Chemistry and Biology of Heparin and Heparan Sulfate
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Chapter 11

Heparin Regulation of the Complement System

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I. Introduction

The complement system consists of approximately 25 proteins that work to complement the activity of antibodies in destroying bacteria, either by facilitating phagocytosis or by puncturing the bacterial cell membrane resulting in bacterial cell lysis (1). Heparin, a clinically used anticoagulant, is a polydisperse, highly sulfated, linear polysaccharide consisting of $1 \rightarrow 4$ linked uronic acid and glucosamine residues (2,3). The biological activities of pharmaceutical heparin are reviewed elsewhere (2–6).

Heparin can bind to a variety of proteins, including growth factors (7), proinflammatory chemokines and cytokines (6), extracellular matrix proteins (8), and complement proteins (9). Binding takes place primarily through electrostatic interactions between heparin's anionic groups (sulfo and carboxyl), and the positively charged amino acid residues (arginine and lysine) of the heparin-binding proteins (10,11). Heparin–protein interactions regulate such diverse processes as coagulation, homeostasis (12), cell adhesion (8), lipid metabolism (13), growth factor signal

transduction (7), and complement-mediated cell lysis (9), which is the subject of this chapter.

II. Background and History

A. Heparin

1. Structure

Heparin is acidic (average negative charge of −100) and polydisperse (chains ranging in molecular weight from 5000 to 40,000) (2,14). It is comprised of a major (75–95%) alternating disaccharide having the structure *N*-sulfo-6-*O*-sulfo-β-D-glucosamine (1→4) 2-*O*-sulfo-α-L-iduronic acid. Minor sequences containing variable levels of *O*- and *N*-sulfo groups and containing glucuronic acid in place of iduronic acid are also found in heparin (15) (Fig. 1A). Heparin, localized intracellularly in the cell granules of mast cells and basophils, is biosynthesized as serglycin proteoglycan (PG, M_r ~1500 kDa), consisting of the central core protein, from which approximately 11 long-linear polysaccharide chains (M_r ~100 kDa) extend (Fig 1B) (16). Once released from core protein, the polysaccharide chains are cleaved by proteases to form peptidoglycan heparin, a single long polysaccharide chain attached to a small peptide (M_r 100 kDa), which is immediately processed by a β-endoglucuronidase to generate a number of smaller polysaccharide chains called glycosaminoglycan (GAG) heparin (12).

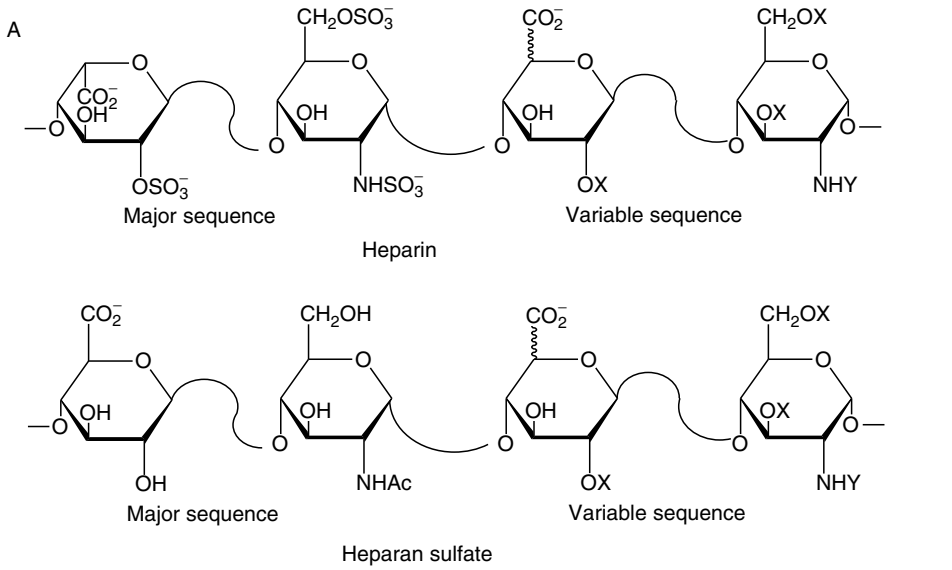
2. Biological Activities and Therapeutic Potential

Heparin has a variety of biological activities, many of which are of interest because of their potential therapeutic utility (17,18). By regulating the activity of heparin-binding proteins, heparin and the related GAG, heparan sulfate, can influence various biological processes giving heparin therapeutic applications as an antithrombotic, antiatherosclerotic, anticomplement, antiinfective, anticancer, and antiinflammatory agent (16,19–29). The heparin-binding proteins that represent therapeutic targets include enzymes, protease inhibitors, lipoproteins, growth factors, chemokines, selectins, extracellular matrix proteins, receptor proteins, viral coat proteins, and nuclear proteins (30).

B. Complement System

1. General Description of the Complement System

Complement is a major defense and clearance system in the bloodstream and is comprised of a series of approximately 25 different proteins (Table 1) (1,30,31). The complement system can be activated in three main ways: the classical pathway, the alternative pathway, and the mannose-binding lectin pathway. The most potent activation occurs when antibody (IgG or IgM) binds to antigen at the surface of a cell (Fig. 2). This results in the immune triggering of the classical pathway.



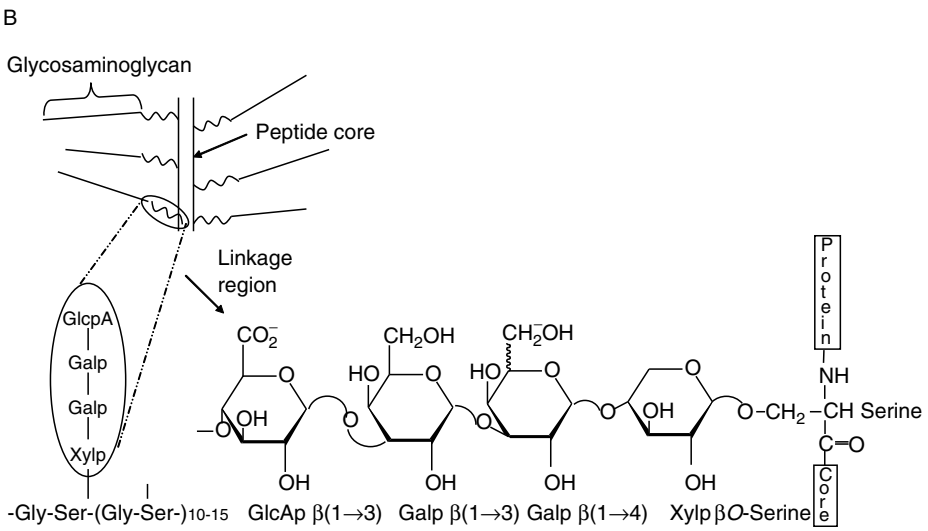


Figure 1 Heparin structure. (A) The sequence of heparin and the related heparan sulfate are shown, where X, SO_3^-, or H and Y, SO_3^-, $COCH_3$, or H. (B) The structure of heparin proteoglycan serglycin and the linkage region between core protein and GAG chain.

