Phospholipid Dependence and Liposome Reconstitution of Purified Hyaluronan Synthase*

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Previous radiation inactivation and enzyme characterization studies demonstrated that the *Streptococcus equisimilis* hyaluronan synthase (seHAS) is phospholipid-dependent and that cardiolipin (CL) is the best phospholipid for enzyme activation. Here we investigated the ability of seHAS, purified in the absence of added lipid, to be activated by synthetic phosphatidic acid (PA), phosphatidylserine, or CL lipids containing fatty acyl chains of different length or different numbers of double bonds. The most effective lipid was tetraoleoyl CL (TO-CL), whereas tetramyristoyl CL (TM-CL) was ineffective. None of the phosphatidylserine species tested gave significant activation. PAs containing C10 to C18 saturated acyl chains were not effective activators, and neither were oleoyl lypo PA, dilinoleoyl PA, or PA containing one oleoyl chain and either a palmitoyl or stearoyl chain. In contrast, dioleoyl PA stimulated seHAS 10-fold, to 20% of the activity observed with TO-CL. The tested acidic lipids such as PA and CL activated the enzyme most efficiently if they contained only oleic acid. Mixing experiments showed that the enzyme interacts preferentially with TO-CL in the presence of TM-CL. Similarly, seHAS incorporated into phosphotidylcholine-based liposomes showed increasing activity with increasing TO-CL, but not TM-CL, content. Inactivation of membrane-bound seHAS by solubilization with Nonidet P-40 was prevented by TO-CL, but not TM-CL. The pH dependence of seHAS in the presence of synthetic or naturally occurring CLs showed the same pattern of lipid preference between pH 6 and 10.5. Unexpectedly, HAS showed lipid-independent activity at pH 11.5. The results suggest that Class I HAS enzymes are lipid-dependent and that assembly of active seHAS-lipid complexes has high specificity for the phospholipid head group and the nature of the fatty acyl chains.

Hyaluronan synthase (HAS) is a membrane-bound enzyme that alternately uses the two precursor sugars UDP-GlcNAc and UDP-GlcUA to assemble HA in the presence of Mg2+ ions (1–3). The Class I HAS enzymes are processive, and when an HA chain is released, it is no longer capable of being elongated. Typical mass ranges of HA products made by HASs are very broad and range up to 8–10 MDa. Unlike the vast majority of glycosyltransferases that make glycoside linkages at the nonreducing end, the streptococcal HASs assemble HA at the reducing end (4–6), which means that the growing chain is always attached to UDP (i.e. UDP-HA). In this mechanism, cleavage of the UDP-HA linkage at the reducing end results in chain termination.

The three streptococcal HASs, which are 417–419 amino acids long (7–9), contain six membrane domains (MDs), with the N-terminal and C-terminal ends inside the cell (10). The eukaryotic HASs contain an additional ~140 amino acids at the C-terminal end, which are predicted to include two MDs. Previous radiation inactivation studies (11) and characterization of two purified enzymes (12) demonstrated that the streptococcal HASs are lipid-dependent and particularly active in the presence of cardiolipin (CL).

The activity of many integral membrane enzymes is dependent on, or influenced by, a specific type of phospholipid, and there are now many cases known in which the activating lipid is CL (13–18). Mitochondrial enzymes involved in oxidative phosphorylation comprise a large fraction of this group, and their ability to form large functional complexes is CL-dependent. The endoplasmic reticulum UDP-GlcNAc-dolichol phosphate, GlcNAc-1-phosphate transferase is another example of an enzyme whose conformation and activity are stimulated by CL (19). This glycosyltransferase makes dolichol-diphospho-GlcNAc, which is utilized in the first steps of the pathway for N-linked oligosaccharide biosynthesis.

CL domains within membranes have been reported in *Escherichia coli* and *Bacillus subtilis* Marburg membranes as assessed using the fluorescent dye 10-N-nonyl-acridine orange (20–22), which interacts with CL. Although the interaction of this dye and CL may be influenced by other factors and not uniquely specific, CL appears to enhance the formation of N-nonyl-acridine orange dimers (23). The possible presence of CL-rich domains within cell membranes has intriguing implications for the localization and function of proteins that interact with CL tightly and specifically.

