Insights into the Mechanism of Action of Hyaluronate Lyase

ROLE OF C-TERMINAL DOMAIN AND Ca2⁺ IN THE FUNCTIONAL REGULATION OF ENZYME

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Hyaluronate lyases (HLs) cleave hyaluronan and certain other chondroitin/chondroitin sulfates. Although native HL from *Streptococcus agalactiae* is composed of four domains, it finally stabilizes after autocatalytic conversion as a 92-kDa enzyme composed of the N-terminal spacer, middle α-, and C-terminal domains. These three domains are independent folding/unfolding units of the enzyme. Comparative structural and functional studies using the enzyme and its various fragments/domains suggest a relatively insignificant role of the N-terminal spacer domain in the 92-kDa enzyme. Functional studies demonstrate that the α-domain is the catalytic domain. However, independently it has a maximum of only about 10% of the activity of the 92-kDa enzyme, whereas its complex with the C-terminal domain in vitro shows a significant enhancement (about 6-fold) in the activity. It has been previously proposed that the C-terminal domain modulates the enzymatic activity of HLs. In addition, one of the possible roles for calcium ions was suggested to induce conformational changes in the enzyme loops, making HL more suitable for catalysis. However, we observed that calcium ions do not interact with the enzyme, and its role actually is in modulating the hyaluronan conformation and not in the functional regulation of enzyme.

Streptococci of serological group B are well known major human pathogens. The organism predominantly colonizes the mucosal surfaces and/or skin as a first step for invasion, and is responsible for diseases like meningitis, septicemia, and other neonatal infections (1, 2). Group B streptococci (GBS) possess a variety of virulence factors. Hyaluronate lyase (HL), a potential virulence determinant, is produced as an extracellular enzyme by most strains of GBS (3). Virulent *Streptococcus agalactiae* isolates associated with invasive disease are known to produce high levels of extracellular hyaluronate lyases (4). HLs form a special group of polysaccharide-degrading enzymes that cleave predominantly the N-acetylgalactosamine (1→4) glycosidic bond of hyaluronan (HA) to produce unsaturated polysaccharides (5). It is part of a large family of enzymes called hyaluronidases, found in many Gram-positive pathogens (6). These enzymes have been postulated to facilitate the spread of bacteria by breaking down HA, a polymer composed of repeating units of D-glucosamine and D-glucuronic acid (7). HA is ubiquitously present in many mammalian tissues including synovial fluid, cartilage, skin, brain, etc. and forms a major part of the extracellular matrix (8). The secondary substrate of HL is chondroitin/chondroitin sulfate, which is involved in cushioning of the surrounding structures and macromolecule transport (9).

The native HLs from *S. agalactiae* and *Streptococcus pneumoniae* are composed of four domains: an N-terminal carbohydrate binding domain, small spacer β-sheet domain, α-domain, and C-terminal domain, which are connected through peptide linkers. Both the homologous enzymes (68% similarity) are initially expressed as 118/111-kDa and 107-kDa proteins, respectively, which are subsequently autodegraded from the N-terminal region and finally stabilized as 92-kDa (SagHL) and 89-kDa (SpnHL) proteins for which crystal structures are also available (10–20). Crystal structures of both the HLs suggest that they are similar in whole structure architecture, and the active site geometry except for the spacer domain that is only present in SagHL (18). Although no calcium binding sites are observed on these enzymes, their enzymatic activities are reported to be calcium-dependent (3, 14, 21). Recently significant emphasis has been put on proposing specific details of the mechanism of HL activity (16–20). In particular, the role of the C-terminal β-domain and the role of Ca²⁺ was suggested to modulate the functional activity of HL. To date no experimental validation of the role of Ca²⁺ is available.

To understand the role of calcium ions in modulating the enzymatic activity of SagHL, and to study the role of different domains and their interactions that are essential for the functional regulation of the enzyme we cloned, overexpressed, and purified the SagHL, SagHL without the spacer domain (SagHL-βI), SagHL without the C-terminal domain (Sag Domain-I), α-domain, and finally the C-terminal domain (SagC). To demonstrate that the structural domains of SagHL are independent folding/unfolding units, the stability characteristics of the recombinant enzyme and independent fragments/domains were also studied.
**EXPERIMENTAL PROCEDURES**

**Materials**—Most of the chemicals were purchased from Sigma. Chelex100 resin was purchased from Bio-Rad, and ultra pure water (LiChrosolv®) was purchased from Merck.

