Kinetic Characterization of the Recombinant Hya-luronan Syntheses from Streptococcus pyogenes and Streptococcus equisimilis*

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The two hyaluronan synthases (HASs) from Streptococcus pyogenes (spHAS) and Streptococcus equisimilis (seHAS) were expressed in Escherichia coli as recombinant proteins containing His6 tails. The accompanying paper has described the purification and lipid dependence of both HASs, their preference for cardiolipin, and their stability during storage (Tlapak-Simmons, V. L., Baggenstoss, B. A., Clyne, T., and Weigel, P. H. (1999) J. Biol. Chem. 274, 4239–4245). Kinetic characterization of the enzymes in isolated membranes gave $K_m$ values for UDP-GlcUA of $40 \pm 4 \mu M$ for spHAS and $51 \pm 5 \mu M$ for seHAS. In both cases, the $V_{max}$ profiles at various concentrations of UDP-GlcNAc were hyperbolic, with no evidence of cooperativity. In contrast, membrane-bound spHAS, but not seHAS, showed sigmoidal behavior as the UDP-GlcNAc concentration was increased, with a Hill number of $-2$, indicating significant cooperativity. The Hill number for UDP-GlcNAc utilization by seHAS was 1, confirming the lack of cooperativity for UDP-GlcNAc in this enzyme. The $K_m$ values for UDP-GlcNAc were $60 \pm 7 \mu M$ for seHAS and $149 \pm 3 \mu M$ for spHAS in the isolated membranes. The kinetic characteristics of the two affinity-purified HAS enzymes were assessed in the presence of cardiolipin after 8–9 days of storage at $-80^\circ C$ without cardiolipin. With increasing storage time, the enzymes showed a gradual increase in their $K_m$ values for both substrates and a decrease in $V_{max}$. Even in the presence of cardiolipin, the detergent-solubilized, purified HASs had substantially higher $K_m$ values for both substrates than the membrane-bound enzymes. The $K_{\text{UDP-GlcUA}}$ for purified spHAS and seHAS increased 2–4-fold. The $K_{\text{UDP-GlcNAc}}$ for spHAS and seHAS increased 4- and 5-fold, respectively. Despite the higher $K_m$ values, the $V_{max}$ values for the purified HASs were only $-50\%$ lower than those for the membrane-bound enzymes. Significantly, purified spHAS displayed the same cooperative interaction with UDP-GlcNAc ($n_H = 2$), whereas purified seHAS showed no cooperativity.

HA is a polysaccharide composed of two alternating sugars, $\beta1,3$-linked glucuronic acid and $\beta1,4$-linked N-acetylgalactosamine (1). Although the structure of HA seems quite simple, the molecule, nonetheless, has unusual physical properties that are important for its numerous biological functions (2–5). For example, HA forms very viscous solutions and gels due to its high molecular mass and its ability to bind cations and to hydrate large amounts of water. This characteristic of HA provides the viscous lubrication of synovial fluid and helps provide cartilage with its viscoelasticity. These characteristics are also ideal for the role HA has in the extracellular matrices (4, 5) of the skin and virtually every vertebrate tissue as well as in the fluid of the vitreous humor of the eye. HA also plays an important role in morphogenesis, wound healing (6–9), and angiogenesis (10, 11). HA receptors and HA-binding proteins, particularly CD44 (12) and the receptor for hyaluronic acid-mediated mobility (RHAMM; Ref. 13), modulate cellular responses to HA.

The first HAS gene to be cloned was from Group A Streptococcus pyogenes (14–16). When the bona fide HAS from Group C Streptococcus equisimilis was later cloned (17), the seHAS protein showed 70 and 72% identities to spHAS at the nucleotide and amino acid sequence levels, respectively. After discovery of the spHAS gene, a related family of homologous cDNAs and enzymes was then found in eukaryotes (18, 19). These HASs include human HAS1 and HAS2 (20–22); murine HAS1, HAS2, and HAS3 (19, 23, 24); chicken HAS2 and HAS3 (19); and Xenopus laevis HAS1, HAS2, HAS3, and HAS-related sequence (19, 25–29). In addition, HASs have also been identified and cloned (30) from chlorella virus PBCV-1 (A9R) and from Pasteurella multocida (31). Although the two streptococcal HASs are very similar, the HAS from P. multocida is quite different structurally. Similarities among the prokaryotic and eukaryotic members of this HAS family have been reviewed (18). These various HAS enzymes comprise a large family of proteins with many common features and regions of amino acid sequence identity or similarity.

