Identification of a membrane-localized cysteine cluster near the substrate-binding sites of the Streptococcus equisimilis hyaluronan synthase

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The membrane-bound hyaluronan synthase (HAS) from Streptococcus equisimilis (seHAS), which is the smallest Class I HAS, has four cysteine residues (positions 226, 262, 281, and 367) that are generally conserved within this family. Although Cys-null seHAS is still active, chemical modification of cysteine residues causes inhibition of wild-type enzyme. Here we studied the effects of N-ethylmaleimide (NEM) treatment on a panel of seHAS Cys-mutants to examine the structural and functional roles of the four cysteine residues in the activity of the enzyme. We found that Cys$^{226}$, Cys$^{262}$, and Cys$^{281}$ are reactive with NEM, but Cys$^{367}$ is not. Substrate protection studies of wild-type seHAS and a variety of Cys-mutants revealed that binding of UDP-GlcUA, UDP-GlcNAc, or UDP can protect Cys$^{226}$ and Cys$^{262}$ from NEM inhibition. Inhibition of the six double Cys-mutants of seHAS by sodium arsenite, which can cross-link vicinyl sulfhydryl groups, also supported the conclusion that Cys$^{262}$ and Cys$^{281}$ are close enough to be cross-linked. Similar results indicated that Cys$^{281}$ and Cys$^{367}$ are also very close in the active enzyme. We conclude that three of the four Cys residues in seHAS (Cys$^{262}$, Cys$^{281}$, and Cys$^{367}$) are clustered very close together, that these Cys residues and Cys$^{226}$ are located at the inner surface of the cell membrane, and that Cys$^{226}$ and Cys$^{262}$ are located in or near a UDP binding site.

Key words: cysteine modification/enzyme inhibition/N-ethylmaleimide/site-directed mutagenesis/sulfhydryl reagents

Introduction

Hyaluronan synthase (HAS) is a membrane-bound enzyme that catalyzes the synthesis of hyaluronan (HA) in both eukaryotes (Ito and Kimata, 2000) and prokaryotes (Weigel, 2002). HA is a linear glycosaminoglycan composed of the repeating heterodiuridiosehexosamine unit: GlcUAβ(1,3)-GlcNAcβ(1,4). HA is a component of extracellular matrices in all vertebrates and is present in large amounts for special functions in cartilage, synovial fluid, dermis, and the vitreous humor of eye (Abatangelo and Weigel, 2000). This glycosaminoglycan plays critical roles during fertilization, embryogenesis, development, and differentiation (Fenderson et al., 1993; Knudson and Knudson, 1993; Fraser et al., 1997; Toole, 1997).

In Group A and Group C streptococcal strains, HA forms a capsule that helps these cells evade the host immune system during infection (Kass and Seastone, 1944; Wessels et al., 1994). Progress in understanding the molecular basis for HA biosynthesis accelerated greatly after 1993, when the Streptococcus pyogenes HA synthase (spHAS) gene was first cloned (DeAngelis et al., 1993). Other members of the HAS family were subsequently identified from mammalian, avian, and amphibian species (DeAngelis et al., 1997, 1998; Fulop et al., 1997; Itano and Kimata, 1995; Shyjan et al., 1996; Spicer et al., 1996; Spicer and McDonald, 1998; Watanabe and Yamaguchi, 1996), and also from Group C Streptococcus equisimilis (seHAS) and Streptococcus uberis (Kumari and Weigel, 1997; Ward et al., 2001).

The HAS proteins are organized into two groups. The Class I enzymes contain all but one reported HAS; the single Class II enzyme (from Pasteurella multocida) differs from all other HASs in its structure and mechanism of HA biosynthesis (DeAngelis, 1999). The Class I HASs from prokaryotes and vertebrates are ~30% identical and share a common membrane topology (Heldermon et al., 2001a; Weigel et al., 1997).

Although Class I HASs function as glycosyltransferases, they are very different from the vast majority of glycosyltransferases in that their UDP-sugar substrates are acceptors rather than donors (Robbins et al., 1967), because sugar addition occurs at the reducing end of growing HA chains (Asplund et al., 1998; Prehm, 1983; Tlapak-Simmons and Weigel, 2002). The growing HA chain remains bound to the enzyme at the cell membrane while it is extruded into the extracellular space and then ultimately shed into the extracellular space or assembled into a pericellular coat around eukaryotic cells or a capsule around bacterial cells. Therefore, despite their relatively small sizes (e.g., seHAS is ~49 kDa), HASs perform multiple discrete functions to synthesize HA (Tlapak-Simmons et al., 1999b; Weigel, 2002). Radiation inactivation studies demonstrated that the streptococcal (Tlapak-Simmons et al., 1998) and amphibian (Pummill et al., 2001) Class I HASs function as protein monomers but require phospholipids for activity, probably in both cases. Cardiolipin, phosphatidylerine, or phosphatidic acid activate purified SPHAS and seHAS, whereas other lipids do not activate these enzymes (Tlapak-Simmons et al., 1999a). The activating lipids for the eukaryotic HASs have not yet been identified.

