the highest hyaluronate synthase activity.

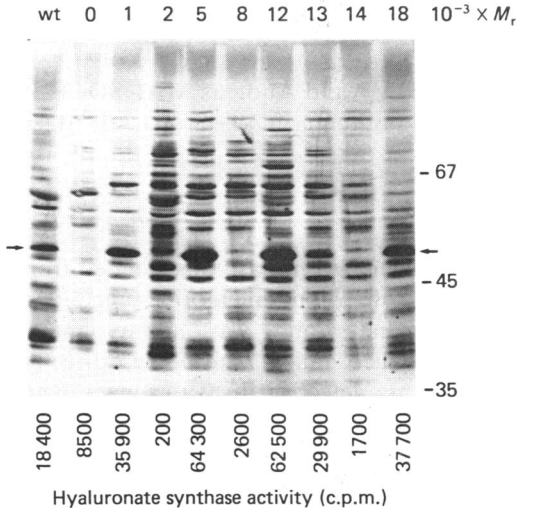

Fig. 2. Membrane proteins of hyaluronate-deficient mutants

Hyaluronate-defective mutants of streptococci were isolated from a culture mutagenized with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine by their glossy appearance (Wilson, 1959). Protoblast membranes were prepared from wild-type (wt) bacteria and mutants (0 to 18) after sonic disruption by differential centrifugation (Sugahara *et al.*, (1979). The synthase activity of the membranes was compared with the protein pattern on polyacrylamide-gel electrophoresis of Triton X-114 detergent extract. A 40 μl portion of 10% (v/v) Triton X-114 was added to 2 mg of membranes from hyaluronate-deficient streptococci in 200 μl of 40 mM-sodium phosphate buffer, pH 6.8, at 4 °C, warmed to 37 °C and centrifuged at 10000 *g* for 3 min. The supernatant was subjected to electrophoresis on 10% (w/v) polyacrylamide gel. The hyaluronate synthase activity of intact membranes was determined as described previously (Prehm, 1980).

RESULTS AND DISCUSSION

Isolation of proteins bound to nascent hyaluronate

The hyaluronate synthase resided in protoblast membranes of streptococci (Markovitz & Dorfman, 1962). Because the enzyme should form a complex with nascent hyaluronate, we used the unique properties of hyaluronate for the isolation of the enzyme. Streptococcal membranes were incubated with UDP-*N*-acetylglucosamine and UDP-glucuronic acid to load the synthase with hyaluronate. When the membranes were solubilized at 4 °C with Triton X-114 and warmed to 37 °C, two phases formed (Bourdier, 1981). The organic phase contained most of the solubilized membrane proteins, and the water phase contained hyaluronate. Hyaluronate was precipitated from the aqueous phase by cetylpyridinium chloride, and the associated proteins were analysed by polyacrylamide-gel electrophoresis (Fig. 1, lane B). Only two proteins were obtained, with M_r values of 52000 and 88000.

For identification of the hyaluronate synthase, the streptococcal membranes were analysed by affinity labelling with periodate-oxidized [^{3}H]UDP-*N*-acetylglucosamine and [^{3}H]UDP-glucuronic acid, which bind covalently to the enzyme (Prehm, 1985). This affinity-labelled proteins were separated by polyacrylamide-gel electrophoresis and located by fluorography (Fig. 1, lanes C and D). The 52000-M_r protein was most prominently labelled.

The role of this protein in hyaluronate synthesis was further confirmed by the analysis of membrane proteins from hyaluronate-deficient mutants. Some of the mutants lacked hyaluronate synthase, as determined in membranes (Fig. 2). When the membranes were subjected to Triton X-114 extraction, the occurrence of the 52000-M_r protein (Fig. 2, arrow) correlated with synthase activity in membranes. Other mutants had an active enzyme in the membranes, although the bacteria did not produce any hyaluronate. These were probably defective in genes responsible for substrate synthesis. These results indicated that the 52000-M_r protein was the synthase.