**Cloning of SagHL, SagHL-βI, Sag Domain-I, α-Domain, and SagC—S. agalactiae culture and chromosomal DNA extractions were carried out as described previously (22).** Gene (GenBank™ accession number- U15050) fragments encoding SagHL (Ser171–Ile984), SagHL-βI (Phe252–Ile984), Sag Domain-I (Ser171–Asn411), α-domain (Phe252–Asn411), and SagC (Lys621–Ile984) were amplified by polymerase chain reaction. The different forward primers used for SagHL/Sag Domain-I, SagHL-βI/α-domain, and SagC were 5′-CTC GAG GAT AGC TAA TTG GTC TGT TTT TGT C and 5′/H11032 with 2 units of Taq DNA polymerase. The amplification condition was 94 °C, 30 s; 55 °C, 30 s; 72 °C, 3 min (for 30 cycles) and final extension for 10 min. All these amplified gene fragments were digested with Nhel and Xhol and then ligated into the pET 21-d vector cut with the same enzymes. Competent *Escherichia coli* DH5α cells for checking the expression of gene fragments. Overexpression and Purification of Proteins—For all the recombinant proteins, cells were initially grown at 30 °C until A600 = 0.5–0.6 and later induced at 16 °C with 1 mM isopropyl-

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**FPLC (GE Healthcare) pre-equilibrated with the respective buffers prepared in water (LiChrosolv®) and purified through a Chelex100 column) for activity measurements. The purity of recombinant proteins were checked and found to be more than 95% pure. Expression and purification of SpnHL was carried out as described earlier (23).

**Assay of Enzymatic Activity**—The activities of the SagHL, SagHL-βI, Sag Domain-I, α-domain, and Sag Domain-I:SagC complex were determined by measuring their ability to break down HA to unsaturated disaccharide units. Briefly 20 μl of the enzyme sample (1 nm, diluted just before the assay) was added to 1 ml of 0.2 mg/ml HA or 0.5 mg/ml chondroitin 6-sulfate in 50 mM sodium acetate buffer containing 10 mM CaCl2 (or stated otherwise) at pH 6.2. The reaction mixture was incubated for 5 min during which the measurements were carried out by monitoring the increase in absorbance at 232 nm at 37 °C (23).

For K_m and V_max determination, the protein concentrations used were 1, 8, and 3.3/1.6 nM for SagHL/SagHL-βI, Sag Domain-I/α-domain, and Sag Domain-I:SagC complexes, respectively. The concentration of polymeric HA (24) was expressed as hexasaccharide units of HA (HA_6), which was prepared by adding 40 units of *Streptococcus hyalurolyticus* HL (Sigma) to a 6 mg/ml solution of HA in 50 mM sodium acetate buffer containing 10 mM CaCl2. The mixture was incubated for 10 min to 6 h (depending upon the products desired) at 37 °C. The digested products (contains mostly tetra/hexa/octa/decasaccharide (HA_4/HA_6/HA_8/HA_10)) were loaded onto a Superdex 200HR 10/30 column pre-equilibrated with 20 mM Tris-Cl, pH 7.2 (prepared in water (LiChrosolv®) and purified through a Chelex100 column) at 25 °C with detection at 232 nm. The products were eluted, and the masses were confirmed by ESI-MS. A millimolar absorption coefficient of 5.5 was used for the disaccharide product (25).

For all enzymatic activity measurements, the required chemicals, buffers, and other components did not contain trace amounts of metal ion contamination. The solutions were further checked by atomic absorption spectroscopy.

**Circular Dichroism Measurements**—CD measurements were made using a Jasco J-810 Spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as the mean residual ellipticity [θ], which is defined as [θ] = 100 × θ_{obs}/(l·c), where θ_{obs} is the observed ellipticity in degrees, c is the concentration in moles of residue per liter, and l is the length of the light path in centimeters. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant or salt under similar conditions. The protein concentration used for the studies was 1 μM.

