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Heparin Binding and Augmentation of C1 Inhibitor Activity

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Heparin and other glycosaminoglycans have profound activity *in vitro* on the regulation of complement activity. The studies reported here examined the mechanism whereby heparin enhances C1 esterase inhibitor (C1INH) activity on C1 esterase (C1). The interaction of heparin and heparan sulfate with C1INH was first examined using surface plasmon resonance. Heparin was immobilized on a biosensor chip in two orientations, at its reducing end and in midchain, and heparan sulfate was immobilized at its reducing end. Heparin immobilized at its reducing end interacted with C1INH, giving an association constant (K_a) value of $1.43 \times 10^7\ M^{-1}$, whereas heparin immobilized in midchain afforded a K_a value of $7 \times 10^6\ M^{-1}$. No interaction between C1INH and heparan sulfate could be observed. Next, the augmentation of C1INH by heparin (M_r (av) 13,000), low-molecular-weight (LMW) heparin (M_r (av) 5000), and heparan sulfate (M_r (av) 11,000) was determined. C1INH alone was at least 10,000 times more active in inhibiting fluid phase C1 compared with erythrocyte-bound C1 (EAC1). When C1 was in the fluid phase, both heparin and LMW heparin were relatively ineffective at augmenting C1INH activity on C1. In contrast, when C1 was present as EAC1, heparin augmented C1INH activity at all C1INH concentrations examined and LMW heparin was up to 1.3 times more effective than heparin. This augmentation only occurred when both C1INH and heparin were present together with the EAC1. Hence, although surface plasmon resonance shows that heparin binds to C1INH, heparin augmentation of C1INH activity appears to require a terniary complex in which cell bound C1 interacts with both heparin and C1INH. This is the first report of LMW heparin augmenting C1INH activity. Heparan sulfate neither interacted with C1INH nor did it augment C1INH activity. © 1999 Academic Press

Key Words: **C1 inhibitor; heparin; surface plasmon resonance.**

C1 inhibitor (C1INH)[2] is a 105,000-D protein that is the only serine protease inhibitor known to regulate activity of the classical complement pathway components C1r̄ and C1s̄ (1). Genetic deficiency of C1INH results in hereditary angioedema, in which patients suffer from recurrent attacks of angioedema, severe abdominal pain, and laryngeal edema (2, 3). Individuals with hereditary angioedema express half-normal levels of functional C1INH, which is insufficient to maintain regulation of C1 esterase production. Historically, as many as 30% of patients with hereditary angioedema may die from laryngeal edema (3). Although hereditary angioedema may, theoretically, be "controlled" by infusion of C1INH concentrate, this material remains unlicensed in the United States. Even if C1INH becomes available, it may be antigenic and its cost may preclude long-term use in patients.

Reports in 1976 first suggested that heparin, a natural product derived from mast cells and basophils, as well as other acidic glycosaminoglycans may augment activity of C1INH on C1r̄ and C1s̄ *in vitro* (4, 5). Rent *et al.* (4) suggested that heparin, at concentrations much lower than those required to inhibit C1, potentiates C1INH activity in plasma as well as in a system using purified proteins. In addition, the report by Na-

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[2] Abbreviations used: C1INH, C1 inhibitor; SPR, surface plasmon resonance; LMW, low molecular weight; EA, antibody-labeled erythrocytes; EAC1, EA containing C1 cellular intermediate; GVB^{--}, half-isotonic veronal-buffered saline, pH 7.5; $DGVB^{++}$, GVB^{--} with dextrose, magnesium, and calcium; C-EDTA, guinea pig serum diluted 1:12.5 in GVB^{--} containing EDTA; MWCO, molecular weight cutoff.

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gaki *et al.* (5) demonstrated that heparin augments C1INH activity on $C1\bar{s}$, but not on plasmin. These early studies showed that other glycosaminoglycans also have capacity to augment activity of C1INH but at higher concentrations than are required for heparin. In the 1980s, Caughman *et al.* (6) observed that heparin inhibits heat-aggregated IgG activation of complement in normal, but not in C1INH-depleted human serum, and suggested that heparin's predominant activity on the classical pathway is through augmentation of C1INH. Kinetic studies revealed that heparin augments C1INH activity by 15- to 30-fold (7, 8). Based upon these *in vitro* observations, Weiler *et al.* (9) and Levine and Stechschulte (10) reported the potential use of inhaled heparin for patients with hereditary angioedema. Patients had a history of typical attacks of hereditary angioedema and either became asymptomatic or experienced a significant decrease in hereditary angioedema symptoms while receiving heparin.

The present studies were designed to examine the ability of heparin and the structurally related glycosaminoglycan, heparan sulfate, to bind to C1INH and to augment its activity. In addition, we examined the mechanism by which heparin augments C1INH activity by comparing the interaction of heparin with C1INH in the presence of fluid-phase C1 and cell-bound C1 (EAC1). Finally, we studied whether heparin and C1INH needed to be present simultaneously or sequentially for heparin to augment C1INH activity.

