Interaction of soluble and surface-bound heparin binding growth-associated molecule with heparin

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Abstract The interaction of heparin with heparin binding growth-associated molecule (HB-GAM) was studied using isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). ITC studies showed that, in solution, heparin bound HB-GAM with a ΔH of −30 kcal/mole corresponding to a dissociation constant (K_d) of 460 nM. The stoichiometry of interaction was 3 moles of HB-GAM per mole of heparin, corresponding to a minimum heparin binding site for HB-GAM of 12–16 saccharide residues. Kinetic measurements of heparin interaction with HB-GAM made by SPR afforded a K_d of 4 nM, suggesting considerably tighter binding when HB-GAM was immobilized on a surface. Affinity chromatography of a sized mixture of heparin oligosaccharides, having a degree of polymerization (dp) of >14 saccharide units, on HB-GAM-Sepharose demonstrated that oligosaccharides having more than 18 saccharide residues showed the tightest interaction.

Key words: Heparin; Heparin binding growth-associated molecule; HB-GAM; Pleiotrophin; Interaction; Isothermal titration calorimetry; Surface plasmon resonance

1. Introduction

Heparin binding growth-associated molecule (HB-GAM), also known as pleiotrophin, is a secretory extracellular matrix-associated protein. It is an 18 kDa protein, rich in basic amino acids, and of its 136 residues, 20.6% are lysine and 3.7% are arginine. These basic residues are clustered in two discrete domains at the protein's N-terminus and C-terminus [1]. HB-GAM is found at high level in developing rat brains [1] and at developing neuromuscular junctions [2]. The HB-GAM sequence is highly conserved across species [3] and shows about 50% homology to midkine [4].

HB-GAM enhances neurite outgrowth [5–10]. While HB-GAM appears to have no mitogenic activity [10], it is involved in providing extracellular tracts to guide neurites in the developing brain [11,12]. HB-GAM has also been shown to induce proliferation arrest in limb development and is also involved in muscle development [13].

N-syndecan (Syndecan-3) has been suggested to be the HB-GAM receptor. This heparan sulfate proteoglycan binds HB-GAM with high affinity (K_d of 0.6 nM) and mediates neurite outgrowth-promoting signal from growing neurites [11]. HB-GAM and N-syndecan are also co-expressed in developing rat brain [4]. The specificity of glycosaminoglycans on this interaction, investigated using a neurite outgrowth inhibition cell assay system [14], demonstrated that heparin and heparan sulfate showed activity while chondroitin sulfate and dermatan sulfate were inactive. Furthermore, the inhibitory effects of heparin were lost on 2-*O*-desulfation. Heparin oligosaccharides having a degree of polymerization (dp) of 10 were required for inhibition of HB-GAM induced neurite outgrowth with an optimal activity observed at dp 18. This activity also correlated with the binding activity of these oligosaccharide fractions with HB-GAM. Recently, chondroitin sulfate brain proteoglycans, phosphacan [15,16] and neurocan [16], have also been shown to bind HB-GAM with high affinity.

The current study was undertaken to examine the thermodynamics and kinetics of heparin binding to HB-GAM and to further establish both the minimum and optimal size of the binding domain in heparin for HB-GAM.

2. Materials and methods

Porcine intestinal mucosal heparin (M_r = 14 000) was obtained from Celsus Laboratories (Cincinnati, OH, USA). Recombinant HB-GAM was prepared using a baculovirus expression system and purified to apparent homogeneity [11]. Heparin lyase I (heparinase I, EC 4.2.2.7) and *N*-hydroxysuccinimidyl (NHS)-activated HiTrap column were from Sigma Chemical (St. Louis, MO, USA). *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(dimethyaminopropyl) carbodiimide were from Pharmacia (Uppsala, Sweden). Ethanolamine was from Fisher Chemical (Fair Lawn, NJ, USA). ^2H_2O (99.96 atom %) was from Isotec Inc. (Miamisburg, OH, USA). Dialysis tubing was purchased from Spectrum Laboratory Products (Houston, TX, USA). All spectrophotometric measurements were carried out on a Shimadzu model UV-21001 PC UV-vis spectrophotometer. Conductivity was determined using a CDM 230 conductivity meter from radiometer (Copenhagen, Denmark). Isothermal titration calorimetry was performed on a Calorimetry Sciences Corporation Model 4200 (Provo, UT, USA). Surface plasmon resonance used a Biacore 2000 instrument at the Biopolymer Facility of Ohio State University (Columbus, OH, USA).

2.1. Isothermal titration calorimetry (ITC)

Titrations HB-GAM (1 ml at 30.7 μM) with 20 5 μl injections of heparin (400 μM) were performed in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl. Experimental details and data processing relied on previously described methods [17].