**Size-exclusion Chromatography (SEC)**—Gel filtration experiments were carried out on a Superdex 200HR 10/30 column on AKTA FPLC. The column was calibrated with various molecular weight standard markers before use. The column was equilibrated and run with 20 mM Tris-HCl of the desired pH. 200 μl of the sample were loaded on the column and run at 25 °C at a flow rate of 0.3 ml/min with detection at 280 nm.
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RESULTS

The three domains present in the crystal structure of SagHL (92 kDa) are: the N-terminal spacer domain or β domain (Ser^{171}–Val^{246}), middle domain or α-domain (Phe^{252}–Asn^{611}), and the C-terminal domain or βII domain (Lys^{621}–Ile^{984}) (18). The spacer domain is connected to the α-domain by a seven residue linker (Thr^{245}–Asn^{252}) and the α-domain is connected to the C-terminal domain by a nine residue linker (Asp^{612}–Leu^{620}). We have cloned, overexpressed, and purified the SagHL and its different fragments/domains (Fig. 1) and studied their structural and functional properties alone or in combination with other domains.

Purification and Characterization of SagHL and Isolated Fragments/Domains—The cloned and overexpressed proteins were purified by the methods described under “Experimental Procedures.” The molecular masses of the purified recombinant SagHL, SagHL-βI, Sag Domain-I, α-domain, and SagC were 92, 83, 50, 41, and 42 kDa, respectively, as determined by SDS-PAGE (Fig. 2A) and MALDI-TOF (data not shown) and were similar to that deduced from their primary amino acid sequences. On a Superdex S-200 column (GE Healthcare), a single peak having retention volumes of 14.1, 14.4, 15.3, 15.7, and 15.2 ml (Fig. 2B), corresponding to the calculated molecular masses of 92, 83, 50, 41, and 51 kDa were observed for SagHL, SagHL-βI, Sag Domain-I, α-domain, and SagC, respectively. The molecular mass of SagC obtained from SEC was slightly higher than that observed from the SDS-PAGE or MALDI-TOF. This discrepancy may be caused by stabilization of the isolated SagC domain in a slightly open conformation resulting in a larger molecular dimension compared with the globular protein of equivalent molecular mass.

Analysis of the thermal denaturation and solvent-induced denaturation using a chaotrophic agent provides a measure of the conformational stability of proteins (26). In the far-UV region, the CD spectrum (27) of SagHL shows the presence of a mixed α-helical and β-sheet conformation (Fig. 3A, inset). For studying the thermal stability and the stability against GdmCl denaturation, we monitored the changes in CD ellipticity at 222 nm for all the proteins at increasing temperatures or GdmCl concentration. Upon thermal denaturation of SagHL, three distinct transitions (Fig. 3A) centered at about 42, 59, and 70 °C were observed. In the case of GdmCl-induced denaturation also showed the respective transitions for these domains corresponding to the transition observed for SagHL (Fig. 3B).

Role of Calcium Ions in Modulating Enzymatic Activity of HLs—To perform enzymatic activity measurements, water (LiChrosolv) and buffer were passed through the Chelex100 column to remove any metal ion contamination. Proteins and substrates were further purified by SEC, pre-equilibrated and run with the buffer that was already passed through a Chelex100 column. Furthermore the solutions were also checked by atomic absorption spectroscopy to confirm the absence of metal ion contamination (data not shown).

As reported earlier, the enzymatic activity of HLs is dependent on CaCl_2 (14, 21). SpnHL in the presence of 10 mM CaCl_2 gives almost equal enzymatic activity with polymeric HA (about 5.8 × 10^6 Da) and with its small hexasaccharide unit (HA_{6}) (about 1200 Da) at equal substrate concentrations (24). Comparative enzymatic activity measurements of SagHL using these substrates were carried out in the presence or absence of CaCl_2, NaCl, and EDTA, and the results are summarized in Fig. 4.