The recombinant streptococcal enzymes have been purified, characterized kinetically, and studied more extensively than other Class I HASs (Tlapak-Simmons et al., 1999a,b, 2004). Yoshida et al. (2000) purified and kinetically characterized...
recombinant mouse HAS1, but no other eukaryotic Class I enzyme has been purified. All the vertebrate and prokaryote recombinant Class I HASs synthesize high-molecular-mass HA, although the three mammalian isozymes produce HA of different size distributions (Brinck and Heldin, 1999; Itano et al., 1999; Koprunner et al., 2000). Generally, cells expressing HAS1 or HAS3 synthesize and secrete HA of molecular mass < 2 MDa, whereas cells expressing HAS2 produce HA of molecular mass > 2 MDa.

We use seHAS as a model enzyme to study structure–
function relationships within the Class I HAS family. The eukaryotic HASs contain about 14 Cys residues, whereas seHAS has only has 4, and these latter Cys residues are generally conserved among the vertebrate HASs. Because investigators have known for many decades (Markovitz et al., 1959) that HASs are sensitive to oxidation, we recently investigated whether one or more of the conserved Cys residues is vital for seHAS function. Surprisingly, although Cys modification inhibits HAS activity, the Cys-null mutants of seHAS and spHAS are active (Heldermon et al., 2001b; Kumari et al., 2002). Also, these enzymes do not contain disulfide bonds. The present study examined the structural and functional roles of the four cysteines in seHAS, using combinations of site-directed mutagenesis, chemical modification, and substrate protection studies. The results were previously reported in preliminary form (Kumari et al., 1999).

Results

N-ethylmaleimide inhibition of single Cys-to-Ala and Cys-to-Ser seHAS mutants

We recently reported that wild-type seHAS and spHAS are inhibited by a variety of sulfhydryl reagents, even though the Cys-null mutants are active (Heldermon et al., 2001b; Kumari et al., 2002). The kinetics of N-ethylmaleimide (NEM) inhibition for both wild-type seHAS and spHAS were complex, indicating that enzyme activity is probably very sensitive to the modification of some Cys residues and less sensitive to the modification of others. To explore the role of particular Cys residues in the inhibition of seHAS by sulfhydryl reagents, we examined the kinetics of NEM inhibition in a complete panel of seHAS Cys-mutants. NEM treatment of the four single Cys-to-Ala (Figure 1) and Cys-to-Ser seHAS (Figure 2) mutants caused variable degrees of inhibition. The kinetics and extent of inhibition of particular single Cys-mutants by NEM were similar to, more sensitive than, or less sensitive than wild type. None of the single Cys-mutants of seHAS was completely insensitive to NEM, which indicates that inhibition is not due to modification of a single Cys residue.

The C367A or C367S and C226A or C226S mutant pairs were similar to wild type in both their initial rates (Figures 1B and 2B) and their final extents (Figures 1A and 2A) of inhibition. The C262A and C262S mutants were inhibited at a greater rate and to a greater extent (> 95%) than wild-type enzyme. The C281A mutant was the least sensitive to NEM inhibition, being only ~ 5% inhibited during the first 10 min (Figure 1B), but was then slowly inhibited by ~ 30% over 80 min (Figure 1A). In contrast, seHAS(C281S) was significantly more sensitive to NEM than the C281A mutant or wild type. One possible explanation for these varied results is that more than one modified Cys residue can be responsible for substantial seHAS inactivation and the order or extent of Cys modification likely determines the residual seHAS activity. The loss of rapid inactivation in seHAS(C281A) indicates that modification of Cys281 greatly affects the accessibility or reactivity of NEM with other Cys residues. The different behavior of seHAS(C281S) suggests that enhanced modification at another Cys

![Fig. 1. Effect of NEM on the activity of seHAS single Cys-to-Ala mutants.](image-url)
A cysteine cluster near the substrate binding site of seHAS residue occurred, causing greater inhibition of activity. This result could be due to the ability of Ser to engage in an altered H-bond or to the less hydrophobic nature of Ser compared to Cys. Changing Cys262 to either Ala or Ser greatly enhanced activity loss, suggesting that NEM modification of Cys262 slows the further kinetics of inhibition (i.e., modification of Cys262 hinders further modification of other Cys residues responsible for a more rapid activity loss).

**NEM inhibition of double seHAS Cys-mutants**

Because it seemed likely that more than one Cys was reactive with NEM, the six possible Cys-to-Ala seHAS double mutants were examined for their sensitivity to NEM. If two specific Cys residues were modified, resulting in activity loss, then some of the double mutants should be NEM insensitive. Surprisingly, none of the double mutants were completely resistant to NEM inhibition (Figures 3A and 3B). The least sensitive seHAS mutants, C(226,281)A and C(281,367)A, were inhibited to a final extent of ~37% and 35%, respectively. Mutant C(262,281)A showed almost no rapid activity loss but still showed a significant slow rate of inactivation. The C(226,262)A and C(262,367)A mutants were more sensitive to NEM than wild type, showing a very rapid inactivation and indicating that Cys281 in these seHAS mutants was readily accessible to react with NEM. The C(262,367)A double mutant was also more sensitive to NEM inactivation, losing >96% of its activity.

