The Streptococcal Hyaluronan Synthases Are Inhibited by Sulfhydryl-modifying Reagents, but Conserved Cysteine Residues Are Not Essential for Enzyme Function*

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Hyaluronan (HA) synthase (HAS) is a membrane-bound enzyme that utilizes UDP-glucuronic acid (GlcUA) and UDP-GlcNAc to synthesize HA. The HAS from Streptococcus pyogenes (spHAS, 419 amino acids) contains six Cys residues, whereas the enzyme from Streptococcus equisimilis (seHAS, 417 amino acids) contains four Cys residues. These Cys residues of seHAS are highly conserved in all Class I HAS family members. Here we investigated the structural and functional roles of these conserved cysteines in seHAS by using site-directed mutagenesis and sensitivity to sulfhydryl modifying reagents. Both seHAS and spHAS were inhibited by sulfhydryl reagents such as N-ethylmaleimide (NEM) and iodoacetamide in a dose-dependent and time-dependent manner. These inhibition curves were biphasic, indicating the presence of sensitive and insensitive components. After treatment of seHAS with NEM, the V_{max} value was decreased ~50%, and the K_m values changed only slightly. All the Cys-to-Ala mutants of seHAS were partially active. The least active single (C226A), double (C226A,C262A), or triple (C226A,C262A,C367A) Cys mutants retained 24, 3.2, and 1.4% activity, respectively, compared with wild-type enzyme. Surprisingly, the V_{max} value of the seHAS\textsuperscript{cys-null} mutant was ~17% of wild-type, although the K_m values for both substrates were increased 3–6-fold. Cys residues, therefore, are not involved in a critical interaction necessary for either substrate binding or catalysis. However, the distribution of HA products was shifted to a smaller size in ~25% of the seHAS Cys mutants, particularly the triple mutants. Mass spectroscopic analysis of wild-type and Cys-null seHAS as well as the labeling of all double Cys-to-Ala mutants with [\textsuperscript{14}C]NEM demonstrated that seHAS contains no disulfide bonds. We conclude that the four Cys residues in seHAS are not directly involved in catalysis, but that one or more of these Cys residues are located in or near substrate binding or glycosyltransferase active sites, so that their modification hinders the functions of HAS.

HAS\textsuperscript{2} is a membrane-bound enzyme that catalyzes the synthesis of HA in both eukaryotes and prokaryotes. HA is a linear heteropolysaccharide consisting of repeating (GlcUA\textsubscript{1,3}-GlcNAc\textsubscript{1,4}) disaccharide units (1, 2). HA is a ubiquitous component of extracellular matrices in vertebrates. This glycosaminoglycan is present in large amounts to serve specialized functions in cartilage, synovial fluid, dermis, and the vitreous humor of the eye (1–4). HA plays critical roles during fertilization and embryogenesis as well as development and differentiation. In many Group A and Group C streptococcal strains, HA forms a capsule that helps these pathogens evade the host immune system during infection (5–7). Substantial progress in understanding HA biosynthesis has been made since the first cloning in 1983 of a HAS from the human pathogen Group A Streptococcus pyogenes (8). Other members of the same HAS family were subsequently identified and cloned from human, mouse, frog, cow, rabbit, and chicken (9–16) and also from prokaryotes, including an algal-specific virus (17), Group C Streptococcus equisimilis (18), the bovine pathogen Streptococcus uberis (19), and the fowl pathogen Pasteurella multocida (20). With the exception of the latter enzyme, which has been designated the only member of the Class II HAS family (14), all of the Class I HASs from prokaryotes and eukaryotes are ~28–30% identical and are predicted to share a common membrane topology (21, 22). The three streptococcal HAS genes and protein sequences are 70–75% identical.

Although HAS functions as a glycosyltransferase, it is different from the vast majority of previously characterized enzymes in that it is a dual transferase with at least six different functions (23, 24). The single HAS polypeptide binds both UDP-GlcNAc and UDP-GlcUA and transfers these two different precursor sugars to the growing HA chain (the bound acceptor) via two different linkages. In addition to these five discrete substrate binding or catalytic functions, HAS is able to extrude or translocate the growing HA chain into the extracellular space while it remains bound to the enzyme at the plasma membrane (25, 26).

Two recombinant prokaryotic HASs, spHAS and seHAS, have been purified and characterized kinetically (24, 27). All recombinant HASs, either from vertebrates or prokaryotes, have been shown to synthesize high molecular weight HA in vitro. The Class I HAS proteins likely have essentially identical topological organizations in their N-terminal regions that are highly homologous with spHAS, the only HAS whose membrane topology has been determined experimentally (22).

