Heparin Inhibits the Intrinsic Tenase Complex by Interacting with an Exosite on Factor IXa†

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ABSTRACT: The specific molecular target for direct heparin inhibition of factor X activation by intrinsic tenase (factor IXa−factor VIIIa) was investigated. Comparison of size-fractionated oligosaccharides demonstrated that an octasaccharide was sufficient to inhibit intrinsic tenase. Substitution of soluble dihexanoic phosphatidylserine (C6PS) for phospholipid (PL) vesicles demonstrated that inhibition by low-molecular weight heparin (LMWH) was independent of factor IXa−factor VIIIa membrane assembly. LMWH also inhibited factor X activation by the factor IXa−PL complex via a distinct mechanism that required longer oligosaccharides and was independent of substrate concentrations. The apparent affinity of LMWH for the factor IXa−PL complex was higher in the absence of factor VIIIa, suggesting that the cofactor adversely affected the interaction of heparin with factor IXa-phospholipid. LMWH did not interact directly with the active site, as it failed to inhibit chromogenic substrate cleavage by the factor IXa−PL complex. LMWH induced a modest decrease in factor IXa−factor VIIIa affinity [$K_{D(app)}$] on PL vesicles that did not account for the inhibition. In contrast, LMWH caused a substantial reduction in factor IXa−factor VIIIa affinity in the presence of C6PS that fully accounted for the inhibition. Factor IXa bound LMWH with significantly higher affinity than factor X by competition solution affinity analysis, and the $K_{D(app)}$ for the factor IXa−LMWH complex agreed with the K_I for inhibition of the factor IXa−PL complex by LMWH. Thus, LMWH binds to an exosite on factor IXa that antagonizes cofactor activity without disrupting factor IXa−factor VIIIa assembly on the PL surface. This exosite may contribute to the clinical efficacy of heparin and represents a novel target for antithrombotic therapy.

Heparin is a complex, heterogeneous mixture of oligosaccharide chains. The specific mechanisms responsible for the clinical efficacy of unfractionated and low-molecular weight heparin preparations in human thrombotic disorders remain incompletely understood. Although the effect of heparin on antithrombin-dependent inhibition of coagulation proteases is well-documented, assembly of these proteases into their respective membrane-bound enzyme complexes results in relative protection from antithrombin inhibition (*1*, *2*). Furthermore, low-affinity (for antithrombin) heparin demonstrates antithrombotic efficacy in a rabbit model of venous thrombosis, suggesting that antithrombin-independent mechanisms are relevant in vivo (*3*). Unfractionated, low-molecular weight, and low-affinity forms of heparin inhibit the intact intrinsic tenase complex in an antithrombin-independent manner (*4*). This effect of heparin appears to be selective for the intrinsic tenase complex, as neither the prothrombinase nor the tissue factor−factor VIIa complexes are inhibited in this manner. The inhibition exhibits a partial, noncompetitive pattern, which is not explained by effects on factor IXa−factor VIIIa assembly on phospholipid vesicles or cofactor stability (half-life) (*4*). Thus, therapeutic concentrations of unfractionated or low-molecular weight heparin (LMWH)[1] directly inhibit factor X activation by the intact intrinsic tenase complex, which may contribute to the clinical efficacy of heparin.

The critical role of the intrinsic tenase complex in propagation of the coagulation response makes it an attractive target for the development of specific antithrombotic agents. Factor X activation by the intrinsic tenase complex is the rate-limiting step for thrombin generation during tissue factor-dependent coagulation (*5*, *6*). Animal models suggest that targeting of the intrinsic tense complex may improve the risk/benefit ratio of antithrombotic therapy. Treatment with active site-blocked factor IXai is as effective as unfractionated heparin in canine coronary thrombosis or murine stroke models and demonstrates significantly less bleeding from a standard abdominal incision (canine model) or intracerebral hemorrhage (murine model) at equivalent therapeutic doses (*7*, *8*). Anticoagulation with factor IXai during cardiopulmonary bypass in the baboon yields a

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[1] Abbreviations: APTT, activated partial thromboplastin time; BSA, bovine serum albumin; C6PS, dihexanoic phosphatidylserine; EG, ethylene glycol; ES, enzyme−substrate complex; ESI, enzyme−substrate−inhibitor complex; Gla, γ-carboxyglutamic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LMWH, low-molecular weight heparin; PC/PS, phosphatidylcholine/phosphatidylserine; PEG-8000, polyethylene glycol (average molecular mass of 8000 Da); PL, phospholipid; PS ODN, phosphorothioate oligonucleotides.

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significant reduction in blood loss compared to heparin, both in the thoracic cavity and from a standard incisional wound (*9*). Similarly, a monoclonal antibody directed against the Gla domain of factor IXa is at least as effective as LMWH in a rat carotid thrombosis model, with markedly reduced activated partial thromboplastin time (APTT) prolongation and blood loss with injury (*10*, *11*). These animal models suggest that selective inhibition of factor IX(a), in the presence of intact tissue factor-induced coagulation, may reduce the bleeding complications of antithrombotic therapy.

Similarities between heparin and other polyanionic inhibitors of the intrinsic tenase complex suggest that a common mechanism may exist. Phosphorothioate oligonucleotides (PS ODN) are polyanionic polymers that prolong the APTT in human plasma via inhibition of factor X activation by intrinsic tenase (*12*). This inhibition exhibits a partial, uncompetitive pattern that is not explained by effects on factor IXa−factor VIIIa affinity or cofactor stability, but requires the binding of PS ODN to an exosite on factor IXa (*13*). In contrast, the molecular target for antithrombin-independent inhibition of intrinsic tenase activity by heparin remains undefined. Although factor IXa, factor VIII(a), and factor X(a) all bind heparin, similarities between the inhibition of intrinsic tenase by heparin and PS ODN lead to the hypothesis that heparin also acts by binding to an exosite on factor IXa. Detailed analysis of this inhibition mechanism was undertaken using LMWH and size-fractionated heparin oligosaccharides, in the presence of either phospholipid vesicles or soluble phospholipid. The results demonstrate that heparin oligosaccharide interacts with an exosite on factor IXa that antagonizes the cofactor activity of factor VIIIa without disrupting factor IXa−factor VIIIa assembly on the phospholipid membrane. The results of this investigation are relevant to the therapeutic efficacy of heparin and the development of exosite-directed inhibitors of the intrinsic tenase complex.