In the present study, we sought to determine in more detail the phospholipid preference of seHAS either reconstituted into defined liposomes or using synthetic lipids containing known fatty acyl chains of different lengths or containing different numbers of double bonds. The results indicate that the enzyme is activated most effectively by CL, but the ability to be activated is very dependent on the nature of the particular fatty acyl chains present.
**EXPERIMENTAL PROCEDURES**

*Materials and Buffers*—Media components were from Difco (Fisher). UDP-[14C]GlcUA (285 mCi/mmol) was from Amer sham Biosciences. Natural or synthetic phospholipids were obtained in sealed vials under inert gas from Matreya, Inc. (Pleasant Gap, PA), Avanti Polar Lipid, Inc. (Alabaster, AL), or Sigma-Aldrich. Other reagents, including *E. coli* and bovine CL, n-octyl β-d-glucopyranoside (OG), UDP-N-acetyl-β-d-glucosamine (UDP-GlcNAc), and UDP-β-d-glucuronic acid (UDP-GlcUA) were supplied by Sigma unless stated otherwise. Assay buffer contains 25 mM Na2KPO4, pH 7.6, 50 mM NaCl, 1 mM dithiothreitol, 1 mM n-dodecyl-β-d-maltoside (DDM), 0.1 mM EGTA, 10% (v/v) glycerol, 1 mM UDP-GlcUA, 1 mM UDP-GlcNAc, and 10 mM MgCl2 (reagents were mixed in the order indicated at room temperature). Column wash buffer contains 25 mM Na2KPO4, pH 7.0, 50 mM NaCl, 1.0 mM dithiothreitol, 0.1 mM EGTA, 10% glycerol, 1 mM DDM, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 46 μg/ml phenylmethylsulfonyl fluoride, and 10 mM histidine. Elution buffer was identical to wash buffer, except that the histidine concentration was 200 mM. Phosphate buffer contains 20 mM Na2KPO4, pH 7.4.

*Expression and Purification of seHAS—E. coli SURE™ cells containing pKK223-3 plasmid encoding seHAS, with a C-terminal His6 fusion, were grown at 30 °C in Luria broth, HAS expression was induced, and membranes containing seHAS were prepared as described earlier (12). The membrane pellets were washed once with phosphate-buffered saline containing 1.3 M glycerol and protease inhibitors, sonicated briefly, aliquoted, recentrifuged at 100,000 × g for 1 h, and stored at −80 °C. The extraction buffer, membrane solubilization procedure, and affinity chromatography over Ni2+-nitrilotriacetic acid resin (Qiagen Inc.) were described previously (12). After loading DDM extract, the column was washed with 10 volumes of wash buffer, and seHAS was released using 3 volumes of elution buffer. No added phospholipids were present during any stage of the purification.

*HAS Activity Assays*—Activity of seHAS was determined in 100 μl (final volume) of assay buffer with or without phospholipids at the concentrations indicated in the figures, as described previously (12, 24, 25). Purified seHAS (~0.3 μg) was added to initiate the enzyme reaction, and the mixtures were gently agitated in a Taitec MicroMixer, model E-36, at 30 °C for 60 min, or for the indicated time. The reactions were terminated by the addition of 20 μl of 12% (w/v) SDS and analyzed by descending paper chromatography using Whatman 3MM paper and elution with 1 M ammonium acetate, pH 5.5 (adjusted with glacial acetic acid), and 95% ethanol (7:13). The values are presented as the means ± S.E. (n = 3–4).

*Phospholipid Preparation*—Portions of stock chloroform or ethanol solutions of each phospholipid were dried under nitrogen, resuspended in 0.05% (w/v) DDM in distilled water, and added to assay buffer to give the desired final concentration. In experiments with combinations of TM-CL and TO-CL, the two lipids were reconstituted in DDM either separately and then mixed when added to assay buffer, or the chloroform solutions were mixed prior to evaporation and resuspension in DDM.