To understand the important role of the HA polysaccharide in normal development and health and in various diseases, it is critical to know more about the HASs, the enzymes responsible for HA synthesis. We need to know how these HASs work to assemble the HA polymer and how the enzymes are regulated.

In the accompanying paper (32), we reported the purification and lipid dependence of active recombinant spHAS and seHAS expressed in Escherichia coli. In the present study, we have determined, for the first time in the absence of other streptococcal proteins or factors, the kinetic constants for both HAS enzymes in membranes and after detergent solubilization and purification. A preliminary report of these findings was reported earlier (33).

EXPERIMENTAL PROCEDURES

Materials, Strains, and Plasmids—Reagents were from Sigma unless stated otherwise. Media component were from Difco. S. pyogenes strain S43/192/4 and Group C S. equisimilis strain D181 were from the Rockefeller University collection. E. coli SURE™ cells were from Strat-
agon. The HAS open reading frames from *S. pyogenes* (15, 16) and *S. equisimilis* (17) were inserted into the pKK223-3 vector (Amersham Pharmacia Biotech) and cloned into *E. coli* SURE™ cells. The spHAS and seHAS proteins (16, 17) contained a C-terminal fusion of 6 His residues. When a comparison was made between seHAS-His in *E. coli* membranes and seHAS in streptococcal membranes, there was no decrease in HAS activity, normalized for HAS protein, due to the His fusion (data not shown).

**Cell Growth and Membrane Preparation**—Membranes from *S. pyogenes* and *S. equisimilis* were obtained by modifications of a protoplast method (34), as reported previously (16). Membranes from *E. coli* were isolated by variation of the protoplast method of Ito et al. (35). The SURE™ cells containing the construct were grown at 30 °C in Luria broth containing 50 mM glucose and trace elements (36) to an *A*ₚ₀ of 1.5 and then induced with isopropyl-β-D-thiogalactoside and grown for an additional 3 h. The cells were harvested by centrifugation at 4 °C for 30 min at 3000 × g, washed twice with phosphate-buffered saline containing 10% glycerol, and then frozen at −80 °C. The cell pellet was thawed and resuspended to 1% of the original culture volume in 20% sucrose, 30 mM Tris, pH 8.2, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 μM leupeptin, and 0.7 μg/ml pepstatin. Lysozyme (4 mg/ml) in 0.1 mM EDTA, pH 8 (0.1% of the initial culture volume), was added, and the suspension was incubated for 40 min on ice with constant mixing. Phenylmethanesulfonyl fluoride was added to a concentration of 46 μg/ml, and the suspension was sonicated three times for 30 s each at 20 watts with a microtip (Model W-380, Heat Systems Ultrasonic, Inc.). MgCl₂ (60 mM) and DNase and RNase (1 μg/ml each) were added to the indicated final concentrations. After 20 min on ice with constant mixing, debris was removed by centrifugation (10,000 × g, 30 min, 4 °C). The lysate was diluted with 1 volume of ice-cold phosphate-buffered saline containing 10% glycerol, 1 mM dithiothreitol, and the above protease inhibitors, and the membranes were harvested at 100,000 × g for 1 h. The membrane pellet was washed once with phosphate-buffered saline containing 10% glycerol, 1 mM dithiothreitol, and the above protease inhibitors by resuspension and centrifugation. The final pellet was stored frozen at −80 °C.

