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THE FUNCTIONAL MOLECULAR MASS OF THE *PASTEURELLA* HYALURONAN SYNTHASE IS A MONOMER*

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Abstract

Hyaluronan (HA), a linear polysaccharide composed of β 1,3-GlcNAc- β 1,4-GlcUA repeats, is found in the extracellular matrix of vertebrate tissues as well as the capsule of several pathogenic bacteria. All known HA synthases (HASs) are dual-action glycosyltransferases that catalyze the addition of two different sugars from UDP-linked precursors to the growing HA chain. The bacterial hyaluronan synthase, PmHAS from Gram-negative *Pasteurella multocida*, is a 972-residue membrane-associated protein. Previously, the Gram-positive *Streptococcus pyogenes* enzyme, SpHAS (419 residues), and the vertebrate enzyme, XIHAS1 (588 residues), were found to function as monomers of protein, but the PmHAS is not similar at the protein sequence level and has quite different enzymological properties. We have utilized radiation inactivation to measure the target size of recombinant full-length and truncated PmHAS. The target size of HAS activity was confirmed using internal enzyme standards of known molecular weight. We found that the *Pasteurella* HA synthase protein functions catalytically as a monomer. Functional truncated soluble PmHAS also behaves as a polypeptide monomer as assessed by gel filtration chromatography and light scattering.

Keywords

glycosaminoglycan; glycosyltransferase; hyaluronan; hyaluronan synthase; *Pasteurella multocida*; radiation inactivation

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INTRODUCTION

Hyaluronan (HA)1 [$\rightarrow$ 4)- β -D-GlcUA(1 $\rightarrow$ 3)- β -D-GlcNAc(1 $\rightarrow$)] is a prominent GAG that plays roles as a structural element and a recognition molecule in vertebrates, and is also employed by certain pathogenic bacteria to enhance infection [1]. The enzymes that catalyze the production of HA, the HA synthases, were the first examples of glycosyltransferases capable of forming the disaccharide repeat of a GAG that were cloned and described at the molecular level. The enzymes utilize UDP-GlcUA and UDP-GlcNAc precursors found in the cytosol and the growing HA chain is extruded out of the cell after polymerization.

The microbial HA synthases, SpHAS of Group A *Streptococcus pyogenes* and PmHAS of Type A *Pasteurella multocida*, are responsible for the polymerization of HA of the extracellular capsule [2,3]. A *Xenopus laevis* (African clawed frog) protein, XIHAS1, originally called DG42, was the first vertebrate HA synthase to be cloned as an abundant transcript during gastrulation, but at the time its function was not known [4–6]. More vertebrate isozymes with similar amino acid sequences were subsequently identified [7,8]. In addition, a HAS from an algal virus, CvHAS, was also discovered [7,9].

The streptococcal, vertebrate, and viral HA synthases (Class I synthases; [7]) appear to have protein sequence similarity especially in the central region of the polypeptides. All of these glycosyltransferase enzymes appear to have a single GT2 family module (<http://afmb.cnrs-mrs.fr/CAZY/fam/GT2.html>) with some similarity to the chitin synthases. Members of this group display integral membrane protein characteristics including 4 to 6 predicted transmembrane helices and 1 or 2 membrane associated regions. The general nature of these regions predicted in silico has been confirmed for SpHAS by a reporter protein fusion approach [10]. The streptococcal enzymes have a lipid (cardiolipin is the most active tested compound) requirement for activity, but it is not known if lipid is a necessity for the vertebrate and viral enzymes [11,12].

In contrast, the *Pasteurella* HA synthase (Class II HA synthase; [7]) does not have strong sequence similarity to the other known HA synthases described above. PmHAS has two GT2 family modules that do not closely resemble the modules found in the chitin synthases. No unambiguous candidates for transmembrane helices or membrane-associated regions are predicted in silico. The PmHAS enzyme appears to be a peripheral membrane protein with neither an intrinsic affinity for bilayers nor a lipid requirement for catalytic activity. Truncation of the carboxyl terminus residues 704–972 results in an active, soluble synthase; this region may be involved in docking peripherally with integral membrane transport proteins common to many encapsulated Gram-negative bacteria [13].