Table 1 Serum Concentrations of Selected Complement Proteins

Complement protein	Concentration in serum (μg/ml)	Concentration in serum (nM)
C1q	75	182
C1r	34	178
C1s	30	344
C2	15–20	130–173
C3	1500	8333
C4	430	2047
C5	75	394
C6	60	468
C7	60	495
C8	80	490
C9	58	734
C1INH	262.5	2500
C4BP	250	454
Factor B	120–300	1290–3225
Factor D	1	41
Factor P	20	90
Factor H	470	3133
Factor I	34	386
Factor J	2.6–8.2	130–410
MBL (mannan binding lectin)	1	1.85

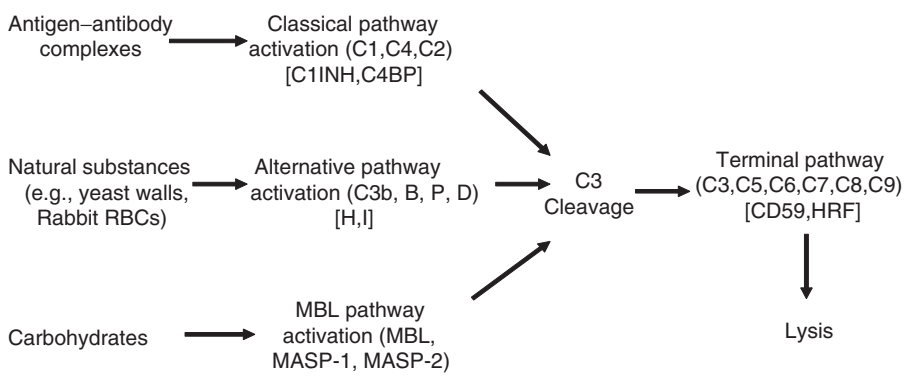


Figure 2 Overview of the three pathways of complement cascade.

The second means of activation, associated with the alternative or properdin pathway, is activated by direct contact with activated C3 that is deposited on a variety of surfaces including pathogens, such as viruses, and fungi, but also host cells in autoimmune disorders (30). The mannose-binding lectin (MBL) pathway, the most recently recognized activation pathway, leads to complement activation after contact with mannose on the cell surface of pathogens such as bacteria (Fig. 2).

In all three cases, a cascade of events follows, in which each step leads to the next. At the center of the cascade are steps, in which the proteolysis of a complement protein (C) leads to a smaller protein and a peptide (usually designated Ca, Cb). The smaller protein remains bound to the complex at the surface of the microorganism, while the peptide diffuses away. Both pathways converge at the fifth complement protein, C5, ultimately leading to the assembly of a multiprotein complex on the bacterial cell membrane known as the membrane attack complex (MAC), which lyses the bacterial cell.

2. Role of Complement in Health and Disease

a. Biosynthesis of Complement Components

Soluble complement components, with the exception C1q, factor D, properdin, and C7, are primarily synthesized in the liver (32). Extrahepatic biosynthesis also has been observed for most components. Complement protein secretion is increased by various mediators of inflammation, such as cytokines/chemokines, including tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6). C1q is biosynthesized primarily in macrophages. The spleen appears to be the major organ involved in properdin biosynthesis. Adipocytes in the fat tissue are responsible for factor D biosynthesis.

b. Disease Associated with Complement System

Complement deficiencies result in frequent infections and immune complex diseases (33). With the exception of C9, deficiencies have been identified in all of the complement factors, including Factor D and properdin. Deficiencies have also been identified in the complement regulatory proteins C1INH, Factors I and H, DAF (complement decay accelerating factor), and HRF (homologous restriction factor). In general, deficiencies in complement components result in increased bacterial infections, especially with *Neisseria* species, resulting from reduced bacterial opsonization and phagocytosis (34).

The immune complex diseases are quite varied. These diseases include rheumatoid arthritis, where an inflammatory response is induced by antibody–antigen complexes in the synovial fluid; types of glomerulonephritis, due to complex trapping within the glomerulus, or retention of antibody–antigen in the glomerulus; extrinsic allergic alveolitis, as in ''farmer's lung'', where antigens are inhaled; filariasis, where antigens released from parasites in the lymphatic vessels; and erythema nodosum, where the chemotherapy treatment of patients with high levels of antibody against the leprosy bacillus results in antigen release and immune complex formation.

A series of inflammatory activities, including the induction of smooth muscle contraction, vasodilation, and an increase in vascular permeability have been attributed in large part to the activation of two peptides (C3a and C5a), released by the proteolytic action of the convertases on C3 and C5 (Fig. 3).

III. Heparin Regulation of the Complement System

A. Early Studies of Heparin-Complement Interaction

Ecker and Gross (35) reported nearly 70 years ago that heparin-like polyanions exhibit anticomplementary activity through the direct interaction of heparin with complement proteins. Ecker and Gross also showed that heparin did not inhibit lysis by binding to red cell membranes (9,35). Rosenberg and coworkers (36) reported in the early 1980s that heparin interacted with C1q, subsequently Kazatchkine and coworkers (37) demonstrated multiple sites in the classical and the alternative amplification pathways of complement, at which heparin may act. In 1993, Sahu and Pangburn (38) examined the binding activity of the normal human serum on heparin-agarose using affinity chromatography. They found that 13 complement proteins (C1q, C2, C4, C4bp, C1INH, B, D, H, P, C6, C8, C9, and vitronectin) bound heparin, while nine complement proteins (C1r, C1s, C3, Factor I, C5, C7, C3b, Ba, and Bb) did not bind heparin. Factor J, a highly glycosylated complement inhibitor glycoprotein, which acts on the classical and the alternative pathways, was also found to strongly interact with heparin and the structurally related heparan sulfate (39). Heparin directly inhibits C1q binding to immune complexes, inhibits the interaction of C1s with C4 and C2, and inhibits the binding of C2 to C4b (40). Heparin also inhibits formation or binding of the complement protein trimolecular complex, C5b67 (41). Heparin inhibits cobra venom factor (CoVF)-dependent C3 inactivation in whole serum, thereby having an impact on the generation and action of the C3 convertase formed from CoVF, factors B and D (42). Edens et al. (9) described the multiple effects of heparin on the classical and

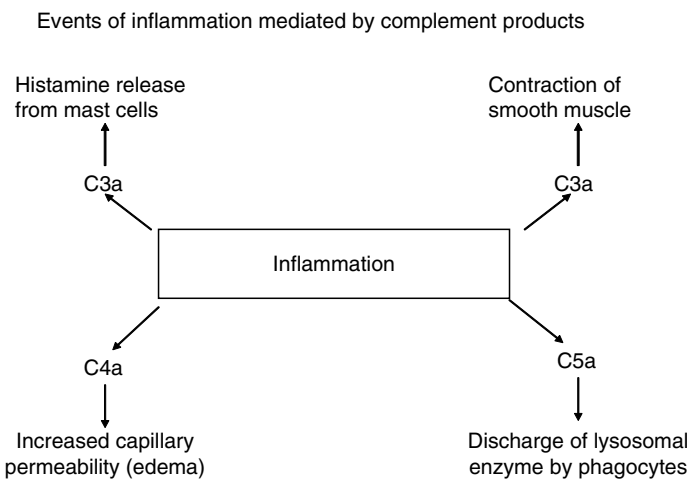


Figure 3 The role of complement components in the events of inflammation.

alternative pathways (Fig. 4). The MBL consists of a carbohydrate recognition domain (CRD) that binds to side chains on glycoconjugates rich in D-mannose and *N*-acetylglucosamine (43). However, little is known about the specific effects of sulfated polysaccharides on the recently described MBL pathway of complement activation.

