Interaction between Glycosaminoglycans and Immunoglobulin Light Chains†

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ABSTRACT: Amyloidosis is a pathological process in which normally soluble proteins polymerize to form insoluble fibrils (amyloid). Amyloid formation is found in a number of diseases, including Alzheimer's disease, adult-onset diabetes, and light-chain-associated amyloidosis. No pharmaceutical methods currently exist to prevent this process or to remove the fibrils from tissue. The search for treatment and prevention methods is hampered by a limited understanding of the biophysical basis of amyloid formation. Glycosaminoglycans (GAGs) are long, unbranched heteropolysaccharides composed of repeating disaccharide subunits and are known to associate with amyloid fibrils. The interaction of amyloid-associated free light chains with GAGs was tested by both size-exclusion high-performance liquid chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis experiments. The results indicated that heparin 16 000 and chondroitin sulfate B and C precipitated both human intact light chains and recombinant light chain variable domains. Although all light chains interacted with heparin, the strongest interactions were obtained with proteins that had formed amyloid. Molecular modeling indicated the possibility of interaction between heparin and the conserved saddlelike surface of the light chain dimer opposite the complementarity-determining segments that form part of the antigen-binding site of a functional antibody. This suggestion might offer a new path to block the aggregation of amyloid-associated light chain proteins, by design of antagonists based on properties of GAG binding. A hexasaccharide was modeled as the basis for a possible antagonist.

Glycosaminoglycans (GAGs) perform numerous structural and regulatory functions in a cell (Jackson et al., 1991). They are ubiquitous components of basement membrane glycoproteins (Iozzo & Murdoch, 1996). One GAG, heparin, is a regulatory cofactor of fibroblast growth factor (Moy et al., 1997) and a well-known inhibitor of thrombin, leading to its use as an anticoagulant. GAGs are composed of long and unbranched heteropolysaccharide chains of repeating disaccharide subunits, one sugar of which is either *N*-acetylglucosamine or *N*-acetylgalactosamine. Carbohydrate has been correlated with amyloid deposits since 1851, when Virchow attempted to observe isomers of starch in humans and introduced the name "amyloid", which means starchlike (Pascali, 1995). There is a growing accumulation of studies implicating GAGs in the pathophysiology of amyloid; whether their role is in the development of amyloid or in its stabilization is not known.

Normally innocuous soluble proteins that polymerize to form insoluble fibrils produce the diverse biochemical conditions referred to as amyloidosis. Amyloid fibrils may be composed of various proteins—monoclonal light chains, transthyretin (prealbumin), β2-microglobulin, β-peptide, and peptide hormones—but all types appear similar under light microscopy with Congo red or metachromatic stains. Under electron microscopy, they appear as rigid, linear, nonbranching fibrils of indefinite length and 7.5−10-nm width. The fibrils associate with plasma and extracellular matrix proteins and proteoglycans to form amyloid deposits, which are an insoluble mass that invades the extracelluar space of organs, destroying normal tissue. Amyloid deposition is a factor in numerous medical conditions and disorders, including rheumatoid arthritis, Alzheimer's disease, familial amyloid polyneuropathy, and metabolic diseases such as adult-onset diabetes, multiple myeloma, and primary light-chain-associated amyloidosis [for review, see Sipe (1992)].

Study of the relationship between the accumulation of GAGs and amyloid deposition in two models of amyloid induction by Snow and Kisilevsky (1985) showed that amyloid-associated GAGs appearing in the tissues together with the amyloid-associated protein are not a function of the tissue type. Nelson et al. (1991) demonstrated that GAGs represented 1−2% by weight of amyloid fibrils that were isolated from the livers and spleens of four patients who had died of light chain (AL) systemic amyloidosis and from one patient with reactive systemic, amyloid A- (AA-) type amyloidosis. Nelson et al. (1991) also demonstrated that these GAGs were tightly associated with the fibrils and hence were not a fortuitous coisolate from the tissues. DeWitt et al. (1993) and Narindrasorasak et al. (1991) showed that chondroitin sulfate proteoglycans are associated with the lesions of Alzheimer's disease and have high affinity for Alzheimer's amyloid precursor proteins. The appearance of GAGs has also been shown to be independent of the nature or length of action of the inflammatory agent but appears to be an integral part of the process involved in the deposition of the AA protein (Husby et al., 1994).