MATERIALS AND METHODS

Materials. Half-isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin (GVB^{--}) (11), and GVB^{--} containing 2.5% dextrose, 0.5 mM magnesium, and 0.15 mM calcium ($DGVB^{++}$) were used as buffers in the hemolytic assays. The C1INH used in these studies was purified to chemical homogeneity from human plasma (12). The C1INH showed a single band on visualization of silver nitrate-stained gels that had been subjected to SDS–PAGE; concentration was determined by use of Coomassie Plus Protein Assay Reagent from Pierce (Rockford, IL). Guinea pig C1 and C2 were isolated as previously described (13); C4 was purchased from Diamedix (Miami, FL). Guinea pig serum, as a source of terminal components for the classical pathway assays, was purchased from Rockland, Inc. (Gilbertville, PA) and diluted 1:12.5 with GVB^{--} containing 40 mM EDTA (C-EDTA). Heparin, semipurified (Lot No. RR-1327, 150 U/mg, M_r (av) 13,000) and purified (Lot No. PH-30896, 177 U/mg, M_r (av) 13,000), and LMW (Lot No. FH 10889, 74 U/mg USP activity, M_r (av) 5000) sodium salts, from porcine intestinal mucusa, were from Celsus Laboratories (Cincinnati, OH). Biotin-X-hydrazide was from Calbiochem (San Diego, CA) and biotin-LC-hydrazide and succinimidyl-6-(biotinamido)hexanoate were from Pierce. Guanidinium chloride, piperazine HCl, *N*-ethylmaleimide, phenylmethanesulphohyl fluoride, trichloroacetic acid, and chondroitinase ABC were obtained from Sigma (St. Louis, MO). Heparin lyases were from Siekagaku America, Inc. (Rockville, MD) and heparin disaccharide standards were from Grampian Enzymes (Aberdeen, Scotland). Ultrapure urea was from Life Technologies (Grand Island, NY). Triton X-100, EDTA, and polyethylene glycol were from Fisher Scientific (Fair Lawn, NJ). Proteinase K was from Boehringer-Mannheim (Indianapolis, IN) DEAE-Sephacel matrix was from Pharmacia Biotech (Uppsala, Sweden). All other reagents were analytical grade. SPR was performed on a BIAcore 2000 biosensor in the Biochemistry Core Facility at the Ohio State University in Columbus, OH.

Purification of heparan sulfate. Peptidoheparan sulfate was prepared from bovine kidney following the procedure for preparing heparan sulfate proteoglycans (14). Briefly, six bovine kidneys were homogenized in 4 M guanidinium chloride containing 2% (v/v) Triton X-100, 50 mM sodium acetate, 0.1 M 6-aminohexanoic acid, 20 mM benzamidine hydrochloride, 10 mM EDTA, 5 mM *N*-ethylmaleimide, and 0.5 mM phenylmethanesulphonyl fluoride, pH 5.0, and filtered through cheese cloth. Protein was removed by precipitation in ice-cold trichloroacetic acid (10% (v/v) final concentration) followed by centrifugation. The supernatant was neutralized with NaOH and concentrated by reverse osmosis (MWCO 1000 or 3500) against polyethylene glycol 8000 overnight. The concentrated extract was dialyzed (MWCO 6000–8000) extensively against 8 M urea solution containing 0.15 M NaCl, 0.5% (v/v) Triton X-100 and 20 mM Tris–HCl, pH 8.0.

The extract was batch adsorbed onto 500-ml DEAE-Sephacel beads equilibrated with 8 M urea containing 0.15 M NaCl, 0.5% (v/v) Triton X-100, and 20 mM Tris–HCl (pH 8.0) and then poured into a column (5 × 19 cm) at room temperature. After washing the column with 2 column volumes of the 8 M urea solution (pH 8.0) and 3 column volumes of 6 M urea containing 0.15 M NaCl, 0.5% (v/v) Triton X-100, 20 mM piperazine–HCl (pH 5.0), the column was eluted using a gradient of 0.15 to 1.0 M NaCl in the 6 M urea solution (pH 5.0). Fractions were monitored for UV absorbance at 280 nm and tested for uronic acid content by carbazole assay (15).

The high-salt eluting (0.8–1.0 M NaCl) fractions were pooled and precipitated with 4 volumes of 1.3% potassium acetate in 95% ethanol. The pellet was redissolved in water to which NaCl was added (16% final concentration). After methanol precipitation (80% (v/v) final concentration) the pellet was washed with ice-cold 80% methanol and air-dried. The pellet was dissolved in 50 mM sodium phosphate buffer (pH 7.6) and digested with chondroitinase ABC (400 mU) in a 37°C water bath for 2.5 h followed by proteinase K (0.43 mg) digestion at 37°C. After 1 h incubation, additional proteinase K was added (0.17 mg) and further incubated for 2 h. The enzyme was deactivated by boiling, and the whole mixture was then applied to macroporous Dowex strong anion-exchanger and eluted with water, 3% NaCl, and 16% NaCl. The peptidoheparan sulfate (M_r (av) 11,000) was recovered from 16% NaCl-eluted fraction by methanol precipitation (as before) and its structure was confirmed using heparin lyases (16, 17).