**RESULTS**

*seHAS Activation by Synthetic Phospholipids*—When seHAS is affinity-purified from DDM extracts of *E. coli* membranes in the absence of exogenous lipids, the enzyme displays very low and variable activity. The purified enzyme was greatly stimulated by mixtures of some, but not all, naturally occurring phospholipids (Fig. 5 in Ref. 12). As noted previously, bovine CL activated seHAS, purified in the absence of added lipids, to a much greater extent than phosphatidic acid (PA), PC, phos-
Phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidylinositol, or phosphatidylserine (PS). PS was the only other lipid that stimulated seHAS activity significantly, to a level severalfold above background and ~25% of the activity with bovine CL. These results indicated a significant lipid dependence for the streptococcal enzyme and confirmed earlier radiation inactivation studies, in which CL was identified as a component of the active synthase (11).

To define better the fatty acyl requirements for HAS activity, we tested the abilities of well defined synthetic lipids to stimulate the activity of lipid-depleted, purified seHAS. The reference lipid in these comparisons was TO-CL, which was previously found to be a better activating lipid than crude bovine or E. coli CL preparations (24). We first tested a series of synthetic PA species (Fig. 1). The activity of purified seHAS was not stimulated by 2 mM of PA variants containing two identical fatty acyl groups ranging from C10 to C18 in length (Fig. 1A). Although the enzyme was several-fold more active in the presence of PA (16:0) than the other members of this series, this level of activity was only ~5% of that in the presence of TO-CL. Based on the activation of seHAS by CL-containing oleoyl groups, we expected PA (18:1) to be a good activating lipid, and it was by far the most effective PA variant tested (~18% of the TO-CL-stimulated activity). PA species containing a single oleoyl chain (lyso) or with a second palmitoyl or stearoyl fatty acyl chain were unable to activate seHAS (Fig. 1A). Fig. 1B shows the concentration dependence for activation of seHAS by dioleoyl PA and TO-CL and the lack of effect with dilinoleoyl PA or distearoyl PA.

We then tested a series of PS variants with two identical saturated fatty acyl chains ranging from C12 to C18 (Fig. 2). Although in this experiment the mean activity of seHAS in the presence of dilauroyl PS was ~3-fold greater than in the presence of TM-CL, the results with this PS lipid were variable and not statistically different from any of the other variants tested except TO-CL. Dilauroyl PS was unable to stimulate seHAS activity (Fig. 2), in contrast to dioleoyl PA (Fig. 2). Lyso-oleoyl PS was also unable to activate the enzyme (Fig. 2). Although a naturally occurring PS mixture stimulated activity (12), none of the synthetic PS variants tested showed the ability to activate the enzyme. Presumably, other PS species that were not tested in this study are capable of activating HAS, or an activating contaminant (e.g. CL) may be present in some commercial PS preparations.

Among the commercially available synthetic PA and CL lipids tested, the two containing only oleic acid were consistently capable of activating purified seHAS (TO-CL >> dioleoyl PA). Dilinoleoyl PA and distearoyl PA showed inconsistent and low levels of stimulation, and some of this variability, as noted above, is likely due to the presence of variable amounts of endogenous tightly bound annular lipids still associated with seHAS (i.e. not removed by the purification procedure).

seHAS Preferentially Interacts with TO-CL Compared with TM-CL—If seHAS and multiple CL molecules form discrete, specific complexes, then the way in which lipid is presented to the lipid-depleted enzyme might affect the kinetics of forming active HAS-lipid complexes and affect the experimental results. To assess this possible complication, the abilities of the above lipids (as well as TM-CL, bovine CL, and E. coli CL) to activate seHAS were tested using two different protocols. In the standard assay, the enzyme is added last to assay buffer containing substrates and lipid. If enzyme and lipid require substantial
time to interact to achieve seHAS activation, then a lag time would occur under these conditions; in this case, a preincubation of enzyme and lipid, prior to the addition of substrate, should decrease such a lag. When this was tested (Fig. 3), in all but one case (with *E. coli* CL), seHAS activity was actually greater under the standard conditions, without a 30-min pre-treatment. This result indicates that seHAS and phospholipids interact quickly, with no apparent kinetic lag, to form enzyme complexes.

An important feature of a lipid requirement for any enzyme is whether the enzyme interaction with preferred lipid is much more favored, and therefore more specific, than for other lipids that are not capable of activating the enzyme. Is the enzyme capable of binding many different types of lipids with relatively little difference in the energetics of binding each lipid, or is there great specificity (presumably indicating a greater $K_{eq}$) for the activating lipid? Since two synthetic CL species were available with dramatically different abilities to activate seHAS, we performed mixing experiments to test whether the purified enzyme was more readily able to interact with the activating TO-CL compared with the inactive TM-CL.