Cofrancesco et al. (44) observed that heparin's inhibitory effect on the complement cascade is time-dependent and reversible. In addition, the size of the heparin chain and its chemical modification can influence and extent of its inhibitory activity (19). However, these previous reports on heparin-complement interactions are generally qualitative and lack kinetic and thermodynamic data, making it difficult to conclude what the real impact heparin has in the complement proteins of the classical and alternative pathways. This is particularly important when comparing the very low endogenous levels of circulating heparin and heparan sulfate with the high conventional therapeutic concentrations of exogenously administered heparin. More recently developed technologies, such as surface plasmon resonance (described later), better permit kinetic and thermodynamic determination of polysaccharide-complement interactions.

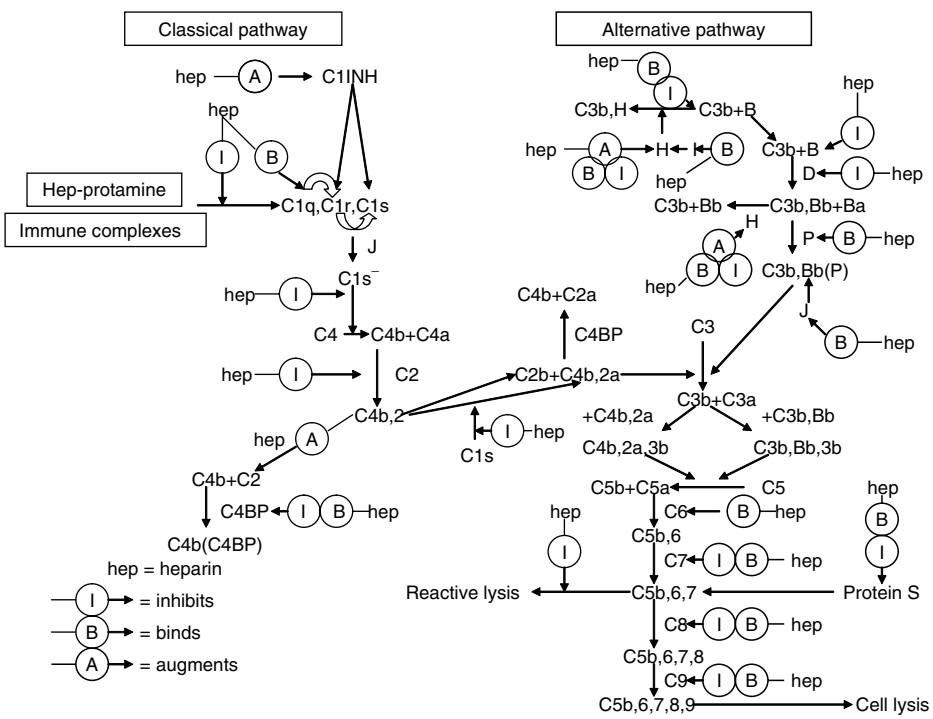


Figure 4 Current view of heparin effects on the classical and alternative pathways.

B. The Effects of Heparin on the Classical Pathway

1. Heparin Inhibition of C1 Activity

The classical pathway is triggered by C1, a protein composed of three functionally different subunits: C1q, C1r, and C1s. The recognition protein C1q ($\sim 75\,\mu g/ml$ in normal adult serum) binds to the Fc portion of an antibody. The catalytic subunit is the tetramer C1s–C1r–C1r–C1s that is formed in a calcium-dependent manner (45). C1s enzymatically cleaves the next complement protein, C4, while C1r acts as a bridge connecting C1q to C1s (46). Most C1-complexing ligands are recognized by the C1q moiety, generating a conformational signal that triggers self-activation of C1r that in turn activates C1s (47).

The interaction of heparin with C1 was examined using both affinity chromatography on heparin-Sepharose and by fluorescence polarization measurement using fluorescein-labeled heparin (48). Almeda et al. (36) measured the interaction of a radiolabeled low molecular weight (LMW) heparin ($M_r = 8500$) (6), and found two K_d values for C1q binding, high-affinity binding constant of 76.6 nM a low-affinity binding constant of 1.01 μM. They also reported that LMW–Hep (2.5 nM) inhibited the ability of C1q (0.5 nM) to recombine with C1r (1.4 nM) and C1s (1.6 nM) to form hemolytically active C1 and at 250 mM, LMW–Hep inhibited the hemolytic activity of reconstituted C1.

In a recent study in our laboratory (49), surface plasmon resonance (SPR) spectrometry was utilized to determine the kinetic and thermodynamic parameters for C1q-heparin interaction. Biotinylated heparin, immobilized to a streptavidin chip, interacted with fluid phase C1q. A sensorgram for interaction of C1 and heparin is shown in Fig 6A. Immobilized heparin interacted with C1, giving an on-rate constant (k_{on}) value of $1.95 \times 10^5\,M^{-1}s^{-1}$, off-rate constant ($k_{off}$) of $5.39 \times 10^{-3}\,M$, and K_d of 27.6 nM (Table 2). The calculated K_d is comparable to the high affinity heparin-binding data for C1q, obtained by Almeda. Therefore, our result confirms that the interaction between C1 and heparin can primarily be attributed to the C1q. Based on the structure of C1 complex (Fig. 5) and 1:1 stoichiometry of the binding, we suggest that heparin binds only to the collagenous stalk region of C1q, showing no interaction with the C1q globular region.

Heparin inhibits C1 functional activity when it binds to C1. Raepple et al. (40) reported that incubation of heparin, or other selected polyanions (including dextran sulfate, polyvinyl sulfate, and chondroitin sulfate), with C1, C4, and C2 separately, reduced most of the hemolytic activity of C1, but had no effect on that of C2 and C4. These experiments demonstrated that all the tested polyanions strongly inactivated C1 and that the extent of this inactivation differed with each polyanion. In addition, all the polyanions examined also inhibited the consumption of C2 and C4 by C1, prevented the uptake of C2a by C4b and promoted the dissociation of C4b2a. This report was further confirmed by Strunk and Colten (50), who found that heparin noncompetitively inhibited the cleavage of C4 and C2 by C1. Heparin has a more pronounced effect on C2 cleavage than on C4 cleavage (50,51).

Table 2 Kinetic and Affinity Constants for Heparin-Complement Protein Binding

Complement protein	Chip surface	$k_{on}(M^{-1}s^{-1})$	$k_{off}(s^{-1})$	K_d (nM)
C1	DEX-SA	1.95×10^5	5.39×10^{-3}	27.6
C2	DEX-SA	4.13×10^3	1.32×10^{-3}	320
C3	DEX-SA	1.83×10^4	5.73×10^{-4}	31.2
C4	DEX-SA	9.64×10^4	3.46×10^{-3}	60.8
C5	DEX-SA	2.43×10^5	1.35×10^{-3}	21.9
C6	DEX-SA	1.72×10^4	5.58×10^{-4}	31.3
C7	DEX-SA	6.25×10^4	9.60×10^{-4}	12.8
C8	DEX-SA	4.17×10^4	5.74×10^{-3}	173
C9	DEX-SA	3.43×10^4	4.51×10^{-3}	131
C1INH	DEX-SA	1.58×10^4	4.64×10^{-4}	29.4
Factor I	DEX-SA	4.45×10^4	1.03×10^{-3}	36.2
Factor H	DEX-SA	1.84×10^4	7.25×10^{-3}	399
Factor B	PEG-NA	2.58×10^5	2.77×10^{-4}	2.10
Factor P	PEG-NA	3.84×10^4	8.74×10^{-5}	26.8

Two chips were used to acquire these data: the dextran-streptavidin (DEX-SA) chip and the poly(ethylene glycol)-neutravidin (PEG-NA) chip.

