The two hyaluronan synthases (HASs) from *Streptococcus pyogenes* (spHAS) and *Streptococcus equisimilis* (seHAS) were expressed in *Escherichia coli* as recombinant proteins containing His$_6$ tails. Both enzymes were expressed as major membrane proteins, accounting for 5–8% of the total membrane protein. Using nickel chelate affinity chromatography, the HASs were purified to homogeneity from n-dodecyl β-D-maltoside extracts. High levels of HAS activity could be achieved only if the purified enzymes were supplemented with either bovine or *E. coli* cardiolipin (CL), although bovine CL gave consistently greater activity. Mass spectrometric analysis revealed that the fatty acid compositions of these two CL preparations did not overlap. The two HAS enzymes showed similar but distinct activation profiles with the 10 other lipids tested. For example, phosphatidic acid showed similar but distinct activation profiles with the CL preparations did not overlap. The two HAS enzymes became inactive after storage for ~5 days at 4 °C. Both purified enzymes also lost activity over ~4–5 days when stored at ~80 °C in the presence of CL, but reached a level of activity that then slowly decreased over a period of months. Although the purified enzymes stored in the absence of CL at ~80 °C were much less active, the enzymes retained this same low level of activity for at least 5 weeks. When both spHAS and seHAS were stored without CL at ~80 °C, even after 2 months, they could be stimulated by the addition of bovine CL to ~60% of the initial activity of the freshly purified enzyme.

Since the discovery of HA over 60 years ago (1), this saccharide polymer, which contains repeating disaccharide units of GlcUAβ(1,3)GlcNAcβ(1,4), has been shown to have numerous biological functions. For example, HA provides the viscous lubrication of synovial fluid in joints and provides cartilage with its viscoelastic properties. HA is involved in a wide variety of cellular functions and behaviors, including cell migration (2, 3), development (4–6), differentiation (7–9), phagocytosis (6), and proteoglycan synthesis (2, 4). As well as being a major structural component of the matrix, HA has wound healing, pharmaceutical, and analgesic effects (10–14) and is also being used as a vehicle for drug delivery (15, 16).

Although cell-free HA biosynthesis was achieved 40 years ago (17) and HAS activity was successfully detergent-solubilized in the 1980s from the plasma membranes of both eukaryotes (18, 19) and bacteria (20, 21), a functional enzyme-encoding gene or cDNA was not cloned until 1993, when we reported on the Group A HAS, spHAS (22–24). At the same time, others reported that a 52-kDa protein from Group C *Streptococcus equisimilis* was the HAS since an antibody to this protein appeared to inhibit HA biosynthesis (25). However, this 52-kDa protein did not have HAS activity, and the cloned gene (26) had no homology to the Group A HAS (23). When a *bona fide* HAS from Group C *S. equisimilis* was cloned (27), this seHAS protein showed 72% identity to spHAS and no similarity to the previously reported Group C gene (26). Although the 52-kDa protein is not a HAS, several studies used antiserum to this unknown 52-kDa protein and erroneously reported on putative HASs (reviewed in Ref. 28).

The HAS reaction is membrane-associated and combines the sugar nucleotides UDP-GlcNAc and UDP-GlcUA in an alternating fashion to polymerize HA in the presence of Mg$^{2+}$ at neutral pH. The enzyme produces a linear nonsulfated polymer with an $M_r > 5 	imes 10^8$. With the discovery of this new family of HAS isoymes (28), there is a clear need for the purification and characterization of an active enzyme. Although there have been numerous attempts to solubilize, identify, and purify HAS from strains of streptococci that produce HA as well as HAS from eukaryotic cells (17–19, 21, 25, 29, 30), this has not yet been accomplished. In this study, we have detergent-solubilized and purified to homogeneity the active recombinant spHAS and seHAS enzymes expressed in *Escherichia coli*. We have also determined that these purified HASs, in the absence of other streptococcal proteins, are lipid-dependent enzymes that are strongly stimulated by CL. A preliminary