The inhibition kinetics of the six double Cys-mutants were complex but fell roughly into four groups: (1) Mutant C(226,367)A behaved most similarly to wild-type enzyme. (2) Two of the mutants in which Cys281 was unaltered, C(226,262)A and C(262,367)A, showed enhanced rapid inactivation compared to wild type. Mutant C(262,367)A also showed the greatest total activity loss. (3) Mutant C(226,281)A retained a normal rapid inactivation, but then was not inactivated further compared to wild type. Mutant C(281,367)A was partially inactivated to the same extent, but more slowly. (4) Mutant C(262,281)A was also only slowly inactivated, but the final level of inactivation was greater than the group 3 mutants and more similar to wild type. These double Cys-mutant data are consistent with the conclusion that modification of Cys262 results in the greatest inhibition of seHAS.

**NEM inhibition of triple seHAS Cys-mutants**

Previous studies demonstrated, as expected, that NEM treatment of Cys-null seHAS has no affect on enzyme activity and does not modify the protein (Kumari et al., 2002). To determine if any of the four Cys residues might not contribute to the NEM sensitivity of seHAS, we examined the NEM inhibition of the four triple Cys-mutants (Figure 4), each of which contains one free Cys residue. Three of these Cys-mutants showed initial rates of inactivation that were similar to wild-type enzyme, whereas the seHAS(Δ3C)C367 mutant was unaffected by NEM treatment (Figure 4B). The seHAS(Δ3C)C262 and seHAS(Δ3C)C281 mutants were inhibited ~25% by NEM during the first 10 min of treatment, but then no further inhibition of activity occurred. In contrast, seHAS(Δ3C)C226 also showed a slower activity loss characteristic of wild-type seHAS, which continued to decrease to ~70% inhibition at 60 min (Figure 4A). The results indicate that Cys281, Cys226, and Cys262 are accessible to react with NEM, that their resulting modification causes inhibition of seHAS activity, and that modification of Cys226 causes the greatest inhibition.

The lack of effect by NEM treatment on the seHAS(Δ3C)C367 mutant could mean that either Cys367 does not react with NEM or modification of this residue by NEM does not alter HAS activity. This issue was examined by

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**Fig. 2.** Effect of NEM on the activity of seHAS single Cys-to-Ser mutants. Membranes containing wild-type seHAS (filled triangles), or the C226S (filled circles), C262S (open triangles), C281S (filled squares), or C367S (open circles) single Cys-mutants of seHAS were treated with 5 mM NEM at 4°C, and the HAS activity of samples was determined at the indicated times as described in Materials and methods. (B) A blowup of the early incubation times shown in A. Results are the mean ± SEM for three separate experiments using different membrane preparations (n = 3).
treating membranes containing the four triple Cys-mutants with $[^{14}\text{C}]\text{NEM}$. Because recombinant seHAS is up to $\sim10\%$ of the total membrane protein and appears in a region of the gel with little background from endogenous proteins (Kumari et al., 2002), we could monitor the radiolabeling of seHAS by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and autoradiography of treated membranes (Figure 5). The results showed that Cys$^{367}$ was not accessible to NEM, whereas Cys$^{281}$, Cys$^{226}$, and Cys$^{262}$ in the three other seHAS Cys-mutants were readily labeled with $[^{14}\text{C}]\text{NEM}$. We conclude from studies of the triple Cys-mutants that modification of Cys$^{281}$, Cys$^{226}$, or Cys$^{262}$ results in decreased enzyme activity and that seHAS function in this series of mutants is more sensitive to modification of Cys$^{226}$ than any other Cys residue. Table I summarizes the NEM inactivation results at 10 min and 80 min of treatment for the complete panel of seHAS Cys-mutants.

Fig. 3. Effect of NEM on the activity of seHAS double Cys-to-Ala mutants. Membranes containing wild-type seHAS (filled triangles), or the 226,262 (open circles), 226,281 (filled upside-down triangles), 262,367 (open triangles), 262,281 (filled squares), 281,367 (filled circles) double Cys-to-Ala mutants of seHAS were treated with 5 mM NEM at 4°C, and the HAS activity of samples was determined at the indicated times as described in Materials and methods. (B) A blowup of the early incubation times shown in A. Results are the mean ± SEM for three separate experiments ($n = 3$).