EXPERIMENTAL PROCEDURES

Reagents. Human factors IXa_B, X, and Xa and thrombin were purchased from Enzyme Research (South Bend, IN). Recombinant human factor VIII (Kogenate FS) was generously provided by J. Brown[2] and A. Mueller-Beckhaus of the Bayer Corp. (Berkeley, CA). Phosphatidylserine (PS), phosphatidylcholine (PC), and dihexanoic phosphatidylserine (C6PS) were purchased from Avanti Lipids (Alabaster, AL). Cholesterol was purchased from Calbiochem (San Diego, CA). Recombinant hirudin and bovine serum albumin were purchased from Sigma (St. Louis, MO). Chromogenic substrates were purchased as follows: S-2765 (*N*-α-benzyloxycarbonyl-D-Arg-Gly-Arg-pNA) from DiaPharma (Franklin, OH), and Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) from Centerchem, Inc. (Stamford, CT). LMWH (dalteparin sodium) was obtained from Pharmacia and UpJohn Co. (Kalamazoo, MI). Heparin oligosaccharides obtained by partial nitrous acid degradation and gel filtration chromatography were purchased from Neoparin, Inc. (San Leandro, CA). Biotinylated LMWH (ardeparin) was purchased from Celsus, Inc. (Cincinnati, OH). Biotin-X-hydrazide was obtained from Pierce Endogen (Rockford, IL). Strepavidin-coated surface plasmon resonance sensor chips were purchased from BiaCore, Inc. (Piscataway, NJ). Phosphatidylcholine/phosphatidlylserine/cholesterol (75/25/1 molar ratio) phospholipid vesicles (PC/PS vesicles) were prepared by extrusion through a 100 nm polycarbonate filter (*14*). The molar concentration of phospholipid was determined with an elemental phosphorus assay (*15*).

Molecular masses (daltons) and extinction coefficients ($\epsilon_{0.1\%}$): 46 000 and 1.43 for for human factor IXa_B, 58 900 and 1.16 for factor X, 46 000 and 1.40 for factor Xa, 36 700 and 1.83 for thrombin, and 280 000 and 1.2 for recombinant human factor VIII, respectively. Average molecular mass for low-molecular weight heparins: 6000 Da for ardeparin and 5000 Da for dalteparin.

Factor X Activation by the Factor IXa−Factor VIIIa Complex. A chromogenic assay for intrinsic tenase complex activity was performed with limiting factor IXa. Recombinant human factor VIII was activated with 40 nM thrombin in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 5 mM $CaCl_2$, and 0.01% Tween 80 for 30 s at room temperature. Thrombin was neutralized with recombinant hirudin (60 nM) and the activation mixture diluted 25-fold into a tenase reaction mix containing final concentrations of 0.1 nM human factor IXa_B, 1.0 nM factor VIIIa, and 100 μM PC/PS phospholipid vesicles, in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL bovine serum albumin, and 0.1% PEG-8000 buffer. Human factor X was added immediately and the reaction mixture incubated between 15 s and 5 min at room temperature depending on the expected rate of factor Xa generation. The reaction was terminated by addition of EDTA and Polybrene to final concentrations of 0.042 M and 0.183 mg/mL, respectively. Triplicate 60 μL aliquots were placed in a 96-well microtiter plate (Greiner Bio-One, Longwood, FL), followed by addition of 100 μL of S-2765 at a final concentration of 300 μM. The amount of factor Xa was determined by comparing the rate of substrate hydrolysis (change in absorbance at 405 nm over the course of 2 min) in a kinetic microtiter plate reader (V_{max} Reader, Molecular Devices Corp.) to a standard curve constructed with purified factor Xa. The initial rate of factor Xa generation by the intrinsic tenase complex was determined by dividing the total amount of factor Xa generated by the incubation time, and was restricted to conditions under which less than 10% total substrate cleavage occurred. PC/PS vesicles were replaced with 0.25 mM dihexanoic phosphatidylserine (C6PS) for additional assays, and reactant concentrations were adjusted to 0.6 nM factor IXa and 1.8 nM factor VIIIa. Kinetic constants for factor X activation were obtained by plotting the rate of factor Xa generation (nanomoles per liter per minute) versus substrate concentration in the presence and absence of LMWH, and $K_{m(app)}$ and $V_{max(app)}$ values were determined by fitting the data by nonlinear regression to the Michaelis−Menten equation.

Factor X Activation by the Factor IXa−Phospholipid Complex. Factor X generation by the factor IXa_B−phospholipid complex in the absence of cofactor was assessed as described above, with the factor IXa concentration adjusted to 40 nM human factor IXa, and an incubation time between 5 and 30 min prior to termination with EDTA and Polybrene.

Determination of the K_I for Inhibition of Factor X Activation by Heparin Oligosaccharides. The rate of factor X generation (nanomoles per liter per minute) was plotted

[2] No longer with Bayer.

versus oligosaccharide concentration, and the data were fit by nonlinear regression to the equation for partial noncompetitive inhibition (V_{max} decreased; K_m was unchanged) to determine the values for $V_{max(app)}$, $V_{imax(app)}$, and K_I for heparin oligosaccharide (*4*, *16*, *17*). $V_{max(app)}$ and $V_{imax(app)}$ represent the maximal velocity in the absence and presence of inhibitor, and K_I is the dissociation constant for the enzyme−inhibitor complex.

Determination of the Affinity of Formation of the Factor VIIIa−Factor IXa Complex. The affinity of the factor IXa_B−factor VIIIa complex was assessed by a functional binding assay. Thrombin-activated factor VIIIa (0.3 nM) was titrated with increasing amounts of factor IXa_B, in the presence of either 100 μM PC/PS vesicles or 0.25 mM C6PS, and 200 nM factor X. The rate of factor Xa generation in the absence of factor VIIIa under these conditions was less than 2% of the total activity. The factor IXa_B concentration was plotted versus the rate of factor Xa generation, and the data were fit by nonlinear regression to a single-site binding model to determine the $K_{D(app)}$. The concentration of the factor VIIIa−factor IXa complex in the absence and presence of LMWH was determined by using the experimentally determined K_D values to solve the quadratic equation

$$[\text{FIXa}-\text{FVIIIa}] = \frac{[\text{FIXa}]_t + [\text{FVIIa}]_t + K_D}{2} - \frac{\sqrt{([\text{FIXa}]_t + [\text{FVIIIa}]_t + K_D)^2 - 4[\text{FIXa}]_t[\text{FVIIIa}]_t}}{2} \quad (1)$$

where $[FIXa]_t$ and $[FVIIIa]_t$ represent the initial concentrations of factor IXa and factor VIIIa, respectively, and K_D represents the dissociation constant for the factor IXa−factor VIIIa complex.

Chromogenic Substrate Catalysis by the Factor IXa−Phospholipid Complex. The effect of increasing LMWH concentrations on hydrolysis of Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) was determined with either 40 or 100 nM factor IXa, in the presence or absence of 30% ethylene glycol, respectively. Assays contained 100 μM PC/PS vesicles, 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL BSA, 0.1% PEG-8000, and 2.5 mM Pefachrome IXa. Substrate hydrolysis was assessed by the change in absorbance at 405 nm over the course of 10 min at room temperature.