2. Heparin Augmentation of C1 Inhibitor (C1INH) Activity

C1 inhibitor (C1INH) is a 105 kDa plasma protein, which is the only serine protease inhibitor known to dissociate the activated C1r and C1s from C1q, thus limiting the time this complex is active (51,52). C1 inhibitor also blocks spontaneous activation of C1 by plasma proteases. A deficiency in C1INH is associated with sudden, life-threatening episodes of angioedema.

As early as 1976, Nagaki and Inai (53) found the presence of heparin in the reaction mixture of C1INH, and C1s substantially enhanced C1INH inhibition of

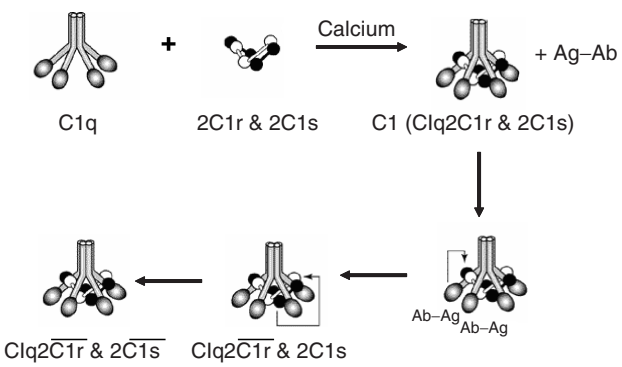


Figure 5 Schematic representation of activation of three components of C1: C1q, C1r, and C1s. The globular region and the collagenous stalk region of C1q were also specified.

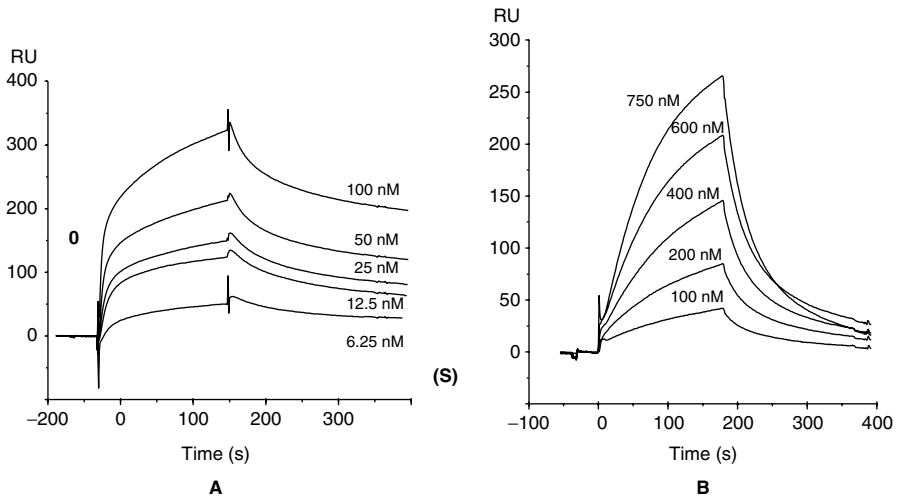


Figure 6 SPR analysis of (A) C1 ($C1qC1r_2C1s_2$)-heparin interaction; (B) C1INH-heparin. Increasing concentrations of C1 (100, 50, 25, 12.5, and 6.25 nM) and C1INH (100, 200, 400, 600, and 750 nM) were injected over sensor chip containing biotinylated heparin, which was bound to immobilized streptavidin. The amount of protein associating with the heparin was measured in resonance units. Identical samples were also injected over a control chip containing streptavidin but no heparin (nonspecific binding), and the sensorgrams shown in this figure were obtained after subtraction of nonspecific binding.

C1s (54–56). Other GAGs including chondroitin sulfate A, B, and C were also found effective in augmenting the C1INH inhibition of C1s, but to a lesser extent than heparin. Kinetic studies indicated that heparin potentiated C1IHN inhibitory activity by up to 15- to 35-fold. The SPR studies of the interaction of heparin and heparan sulfate with C1INH were first reported by our laboratory in 1999 (57). Heparin, immobilized on a biosensor chip gave a K_a value of $7 \times 10^6\,M^{-1}$. These data showed somewhat weaker binding than our recently obtained results (49) (Fig. 6B), which affords a K_a of $3.41 \times 10^7\,M^{-1}$ and K_d of $2.94 \times 10^{-8}\,M$, respectively (Table 2). The discrepancy in these results may be caused by differences in the heparin–biotin conjugates or the surface density of the immobilized heparin. The proximity of the protein-binding site to the point of immobilization might interfere with protein binding, having a major impact on the measured affinity constants.

The order of interaction between C1, C1INH, and heparin was also determined by protease inhibition experiment (57). The results suggest that 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. Summarily, heparin interacts with C1INH and augments its inhibition of C1. The concentrations of heparin required for binding and augmentation are within those achieved by inhalation therapy in patients (58,59). Future studies will be required to determine if the heparin–C1INH

interaction has biological significance *in vivo* and, if this is the case, suggests a potential new therapy for treating hereditary angioedema.

C. Effect of Heparin on C2 and C4 Activity

C2 is one of the least abundant of the complement components in the human plasma, present at a concentration of 15–20 μg/ml (Table 1) (60). C2 is a single chain glycoprotein with an M_r of 100 kDa (61). C4 is the second complement protein to undergo reaction in the classical pathway of the complement cascade. C4 is a β-globulin with an M_r of 240 kDa and is found at a serum concentration of 430 μg/ml (Table 1) (62).

The role of C2 and C4 in the classical pathway has been reviewed extensively (9,54,60,61). C4 is cleaved by the C1s domain of the membrane bound C1qC1rC1s complex with the release of a small and inactive peptide, C4a. The larger C4b ($M_r = 198$ kDa) fragment binds covalently to sugar residues on cell-surface glycoproteins through ester bond formation (63). C4b serves as a receptor for C2 by forming an Mg^{2+}-dependent complex with C2. C2 is then rapidly cleaved by C1s with the loss of hemolytic activity to yield a small, inactive, fragment of C2b ($M_r = 34$ kDa) that diffuses away. C2a ($M_r = 73$ kDa) binds noncovalently to a site on membrane-bound C4b. The complex of C4b2a is referred to as ''C3 convertase'' since it cleaves C3 into C3a and C3b (Fig. 7).