HA synthases apparently perform many functions including binding UDP-GlcNAc and UDP-GlcUA precursors, catalyzing formation of glycosidic bonds, and potentially the transport of the polysaccharide through the plasma membrane. Immunopurification of the SpHAS [12] or the murine HAS1 [14] polypeptide indicates that a single polypeptide species is responsible for the catalytic transfer of both GlcNAc and GlcUA to the growing polymer chain. The Class II PmHAS protein was recently shown by molecular genetic means to have two active sites in one polypeptide [13]. In contrast to the known HA synthases, the vast majority of other glycosyltransferases can only perform the transfer of one type of sugar for any given polypeptide species.

Radiation inactivation studies of SpHAS and SeHAS, the homologous enzymes from Group A and C *Streptococcus*, respectively, have indicated that ~16 molecules of lipid and a monomer of protein comprise the functional HA polymerization unit [15]. The polymerization of HA polysaccharide by XIHAS1 requires a single monomer but a role for lipid was neither ruled out nor supported [16]. Here, we report that the PmHAS enzyme also functions as a monomer.

MATERIALS AND METHODS

Production of Recombinant Full-Length PmHAS Proteins

All reagents were from Sigma or Fisher unless noted otherwise. The construction and the use of the full-length PmHAS expression plasmid for studies in *E. coli* were described [3]. Basically, the open reading frame of 972 residues was cloned into the pKK223-3 vector (Pharmacia) under control of the *tac* promoter. Crude membranes were prepared by sonication and ultracentrifugation [17]. The membrane pellets were suspended in 50 mM Tris or HEPES buffer, pH 7.2, 10% glycerol, 1 mM EDTA, 0 or 1 mM β -mercaptoethanol, 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM benzamidine, 5 μ g/ml pepstatin, leupeptin, and 0 or 1 μ M E-64. Protein was quantitated by the Bradford Coomassie dye-binding assay [18] (Pierce, Rockford, IL) using a bovine serum albumin standard. The total protein concentration of samples destined for radiation inactivation was adjusted to 8–15 mg/ml.

A standard enzyme with a known target size, glucose-6-phosphate dehydrogenase (G6P-DH), was added to the membrane preparation (~25 units/ml final). Two sources of G6P-DH enzyme were used in different experiments: *Saccharomyces cerevisiae* (dimer of two 57.5 kDa subunits or 115 kDa; ref. [19] or *Leuconostoc mesenteroides* (dimer of two 54.4 kDa subunits or 109 kDa; ref. [20]). A series of identical aliquots (0.3 to 0.5 ml of membranes each) were frozen on dry ice and flame-sealed in 2-ml glass vials. The vials were kept at -80°C or lower throughout all procedures until assayed.

Production of Recombinant Truncated PmHAS Protein

Soluble active recombinant PmHAS^{1–703} enzyme was produced in *E. coli* Xja host cells (Zymo Research Corp., Orange, CA) grown in Superior broth (Athena ES, Baltimore, MD) with ampicillin selection at 30°C . Synthase production was induced by adding isopropylthiogalactoside (0.2 mM final) at mid-log phase. The phage lysin residing on the *E. coli* Xja chromosome was induced with L-arabinose (3.3 mM final) present during the entire growth period. After overnight growth, the cells were harvested by centrifugation and frozen at -80°C . After two freeze/thaw cycles, Xja cells readily release their cytoplasmic contents. The bacterial lysates were clarified by high-speed centrifugation, mixed with G6P-DH, and flame sealed as above.