Equilibrium Binding Analysis of Factor IXa−LMWH and Factor X−LMWH Affinity Using Surface Plasmon Resonance. Preliminary experiments with the BiaCore 2000 instrument demonstrated that binding of human factors IXa and X to immobilized LMWH was limited by mass transport. Association rates varied in proportion to flow rate even at a low surface density of LMWH, and dissociation rates were markedly accelerated by addition of 2 μM LMWH to the buffer, indicating rebuilding of the interaction. Thus, a solution affinity approach was performed using a soluble LMWH competitor. Flow cells on a BiaCore SA chip were prepared as follows: 1.0 M NaCl and 50 mM NaOH were injected at 30 μL/min for 60 s three times to activate the strepavidin surface, followed by two 60 μL injections of 2 M NaCl, 20 mM HEPES (pH 7.4), and 0.05% Tween 20 (regeneration buffer). The reference surface was created by injecting 20 μL of 4 μM biotin-X-hydrazide (Pierce Endogen) at 10 μL/min in 0.3 M NaCl, 20 mM HEPES (pH 7.4), and 0.05% Tween 20 (injection buffer), resulting in an ~45 RU signal that remained stable after repeat injections of regeneration buffer (cell 1). A high-capacity LMWH surface was created by injecting 20 μL of 200 μg/mL biotin-bound low-molecular weight heparin (ardeparin) at 10 μL/min in the injection buffer, resulting in an ~250 RU signal (cell 2) that was stable after repeat injections of regeneration buffer. Human factor IXa (0−250 nM) or factor X (0−4 μM) was serially injected at 5 μL/min for 120 s (association phase) in 0.15 M NaCl, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4), and 0.05% Tween 20 (running buffer), followed by 2 μM LMWH (ardeparin) in running buffer for 120 s (dissociation phase). Final sensograms were obtained by subtracting the reference surface from the heparin surface signal, averaging replicate determinations, and subtracting the mean sham injection (buffer only) signal (*18*). The sensogram response at 90 s postinjection was plotted versus the factor IXa or factor X concentration, yielding linear calibration curves for the binding of each protein. To determine the EC_{50} for the solution interaction, increasing concentrations (1 nM to 100 μM) of LMWH (dalteparin) were incubated with 250 nM factor IXa or 2.5 μM factor X for 20 min prior to injection over the sensor chip exactly as described for the calibration curves. For each LMWH concentration, the sensogram response at 90 s postinjection was converted to a concentration of free factor IXa or factor X with the appropriate calibration curve. The concentration of the LMWH competitor was plotted versus the proportion of remaining free factor IXa or factor X, and the EC_{50} was determined by fitting the data to the equation

$$B = \frac{EC_{50}{}^n}{EC_{50}{}^n + [I]^n} \quad (2)$$

where B represents the fractional specific binding, [I] represents the concentration of LMWH used as a competitor, EC_{50} represents the concentration of LMWH that causes a 50% reduction in the surface plasmon binding response, and n represents the pseudo-Hill coefficient (*19*). The data were fit to this functional binding equation because of the multivalent nature of heparin−protease interactions, consistent with the relatively shallow slopes of the competition curves and pseudo-Hill coefficients of <1 (*20*). Since competition curves were determined at protein concentrations lower than the EC_{50}, the K_D of the protease−heparin interactions can be estimated by assuming that the K_I for soluble LMWH is equal to the K_D for immobilized LMWH, and converting the EC_{50} as described below:

$$EC_{50} = K_I(1 + [L]/K_D) \quad (3)$$

where [L] is the factor IXa or factor X concentration.

RESULTS AND DISCUSSION

Inhibition of the Intrinsic Tenase Complex by Heparin Oligosaccharides. Therapeutically relevant concentrations of unfractionated, low-molecular weight, and low-affinity heparins inhibit factor X activation by the intrinsic tenase complex in a partial, noncompetitive pattern (*4*). However, since factor IXa, factor VIIIa, and factor X all bind heparin,

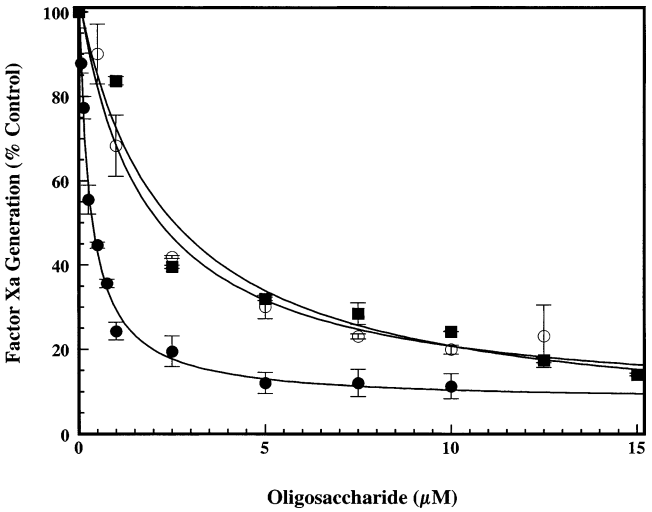

FIGURE 1: Effect of LMWH (dalteparin) and size-fractionated heparin oligosaccharides on factor X activation by the intrinsic tenase complex. Increasing concentrations of low-molecular weight heparin (●), heparin tetradecasaccharide (○), or octasaccharide (■) were added to a reaction mixture containing final concentrations of 1.0 nM factor VIIIa, 0.1 nM factor IXa, 200 nM factor X, and 100 μM PC/PS vesicles (75/25) in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL BSA, and 0.1% PEG-8000. The rate of factor X activation (nanomoles per liter per minute) by the intrinsic tenase complex was determined in a chromogenic assay as described in Experimental Procedures. Mean values (% control) were plotted with error bars representing the standard deviation ($n = 6$). The inhibition constant (K_I) for heparin oligosaccharides was determined by fitting the data by nonlinear regression to the equation for partial, noncompetitive inhibition. The K_I values ± standard error were 0.30 ± 0.03 μM for LMWH, 1.7 ± 0.4 μM for heparin tetradecasaccharide, and 2.5 ± 0.7 μM for heparin octasaccharide.

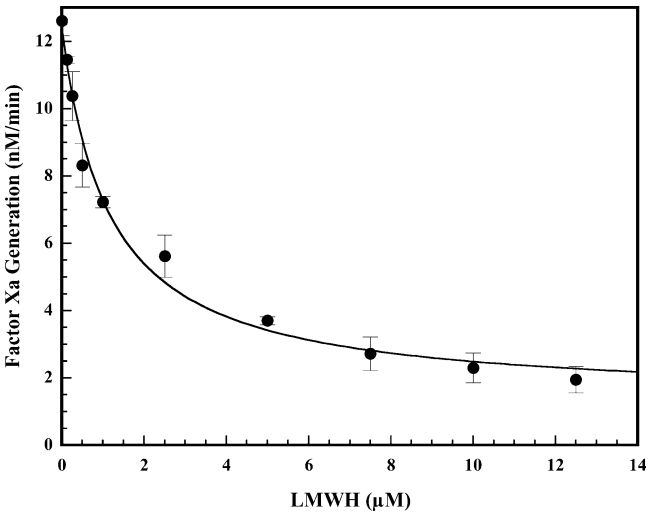

FIGURE 2: Effect of LMWH on factor X activation by the factor IXa−factor VIIIa complex in the presence of soluble phospholipid (C6PS). Increasing concentrations of low-molecular weight heparin were added to a reaction mixture containing final concentrations of 1.8 nM factor VIIIa, 0.6 nM factor IXa, 200 nM factor X, and 0.25 mM C6PS in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL BSA, and 0.1% PEG-8000. The rate of factor X activation (nanomoles per liter per minute) by the intrinsic tenase complex was determined as described in Experimental Procedures. Mean values were plotted with error bars representing the standard deviation ($n = 4$). The inhibition constant for LMWH ($K_I = 1.2 \pm 0.2$ μM) was determined by fitting the data by nonlinear regression to the equation for partial, noncompetitive inhibition.