**HAS Activity**—The standard determinations for HAS activity were carried out 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 EDTA, 15–20% glycerol, 1 mM UDP-GlcUA (Fluka), and 0.8 μM UDP-[¹⁴C]GlcUA (267 mCi/mmol; NEN Life Science Products). When assaying spHAS, 1.5 mM UDP-GlcNAc was used, whereas 1.0 mM UDP-GlcNAc was used to assay seHAS. n-Dodecyl β-D-maltoside (0.98 mM) and 2 mM CL were also present in assays with the purified HAS, which was purified as described in the accompanying paper (32). To initiate the enzyme reaction, 3 μg of whole membrane protein or 0.5–0.75 μg of pure HAS protein, and the mixtures were gently mixed in a Micro-mixer (Taitec) at 30 °C for 1 h. Reactions were terminated by the addition of SDS to 2% (w/v) final concentration at room temperature. Incorporation of [¹⁴C]GlcUA into high molecular mass HA was measured by descending paper chromatography using Whatman No. 3MM paper developed in 1% ammonium acetate, pH 5.5, and ethanol (17:1). After cutting out the origins, the amount of radioactivity present was assessed using 1 ml of H₂O and 5 ml of Ultimagold scintillation fluid (Packard) and a Packard Model A2300 scintillation counter.

**Determination of Michaelis-Menten Constants (**$K_m$**)**—The *K_m* values for both UDP-GlcUA ($K_m^{UDP-GlcUA}$) and UDP-GlcNAc ($K_m^{UDP-GlcNAc}$) were determined by varying one substrate while holding the other constant. The incorporation of substrate was monitored as described above. As noted previously in studies of HA chain elongation (17), seHAS appeared to be 2-fold more catalytically active than spHAS. The $K_m^{UDP-GlcUA}$ of both membrane-bound enzymes differed only slightly, 40 ± 4 μM for spHAS (Table I) and 51 ± 5 μM for seHAS (Table II), at saturating UDP-GlcNAc concentrations. The $V_{max}$ saturation profiles, with respect to UDP-GlcUA utilization, for both enzymes in *E. coli* membranes were hyperbolic (Figs. 1 and 2), and the Lineweaver-Burk plots were linear (data not shown).

For both spHAS (Fig. 1B) and seHAS (Fig. 2B), the specific enzyme activity at lower concentrations of UDP-GlcUA increased and then decreased as the concentration of UDP-GlcNAc increased. For example, the spHAS enzyme was roughly twice as active in response to the UDP-GlcUA concentration at 0.2 mM UDP-GlcNAc compared with 2.0 mM UDP-GlcNAc (Fig. 1B). This biphasic behavior probably reflects the partial competition of UDP-GlcNAc at high concentrations, for the UDP-GlcUA-binding site, although there is no evidence that other

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**RESULTS**

**Kinetic Characterization of Recombinant Membrane-bound spHAS and seHAS**—The kinetic behaviors of the two recombinant HAS isoforms in isolated *E. coli* membranes were compared using the paper chromatography assay for incorporation of radiolabeled precursors into HA (Figs. 1–4 and Tables I and II). As noted previously in studies of HA chain elongation (17), seHAS appeared to be 2-fold more catalytically active than spHAS. The $K_m^{UDP-GlcUA}$ of both membrane-bound enzymes differed only slightly, 40 ± 4 μM for spHAS (Table I) and 51 ± 5 μM for seHAS (Table II), at saturating UDP-GlcNAc concentrations. The $V_{max}$ saturation profiles, with respect to UDP-GlcUA utilization, for both enzymes in *E. coli* membranes were hyperbolic (Figs. 1 and 2), and the Lineweaver-Burk plots were linear (data not shown).

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UDP-GlcUA, the Hill analysis (Fig. 3B). When UDP-GlcNAc was varied at a given concentration of UDP-GlcUA, the Hill analysis (Fig. 3C) revealed Hill numbers of ~2 (Table I). A Hill number >1 indicates a degree of cooperativity associated with the spHAS enzyme’s ability to bind and utilize UDP-GlcNAc at fixed UDP-GlcUA concentrations. Hill analysis gave a $K_{	ext{m}}$ for UDP-GlcNAc at $V_{	ext{max}}$ is 0.005–2.0 $\mu$M for spHAS at saturating UDP-GlcUA concentrations.