For MALDI-ToF MS and SEC-MALLS analyses, a preparation of PmHAS^{1–703} was purified by chromatography on Toyopearl Red AF resin (Tosoh Corp., Tokyo, Japan) using salt elution (50 mM HEPES, pH 7.2, 1 M ethylene glycol with 0 to 1.5 M NaCl gradient in 1 hour) as described previously [21]. The fractions containing the target protein (~95% pure by SDS-PAGE/Coomassie-staining) were pooled and concentrated by ultrafiltration. Typically, PmHAS^{1–703} preparations had specific activities of ~0.4 nmoles monosaccharides transferred per minute per microgram protein.

Irradiation Procedure

Samples were irradiated at -135°C in the gas phase of evaporating liquid nitrogen with a beam of 13-MeV electrons produced by a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda MD or the National Institute of Standards and Technology, Gaithersburg MD). Two to four aliquots were not irradiated to serve as the zero dose control. Radiation dose was determined with thermoluminescent or alanine dosimeters. Typical exposure rates were 10–30 Mrad/hr. Upon opening the vials, but before thawing, nitrogen gas was used to purge any remaining ozone generated by the intense irradiation [22]. The volume in each vial was then divided into several small aliquots that were stored at -80°C until assayed.

HA Synthase Assays

The incorporation of sugars into high molecular weight HA polysaccharide was monitored by using UDP-[^{14}C]GlcUA (NEN, Perkin Elmer, Boston, MA) precursor as described previously [13]. The enzyme preparations were incubated at 30°C for 30 min in 40–100 μl buffer containing 50 mM Tris, pH 7.2, 20 mM MnCl_2 , 10–100 $\mu\text{g/ml}$ HA tetramer, 200–300 μM UDP-GlcUA (0.02–0.08 μCi), and 600–700 μM UDP-GlcNAc. Some assays also included 0.1–1 mM β -mercaptoethanol or 0.1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 M ethylene glycol as stabilizers. Reactions were terminated by the addition of SDS to 2% (w/v). Descending paper chromatography (65:35, ethanol/1 M ammonium acetate, pH 5.5) was utilized to separate products from substrates; the radioactive polymers at the origin of the paper chromatogram were detected by liquid scintillation counting. All assays were performed in duplicate and the values were averaged. To assess nonspecific incorporation, results from parallel control reactions without UDP-GlcNAc, a required precursor for HA polymerization, were subtracted from the HAS assay results. Assays for quantitation of PmHAS synthase activity were set so that < 5% of the radiolabeled substrate was consumed and the enzyme concentration was in the linear range.

G6P-DH Assays

The G6P-DH standard enzyme activity was measured at 25°C in 1 ml assays containing 56 mM Tris, pH 7.8, 3.4 mM MgCl_2 , 0.2 mM NADP, 3.3 mM glucose-6-phosphate (G6P), and 0.5 to 50 μl of the irradiated enzyme preparations [23]. Reaction rates were obtained by monitoring the change in OD_{340} over time in duplicate assays with two different amounts of irradiated enzyme, for a total of at least four determinations for each radiation dose. At each radiation dose, reactions with the same amount of enzyme extract, but lacking the G6P, were used to obtain baseline reaction rates.

Radiation Inactivation Molecular Mass Determination

The fraction of the surviving HA synthase activity [A/A_0] was plotted on a logarithmic scale as a function of radiation dosage. The data from each series of irradiated samples were fit to a line by least squares analysis. The slope of the line (k) yields the molecular mass in kDa = $1792 k$ [16,23,24]. In the case of measuring enzyme activity, the target size corresponds to the mass of the enzyme's functional unit (strictly defined as the sum of the masses of all the structures required to express activity in the assay).

Mass Spectrometry

For MALDI-ToF MS, 1 μl of 2.7 mg/ml of purified PmHAS^{1–703} protein was spotted onto the target plate followed by 1 μl of matrix solution (saturated 2',4',6'-trihydroxyphenone monohydrate (Aldrich) in 50% acetonitrile, 49.9% water, 0.1% trifluoroacetic acid), mixed by pipetting, and vacuum dried. The samples were analyzed in the positive ion, linear mode on a Voyager Elite DE mass spectrometer (25 kV acceleration, low mass gate 4 kDa, delayed extraction 300 ns). The data from two samples were averaged. BSA was employed as a standard.