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* This work was supported in part by NIGMS Grant GM35978 from the National Institutes of Health. Work carried out in the Oklahoma Laser Mass Spectrometry Facility was supported by a National Science Foundation EPSCoR grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Materials, Strains, and Plasmids—Reagents were supplied by Sigma unless stated otherwise. Media components were from Difco. Mucoid Group A Streptococcus pyogenes strain S43/192/4 and Group C S. equisimilis strain D181 were obtained from the Rockefeller University collection. The E. coli host strain SURE™ cells were from Stratagene. The HAS open reading frames from S. pyogenes (23, 24) and S. equisimilis (27) were inserted into the pKK223-3 vector (Amersham Pharmacia Biotech) and cloned into E. coli SURE™ cells by the method of DeAngelis and Weigel (24). The pKK223-3 vector contains the strong tac promoter that can be regulated by the lac repressor and induced with isopropyl-β-D-thiogalactoside. To facilitate purification of spHAS and seHAS, a C-terminal fusion of 6 His residues was introduced into each construct. This modification does not significantly alter enzyme activity (24). Membranes from S. pyogenes and S. equisimilis were obtained as described in the accompanying paper (32). Lipids were obtained in sealed vials from Matreya, Inc. (Pleasant Gap, PA).

HAS Extraction and Purification—The thawed membrane pellets were solubilized using 9.8 mM DDM (0.5%, w/v) in extraction buffer (50 mM sodium and potassium phosphate, pH 7.0, 150 mM NaCl, 10 mM MgCl₂, 1.0 mM β-mercaptoethanol, 20% glycerol, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 46 μg/ml phenylmethylsulfonyl fluoride) at 4 °C for 2 h with gentle mixing in a Micromixer (Taitec). Insoluble membrane components were sedimented by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatant was then added to the supernatant to a final concentration of 20 mM to minimize nonspecific binding of E. coli proteins to the Ni²⁺-nitrilotriacetic acid resin (QIAGEN Inc.). The final extract was applied directly to a mini-spin column (Bio-Rad) containing Ni²⁺-nitrilotriacetic acid resin, which had been equilibrated with extraction buffer lacking MgCl₂. The enzyme was incubated with the resin for 90 min at 4 °C with constant mixing. After incubation, the flow-through fraction from the Ni²⁺-nitrilotriacetic acid resin was passed over the resin four times (on ice). The resin was then washed with 10 volumes of extraction buffer, and the HAS was eluted with 25 mM sodium and potassium phosphate, pH 7.0, 50 mM NaCl, 1.0 mM dithiothreitol, 20% (v/v) glycerol, 0.98 mM DDM, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 46 μg/ml phenylmethylsulfonyl fluoride, and various concentrations of histidine. For certain experiments, this elution buffer also contained CL. spHAS and seHAS were solubilized, purified, and stored with or without the addition of bovine heart or E. coli CL depending on the type of experiment. Exposure to, or the presence of, histidine did not affect the activity of either HAS. Protein concentrations were determined with the Coomassie protein assay reagent (Fierce) using bovine serum albumin as the standard (33).

HAS Activity—HAS activity was determined in 100 μl of 25 mM sodium and potassium phosphate, pH 7.0, containing 50 mM NaCl, 20 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EGTA, 15-20% (w/v) polyacrylamide gels. The gels were electrophoresed using a Bio-Rad Mini-PROTEAN II at 25 mA until the dye reached the running gel interface, at which point, the current was held constant at 40 mA. The gels were stained with Coomassie Brilliant Blue R-250 for 30 min and destained with 25% methanol and 10% acetic acid. After SDS-PAGE, proteins were transferred to nitrocellulose (0.1 μm; Schleicher & Schuell) using a Bio-Rad Mini-Transblot device at 90 V for 2 h in buffer with 20% methanol and 0.1% (w/v) SDS as described by Towbin et al. (36). The blots were blocked, incubated with a specific polyclonal antibody to the spHAS sequence Glu(147–Thr(155)), and developed as described previously (24).

RESULTS

spHAS and seHAS, containing C-terminal His₉ tags, were affinity-purified using nickel chelate chromatography. Conditions were chosen so that at least 95% of each protein was solubilized from isolated membranes and recovered in the DDM detergent extracts. Overall, only a 15–17-fold purification was needed to obtain homogeneous preparations (Table I) since at least 5–8% of the total E. coli membrane protein can be the recombinant HAS (27). Based on SDS-PAGE followed by Coomassie Blue staining (Fig. 1) or Western analysis (data not shown), the affinity-purified spHAS and seHAS were >99% pure. Both spHAS and seHAS migrated similarly fast on SDS-PAGE and give relative molecular masses of 42–43 kDa, although the calculated molecular masses based on amino acid sequence were 48,677 and 48,600 Da, respectively, for the two enzymes containing a His₉ tail. These predicted molecular masses were, in fact, found to be accurate within 0.07% by MALDI-TOF mass spectrometry (34). Despite their almost identical masses, we observed that seHAS consistently migrated slightly slower than spHAS, giving a greater apparent molecular mass of ~1 kDa (Fig. 1, lanes 8 and 9).