2.2. Immobilization of HB-GAM on biocore sensor chip

HB-GAM was covalently bound to the sensor surface via primary amino groups using the manufacturer's protocol. Briefly, the carboxymethylated dextran matrix (CM 5% sensor chip) was first activated using a 7 min (35 μl) injection pulse of an equimolar mix of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(dimethyaminopropyl) car-

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bodiimide (final concentration is 0.05 M, mixed immediately prior to injection). A 35 μl volume of HB-GAM (typically 5 μg/ml in 5 mM sodium phosphate buffer, pH 7.4) was then flowed over the activated surface. Excess unreacted sites on the sensor surface were blocked with a 35 μl injection of 1 M ethanolamine. 1500 RU of HB-GAM were immobilized on the sensor chip.

2.3. Kinetic measurements of HB-GAM heparin interactions via surface plasmon resonance

A 15 μl injection of heparin, in the concentration range of 100–1000 nM in 50 nM sodium phosphate, pH 7.4, was made at a flow rate of 5 μl/min. At the end of the sample plug, the same buffer was flowed over the sensor surface to facilitate dissociation. After a suitable dissociation time, the sensor surface was regenerated for the next sample using a 10 μl pulse of 2 M NaCl. The response was monitored as a function of time (sensorgram) at 25°C. Kinetic parameters were evaluated using the BIA Evaluation software (Pharmacia Biosensor) according to manufacturer's methods by first evaluating the dissociation rate constant k_d using $dR/dt = -k_d R$ where R represents the sensor response. The association rate constant was determined from the slope of a plot of the observed association rate constant k_s as a function of ligand concentration, according to $k_s = k_d + k_a C$ where k_a is the association rate constant and C represents the ligand concentration. The dissociation constant, K_d, was obtained from the ratio k_d/k_a.

2.4. Preparation of HB-GAM-Sepharose

HB-GAM (1 mg) was dissolved in the coupling buffer (100 mM sodium bicarbonate containing 500 mM NaCl, pH 8.4) and injected onto a 1 ml NHS-activated HiTrap column that had been washed with cold 1 mM HCl. The column was sealed and allowed to stand at 4°C overnight. Deactivation of any excess active groups, which had not coupled to the ligand, was performed by repeated washings with 3 M ethanolamine, pH 8.6 and 100 mM acetic acid buffer containing 1 M NaCl buffer at pH 4.5.

2.5. Preparation of oligosaccharides

Heparin (10 g) was digested to 30% reaction completion using heparin lyase I as previously reported [18] and fractionated by Sephadex G50 gel exclusion chromatography into sized oligosaccharide mixtures. A mixture of tetradecasaccharides and higher oligosaccharides, prepared by repeated gel permeation chromatography [18] and characterized by gradient polyacrylamide gel electrophoresis (PAGE) [19] was used for affinity chromatography. A single tetradecasaccharide was purified from the mixture using strong anion exchange high performance liquid chromatography and its purity was assessed as >90% by gradient PAGE [19] and capillary electrophoresis (CE) [18]. One-dimensional (1D) ^{1}H-NMR and 2D COSY NMR were used to examine the structure of this tetradecasaccharide [18].

2.6. HB-GAM-Sepharose affinity chromatography of a sized heparin oligosaccharide mixture

Excess oligosaccharide mixture (500 μg) was loaded onto the HB-GAM-Sepharose column, equilibrated with 200 mM NaCl in 5 mM sodium phosphate at pH 7.4 buffer. The column was washed with 10 ml of the same buffer and eluted with 30 ml gradient of 0.2 to 2 M NaCl in sodium phosphate buffer, pH 7.4. The eluent was monitored at 232 nm and salt concentrations were determined by measuring the conductivity. Fractions corresponding to the interacting peak were collected and exhaustively dialyzed (1000 molecular weight cut-off membranes) for 4 days against 4×20 l distilled water and lyophilized.

2.7. Polyacrylamide gel electrophoresis

Fractions from the affinity chromatography experiments were analyzed by gradient PAGE. Samples were dissolved in 10–15 μl of distilled water and 15 μl of a 50% (w/v) sucrose solution and subjected to electrophoresis on a 20 cm long linear gradient (12 to 22% total acrylamide) gel for 5 h at 400 V or through a 32 cm long gradient gel for 18 h at 400 V. The gels were stained with alcian blue (0.5% in 2% acetic acid) for 30 min, destained by washing with distilled water for 24 h, and silver stained.