the molecular target for this inhibition was undefined. To assess the contribution of oligosaccharide chain length to inhibition of factor X activation by the intrinsic tenase complex, size-fractionated heparin oligosaccharides were compared to LMWH (dalteparin, average molecular mass of 5000 Da). Increasing concentrations of LMWH, size-fractionated heparin hexadecasaccharide, and octasaccharide each partially inhibited factor X activation by the intrinsic tenase complex (Figure 1). The apparent affinity of heparin oligosaccharides for the intrinsic tenase complex was determined by fitting the data to the equation for partial, noncompetitive inhibition (*4*). The apparent affinity of LMWH for the intrinsic tenase complex was significantly higher than that of the size-fractionated oligosaccharides, as demonstrated by the K_I values for octasaccharide (2.5 μM), tetradecasaccharide (1.7 μM), and LMWH (0.3 μM). The increase in apparent affinity with oligosaccharide chain length is consistent with the expected behavior for linear polyelectrolyte binding (*21*). Residual intrinsic tenase activity at maximally inhibitory oligosaccharide concentrations was approximately 10% for LMWH, and approached 10−20% of control values for both the heparin tetradecasaccharide and octasaccharide (Figure 1). Thus, size-fractionated heparin tetradecasaccharide and octasaccharide were sufficiently long to inhibit the intrinsic tenase complex, and maximally inhibitory oligosaccharide concentrations demonstrated a similar magnitude of inhibition (Figure 1).

Effect of Low-Molecular Weight Heparin on Factor X Activation by the Factor IXa−Factor VIIIa Complex in the Presence of Soluble Phospholipid. Soluble dihexanoic phosphatidylserine (C6PS) was substituted for PC/PS (75/25) vesicles to assess the contribution of membrane assembly to heparin inhibition of factor X activation by the factor IXa−factor VIIIa complex. A submicellar concentration (0.25 mM) of C6PS accelerates factor X cleavage via a 25-fold enhancement in k_{cat} and a 30-fold increase in the affinity of the factor IXa−factor VIIIa complex, relative to the absence of phospholipid (*22*). LMWH partially inhibited factor X activation by the factor IXa−factor VIIIa complex in the presence of 0.25 mM C6PS, similar to the inhibition observed on phospholipid vesicles (Figure 2). This result demonstrates that membrane assembly of the enzyme complex is not required for inhibition of factor X activation by heparin oligosaccharide.

Substrate−membrane interactions dominate the apparent affinity of the macromolecular substrate [$K_{m(app)}$] for membrane-bound coagulation complexes (*23*−*25*). By eliminating the phospholipid surface, substitution of soluble C6PS for phospholipid vesicles emphasizes the contribution of protein−protein (as opposed to protein−membrane) interactions to the apparent affinity of the factor IXa−factor VIIIa complex for factor X. Consistent with these observations, the apparent affinity of the factor IXa−factor VIIIa complex for factor X was reduced approximately 7-fold [$K_{m(app)} = 0.14$ μM] with C6PS compared to the reaction on phospholipid vesicles (not shown). To classify the inhibition mechanism, the kinetics of factor X activation by the factor IXa−factor VIIIa complex in 0.25 mM C6PS were examined in the absence and presence of increasing concentrations of LMWH. Addition of LWMH progressively reduced the $V_{max(app)}$ to approximately 50% of control, with no significant change in the $K_{m(app)}$ for factor X activation by the factor IXa−factor VIIIa complex (Figure 3). These results are qualitatively

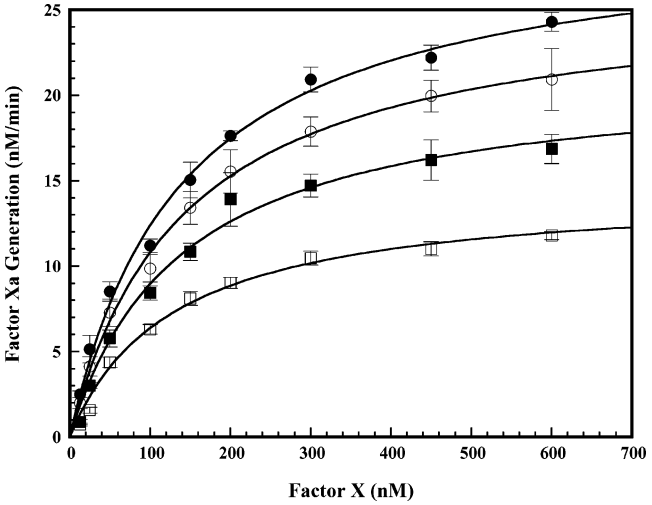

FIGURE 3: Effect of LMWH on the kinetics of factor X activation by the factor IXa−factor VIIIa complex in the presence of soluble phospholipid (C6PS). The rate of factor X activation for reaction mixtures containing 1.8 nM factor VIIIa, 0.6 nM factor IXa, 12.5−450 nM factor X, and 0.25 mM C6PS in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL BSA, and 0.1% PEG-8000 was determined in the absence (●) or presence of 0.75 (○), 1.5 (■), or 3.0 μM (□) low-molecular weight heparin. Mean values were plotted with error bars representing the standard deviation (n = 2−3). The $K_{m(app)}$ and $V_{max(app)}$ for factor X activation were determined by fitting the data by nonlinear regression to the Michaelis−Menten equation. $K_{m(app)}$ values ± standard error for 0, 0.75, 1.5, and 3.0 μM LMWH were 141 ± 13, 143 ± 10, 139 ± 18, and 128 ± 16 nM, respectively. $V_{max(app)}$ values ± standard error for 0, 0.75, 1.5, and 3.0 μM LMWH were 29.8 ± 1.0, 26.1 ± 0.7, 21.3 ± 1.0, and 14.5 ± 0.6 nM/min, respectively.

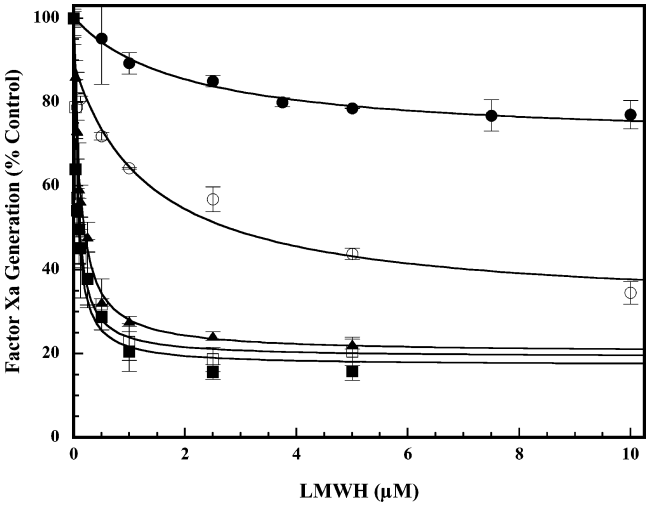

FIGURE 4: Effect of LMWH and size-fractionated heparin oligosaccharides on factor X activation by the factor IXa−phospholipid complex. Increasing concentrations of heparin octasaccharide (●), tetradecasaccharide (○), or LMWH (□) were added to a reaction mixture containing final concentrations of 40 nM factor IXa, 300 nM factor X, and 100 μM PC/PS vesicles (75/25) in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL BSA, and 0.1% PEG-8000. The effect of LMWH was also examined at 150 (■) and 450 nM (▲) factor X. The rate of factor X activation (nanomoles per liter per minute) by the factor IXa−phospholipid complex was determined as described herein (see Experimental Procedures). Mean values (% control) were plotted with error bars representing the standard deviation (n = 2−4). The inhibition constant (K_I) for heparin oligosaccharides was determined by fitting the data by nonlinear regression to the equation for partial, noncompetitive inhibition. The K_I values ± standard error were 2.0 ± 0.4 μM for heparin octasaccharide, 1.4 ± 0.80 μM for heparin tetradecasaccharide, and 0.059 ± 0.010, 0.062 ± 0.009, or 0.107 ± 0.12 μM for LMWH in the presence of 150, 300, or 450 nM FX, respectively.