A better understanding of the interactions between GAGs and amyloidogenic proteins may have clinical implications,

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Table 1: Summary of the Interaction of Light Chains with GAGs

			fractional recovery[c]				
protein[a]	isotype	pathology[b]	heparin 16 000[d]	heparin 6000[e]	chondroitin sulfate B	chondroitin sulfate C	heparin sulfate
Alb	control		1.00				
Chy	control		0.03				
Rib	control		0.16				
Cro	κI	amyloid	0.30				
Gri	κI	amyloid	0.03	0.95	0.61	0.66	1.00
Mev	κI	amyloid	0.06	0.70	0.87	0.93	0.93
Pfu	λI	amyloid	0.03	0.44	0.18	0.52	0.83
Kel	κIII	basement	0.27	0.52	0.80	0.82	1.00
Cox	λI	basement	0.38	0.75	0.54	0.74	1.00
Hol	κI	casts	0.20				
Pri	κI	casts	0.20				
Len	κIV	none	0.32	0.86	0.54	0.62	0.86
Borf	κI	none	0.38				
Kin	κI	none	0.62				
Kir	λIII	none	0.56				
r_Rei	κI	unknown	0.54	0.99	0.84	0.80	0.80
r_Len	κIV	none	0.64	0.66	0.93	0.70	1.00
r_Sma	κIV	amyloid	0.84		0.78	0.44	

[a] rRei, rLen, and rSma were recombinant light chain V domains. Others were obtained from patients as encoded by three- or four-letter designators for each monoclonal protein. Concentration of protein was 1.24 mg/mL and GAG concentration was 2.5 mg/mL. [b] Basement refers to nonfibrillar deposition of protein on basement membranes; cast indicates proteins that formed amorphous precipitates within nephron tubules. [c] Fractional recovery at a GAG concentration of 2.5 mg/mL. Heparin 16 000 concentration series were obtained for proteins Borf, Cro, Gri, Hol, Kin, Kir, Len, Mev, Pfu, Pri, and rRei; other data were obtained in single-concentration experiments. Alb, Chy, and Rib refer to control proteins albumin, chymotrypsinogen, and ribonuclease, respectively. [d] High molecular weight heparin. [e] Low molecular weight heparin.

as GAGs already have therapeutic applications in other conditions. GAGs have been tested in rats as a treatment for diabetic nephropathy; long-term administration of GAGs prevented renal morphological and functional alterations in diabetic rats (Gambaro et al., 1994). In a patient with abnormal amounts of protein in the urine, researchers suggested that GAGs have an antiproteinuric effect (Baggio et al., 1993). More recently, Kisilevsky et al. (1995) prepared a series of simple anionic sulfonate or sulfate compounds of molecular weight 135−1000 as mimetics of GAGs. These compounds were orally administered to mice, with the result that splenic AA amyloid progression was reduced.

With respect to AL amyloidosis, the availability of both human and recombinant immunoglobulin proteins offers an opportunity to characterize the interaction of free protein with GAGs. Only one related study has been reported for this type of interaction: 8 out of 100 intact antibody proteins tested from patients with multiple myeloma were shown to interact with heparin (Levy et al., 1981). However, we are not aware of any study of the interaction of free light chains with GAGs. If the interaction of GAGs with free light chain can be demonstrated and, further, be understood, it could lead to the design of inhibitors using GAGs as a template. Since GAGs are negatively charged molecules, a potential site of interaction of GAGs with a light chain dimer could be on the "bottom" of the complex, where conserved positively charged lysines are located. This site was previously proposed to interact with the complementarity-determining regions of a second dimer to form a tetramer, leading to assembly of the fibril in an amyloid formation model (Stevens et al., 1995). In this report, interactions between light chain proteins and recombinant V domain proteins with five common GAGs are described. We also present a model of one potential mode of interaction between GAGs and a light chain dimer.

MATERIALS AND METHODS

Protein Preparation and Characterization. The human proteins Borf, Cle, Cox, Cro, Hol, Kel, Kin, Kir, Len, and Pri were provided by Dr. Alan Solomon (University of Tennessee Medical Center, Knoxville) and are identified by first three or four letters of the patient's name. They were isolated and purified from the urine of patients with multiple myeloma or monoclonal gammopathy of uncertain significance, as described (Solomon et al., 1985). Protein Len was from a patient who produced 50 g of protein daily with no indication of amyloid deposits or other pathology. Human proteins Gri, Mev, and Pfu were provided by Dr. Manfred Eulitz of the GSF Institute of Clinical Molecular Biology (Munich, Germany). These proteins were isolated and purified from the urine of patients with AL amyloidosis. The recombinant variable domains rRei, rLen, and rSma were prepared as described by P. W. Stevens et al. (1995). The pathologies of the patients correlated with the protein isotypes are summarized in Table 1. The molecular forms of the Bence Jones proteins and recombinant variable domains were determined by SDS-polyacrylamide gel electrophoresis and gel filtration. Each sample was free of high-molecular-weight contaminants. Proteins were freshly dissolved in 20 mM sodium phosphate and 100 mM NaCl, pH 7.0, one day before the experiments.

Chymotrypsinogen A, ribonuclease A, and albumin were from the Pharmacia low molecular weight gel filtration calibration kit, and their p*I*s were obtained by running IEF3−9 PhastGel of Pharmacia.