In the first experiment (Fig. 4), seHAS was mixed with either 1 mM TM-CL or 1 mM TO-CL and increasing amounts of the second CL species. If TM-CL was fixed at 1 mM, then seHAS activity was initially very low as expected but increased sharply as the TO-CL concentration increased from 0.25 to 1 mM. TO-CL was able to activate seHAS in the presence of a greater or equal concentration of TM-CL. In contrast, when the fixed lipid was TO-CL, the addition of TM-CL, even up to 2 mM, did not decrease the ability of TO-CL to activate seHAS. These results indicate that seHAS interacts preferentially with TO-CL compared with TM-CL.
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In a second kinetic experiment (Fig. 5), we assessed the effects on the rate of HA synthesis of 1 mM TO-CL or TM-CL alone or as mixtures of the two CL species. The behavior of the enzyme was different depending on whether the two lipids were premixed and dried from organic solvent before aqueous suspension (Fig. 5A) or were mixed separately from aqueous suspensions (Fig. 5B). The activity of seHAS in the presence of either TM-CL or TO-CL alone was not affected by the method of lipid preparation (Fig. 5, solid circles and squares). When the lipids were premixed prior to suspension, the addition of 1 mM TM-CL had no effect on the rate of seHAS activity in the presence of 1 mM TO-CL (Fig. 5A, open circles), consistent with the results in Fig. 4. However, the enzyme was less active in this same lipid mixture when the lipids were presented to the protein as separate aqueous suspensions (Fig. 5B, open circles). Interestingly, after a lag time of ~20–25 min, the steady-state rate of HA synthesis in the presence of the two CL species was identical to that with TO-CL alone. These results indicate that when the two lipids are presented as a mixture from separate aqueous suspensions, seHAS interacts with each of the CL species (e.g. as micelles or liposomes) in a kinetically different way. The protein-lipid interactions are dynamic (i.e. kinetically reversible), and the interaction with TO-CL is probably more favorable thermodynamically than with TM-CL.

seHAS Shows the Same Order of CL Preference between pH 6 and 10.5—In a previous characterization of the purified seHAS, we noted an unusually broad pH range for enzyme activity, from pH 6 to pH 10.5–11.0 (24). Because this result was obtained in the presence of BCL, we wanted to determine whether the enzyme behaved any differently with the more effectively activating TO-CL or whether the inability of TM-CL
to activate seHAS was pH-dependent (Fig. 6A). As expected, lipid-depleted purified seHAS had very low activity with TM-CL from pH 4.5 to 10.5, although a very small peak of activity was observed between pH 7.5 and 8.5. The bovine, E. coli, and TO-CL preparations showed the same overall pattern of pH dependence, and their order of effectiveness did not change with pH. In each case, an interesting multi-component profile was observed between pH 6 and 10.5 showing two distinct peaks with maximum activities at pH ~7.5–8 and pH 9–10. This result was observed in three independent experiments, although the decreased activity observed in the region at pH 8.5 was variable. In all cases, the enzyme was essentially inactive at pH 10.5, but another small peak of activity was found between pH 11 and 12, an unusually high pH for retention of enzyme activity. The results show that the lipid requirement for seHAS activity and the preference for TO-CL are still present and consistent over a broad range between pH 6 and pH ~10.5.

seHAS Becomes Lipid-independent above pH 11—The additional peak of enzyme activity seen in Fig. 6A above pH 10.5–11 was unexpected and seemed unusual. This activity was reproducible, but on further examination, we found that phospholipids were not required at this very high pH for seHAS to make HA (Fig. 6B). Treatment with Streptomyces hyaluronidase verified that the enzyme synthesized normal HA products (not shown). In multiple experiments, the level of HAS activity at pH 11.5 without lipid ranged up to ~10% of that at pH 7.6 with BCL.