3. Results

Isothermal titration calorimetry was used to characterize the HB-GAM heparin interaction (Fig. 1). This technique affords ΔH, K_d and n (the number of ligand interactions per mole of macromolecule). The fitted heats of interaction for titrations of heparin into HB-GAM yielded a K_d of 460 nM, ΔH of −30 kcal/mole and an n of 3 moles of HB-GAM per mole of heparin at 25°C in 50 mM sodium phosphate, 100 mM NaCl, pH 7.4. This n corresponds to a heparin binding site for HB-GAM of 12 to 16 saccharide residues.

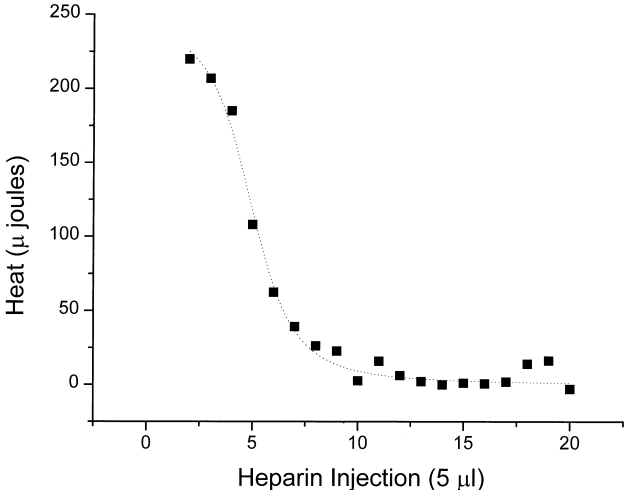

Fig. 1. Binding isotherm of the interaction between HB-GAM and heparin. HB-GAM (in the cell) was titrated with heparin (in the syringe). The heat released afforded peaks that were integrated and the total heat per injection (peak area) are plotted as a function of injection number.

Negatively charged carboxymethylated dextran was selected as the support on the sensor surface of the plasmon resonance chip for immobilization of HB-GAM to prevent a favorable contribution from the surface on the heparin HB-GAM interaction being determined. A control experiment demonstrated no interaction took place between the carboxymethylated dextran coated sensor surface and HB-GAM (not shown). When HB-GAM was immobilized to the carboxymethylated dextran on the sensor chip, and heparin flowed over this surface, a strong interaction was observed (Fig. 2). A k_a (on-rate) of 6.6×10^5 ($\pm 8\times10^4$) M^{-1} s^{-1} and a k_d (off-rate) of 2.4×10^{-3} ($\pm 1\times10^{-3}$) s^{-1} was observed. The dissociation rate constant, obtained from multiple traces, was combined with the slope of the linear portion of the observed association rate constant as a function of ligand concentration (inset) to obtain the dissociation constant (K_d) of 4($\pm$2) nM. Despite the negatively charged underlying surface coating of carboxymethylated dextran, SPR gave a 100-fold stronger binding affinity than that measured in solution using ITC.

ITC showed that the minimum binding site in heparin contained 12 to 16 saccharide residues, consistent with the report that oligosaccharides of 14 saccharide residues gave inhibition of HB-GAM [14]. A heparin tetradecasaccharide contained in an interacting mixture was selected and purified to homogeneity by gel permeation chromatography and strong anionic exchange high pressure liquid chromatography [18]. PAGE and CE analysis demonstrated this tetradecasaccharide showed it to be >90% pure. 1D ^{1}H-NMR and 2D COSY NMR were used to identify it as a tetradecasaccharide containing an internal antithrombin III binding domain, similar