Glycosaminoglycans. All GAGs (heparin 16 000, heparin 6000, heparan sulfate, and chondroitin sulfate B and C), were from Sigma and were used without further purification. According to the catalog and technical information from Sigma, heparin 16 000 was from porcine intestinal mucosa with typical activity ∼170 USP units/mg and average molecular weight ∼16 000. Heparin 6000 was also from porcine intestinal mucosa and produced by enzymatic de-

polymerization (auto-Xa activity >90 USP units/mg, APTT activity <100 USP units/mg, average molecular weight ~6000). Heparan sulfate was from bovine intestinal mucosa with average molecular weight ~7500. Chondroitin sulfate B was from bovine mucosa and composed of 80% chondroitin sulfate B with the balance being chondroitin sulfate A and C. Chondroitin sulfate C was from shark cartilage (90%) and the balance chondroitin sulfate A. The average molecular weight was ~50 000. All GAGs were in the form of the sodium salt.

Size-Exclusion Chromatography. Size-exclusion chromatography was performed as described by Myatt et al. (1994). The running buffer was 20 mM sodium phosphate and 100 mM NaCl, pH 7.0. The flow rate was 0.06 mL/min, and a 50 μL sample was injected into a 5 μL sample loop for sample introduction.

SDS−Polyacrylamide Gel Electrophoresis and Data Analysis. The PhastSystem and a gradient 8−25% PhastGel from Pharmacia LKB Biotechnology were used for gel electrophoresis experiments. In SDS−polyacrylamide gel experiments, the samples were prepared by mixing protein (nonreduced and reduced) and GAG to a final concentration of protein of about 1.0 mg/mL, of GAG 2.5 mg/mL, and a total volume of 12 μL, incubated at 37 °C in a water bath for 2 h, and then centrifuged at 12 000 rpm for 5 min. The 7.5 μL supernatant was transferred to a fresh microcentrifuge tube, to which a 2.5 μL mixture of 10% SDS and 20% mercaptoethanol was added. The mixture was incubated in a boiling water bath for 5 min. Samples (1 μL) were loaded for a PhastSystem 8−25% gradient gel. Reduction was done by adding 0.46 mg of dithiothreitol to 1 mg of protein and allowing the solution to stand at room temperature for 2 h. Salt concentration was controlled by adding stock sodium chloride or sodium sulfite solution from different experiments. The SDS−polyacrylamide gels were scanned by Adobe Photoshop 3.0 and the integrated density of bands was determined by the scanning program NIH image 1.59/ppc.

Heparin Depletion Assay. A variation of the assay described above was used to confirm the removal of heparin from solution as a result of interaction with protein. Three samples of heparin (2.5 mg/mL) containing 0.0, 0.25, and 0.50 mg/mL light chain, respectively, were incubated at 37 °C for 2 h. Following centrifugation to remove insoluble protein−heparin complexes, additional light chain was then added to restore the initial protein concentration. Protein was added to the control to a final concentration of 0.25 mg/mL.

Modeling. The light chain Rei V-domain crystal structure (Epp et al., 1974), a 12-sugar-unit heparin NMR structure (Mulloy et al., 1993), and a 6-sugar-unit chondroitin sulfate B structure (Winter et al., 1978) from Brookhaven Protein Data Bank (pdb1rei.ent, pdb1hpn.ent, and pdblc4s.ent, respectively) and INSIGHT II (95.0)/DISCOVER (version 2.97) molecular modeling package from Molecular Simulations Inc. (San Diego, CA) were used for modeling. The default force field of the DISCOVER program, cvff (version 3.0) with cut distance 12 Å, cutoff distance 14 Å, and switch distance 2.0 Å was used for all molecular mechanical calculations. Energy minimization calculations used 1000 steps of steepest descent followed by 5000 steps of complex conjugate minimization.

A distance-dependant dielectric constant (1.0*r*) was used for all molecular mechanical calculations (except five minimizations with a 5 Å water layer used for comparison purposes). Charges were assigned to all amino acids and heparin sulfate groups with the assumption that pK_a values were unaffected by ionic proximity.

The hexasaccharide fragment of heparin from sugar 2 through sugar 7 of Mulloy's 12-sugar NMR structure was chosen as a starting fragment based on structural analysis. The NMR structure analyses found that the distance between the positively charged side-chain nitrogen atoms of the two position-42 lysines was 22.5 Å. The distance between sulfur atoms of the SO_3^- groups from the more negatively charged side of heparin, i.e., the distance of the S atom in the O−SO_3^- group of the second sugar (iduronic acid) to the S atom in the N− SO_3^- group of the seventh sugar (glucosamine), was 23.6 Å. The distances between positively charged protein groups and the negatively charged sugar groups (22.5 and 23.6 Å, respectively) corresponded reasonably.

The hexasaccharide−heparin fragment was first manually docked into the potential binding site visually with one of the more negatively charged sides adjacent to the two position-42 lysines. This protein and hexasaccharide represented initial complex I for minimization. The hexasaccharide was rotated 45° about its axis and the molecules were merged to generate initial complex II. Initial complex III was generated by rotating the hexasaccharide in initial complex II another 45°. The three initial complexes were energy-minimized to a derivative of 1 kcal/Å and their intermolecular energies and total energies were calculated.