We recently found that the activity of recombinant spHAS or
**Purification of Streptococcal Hyaluronan Synthases**

seHAS is stimulated when exogenous CL is added to purified *E. coli* membranes (34). Triscott and van de Rijn (21) also found that digitonin-solubilized seHAS was stimulated by CL. Based on these results, we investigated the influence of CL and other lipids on HAS activity as well as on HAS purification and stability during storage.

When either spHAS (Fig. 2) or seHAS (Fig. 3) was purified under CL-deficient conditions, the re-addition of CL caused a large increase in enzyme activity. As the CL concentration was increased, the specific activity of both enzymes also increased. In both cases, there was an apparent critical concentration of ~0.5 mM CL required before HAS activity was stimulated. The stimulation of HAS activity with CL occurred almost immediately, in <1 min (data not shown). With spHAS, bovine or *E. coli* CL gave similar results (Fig. 2), although bovine CL consistently resulted in a significantly higher specific activity: a 11.5-fold stimulation compared with a 9-fold increase at 3 mM. The seHAS enzyme behaved in a qualitatively similar manner (Fig. 3), although the difference between bovine and *E. coli* CL was greater, and the overall stimulation of HAS activity was less than that found for spHAS. Consistent with this result, the residual HAS activity without exogenous CL was always considerably higher (e.g., ~7-fold) for seHAS compared with spHAS.

The difference in the ability of the bovine and *E. coli* CL preparations to stimulate either seHAS or spHAS was unexpected, especially since the enzyme was expressed in and purified from *E. coli*, which is known to contain CL (37). To assess the molecular basis for this different ability to stimulate, we examined the composition of these lipids using MALDI-TOF mass spectrometry (Fig. 4). Interestingly, the two CL preparations were quite different and, in fact, did not share any of the same CL species. Since CL contains four fatty acyl chains and there are dozens of different possible fatty acids that can be linked at each position, there are many possible CL isoforms. Bovine CL contained essentially one species (>90%) with the major mass peak at 1447.93, which was not present in the...

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**FIG. 3.** Effect of cardiolipin on the activity of purified seHAS. seHAS was purified as described under “Experimental Procedures,” except that exogenous CL was omitted at each step. The freshly purified enzyme was then assayed in the presence of the indicated final concentrations of either bovine (□) or *E. coli* (■) CL.

**FIG. 4.** MALDI-TOF mass spectroscopic analysis of bovine and *E. coli* cardiolipin. Samples of each CL were analyzed using a modification of the procedure of Papac et al. (50). CL was dissolved in deionized water at a concentration of 10 pmol/μl, and 1 μl was spotted to the target followed by 1 μl of matrix. The matrix compound was 6-aza-2-thiothymine dissolved at a concentration of 3 mg/ml in 50% acetonitrile containing 0.1% trifluoroacetic acid. The samples were then rapidly dried (<30 s) under vacuum. The MALDI-TOF mass spectrometer used was a Voyager Elite (Perceptive Biosystems, Framingham, MA) equipped with an N2 laser (337 nm), located in the Oklahoma Laser Mass Spectrometry Facility. Samples were analyzed in the linear negative-ion mode with a delayed extraction of 150 ns and were subject to a 20-kV accelerating voltage.
bacterial CL. *E. coli* CL was much more complex, containing at least seven different CL species ranging in mass from 1335.91 to 1432.09. These seven species constitute a series with each member differing in mass by 14 or one -CH₂ group.

A variety of other phospholipids were also tested for their ability to stimulate HAS activity. Freshly purified spHAS (Fig. 5) and seHAS (Fig. 6) were both stimulated to the greatest extent by bovine CL and to the next greatest extent by phosphatidylserine. spHAS showed the greater specificity for CL since none of the other 10 lipids tested gave a specific activity of greater than 30% of that with bovine CL (Fig. 5). The only other lipids to give a moderate stimulation of spHAS activity were lysolecithin, cerebrosides, and phosphatidylserine. Phosphatidylserine was the second best activating lipid, with a 2.5-fold stimulation of activity. The basal activity of purified spHAS was actually inhibited in the presence of phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, sphingomyelin, and sulfatides (Fig. 5).