Lipid Dependence of seHAS Incorporated into Proteoliposomes—We next wanted to characterize the lipid dependence of seHAS incorporated into liposomes of defined size and composition. Proteoliposomes were prepared by disrupting preformed PC-based liposomes with OG, adding purified seHAS, and then removing the detergent. Transmission electron microscopy (Fig. 7A) showed single intact liposomes of relatively uniform size (~50 nm). To determine the efficiency of seHAS incorporation into liposomes, the protein remaining in the supernatant after ultracentrifugation was precipitated using trichloroacetic acid and analyzed by SDS-PAGE and Western blotting (Fig. 7B). Only trace amounts of seHAS were detected in the supernatant after centrifugation, demonstrating that essentially complete incorporation of purified recombinant seHAS protein into liposomes was achieved. Consistent with the above and previous (12) results, seHAS had little or no activity in proteoliposomes containing only PC (not shown).

We then tested the effect of different total lipid-to-protein molar ratios on seHAS activity in proteoliposomes containing TO-CL. When liposomes were prepared with a constant PC:TO-CL molar ratio (4:1) and different amounts of purified seHAS, enzyme activity increased with increasing protein-to-lipid ratios (Fig. 7C). To assess whether seHAS showed the same specific activation by TO-CL in proteoliposomes as in the above experiments,
we varied the TO-CL:PC molar ratio, while keeping the lipid-to-protein molar ratio constant. As a control to assess the potential activity of the enzyme, we assayed the activity of seHAS in proteoliposomes in the presence or absence of 2 mM BCL. seHAS showed much higher activity in liposomes produced using a 1:1 molar ratio rather than a 1:2 ratio of TO-CL:PC (Fig. 8A). Inclusion of exogenous BCL had a strong stimulatory effect on seHAS activity if the liposome TO-CL content was 33 mol % but did not further increase the enzyme activity when the TO-CL content was 50 mol %. This result indicates that proteoliposomes with a PC-to-TO-CL molar ratio of 1:1 provide sufficient opportunity for seHAS to be activated optimally by TO-CL (or conversely, to be not inhibited by interaction with PC). This finding was valid for proteoliposome preparations with a range of seHAS-to-lipid molar ratios. HAS activity was then determined in the presence (solid bars) or absence (open bars) of 2 mM BCL at 30 °C.

TO-CL Rescues the Activity of Detergent-solubilized seHAS—Finally, we examined the ability of TM-CL or TO-CL to prevent the apparently irreversible inactivation of HAS that typically occurs during extraction with nonionic detergents (Fig. 9).

Based on a titration experiment (not shown) to assess the sensitivity of membrane-bound HAS to detergent, we found that activity was completely inhibited by ≥0.01% Nonidet P-40. We then assessed the effect of increasing concentrations of TO-CL or TM-CL on inactivation of seHAS with 0.01% Nonidet P-40. HAS inactivation was completely prevented by including 0.25 mM TO-CL during treatment with detergent, whereas 0.25 mM TM-CL had only a minor effect. As the concentration of TO-CL increased to 2 mM, there was actually a further activation of seHAS to a level that was ~151% of the original membrane activity. In contrast, in the presence of 2 mM TM-CL, the enzyme showed only ~15% of its initial membrane activity. Thus, membrane-bound seHAS was solubilized with excellent retention of activity, only when the best activating phospholipid was present during extraction.

DISCUSSION

This is the first report in which a HAS has been purified and reconstituted in an active form into liposomes. The characteristics of purified seHAS reconstituted in proteoliposomes were similar to and very consistent with the phospholipid dependence and preference for TO-CL, rather than TM-CL, demonstrated when the enzyme was simply mixed with the lipids in suspension. Another major finding is that membrane-bound seHAS could be solubilized without inactivating the enzyme, if the best activating phospholipid was present during extraction.

Streptococcal HASs have been amenable to detailed study because these enzymes can be solubilized by the mild detergent DDM with retention of activity and purified (12, 24). However, purification and study of the eukaryotic enzymes has been more difficult because these HASs are generally inactivated in the presence of any detergent, including DDM (3). This lability of
HAS to detergent solubilization has hindered progress in the field, because no one has been able to study an active purified mammalian HAS. The only exception is a study by Yoshida et al. (30) in which mouse FLAG-HAS1 was extracted from membranes with 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonic acid, purified by affinity chromatography, and reconstituted in an active form by layering onto buffer containing a high concentration of the same detergent. This two-phase system apparently mimics features of the lipid bilayer required by HAS.