To assess the relative validity of the initial docking, three independent visual docking replicates with heparin fragment were conducted, using the arrangement of initial complex I to generate three complexes. Three complexes were energy-minimized to a derivative of 1 kcal/Å. Rei dimer and heparin fragment, both alone, were energy-minimized under the same conditions as controls. The final conformations of the Rei dimers after minimization as a complex were compared to each other as well as against the independently minimized Rei dimer by superimposing the Cα atoms. The final conformations of the heparin component after minimization in complex were also compared to each other and to heparin minimized in isolation by superimposing all atoms.

Molecular dynamics on the energy-minimized initial complex I was performed for 50 ps at 300 K and the conformation was written out every 2.5 ps. The intermolecular energies (without minimization) of the resulting 21 conformers were calculated by the docking/intermolecular procedure of InsightII.

RESULTS

HPLC Size-Exclusion Chromatography and SDS−PAGE Study. Gri is an intact light chain protein from a patient who had AL amyloidosis. Because of the quantities available, Gri was used as the reference protein for characterization of the interaction between protein and GAGs. Gri samples of 0.05−2.5 mg/mL were run in the HPLC size-exclusion chromatography system at both 23 and 37 °C. Elution profiles at all concentrations exhibited two peaks, which represented high-molecular-weight aggregates (10−15%) and dimer. The stability of Gri was tested at both 23 and 37 °C by injecting samples after 1 h and 6 h of

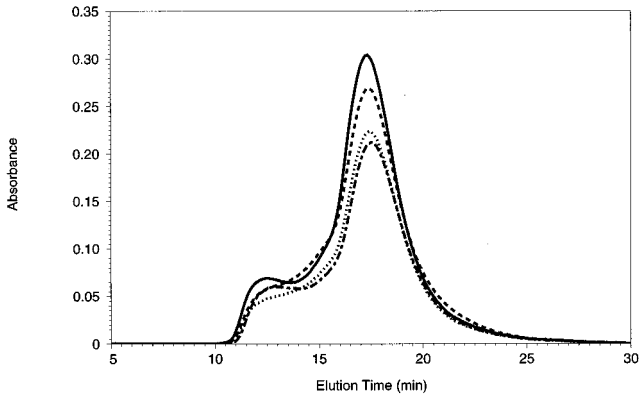

FIGURE 1: Gri elution patterns compared to profiles obtained for mixtures of Gri and chondroitin sulfate A, B, and C, showing decreased recovery of protein. (—) Gri; (- -) Gri + chondroitin sulfate A; (•••) Gri + chondroitin sulfate B; (-•-) Gri + chondroitin sulfate C.

incubation at each temperature. The elution profiles were reproducible. Gri was stable at each temperature during the incubation period.

Recovery of Gri protein from the supernatants of mixtures of Gri and GAGs decreased relative to that from solutions of Gri alone under the same conditions. An example of modest interaction between protein Gri and various chondroitin sulfates is shown in Figure 1. Supernatants of mixtures of Gri and GAGs with incubation times from 40 to 190 min and with GAG concentrations from 0.8 to 4.2 mg/mL were tested (not shown). The recovery of protein decreased as a function of both incubation time and GAG concentration. On the basis of these results, an intermediate GAG concentration of 2.5 mg/mL and a 2-h incubation time at 37 °C were chosen as standards for further experiments. Loss of protein in solution suggested the possible formation of an insoluble protein−GAG complex.

In addition to Gri, other κ (Borf, Cro, Kel, Kin, Len, and Mev) and λ proteins (Cox, Kir, and Pfu) from patients with selected light chain pathologies were chosen for further experiments using SDS−polyacrylamide gel electrophoresis to evaluate the generality of intact light chain interactions with GAGs. Five GAGs (chondroitin sulfates B and C, heparan sulfate, heparin 6000, and heparin 16 000) were used in mixtures with light chain proteins. All of the human proteins tested, regardless of pathology and isotype, exhibited substantially decreased protein recoveries when treated with heparin 16 000 (Table 1). The extent of interaction varied from protein to protein, presumably reflecting structural factors unique to the protein produced by each individual patient. Smaller decreases in recovery were generally found with heparin 6000 and chondroitin sulfates B and C. Heparan sulfate appeared to have the least interaction with the proteins tested.

Heparin 16 000, at concentrations varying from 0.5 to 4.7 mg/mL, was mixed with light chain proteins, and the supernatants were tested after 2 h of incubation. The decrease in protein recovery was dependent on the heparin 16 000 concentration (Figure 2). Moreover, although each protein tested interacted with heparin 16 000, the degree of interaction was protein-specific. Fractional recoveries ranged from 3% (protein Gri and Pfu) to 62% (protein Kin). This variation was not principally due to differences in free monomer and covalent dimer composition of the samples