In the present study we investigated the possibility that one or more of the conserved Cys residues in seHAS may be necessary for one of the six discrete HAS functions. Beginning in the 1950s almost all studies of prokaryotic or eukaryotic HAS function have used isolated membranes, in part because most of the eukaryotic HAS enzymes cannot be detergent-solubilized with retention of activity. Therefore, we performed a combination of site-directed mutagenesis, chemical modification, and...
Synthetic oligonucleotides used to make seHAS mutants

| seHAS mutant | Sequence |
|--------------|----------|
| C262A        | 5'-GGTAAATCTTGGTCGCAGTGGGCTTTACG-3' |
| C262S        | 5'-GGTAAATCTTGGTCGCAGTGGGCTTTACG-3' |
| C262A        | 5'-ATTGGTGTACGGCGCTTCCAGGCAACTATG-3' |
| C262S        | 5'-ATTGGTGTACGGCGCTTCCAGGCAACTATG-3' |
| C262A        | 5'-AACTTCAACGGGCCTTCCCCAGGCAACTATG-3' |
| C262S        | 5'-AACTTCAACGGGCCTTCCCCAGGCAACTATG-3' |
| C267A        | 5'-CTATTTGTTGGCCCTGCGGAACATTCATTAC-3' |
| C267S        | 5'-CTATTTGTTGGCCCTGCGGAACATTCATTAC-3' |

kinetic analyses using isolated membranes to characterize the structural and functional role(s) of Cys in seHAS.

**Experimental Procedures**

**Vectors, Primers, and Reagents**—The expression vector pK223 was from Amersham Biosciences, Inc. Escherichia coli SURE cells were from Stratagene. The QuikChange™ site-directed mutagenesis kit was obtained from Stratagene. All of the mutagenic oligonucleotides were synthesized by Geneset Biotechnologies, Inc. (Spring, TX) and were purified by reverse-phase chromatography. Cy-5-fluorescent sequencing primers were synthesized by the Molecular Biology Resource Facility, Oklahoma University Health Sciences Center. Other oligonucleotide primers were synthesized by The Great American Gene Co. (Ransom Hill Bioscience, Inc., CA). UDP-GlcUA and UDP-GlcNac were from Fluka and Sigma, respectively. UDP-[14C]GlcUA (300 mCi/mmol) and [14C]NEM (40 mCi/mmol) were from PerkinElmer Life Sciences. NEM and biotin-PEO-maleimide were from Sigma. All other reagents were of the highest grade available from Sigma unless otherwise noted.

**Site-directed Mutagenesis**—The seHAS gene with a fusion at the 3’ end encoding a His6 tail (seHAS-His6) was cloned into pK2K33 as described earlier (27). Mutagenic primers were designed to change the cysteines to either Ala or Ser at positions 225, 262, 291, and 367. Two complementary oligonucleotide primers encoding the desired mutations were used to create the single Cys mutations (Table I). Mutagenesis was carried out using the QuikChange method according to the manufacturer’s instructions. The pK2K33 plasmid containing the seHAS-His6 gene was grown in SURE cells, purified using a Spin Miniprep kit (Qiagen), and analyzed by agarose gel electrophoresis to verify the correct size. The purified pDNA was used as the template for the primer extension reaction with a pair of mutagenic primers. The PCR amplification conditions for PCR, using Ffi DNA polymerase, were 16 cycles of the following: 95 °C for 1 min, 58 °C for 1 min, and 68 °C for 18 min. This amplification generated mutated plasmids with staggered nicks, which were then treated with DpnI to digest the methylated and hemimethylated parental DNA. The digested pDNA was transformed into SURE cells, and colonies were screened for the desired mutations by sequencing the isolated plasmid DNA using fluorescently labeled terminators (ABI Prism 377 MODEL program, v2.1.1). The complete open reading frames of selected mutants were confirmed by sequencing in both directions with Cy-5-labeled vector primers on a Amersham Biosciences, Inc. ALF Express DNA Sequence. Data were analyzed using ALF Manager, v3.02. The double, triple, and null Cys mutants of seHAS-His6 were made using the appropriate single, double, or triple Cys mutant plasmid DNA as the template, respectively.

**Effect of Sulfhydryl Reagent Treatments on seHAS and spHAS Activity and Determination of the Kinetic Constants of seHAS Cys Mutants**—E. coli SURE cells transformed with plasmids containing various seHAS mutants were grown in LB medium with vigorous shaking at 32 °C to a A600 of ~0.8 and induced with 1 mM isopropl-β-thiogalactoside for 3 h. Cells were harvested, and membranes were prepared as described previously (28). The initial activities for HAS were determined at 37 °C in 100 μl of 50 mM sodium and potassium phosphate, pH 7.0, with 20 mM MgCl2, 1 mM DTE, 240 μM UDP-GlcUA, 0.2 μM UDP-[14C]GlcUA, and 0.05 μM UDP-[14C]GlcNac. The kinetic constants for both UDP-GlcUA and UDP-GlcNac were determined in 100 μl of 25 mM sodium and potassium phosphate, pH 7, 0.8 mM UDP-[14C]GlcUA, and 0.05 μM UDP-[14C]GlcNac. The amounts of one substrate (0.01–4 mM) while holding the other constant at 1 mM. Some assays also contained 0.1 mM EDTA and 20% glycerol (v/v). To initiate the enzyme reaction, ~0.5–40 μg of membrane protein was added, and the mixtures were gently shaken in a MicroMixer X-36 (Taitai) at 30 °C for 1–2 h. The reactions were terminated by the addition of SDS to a final concentration of 2% (w/v). The incorporation of radioactive [14C]GlcUA was determined by descending paper chromatography, and the Km and Vmax values were determined as described by Tlapak-Simmons et al. (24). Data were analyzed by the methods of Michaelis-Menten (29) or Hill (30). The protein content was determined by the method of Bradford employing the Ponceau-S as the standard. All Cys mutant or sulfhydryl-treated seHAS samples were assayed in duplicate or triplicate using two or three independent membrane preparations. The results are presented as the mean ± S.E. All enzyme assays were performed under conditions that were linear with respect to time and protein concentration. None of the seHAS variants exhibited an unstable undetectable enzyme activity under these steady-state conditions (18) so that each enzyme molecule on average synthesizes >10 HA chains during the incubation. The reactions were terminated by heating at 95 °C for 1 min, the mixtures were then centrifuged at high speed, and the HA-containing supernatants were recovered. The samples were concentrated to ~10-fold using Microcon YM-3 filters (Amicon Bioseparations, Inc.) and treated with DNase and RNase (4 μg/ml each) in the presence of 60 mM MgCl2 for 30 min at 20 °C. The reactions were stopped by adding DTE to a final concentration of 1.3% (w/v) agarose gel, 80–90 V. The gels were dried without heating and exposed to Biomax-MR film (Eastman Kodak Co.) for 1–4 weeks. The autoradiograms were scanned to create digital files using a Fluorchem TM8000 (Alpha Innotech Corp.) image analysis station. As a control, samples were treated with Streptomyces hyaluronate lyase (80 units) at 37 °C overnight, which resulted in the complete loss of radiolabeled bands.