Kimata and co-workers3 recently purified recombinant mouse HAS2 expressed in insect cells using a baculovirus expression vector. Significantly, they found that the best detergent was DDM, and DDM-solubilized HAS2 was activated by subsequent addition of one particular phospholipid. Thus, it seems likely that all of the HAS family members are lipid-dependent, as reported originally for the streptococcal enzymes (12). The single exception, the Pasteuella enzyme, is structurally and functionally different from all other HAS proteins (2, 31).

We note that the rescue of HAS activity by TO-CL after Nonidet P-40 treatment (Fig. 9) is the first successful solubilization of a Class I HAS using a nonionic detergent (other than DDM) under conditions that retain or rescue activity of HAS suitable for enzyme purification. These results support the conclusion that detergent inactivation of HAS is due to removal of activating lipids associated with the enzyme. If these lipids are replaced, as in the case of the lipid-depleted purified seHAS, or not removed completely, then HAS can remain active. Taking advantage of this strategy should allow other mammalian HASs to be purified and characterized.

Lipids associated with membrane proteins are found in three general types of interactions: tightly bound in an annular shell, nonannular in cavities of the protein surface, and as integral proteins (2, 31). In preliminary mass spectrometry experiments (not shown), we detected CL m/z signature profiles in seHAS that had been DDM-solubilized and purified without added lipid. These residual CL molecules likely represent tightly bound annular lipids associated with the enzyme. If these lipids are replaced, as in the case of the lipid-depleted purified seHAS, or not removed completely, then HAS can remain active. Taking advantage of this strategy should allow other mammalian HASs to be purified and characterized.

Some of the proteins and enzymes known to require CL for activity or stability include the sodium/proton antiporter, cytochrome bc1 complex, ATP synthase, bacteriorhodopsin, and the EmrE multidrug transporter. In general, the membrane proteins known to be CL-dependent are channels or transporters that use multiple membrane domains to constitute a pore within the bilayer. An interesting exception is MurG, an E. coli glycosyltransferase involved in peptidoglycan biosynthesis (14). Overexpression of this peripheral membrane protein results in elevated CL content in cell membranes and association of the protein with unusual intracellular vesicles.

The distinct bimodal pH optima for seHAS are unusual for a single enzyme. HAS, however, is actually a multifunctional enzyme with four distinct donor/acceptor-binding sites, a hyaluronyl-UDP translocation activity, and two separate glycosyltransferase activities (31). Unlike the vast majority of known glycosyltransferases, HASs that elongate at the reducing end use UDP-GlcUA and UDP-GlcNAc as acceptors rather than donors. The donors in these two reactions are the hyaluronyl-UDP products with either GlcNAc or GlcUA attached to UDP at the reducing end. Further studies are needed to determine whether the bimodal pH optima reflect distinct partial reactions catalyzed by HAS. Despite the phospholipid requirement for activity over a broad pH range, HAS showed unexpected activity at pH 11–12 in the absence of any added lipid. This high pH is well above the pKa values for Glu, Asp, Lys, Arg, and His residues in HAS and also likely near or above the pKa values for Ser, Thr, Cys, and Tyr. Interestingly, this pH range is also likely to deprotonate many of the -OH groups in GlcNAc and GlcUA residues in HA and, in HA-UDP, GlcNAc-UDP and GlcUA-UDP. Thus, in this lipid-independent mode at high pH, the enzyme and substrates are all expected to be highly negatively charged. The enzyme-activating phospholipids (e.g. CL) are also negatively charged, which is consistent with the possibility that negative charge is important for HAS activity.

The finding that HAS activity is lipid-independent at high pH levels is not only surprising, but it could also be very significant for new approaches to characterize the structure, function, and mechanism of HAS. The ability to be active in the absence of lipid means that it might be feasible to study purified streptococcal HASs using biophysical techniques typically employed to study protein structure (e.g. circular dichroism, fluorescence spectroscopy, ultracentrifugation, or calorimetry). Such techniques could not be easily used before, because of the presence of large amounts of lipid needed for activity.