Reducing-end biotinylation of heparin and heparan sulfate. The *N*-succinimidal ester of biotin was reacted with the free amino groups of peptidoglycan (semi-purified) heparin or peptidoglycan heparan sulfate as follows. Peptidoglycan heparin or peptidoglycan heparan sulfate 10 mg (0.7 μmol) was dissolved in 1 ml of 50 mM sodium bicarbonate (pH 8.3) and mixed with 10 μl of dry *N,N'*-dimethylformamide containing 2 mg succinimidyl-6-(biotinamido)hexanoate (4.3 μmol). The reaction was incubated on ice for 2 h. The reaction mixtures were exhaustively dialyzed using 3500 MWCO membranes against 3 × 4 liters of distilled water over 36 h to remove unreacted biotin. Following dialysis, the biotinylated peptidoglycans were freeze-dried and stored at −20°C.

Midchain biotinylation of heparin. Purified heparin (180 mg) was dissolved in 40 ml of 0.1 M sodium acetate (pH 5.5) containing 3.3 mM sodium periodate. The mixture was allowed to stand at room temperature for 1 h. Sodium sulfite was added and brought to a concentration of 10 mM followed by addition of a sixfold molar excess of biotin-X-hydrazide followed by a second room temperature incubation of 2 h. The mixture was then applied to a macroporous Dowex strong anion-exchanger and eluted with 16% NaCl. The eluent was methanol precipitated (as in end-chain labeling), centrifuged, redissolved in water, and then exhaustively dialyzed, freeze-dried, and stored at −20°C.

Immobilization of glycosaminoglycans. A streptavidin sensor surface (SA sensor chip, Pharmacia AB) was pretreated with three 5-μl injections of 50 mM NaOH in 1 M NaCl, to remove any nonspecifically bound contaminants. A 5-μl injection of reducing-end biotinylated heparin in Hepes running buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, pH 7.4, containing 0.005% (v/v) P-20 surfactant) was made, followed by a 10-μl injection of 2 M NaCl. The other three flow cells of the sensor chip were similarly treated to immobilize nothing (buffer only to serve as a control), reducing-end biotinylated heparan sulfate, or midchain biotinylated heparin. Approximately 100 RU of biotinylated material was immobilized in each of the three glycosaminoglycan containing flow cells.

Kinetic measurement of protein–glycosaminoglycan interactions. Typically, a 20-μl injection of C1INH (in the concentration range 0.1–1 μM in 50 mM sodium phosphate, pH 7.4) into the BIAcore 2000 was made at a flow rate of 5 μl/min. At the end of the sample plug, the same buffer was flowed past the sensor surface to allow dissociation. After a suitable dissociation phase, the sensor surface was regenerated for the next sample using a 10-μl pulse of 2 M NaCl. The same solution was flowed past all four sensor surfaces in sequence and the response was monitored as a function of time (sensorgram) at 25°C. The control cell was used to subtract the contribution of nonspecific interaction with the dextran matrix itself. Kinetic analyses of the association and dissociation phases permitted accurate (±1 s) alignment of the control cell with the experimental cell trace as well as removal of the spikes (observed at the change of buffer) without compromising the data. Kinetic parameters were evaluated using the BIA Evaluation software according to the 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 of K_d/K_a.

Complement assays. Antibody labeled erythrocytes (EA) were prepared as described earlier (18). EA containing C1 (EAC1 cellular intermediates) were prepared by incubating 2×10^{10} EA with 2 μl guinea pig C1 (0.67 μg/ml) for 90 min at 0°C in $DGVB^{++}$. Then, excess C1 was removed by washing the cells with $DGVB^{++}$. In each experiment using these cellular intermediates, inhibition was based upon a noninhibited control tube, which contained one hemolytic site per cell (1 Z of lysis) (19).

Effect of C1INH and/or glycosaminoglycan on fluid-phase C1 activity. The regulatory activity of C1INH and/or glycosaminoglycan on the fluid-phase classical pathway was assessed by incubating C1 (22 ng) with and without 0.05, 0.1, or 0.2 ng of C1INH, with and without 10, 100, or 1000 ng heparin, LMW heparin, or 100 or 1000 ng heparan sulfate in 0.1 ml $DGVB^{++}$ for 10 min at 30°C in a shaking water bath. Then, 1×10^7 EA in 0.1 ml $DGVB^{++}$ were added and the incubation was continued for an additional 10 min at 30°C. The tubes were then centrifuged and the cells were washed twice with 1.0 ml $DGVB^{++}$. Next, the cells were suspended in 0.2 ml $DGVB^{++}$ (reagent blank and 100% lysis tubes) or 0.2 ml $DGVB^{++}$ containing C4 (10 units) and guinea pig C2 (33 μg) in amounts required to produce 1 Z of lysis and were incubated for 10 min at 30°C. Then, 0.1 ml C-EDTA was added to all tubes and incubation was continued for an additional 30 min at 37°C in a shaking water bath. Next, 1.2 ml saline was added to each tube, except the 100% lysis tube, which received 1.2 ml water. The tubes were shaken and centrifuged, and lysis of the cellular intermediates was determined by measuring the optical density of the supernatant at 414 nm. The control tubes included an uninhibited control (EA, C1, C2, C4, and C-EDTA), a C1INH alone control (uninhibited control + C1INH), and a glycosaminoglycan alone control (uninhibited control + glycosaminoglycan).

