Solubilization of Hyaluronic Acid Synthetic Activity from Streptococci and Its Activation with Phospholipids*

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Hyaluronic acid is a ubiquitous nonbranching acid mucopolysaccharide composed of β 1-4-linked repeating disaccharide units of glucuronic acid β 1-3 linked to N-acetylgalactosamine. Even though the hyaluronic acid biosynthetic system was one of the first membrane heteropolysaccharide synthetase pathways studied (1), our understanding of the mechanism of synthesis of hyaluronic acid is still incomplete. This lack of knowledge is attributed to an inability to solubilize the synthetic system from either eucaryotic or procaryotic sources.

Many investigators have taken advantage of the abundance and exclusive production of the glycosaminoglycan hyaluronic acid by groups A and C streptococci (2-5). Other workers have investigated hyaluronic acid production in mammalian systems using intact membranes or whole cells (6-9). These studies, using particulate enzyme preparations, demonstrated the activity from the digitonin extract, particularly phosphatidylethanolamine and phosphatidylyserine. In system, the specific activity of hyaluronic acid synthetase was increased up to 63 times that of the system of the intact membrane. Furthermore, the total activity of the reconstituted system was 4.9 times greater than that of intact membranes. The soluble enzyme system showed similarities to the membrane-bound synthetase in the kinetics of production of trichloroacetic acid-soluble and -insoluble hyaluronic acid, and the hyaluronic acid produced was of comparable molecular weight.

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To date all hyaluronic acid synthetin systems have been of a particulate nature, and attempts at solubilization have been unsuccessful. This has hampered attempts to elucidate the mechanism by which hyaluronic acid is produced. In this paper we demonstrate that the hyaluronic acid synthetic activity from group C streptococcal membranes was solubilized using 2% digitonin and that the activity was optimized by reconstitution with cardiolipin at an optimum phospholipid/protein ratio (μg/μg) of 5:1. Furthermore, chromatography of the solubilized synthetase demonstrated that it eluted after the void volume of a Sepharose CL-6B column. CHAPSO, octyl glucopyranoside, sodium cholate, Triton X-100, and zwittergent 314 either inhibited or failed to solubilize the synthetase activity. Phospholipids other than cardiolipin also reconstituted the activity from the digitonin extract, particularly phosphatidyl-ethanolamine and phosphatidylyserine. In system, the specific activity of hyaluronic acid synthetase was increased up to 63 times that of the system of the intact membrane. Furthermore, the total activity of the reconstituted system was 4.9 times greater than that of intact membranes. The soluble enzyme system showed similarities to the membrane-bound synthetase in the kinetics of production of trichloroacetic acid-soluble and -insoluble hyaluronic acid, and the hyaluronic acid produced was of comparable molecular weight.

Hyaluronic acid is a ubiquitous nonbranching acid mucopolysaccharide composed of β 1-4-linked repeating disaccharide units of glucuronic acid β 1-3 linked to N-acetylgalactosamine. Even though the hyaluronic acid biosynthetic system was one of the first membrane heteropolysaccharide synthetase pathways studied (1), our understanding of the mechanism of synthesis of hyaluronic acid is still incomplete. This lack of knowledge is attributed to an inability to solubilize the synthetic system from either eucaryotic or procaryotic sources.

Many investigators have taken advantage of the abundance and exclusive production of the glycosaminoglycan hyaluronic acid by groups A and C streptococci (2-5). Other workers have investigated hyaluronic acid production in mammalian systems using intact membranes or whole cells (6-9). These studies, using particulate enzyme preparations, demonstrated that certain factors were essential for the reconstitution of activity of this solubilized synthetic system. In the past, attempts to solubilize the enzyme system with detergents (6) or organic solvents (18) resulted in complete inactivation of the synthetase. In this report we demonstrate the first successful solubilization of the streptococcal hyaluronic acid synthetic system using the detergent, digitonin. In addition, phospholipids were required for the reconstitution of activity of this solubilized synthetic system, and their presence increased the total activity above that of intact membranes. When compared to intact membranes and the particulate enzyme systems previously described, the solubilized synthetase produced hyaluronic acid at an equivalent rate and in the same molecular weight range, and a specific activity of 2406 nmol of glucuronic acid transferred/h/mg of protein was demonstrated for the reconstituted system.