Purification of the enzymically active synthase

Since solubilization of the streptococcal membranes with Triton X-114 destroyed enzyme activity, the enzyme

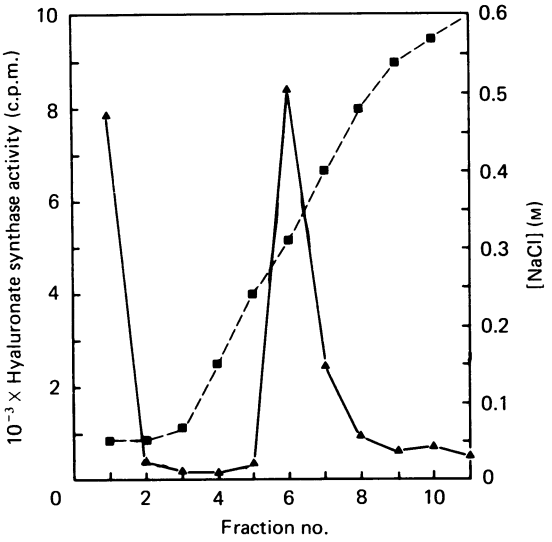

Fig. 3. Purification of hyaluronate synthase in active form

Streptococcal membranes were solubilized by cholate and the proteins were separated by ion-exchange chromatography on DEAE-Sephacel as described in the Materials and methods section. ▲, Hyaluronate synthase; ■, salt concentration.

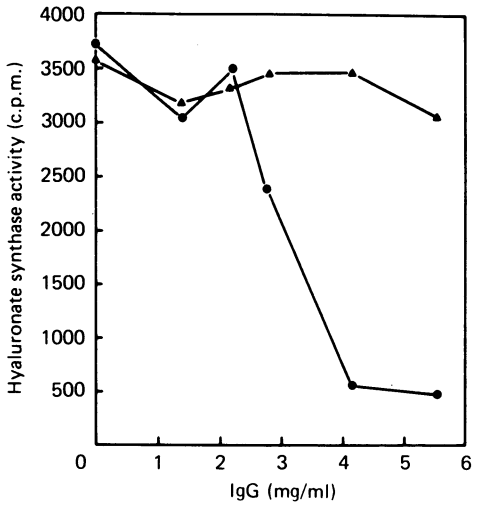

Fig. 4. Inhibition of hyaluronate synthase by antibodies

Streptococcal membranes (25 μg) in 25 μl of 40 mM-sodium phosphate buffer, pH 6.8, were incubated with the IgG fraction of pre-immune serum (▲) or of antiserum against the 52000-M_r protein (●) for 2 h at the concentration indicated. Hyaluronate synthase activity was determined as described previously (Prehm, 1980).

was loaded with hyaluronate by incubation with UDP-*N*-acetylglucosamine and UDP-glucuronic acid, and the membranes were solubilized with cholate. The synthase could be separated in active form from most other proteins by ion-exchange chromatography on DEAE-Sephacel (Fig. 3). The enzyme activity was eluted together with hyaluronate at a salt concentration of 0.3 M-NaCl at pH 7.5. Analysis of the eluted proteins by polyacrylamide-gel electrophoresis showed again the 52000-M_r and 88000-M_r bands, as well as other proteins with M_r values of 75000, 47000, 42000 and 24000. The protein pattern of the most active fraction from the ion-exchange column is shown in Fig. 1 (lane E).

Antibodies against the 52000-M_r protein

The 52000-M_r protein was isolated by Triton X-114 extraction and cetylpyridinium chloride precipitation, and further purified by preparative polyacrylamide-gel electrophoresis. The Coomassie Blue-stained band was cut out and used to immunize rabbits. IgG was prepared from the antiserum by affinity chromatography on Protein A–Sepharose. The purified IgG inhibited hyaluronate synthase of streptococcal membranes in a concentration-dependent manner (Fig. 4). IgG from pre-immune serum had no effect.

Because the 88000-M_r protein was not affinity-labelled (Fig. 1) and its occurrence in mutants did not correlate with the synthase activity (Fig. 2), it could not represent the active enzyme and was not analysed further. These results suggest that the 52000-M_r protein was the hyaluronate synthase.

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