Effect of C1INH and/or glycosaminoglycan on cell-bound C1 activity. The regulatory activity of C1INH and/or glycosaminoglycan on the cell-bound classical pathway was assessed by incubating 1×10^7 EAC1 cellular intermediates with and without 7.8, 15.6, 31.25, 62.5, 125, 250, or 500 ng C1INH (7×10^{-5} to 4.5×10^{-3} nmol), with and without 10, 100, or 1000 ng glycosaminoglycan (8×10^{-4} to 8×10^{-2} nmol heparin or 2×10^{-3} to 2×10^{-1} nmol LMW heparin or 9×10^{-2} nmol heparan sulfate) in 0.2 ml $DGVB^{++}$ for 20 min at 30°C. The tubes were then centrifuged and the cells were washed twice with 1.0 ml $DGVB^{++}$. Next, the cells were suspended in 0.2 ml $DGVB^{++}$ (reagent blank and 100% lysis) or 0.2 ml $DGVB^{++}$ containing 8 units C4 and 1.2 μg guinea pig C2 in amounts required to produce an average of 1 Z of lysis. The cells were then incubated at 30°C for an additional period of time determined by a T_{max} assay of EAC1 cellular intermediates. Then, 0.1 ml C-EDTA was added and incubation was continued for an additional 60 min at 37°C. Finally, the cells were diluted and optical densities were determined as described in the previous experiment. Control tubes included the uninhibited control (EAC1, C2, C4, and C-EDTA), a C1INH alone control (uninhibited control + C1INH), and a glycosaminoglycan alone control (uninhibited control + glycosaminoglycan).

Effect of sequential addition of C1INH and heparin to EAC1 cellular intermediates on the ability of heparin to regulate C1INH and classical pathway activity. The regulatory activity of C1INH and heparin on the classical pathway was assessed to determine the order in which addition of these substances permits heparin to enhance C1INH activity.

EAC1 cellular intermediates (1×10^7) were incubated with C1INH (150 ng) and heparin or LMW heparin (10, 100, or 1000 ng) (Experiment A), C1INH alone (Experiment B), or with heparin alone (Experiment C) in a total volume of 0.2 ml $DGVB^{++}$ for 20 min at 30°C. The tubes were then centrifuged and the cells were washed twice with 1.0 ml $DGVB^{++}$. Next, the cells were suspended in 0.2 ml $DGVB^{++}$ (Experiment A), 0.2 μl $DGVB^{++}$ containing 10, 100, or 1000 ng heparin (Experiment B), or 150 ng C1INH (Experiment C) and were incubated an additional 20 min at 30°C. The tubes were then centrifuged and the cells were washed twice with 1.0 ml $DGVB^{++}$ and resuspended in 0.2 ml $DGVB^{++}$ (reagent blank and 1000% lysis tubes) or in 0.2 ml $DGVB^{++}$ containing sufficient (8 units) C4 and 2 μg guinea pig C2 to produce an average of one 1 Z of lysis. The cells were then incubated at 30°C for an additional period of time determined by a T_{max} assay of EAC1 cellular intermediates. Then, 0.1 ml C-EDTA was added to all tubes and incubation was continued for an additional 60 min at 37°C. Next, 1.2 ml saline was added to each tube, except the 100% lysis tube, which received 1.2 ml water before lysis was assessed.

RESULTS

Binding of Glycosaminoglycans to C1INH

Sensorgrams for the binding of C1INH to reducing-end and midchain immobilized heparin and reducing-end immobilized heparan sulfate are presented in Fig. 1. The initial part of each curve corresponds to buffer flowing past the sensor surface, the second, rising portion corresponds to the response of the sensor surface following injection of C1INH, and the final portion of each curve shows the dissociation of bound C1INH when buffer (containing no C1INH) flows past the sensor surface again. The dissociation rate constant, k_d, was evaluated from each trace at multiple C1INH concentrations and used to calculate the observed association rate constant (k_s) (Fig. 2). A plot of k_s as a function of ligand concentration (see Fig. 2, inset) yields a