**EXPERIMENTAL PROCEDURES**

**Materials** - Zwittergent 314, octyl-β-D-glucopyranoside, CHAPSO, and 98% pure digitonin were purchased from Calbiochem Behring. Digitonin (80% pure), sodium cholate, Triton X-100, phosphatidylethanolamine (type V, bovine brain), phosphatidylserine (bovine brain), phosphatidyglycerol (egg yolk lecithin), cardiolipin (bovine heart), uridine diphosphoglucuronic acid, and uridine diphospho-N-acetylgalactosamine were purchased from Sigma. Phosphatidylinositol (pig liver) was purchased from Serdary Research Laboratories and UDP-[U-14C]glucuronic acid (specific activity 325.0 mCi/mmol) from New England Nuclear.

**Bacteria, Media, and Membranes** - Group C streptococci (strain D181) were grown in chemically defined medium as described by van de Rijn and Kessler (13) and served as the source of membranes with hyaluronic acid synthetase activity. Membranes were prepared using phase-associated lysozyme as previously described (14). Briefly, the organism was grown in 15 liters of chemically defined medium to mid

$$**^{1}** The abbreviation used is: CHAPSO, 3-[3-cholamidopropyl-di-methylammonio]-1-[2-hydroxy-1-propanesulfonate]-2H_2O.
exponential phase (0.4 absorbance units at 650 nm), treated with hyaluronidase (bovine tissues, Worthington; 3 mg/liter) and harvested in a continuous flow centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, PA.). The bacteria were washed in cold 0.15 M NaCl and resuspended to 10% (w/v) in protoplasting buffer (0.05 M Na2HPO4/KH2PO4, pH 6.1, 0.5 mM MgCl2, 0.5 mM dithioerythritol) containing 100 units/ml of lysozyme and sedimented at 9000 g for 10 min. The protoplasts were finally lysed in hypotonic buffer (0.05 M Na2HPO4/KH2PO4, pH 7.0, 10 mM MgCl2, RNase (10 μg/ml), DNase (10 μg/ml) and incubated at 37 °C for 1 h. The bacterial preparation was then incubated for 1 h at 37 °C, checked by Gram stain for complete protoplast formation, and sedimented at 7,500 × g for 8 min at 22 °C. The protoplasts were then resuspended to 30% (w/v) in protoplasting buffer without lysozyme and sedimented at 9000 × g for 10 min. The protoplasts were digested with a solution of 100,000 units of hyaluronic acidase (Sigma) in 100 ml of cold phosphate buffer, pH 7.4, at 37 °C for 1 h. The bacterial preparation was then adjusted by centrifugation at 13,000 × g for 1 h and resuspended to their original volume in the appropriate detergent. The membranes were then extracted at 4 °C for 1 h with stirring. Once the membranes were extracted, the mixture was sedimented by centrifugation at 15,000 × g for 3 min, the supernatant was decanted, and the pellet was resuspended for 1 h at 196,000 × g. After centrifugation at 196,000 × g at 37 °C for 1 h. The bacterial preparation was then incubated for 1 h with trypsin (2 mg/ml) treatment of detergent extracts (0.025 mg of protein/ml) was carried out in 0.05 M phosphate buffer, pH 7.4, with 0.15 M NaCl. The reaction mixture was incubated at 37 °C for 2 h and then terminated by the addition of soy bean trypsin inhibitor to a final concentration of 3 mg/ml. The total lipids extracted from the membrane were prepared according to the method of Bligh and Dyer (15). The extracts were reconstituted in buffer A containing 0.45% CHAPSO.