With polymeric HA as substrate, about 10-fold enhancement in enzymatic activity of SagHL was observed when 0–10 mM CaCl_2 was added (Fig. 4A). However, when HA_{6}/HA_{8}/HA_{10} was used as a substrate, SagHL showed maximal activity even

FIGURE 1. Diagrammatic representation of recombinant SagHL and SpnHL shows the respective domains. The figure was generated from the structure coordinates of SagHL (1F1S) and SpnHL (1C82) accessible from the Protein Data Bank.
without adding any CaCl$_2$, and the presence of even 10 mM CaCl$_2$ made no difference in its activity. In the presence of 0.1 M NaCl, with polymeric HA about 30% reduction in the SagHL activity was observed; however, with HA$_6$/HA$_8$/HA$_{10}$ again no effect of NaCl on enzymatic activity was seen. The effect of EDTA (5 mM) on the enzymatic activity (Fig. 4B) was only observed with the polymeric hyaluronan, and with the other substrates, the activity was completely independent of the EDTA. No effect of EDTA was observed on the background activity (where HA was used as a substrate and no Ca$^{2+}$ was added in the reaction mixture) of SagHL. This also suggested that the solutions we used for our experiment were devoid of any metal ion contamination. Similar results were also obtained with SpnHL (data not shown).

In Vitro Complex Formation of $\alpha$- and $C$-terminal Domains of SagHL—The interface between the $\alpha$-domain and $C$-terminal domain in closely related enzymes like SpnHL and Flavobacterium heparinum chondroitin AC lyase is spread over a large
area (up to 15% of the total surface area) and have significant interactions (16, 28). From the crystal structure of SagHL, it is observed that the interface between these two domains is spread over a significant area (Fig. 5A) and is composed of residues from both domains making several interactions between them (18–19).

Studies on the interactions between Sag Domain-I and SagC were carried out by incubating these domains together in vitro under specified conditions and analyzing the stabilization of the Sag Domain-I:SagC complex. Fig. 5B shows the SEC profile of the sample in which Sag Domain-I and SagC were incubated at a ratio of 1:2 (μM) at pH 7.6 for 2 h before loading onto the column. In Fig. 5B (profile 2) the observed two peaks x and y having retention volumes of 14.2 and 15.2 ml correspond to the protein molecular mass of about 91 and 50 kDa, respectively. Because the observed elution volume of peak x (14.2 ml) is similar to that observed for SagHL (15.2 ml), the protein sample under peak x probably corresponds to the Sag Domain-I:SagC complex, which is stabilized under these conditions. The protein sample under peak y corresponds to the noninteracted free Sag Domain-I and/or SagC.

The stabilization of the Sag Domain-I:SagC complex was supported by PAGE analysis of the incubated samples. The pI of the SagHL, Sag Domain-I, and SagC are 8.52, 7.01, and 8.84, respectively, so their migration on PAGE differed accordingly (Fig. 5C). In the incubated sample containing mixtures of the stabilized complex as well as noninteracted free Sag Domain-I and SagC, three separate bands corresponding to these three species were observed (lane 4). However, for the purified complex (sample from peak x of profile 2), a single band having a migration equivalent to the SagHL was observed.

The stability of the Sag Domain-I:SagC complex was further checked by subjecting the purified complex (sample from peak x of profile 2) to SEC. No alteration in the retention volume of the sample was observed on re-injection. These results demon-
strate that the Sag Domain-I:SagC complex formed on in vitro incubation of the two domains is a stable heterocomplex.

In the above-presented studies, the complex formation was carried out at 4 °C to minimize the random motion/movement of the molecules. The domain complexes once stabilized were found to be structurally and functionally stable for at least 12 h at room temperature. No stable complex formation was observed at room temperature. For maximum complex formation, the effect of varying ratio of individual domains, and the different pH values of incubation were studied. The most suitable conditions observed for the complex formation was Sag Domain-I:SagC ratio of 1:1 or 1:2 (H9262 M) and pH 7.6 (supplemental Fig. S1).