The membrane-bound seHAS enzyme, however, did not respond to UDP-GlcNAc in the same manner. Rather than showing a sigmoidal $V_{\text{max}}$ saturation profile with respect to UDP-GlcUA, seHAS showed a typical hyperbolic curve (Fig. 4A). The Lineweaver-Burk analysis for seHAS was linear and gave a $K_{\text{m}}$ for UDP-GlcNAc of 60 ± 7 $\mu$M at saturating UDP-GlcUA concentrations (Table II). Hill plots for the seHAS interaction with UDP-GlcNAc gave Hill numbers of ~1, indicating no cooperativity (Table II). As also noted in Figs. 1B and 2B, when the UDP-GlcUA concentration was increased, the specific activity of seHAS first increased and then decreased (Fig. 4B). This biphasic behavior at low concentrations of UDP-GlcNAc could reflect the competitive interactions of UDP-GlcUA at the UDP-GlcNAc-binding site.

**Table I**

Michaelis-Menten constants for spHAS in E. coli membranes

| UDP-GlcNAc $\mu$M | $K_{\text{m}}$ for UDP-GlcUA $\mu$M | $V_{\text{max}}$ nmol/µg protein/h |
|-------------------|-------------------------------------|-----------------------------------|
| 0.1               | 17 ± 2                              | 0.6 ± 0.2                         |
| 0.2               | 25 ± 1                              | 1.4 ± 0.1                         |
| 0.5               | 27 ± 2                              | 1.9 ± 0.3                         |
| 1.0               | 35 ± 4                              | 2.2 ± 0.1                         |
| 1.5               | 40 ± 4                              | 2.3 ± 0.3                         |

**Table II**

Michaelis-Menten constants for seHAS in E. coli membranes

| UDP-GlcUA $\mu$M | $K_{\text{m}}$ for UDP-GlcNAc $\mu$M | $V_{\text{max}}$ nmol/µg protein/h |
|------------------|-------------------------------------|-----------------------------------|
| 0.01             | 5 ± 3                               | 0.6 ± 0.1                         |
| 0.05             | 17 ± 2                              | 2.6 ± 0.1                         |
| 0.1              | 20 ± 4                              | 3.0 ± 0.1                         |
| 0.5              | 51 ± 5                              | 4.2 ± 0.6                         |
| 1.0              | 76 ± 5                              | 4.2 ± 0.5                         |

**Figure 2**

Effect of UDP-GlcUA concentration on the activity of membrane-bound seHAS at various concentrations of UDP-GlcNAc. *A*, membranes containing recombinant seHAS were incubated with the indicated concentrations of UDP-GlcUA (UDP-GlcA) and 0.01 mM (●), 0.05 mM (○), 0.1 mM (●), 0.5 mM (■), 1.0 mM (▲), or 1.5 mM (▲) UDP-GlcNAc, and HA activity was measured as described under “Experimental Procedures.” The saturation profiles of all curves were hyperbolic and gave linear Lineweaver-Burk plots (data not shown). *B*, a blowup of the initial concentration range of the saturation profile in *A* shows an increase and then a decrease in activity at low concentrations of UDP-GlcUA as the concentration of UDP-GlcNAc increases.
FIG. 3. Effect of UDP-GlcNAc concentration on the activity of recombinant membrane-bound spHAS at various concentrations of UDP-GlcUA. A, membranes containing recombinant spHAS were incubated with the indicated concentrations of UDP-GlcNAc and 0.01 mM (●), 0.05 mM (○), 0.1 mM (■), 0.5 mM ( □), or 1.0 mM (▲) UDP-GlcUA (UDP-GlcA). The saturation profiles of all the curves showed sigmoidal behavior with respect to UDP-GlcNAc at various UDP-GlcUA concentrations. B, a blowup of the initial concentration range in A shows an increase in the sigmoidal behavior of the enzyme toward UDP-GlcNAc as the UDP-GlcUA concentration increases. C, the Hill analysis of the data from A yielded numbers that are approximately equal to 2, thus indicating cooperativity associated with the ability of spHAS to utilize UDP-GlcNAc in the presence of UDP-GlcUA.