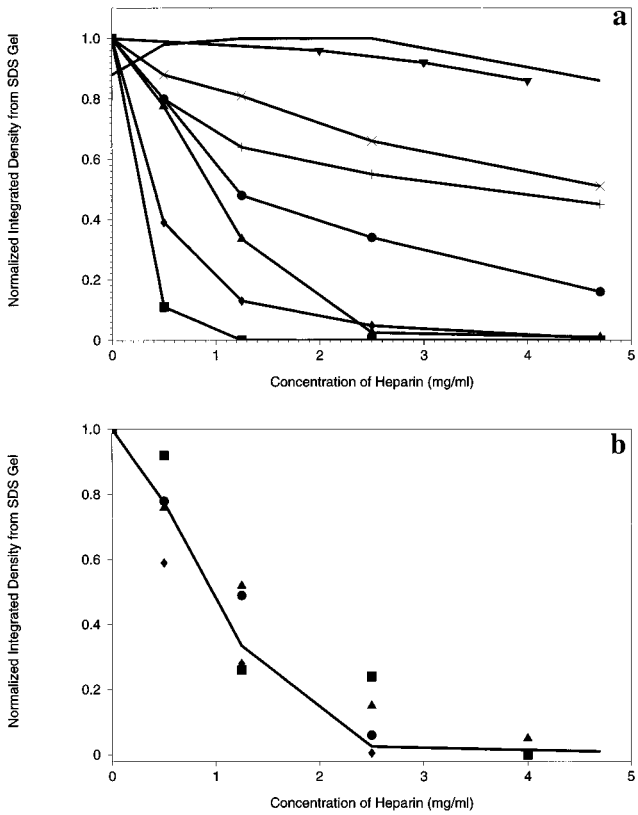

FIGURE 2: (a) Concentration dependence of light chain interaction with heparin. The concentration of intact light chain was 1 mg/mL and that of recombinant light chain V domain was 0.5 mg/mL. The light chain concentration in the supernatant of a mixture of Gri and heparin 16 000 decreased with increasing heparin 16 000 concentration. Intact light chains were generally more sensitive to GAGs than recombinant V domains. Amyloid-forming proteins tended to show less recovery than non-amyloid-forming proteins. There was no decrease in the supernatant of a mixture of albumin and heparin 16 000 in the concentration range tested. (—) albumin; (●) Gri + heparin 6000; (×) r_Len + heparin 16 000; +, r_Rei + heparin 16 000; (◆) Len + heparin 16 000; (■) Gri + heparin 16 000; (▼) Mev + heparin 16 000; (▲) Pfu + heparin 16 000. (b) Concentration dependence of light chain interaction with heparin under both varying salt and salt concentration. Concentrations of protein and heparin were as in panel a. Results showed that Gri recovery is relatively insensitive to salt concentration and is not sensitive to the substitution of the sulfate ion for chloride. (—) Gri + heparin 16 000 (100 mM NaCl); (▲) Gri + heparin 16 000 (50mM NaCl); (●) Gri + heparin 16 000 (25 mM NaCl); (■) Gri + heparin 16 000 (0 mM salt); (♦) Gri + heparin 16 000 (300 mM Na_2SO_3).

because similar recovery was observed when reduced Gri was treated by heparin 16 000 (data not shown).

In order to test the possibility that proteinase contamination of the GAG preparations was responsible for loss of protein, three control proteins (albumin, chymotrypsinogen A, and ribonuclease A) were mixed with different concentrations of heparin 16 000 and incubated for 2 h at 37 °C. After centrifugation, the supernatants were tested as above. Chymotrypsinogen A and ribonuclease A showed decreased protein recovery with increasing heparin 16 000 concentration, similar to the reaction pattern of human intact light chains. In contrast, heparin 16 000 did not show any effect on albumin; i.e., the albumin recoveries were independent of the concentration of heparin 16 000 (Figure 2a). In addition, no low-molecular-weight fragments were observed in the gels. This result suggested that there was no proteinase in heparin 16 000 and that the observed loss of light chains

Table 2: Depletion of Heparin

	protein concentration					
	0.25 mg/mL		0.50 mg/mL		0.25 mg/mL	
inc[a]	I.D.[b]	% R[c]	I.D.	% R	I.D.	% R
first	1.8	3	17.9	17		
second	11.1	18	47.2	47	0.4	1

[a] Incubation series. [b] Integrated density of protein band in gel. [c] Percent recovery.

was from the formation of insoluble complexes with heparin 16 000.

Gri interaction with heparin 16 000 under NaCl concentrations of 20−700 mM was tested. The interactions did not depend on salt concentration under these experimental conditions. The results may reflect the dominance of the high local concentration of negative charges on the surface of heparin and the cooperativity of multiple salt bridges between heparin and the light chain. Gri interaction with heparin 16 000 in the presence of 300 mM Na_2SO_3 was tested (Figure 2b) and was similar to that observed in 150 mM NaCl, indicating that the decrease in light chain solubility in the presence of heparin was not due to a simple ionic effect of SO_3^- groups.

The human proteins used in this study were intact proteins that included both constant and variable domains. The human light chain samples also contained varying ratios of monomeric light chain and dimers covalently linked by a disulfide bond between cysteine residues located near the C-terminus of the constant domain. The precipitation of light chain proteins by GAGs might involve either V or C domain interaction or both. Our recombinant light chain proteins (Stevens, P. W., et al., 1995) are V-domains and there exists no covalently linked dimer. rLen, rRei, and rSma proteins, at the same molar concentrations and experimental conditions as intact human light chains, were mixed with different GAGs. Heparin 16 000, and chondroitin sulfates B and C showed interactions with the light chain V-domain. rRei with concentrations of heparin 16 000 from 0.5 to 4.2 mg/mL showed that solubility was heparin concentration-dependent. Although similar reaction patterns were found for recombinant proteins as for intact human light chains, recombinant V domain light chains were not as effectively precipitated as the intact human proteins (Figure 2a). Further studies will be required to identify the relative contributions of the V and C domains to the interactions with GAGs.