**Determination of HA Size Produced by seHAS Variants**—The relative Mw of the HA synthesized by wild-type seHAS or the Cys mutants was determined by agarose gel electrophoresis (32) of [14C]-labeled HA products synthesized under the assay conditions described above. The wild-type seHAS synthesizes and releases an HA chain in <5 min under these steady-state conditions (18) so that each enzyme molecule on average synthesizes >10 HA chains during the incubation. The reactions were terminated by heating at 95 °C for 1 min, the mixtures were then centrifuged at high speed, and the HA-containing supernatants were recovered. The samples were concentrated to ~10-fold using Microcon YM-3 filters (Amicon Bioseparations, Inc.) and treated with DNase and RNase (4 μg/ml each) in the presence of 60 mM MgCl2 for 30 min at 20 °C. The reactions were stopped by adding DTE to a final concentration of 1.3% (w/v) agarose gel, 80–90 V. The gels were dried without heating and exposed to Biomax-MR film (Eastman Kodak Co.) for 1–4 weeks. The autoradiograms were scanned to create digital files using a Fluorchem TM8000 (Alpha Innotech Corp.) image analysis station. As a control, samples were treated with Streptomyces hyaluronate lyase (80 units) at 37 °C overnight, which resulted in the complete loss of radiolabeled bands.

**Determination of seHAS Protein Concentration in Membranes and Normalization from seHAS Activity**—The recombinant seHAS protein in isolated membranes is a major component comprising ~5–8% of the total protein, is well separated from other major proteins by SDS-PAGE, and can be readily identified in Coomassie Blue-stained gels (18). E. coli membranes containing wild-type or mutant seHASs were solubilized and electrophoresed on 10% (w/v) gels following the procedure of Laemmli for SDS-PAGE (33). The amount of seHAS protein in each membrane preparation was quantitated by image analysis of the stained gel using a Fluorchem TM8000 (Alpha Innotech Corp). The linearity of Coomassie Blue-stained seHAS bands was verified by loading different amounts of membrane protein. To generate a standard curve, seHAS samples were affinity purified, stained with anti-seHAS antibodies, and blotted onto PVDF membranes. The integrated intensity of the bands was then determined. The seHAS activity was normalized to the membrane prepa-
was eluted with distilled water containing 0.5% (v/v) trifluoroacetic acid and 0.02% (w/v) dodecylmaltoside. The degree of modification of Cys residues in treated seHAS samples was determined using a MALDI-TOF Voyager Elite mass spectrometer (Applied Biosystems, Framingham, MA) that was equipped with a Nd laser (337 nm), located in the National Science Foundation EPSCoR Oklahoma Laser Mass Spectrometry Facility. The sample (1 μl) was spotted to a sample plate followed by matrix solution (1 μl) and allowed to air dry. The matrix used was a 20 mg/ml solution of 2,4,6-trihydroxyacetophenone in 50% acetonitrile containing 0.1% trifluoroacetic acid and 0.05% (w/v) dodecylmaltoside. Samples were analyzed in the linear, positive ion mode using a delayed extraction of 300 ns and a grid voltage of 87.8% and were accelerated to a 25 KV accelerating voltage. Calibration and internal calibrations were routinely performed using horse aponyoglobin and bovine serum albumin (16,951 and 66,430 Da, respectively). Spectra were an average of 80–120 scans and were processed using the 19-point Savitsky-Golay smoothing option included in the software provided by the manufacturer.