Fig. 4. Effect of NEM on the activity of seHAS triple Cys-to-Ala mutants. Membranes containing wild-type seHAS (filled triangles), or the (3ΔC)$^{226}$ (filled circles), (3ΔC)$^{262}$ (open triangles), (3ΔC)$^{281}$ (filled squares), or (3ΔC)$^{367}$ (open circles) triple Cys-mutants of seHAS were treated with 5 mM NEM at 4°C, and the HAS activity of samples was determined at the indicated times as described in Materials and methods. (B) A blowup of the early incubation times shown in A. Results are the mean ± SEM for three separate experiments ($n = 3$).
Cys262 and Cys281 are in very close proximity and that inhibition, a sensitivity to NEM that was intermediate to cross-link two vicinyl Cys residues; two SH groups that are adjacent or spatially very close in the folded protein. Sodium arsenite can react very close in the active enzyme. The results suggest that inactivation by UDP-sugars

Many of the seHAS Cys-mutants are protected from NEM inactivation by UDP-sugars

All of the seHAS Cys-mutants are active (Kumari et al., 2002), although double mutant C(226,262)A and triple mutant (Δ3C)C281 have only ~2% activity relative to wild-type 40

Double and triple seHAS Cys-mutants show differential sensitivity to sodium arsenite

The results show that the presence or absence of an NEM-modified Cys residue may influence the subsequent reactivity of other Cys residues in seHAS, indicating that some combination(s) of Cys226, Cys262, or Cys281 are spatially very close in the active enzyme. Sodium arsenite can react to cross-link two vicinyl Cys residues; two SH groups that are adjacent or spatially very close in the folded protein (Bhattacharjee and Rosen, 1996; Chakraborti et al., 1993; Stancato et al., 1993). Wild-type seHAS was inhibited ~41% by treatment with 10 mM sodium arsenite (Table II). To assess whether specific pairs of Cys residues in seHAS are close together and could account for this sensitivity of wild-type enzyme, we assessed the effect of sodium arsenite treatment on the activity of the six double Cys-mutants. The four triple Cys-mutants, which served as controls for the effects of possible reaction with individual Cys residues, were inhibited only 1–10% by the same sodium arsenite treatment. Four of the double Cys-mutants were also inhibited by <10%. In contrast, the C(226,367)A mutant in which Cys262 and Cys281 are present was inhibited 45%, essentially the same as wild-type seHAS. The C(226,262)A mutant in which Cys281 and Cys367 are present showed 19% inhibition, a sensitivity to NEM that was intermediate between the controls and wild type. The results suggest that Cys262 and Cys281 are in very close proximity and that Cys281 and Cys367 are also close enough to be cross-linked by arsenite, although to a lesser extent.

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type. The Cys-null seHAS mutant is substantially more active (~20% of wild type) than these latter two mutants. Thus, none of the four Cys residues is critical or necessary for enzyme activity, although the results showed that modification of Cys226, Cys262, or Cys281 by NEM caused inhibition and the arsenite sensitivity indicates that Cys262, Cys281, and Cys367 are very close together. To determine the effect of substrates on NEM inactivation, membranes containing wild-type or mutant seHASs were preincubated with UDP, UDP-GlcUA, or UDP-GlcNAc prior to and during treatment with NEM. Changes in the rate of NEM inhibition were evaluated during the first 10 min.

Wild-type seHAS was protected from NEM inhibition by either UDP-GlcUA or UDPGlcNAc and also somewhat by UDP (Figure 6). The single Cys-mutants C226A, C262A, C281A, and C367A were also protected to varying degrees from NEM inhibition by UDP, UDP-GlcUA, and UDPGlcNAc, although seHAS(C226A) was less protected than the other three mutants by any of the substrates. For the C281A and C367A mutants, any of the substrates provided ≥50% protection from inactivation by NEM. The C262A mutant showed a dramatic protection of ~75% by UDP-sugars. The finding that the UDP-sugars protected all four single Cys-mutants from NEM inhibition is consistent with the conclusion that more than one Cys residue reacts with the sulfhydryl reagent and several of the NEM-reactive cysteines are located either in or very close to a UDP-sugar binding pocket. Limited studies with the double Cys-mutants confirmed these general conclusions (data not shown).

The substrate protection characteristics of the three triple Cys-mutants that were sensitive to NEM inhibition were examined in more detail (Figure 7). Mutants seHAS(∆3C)226 and seHAS(∆3C)262 were protected from NEM inhibition by both UDP-sugar substrates, whereas the seHAS(∆3C)281 mutant was not protected. The protection from NEM inhibition in the seHAS(∆3C)262 and seHAS(∆3C)281 mutants indicates that Cys226 and Cys262 are close enough to a UDP binding site of a substrate that they are not accessible to react with NEM when the site is occupied.