Role of Different Domains of SagHL in Functional Activity—To look into the earlier proposed mechanism of HA degradation by HLs and to study the role of different domains in modulating functional activity of the enzyme (29), comparative enzymatic activity measurements of SagHL, SagHL/β, Sag Domain-I, α-domain, SagC, and purified (after SEC) Sag Domain-I:SagC complex with HA or HA6/HA8/HA10 were carried out, and the results are summarized in Fig. 6. SagHL and SagHL/β showed almost similar enzymatic activity with HA and HA6/HA8/HA10. The Sag Domain-I and α-domain showed about 10% enzymatic activity compared with full-length enzyme with all the substrates whereas SagC did not show any enzymatic activity. The stabilized Sag Domain-I:SagC complex with HA showed about 31% enzymatic activity, which is about 2.51-fold higher than that observed for the isolated Sag Domain-I/α-domain. However, with HA6/HA8/HA10 as substrate, the complex showed 6-fold higher activity (about 60%). These observations clearly demonstrate that the interaction of the C-terminal domain of SagHL with Sag Domain-I results in significant enhancement in enzymatic activity. Furthermore for all

FIGURE 4. Relative enzymatic activity of SagHL. A, enzymatic activity with HA and HA6/HA8/HA10 in the presence or absence of CaCl2 and NaCl. The filled or hollow squares represent activity of SagHL with HA in the presence or absence of CaCl2, respectively. Regular or inverted triangles represent activity with HA6/HA8/HA10 in the presence or absence of CaCl2, respectively. The filled or hollow circles are activity in the presence of 0.1 M NaCl with HA and HA6/HA8/HA10, respectively containing 10 mM CaCl2. B, enzymatic activity with HA and HA6/HA8/HA10 in the presence or absence of EDTA. The filled or hollow squares represent activity of SagHL with HA in the presence or absence of 5 mM EDTA, respectively containing 10 mM CaCl2. Regular or inverted triangles represent activity with HA6/HA8/HA10 in the presence or absence of 5 mM EDTA, respectively. The filled circle represents activity of SagHL with HA in the absence of CaCl2 but in the presence of 5 mM EDTA. Activity was measured by monitoring the rate of increase in absorbance at 232 nm for 5 min at 37 °C. The data presented are of single measurements only as the mean value of even three data in the case of enzymatic activity with HA6/HA8/HA10 (in any condition) and with HA (in the presence of 10 mM CaCl2) if plotted almost overlaps. The inset shows the activity of SagHL with chondroitin sulfate in the presence (filled square) or absence (hollow square) of CaCl2.
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[A] C-terminal domain

[B] Absorbance at 280 nm

80 kDa
40 kDa

Elution Volume (mL)

[C] 1 2 3 4 5
the proteins studied, the final degradation product was found to be only disaccharide (Fig. 6, inset).

In a control experiment in the presence of neutral proteins like ovalbumin and bovine serum albumin in vitro, no change in the enzymatic activity of Sag Domain-I/α-domain was observed (data not shown). In another experiment, a mixture (Fig. 6) just composed of Sag Domain-I and SagC at 37 °C also did not show any enhancement in activity. These observations suggest that the enhancement in activity of Sag Domain-I in the presence of the C-terminal domain is only due to the formation of an active complex between them and not because of any stabilization/preservation effect of the Sag Domain-I in the presence of other proteins.

DISCUSSION

The activity reported for the 111-kDa HL compared with the truncated 92-kDa HL (SagHL) has only about a 3% difference (15). Interestingly, our results demonstrate that even further truncated SagHL-β (83 kDa) has almost equal stability and enzymatic properties as SagHL.

It is well documented that calcium ions are essential for the enzymatic activity of HLs; however, the exact reason for this is not known. It has been reported that polymeric HA (~25,000 disaccharide units) forms a stable reversible tertiary structure in solution, and Ca²⁺ induces HA to assume an extended conformation with three disaccharides per helix by making salt bridges between two carboxylic groups from its adjacent polyanionic strand. NaCl induces a compressed 4-fold helical conformation of HA (17, 30, 31). We did comparative enzymatic activity studies of SagHL/SpnHL with polymeric HA and with the small extended hexa/octa/decasaccharide (HA₆/HA₈/HA₁₀) in the presence or absence of CaCl₂ as well as NaCl and observed that although the enzymatic activity with HA is significantly modulated in the presence of CaCl₂ (significantly enhanced) and NaCl (significantly reduced) there is no effect of CaCl₂ and NaCl on the enzymatic activity of SagHL/SpnHL with HA₆/HA₈/HA₁₀. The results therefore suggest that calcium ions do not activate HLs, and the enhancement and reduction in its enzymatic activity in the presence of CaCl₂ and NaCl, respec-