**RESULTS**

**Sulfhydryl Reagents Inhibit the Activity of seHAS and spHAS—**SeHAS is the smallest HAS protein (417 amino acids) and contains four Cys residues. The four cysteines of seHAS are completely conserved among the three prokaryotic HASs (excluding *P. multocida* HAS) and are conserved positionally among all the vertebrate HASs (Fig. 1). To explore the possible role of cysteines in the function of HAS, the activities of seHAS and spHAS were assayed in the presence of different sulfhydryl-modifying reagents (Table II). Almost identical sensitivities were observed for the two enzymes. For example, seHAS activity was inhibited >93% by methylmethanethiosulfonate (0.05 mM) and ~70% by NEM (5 mM), whereas iodoacetamide inhibited only 15%. Sodium arsenite and 5,5'-dithiobis(2-nitrobenzoic acid) also inhibited each HAS activity. These results indicate that one or more Cys residues are important for the overall HA synthesis activity of the seHAS and spHAS proteins. The inhibition of each HAS by NEM was examined in more detail with respect to the time of incubation and NEM concentration (Fig. 2). Both seHAS and spHAS were inhibited in a biphasic manner with respect to incubation time or NEM concentration. Although the extent of inhibition varied from experiment to experiment, a 60–70% effect was typical. About half of the observed inactivation occurred at ~1 mM NEM, whereas the remaining inactivation occurred from 1 to 6 mM (Fig. 2A). Kinetically, there was a fast initial inactivation and then a much slower phase of inhibition; again, each of the phases involved about half of the affected activity (Fig. 2B). A potential complication in the above NEM studies is that the effects of a sulfhydryl-modifying reagent could be due to secondary effects caused by modification of other molecules in the membranes being tested. Although this possibility is highly unlikely since seHAS is the only protein necessary for HA biosynthesis (21, 27), we also examined the effect of sulfhydryl reagents on the seHAS Cys-null mutant in isolated membranes under the conditions shown in Fig. 3. The activity of seHAS Cys-null was not affected (~1%) by treatment with NEM, iodoacetamide, or sodium arsenite, which eliminates the possibility that modified secondary proteins in the membranes preparations were responsible for the altered HAS activity.

Modification of the protein by NEM could affect any one or several of the six discrete functions that HAS must perform to synthesize HA (23, 24). To determine whether one of the nucleotide-sugar binding sites was affected by NEM, we examined the UDP-GlcUA and UDP-GlcNAc saturation profiles for treated and untreated seHAS (Fig. 3 and Table III). The *K*$_m$ values for either UDP-GlcNAc (Fig. 3A) or UDP-GlcUA (Fig. 3B) were not altered significantly by treatment with NEM or sodium arsenite, whereas the maximum enzymatic velocity was reduced by up to ~70%.
TABLE II

| Sulfhydryl reagent | Inhibition of HAS Activity |
|--------------------|---------------------------|
|                    | seHAS | spHAS |
| N-Ethylmaleimide   | 70 ± 4.8 | 60 ± 3.5 |
| Iodoacetic acid    | 15 ± 6.0 | 13 ± 5.4 |
| 5,5'-Dithiobis-(2-nitrobenzoic acid) | 52 ± 6.5 | 52 ± 4.9 |
| Methylmethanethiosulfonate | 93 ± 5.4 | 89 ± 5.5 |
| Sodium arsenite    | 40 ± 4.0 | 46 ± 5.1 |

Fig. 2. Effect of NEM concentration and incubation time on the activity of seHAS and spHAS. A, E. coli membranes containing recombinant seHAS or spHAS were incubated at 4 °C for 1 h with PBS alone (minus NEM control) or PBS containing different concentrations of NEM. The unreacted NEM was quenched by the addition of DTE to a final concentration of 1–6 mM, and the samples were assayed for HAS activity as described under “Experimental Procedures.” B, the effect of incubation time on seHAS and spHAS activity was assessed by incubating the membranes with 5 mM NEM at 4 °C for the indicated times. Aliquots were removed into assay buffer containing 5 mM DTE, and HAS activities were determined. HAS activity in control untreated membranes was stable for 1 h at 4 °C. The inhibition of HAS activity is expressed as percent relative to the controls.

Cys residue for enzyme activity. The seHAS(C367A) variant was actually more active than wild-type (~145%), and the seHAS(C367S) variant was not significantly altered. In each of the four cases, the Cys-to-Ala change resulted in a variant with greater activity than the Cys-to-Ser change. The least tolerated single Cys change was C226S; this mutant was inhibited >90%.

We next constructed and examined all the possible Cys-to-Ala double mutants (C226A,C262A; C226A,C281A; C226A,C367A; C262A,C281A; C262A,C367A; C281A,C367A) as well as the triple mutants and the Cys-null mutant. For simplicity, we designate the triple Cys mutants by a convention that indicates which of the four Cys residues remains unaltered. For example, the triple mutant containing C226A,C281A,C367A changes is seHAS(C367A)C281A, which has only one Cys at position 262 as in the wild-type protein. The HA synthase activities of these multiple-Cys seHAS mutants were then determined under saturating conditions for each substrate and normalized to the amount of seHAS protein present in the isolated membranes (Fig. 5). The least active double mutant was C226A,C262A, which had only 2–3% of the specific activity of the wild-type enzyme. All three double mutants in which Cys226 was changed to Ala had lower activity compared with the other three double mutants. Two of the triple mutants, seHAS(C367C)C262 and seHAS(C262C)C367, were significantly more active (~3–30-fold) than the other two triple mutants, seHAS(C367C)C281 and seHAS(C281C)C367.