![Fig. 6. Effect of substrates on NEM inhibition of seHAS single Cys-to-Ala mutants.](image)

![Fig. 7. Substrate protection from NEM inhibition of seHAS triple Cys-mutants.](image)
Discussion

Cysteines play a variety of catalytic, structural, and functional roles in many types of proteins (Carugo et al., 2003; Jose-Estanyol et al., 2004; Saito, 1989). Although reducing agents have been included in assay buffers since the first report identifying HAS activity (Markovitz et al., 1959), it was not until recently that these enzymes are to inhibition by sulfhydryl reagents (Kumari et al., 2002; Pummill and DeAngelis, 2002). We previously found that seHAS and spHAS do not contain disulfide bonds and that their Cys-null mutants are active. Thus the role of cysteines in Class I HAS function is not straightforward, because they are not absolutely required for activity, yet their modification results in substantial activity loss. To understand this intriguing result and determine the function of Cys residues in this simplest member of the family, we studied a panel of 18 seHAS Cys-mutants that were characterized earlier in terms of protein expression levels and kinetic parameters (Kumari et al., 2002).

Our present findings show complicated patterns of NEM inactivation of the seHAS Cys-mutants that presumably reflected the reactivity of their cysteines with NEM. Only in a few cases was a Cys-mutant unaffected by NEM treatment. The question of which Cys residues were modified by NEM was addressed by determining the ability of the triple Cys-mutants to be labeled by $^{[14]C}$NEM (Figure 5). Of the four Cys residues in seHAS, only Cys$^{367}$ was not covalently modified by $^{[14]C}$NEM. Cys$^{367}$ is either inaccessible to or does not react well with NEM (137 Da), yet it does react with the slightly smaller arsenite (AsO$_2^-$; 107 Da). This differential reactivity is consistent with the localization of Cys$^{367}$ at the membrane–cytoplasm junction of MD5 (Heldemon et al., 2001a), and indicates that this Cys residue may be partially buried within MD5 (Figure 8A). Because modification of Cys$^{226}$, Cys$^{262}$, or Cys$^{281}$ in the triple Cys-mutants caused inhibition, we conclude that inhibition of the single and double seHAS Cys-mutants results from the NEM modification of any of these three residues, either singly or in combination.

Although the reactivity kinetics of individual Cys residues may be altered in some of the Cys-mutants, it is likely that the same cysteines are modified in the wild-type enzyme. The much greater labeling of wild-type seHAS compared to the individual triple Cys-mutants (Figure 5) also supports the conclusion from the activity inhibition studies (Figures 1–4) that multiple Cys residues are modified. Based on all of the results, we conclude that Cys$^{226}$, Cys$^{262}$, and Cys$^{281}$ in wild-type seHAS can react with NEM, resulting in inhibition, whereas Cys$^{367}$ essentially does not react with NEM (i.e., the reaction rate is extremely slow; Figure 4A). The overall pattern of modification (i.e., the combinations of modified Cys residues) and the resulting effect on activity is likely dependent on which residue is modified first.

The Cys-to-Ala and Cys-to-Ser mutant pairs at positions 226, 262, and 367 showed similar changes in sensitivity to NEM relative to wild type. Interestingly, however, changing Cys$^{281}$ to Ser$^{281}$ made the enzyme very susceptible to NEM inhibition, whereas the Cys$^{281}$-to-Ala$^{281}$ change made it resistant. We interpret this result to indicate that the nature of the side chain at position 281 influences the accessibility or reactivity of other Cys residues to NEM. Although the atomic volume difference between the side chains of Ala and Ser is relatively small, the ability of Ser to interact differently than Cys with a nearby H-bond acceptor or donor might stabilize a conformation in which NEM access to the other Cys residues is enhanced. Alternatively, these opposite effects could be due to the difference in hydrophobicity between Ala and Ser.

Because the NEM inhibition kinetics of seHAS(C226A) and seHAS(C367A) were similar to wild type, it is likely that Cys$^{262}$ and Cys$^{281}$ are accessible to and modified by NEM in all three seHAS variants. Because we know that Cys$^{367}$ does not react with NEM, we also conclude from this result that Cys$^{226}$ may not be responsible for the sensitivity

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**Fig. 8.** Model for the topology of seHAS and the orientation of cysteines relative to the membrane. (A) The topological scheme shows the location of the six membrane domains (numbered 1–6) and the four cysteines (circled in white). The amino acid predicted to be at each membrane junction is indicated on the cytoplasmic side of the membrane. The dashed lines between Cys$^{262}$–Cys$^{281}$ and Cys$^{281}$–Cys$^{367}$ indicate that these Cys pairs are close enough to be cross-linked by sodium arsenite. The shaded oval indicates a UDP-sugar binding site. The small box indicates the DXD motif (DSD$^{161}$) in seHAS that is characteristic of UDP binding sites in glycosyltransferases. Details of the model are described in the text. (B) The schematic illustrates that all four Cys residues of seHAS are clustered in or near a UDP-sugar binding site (either one or two) located at the interface between the inner membrane and the protein.
of wild-type seHAS to NEM inhibition. In contrast, seHAS(C262A) and seHAS(C262S) were almost completely inhibited by NEM, indicating that altering Cys262 actually made Cys226, Cys281, or both residues more accessible to or reactive with NEM, resulting in greater enzyme inactivation. Our interpretation of these results is that Cys226 can react with NEM in many of the Cys-mutants, although in wild-type seHAS the NEM modification of Cys262 or Cys281 inhibits the NEM modification of Cys226. When Cys262 is changed to Ala or Ser, NEM can then react with Cys226 as well.