The inference that the loss of light chains from solution containing GAGs results from formation of insoluble complexes containing both molecules requires evidence that GAG is also removed from solution. This evidence was obtained by demonstrating a reduction in the ability of the heparin solution to precipitate light chain. A solution of 2.5 mg/mL heparin, although in molar excess, was insufficient to completely precipitate light chains of concentrations of 0.25 mg/mL and above. Thus, decreases in heparin concentration below 2.5 mg/mL result in increased light chain recovery.

After 2 h of incubation with heparin (2.5 mg/mL), samples of GRI at initial concentrations of 0.25 and 0.50 mg/mL were found by gel electrophoresis to have recoveries of 3% and 17%, respectively (Table 2). Small volumes of concentrated stock solution were added to the resulting supernatant solutions to restore the initial protein concentrations. After a second 2 h incubation, light chain recoveries were found to be 18% and 47% respectively. Increased recovery was not the result of an "inactivation" of heparin during the prior incubation at 37 °C. A control sample in which a protein-free heparin solution was incubated at 37 °C for 2 h was found to be fully active when treated with a 0.25 mg/mL sample of protein GRI. Although the apparent recovery of the 0.50 mg/mL sample following the second incubation is slightly inflated due to carryover of a small amount of protein from the first incubation, it is clear that the ability of the heparin solution to decrease the solubility of the light chain was diminished by pretreatment with protein, suggesting removal of heparin from solution.

Table 3: Intermolecular Energy of the Three Initial Complexes

	intermolecular energy (kcal/mol)		
initial complex	total	electrostatic	van der Waals (vdW)
I	−83	−63	−20
II	−73	−56	−17
III	−67	−53	−14

In modeling of three potential modes of docking, the intermolecular energy calculation (Table 3) showed that initial complex I was most energetically favored in agreement with our prediction. However, when the energies of the various biomolecular complexes are compared to the sum of the energies of two molecules minimized independently, it appears that all complexes are favorable with ΔE values that range from −133 to −158 kcal/mol (Table 4), but that no complex configuration is obviously dominant. This observation may be consistent with an interaction that is rather nonspecific and electrostatically driven. Note that the quantitative energies calculated here are not intended to represent more than an indication of favorable interaction. An attempt to rigorously calculate energetics of interaction would require knowledge of the structure of a light chain−heparin complex, as well as inclusion of a larger heparin fragment, water, and ions in the model. A more detailed representation of the ionic groups would also be important.

In our docking stability study, RMS deviation between Rei dimers from three replicate complexes after minimization reached relatively small values (0.07−0.13) compared to RMS values obtained by superimposing the Rei dimer from complexes on the control (0.34−0.39). Conformational changes in the heparin fragment were reflected by an average RMS deviation of 0.49 by comparing the three conformations of energy-minimized heparin in the complex to that minimized independently.

For the dynamics simulation, the intermolecular energy reached as low as −168 kcal/mol and appeared to stabilize at about −150 kcal/mol, suggesting the possibility that the hexasaccharide might bind the light chain dimer with significant affinity. During molecular dynamics, the initial extended heparin structure underwent substantial rearrangement. The resulting curved heparin conformation appears aligned to interact well with the A103, B103, and A42 lysines, leaving B42 lysine without a close contact. The distances of the A103 side-chain NZ atom to the S atoms of two SO_3^- groups from sugar 7 and one SO_3^- group from sugar 5 are all approximately 7 Å. The distances of the A42 NZ atom to the S atoms from the two SO_3^- groups of sugar 5 are ∼6 Å. The distance of B103 NZ from the S atoms of SO_3^- of sugar four is ∼7 Å. Seven SO_3^- groups out of nine are in good contact with lysines. Only sugar 2 (the first

Table 4: Energy Comparison of Initial Complex vs Controls[a]

compound	total[b]	total[c]	electrostatic	vdW	bond	θ	ϕ	out of plane
Rei[d]	1612	1226	−901	446	609	801	260	10
heparin[e]	176	156	38	32	18	43	26	0
Rei + heparin[f]	1788	1382	−863	478	627	844	286	10
Rei + heparin I[g]	1691	1240	−1023	460	630	854	309	11
Rei + heparin II[h]	1737	1224	−1030	451	631	853	308	11
Rei + heparin III[i]	1687	1249	−996	451	629	853	300	11

[a] All energies are given in kilocalories. [b] Minimized energies with 5 Å water shell. [c] Minimized energies using distance -dependent dielectric. [d] Minimized energies of Rei itself. [e] Minimized energies of heparin itself. [f] Sum of *d* and *e*. [g] Minimized energies of complex I. [h] Minimized energies of complex II. [i] Minimized energies of complex III.