In 1976, Loos and coworkers (40,64) demonstrated that heparin interfered with the components of the classical pathway in at least three different ways. Heparin binds to C1q, preventing the consumption of C4 and C2 by C1s by interfering with its C4 and C2 binding sites, and inhibiting the binding of C2 to C4b by sequestering the Mg^{2+}. Almost a decade later, Sahu and Pangburn (38) determined a relative affinity of C2 for heparin represented as an IC_{50}

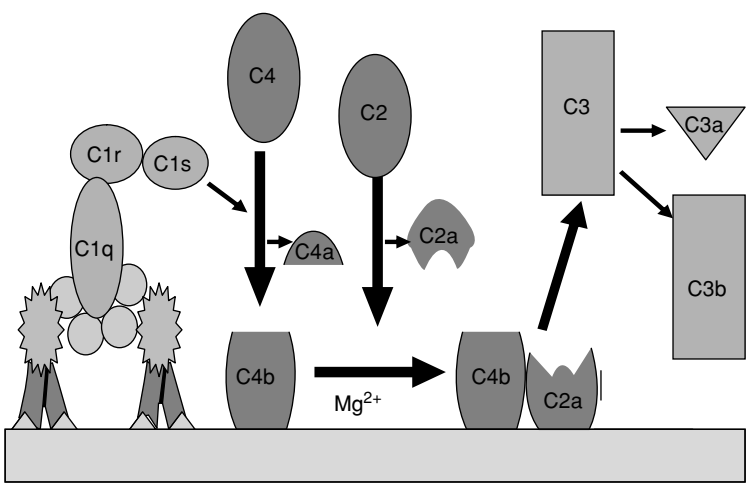


Figure 7 The formation of C3 convertase (C4b2a) and its cleavage of C3.

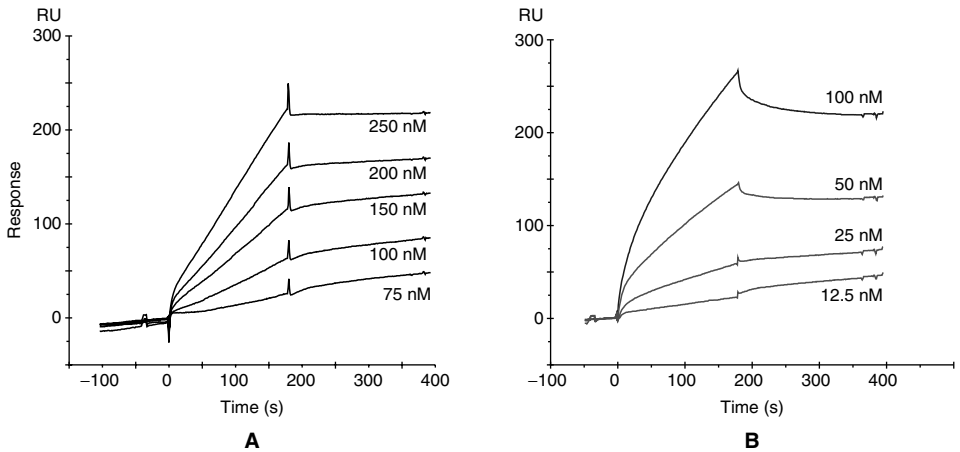


Figure 8 Sensorgram shows the interaction of biotinylated heparin on a streptavidin chip with: (A) soluble complement C2 at five representative concentrations (75, 100, 150, 200, and 250 nM) and (B) C3 at 12.5, 25, 50, 75, and 100 nM.

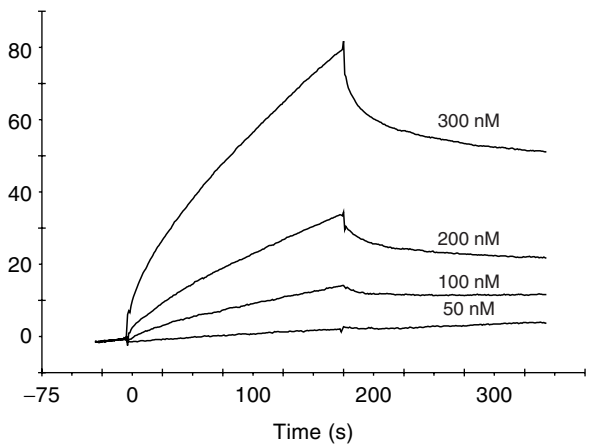


Figure 9 SPR sensorgrams of interaction between C4 at 50, 100, 200, and 300 nM with biotinylated heparin immobilized on a streptavidin (SA) chip.

(concentration of free heparin needed to inhibit C2 protein binding to heparin-agarose by 50%) of 12 μg/ml. In contrast, other studies performed by incubating C4 and C2 with heparin, and determining their residual activity showed that heparin had no direct effect on purified C4 and C2. Our laboratory recently determined the kinetic interactions of heparin with C4 using SPR (49). We measured the kinetic interaction of heparin with C2 and C4 using SPR. Sensorgram (Figs. 8A and 9) clearly show that *in vitro* heparin binds C2 with K_d of 320 nM; binds C4 with C4 with a K_d of 60.8 nM (Table 2) a K_d of 6.08 nM (Table 2).

D. Effect of Heparin on C4 Binding Protein (C4BP)

C4 binding protein (C4BP) is a glycoprotein that is capable of specifically binding to C4b. The main function of C4BP is believed to be the inhibition of the activity of the C3 convertase (C4b2a) of the classical pathway. There are two forms of C4BP having similar functional properties present in human plasma. These forms differ in molecular weight (590 and 540 kDa) and net charge (65). C4 binding protein contributes to the regulation of the classical pathway of the complement system and plays an important role in blood coagulation. It binds specifically to C4b to control the assembly and function of the C3 convertase (C4b2a), and accelerates the decay of this convertase in a concentration-dependent fashion (66).

Scharfstein et al. (67) first reported that the formation of C4/C4BP complex could be prevented in the presence of heparin, suggesting that heparin bound to C4BP. Schwalbe et al. (68) reached the same conclusion by examining heparin's effect on serum amyloid protein (SAP) interaction with C4BP. Subsequently, Hessing et al. (69) reported that the heparin-binding domain was localized on, or close to the C4b-binding site in C4BP. The interaction between heparin and C4BP was first studied using SPR by Blom et al. (70). They found C4BP interaction with heparin showed a high-association rate constant (k_{on}), and low-dissociation rate constant (k_{off}), suggesting this was a high-affinity interaction (70). However, in these experiments, the interaction between the fluid phase multimeric C4BP and the immobilized heterogeneous heparin was too complex to calculate meaningful affinity constants for this interaction.

E. Effect of Heparin on C3 and C3 Convertase

C3, a prominent complement protein, with the plasma level of 1500 μg/ml (Table 1) holds a key position in the complement cascade since it is found at the convergence of the classical and the alternative pathways (Fig. 10).

C3 has an M_r of 180 kDa and is made up of two different polypeptide chains held together through disulfide bonds and noncovalent interactions. The larger α-chain has an M_r of 110 kDa and the smaller β-chain an M_r of 70 kDa (71,72).

In the classical pathway C3 convertase, C4b2a cleaves an arginine–serine peptide bond at position 77 of the C3 α-chain (Fig. 7) to generate the 9 kDa C3a and the 171 kDa C3b. In the alternative pathway, the same fragments are formed from C3 through the action of a complex of C3b and factor B fragment Bb (C3bBb). Subsequently, C3b binds to the C4b2a of classical pathway, or C3bBb of alternative pathway already attached to a biological membrane to form a new membrane-bound enzyme complex that recognizes C5 as its substrate. In 1981, Sim et al. (73) established the mechanism, by which C3b formed a covalent bond with surfaces, such as protein or glycoprotein, carbohydrate, and phospholipids (Fig. 11).