Surprisingly, the Cys-null seHAS mutant was more active than the two least active triple Cys mutants and two of the six double Cys mutants (Fig. 5). The decreased activities of the single and multiple Cys mutants are consistent with the inhibition of seHAS or spHAS by sulfhydryl reagents described above. Based on the lower specific activities of most of these Cys mutants, we conclude that no particular cysteine residue in seHAS is required for a critical step during HA synthesis. Nonetheless, these data also support the conclusion that Cys226 and C281 may play a role in or at least influence one or more of the six sub-activities required for the overall activity of HAS. At least the alteration or modification of these latter two residues hinders the enzyme and results in apparently lower $V_{max}$ values.

Enzymatic Analysis of seHAS Cys Mutants—To determine which sub-activities of seHAS might be altered by mutating its Cys residues, we performed kinetic analyses of the wild-type enzyme and all the Cys mutants and calculated their respective $K_m$ and $V_{max}$ values (Tables IV–VI). A comparison of the $V_{max}$ values for each of the single, double, and triple Cys-to-Ala

*Fig. 3. Effect of NEM or sodium arsenite treatment on the utilization of UDP-GlcUA and UDP-GlcNAc by wild-type seHAS. E. coli membranes containing seHAS protein were incubated at 4 °C for 1 h in PBS containing 5 mM NEM or 10 mM sodium arsenite, and the control membranes were incubated with PBS alone. Michaelis-Menten constants ($K_m$) were calculated from the activities of seHAS at varying concentrations of UDP-GlcUA or UDP-GlcNAc.*

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From the above results we conclude that functional constraints are put on the enzyme by particular alterations of some of its Cys residues. Because the C226A and C226S mutants were the least active, Cys$^{226}$ may be the most important
NEM Inhibition and the Role of Cys in HAS Function

TABLE III

Effect of NEM or sodium arsenite treatment on the utilization of UDP-GlcUA and UDP-GlcNAc by wild-type seHAS

|             | UDP-GlcUA | UDP-GlcNAc |
|-------------|-----------|------------|
|             | Control   | NEM        | Sodium arsenite |
|             | Control   | NEM        | Sodium arsenite |
| K_m (µM)    | 85 ± 10   | 57 ± 5     | 67 ± 23         |
| V_max (nmol/µmol/h) | 14.9 ± 3.6 | 7.3 ± 2.2 | 9.5 ± 1.1 |

TABLE IV

Michaelis-Menten constants for single Cys-Mutants of seHAS

| seHAS mutants | V_max a (nmol/pmol/h) | K_m UDP-GlcUA (µM) | K_m UDP-GlcNAc (µM) |
|---------------|-----------------------|--------------------|---------------------|
| Wild type     | 5.60 ± 0.48           | 77 ± 5             | 74 ± 7              |
| C226A         | 1.34 ± 0.21           | 88 ± 17            | 154 ± 23 b          |
| C262S         | 0.45 ± 0.08           | 44 ± 7 b           | 232 ± 0.2           |
| C262A         | 3.36 ± 0.26           | 146 ± 45           | 186 ± 26 b          |
| C262S         | 1.56 ± 0.05           | 96 ± 10            | 153 ± 0.5           |
| C281S         | 3.50 ± 0.37           | 40 ± 9 b           | 130 ± 12            |
| C367A         | 2.29 ± 0.40           | 56 ± 0.6 b         | 98 ± 11             |
| C367S         | 5.17 ± 0.32           | 85 ± 12            | 90 ± 10             |
| C367T         | 5.37 ± 0.26           | 79 ± 10            | 91 ± 1              |

a All values were significantly different from wild type (p ≤ 0.05) except for C367S.

b Significantly different from wild type (p ≤ 0.05).

c Significantly different from wild type (p > 0.006).

d Significantly different from wild type (p ≥ 0.005).

e Significantly different from wild type (p ≥ 0.05).

The K_m values for UDP-GlcUA for all the Cys mutants (Tables IV–VI) differed by no more than 2–3-fold from wild-type seHAS. For most of the Cys mutants, the K_m values for UDP-GlcNAc also did not change dramatically (within 1–3-fold). These relatively modest changes indicate that the altered Cys residues in these seHAS variants play a relatively minor role in how the enzyme binds and uses each nucleotide-sugar. However, some combinations of Cys mutations had more dramatic effects on UDP-GlcNAc utilization. For example, the K_m UDP-GlcNAc value for seHASΔ3C228 was 4-fold higher (Table VI). The K_m UDP-GlcNAc values for the C262A,C281A mutant (Table V) and the seHASΔ3C281, seHASΔ3C238, and Cys-null mutants (Table VI) were even more affected; they were 6–9-fold more than wild-type. These latter mutants were
clearly less efficient in their utilization of UDP-GlcNAc than the wild-type seHAS. Interestingly, these mutants also had Hill numbers >1.5, compared with a value of 1.0 for the wild-type enzyme, indicating that they had acquired a new level of cooperativity in their utilization of UDP-GlcNAc. All of the above kinetic results indicate a potentially important, although not absolutely essential, role for Cys226 and Cys262 in seHAS activity.