**RESULTS**

**Detergent Extraction of Membranes**—In preliminary experiments used to determine conditions for solubilization of the hyaluronic acid synthetic enzyme, streptococcal membranes were prepared and extracted with detergents as described under “Experimental Procedures.” A range of concentrations were used for each detergent, and the concentration giving peak activity for hyaluronic acid synthetic activity is shown in Table I. Analysis of the 13,000 × g pellets demonstrated that only 2.7–7.2% of the enzymatic activity remained associated with the membrane pellet after detergent extraction except for the pellets from CHAPSO and sodium cholate extractions (105 and 61.2%, respectively). Analysis of the 196,000 × g pellet fractions indicated that 3.1–163% of the activity was associated with the high speed pellets. No activity was found in any of the 196,000 × g supernatants indicating that the synthetase was not solubilized in an active form at the detergent concentrations tested.

The total recovery of enzymatic activity from each extraction protocol demonstrated that only CHAPSO enhanced the enzymatic activity as compared to intact membrane controls (290.3 nmoles/h, combined fractions; 162.2 nmoles/h, CHAPSO control; 108.3 nmoles/h, intact membrane), indicating that the detergent either destroyed or inhibited the enzymatic activity. Furthermore, the specific activity of the 196,000 × g pellet was more than 36-fold that of the membrane control with 163% of the original total activity. Attempts were made to solubilize the activity from the 196,000 × g pellets using the same detergent, different detergents, and detergent mixtures; however, no activity was found in any of the 196,000 × g supernatants obtained from these experiments.

**Reconstitution of Hyaluronic Acid Synthetic Activity from Solubilized Membranes**—Since less than 100% of the enzymatic activity was recovered from any of the detergent extracts other than the CHAPSO extract, the possibility existed that the detergents exhibited an inhibitory effect on the enzymatic activity. Furthermore, the specific activity of the 196,000 × g pellet was more than 36-fold that of the membrane control with 163% of the original total activity. Attempts were made to solubilize the activity from the 196,000 × g pellets using the same detergent, different detergents, and detergent mixtures; however, no activity was found in any of the 196,000 × g supernatants obtained from these experiments.
Detergent solubilization of hyaluronic acid synthesis enzyme(s) from group C streptococcal membranes

Membranes were prepared as described under "Experimental Procedures" (3-4 mg of protein/ml). These intact membrane preparations were aliquoted (200 μl) and centrifuged for 3 min at 13,000 x g. The listed detergent solutions were prepared in buffer A. The pelleted membranes were resuspended to their original volumes in the detergent solutions and extracted at 4 °C for 1 h with stirring. Residual membranes were removed by centrifugation at 13,000 x g for 3 min, and the supernatant was then sedimented for an additional hour at 196,000 x g. Only the top half (100 μl) of the supernatant was removed and stored at 4 °C while the remainder was discarded. The pellet was next resuspended in 200 μl of buffer A. These two preparations were termed the supernatant and pellet, respectively. The preparations were then tested for hyaluronic acid synthetic activity as described under "Experimental Procedures." No activity was found in any of the supernatants, and the results below show the detergent concentration at which optimum activity was obtained in the pellet. The total synthetic activity of whole membrane was 108.3 nmol glucuronic acid transferred from UDP-glucuronic acid/h/mg of protein with a specific activity of 38.1. The values in parentheses are the percentage of activity as compared to intact membrane.

| Detergent       | Optimum conc | 13,000 x g pellet total activity (%) | 196,000 x g supernatant total activity | 196,000 x g pellet total activity (%) | Total recovery (%) |
|-----------------|--------------|------------------------------------|--------------------------------------|--------------------------------------|--------------------|
| CHAPSO          | 0.5          | 113.4 (105)                        | 136.0                                | 176.9 (163)                          | 268                |
| Digitonin       | 2.0          | 7.8 (7.2)                          | 204.3                                | 4.1 (3.8)                            | 11.0               |
| Octyl-β-D-glucopyranoside | 0.5          | 2.9 (2.7)                          | 161.1                                | 12.8 (11.8)                          | 14.5               |
| Sodium cholate  | 0.3          | 66.3 (61.2)                        | 129.6                                | 10.3 (9.5)                           | 70.7               |
| Triton X-100    | 0.1          | 3.2 (3.0)                          | 11.0                                 | 3.4 (3.1)                            | 6.1                |
| Zwittergent 314 | 0.5          | 5.1 (2.8)                          | 4.4                                  | 5.3 (4.9)                            | 7.5                |

% Specific activity; nmol glucuronic acid transferred from UDP-glucuronic acid/h/mg of protein.