Four decades of studies have established that C3 functions like a double-edged sword: on one hand it promotes phagocytosis, inducing inflammatory responses against pathogens; on the other hand, unregulated activity of C3 resulting in host cell damage. C3b also serves the important function of distinguishing between cells of different species. Hence, the human alternative and MBL pathways can only

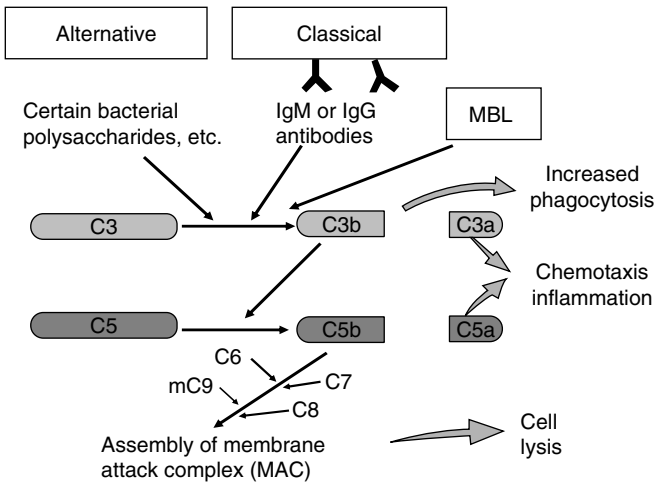


Figure 10 C3 is a central component in all three pathways of the complement activation.

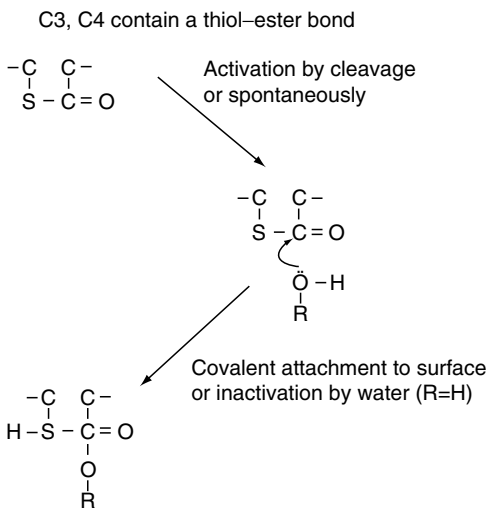


Figure 11 Schematic representation of the chemistry taking place upon C3 immobilization on the cell surface.

be activated by cell walls of other species and by certain bacteria, but not by human erythrocytes.

The effect of heparin on C3 activity has been examined both *in vivo* (20) and *in vitro*. Heparin inhibitory activity on the alternative pathway convertase formation (Fig. 4) is independent of its anticoagulant activity and requires the presence of *O*-sulfo groups (74). Pangburn (75) observed that C3b binding to polysaccharide caused a reduction of C3 activity, which depended in part on the size of the

oligosaccharide. Monosaccharide and disaccharides with structural similarities to dextran did not cause a detectable decrease in C3b-factor H binding, while sugar polymers caused large decreases in the affinity between C3b and factor H, due to the polysaccharide occupying the binding site in C3b or in factor H, preventing their interaction. An earlier finding by Weiler et al. (76) suggested that heparin could reversibly inhibit C3b binding to factor B. They believed that the inhibiting action of heparin had nothing to do with the chelation of Mg^{2+}, but came about preventing B utilization during the fluid phase interaction of C3b, B, and D.

SPR was used to characterizing the heparin and C3 interaction. The SA chip immobilized with biotinylated heparin was also used in this experiment. Sensorgram (Fig. 8B) was fitted with the Langmuir 1:1 binding model to afford a k_{on} value of $1.83 \times 10^4\,M^{-1}s^{-1}$ and k_{off} value of $5.73 \times 10^{-4}\,s^{-1}$. Thus, the overall approximate K_d for heparin–C3 binding is 31.2 nM.

Recent studies on the antimicrobial activities of heparin-binding peptides by Andersson et al. (77) showed that the heparin-binding peptides derived from complement factor C3 exerted antimicrobial activities against Gram-positive and Gram-negative bacteria. An improved understanding of the features of GAG-complement protein binding, such as heparin–C3 binding, may aid in the search for endogenous antimicrobial peptides from complex biological sources. It may also provide a logical rationale for evaluating possible antimicrobial properties of GAG-binding proteins or peptides not yet considered as antimicrobials.

F. The Effects of Heparin on the Terminal Pathway

1. *Effect of Heparin on the Membrane Attack Complex (MAC) and Reactive Lysis*

The final step of the complement cascade common to all three activation pathways is referred to as the terminal pathway. C5 convertases are membrane assemblies of C4b2a (classical pathway) or C3bBb (alternative pathway), and additional C3b molecules corresponding to C4b2a3b and C3bBb3b, respectively (78). The major difference observed between the two convertases is in their rates of C5 cleavage. The classical pathway C5 convertase cleaves C5 about six to nine times faster than does the alternative pathway C5 convertase. C5, present at a plasma concentration of 0.37 μM (Table 1) (79), is cleaved by these convertases into two biologically important products, corresponding to the last enzymatic step in the complement activation cascade (54). The smaller fragment, C5a, is chemotactic, regulating inflammatory responses by stimulating neutrophils and phagocytes. The larger fragment, C5b, initiates the formation of the MAC (C5b-9) (Fig. 12), resulting in the lysis of bacteria and other pathogens (80). Activated C5 provides the nucleus for the sequential and essentially nonreversible addition of single copies of components C6, C7, and C8, and multiple copies of C9 to produce the MAC at the targeted site. The MAC is regulated by S protein, also called vitronectin, which controls the activity of C5b67. MAC assembly is also regulated by homologous restriction factor (HRF), SP40, and CD59, which regulates C8, C9 activity.

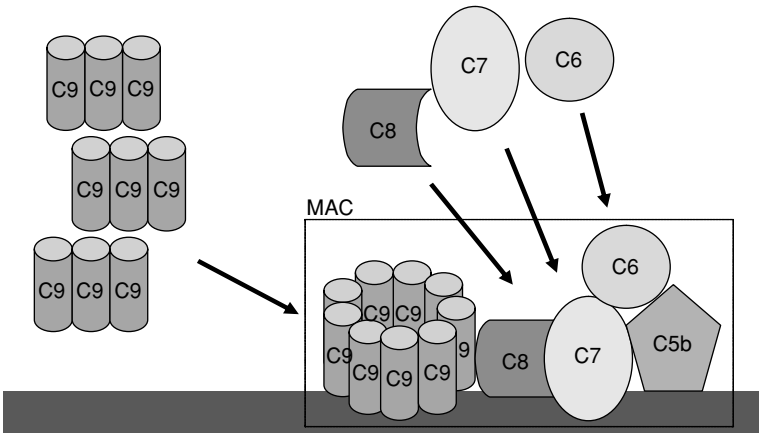


Figure 12 The formation of the membrane attack complex (C5b–9).

Baker et al. (41) first reported that heparin and other polyanions including polyanethol sulfonate and dextran sulfate, inhibited MAC promoted reactive lysis. By contrast, polycations, including polybrene, protamine, and polyornithine, potentiated the formation of C567, which can attach to erythrocyte membranes rendering them susceptible to lysis in the presence of C8 and C9. They observed that the heparin polyanion inhibited the formation of C567 from C56, C7, and sheep erythrocytes, suggesting that the reaction C567 → EAC567 (erythrocytes sensitized by antibody and the C567 complex) was susceptible to manipulation by a spectrum of polymers based on their charge and size (41). Heparin at 2 μg/ml causes 50% inhibition of the formation of C5b67 while as much as 250 μg/ml of chondroitin sulfates A or B or C or hyaluronic acid (a nonsulfated GAG) has no effect on the formation of C5b67. These findings were confirmed by Tschopp and Masson (81), who found that negatively charged GAGs, such as heparin, inhibited hemolytic activity by blocking the lytic activities of C6, C7, C8, and C9 by interfering with the incorporation of these terminal components into the MAC.