The NEM inhibition of wild-type seHAS and some of the Cys-mutants tested were partially prevented by UDP-GlcNAc or UDP-GlcUA and even UDP. Because triple Cys-mutant seHAS(ΔC)C281 was not protected by any of these substrates, we conclude that Cys281 is not in or close enough to a UDP-sugar binding site. However, substrate protection was observed for Cys226 and Cys262, indicating that these cysteines are located close enough to one or more substrate binding sites of seHAS that NEM reactivity is lost when the site is occupied. Because both substrates protected the enzyme from NEM inhibition, we conclude that either there is only one UDP-sugar binding site, which alternates its sugar specificity, or there are two very close binding sites so that occupancy of one site blocks access to other Cys residues in or near the second binding site.

An alternative possibility is that binding of a UDP-sugar may cause a conformation change that hinders NEM access to other Cys residues that are not spatially close to the UDP-sugar binding site. However, we previously noted that all the UDP-containing molecules tested (e.g., UTP and UDP-sugars) inhibit seHAS activity, although there is no evidence for misincorporation of other sugars by the enzyme (Kumari and Weigel, 1997; Tlapak-Simmons et al., 1999b). We previously found that the kinetics of HA synthesis is also slowed if one of the normal UDP-sugar substrates is in large excess over the other. The UDP portion of any of these molecules is apparently able to bind to either UDP-sugar binding site. In a similar study, a broad range of pyrophosphoryl-containing compounds protected xHAS1 from inactivation by NEM (Pummill and DeAngelis, 2002).

The present observation that substrates protect HAS from inactivation by NEM provides a rationale to explain the effect of sulfydryl modification on HAS activity. We found previously that the $K_m$ values for UDP-sugar use by seHAS Cys-mutants were changed only slightly by NEM inhibition, but the $V_{max}$ value decreased substantially (Kumari et al., 2002). The current findings are consistent with the earlier conclusion that sulfhydryl group modification results in hindered substrate utilization by the enzyme, which slows the overall polymerization rate of substrates into HA. Inhibition could be caused by several effects of NEM-modified Cys residues. For example, they could affect enzyme function by altering interactions with a bound substrate, changing a conformation of the protein, or a local chemical environment needed for one of the enzyme’s multiple functions.

Other proteins and enzymes have been reported to contain clusters of Cys residues involved in the binding of ligands or substrates, including the retinoic acid receptor protein (Wolfgang et al., 1997), glutathione synthetase (Kato et al., 1988), the glucocorticoid receptor (Stancato et al., 1993), and the ArsA ATPase (Bhattacharjee and Rosen, 1996). Single Cys residues are often part of a substrate binding site, such as in the lactose permease of Escherichia coli, in which Cys148 interacts weakly and hydrophobically with the galactosyl moiety of the substrate (Jung et al., 1994). In Class I HAS family members, Cys226 is adjacent to a conserved motif, (S/G)GPL, which is associated with β-GlcUA transferase activity in mouse HAS1 (Yoshida et al., 2000). Our present results could support the involvement of Cys226 either in UDP-GlcUA binding or hyaluronyl transfer from HA-UDP to UDP-GlcUA. Cys262 is also in the middle part of conserved motif GDDR(CH)LTN, found in all HAS 1 family members. The function of this region is not yet known.

A model that explains the present results is that Cys281 is near the entrance to a groove or pocket within the enzyme that contains Cys226 and Cys262 and access to the interior of this groove is limited (Figure 8B). This suggestion is also consistent with the organization of the three NEM-sensitive Cys residues within the enzyme and relative to the membrane. Based on the experimentally determined topology of spHAS (Helderson et al., 2001a), Cys226, Cys262, and Cys281 in seHAS are located in the large central subdomain (Figure 8A) between MD3 (which does not traverse the membrane) and MD4 (a transmembrane domain). The adjacent central subdomain between MD2 and MD3 contains a D-X-D sequence (DDSD16), which is a conserved motif involved in UDP-sugar binding in β-glycosyltransferases (Breton and Imberty, 1999). Cys367 is at the C-terminal intracellular side of MD5, which spans the membrane. Interestingly, Cys226 is the only Cys residue that is absolutely conserved among all family members, and this residue is within MD3, an amphipathic helix.