FIG. 4. Effect of UDP-GlcNAc concentration on the activity of recombinant membrane-bound seHAS at various concentrations of UDP-GlcUA. A, membranes containing recombinant seHAS were incubated with the indicated concentrations of UDP-GlcNAc and 0.01 mM (●), 0.05 mM (○), 0.1 mM (■), 0.5 mM ( □), or 1.0 mM (▲) UDP-GlcUA (UDP-GlcA). The saturation profiles of all the curves were hyperbolic and gave linear Lineweaver-Burk plots (data not shown). B, a blowup of the initial concentration range in A shows a decrease in enzyme activity at low concentrations of UDP-GlcNAc as the UDP-GlcUA concentration increases.
Kinetic Characterization of Purified Streptococcal HASs—
The purification, storage, and stability of seHAS and spHAS and the effects of CL on their activities have been described in the accompanying paper (32). The kinetic characterization of both purified enzymes was performed after 8–9 days of storage at −80 °C in the absence of CL. The activities of the two affinity-purified synthases were relatively stable from about day 6 to at least 4 weeks under these conditions. Bovine CL was present at 2 mM in all the kinetic assays since both enzymes are lipid-dependent and highly stimulated by CL.

The kinetic characteristics observed for the pure enzymes were very similar to those seen in Figs. 1–4 for the membrane-bound HASs. Both spHAS and seHAS showed hyperbolic responses to UDP-GlcUA in the presence of UDP-GlcNAc. Most notably, purified spHAS showed sigmoidal behavior for its utilization of UDP-GlcNAc (Fig. 5A), but the response of seHAS to UDP-GlcNAc was hyperbolic, not sigmoidal (data not shown). Therefore, purified spHAS retained this distinct characteristic of cooperativity displayed by the membrane-bound enzyme. The Michaelis-Menten constants for utilization of both sugar nucleotides by the purified enzymes were significantly increased when compared with the membrane-bound enzymes. The saturation profiles, however, remained very similar. For spHAS, $K_{\text{UDP-GlcUA}}$ and $K_{\text{UDP-GlcNAc}}$ were 40 and 149 μM for the membrane-bound enzyme, but increased to 175 and 434 μM, respectively, for purified spHAS (Table III). Likewise, for seHAS, $K_{\text{UDP-GlcUA}}$ and $K_{\text{UDP-GlcNAc}}$ were 51 and 60 μM for the membrane-bound enzyme, but increased to 274 and 251 μM, respectively, for purified seHAS (Table IV).

Although the $K_m$ values for the two substrates increased 4–10-fold in the purified HASs, the overall catalytic efficiencies of the two enzymes were very similar to those of the membrane-bound enzymes. Based on their specific activities at saturation and their abundance in the E. coli membranes used for the purification (32), we estimate that the $V_{\text{max}}$ for the pure HASs, after 8–9 days of storage, is at least 50% of the $V_{\text{max}}$ for the membrane-bound enzymes.

Another difference noted in the purified compared with the membrane-bound enzymes is that both purified HASs were more sensitive to substrate inhibition, especially spHAS (Fig. 5). This increase in the degree of inhibition with increasing sugar nucleotide concentration was apparent when either substrate was present at or over saturating concentrations. At low concentrations of the first sugar nucleotide, spHAS showed a plateau and then a decrease in velocity as the second sugar nucleotide concentration increased. The $V_{\text{max}}$ for spHAS increased and then dramatically decreased as the UDP-GlcNAc concentration was varied from 5 μM to 2 mM at fixed concentrations of UDP-GlcUA (Fig. 5A). Likewise, the same inhibition pattern occurred when the concentration of UDP-GlcUA was varied from 5 μM to 2 mM at fixed concentrations of UDP-GlcNAc (Fig. 5B). This dramatic inhibition of the purified HASs at high sugar nucleotide concentrations was not as apparent in the membrane-bound species. These results indicate that the recognition of the normal substrates is affected by other sugar nucleotides and that “cross-talk” occurs between the two sugar-binding sites and both substrates.

**DISCUSSION**

Historically, the first cell-free studies of HA biosynthesis used Group A streptococcal bacteria. The pioneering work of Dorfman and co-workers (39, 44) in the 1950s and 1960s showed that the streptococcal HAS was located in the cell membrane, required Mg$^{2+}$ ions, and used the two sugar nucleotide substrates UDP-GlcUA and UDP-GlcNAc to polymerize a HA chain. Subsequently, however, these and many other workers were unable to solubilize the enzyme in an active and stable form or to purify it. Similarly, eukaryotic HASs have not yet been purified. Isolation of the Group A and Group C streptococcal HAS genes has now allowed us to express these proteins in large amounts in E. coli (32).