Surface plasmon resonance studies were conducted to better understand the numerous interactions between heparin and components of terminal pathway (49). Figures. 13A–D and 14 show SPR sensorgrams for a surface immobilized heparin pulsed with varying concentrations of C5, C6, C7, C8, and C9. The equilibrium and kinetic constants from SPR for the interaction of C5, C6, C7, C8, and C9, as well as all other C proteins are listed in Table 2.

2. Heparin Interaction with Vitronectin

Vitronection is a 70 kDa protein found in both the extracellular matrix as well as serum. Vitronectin regulates the complement terminal pathway in two ways, by binding soluble C5b67 promoting its formation and attenuating its ability to bind to membranes and by preventing C9 polymerization (81). Heparin binds vitronectin

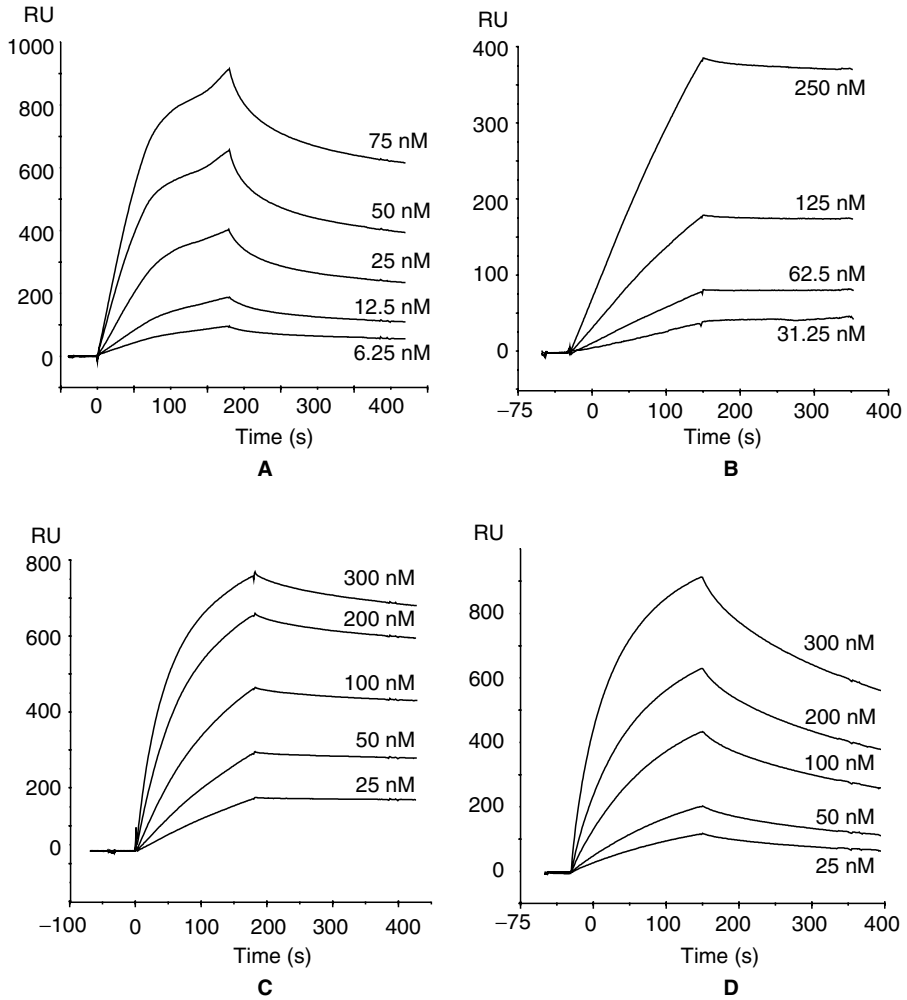


Figure 13 Kinetic binding studies of heparin and complement proteins C5, C6, C7, and C8. Biotinylated heparin was immobilized on a streptavidin-coated sensor chip and complement protein was injected at increasing concentrations (indicated in the legend box of each sensorgram) over the surface. The curves represent injections performed at 30 μl/min for 90 s, followed by 3 min of buffer flow. Injections (repeated at least four times) were aligned at $t = 0$. (A) C5 at 6.25, 12.5, 25, 50, and 75 nM; (B) C6 at 31.25, 62.5, 125, and 250 nM; (C) C7 at 25, 50, 100, 200, and 300 nM; and (D) C8 at 25, 50, 100, 200, and 300 nM. All the complement components listed here nearly have the same association rate, while compared with other complement proteins; C6 is described by a much slower dissociation rate (as seen by the nearly flat in signal during the dissociation phase).

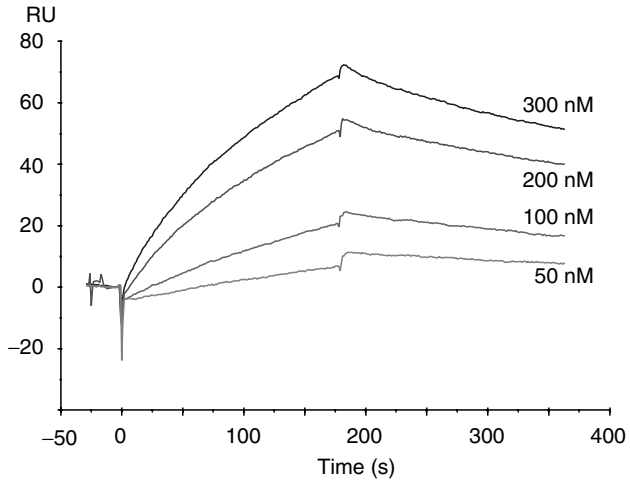


Figure 14 Interaction of C9 with heparin as studied using SPR, C9 at 50, 100, 200, and 300 nM.

and diminishes its inhibition of complement activity (82). The highly basic region of vitronectin ($Ala^{341}-Arg^{379}$) is important in its interaction with heparin. Using an affinity electrophoresis technique, Edens et al. (8) demonstrated that specific heparin chain of $M_r > 8000$-bound vitronectin with high affinity ($K_d \sim 6.7 \times 10^{-6}$ M), whereas most high molecular weight chains showed little, or no affinity for vitronectin. Previous K_d reports for the interation of heparin with vitronectin ranged from 4 to 40×10^{-6} M with most results centered around 5×10^{-6} M (83).