We suggested previously that the growing HA chain is made at or within the membrane and translocated through the Class I HAS and the bilayer to the cell exterior (Tlapak-Simmons et al., 1999a). An alternative explanation for how HA is delivered to the cell exterior in streptococcal cells is that an ABC transport system for HA is required (Ouskova et al., 2004). Such ABC transporter systems are needed for the export of many different bacterial polysaccharides. However, if extracellular HA requires a transport system, it must not be specific for just HA, since Enterococcus faecalis (Deangelis et al., 1993) and Bacillus subtilis (unpublished data), bacteria that do not make HA, can synthesize and secrete large amounts of HA when transformed with the spHAS or seHAS gene, respectively. Also, the lipid dependence of the streptococcal Class I enzymes and the present finding that UDP-sugar binding sites are at the inner membrane surface are consistent with the growing HA chain being made at or within the membrane and then transported through the enzyme to the exterior. If a separate transporter system is needed to deliver HA across the membrane, it is unclear why Class I HASs would have evolved to contain six to eight transmembrane and membrane-associated domains instead of a single membrane domain, like the vast majority of glycosyltransferases—including the Class II HAS. Further studies are clearly required to define the mechanism by which HA is transferred across the cell membrane. The present findings suggest that Cys residues
could be involved in this process, although this cannot yet be assessed.

The arsenite sensitivity results indicate that Cys

\[ ^{367} \text{Cys} \]

can be cross-linked to Cys

\[ ^{281} \text{Cys} \]

and that Cys

\[ ^{281} \text{Cys} \]

can also be cross-linked to Cys

\[ ^{262} \text{Cys} \]

Two Cys residues can react with arsenite if their -SH groups are within about 3–5 Å (Bhattacharjee and Rosen, 1996; Sowerby, 1994). Based on our results, Cys

\[ ^{281} \text{Cys} \]

is probably slightly closer to Cys

\[ ^{262} \text{Cys} \]

to Cys

\[ ^{367} \text{Cys} \]

Because Cys

\[ ^{367} \text{Cys} \]

is located at the membrane junction, Cys

\[ ^{281} \text{Cys} \]

Cys

\[ ^{262} \text{Cys} \]

must also be very close to the membrane, as illustrated in Figure 8B. Therefore, three of the four Cys residues in seHAS are clustered very close together near substrate binding sites, and all four Cys residues are very close to or at the inner surface of the cell membrane. Because the four Cys residues in seHAS are generally conserved within the Class I HAS family, these enzymes also likely contain a similar Cys cluster near the membrane and near or in a substrate binding site.

Materials and methods

Vectors, primers, and reagents

The expression vector pKK233 was from Pharmacia Biotech (Uppsala, Sweden). E. coli SURE cells were from Stratagene (La Jolla, CA). QuickChange Site-Directed Mutagenesis Kits were from Stratagene. All mutagenic oligonucleotides were synthesized by Genosys Biotechnologies (Spring, TX) and 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, CA). UDP-GlcUA and UDP-GlcNAc were from Fluka and Sigma (St. Louis, MO), respectively. UDP-[\(^{14}\)C]GlcUA (300 mCi/mmole) and [\(^{14}\)C]NEM (40 mCi/mmole) were from Perkin Elmer Life and Analytical Sciences (Boston, MA). NEM and all other reagents were the highest grade available from Sigma unless otherwise noted. Phosphate buffered saline (PBS) was prepared according to the Gibco (Grand Island, NY) formulation.

Site-directed mutagenesis

The seHAS gene with a fusion at the 3’ end encoding a His

\[ ^{6} \text{His} \]

tail (seHAS-His

\[ ^{6} \text{His} \]
)

could be cloned into pKK233 as described earlier (Kumari and Weigel, 1997). The following mutagenic primers were designed to change Cys to either Ala or Ser at positions 226, 262, 281, and 367 (primers are shown in the sense orientation with the altered codon in boldface).

- C226A: 5’-GGTAATATCCTTTGGCTCCTCGGTTCCGCTTAGC
- C226S: 5’-GGTAATATCCTTTGGCTCCTCGGTTCCGCTTAGC
- C262A: 5’-ATTGGGTGATGACAGGGCTTGGACCAACTATGCA
- C262S: 5’-ATTGGGTGATGACAGGGCTTGGACCAACTATGCA
- C281A: 5’-CAATCCACTGCTAAAAGCTATTACAGATGTTCCCT
- C281S: 5’-CAATCCACTGCTAAAAGCTATTACAGATGTTCCCT
- C281A: 5’-CAATCCACTGCTAAAAGCTATTACAGATGTTCCCT
- C367A: 5’-TTCATATTGTGCCCTGCTCAGACCATATTAC
- C367S: 5’-TTCATATTGTGCCCTGCTCAGACCATATTAC