All of the known enzymes catalyze reactions that use one or two (or, rarely, three) substrates and produce one or two prod-
TABLE III
Michaelis-Menten constants for purified spHAS after storage

| UDP-GlcNAc | K_m for UDP-GlcNAc | V_max | Hill No. |
|------------|---------------------|--------|---------|
| 0.1        | 68 ± 4.2            | 1.8 ± 0.1 | 1.7 ± 0.0 |
| 0.05       | 80 ± 1.1            | 6.8 ± 0.6 | 1.7 ± 0.1 |
| 0.1        | 126 ± 23.0          | 11.4 ± 0.9 | 1.9 ± 0.1 |
| 0.5        | 335 ± 23.0          | 22.2 ± 1.1 | 2.0 ± 0.1 |
| 1.0        | 434 ± 11.0          | 29.7 ± 0.7 | 2.0 ± 0.1 |
| 2.0        | 605 ± 49.0          | 24.4 ± 2.8 | 2.2 ± 0.1 |

* The concentration of UDP-GlcUA in each case ranged from 5 μM to 20 mM.

* The concentration of UDP-GlcNAc in each case ranged from 5 μM to 5.5 mM.

TABLE IV
Michaelis-Menten constants for purified seHAS after storage

| UDP-GlcNAc | K_m for UDP-GlcNAc | V_max | Hill No. |
|------------|---------------------|--------|---------|
| 0.05       | 22 ± 1.4            | 6.7 ± 2.2 | 1.7 ± 0.0 |
| 0.1        | 40 ± 6.3            | 11.7 ± 2.6 | 1.4 ± 0.1 |
| 0.5        | 90 ± 12.0           | 16.3 ± 1.4 | 1.4 ± 0.1 |
| 1.0        | 163 ± 17.0          | 27.7 ± 3.6 | 1.8 ± 0.1 |
| 1.5        | 274 ± 28.0          | 37.4 ± 0.6 | 1.2 ± 0.1 |
| 2.0        | 370 ± 8.5           | 35.3 ± 1.2 | 1.2 ± 0.1 |

* The concentration of UDP-GlcUA in each case ranged from 5 μM to 2 mM.

* The concentration of UDP-GlcNAc in each case ranged from 5 μM to 3.5 mM.

products. HASs are unique among the enzymes characterized to date. The HAS has two different enzyme activities (i.e. glyco-syltransferases) in the same protein, and the HA product after each sugar addition becomes the acceptor for the next sugar addition. HASs are also membrane enzymes. The overall reaction for the synthesis of one HA disaccharide unit is shown in Equation 1,

\[
\text{UDP-GlcUA} + \text{UDP-GlcNAc} + (\text{HA})_n \xrightarrow{\text{HAS}} (\text{HA})_{n+1} + 2 \text{UDP} \quad \text{(Eq. 1)}
\]

where \( n \) is the number of disaccharide units. Although it seems straightforward, the enzyme must possess at least six (and probably seven) different functions to perform this overall reaction, as shown in Fig. 6. Numerous questions about the details and mechanism of this complex reaction can be answered now that the streptococcal HAS enzymes have been purified.

HAS enzymes are unique in an additional respect because the two sugar nucleotide substrates are structurally so similar, they each have the possibility of competing with the other for the appropriate UDP-sugar-binding site on the enzyme. This cross-talk hypothesis is illustrated in Fig. 6 by the dashed lines that show, for example, UDP-GlcUA interacting with the UDP-GlcNAc-binding site. Although the HASs do not misincorporate other sugar nucleotides into the growing HA chain, other UDP-sugars may transiently occupy a binding site and thus be competitive inhibitors. Initial experiments have indicated that 0–1 mM concentrations of sugar nucleotides like UDP-Glc or UDP-GalUA or even UDP alone decrease the rate of HA synthesis by spHAS or seHAS.2 With high concentrations of one or both of the correct substrates, we observed enzyme inhibition in the presence of a third sugar nucleotide. Even with just the two normal substrates, the rate of HA synthesis becomes biphasic if the concentration of one UDP-sugar is much greater than the other (e.g. Fig. 2A at a ratio of 100:1). There are no previous reports of this cross-talk phenomenon affecting HA biosynthesis. Observation of this kinetic behavior reflects the advantage of studying purified HAS free from other sugar nucleotide-binding proteins orglycosyltransferases.