Relative Size Distributions of HA Synthesized by Various Cys Mutants of seHAS—HASs from different species synthesize HA products with a characteristic and often different distribution of sizes. To determine whether any of the Cys mutants of seHAS synthesize HA having an altered size distribution compared with wild-type seHAS, we used agarose gel electrophoresis to fractionate the radiolabeled HA products made by each variant enzyme (Fig. 6). The majority of the single (Fig. 6A) and double Cys mutants (Fig. 6B) synthesized HA of essentially identical size compared with the wild-type enzyme. The C281A and C367S single mutants and the C262A,C281A and C281A,C367A double mutants made smaller products. Three of the four triple mutants (all except seHAS(ΔC)C281) and the Cys-null mutant made smaller HA products (Fig. 6B). The smallest relative HA size distribution was made by the triple mutant seHAS(ΔC)C281. Interestingly, the HA size distributions of the seHAS mutants C226S, C226A,C262A, and (ΔC)C281 were similar to that of the wild-type enzyme, even though these mutants had the lowest activity (1.4–8% wild-type) and, therefore, the lowest HA elongation rates. Overall, these results clearly show that mutations of various combinations of Cys residues cause seHAS to synthesize shorter HA chains than the wild-type enzyme, indicating that Cys residues can influence the HA size distribution made by seHAS.

Assessment of Disulfide Bond Formation in seHAS—To understand the potential role of Cys residues in the function of seHAS, it is necessary to determine whether any of its four cysteines are involved in the formation of disulfide bonds. We undertook two approaches to answer this question. In the first approach, we treated E. coli membranes containing recombinant seHAS with [14C]NEM to determine whether the wild-type or Cys mutant seHAS proteins could be radiolabeled and then identified by autoradiography after SDS-PAGE (Fig. 7). We used this NEM reactivity to indicate the presence of free cysteines, which are not involved in disulfide bond formation. Each of the six Cys-to-Ala double Cys mutants of seHAS was radiolabeled by [14C]NEM. The labeling was specific because the vector-alone control and the Cys-null mutant did not show significant labeling. These results indicate that none of the Cys residues in seHAS are involved in disulfide bonds. A 31-kDa band, which was present in the mixture of NEM-labeled proteins from the wild-type and several double-Cys mutants, could be a degradation product of HAS, since it was not present in the vector-alone controls. Such a fragment is expected to be inactive and illustrates the importance of normalizing the kinetic data to the amount of intact HAS protein, as assessed by protein staining of SDS-PAGE gels.

In the second approach to assess the presence of disulfide bonds, we treated the purified enzyme with biotin-PEO-maleimide, and the modified protein products were then analyzed by MALDI-TOF mass spectrometry (Fig. 8). For each biotin-PEO-maleimidy group added, the mass of the seHAS derivative would increase by 525.6 Da. The treated wild-type seHAS contained a distribution of derivatized products with increased masses equal to the addition of one-to-four biotin-PEO-maleimide groups per seHAS (Fig. 8A). Most of the proteins were modified by the addition of 3 or 4 groups, demonstrating that the enzyme has no disulfide bonds. The observed mass values for the three largest adducts differed from the predicted values by <0.005%. The degree of modification was only slightly higher when the wild-type seHAS was treated with biotin-PEO-maleimide in the presence of 6 g guanidinium hydrochloride (not shown). This latter result indicates that none of the four Cys residues is substantially buried in the native enzyme; they are all accessible to react with the relatively large modifying reagent. The seHAS(Cys-null) protein was also treated with biotin-PEO-maleimide as a control to verify that no derivatized enzyme products could be formed in the absence of Cys groups (Fig. 8B). The result confirms that the modifying reagent does not react with any other amino acid side chains and is specific for Cys; no covalent adducts were formed with the Cys-null protein.
The HAS enzymes are unique in that they polymerize two sugars, GlcUA and GlcNAc, in an alternate fashion and simultaneously extrude the growing HA chain through the plasma membrane (21, 23). The streptococcal HASs are the smallest members of the Class I HAS family and perform all the functions required for HA synthesis and secretion from cells. Unlike the eukaryotic HAS enzymes, with which they share substantial homology and probably an identical topological organization in their common regions, the streptococcal enzymes have been easier to study because they can be readily overexpressed, purified, and characterized. To date, only one eukaryotic enzyme, mouse HAS1, has been overexpressed, purified, and characterized kinetically (34). One of our goals has been to understand how the Class I HAS enzymes function by using the streptococcal enzymes as a model.

We initially focused on the importance of Cys residues in seHAS for three reasons. First, Cys residues play important structural and functional roles in many proteins (e.g. Ref. 35). Second, the four Cys residues in seHAS at positions 226, 262, 281, and 367 are completely conserved in the two other streptococcal enzymes, Streptococcus uberis HAS and spHAS, and are generally conserved in all the other eukaryotic HASs (Fig. 1). Finally, p-chloromercuribenzoate had been reported to inhibit HA biosynthesis by the Group A spHAS in a cell-free system (36). Although no further studies on the role of sulfhydryls in HAS function had appeared since that report, we decided to investigate the possibility that Cys residues may be required for HAS activity.

Our present results demonstrate that a variety of sulfhydryl reagents inhibit both the spHAS and seHAS enzymes. This inhibition could reflect an important role of Cys in the function of these bacterial HAS proteins. However, interpretation of these results is complicated by the fact that Cys modification creates two changes in the enzyme; the S-H group is eliminated, but a new S-R group is also introduced, where R depends on the sulfhydryl reagent used. The samples were heated at 95 °C for 3 min and subjected to SDS-PAGE. The gels were processed and analyzed as described under “Experimental Procedures.”