Size Exclusion Chromatography

SEC-MALLS was employed to determine the absolute molecular mass of purified PmHAS^{1–703} in aqueous solution. Enzyme (80 μg ; 40 μl injection) was separated on a Pharmacia Superdex 200 column (10 X 300 mm, 24 ml) eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7 at 0.5 ml/min. MALLS analysis of the eluant was performed by a DAWN DSP Laser Photometer in series with an OPTILAB DSP Interferometric Refractometer (632.8 nm; Wyatt Technology, Santa Barbara, CA). The ASTRA software package was used to determine the absolute average molecular mass using a dn/dc coefficient of 0.18 ml/g. The

M_w and polydispersity values are the average of data from two SEC-MALLS runs. The monomer and dimer forms of BSA were used as standards.

RESULTS AND DISCUSSION

It had previously been shown that radiation damage occurred throughout a natural polypeptide no matter where the initial primary ionization had occurred [25]. Target analysis of surviving activity will yield a mass equal to that of the entire polypeptide. If a protein functions as an oligomer, then the target size will correspond to the mass of the entire complex.

In our radiation inactivation studies, we employed an internal standard, G6P-DH, to calibrate and refine our target size of PmHAS. The calculated standard target size for each irradiation run is shown in Table 1. The sizes obtained for PmHAS from three different irradiation runs and for PmHAS¹⁻⁷⁰³ from two different irradiation runs are also shown in Table 1. A graph of the results from one of the runs on each enzyme is shown in Figure 1. The PmHAS target may also be evaluated by comparison to the internal control [16,22,26]. The experimental target size, 95 ± 8 (or 118 ± 11 , as determined by G6P-DH adjustment) corresponds well with the predicted full-length PmHAS mass (112 kDa). Similarly, the truncated PmHAS¹⁻⁷⁰³ gave a value of ~66 kDa (or ~96 kDa by G6P-DH adjustment), which corresponds well to the predicted mass of 80.5 kDa.

In addition to enzymological characterization of PmHAS, we performed physical analyses of the soluble, active truncated PmHAS¹⁻⁷⁰³. MALDI-ToF MS gave a value of 80.18 kDa, which compares very well to the predicted mass of 80.49 kDa for PmHAS¹⁻⁷⁰³ (for the BSA control: predicted 66.48 kDa, observed 66.43 kDa). Therefore, no significant post-translational modifications are present in this HAS species. The missing ~300 Da may be due to several missing carboxyl terminal residues, based on Edman sequencing data yielding the expected amino terminus sequence for these PmHAS preparations (data not shown). SEC-MALLS yielded a size of 82.7 kDa with a polydispersity value of 1.001 (Fig. 2; for reference, 1 is the value for an ideal polymer) thus no oligomerization was detected, thus supporting the radiation inactivation analyses.

The enzyme kinetics analyses of the PmHAS wild-type enzyme and a panel of point mutants strongly suggested that two active sites coexist in one polypeptide [27,28]. In an in vitro complementation study, a pair of PmHAS mutant polypeptide molecules disrupted at or near the two active sites, acted together to polymerize HA chains in a surprisingly rapid fashion approaching rates observed for the undisrupted wild-type sequence enzyme. One possible explanation for this behavior was that PmHAS acts as a homodimer in solution, but the radiation inactivation experiments presented here rule out this possibility. Thus a better explanation for the observed complementation results is that the HA chain must be released by one mutant enzyme to be acted on by the other mutant enzyme. Overall, PmHAS appears to be a polypeptide with two active sites involved with coordinated but intrinsically nonprocessive activities. In several indirect tests, including in vitro syntheses of oligosaccharides [21] and polysaccharides [29], PmHAS appears to operate in a nonprocessive fashion. In vivo, PmHAS is probably docked with the membrane transport machinery which may not allow the HA chain to diffuse far away from the microenvironment of the two active sites. If the HA chain were released completely from PmHAS, then the HA chain would escape from the cell and could not be elongated into the long chains actually observed.