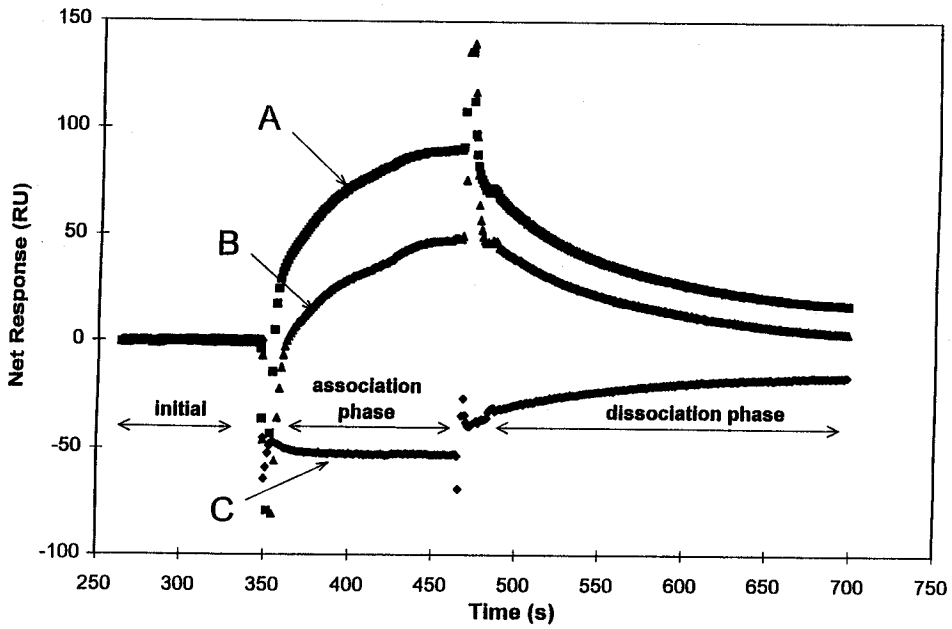

FIG. 1. SPR sensorgrams of C1INH binding to and eluting from 100 RU (15 fmol) of immobilized glycosaminoglycan: heparin immobilized at its reducing-end (A), heparin immobilized at its mid-chain (B), and heparan sulfate immobilized through its reducing end (C).

slope of the association rate constant, k_a. The ratio of the two generates the overall association constant or dissociation constant. Kinetic analysis of the interaction between C1 inhibitor and end-immobilized heparin yielded a K_a value of 1.43×10^7 M^{-1} (K_d of 7.0×10^{-8} M) (Fig. 2).

C1 inhibitor was found to interact somewhat less tightly when heparin was immobilized through a biotin located in the middle of the heparin chain, displaying a K_a value of 7.0×10^6 M^{-1} (Fig. 1). No binding was observed when heparan sulfate was immobilized at its reducing-end.

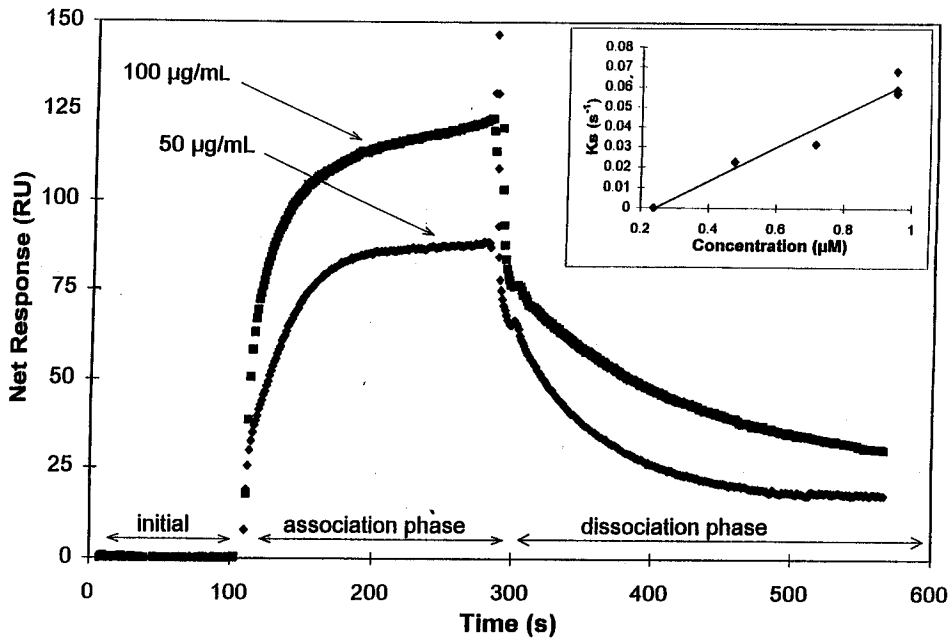

FIG. 2. SPR sensorgrams of C1INH at two representative concentrations (50 and 100 μg/ml) binding to and eluting from heparin immobilized at its reducing end. The inset shows a plot of K_s (S^{-1}) as a function of C1INH concentration (μM). The slope obtained is used to calculate K_a.