In order to test the hypothesis that the hyaluronic acid synthetic activity was dissociated by the extraction systems, the CHAPSO and digitonin extracts (196,000 x g supernatants) were added together, and dialyzed against buffer A, and then assayed for hyaluronic acid synthetic activity. An enhancement of the total activity (Table II, 29.4 nmol transferred/h, 147.7%) was observed when compared to the total activity for the CHAPSO and digitonin extracts (6.6 and 13.3 nmol/h, respectively).

Each of the extracts was treated with trypsin to determine whether the active component was protein in nature. After the trypsin treatment of the digitonin extract, 71.8% (from 29.4 to 8.3) of the activity was lost. However only 35% (from 29.4 to 19.1) of the activity was lost when the CHAPSO supernatant was treated with trypsin. Since the data indicated that the CHAPSO extract contained a trypsin-resistant component of the hyaluronic acid synthetic system possibly of a lipid nature, a total lipid extract of the CHAPSO supernatant was prepared and added to the digitonin supernatant. A total activity of 287.9 nmol/h was observed for this preparation. In comparison to intact membranes, the lipid-reconstituted digitonin extract produced 266% of the total activity with a specific activity 12.6 times greater than the membrane, indicating that components of the lipid fraction of the membrane were required for total enzymatic activity. No synthetase activity was demonstrated associated with the lipid extract.

Next, in order to demonstrate that the hyaluronic acid synthetic activity was indeed soluble, a digitonin 196,000 x g supernatant was loaded onto a column of Sepharose CL-6B and eluted with sodium phosphate buffer containing 0.2% digitonin (Fig. 1). The enzymatic activity eluted after the void volume. Even though an accurate molecular size was not determined due to the presence of digitonin, it appears that the activity resides as an enzyme complex.

Reconstitution of Hyaluronic Acid Synthetic Activity with Phospholipids—Since a total lipid extract of the CHAPSO extract reconstituted the hyaluronic acid synthetic activity of the digitonin extract, attempts were made to reproduce the reconstitution using phospholipids. A range of phospholipid/
FIG. 1. Sepharose CL-6B gel filtration of digitonin 196,000 × g supernatant. Membranes were prepared, solubilized, and sedimented at 196,000 × g for 1 h as described under "Experimental Procedures." The high speed supernatant (0.5 ml) was applied to a column of Sepharose CL-6B (1.5 × 28 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, containing 4 mM dithioerythritol, 1 mM MgCl₂, and 0.2% digitonin. The buffer was clarified by centrifugation before use. Fractions (0.75 ml) were collected at a flow rate of 10 ml/h, aliquots (0.5 ml) were repurified with cardiolipin, and assayed for hyaluronic acid synthetic activity as described under "Experimental Procedures." The elution profiles for enzyme activity (⊙) and protein (●) are shown.

TABLE III

Effectiveness of different phospholipids in reconstituting hyaluronic acid synthetic activity in digitonin extracts from group C streptococcal membranes

| Phospholipid       | Optimum ratio phospholipid/ protein | Specific activitya | Total activity (%)b |
|--------------------|-------------------------------------|--------------------|---------------------|
| Cardiolipin        | 5.0/1                               | 2405.7             | 529.3 (3980)        |
| Phosphatidylserine | 5.0/1                               | 742.8              | 237.7 (1787)        |
| Phosphatidylethanolamine | 20.0/1                            | 494.2             | 207.6 (1561)        |
| Phosphatidylinositol | 0.6/1                             | 264.6              | 66.2 (498)          |
| Phosphatidylglycerol | 0.5/1                            | 183.6              | 58.8 (442)          |
| Phosphatidylvcholine | 0.5/1                            | 79.7               | 23.9 (180)          |
| None               |                                    |                    | 41.7               |

a Specific activity; nmol glucuronic acid transferred from UDP-glucuronic acid/h/mg of protein.
b Per cent of control value.