**DISCUSSION**

The HAS enzymes are unique in that they polymerize two sugars, GlcUA and GlcNAc, in an alternate fashion and simultaneously extrude the growing HA chain through the plasma membrane (21, 23). The streptococcal HASs are the smallest members of the Class I HAS family and perform all the functions required for HA synthesis and secretion from cells. Unlike the eukaryotic HAS enzymes, with which they share substantial homology and probably an identical topological organization in their common regions, the streptococcal enzymes have been easier to study because they can be readily overexpressed, purified, and characterized. To date, only one eukaryotic enzyme, mouse HAS1, has been overexpressed, purified, and characterized kinetically (34). One of our goals has been to understand how the Class I HAS enzymes function by using the streptococcal enzymes as a model.

**FIG. 7. Reactivity of [14C]NEM with the Cys-to-Ala double mutants of seHAS.** E. coli membranes containing wild-type (WT) or double Cys-mutants of seHAS were incubated in two separate experiments (panels A and B) with 2.5 mM [14C]NEM (8 × 10^6 dpm) at 4 °C for 10 min. The excess of [14C]NEM was quenched by the addition of 40 mM DTE and incubation for 5 min at 4 °C. Trichloroacetic acid was added to a final concentration of 10%, and the samples were incubated at 4 °C overnight. The membrane pellet was washed by centrifugation 3 times with 5% trichloroacetic acid, suspended in 20 μl of Laemmli sample buffer (33), and neutralized with sodium hydroxide. The samples were heated at 95 °C for 3 min and subjected to SDS-PAGE. The gels were processed and analyzed as described under “Experimental Procedures.”

**FIG. 8. MALDI-TOF mass spectrophotographs of seHAS-His6 derivatives covalently modified by a sulfhydryl reagent.** Wild-type seHAS-His6 (panel A) or seHAS-His6<sub>Cys-null</sub> (panel B) were incubated with (the upper traces in each panel) or without (lower traces in each panel) biotin-PEO-maleimide, and the eluted proteins were then prepared for mass analysis as described under “Experimental Procedures.” The predicted mass-to-charge ratios for covalent adducts containing 2, 3, or 4 biotin-PEO-maleimide groups per wild-type enzyme molecule (in parentheses), and the observed centroid mass-to-charge ratios are indicated above the peaks. The predicted m/z ratio for the (MH)<sup>+</sup> ion of unmodified seHAS<sub>Cys-null-His6</sub> (with 4 Ala residues replacing the four Cys residues) is 48,473.1.
ent reactivity toward Cys residues, which would depend upon their size, charge, or polarity. The use of site-directed mutagenesis to alter the native Cys residues, although subject to the same concerns noted above, provides a complementary approach to determine the importance of Cys residues in HAS function. Both approaches show that although HAS activity is decreased by altering Cys residues, it is not eliminated; the completely modified Cys-null enzyme was still able to perform all of the functions needed for HA synthesis.

A fundamental question in the present study was whether seHAS contains any disulfide bonds. We addressed this issue in two ways, by mass spectrometric characterization of chemically modified, affinity-purified wild-type seHAS and also by the ability to radiolabel free Cys residues in each of the six double Cys-mutants of seHAS. Both approaches demonstrated clearly that the seHAS enzyme does not contain any disulfide bonds. It is reasonable to conclude, therefore, that the streptococcal HAS proteins do not have disulfide bonds. It may be more difficult to determine whether the eukaryotic HAS proteins contain disulfide bonds, since these proteins are difficult to purify in high yield (34) and contain more Cys residues (14) than the streptococcal proteins (21).

All HAS enzymes make a broad size range of HA rather than a discrete size. This heterogeneity of product size may be important biologically for particular functions of the three vertebrate HAS enzymes. In addition, the HA size distribution made varies among the streptococcal HASs (18) and also among the three mammalian HAS isoforms (37, 38). These enzymatic differences in the size distribution of HA products, which have only been observed in vitro (e.g., in isolated cells or membrane preparations), could have very significant biological consequences if they also occur in vivo in various eukaryotic cells and tissues. Numerous studies during the last decade demonstrate that HA is not simply a structural component of the extracellular matrices of most vertebrate tissues but also a cell signaling molecule capable of modifying important aspects of cell behavior including migration and adhesion (1, 2). The most interesting and surprising aspect of this new paradigm regarding the biological functions of HA is that many cells respond similarly if they also occur if they also occur.

An intriguing finding in the present study is that some, but not all, combinations of Cys mutations in seHAS cause the enzyme to synthesize smaller HA products. Eight of the 19 Cys mutants examined synthesized HA with an apparently normal distribution of sizes that were shifted to varying degrees to smaller masses. There was no apparent correlation between changes in HA elongation rate (V_{max} values) and HA size distribution among these Cys mutants. The least active seHAS variants nonetheless made HA products that were similar in size to the HA made by the wild-type enzyme. We have not estimated the average masses for the HA produced by the various mutants because the agarose gel electrophoresis technique is more suitable for obtaining a qualitative, rather than quantitative, assessment of size differences. It is very difficult to assess the reliability of size assessments outside the narrow range in which migration is proportional to size. For example, several Cys mutants (e.g., one each of the single, double, and triple mutants in Fig. 6) may actually synthesize substantially larger HA than wild type, but the migration differences compared with wild type are very small. For these reasons, we are just beginning a study to characterize the HA size distributions of these seHAS mutants using gel permeation chromatography coupled to dynamic light scattering. Further studies will also be required to assess more thoroughly any possible relationships between altered HA elongation rates and HA product sizes in the Cys mutants presented here. Future studies will also assess the possibility that the functional characteristics of some of the seHAS Cys mutants described here in isolated membranes could be different after detergent solubilization. Nonetheless, the present study demonstrates a role of Cys residues in controlling HA chain length. In particular, the single Cys mutant C281A makes much smaller HA, whereas the C281S mutant makes HA products very similar in size compared with wild-type seHAS.