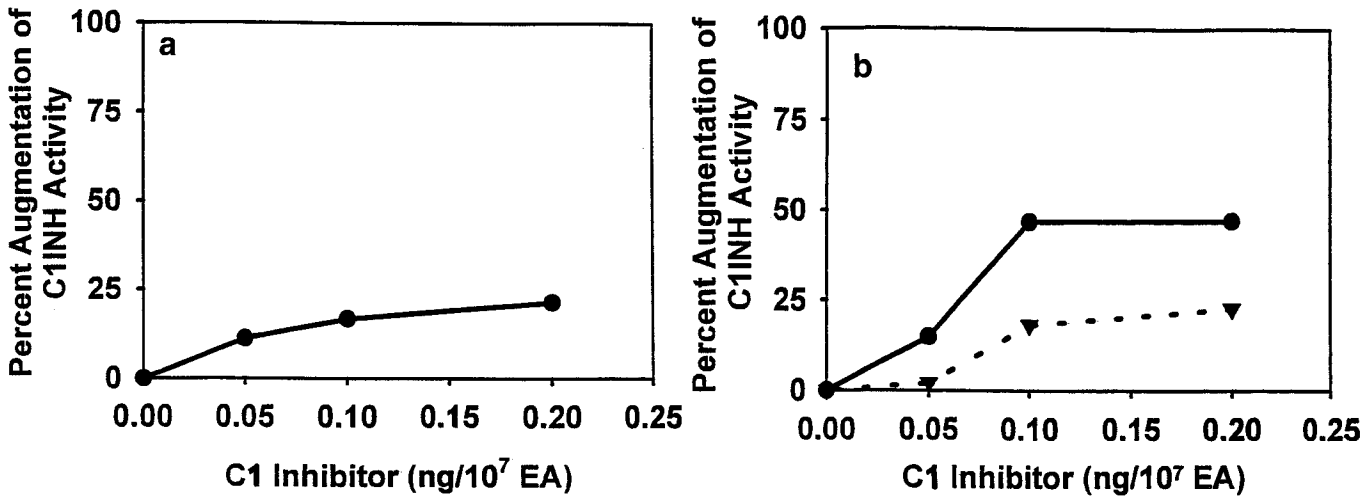

FIG. 3. Heparin percentage of augmentation of C1INH activity on fluid-phase C1. (a) Heparin (●) at 1000 ng/10^7 EA. (b) LMW heparin (●) at 1000 ng/10^7 EA and (▼) at 100 ng/10^7 EA.

C1INH Activity on Fluid-Phase and Cell-Bound C1

Preliminary experiments were conducted to establish the concentration of C1 and C1INH necessary to study the ability of heparin to augment C1INH activity when C1 was in the fluid phase or was cell bound. When C1 was in the fluid phase, C1INH at 0.05 ng/10^7 EA inhibited lysis by 39%. In contrast, the cell-bound C1 assay required C1INH at 500 ng/10^7 EAC1 to inhibit lysis by 41%. This experiment indicates that approximately 10,000 times more C1INH was required to inhibit C1 activity when C1 was cell bound than when C1 was in the fluid phase.

Glycosaminoglycan Augmentation of C1INH

The ability of heparin and heparan sulfate to augment C1INH activity was measured using both fluid-phase and cell-bound C1. These experiments were conducted by two individuals and repeated a total of three or more times to establish reproducibility. A representative set of experiments is presented in Figs. 3 and 4 for heparin and LMW heparin. Heparan sulfate showed no measurable C1INH augmentation.

Neither heparin nor LMW heparin had more than minimal effect upon the activity of C1INH on fluid-phase C1 (Figs. 3a and 3b). At a C1INH concentration of 0.2 ng/10^7 EA and a heparin (or LMW heparin) concentration of 1000 ng/10^7 EA, heparin was in 38,450 molar excess of C1INH (and LMW heparin was in 100,000 molar excess). Even with these high excesses, heparin and LMW heparin were only able to augment C1INH activity on fluid phase C1 by 21 and 47%, respectively. At the two lower heparin and LMW heparin concentrations tested (100 and 10 ng/10^7 EA), only LMW heparin at 100 ng augmented C1INH activity in the fluid-phase C1 (increasing inhibition by 22% at 0.2 ng C1INH/10^7 EA).

In contrast, heparin and LMW heparin markedly augmented C1INH activity on cell bound C1 (EAC1), as shown in Figs. 4a and 4b. Neither heparin nor LMW heparin alone had much capacity to inhibit lysis of the EAC1 (Fig. 4a); in the presence of heparin or LMW heparin at 1000 ng/10^7 EAC1, Z′ (the amount of inhibition that was seen) was only 0.249 and 0.161, respectively (20). Similarly, at the highest concentrations tested, C1INH only modestly inhibited lysis of the EAC1, as shown in Fig. 4a (inset); at 250 ng C1INH/10^7 EAC1, Z′ was only 0.249. However, when C1INH and heparin were present together there was a tremendous increase in inhibitory activity (Figs. 4a and 4b). For example, at 250 ng C1INH and 1000 ng heparin per 10^7 EAC1, augmentation of C1INH activity was 611 and 804%, for heparin and LMW heparin, respectively (Fig. 4b); Z′ for both was 3.706 (Fig. 4a). In this experiment, heparin was in 34 molar excess of C1INH and LMW heparin was in 84 molar excess. Figure 5 is a representative experiment illustrating the inhibition of cell lysis (Z′) which resulted when three LMW heparin concentrations were present in combination with a variety of C1INH concentrations. Clearly there was augmentation of inhibition when both LMW heparin and C1INH were present together over the very modest inhibition that was seen when either was present alone. Similar results were observed for heparin and C1INH (results not presented).

Order of Interaction for Heparin Augmentation of C1INH Activity on Cell-Bound C1

The order in which heparin and C1INH (150 ng/10^7 EAC1) were added to cell bound C1 was varied to examine the interaction leading to heparin augmenta-