Protein ratios (μg/μg) from 0.1:1 to 50:1 in buffer A containing 0.45% CHAPSO were tested for each phospholipid, and the data are presented in Table III. Cardiolipin, phosphatidylethanolamine, and phosphatidylserine reconstituted the hyaluronic acid activity of the digitonin supernatant (529.3, 207.6, and 237.7 nmol/h, respectively, as compared to the membrane control 108.3 nmol/h). Furthermore, cardiolipin enhanced the total activity of the digitonin supernatant 4.9-fold over the activity obtained from intact membranes with a 63.1-fold increase in specific activity. Phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol increased the total activity of the digitonin supernatant but to a more limited extent. Combinations of phospholipids did not enhance the activity over that of cardiolipin reconstituted system.

In order to determine whether digitonin was the optimum detergent for extraction of the hyaluronic acid synthetic system, extraction of membranes with other detergents was repeated and the 196,000 × g supernatants were reconstituted with the optimum concentration of cardiolipin (Table IV).

TABLE IV

Effectiveness of reconstitution of hyaluronic acid synthesis activity with cardiolipin for different detergent extracts of group C streptococcal membranes

| Detergent        | Optimum conc | Specific activity | Total activity |
|------------------|--------------|------------------|---------------|
| CHAPSO           | 0.5          | 60.9             | 24.4          |
| Digitonin        | 2.0          | 2405.7           | 529.3         |
| Octyl-β-D-glucopyranoside | 0.5     | 27.7             | 3.3           |
| Sodium cholate   | 0.3          | 27.0             | 4.3           |
| Triton X-100     | 0.1          | 14.7             | 2.9           |
| Zwittergent 314  | 0.5          | 4.5              | 2.5           |

*Specific activity; nmol glucuronic acid transferred from UDP-glucuronic acid/h/mg of protein.

Cardiolipin did not reconstitute any of the other 196,000 × g supernatants when various detergents and detergent concentrations were tested (2.5–24.4 nmol/h total activity versus 108.3 nmol/h for intact membrane). This experiment was repeated using phosphatidylethanolamine-reconstituted detergent extracts and a similar pattern of synthetic activity was observed.

Confirmation of Hyaluronic Acid Synthetic Activity Product—In order to determine the molecular weight range of the product of phospholipid-reconstituted hyaluronic acid synthetic activity, the sodium dodecyl sulfate-released product (see "Experimental Procedures") was chromatographed over Sepharose 2B (Fig. 2). The elution profile shows that high molecular weight product eluted at the void volume of the column and that a range of smaller molecular weight products were also produced. When the fractions containing [14C]glucuronic acid were pooled, treated with hyaluronidase (see "Experimental Procedures"), and rechromatographed, all of the radioactivity was found to elute at the total volume (Fig. 2). This demonstrated that [3H]glucuronic acid was incorporated into high molecular weight hyaluronic acid by the phospholipid-reconstituted system.

Rate of Product Formation by Reconstituted Hyaluronic Acid Synthetic Activity—In the rate study performed by Stollmuller and Dorfmann (5) and Sugahara et al. (2) demonstrated that the streptococcal hyaluronic acid synthetic system produced both trichloroacetic acid-insoluble and -soluble hyaluronic acid. Fig. 3 shows that this also is the case with the cardiolipin-reconstituted system. The production of trichloroacetic acid-insoluble material reaches a plateau at 30 min, whereas production of the soluble fraction reaches a plateau at 70 min. No appreciable soluble material was observed before 7 min.