similar to those obtained for LMWH in the presence of phospholipid membrane, demonstrating partial, noncompetitive inhibition of factor X activation. Fitting the data to the equation for partial, noncompetitive inhibition demonstrates that the apparent affinity of LMWH for the factor IXa−factor VIIIa complex (K_I = 1.2 ± 0.2 μM) under these conditions was reduced 4-fold (Figure 2) compared to that in the reaction on phospholipid vesicles (Figure 1). These results demonstrate that inhibition by LMWH is independent of membrane assembly of the enzyme complex, and occurs primarily through a reduction in the $V_{max(app)}$ for factor X activation. The lack of a competitive pattern (increased K_m) for inhibition of macromolecular substrate catalysis, in either the presence (PC/PS vesicles) or absence (C6PS) of substrate−membrane binding, suggests that heparin and factor X bind to distinct sites on the factor IXa−factor VIIIa complex.

Effect of Heparin Oligosaccharide on Factor X Activation by the Factor IXa−Phospholipid Complex. The effect of heparin oligosaccharide on factor X activation by the factor IXa−phospholipid complex was compared to that of intrinsic tenase (Figure 1) in an effort to assess the potential contribution of factor VIIIa to inhibitor binding. LMWH partially inhibited factor X activation by the factor IXa−phospholipid complex, with a residual activity that is approximately 20% of control values. Size-fractionated oligosaccharides also partially inhibited factor X activation by the factor IXa−phospholipid complex but demonstrated significantly higher residual activity at maximally inhibitory concentrations. Residual activity was approximately 40% of the control for heparin tetradecasaccharide, and 75% of control for octasaccharide (Figure 4). The inhibition of factor X activation by LMWH in the presence (Figure 1) or absence (Figure 4) of factor VIIIa indicates that cofactor is not required for oligosaccharide binding. However, the markedly inhibited decreased ability of the shorter oligosaccharides to inhibit factor X activation by the factor IXa−phospholipid complex suggests that mechanisms for cofactor-independent and -dependent inhibition are distinct.

To classify the inhibition mechanism for the factor IXa−phospholipid complex, the kinetics of factor X activation were examined in the absence and presence of 1.0 μM LMWH. In the presence of LMWH, a marked reduction in $V_{max(app)}$ was observed, but activity of the factor IXa−phospholipid complex was insufficient to adequately describe the $K_{m(app)}$ for factor X under these conditions (data not shown). Conversely, the effect of substrate on the ability of LMWH to inhibit the factor IXa−phospholipid complex was addressed by varying the factor X concentration [$K_{m(app)}$ = 170 μM in the absence of heparin]. Increasing the concentration of factor X from 150 to 450 nM did not significantly affect inhibition of the factor IXa−phospholipid complex by LMWH (Figure 4), suggesting that the initial interaction of factor X with the factor IXa−phospholipid complex does not directly compete with binding of LMWH. Fitting these inhibition curves to the equation for partial, noncompetitive inhibition demonstrated that the apparent affinity of oligosaccharide inhibitors for the factor IXa−phospholipid complex increased with chain length (Figure 4), similar to the situation with the intrinsic tenase complex (Figure 1). Interestingly, the apparent affinity of LMWH for the factor

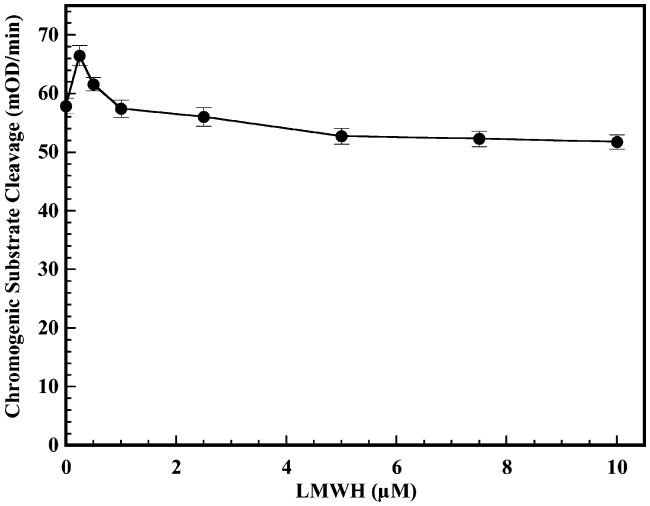

FIGURE 5: Effect of LMWH on chromogenic substrate cleavage by the factor IXa−phospholipid complex. Increasing concentrations of low-molecular weight heparin were added to 40 nM factor IXa, 100 μM PC/PS (75/25) vesicles, and 2.5 mM Pefachrome IXa (CH_3-SO_2-D-CHG-Gly-Arg-pNA) in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1 mg/mL BSA, 0.1% PEG-8000, and 30% (v/v) ethylene glycol. The rate of chromogenic substrate cleavage was determined by monitoring the change in absorbance at 405 nm over the course of 10 min. Mean values were plotted with error bars representing the standard deviation ($n = 3$).

IXa−phospholipid complex (Figure 4) was 3−5-fold higher than in the presence of factor VIIIa (Figure 1). These data suggest that the presence of cofactor reduces the apparent affinity of LMWH for the factor IXa−phospholipid complex. In contrast, varying the factor X concentration produced minimal change (less than 2-fold) in the apparent inhibitor affinity for LMWH. Inhibition of factor X activation by the intact intrinsic tenase complex is clearly the physiologically relevant effect of LMWH, since the factor IXa−phospholipid complex possesses less than 1% of the activity of the intact complex. However, the ability of LMWH to inhibit residual factor X activation in the absence of cofactor suggests an additional inhibitory mechanism for the longer oligosaccharide chains.