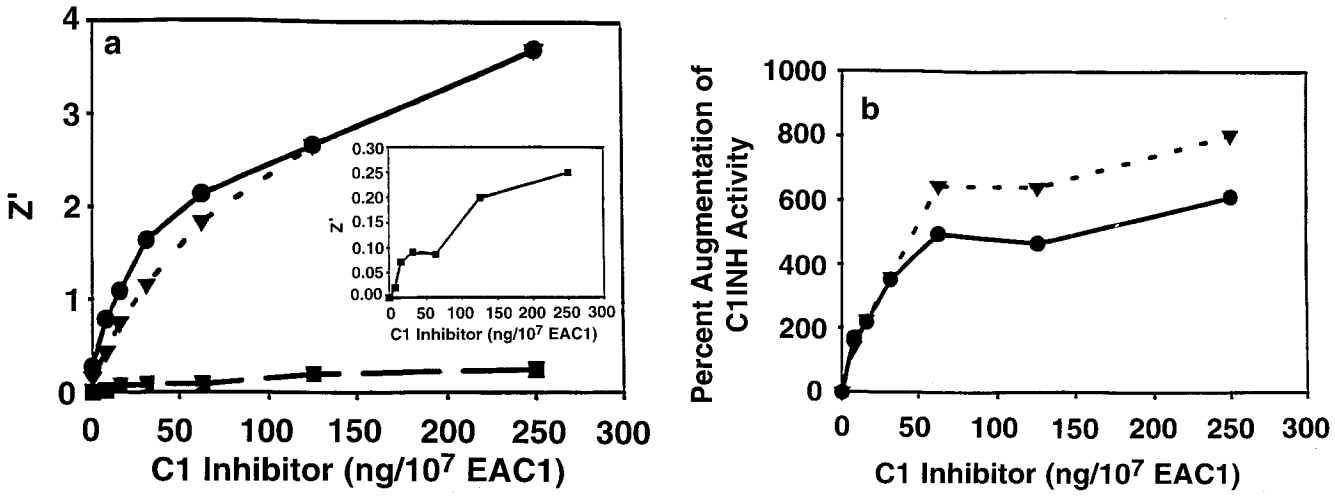

FIG. 4. Heparin effect on C1INH inhibitory activity toward cell-bound C1. (a) The effect of heparin at 1000 ng/10^7 EAC1 on Z′, the inhibition of cell lysis. Heparin (●), LMW heparin (▼), and C1INH alone (■). (b) C1INH activity augmented by heparin (●) and LMW heparin (▼) at 1000 ng/10^7 EAC1.

tion of C1INH activity. Two representative experiments are presented in Fig. 6. When C1INH was incubated independently with EAC1 and the cells washed before adding heparin (or LMW heparin), no heparin augmentation was observed (Fig. 6). Conversely, when heparin (or LMW heparin) was incubated independently with EAC1 and the cells were washed before adding C1INH, the heparin minimally augmented C1INH activity. Only with the highest concentration of heparin (1000 ng/10^7 EAC1) and LMW heparin was 29 and 3% augmentation observed, respectively. When both heparin or LMW heparin (1000 ng/10^7 EAC1) and C1INH (150 ng) were incubated together with the EAC1, augmentation of C1INH activity was 417 and 416%, respectively (Figs. 6a and 6b).

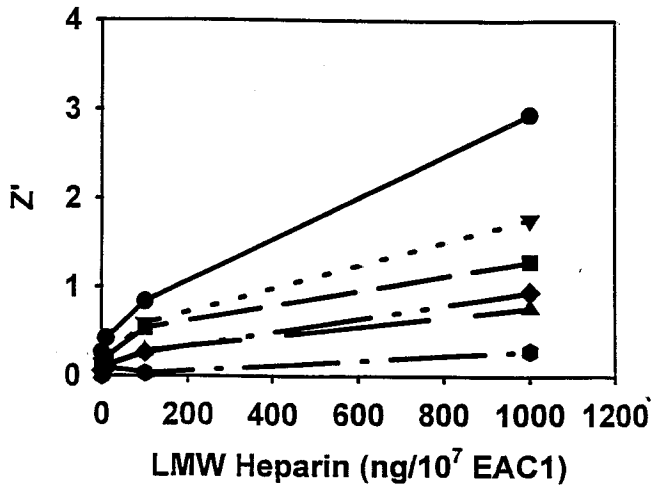

FIG. 5. LMW heparin effect on C1INH inhibition of cell lysis (Z′). C1INH at (●) 125 ng/10^7 EAC1, (▼) 62.5 ng/10^7 EAC1, (■) 31.25 ng/10^7 EAC1, (◆) 15.6 ng/10^7 EAC1, (▲) 7.8 ng/10^7 EAC1, and (●) 0 ng/10^7 EAC1.

DISCUSSION

Experiments in this report were first aimed at examining the interaction of heparin and heparan sulfate with C1INH. The sensorgrams shown in Fig. 1 clearly demonstrate that heparin interacts with C1INH but heparan sulfate does not. It is likely that the observed differences between reducing-end immobilized heparin and midchain immobilized heparin are the result of steric hindrance. C1INH is a large protein (M_r 105 kDa) that may encounter difficulty interacting with a heparin chain that is attached to a surface through a midchain attachment when compared to an end-point attachment. Similar steric hindrance has been previously reported for the interaction between proteins and heparin immobilized in different orientations to microtiter plates (21).