DISCUSSION

The studies presented demonstrate that the hyaluronic acid synthetic system was solubilized as an enzyme complex by extraction of streptococcal membranes with 2% digitonin and reconstituted with cardiolipin for optimum activation. Previous investigators of this synthetic system have attempted solubilization with detergents (6, 17) and organic solvents (2, 18) but were unsuccessful. Because of this failure to solubilize the system, many facets of the production mechanism remain incompletely understood. For example the involvement of a lipid intermediate is uncertain in eucaryotes (11, 12). In addition, the role of primer, the number of proteins involved in the synthesis, the regulation of synthesis, and the mecha-
The original experiments on detergent solubilization showed no activity in 196,000 × g supernatants from any of the detergents tested which confirmed previous reports (6). Analysis of our detergent extraction data indicated that octyl glucopyranoside, Triton X-100, and zwittergent 314 either denatured or inhibited the enzymatic activity of the hyaluronic acid biosynthetic system. CHAPSO did not extract any appreciable enzymatic activity, but it did enhance the activity of the intact membrane. The specific role of CHAPSO in this enhancement of synthetic activity remains to be determined even though it is likely that CHAPSO is opening up membrane vesicles, thereby allowing the enzyme access to the substrates.

The inhibitory effect of a number of detergents was still apparent upon dialysis of the detergent extracts against buffer A. The digitonin extract exhibited the greatest quantity of hyaluronic acid synthetase activity when compared to the other detergent extracts but was still low in comparison to intact membranes (12.3%). However, addition of cardiolipin reconstituted the system completely and showed an enhancement of 4.9-fold in total activity over that of intact membranes. When other detergents were used in an effort to solubilize the hyaluronic acid synthetic activity from the membrane and the extracts were reconstituted under optimal conditions, digitonin was found to be 21.7 times more effective than the next detergent, CHAPSO. The mechanism of digitonin solubilization of eucaryote membranes is through the removal of sterols. However, this class of molecules is not present in streptococcal membranes, and, therefore, digitonin must solubilize the hyaluronic acid synthetic activity through another mechanism. Aloni et al. (19) recently reported that digitonin solubilized cellulose synthetase from Acetobacter xylinum, another microorganism lacking sterols, indicating that other mechanisms of membrane solubilization by digitonin occurred.

The studies by Sugahara et al. (2) and Stoolmiller and Dorfman (1) demonstrated that a lipid intermediate was not involved in the biosynthesis of streptococcal hyaluronic acid, however, the stabilizing role of phospholipids was not recognized. Our studies indicated that phospholipids were necessary for the expression of synthetic activity by this system. Cardiolipin, phosphatidylethanolamine, and phosphatidylserine reconstituted and enhanced the total activity of the digitonin extract over that of the intact membrane. Combinations of these phospholipids did not improve the enzymatic activity of the cardiolipin-reconstituted system. The reconstitution of the enzyme complex with these phospholipids is not unexpected when one considers that Gram-positive bacterial membranes usually contain phosphatidylglycerol, cardiolipin, traces of phosphatidylserine, and occasionally phosphatidylethanolamine (20, 21). Phospholipids have previously been demonstrated to play a role in the activation and stabilization of membrane proteins. For example, in E. coli, the enzymes producing peptidoglycan (22), and in Salmonella typhimurium, the galactosyl transferase system (23) require phospholipid for activity. The phospholipid dependence of a guinea pig liver microsomal UDP-glucuronosyltransferase has also been demonstrated (24).

Investigations into the size of the product of the reconstituted hyaluronic acid synthetic enzyme system showed that a variety of high molecular weight products were synthesized.
The variation in polymer sizes was consistent with the release of chains of polymer at different stages of completion. The size range also was similar to the hyaluronic acid produced in whole cells of group A and C streptococci and their membranes. The kinetic studies on the production of hyaluronic acid using the reconstituted soluble system were similar to results presented by Sugahara et al. (2) for their particulate enzyme preparations.

Finally, the ability to solubilize and reconstitute the hyaluronic acid synthetic system is an important step in the purification of this enzyme. The 63-fold increase in specific activity of the cardiolipin-reconstituted hyaluronic acid synthetic system over intact membranes indicates that this is a suitable starting point in purification of the complex. With a purified system the complete mechanism of hyaluronic acid biosynthesis in procaryotes and eucaryotes can be elucidated.

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