Effect of LMWH on Chromogenic Substrate Cleavage by the Factor IXa−Phospholipid Complex. The active site-directed inhibitor benzamidine inhibits prethrombin-2 activation by the prothrombinase complex in a noncompetitive pattern, reflecting the dominant role of exosite interactions in macromolecular substrate recognition by coagulation proteases (*26*, *27*). To directly assess the effect of oligosaccharide on the active site of the factor IXa−phospholipid complex, the effect of LMWH on cleavage of the chromogenic substrate Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) by the factor IXa−phospholipid complex was examined in the presence and absence of 30% ethylene glycol (EG). In the presence of 30% EG, under conditions similar to those used for factor X activation, LMWH did not significantly inhibit chromogenic substrate cleavage by the factor IXa−phospholipid complex (Figure 5). A modest stimulatory effect was noted at low oligosaccharide concentrations, but no inhibition of chromogenic substrate activity was observed over the entire concentration range that produced maximal inhibition of factor X activation by either the factor IXa−factor VIIIa or factor IXa−phospholipid complex. In the absence of EG, with the concentration of factor IXa increased to 100 nM to enhance the baseline rate, LMWH similarly did not demonstrate any significant inhibition of chromogenic substrate cleavage (data not shown). Thus, heparin oligosaccharides do not appear to bind directly to the active site or S4/S3-S1 subsites of factor IXa. The partial, noncompetitive inhibition pattern observed for intrinsic tenase, and the partial inhibition of the factor IXa−phospholipid complex with an apparent lack of competition between substrate and LMWH, suggest that LMWH binds to a site distinct from the macromolecular substrate binding site, and that the enzyme−substrate−inhibitor complex (ESI) is productive. Together, these findings indicate that LMWH inhibits factor X activation by interaction with an enzymatic exosite on the factor IXa−phospholipid complex, which is distinct from both the active site and factor X binding (exo)-sites.

Effect of LMWH on Factor IXa−Factor VIIIa Affinity. To address whether the decrease in $V_{max(app)}$ for factor X activation was due to effects on enzyme complex assembly (decreased effective enzyme concentration) or reduced catalytic activity of the ESI complex, the effect of LMWH on factor IXa−factor VIIIa affinity was assessed in a functional binding assay. On phospholipid vesicles, the maximal activity (B_{max}) for the enzyme complex was significantly reduced even at high factor IXa concentrations in the presence of 3 μM LMWH (Figure 6A). However, the $K_{D(app)}$ for the factor IXa−factor VIIIa complex was only modestly increased in the presence of LMWH (from 5.6 to 10.0 nM). This modest reduction in affinity is consistent with the results of direct binding studies in which porcine fluorescein-maleimidyl-Phe-Pro-Arg-factor IXa (Fl-M-FPR-fIXa) and size-fractionated heparin were employed (*4*). Under the experimental conditions that were employed, this reduced affinity predicts that the concentration of the factor IXa−factor VIIIa complex would be 60% of control values (eq 1). This reduction in the concentration of the factor IXa−factor VIIIa complex was insufficient to explain the degree of inhibition observed under these conditions (10−15% residual activity). These results suggest a direct effect of LMWH on factor X catalysis by the factor IXa−factor VIIIa complex bound to the phospholipid surface.

In contrast, increasing amounts of LMWH shifted the binding curve significantly to the right in the presence of soluble phospholipid (C6PS), indicating a marked reduction in the apparent affinity of the factor IXa−factor VIIIa interaction (Figure 6B). The progressive increase in $K_{D(app)}$ (from 1.9 to 9.6 nM) under these conditions predicts that the concentration of the factor IXa−factor VIIIa complex (eq 1) would be approximately 73, 43, and 34% of control values in the presence of 0.75, 1.5, and 3 μM LMWH, respectively. Thus, the predicted reduction in the concentration of the factor IXa−factor VIIIa complex with an increasing amount of LMWH is sufficient to explain the magnitude of the observed inhibition. In further contrast to the reaction on phospholipid vesicles, the maximal activity (B_{max}) of the enzyme complex in the presence of LMWH was similar to control values, suggesting competition of the LMWH interaction at high factor IXa concentrations. Thus, in solution, the inhibition of factor X activation by LMWH occurred via a reduction in the extent of factor IXa−factor VIIIa complex formation that was largely overcome in the presence of excess factor IXa. This reduction in the apparent

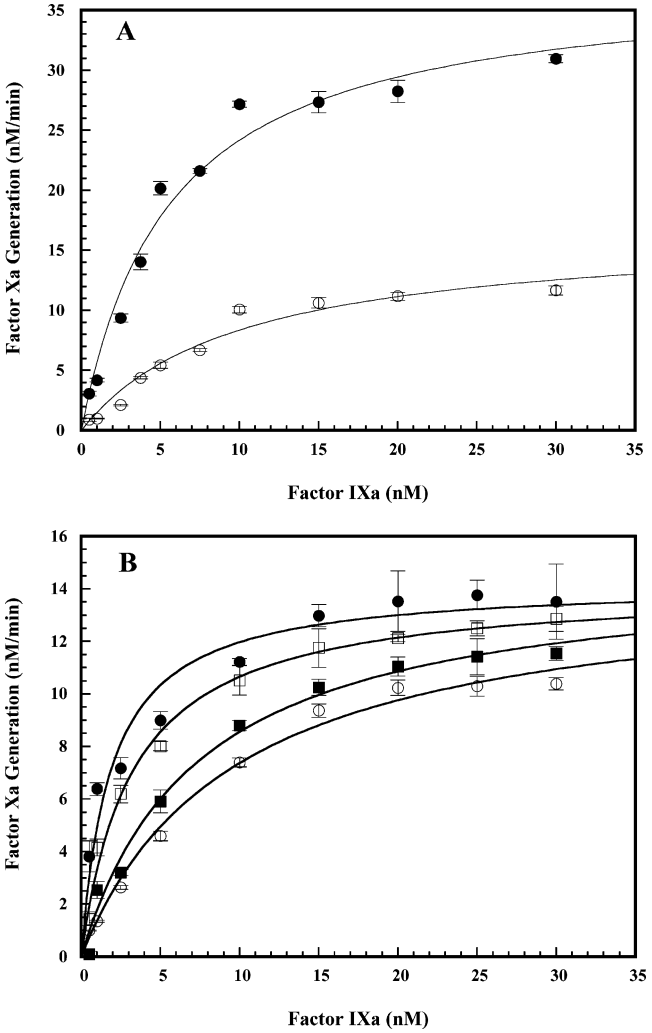

FIGURE 6: Effect of LMWH on the affinity of the factor IXa−factor VIIIa complex in the presence of (A) phospholipid vesicles or (B) soluble phospholipid (C6PS). The apparent affinity [$K_{D(app)}$] of the factor IXa−factor VIIIa interaction was determined using enzymatic activity to detect complex formation. The rate of factor X activation by the factor IXa−factor VIIIa complex was determined by titration of factor IXa (0−30 nM) into a reaction mixture containing 0.3 nM factor VIIIa, 200 nM factor X, and either 100 μM PC/PS vesicles (A) or 0.25 mM C6PS (B). Titrations were done in the absence (●) or presence of 0.75 (□), 1.5 (■), or 3.0 μM (○) LMWH. Mean values were plotted with error bars representing the standard deviation ($n = 3-4$). The $K_{D(app)}$ was determined by fitting the data to a single-site binding model. For PC/PS vesicles, the $K_{D(app)}$ and B_{max} values ± standard error in the absence of heparin were 5.6 ± 1.0 nM and 38 ± 2 nM/min, respectively, and in the presence of LMWH 10.0 ± 2.4 nM and 17 ± 2 nM/min, respectively. For soluble C6PS, the $K_{D(app)}$ and B_{max} values ± standard error in the absence of heparin were 1.9 ± 0.4 nM and 14.2 ± 0.6 nM/min, respectively. In the presence of 0.75, 1.5, or 3.0 μM LMWH, the values were 3.3 ± 0.3 nM and 14.1 ± 0.4 nM/min, 7.4 ± 1.2 nM and 14.9 ± 0.8 nM/min, or 9.6 ± 1.4 nM and 14.4 ± 0.8 nM/min.

factor IXa−factor VIIIa affinity with LMWH present is consistent with the observed reduction in the apparent affinity of LMWH for the factor IXa−phospholipid complex when factor VIIIa was present (Figures 1 and 4). Likewise, a reduction in the apparent affinity of LMWH for the factor IXa−factor VIIIa complex in the presence of C6PS (1.2 μM) versus PC/PS vesicles (0.3 μM) is consistent with the increased affinity of the factor IXa−factor VIIIa complex in the presence of C6PS (1.9 nM) versus phospholipid vesicles (5.5 nM). These results indicate that the binding of either LMWH or factor VIIIa to the factor IXa−phospholipid complex reduces the apparent binding affinity for the other ligand. Thus, factor VIIIa and LMWH must compete for overlapping binding sites on factor IXa, or induce opposing protease conformations.