A somewhat unusual methodology was employed to examine heparin and heparan sulfate interaction with C1INH. The ligand solution containing C1INH was flowed over all four flow cells (endpoint heparin, midchain heparin, heparan sulfate, and control) in series. This represents a convenient means of comparing the ligand binding affinities for different immobilized glycosaminoglycan in real time. Mass transport limitations and the depletion of ligand concentration in the flowing volume were avoided by keeping the amount of immobilized glycosaminoglycans low (i.e., 100 RU or 15 fmol of glycosaminoglycan). Additionally, the system was maintained at a constant flow of 5 μl/min, ensuring that the flow cell volume (60 nl) was replenished at least once per second. Upon initial exposure of the ligand solution to the flow cells, there was a very rapid

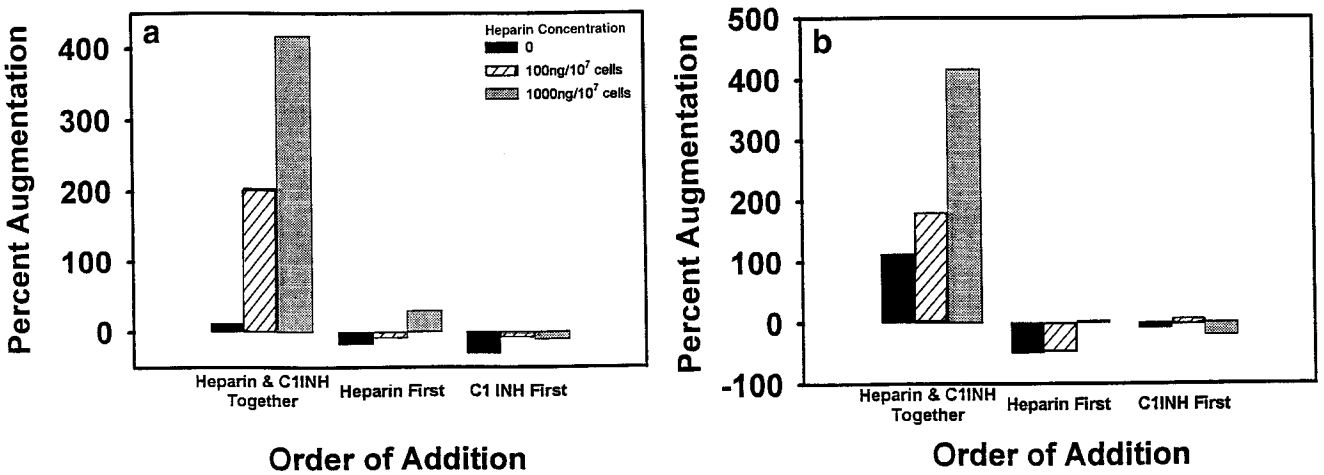

FIG. 6. Effect of heparin and C1INH addition order on the augmentation of C1INH activity on cell-bound C1. C1INH at 150 ng/10^7 EAC1. (a) Heparin. (b) LMW heparin.

rise in signal seen in all the cells, likely as a result of electrostatic uptake and/or interactions of the ligand with the dextran matrix itself. Interaction with the sensor surface continued beyond this initial period reflecting the specific interaction with the immobilized glycosaminoglycans, and it is from this time region which the kinetic data were evaluated. Within this second region, the rise in signal was maximally ~1 RU in 1 s, correlating to 0.001 ng/mm^2, based on a conversion factor of 1000 RU = 1 ng/mm^2, supplied by the manufacturer. We expect the last flow cell to experience the most potential ligand depletion. Based on a surface area of 2 mm^2 in each of three previous flow cells and a flow cell volume of 60 nl, the maximum loss of ligand corresponds to only 0.006 ng or $\frac{1}{1000}$ of the total amount of ligand present in the injected volume. Thus, the sequential exposure of the same ligand solution to a series of flow cells did not adversely affect analysis.

The analyses presented clearly demonstrate the strong interaction of heparin with C1INH affording a K_a of 1.4×10^7 M^{-1} (endpoint attached) and 7×10^6 M^{-1} (midchain attached). Furthermore, endpoint-attached heparan sulfate showed no measurable interaction with C1INH, suggesting either that it lacked the requisite structure or sequence for interaction or that its charge density (~2/disaccharide repeat) was insufficient for binding to C1INH.

Next, the augmentation of C1INH by heparin was examined. The goal of these experiments was to study the mechanism for heparin and LMW heparin augmentation of C1INH activity on C1. Initially, baseline C1INH activity on fluid phase and cell bound C1 was established. C1INH was approximately 10,000 times more active on fluid phase C1 than on cell bound C1. Experiments with C1 in the fluid phase demonstrated little or no effect of heparin or LMW heparin on the ability of C1INH to inhibit cell lysis. Conversely, when C1 was cell bound, both heparin and LMW heparin were quite effective at augmenting C1INH activity (Figs. 4 and 5). At 250 ng (0.0024 nmol) C1INH and 1000 ng (0.074 to 0.2 nmol) heparin per 10^7 EAC1, heparin was approximately 75% as active as the LMW heparin (611% vs 804% augmentation, Fig. 4b).

The order of interaction between C1, C1INH, and heparin was also determined (Fig. 6). When C1INH and heparin were incubated separately with cell-bound C1, augmentation of C1INH activity was not observed. Heparin augmentation of C1INH was only observed when C1INH and heparin were present together with cell bound C1. This suggests heparin must interact with C1 and with C1INH prior to or at the same time that C1INH interacts with C1 for heparin augmentation to occur.

In summary, heparin interacts with C1INH and augments its inhibition of C1. The studies reported here are the first to demonstrate that LMW heparin augments C1INH activity. The concentrations of heparin required for binding and augmentation are within those achieved by inhalation in patients (9, 10). Future studies will be required to determine if this interaction has biological significance and may occur *in vivo* as a potential therapy for hereditary angioedema.

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