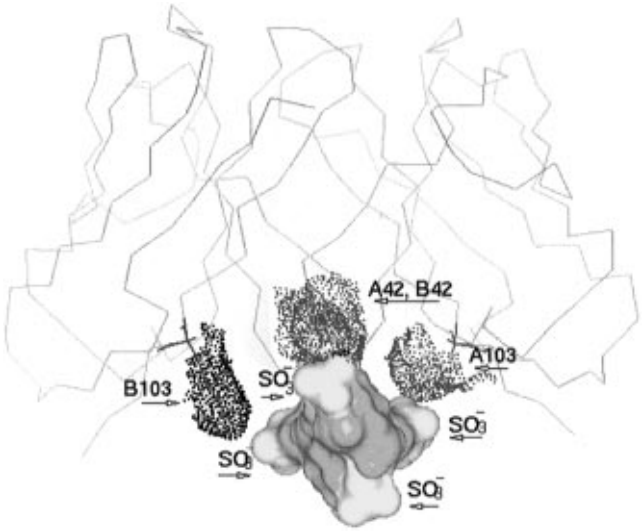

FIGURE 3: Representation of a V_L dimer (based on protein Rei, pdb1rei) and an axial view of a hexasaccharide (heparin, pbd1hpn). The concave lower surface of the V_L is referred to as the "saddle" in the text; Lys 103 residues are observed extending from the sides toward the hexasaccharide. The SO_3^- groups of heparin are arranged periodically in an orthogonal cross through the center.

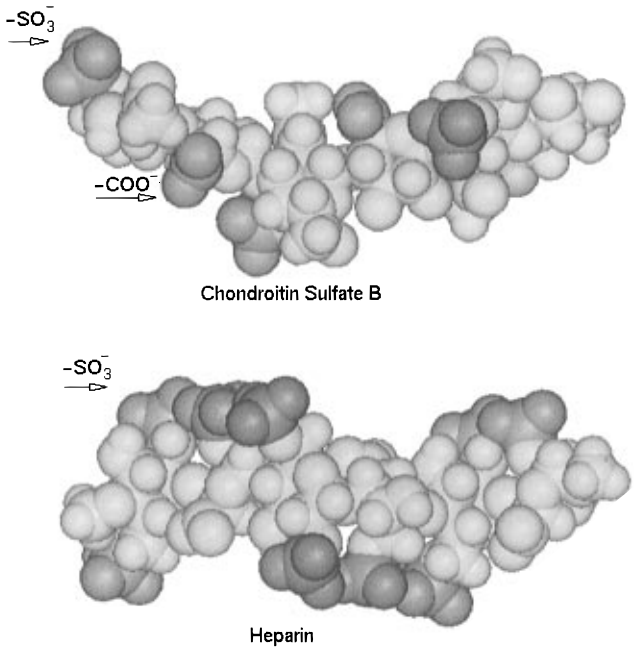

FIGURE 4: Structural comparison of heparin and chondroitin sulfate B. Heparin has a higher local negative charge concentration.

sugar fragment) is unaligned, with its SO_3^- distant from all lysines (the closest is 9 Å) (Figure 3).

DISCUSSION

This study of the interaction between light chains and GAGs showed that heparin with molecular weight ~16 000 at 2.5 mg/mL decreased the solubility of both intact light chains (C + V domains) and V domain fragments. The amyloid-forming light chains and cast-forming light chains appeared to interact more strongly with heparin 16 000 than non-amyloid-forming light chains, although the data are too limited to draw general conclusions. For the same proteins, intact light chain interacted more strongly with heparin 16 000 than did the light chain V domain alone. Heparin 6000 at 2.5 mg/mL had a smaller effect on the solubility of both complete light chains and the recombinant V domains. Thus, the interaction of light chain with heparin was dependent upon both concentration and molecular weight. Chondroitin sulfates B and C at 2.5 mg/mL showed a reaction pattern similar to that of heparin 16 000 but appeared to be less potent. Heparan sulfate at the same concentration showed much smaller effects than other GAGs. Available structural data for GAGs indicates that the negatively charged surface concentration varies, with heparin having the higher charge density and with chondroitin sulfate B lower (Figure 4). This trend is consistent with our experimental results. This apparent agreement suggests the possibility that electrostatic forces are important in the interpretation of light chain−GAG interactions. Reduced Gri reacted similarly to nonreduced Gri, which indicated that covalent linkage between domains was not required for the precipitation of protein by GAG interaction.

Heparin was reported to bind to antithrombin III through five sugar units (Petitou et al., 1988). Consensus sequences for GAG recognition from 12 known heparin-binding sites were determined as [-X-B-B-X-B-X-] and [X-B-B-B-X-X-B-X-], where B is a basic residue and X is a hydrophobic residue (Cardin & Weintraub, 1989). A common feature of known heparin-binding sequences is clusters of basic residues (Jackson et al., 1991). These data indicate that the major binding force between heparin and light chains is most likely electrostatic.