The ability of heparin to antagonize cofactor function on the phospholipid surface without significantly disrupting the apparent affinity of the factor IXa−factor VIIIa interaction is consistent with structure−function analysis of the individual factor VIIIa domains. On the phospholipid surface, the A3−C1−C2 domain of factor VIIIa binds to factor IXa with relatively high affinity ($K_D = 14$ nM) but possesses no cofactor activity for factor X activation (*28*, *29*). In contrast, the A2 domain of factor VIIIa binds factor IXa on the phospholipid surface with low affinity ($K_D = 300$ nM) but possesses significant cofactor activity (*30*). This cofactor activity is enhanced by the A1 domain, which stabilizes the interaction of the A2 domain with the protease (*31*). Site-directed mutagenesis of the factor VIIIa A2 domain markedly reduces maximal cofactor activity (B_{max}) for factor X activation in a functional binding assay, without dramatically affecting the apparent affinity of the factor IXa−factor VIIIa complex on phospholipid vesicles (*32*). This pattern is similar to that observed with LMWH on phospholipid vesicles. Thus, heparin may antagonize A2 domain cofactor activity in a tertiary factor IXa−factor VIIIa−LMWH complex without disrupting the interaction with the A3−C1−C2 domain that is largely responsible for the affinity of factor VIIIa−factor IXa binding on the phospholipid surface. In the presence of C6PS, elimination of protein−membrane interactions enhances the ability to detect disruptions in specific protein−protein interactions by LMWH, resulting in more ideal behavior in the functional binding assay.

Assessment of the Affinity of Factor IXa and Factor X for Low-Molecular Weight Heparin. The ability of LMWH to inhibit factor X activation in the presence and absence of factor VIIIa or phospholipid membrane suggests that either factor IXa or the factor IXa−factor X complex represents the primary molecular target. The relative affinity of LMWH for factor IXa and factor X was assessed by a competition solution affinity approach using surface plasmon resonance to detect free ligand (*33*, *34*). This approach allowed determination of an EC_{50} for solution competition by LMWH and avoided the difficulties in determining equilibrium binding constants from surface association and dissociation rates with mass transport-limited data (*18*, *33*). Factor IXa or factor X was injected over a streptavidin sensor chip containing an immobilized biotin-X-hydrazide reference surface, and a high-density immobilized biotin-LMWH surface (see Experimental Procedures). The dose response for factor IXa demonstrated a surface plasmon resonance binding response markedly higher than that of factor X, which required significantly higher protein concentrations despite a larger molecular mass (molecular mass of factor X = 58 900 Da, and molecular mass of factor IXa = 46 000 Da) (Figure 7A,B). Since the surface plasmon resonance response is directly proportional to the mass of bound protein, factor IXa clearly bound immobilized LMWH with a higher affinity than factor X.

Competition curves were determined at ligand concentrations less than or equal to the observed EC_{50} values.

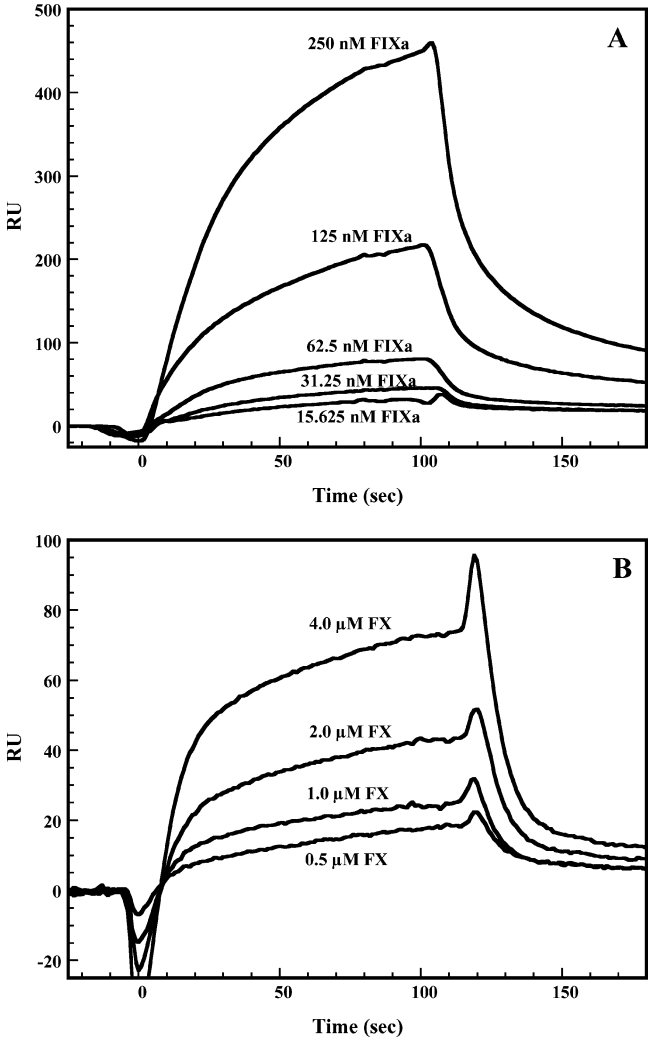

FIGURE 7: Surface plasmon resonance dose response for human factor IXa (A) and factor X (B). Increasing concentrations of factor IXa (0−250 nM) and factor X (0−4 μM) were injected over the reference and immobilized LMWH surfaces at a rate of 5 μL/min in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 0.05% Tween 20 surfactant for a 120 s association phase, followed by buffer with 2 μM LMWH for a 180 s dissociation phase. Final sensogram responses were obtained by subtraction of the reference surface and sham (buffer only) responses from the LMWH surface response. Sensograms are labeled with individual protease concentrations. The binding response at 90 s postinjection was used to construct a standard curve for measurement of the concentration of free factor IXa and factor X, respectively, in the competition assay.