The results with seHAS were qualitatively similar, but quantitatively different (Fig. 6). The basal activity of purified seHAS was also inhibited by phosphatidylcholine, sphingomyelin, and sulfatides. Both enzymes were essentially inactive in the presence of this latter anionic lipid. Like spHAS, seHAS was moderately stimulated by lysolecithin and cerebrosides, but unlike spHAS, seHAS was also weakly stimulated by phosphatidylinositol and phosphatidylethanolamine. Perhaps the biggest difference between the two enzymes was the effect of phosphatidic acid, which stimulated seHAS by 4-fold (Fig. 6), but inhibited spHAS (Fig. 5). We conclude from these data that seHAS and spHAS are very sensitive to the phospholipids in their environment and are essentially dependent on CL.

The large increase in synthase activity with CL could be due to a CL-dependent effect on the enzyme that decreases the *K_m* values for the sugar nucleotide substrates or that only increases *V_max*. To determine this, the kinetic profiles of both purified enzymes were measured in the absence and presence of bovine CL (Fig. 7 and Table II). In the absence of CL, both freshly purified enzymes had responses to increasing UDP-GlcUA similar to those shown in Figs. 2 and 4 for the membrane-bound enzymes, but they had very different responses to UDP-GlcNAc (Fig. 7). Detergent-solubilized, purified spHAS without CL showed the same sigmoidal response to increasing UDP-GlcNAc as observed for the membrane-bound enzyme (32). The Hill number in this case was 2.0, whereas the value for pure seHAS without CL was 1.1, indicating no cooperativity for UDP-GlcNAc utilization (Table II). 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V_max of both enzymes 3.6–6-fold. A more complete kinetic characterization of the HASs in the presence of CL is presented in the accompanying paper (32).

We also investigated conditions that would allow the long-term storage of the purified HAS enzymes with good retention of activity. At 4 °C, with or without CL, the enzymes were inactive by day 5 (data not shown). When affinity-purified spHAS (Fig. 8A) or seHAS (Fig. 8B) was stored for weeks with or without CL at −80 °C, enzyme activity was retained, but changed substantially. Most typically, at −80 °C with CL, both enzymes lost 20–50% of their activity within 4–5 days. Storage of purified seHAS or spHAS at −80 °C with 2 mM bovine CL usually yielded a relatively stable level of HAS activity from day 5 through at least day 34. For both enzymes, storage in the presence of bovine CL was better than with E. coli CL.

Both enzymes consistently displayed an unusual biphasic, changing activity pattern during the first week of storage at −80 °C. This biphasic loss and then recovery of activity with storage time occurred consistently in the presence of bovine CL, not with E. coli CL. The increase noted around days 3 and 4 in Fig. 8 is reproducible, although the time at which these changes occurred and the magnitude of the activity change varied considerably among different enzyme preparations. For example, in another experiment (data not shown), enzyme activity declined −70% over the first 5 days and then rebounded by day 10 to a level of only 20% less activity than at day 0. Typically, either HAS then loses activity irreversibly with a half-life of 2–3 months (data not shown).

If the enzymes were stored at −80 °C without CL, they also had a much lower, but fairly constant level of activity when assayed without CL (Fig. 8). Interestingly, spHAS (Fig. 8A) or seHAS (Fig. 8B) stored at −80 °C in the absence of CL was capable of being restored to a high level of activity by the subsequent addition of CL. The levels of enzyme activity for both spHAS and seHAS attained by this protocol were essentially identical to the activity of the enzymes that had been stored in the continuous presence of either bovine or E. coli CL.

TABLE II
Effect of cardiolipin on the kinetic properties of purified spHAS and seHAS

The HASs were purified in the absence of CL and then assayed within 24 h for HAS activity in the presence or absence of CL as described under “Experimental Procedures” and in the accompanying paper (32). The second substrate concentration was held constant at 1.0 mM, except for the determination of K_{UDP-GlcNAc} for spHAS, which was at 1.5 mM UDP-GlcUA.

| UDP-GlcUA | K_m | V_max | Hill No. |
|-----------|-----|-------|---------|
| spHAS     | 117 ± 23 | 19.8 ± 3.3 | 17.9 ± 19.9 ± 1 |
| +2.0 mM CL | 315 ± 52 | 4.3 ± 1.0 | |
| seHAS     | 44 ± 17 | 8.8 ± 1.1 | 9.4 ± 1.4 ± 1.1 ± 0.1 |
| +2.0 mM CL | 173 ± 22 | 31.7 ± 5.6 | |

FIG. 7. Effect of cardiolipin on K_{UDP-GlcNAc} for purified seHAS and spHAS activities. spHAS (■, □) and seHAS (●, ○) were purified in the absence of CL and assayed in the presence (○, □) or absence (●, ■) of bovine CL as described under “Experimental Procedures.” The concentration of UDP-GlcUA was 1.0 mM.