In summary, Nature has evolved at least two distinct classes of HA synthases based on amino acid sequence and enzymological data, but the functional catalytic glycosyltransferase unit of all known HA synthases is a monomer of polypeptide.

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The abbreviations used are

HA	hyaluronan, hyaluronate, or hyaluronic acid
HAS	HA synthase
GlcUA	glucuronic acid
GlcNAc	N-acetylglucosamine
GAG	glycosaminoglycan
GT2	glycosyltransferase family 2
PCR	polymerase chain reaction
Tris	tris(hydroxymethyl)aminomethane
SDS	sodium dodecylsulfate
G6P-DH	glucose-6-phosphate dehydrogenase
BSA	bovine serum albumin
kDa	kiloDaltons
SEC-MALLS	size exclusion chromatography/multi-angle laser light scattering

MALDI-ToF MS

matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Fig. 1. Target size analysis of *Pasteurella* HAS

Residual HAS activity was determined for each radiation dose a minimum of four times. These results were then graphed logarithmically versus radiation dose and the slope of the least-squares line (k) determined. The plots for Run #1 (Table 1; *circles and solid line*) and Run #4 (Table 1; *squares and dashed line*) are depicted.

Fig. 2. Chromatographic size analysis of PmHAS¹⁻⁷⁰³

The truncated, active form of PmHAS (80 μ g; expected 80.49 kDa) was separated by SEC coupled to MALLS and refractive index (RI) detectors. BSA (expected 66.43 kDa) was analyzed for comparison; the elution times of the monomer (*m*) and dimer (*d*) BSA species are marked with **arrows** (void volume = 7.2 ml). PmHAS eluted at a time appropriate for a monomer (14.7 ml) and light scattering data yielded an average size of 83.2 kDa (molar mass, **solid circles**). No evidence for a dimer or a higher oligomer was observed.

Table 1

Target size analysis of *Pasteurella* HAS. Enzymes (either full length PmHAS¹⁻⁹⁷² in membranes or truncated PmHAS¹⁻⁷⁰³ in soluble extracts) were irradiated and the residual HAS activity determined and graphed as indicated in the *Experimental Procedures*. For full length PmHAS, the results of three independent irradiation experiments are presented, along with the averages and standard deviations of the three experiments. For truncated PmHAS¹⁻⁷⁰³, the results of two independent irradiation experiments are presented. Glucose-6-phosphate dehydrogenase (G6P-DH) from *L. mesenteroides* (*L.m.*) was used as an internal standard for runs 1, 2, and 4. Runs 3 and 5 utilized G6P-DH from *S. cerevisiae* (*S.c.*) for the internal standard. Radiation exposure was determined by thermoluminescent or alanine dosimeters [23]. Target sizes are also given based on a correction for the deviation of the observed G6P-DH target size from the known size ("G6P-DH Adjusted Target Size"). A monomer of PmHAS polypeptide is the HA synthase functional unit.

Run #	Enzyme	Dosimetry Target Size (kDa)	G6P-DH Adjusted Target Size (kDa)	Predicted Size (kDa)
1	<i>L.m.</i> G6P-DH	100	—	109
	PmHAS ¹⁻⁹⁷²	96	105	111
2	<i>L.m.</i> G6P-DH	91	—	109
	PmHAS ¹⁻⁹⁷²	103	123	111
3	<i>S.c.</i> G6P-DH	80	—	115
	PmHAS ¹⁻⁹⁷²	87	125	111
Average± S.D.	PmHAS ¹⁻⁹⁷²	95 ± 8	118 ± 11	111*
4	<i>L.m.</i> G6P-DH	67	—	109
	PmHAS ¹⁻⁷⁰³	54	88	80
5	<i>S.c.</i> G6P-DH	88	—	115
	PmHAS ¹⁻⁷⁰³	79	103	80
Average ± S.D.	PmHAS ¹⁻⁷⁰³	66	96	80*

* Monomer size predicted from the deduced DNA sequence.