Increasing concentrations of LMWH were incubated with either 250 nM factor IXa or 2.5 μM factor X prior to injection, and the remaining free factor IXa or factor X concentration was determined by comparison with their respective calibration curves. The concentration of competing LMWH was plotted versus the proportion of remaining free factor IXa or factor X, and the data were fit to eq 2 to determine the EC_{50} for the LMWH competitor. Fitting of the data demonstrated EC_{50} values of 0.28 μM for the factor IXa−LMWH interaction and 3.5 μM for the factor X−LMWH interaction (Figure 8). Thus, the EC_{50} values obtained from competition curves also demonstrated that factor IXa bound to LMWH in solution with higher affinity than factor X. The slope of the competition curves was relatively shallow and yielded pseudo-Hill coefficient values of <1, consistent with the expected multivalent nature of the protease−heparin interaction (*20*). The reduced direct binding response of factor X relative to factor IXa, and the significantly higher EC_{50} for competition of factor X with soluble LMWH, suggests that factor IXa has a significantly higher affinity for LMWH than factor X.

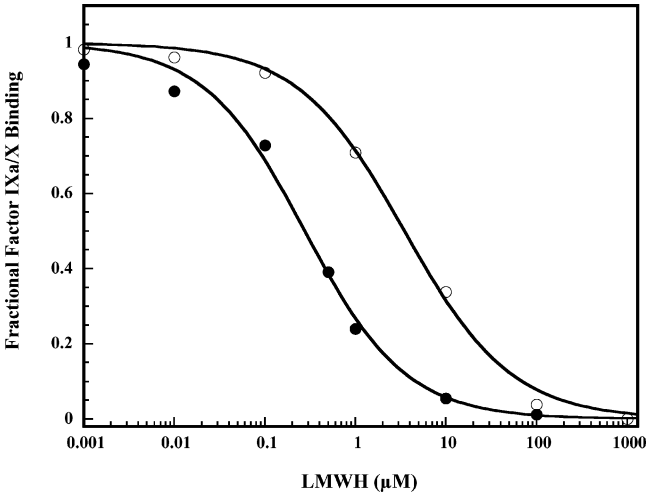

FIGURE 8: Competition solution affinity of low-molecular weight heparin for factor IXa and factor X. Increasing concentrations of LMWH were preincubated for 20 min with either 250 nM factor IXa (●) or 2.5 μM factor X (○) prior to injection over reference and immobilized LMWH surfaces on the BiaCore 2000 instrument. Injections were performed and sensograms generated as described in the legend of Figure 7. The concentration of free factor IXa or factor X was determined by the binding response 90 s after injection, with comparison to standard curves generated under identical conditions (A and B). The concentration of low-molecular weight heparin was plotted vs the proportion of remaining free factor IXa or factor X and fit by nonlinear regression to eq 2 (see Experimental Procedures) to obtain the EC_{50} for competition by low-molecular weight heparin. The fitted values for the EC_{50} ± standard error were 0.28 ± 0.04 μM for factor IXa and 3.5 ± 0.4 μM for factor X.

Immobilized LMWH was lightly biotinylated (one or two biotin groups estimated per oligosaccharide chain), and the competition was performed at a low factor IXa binding density, suggesting that $K_D \cong K_I$ is a reasonable assumption for the LMWH−factor IXa interaction. Using this assumption, the observed EC_{50} from the LMWH competition results in a $K_{D(app)}$ of ≈0.03 μM (eq 3). This value agrees with the apparent affinity ($K_I = 0.059$ μM) observed for inhibition of the factor IXa−phospholipid complex by LMWH in kinetic assays (Figure 4). This estimate represents a functional (not a site-specific) K_D, as it does not account for a multivalent, nonspecific electrostatic model of protease binding to the linear heparin polymer (*20*, *21*). However, this functional K_D is an appropriate comparison for the apparent inhibitor affinity (K_I) obtained in kinetic assays. Although a contribution of the factor X or factor Xa heparin binding site to the inhibition cannot be completely excluded, the kinetic and equilibrium binding analysis suggest that a heparin-binding exosite on factor IXa is the primary molecular target for inhibition by LMWH.

Proposed Model for Heparin Inhibition of the Intrinsic Tenase Complex. Type II hemophilia B mutations and site-directed mutagenesis of factor IXa have identified factor VIIIa interactive regions in the α-helix of residues 162−170 (c162−170 α-helix) and the loop of residues 132−134 of the catalytic domain (chymotrypsin numbering) (*35*, *36*)

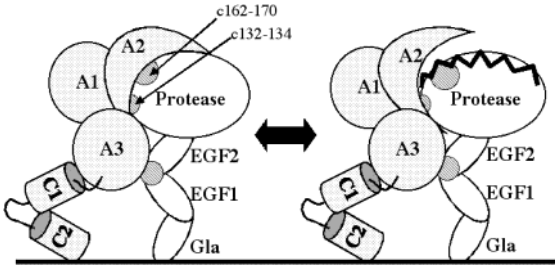

FIGURE 9: Model for antithrombin-independent inhibition of the intrinsic tenase complex by heparin oligosaccharide. Schematic representation of factor IXa (white) and factor VIIIa (stippled) assembled on the phospholipid surface. Domain structures of factor IXa (Gla, EGF1, EGF2, and protease) and factor VIIIa (A1, A2, and A3−C1−C2) are labeled. Putative factor VIIIa interactive sites on factor IXa are highlighted (crosshatched areas). Heparin oligosaccharide bound to the C-terminal α-helix of factor IXa is represented with a thick black chain, with displacement of the factor VIIIa A2 domain.

and the EGF1−EGF2 region of the light chain (*37*−*39*). Modeling the factor IXa−factor VIIIa interaction suggests that the factor VIIIa A2 domain interacts with the c162−170 α-helix, and surface contacts extend toward the C-terminal α-helix of the protease domain (*40*). An exosite extending across the protease surface from the c162−170 α-helix along the C-terminal α-helix has recently been implicated in heparin-dependent acceleration of gla-domainless factor IXa inhibition by antithrombin. (*41*). This region is homologous to heparin-binding exosites identified by site-directed mutagenesis of factor Xa and thrombin (*42*, *43*). Consistent with our findings, these studies suggest that binding sites for the A2 domain and heparin overlap on the factor IXa protease domain. The binding of heparin oligosaccharide to an exosite involving the C-terminal α-helix would also block interaction of the factor VIIIa A2 domain with the c162−170 helix of factor IXa (Figure 9). Disruption of this interaction has only a modest effect on the affinity of factor IXa−factor VIIIa binding on the phospholipid surface, as the interaction of the factor IXa light chain with the factor VIIIa A3−C1−C2 domain is unaffected (*37*). In contrast, oligosaccharide bound to this exosite would be well positioned to antagonize the cofactor activity of the factor VIIIa A2 domain. Consistent with this model, factor IXa R170A (located in the c162 α-helix) demonstrates relative resistance to inhibition by unfractionated heparin in the intrinsic tenase complex, suggesting that this surface residue may participate in the antithrombin-independent inhibition of intrinsic tenase (*44*). Phosphorothioate oligonucleotides also inhibit factor X activation by intrinsic tenase via a similar interaction with an exosite on factor IXa (*13*). Additionally, longer oligosaccharide chains (unfractionated or low-molecular weight heparin) may have extended interactions on the factor IXa surface that inhibit factor X activation by interfering with the transfer of the exosite-bound substrate to the protease active site. Defining the structural correlates of these macromolecular binding sites on factor IXa will identify novel molecular targets for antithrombotic therapy. Partial inhibition of intrinsic tenase activity via these exosite-mediated mechanisms may yield antithrombotic agents with improved specificity and a larger therapeutic window.

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