FIG. 8. Effect of cardiolipin on seHAS and spHAS activities during storage. spHAS (A) and seHAS (B) were purified in the absence of added CL, supplemented with nothing ( ), or with 2 mM bovine (○) or E. coli (△) CL, immediately aliquoted, and frozen at −80 °C. Samples were removed on the indicated day, thawed on ice, and assayed for HAS activity as described under “Experimental Procedures” for the standard conditions. Samples that had been stored without CL were assayed in the absence of CL ( ) or in the presence of either 2 mM E. coli (△) or bovine (○) CL.
The streptococcal and eukaryotic HASs are thought to be organized in the membrane in the same way (28). The majority of the protein, including both the amino and carboxyl termini, is inside the cell. Four of the streptococcal HAS membrane domains appear to pass through the membrane, giving only two small loops of the protein exposed to the extracellular side. Ongoing studies have indicated that another two or more regions of the protein, including portions of the large central domain (24), may associate with the membrane as amphipathic helices or reentrant loops that do not span the membrane. There is a paradox or dilemma, related to the HA transfer function, posed by this topology model and the finding that no protein other than the HAS is needed for HA biosynthesis, at least when the enzyme is solubilized in detergent. How can we account for the ability of this relatively small protein to transfer the growing HA chain, which has some hydrophobic character but which is mostly hydrophilic, across the hydrophobic lipid barrier of the plasma membrane? Other membrane transporters that shepherd small molecules, like sugars, across membranes usually contain 12 or more transmembrane domains and create a pore (49). The HAS protein is not large enough and does not have the necessary number of transmembrane domains to form such a pore. So, how does it guide HA across the membrane?

One solution to this HA transfer dilemma would be if the HAS could only synthesize HA if it forms a dimer or larger oligomer, so that enough transmembrane domains from each HAS protein could be arranged to form a pore. To determine if the active HAS is a monomer or a larger oligomer, we recently used radiation inactivation analysis (34). Both active streptococcal HASs contained a single HAS protein, but the protein was associated with an additional mass of ~23 kDa. Based on mass spectrometry of the purified enzymes, neither spHAS nor seHAS is disulfide-bonded to other proteins or post-translation-

FIG. 9. Model of the cardiolipin-HAS complex. The scheme depicts the close association of 16 CL molecules (red dots) with one HAS protein to create an active enzyme. The figure represents a hypothetical plane parallel to the plasma membrane (purple) and viewed from outside the cell so that the cytoplasm (white) is seen through the enzyme. The growing HA chain would be transferred through this pore-like opening. The membrane domains of the HAS are labeled as transmembrane (TMD) or membrane-associated (MAD) domains according to preliminary studies on the enzyme's topology (48).
ally modified (34). Interestingly, this extra mass was identified as CL. Thus, the active enzyme is a complex of one HAS protein with ~16 molecules of CL. Our interpretation of these results is depicted in Fig. 9. We propose that CL molecules help the enzyme to solve the HA transfer dilemma by creating a pore-like passage within the enzyme through which the growing HA chain passes. The lipid portion of CL could interact with the lipid bilayer and hydrophobic patches of the HA chain, whereas the acidic head groups of CL molecules could interact with the enzyme and hydrophilic portions of the HA chain.

Consistent with this radiation inactivation analysis, we found in the present study that either purified streptococcal HAS had very low activity in the absence of CL. When this phospholipid was added back, however, enzyme activity increased up to 10-fold. In particular, sPHAS was very specifically activated and dependent on CL. The CL dependence of sHAS seemed less stringent since phosphatidic acid or phosphatidylethanolamine, phosphatidylglycerol, or phosphatidylserine, contain a single free -OH group. This hydroyxyl may be important as a hydrogen bond acceptor or donor. It may be relevant that the two most effective phospholipids, CL and the HAS-activating lipids are anionic, but not cationic. It may possibly under any of these conditions, the HAS enzyme unactivated with a half-life of several months. The decline in enzyme activity to be elucidated.

Acknowledgments—We thank Anil Singh for technical support, Coy Heldermon and Dr. Kehama Kumari for helpful discussions, Debbie Blevins for help preparing the manuscript, and Dr. Paul DeAngelis for preparation of the spHAS-H_{2} construct.

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J. Biol. Chem. 1999, 274:4239-4245.
doi: 10.1074/jbc.274.7.4239

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