Although NEM treatment of seHAS caused the velocity maximum (V_{max}) of the enzyme to decrease, it did not substantially change the K_{m} values for either nucleotide-sugar compared with untreated seHAS. These results indicate that the ability of the NEM-treated enzyme to bind each substrate is not greatly decreased by modification of its Cys residues, but the overall catalytic rate is slowed. In contrast, some of the site-specific Cys mutants showed greater changes in their kinetic constants.

None of the single Cys-to-Ala or Cys-to-Ser mutants of seHAS are inactive, indicating that no single Cys residue plays a critical, necessary role in HA synthesis. The specific HAS activity remaining in the single, double, triple, and Cys-null mutants confirms that Cys is not required at any position within the enzyme for a critical step in HA synthesis. Nonetheless, Cys-226 and Cys-262 together appear to play an important role in the activity of seHAS, since the double mutant seHAS(C226A,C262A) was the least active Cys mutant, with only 2–3% wild-type activity. Despite its low activity, this double mutant nonetheless synthesized HA of normal size. The triple Cys-mutant seHAS(D3C)C281 also had very low activity, similar to the double Cys-mutant seHAS(C226A,C262A), and also synthesized normal size HA. These results indicate that alteration of Cys-281 has little or no effect on HAS activity and are consistent with the single Cys mutant results in Table IV.

Interestingly, the lower functionality of seHAS(C226A, C262A) was substantially relieved by the introduction of a C281A change to create seHAS(D3C)C281 the triple Cys-mutant. Possibly a structural or functional constraint, perhaps related to HA chain length, brought about by mutating Cys-226 and Cys-262 to Ala, is substantially relieved by simultaneously mutating Cys-281. The triple mutant seHAS(D3C)C281 and the Cys-null mutant had similar activities and HA product sizes, suggesting a similar degree of compensation for the otherwise deleterious Cys-226/Cys-262 double mutation. The Cys-null mutant of seHAS retained ~20% of wild-type activity. The results indicate that Cys-226 and Cys-262 play an important role in the overall activity and kinetic characteristics of seHAS, but Cys-281 may play a role in regulating HA size. Based on the recently determined topology of spHAS (22) and its high level of homology with seHAS (72% identical plus 10% similar residues), we know that Cys-226, Cys-262, and Cys-281 are present in the central domain region of seHAS (Fig. 1), which is the region that contains β-glycosyltransferase family motifs (40). The topological model predicts that Cys-281 is very close to transmembrane domain 4 and is probably not near the glycosyltransferase motifs.

Based on the NEM modification and Cys mutagenesis results, it appears that one or more Cys residues may be located close to the nucleotide-sugar binding sites of the seHAS enzyme. This possibility provides a rationale to explain why modification or alteration of these Cys residues interferes with enzyme function and lowers enzyme activity. Preliminary results from ongoing studies suggest that either substrate,
UDP-GlcUA or UDP-GlcNAc, can protect seHAS from inhibition by NEM, supporting the premise that at least one Cys residue is located in or near a nucleotide-sugar binding pocket. Substrate binding to this site appears to interfere with the reaction between NEM and the nearby Cys residue(s). Similar conclusions about the proximity of Cys residues to substrate binding sites have been reported for several other proteins, including the lactose permease (41), glutathione synthetase (42), glucocorticoid receptor (43), retinoic acid receptor β (44), and plasma membrane proton-ATPase (45). All of these studies found that modification of Cys residues by sulfhydryl reagents decreased the activity of the protein, even though Cys mutagenesis did not inactivate the protein. Another recent study found that modification of Cys residues by sulfhydryl reagents impaired the interactions and proximity of various domains within the protein. Cys-scanning mutants (47) of seHAS containing a single unique Cys residue at a desired position could allow us to employ electron paramagnetic resonance studies by modifying this Cys residue with a suitable probe. This approach, for example, allowed Voss et al. (48) to determine the proximity of that modified residue to another region of the Lac permease. Similarly, one can chemically modify a single unique Cys residue with a fluorescent probe and systematically analyze the local environment in different regions of the protein (49). Interacting or proximal domains within seHAS may also be determined by assessing the formation of disulfide bonds in specific mutants containing two Cys residues (50). Such approaches may help elucidate the structure and function of seHAS and increase our understanding of how the HAS family is able to synthesize HA.

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The Streptococcal Hyaluronan Synthases Are Inhibited by Sulphydryl-modifying Reagents, but Conserved Cysteine Residues Are Not Essential for Enzyme Function
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