Our results indicate there is something special about 18:1 Δ9 oleoyl chains for seHAS activation, because the only lipids able to activate the enzyme contained this fatty acid. A possible explanation is that the fluidity of this acyl chain provides advantages for sealing the mobile bilayer-protein interface during domain movement of HAS as it polymerizes HA. It will be interesting, although difficult, to determine whether the fatty acyl groups are any different for the annular, nonannular, or integral lipids associated with native protein. Mixing experiment results (Fig. 5) indicate that seHAS interacts with either TM-CL or TO-CL in a dynamic, reversible way. If the enzyme first associates with TM-CL, then it takes time for it to dissociate from TM-CL and bind sufficient TO-CL molecules to become active. This result indicates that seHAS interacts with TO-CL in a thermodynamically more favorable way than with TM-CL.

None of the known CL-dependent proteins is reported to require as many CL molecules associated with the protein as HAS, although few investigations have been able to ascertain the molar ratios within protein-lipid complexes. Radiation inactivation studies of Streptococcus pyogenes and Streptococcus equisimilis membranes containing HAS as well as E. coli membranes containing each of the two recombinant streptococcal HASs revealed that the active unit is a HAS monomer associated with an additional mass of ~23 kDa (11). This addi-

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3 A. Murakawa and K. Kimata, personal communication.
As the UDP-HA chain is elongated, the alternating domain bind a UDP-sugar acceptor, the other is active as a transferase. These activities are reciprocal, and when one domain is able to inactive or active, and only one domain can be active at a time. Different positions in which its transferase function is either conserved within the HAS family, interact directly with each other or with HA during synthesis (37). Fig. 10 illustrates the proximity of MD2 and MD4 in the context of the proposed HAS-lipid complex. In the original model (25), we referred to membrane-associated domains 1 and 2 and transmembrane domains 1–4 differently, with a different numbering system and assumed that their order of interaction in the protein was sequential. Now we know that MD2 and MD4 are partners (37). In fact, all the HAS MDs are likely close together, and specific MDs interact with each other. The organization of HAS domains might be similar to that of many transporter or channel proteins whose folding and topology within the membrane create an intra-protein space important for moving the ligand or ion across the membrane bilayer. The molecular mechanism that appears most consistent with the topological and structural features of these HAS enzymes is the formation of a pore through which the HA product moves.

Reconstitution of purified HAS into defined liposomes will allow us to address some specific questions about the above pore model of HAS and the vectoral nature of HA synthesis. Because proteoliposomes have an internal lumen, transport studies can be performed, and inside and external components can be separated. The method used to prepare proteoliposomes (26) is designed to give an orientation of intracellular domains out (i.e. facing the medium rather than the lumen), although this has yet to be confirmed for seHAS. If this is the orientation of most incorporated seHAS molecules, then HA synthesis might proceed by use of external substrates and translocation of HA product to the proteoliposome interior. Lumenal overaccumulation of HA might result in either disruption of proteoliposomes (and continued HA synthesis) or a time-dependent inhibition of HA synthesis if the proteoliposomes remained intact. Studies are in progress to answer these and other similar questions.

Based on many of these above considerations, we proposed the Pendulum Hypothesis model (38) for polysaccharide synthesis. This model has several conceptually similar variants. In one variant of the Pendulum Hypothesis, the HASs have two functional domains that move, and each domain contains one of the two UDP-sugar-binding sites, a binding site for one of the two donor HA-UDPs, and performs one of the two hyaluronidase unit) at the reducing end. The very nature of such a translocation function for HAS likely requires a mechanism involving the movement of multiple protein domains.

In support of this intra-protein pore model, we recently found that two polar residues within MD2 and MD4, which are conserved within the HAS family, interact directly with each other or with HA during synthesis (37). Fig. 10 illustrates the proximity of MD2 and MD4 in the context of the proposed HAS-lipid complex. In the original model (25), we referred to membrane-associated domains 1 and 2 and transmembrane domains 1–4 differently, with a different numbering system and assumed that their order of interaction in the protein was sequential. Now we know that MD2 and MD4 are partners (37). In fact, all the HAS MDs are likely close together, and specific MDs interact with each other. The organization of HAS domains might be similar to that of many transporter or channel proteins whose folding and topology within the membrane create an intra-protein space important for moving the ligand or ion across the membrane bilayer. The molecular mechanism that appears most consistent with the topological and structural features of these HAS enzymes is the formation of a pore through which the HA product moves.

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synthesis would not be well tolerated if water, ions, or small molecules were able to enter or leave the cell because of leakiness. Studies are in progress to test this model and the role of CL in activating HAS.

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