The scheme in Fig. 6 does not indicate whether sugars are added to the growing HA chain individually or in a coordinated manner as a disaccharide unit as proposed by Saxena et al. (40) for β-glycosyltransferases predicted to contain two functional domains by hydrophobic cluster analysis. Our present kinetic data for the streptococcal HASs do not allow discrimination between these two models. The model of Saxena et al. (40) predicts that HA synthesis occurs by addition to the reducing end, as suggested by Prehm (41). However, this model is not consistent with recent reports that HA synthesis (42) and Type 3 polysaccharide synthesis, mediated by the related synthase from Streptococcus pneumoniae (43), occur from the nonreducing end of the growing polysaccharide chain. Definitive answers regarding the direction of polysaccharide chain growth for the HASs and related syntheses may require the identification, perhaps utilizing mass spectrometry, of putative UDP-polymer intermediates with the UDP attached at the reducing end.

Stoolmiller and Dorfman (44) reported the apparent K_m for UDP-GlcNAc and K_m for UDP-GlcUA as 50 μM and 0.5 μM, respectively, in isolated S. pyogenes membranes. Their UDP-GlcUA kinetic results gave a linear Lineweaver-Burk plot. They noted, however, that the kinetics of UDP-GlcNAc utilization did not behave in the same linear manner when plotted in double-reciprocal form. These investigators were therefore the first to note sigmoidal behavior of spHAS in streptococcal membranes in response to increasing UDP-GlcNAc concentration. van de Rijn and Drake (34) reported, for detergent-solubilized spHAS, values of 39 μM for K_m for UDP-GlcUA and 150 μM for K_m for UDP-GlcNAc, but did not detect a sigmoidal response of the velocity saturation profile when UDP-GlcNAc was varied. They also reported the specific activity of spHAS as 19.4 nmol of UDP-GlcUA/h/mg of extracted membrane protein. We have reported in the accompanying paper (32) that the specific activities of purified spHAS and seHAS are 5,500 and 12,000 nmol/h/mg, respectively. These values are consistent with spHAS composed only of the membrane protein in S. pyogenes cells (17). The addition of bovine CL substantially increased the specific activity of purified spHAS and seHAS, respectively, to 20,000 and 35,000

\[ V = \frac{V_{\text{max}} [S]}{K_m + [S]} \]

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nmol/h/mg. The catalytic constants for purified spHAS and seHAS in the presence of bovine CL at 30 °C were, respectively, 22 and 36 monosaccharides/s. These values are in close agreement with those reported for seHAS assayed at 37 °C in whole membranes (17).

The above apparent Michaelis-Menten values for the two substrates were determined using either crude streptococcal membranes or detergent-solubilized membrane extracts, both of which contain other sugar nucleotide-binding proteins and glycosyltransferases and which could contain potential regulatory factors for the HAS enzyme. Here, for the first time, we have characterized the kinetic behavior of purified spHAS and seHAS and these enzymes in membranes containing no other streptococcal proteins. The results demonstrate that the n-dodecyl β-D-maltoside-solubilized, purified enzymes behave very similarly to the membrane-bound enzymes. This is an important finding because many studies have reported that HAS activity is irreversibly lost upon solubilization of the protein in a wide variety of nonionic detergents (45). The $K_{\text{UDP-GlcUA}}$ values for membrane-bound spHAS and seHAS were 40 and 51 μM, respectively. The $K_{\text{UDP-GlcNAc}}$ values for membrane-bound spHAS and seHAS were 149 and 60 μM, respectively.

Detergent-solubilized, purified HASs showed essentially the same kinetic characteristics as the membrane-bound enzymes, with the exception of their $K_m$ values. The $K_{\text{UDP-GlcUA}}$ values increased ~4-fold for purified spHAS and seHAS, although the latter enzyme was not saturated even at 1.5 mM. Both enzymes also displayed increased $K_m$ values for UDP-GlcNAc after purification; the $K_{\text{UDP-GlcNAc}}$ values increased ~4-fold for spHAS and ~5-fold for seHAS. We also noted that upon storage at ~80 °C in the absence of CL, these enzymes slowly lost activity ($t_{1/2}$ ~2–3 months), and the biggest change appeared to have occurred in $K_{\text{UDP-GlcNAc}}$, which got progressively larger with time of storage. The stimulation of either HAS by CL is due to a large decrease in $K_{\text{UDP-GlcNAc}}$ and an increase in $V_{\text{max}}$ (32).