FIGURE 5. C-terminal and α-domain of the SagHL show strong interactions. A, figure showing residues present at the interface (4 Å difference) between the C-terminal (green) and α-domain (red) of SagHL involved in making interactions between them. B, size-exclusion chromatographic profiles of SagHL (profile 1), Sag Domain-I:SagC complex formed at 1:2 (μM) ratio at pH 7.6 and 4 °C (profile 2) and the re-injected sample from peak x of profile 2 (profile 3). The inset shows the SDS-PAGE profile of the samples under peak x and y of profile 2. Lanes 1–5 represent molecular weight markers, purified Sag Domain-I, SagC, sample under peak x, and sample under peak y, respectively. C, PAGE analysis (8%) of the different proteins made and run at pH 9.15 for 12 h at 4 °C. Lanes 1–5 represent SagHL, Sag Domain-I, SagC, incubated mixture containing Sag Domain-I and SagC at pH 7.6 and 4 °C, stabilized Sag Domain-I:SagC complex from peak x of profile 2, respectively.

FIGURE 6. The C-terminal domain modulates the enzymatic activity of α-domain. Enzymatic activity profile of different proteins at 37 °C with HA and HA₆/HA₈/HA₁₀. The enzymatic activity is represented with respect to the concentration of Sag Domain-I, and the activity of native SagHL was taken as 100%. The last column represents the mixture composed of Sag Domain-I and SagC at 37 °C. The inset shows the SEC profile of HA (2 mg) cleaved by SagHL, Sag Domain-I:SagC complex, and Sag Domain-I at equal molar concentrations (5 nm each).
is negatively charged at neutral pH, which possibly facilitates its catalytic activity with chondroitin 6-sulfate even in the absence of CaCl₂. Hence, possibly the final structure of the respective substrates present in solution is actually responsible for its catalytic degradation by HLs.

In all the lyases, the catalytic cleft contains a number of positively charged residues (29). Because the pKₐ of the glucuronic acid carboxylate group is about 3.2, glycosamines (GAGs) are negatively charged at neutral pH, which possibly facilitates its interaction with the positively charged cleft. As reported in this article, isolated Sag Domain-I/α-domain/stabilized Sag Domain-I:SagC complex was able to attract HA and perform catalysis. Furthermore, in the case of the complex, the interaction of the C-terminal domain with Sag Domain-I possibly facilitates attaining the right geometry of the catalytic cleft and also increases its positive potential; a significant attraction of substrate in a suitable position occurs resulting in an enhanced enzymatic activity.

Because the interacting domain complex showed significantly higher enzymatic activity compared with the Sag Domain-I/α-domain, we carried out a comparative kinetic analysis of these proteins (Table 1). The Kₘ observed for SagHL and the stabilized complex (Sag Domain-I:SagC) were similar; however, differences exist in their Vₘₐₓ, suggesting that although the binding of substrate to the catalytic cleft is similar in both cases, their subsequent translocation is probably different. This seems feasible because in SagHL, the α- and C-terminal domains are connected through a linker so a regulated translocation is possible whereas, in the case of the complex, this process will be hampered. This also may be a reason why we did not observe an absolute activity in the case of the stabilized complex. Furthermore, the observed higher Vₘₐₓ in the case of the complex for the small HA₆ than the polymeric HA suggests that the rate of release of the disaccharide product is significantly higher with HA₆ than polymeric HA. The relatively higher Vₘₐₓ with HA₆ may be because HA₆ is the longest unit of the HA that would completely fit with in the catalytic cleft of the enzyme, and as reported nearly all the residues of the α-domain alone interact directly or indirectly with the hexasaccharide substrate. In the case of the Sag Domain-I/α-domain, both the Kₘ and Vₘₐₓ were different than those of SagHL, suggesting that either the substrate access is insufficient or both the translocation of substrate and release of disaccharide product are affected in the absence of the C-terminal domain.

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