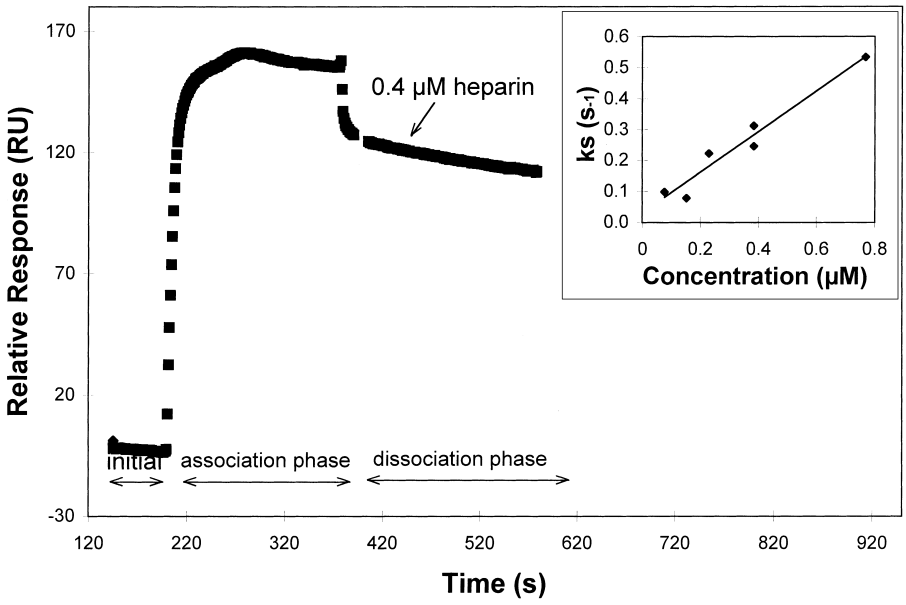

Fig. 2. SPR sensorgram of HB-GAM heparin interaction. HB-GAM was immobilized onto a sensor chip and different concentrations of heparin were flowed over the chip (association phase) followed by buffer (dissociation phase). The association phase and the dissociation phase were used to calculate the K_d. The inset shows the observed association rate as a function of ligand concentration used to calculate the K_d.

to the structure of an analogous decasaccharide of defined structure previously established by our laboratory [19]. Affinity chromatography of this tetradecasaccharide demonstrated that it bound to a HB-GAM-Sepharose column and eluted at 1 M NaCl (not shown), confirming that a tetradecasaccharide was sufficiently large to bind tightly to HB-GAM. Next, the optimum heparin oligosaccharide size for HB-GAM binding was examined using a mixture of large oligosaccharides (degree of polymerization (dp) >14). The column was loaded with excess oligosaccharide mixture (550 μg) and eluted with a salt gradient. Multiple peaks were observed in the run-through but a single peak was observed eluting at 1.2 M NaCl (Fig. 3). This peak, containing high affinity oligosaccharides, was dialyzed and freeze-dried. Analysis by gradient PAGE showed that this peak contained only very large oligosaccharides (dp >18) having the highest affinity for HB-GAM.

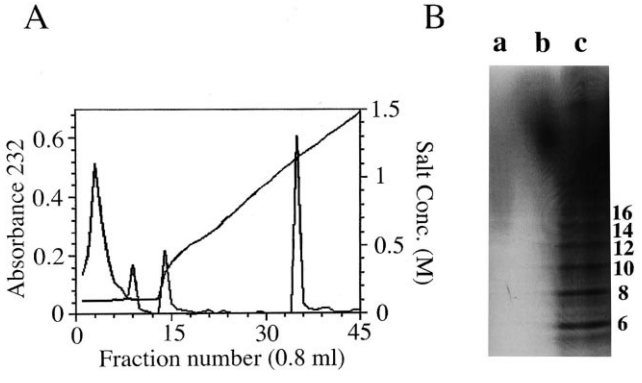

Fig. 3. Affinity chromatography of heparin oligosaccharides on HB-GAM-Sepharose. Panel A: Absorbance (232 nm) (left axis) plotted as a function of fraction number (0.8 ml/fraction) shows the elution of oligosaccharides. The salt concentration (right axis) was measured by conductivity. Panel B: Gradient PAGE analysis of the oligosaccharide sample loaded onto the column (lane a) was identical to that eluting in fraction 5 (not shown). The high affinity oligosaccharides eluting in fraction 35 (lane b) had an average dp of >18 as shown by the oligosaccharide markers (lane c, dp labeled on right).

4. Discussion

Previous studies have demonstrated that heparin had high affinity for HB-GAM and suggested that the heparan sulfate proteoglycan N-syndecan might be an endogenous ligand [11]. Studies on mixture of heparin derived oligosaccharides showed that a minimum heparin binding site having 10 saccharide residues with optimum binding occurred between 14 and 18 saccharide residues [14]. In the current study, two different methods were used to examine heparin interaction with HB-GAM. ITC is a thermodynamic, solution phase measurement of the heat of interaction and SPR is a two-phase kinetic measurement of interaction. Heparin showed a strong binding affinity to HB-GAM in solution as determined by ITC but an even stronger binding affinity to HB-GAM immobilized on a surface as determined by SPR. The difference in the binding constant obtained using these two methods might be due to the multivalency of HB-GAM and the cooperative nature of this interaction when measured on a surface. The size of the minimum binding site within heparin for HB-GAM was determined to be 14 saccharide units by ITC. The interaction of a pure tetradecasaccharide with HB-GAM immobilized on Sepharose confirmed the size of the minimum HB-GAM binding site. Finally, when a mixture of oligosaccharides of dp >14 were added in excess to this HB-GAM column only very large oligosaccharides of dp >18 bound, suggesting that these extended sequences represented an optimal binding domain within heparin. The large size of this binding domain and the high affinity of this interaction suggest that both the N-terminal and C-terminal clusters of basic amino acid residues are involved in this interaction, thus requiring a long heparin sequence to span these sites.

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