Based on our recent findings, the sensitivity of HAS to detergent solubilization can now be explained. Radiation inactivation analysis revealed that the active spHAS and seHAS species are monomers of the HAS protein in complex with ~16 CL molecules (46). This conclusion is supported by the results in the accompanying paper (32) showing that the activity of affinity-purified HAS, which has been depleted of CL, is very low. The HAS enzymes are highly lipid-dependent and are most effectively stimulated by CL. Preliminary mass spectroscopic analysis indicated that even when purified in the absence of exogenous CL, the enzymes still contain residual associated CL. Therefore, the likely reason for why most detergents inactivate HAS (47–49) is that these detergents displace the CL required for enzyme activity. Even with CL present, most nonionic detergents will compete more efficiently than CL for interaction with the protein. The mild detergent n-dodecyl β-D-maltoside is apparently strong enough to solubilize HAS from membranes, but not so strong that the enzyme is stripped of CL. Identification of n-dodecyl β-D-maltoside as a useful detergent for solubilizing HAS was a substantial contribution (45).

Two substantial differences are apparent between the two enzymes. First, the seHAS enzyme is intrinsically about twice as active as spHAS. This was apparent in this and the accompanying study (32) with both membrane-bound and purified enzymes and in an earlier study (17) that examined the rates of HA chain elongation by gel filtration analysis. Since the Group C HA capsule is typically larger than the Group A capsule, this difference could be due to the $V_{\text{max}}$ between the two HASs. Second, spHAS, but not seHAS, is complexly regulated by UDP-GlcNAc. The spHAS interaction with this substrate shows a cooperative activation of the enzyme as the UDP-GlcNAc concentration increases. This sigmoidal behavior indicates that spHAS has a second binding site for UDP-GlcNAc that is involved in regulation rather than catalysis. Such allosteric-like regulation is usually observed in enzymes that function as oligomers, not enzymes that are active as a monomeric species such as HAS.

For a bacterium to synthesize a HA capsule, three different genes must usually be present. These genes, which encode three different enzymes, are arranged in an operon designated the HA synthesis (or has) operon (50, 51). Two of the enzymes are needed for the cell to produce large amounts of the two UDP-sugar precursors, and the third enzyme is HAS, encoded...
by the gene hasA. UDP-Glc dehydrogenase (whose gene is designated hasB) is required to make UDP-GlcUA from UDP-Glc in an oxidation reaction that utilizes 2 mol of NAD\(^7\)/mol of UDP-Glc. UDP-Glc pyrophosphorylase (the hasC gene) creates UDP-Glc from UTP and Glc-1-P. Since UDP-Glc is the precursor from which many of the other sugar nucleotides are made, the amount of UDP-Glc produced by a cell will regulate the total amount of all the cell’s sugar nucleotides. Bacteria like Group A Streptococcus that make HA capsules usually have two different genes for this pyrophosphorylase enzyme to increase greatly the total amount of cellular sugar nucleotides and thereby to support synthesis of the large amount of HA in the extracellular capsule.

If the bacterial cells did not greatly expand their sugar nucleotide pool, the very active HAS would make HA and deplete the cell of UDP-GlcUA and UDP-GlcNac. Since the latter is also needed for cell wall synthesis, such depletion would stop cell growth. To ensure that cell growth is not impaired by the production of the HA capsule, UDP-GlcNac concentration in a sigmoidal manner, the enzyme cannot attain its V\(_{\text{max}}\) until this concentration is very high. This kinetic regulation of spHAS may ensure that cell wall synthesis does not compete with capsule production for the necessary UDP-GlcNac. Since the seHAS enzyme is not similarly regulated by UDP-GlcNAc or if this lack of regulation is, similarly regulated by UDP-GlcNAc or if this lack of regulation is, thereby to support synthesis of the large amount of HA in the extracellular capsule.

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