Two complementary oligonucleotide primers encoding the desired mutation were used to create the single Cys mutations using the Quick Change method according to the manufacturer’s instructions. The pKK233 plasmid containing the seHAS-His

\[ ^{6} \text{His} \]

gene was amplified in SURE cells, purified using a Spin Miniprep Kit (Qiagen, Valencia, CA) and analyzed by agarose gel electrophoresis to verify the correct size. The purified pDNA was used as the template for the primer extension reactions with each pair of mutagenic primers. Polymerase chain reaction, using pfu DNA polymerase, was performed for 16 cycles; 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 hemi-methylated parental DNA. The digested pDNA was transformed into SURE cells and colonies were screened for the desired mutations by sequencing isolated pDNA using fluorescent terminators (ABI Prism 377 MODEL program, v2.1.1). The promoter and complete open reading frame of selected mutants were confirmed by sequencing in both directions with Cy-5-labeled vector primers using a Pharmacia ALF Express DNA Sequencer. Data were analyzed using ALF Manager, v3.02. The double, triple, and null Cys-mutants of seHAS-His

\[ ^{6} \text{His} \]

were made using an appropriate single, double, or triple Cys-mutant pDNA as the template, respectively. The designation of triple Cys-mutants as seHAS(A3C)\(^{367}\) denotes the deletion of three Cys residues leaving one free Cys at position yyy.

Effect of NEM modification on seHAS activity

Stock solutions of NEM (100 mM) were made in PBS. Membrane suspensions containing seHAS variants were incubated with 5 mM NEM at 4°C and aliquots were removed at different times, and added to assay buffer containing 10 mM dithioerythritol (DTE) to quench unreacted NEM. The seHAS activity was determined at 37°C in 100 µl of 50 mM sodium and potassium phosphate, pH 7.0, with 20 mM MgCl

\[ _{2} \]

1 mM dithiothreitol, 240 µM UDP-GlcUA, 0.7 µM UDP-[\(^{14}\)C]GlcUA, and 600 µM UDP-GlcNAc. Reactions were terminated after 1 h by the addition of SDS to a final concentration of 2% (w/v). The incorporation of [\(^{14}\)C]GlcUA was determined by descending paper chromatography (Markovitz et al., 1959). Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

All seHAS Cys-mutants were assayed in duplicate or triplicate in two or three experiments using independent membrane preparations. Different amounts of membrane protein were used for each seHAS variant, so that similar levels of HAS activities were compared; all untreated samples showed good and similar HA synthesis (at least several thousand cpm). For each mutant or wild-type seHAS, the activity at zero time (no NEM treatment) was set at 100%.
(control), and the remaining activities at different times are presented as a percentage of this control HAS activity. Results are presented as the mean ± SEM. All enzyme assays were performed under conditions that were linear with respect to time and protein concentration, and all the seHAS variant enzymes were stable under the conditions used.

Substrate protection of NEM inhibition

Different amounts of E. coli membrane protein containing seHAS variants, as already noted, were preincubated at 4°C for 1 h in 50 mM sodium and potassium phosphate, pH 7.0, containing 10 mM MgCl₂ and either 600 µM UDP-GlcUA, 1.5 mM UDP-GlcNAc, or 1.5 mM UDP and then treated for 10 min at 4°C with or without 5 mM NEM. The reaction with NEM was terminated by addition of 10 mM DTE and HAS activity was then determined at 37°C in the assay buffer already described. The membrane samples were diluted 40-fold in the final assay buffer, so that the final concentration of unlabeled UDP-GlcUA was reduced to 15 µM. Because the concentration of radiolabeled UDP-GlcUA in the assay was 240 µM, the final change in specific radioactivity was small enough that it was ignored. Also because this dilution effect would cause a slight apparent inhibition of HA synthesis, our results slightly underestimate (rather than overestimate) any protective effects for UDP-GlcUA. Under these conditions, there were also no effects of UDP or the other unlabeled UDP derivatives on seHAS activity in the absence of NEM.

Labeling of seHAS with [¹⁴C]NEM

Isolated membranes containing wild-type seHAS or one of the four triple Cys-mutants of seHAS were incubated at 4°C in PBS with 2.5 mM [¹⁴C]NEM (≈ 8 × 10⁵ dpm) for 5 min. The reactions were terminated by the addition of DTE to a final concentration of 5 mM. Membrane proteins were precipitated by incubation with 10% (w/v) trichloroacetic acid overnight at 4°C, and free [¹⁴C]NEM was then removed by two cycles of centrifugation and resuspension with 5% trichloroacetic acid. The precipitated proteins were dissolved in 1× Laemmli (1970) sample buffer, neutralized by the addition of 0.1 N NaOH, and analyzed by SDS-PAGE using a 10% gel. Coomassie blue–stained gels were scanned using a Model PDSIP60 densitometer (Amersham Biosciences, Piscataway, NJ), treated with scintillants, and subjected to fluorography using Biomax-MR (Kodak, Rochester, NY) film and an exposure of about 1 week. E. coli membranes prepared from cells transformed with vector alone, containing no seHAS, were included as a control.

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Abbreviations

DTE, dithioerythritol; HA, hyaluronan; HAS, hyaluronan synthase; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; seHAS, Streptococcus equisimilis hyaluronan synthase; spHAS, Streptococcus pyogenes hyaluronan synthase.

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