The crystal structures of chymotrypsinogen A and ribonuclease A from the Brookhaven Protein Data Bank (pdb1chg.ent and pdb3rn3.ent, respectively) were examined. There was an extended positively charged cluster on the surface of chymotrypsinogen A and a positively charged site similar to the Rei structure on ribonuclease A. Overall, both chymotrypsinogen A and ribonuclease A have more positively charged residues on their surface than does Rei. The structure of albumin is not known and, therefore, albumin could not be examined structurally. The p*I*s of chymotrypsinogen A and ribonuclease A are 9.1 and 9.2, respectively, while that of bovine serum albumin is 4.7. Thus, the

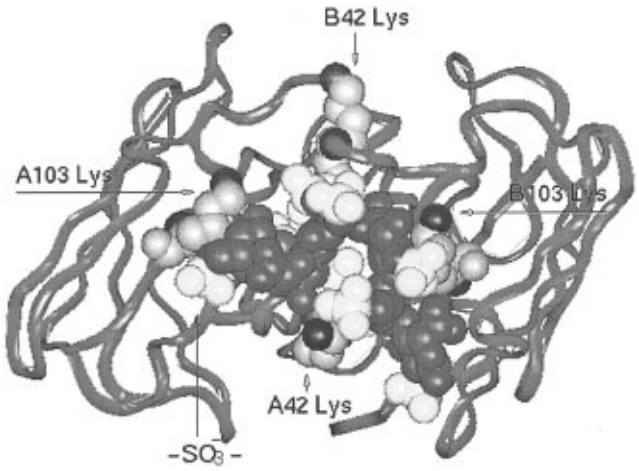

FIGURE 5: Hexasaccharide docked into light chain dimer after 40 ps of molecular dynamics simulation.

observed interactions of heparin with chymotrypsinogen A and ribonuclease A, and the lack of interaction with albumin, are consistent with their electrostatic features.

During the docking of protein and GAG, electrostatic forces might first bring the positively charged end of light chain dimers to "sit on" the horseback of the negatively charged heparin. Since heparin in solution has an extended helical conformation, multiple light chain dimers might be arranged regularly on heparin and form a oligomolecular complex. Accumulation of a sufficient mass of dimers or light chains could lead to precipitation. There might be more than one pattern in which multiple dimers can be arranged on heparin, ranging from adjacent dimers on alternate surfaces to having all the dimers on one side. The fact that the interaction with chondroitin sulfate was weaker than that with heparin supported the premise that electrostatic forces play an important role during the interaction, since chondroitin sulfate disaccharide has fewer negatively charged groups than heparin disaccharide.

Gri recovery was similar in solutions of both low and high salt concentration. NaCl concentration as high as 700 mM did not interrupt the interaction between intact light chain and heparin 16 000. A calculation considering heparin as a cylinder, with the distance between two helical repeats as the length of the cylinder, showed that the local concentration of SO_3^- is about 8.4 M, which is more than 10 times higher than the salt concentration we used. Lack of inhibition by salt indicates that the interaction between the light chain and the GAG is strong.

Modeling was based on the Rei light chain V domain structure (Epp et al., 1974). A sequence comparison of Rei with other protein V domains showed that, typically, other light chain V domains are even more positively charged than Rei in the proposed binding area. Therefore, modeling with Rei was a conservative representation of the interaction between a light chain and GAGs.

In the docked model for interaction between heparin and light chain dimers, the positively charged bottom of the dimer was the site of interaction with heparin and was the same site for interaction with another dimer to form an amyloid fibril proposed by the model of Stevens et al. (1995). Further, molecular modeling calculations indicated that a hexasaccharide might be a functional fragment for binding a dimer (Figure 5). Thus, when excess hexasaccharide fragments or analoguess are combined with light chains, these ligands could occupy fibril formation sites and interfere with amyloid fibril formation. Since hexasaccharides can be synthetically modified, GAGs might be useful as leads for further study to identify properties of related molecules that may be suitable for inhibition of fibril development.

The molar ratio of GAG to light chain required to provide a loss of protein solubility is substantially higher than that implied by the GAG's 1−2% contribution to the mass of physiologically derived amyloid fibrils (Nelson et al., 1991). The approximate stoichiometric ratio required for the *in vitro* decrease in solubility of free light chains may reflect undetermined aspects of fibril−fibril interaction. In vivo, low stoichiometries of GAGs may be involved in initiating or capping fibrils, presuming that the end points of fibrils represent sites of unique structure to which GAGs may be able to establish multiple noncovalent interactions, resulting in a high effective affinity of the GAG for the fibril but low total mass incorporation.

In summary, this paper reports on interaction between amyloid-related light chain protein and GAGs, particularly heparin. A model based on electrostatic forces is proposed to rationalize the interaction, and hexasaccharide or mimetics are suggested as possible antagonists. Although this model represents a feasible mode of interaction, it should be noted that independent experimental data in support of the model or the hypothesis of a direct interaction is, to date, not available. Studies into the detailed mechanisms of light chain−GAG interactions might offer new paths for research to block the aggregation of amyloid-associated proteins.

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