IV. The Effects of Heparin on the Alternative Pathway

In the alternative pathway, complement is activated directly by microbial components (Fig. 15). This is an important and biologically primitive form of complement activation. The alternative pathway begins with spontaneous self-activation of C3. As previously discussed, C3bBb is the C3 convertase of the alternative pathway of complement. The enzyme is controlled by the serum proteins, factors H and I, and properdin. The formation of the enzyme requires C3b, factors B and D, and Mg^{2+}. In solution, C3 undergoes a slow conformational change, similar to that produced by cleavage or hydrolysis, exposing its reactive thioester bond. This activated fluid-phase C3* resembles C3b and is rapidly inactivated by factors H and I. Its lifetime is prolonged by binding to an ''activator surface,'' such as a bacteria capsule. There it may persist long enough to bind factor B and allow cleavage and activation by factor D. The activated Bb subunit of the resulting C3bBb complex can bind and activate additional molecules of C3. This C3 convertase of the alternative pathway is stabilized by the binding of a protein, properdin (P). The C3bBbP will cleave

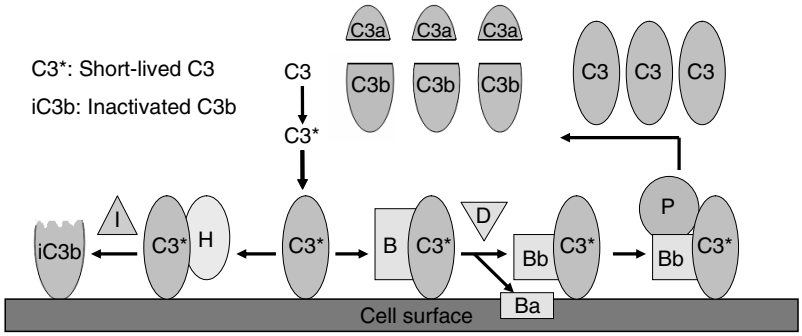


Figure 15 Cartoon showing the activation of C3 in the alternative pathway.

more C3 molecules and form a C3bBbP ($C3b_n$) complex, which in turn binds and activates C5.

There are multiple sites in the alternative pathway where heparin exerts regulatory activity (84). The ability of heparin to mediate the alternative pathway can be correlated to its size and degree of sulfation (85). At a given concentration, the inhibitory activity of heparin on the alternative pathway is much greater than its inhibitory activity on the classical pathway (63). Weiler (86) reported a comparison of a series of polyanions with a series of polycations for ability to inhibit either the classical or the alternative pathways. They also observed that polyanions have more activity on the alternative pathway, while polycations have more activity on the classical pathway on a weight basis. It remains unclear why polycations preferentially inhibit the classical pathway and polyanions preferentially inhibit the alternative pathway.

A. Heparin Interaction with Factors H and I

There are six complement glycoproteins: C3 and factors B, D, H, I, and P. Factor H is an elongated $\approx$ 150 kDa plasma glycoprotein composed of a single polypeptide chain containing 9.3% carbohydrate. C3b and factor H are directly involved in the discrimination between activators and nonactivators (87–89). Whether C3b is inactivated or initiates its amplification most frequently depends on the affinity of bound C3b for regulatory factor H. This affinity is mediated by the interaction of factor H with polyanions, such as heparin and sialic acids, on glycoproteins and glycolipids (90,91). Heparin's effect on factor H seems to be inconsistent because heparin has been reported to activate both pathways of complement (92), and also inhibit alternative pathway activation, suggesting that heparin simultaneously has opposing effects on the functions of factor H. Heparin in a concentration range of $0.54-8.66\,\mu g$ per 10^7 erythrocytes inhibits the activity of factor H to accelerate the decay of unstabilized convertase (90). In contrast, polyanions-like heparin on surfaces can prevent activation of the alternative pathway by enhancing the binding of factor H to C3b. The reason for such apparent discrepancy is likely because of a

difference in experimental design. Studies investigating the classical or alternative pathways, examining effects of fluid-phase heparin, or of immobilized heparin led to different outcomes. In addition, the varied concentrations used in these different studies might also have caused this apparent discrepancy (93).

In 1994, Meri and Pangburn (94) compared the ability of heparin and chemically modified heparins ($M_r \sim 11$kDa) to enhance factor H binding to zymosan (an insoluble polysaccharide fraction of yeast cell walls used as a support) C3b by measuring the binding of radiolabeled factor H to activator-bound C3b in the presence of heparin. They found the magnitude of this enhancement effect was much reduced in the case of LMW heparin, and the effect also decreased in a concentration-dependent manner if *N*-sulfo groups were removed from heparin, indicating that these negatively charged *N*-sulfo groups were essential for heparin's activity (Fig. 16). Although the heparin-binding domain on factor H has been identified (95,96), the mechanism of heparin augmentation of factor H function is still not clear. One assumption is that heparin causes a conformational change in factor H, which in turn facilitates a high-affinity interaction between factor H and surface-associated C3b (97).

Factor I, also referred to as C3b inactivator, is composed of two disulfide-linked polypeptide chains (M_r 50 and 38 kDa, respectively), and is found in plasma at a concentration of $\sim 34\,\mu g/ml$ (Table 1). Factor I is a regulatory protein of the alternative pathway exerting its control with the help of its cofactor, factor H, by the enzymatic inactivation of soluble C3b, cleaving the α' chain of C3b into two fragments (M_r 67 and 40 kDa) (98,99). If C3b is attached to particulate activators (polysaccharides, fungi, bacteria, viruses, certain mammalian cells, and aggregates of immunoglobulins), C3b can be cleaved by factor I alone, but the rate of cleavage is enhanced 30-fold by factor H (97).

Meri and Pangburn (89) demonstrated that heparin not only augmented the binding of factor H to zymosan-bound C3b, but at the same concentration it also enhanced the cofactor function of factor I with factor H, on C3b linked to soluble dextran support. In another report, Koistinen (100) showed that neither heparin nor LMW dextran sulfate had regulatory effect on the activity of factor I cofactor, using C3b bound to Sepharose 4B as a support. In contrast, high molecular weight

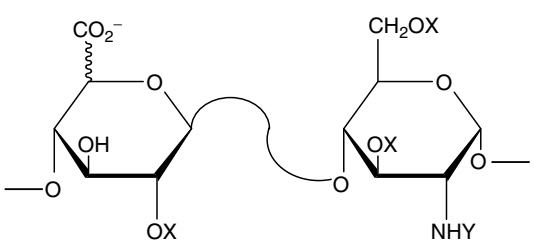


Figure 16 Summary of the structural requirements of heparin acting on complement. Some X substituents must be sulfo and Y substituents can be sulfo or acetyl. If no X is sulfo, or if Y is a hydrogen, no complement inhibitory activity is observed.

dextran sulfate strongly inhibited factor I. However, when Koistinen (100) incubated fluid phase C3b with factors I and H, either in the presence or absence of polyanions, no effect was observed on factor I. This discrepancy may also result from different experimental parameters. No direct kinetic binding data for heparin and factor I had been reported.

Recent studies in our laboratory (49) performed kinetic analysis to study the binding of heparin and factors I and H (Fig. 17A and B), the kinetic constants obtained for factor I are k_{on} ($M^{-1}s^{-1}$) $= 4.45 \times 10^4$, k_{off} (s^{-1}) $= 1.03 \times 10^{-3}$, K_d (M) $= 3.62 \times 10^{-8}$; for factor H are k_{on} ($M^{-1}s^{-1}$) $= 1.84 \times 10^4$, $k_{off}(s^{-1})$ $= 7.25 \times 10^{-3}$, K_d (M) $= 3.99 \times 10^{-7}$ (Table 2).

B. Heparin Activity on Factors B and D

Factor B, also called C3 proactivator, is a glycine-rich, heat-labile, β-glycoprotein with an M_r of 93 kDa found in blood at concentrations of 120–300 μg/ml (Table 1) (101). Factor B, the precursor of the catalytic subunit of the C3 convertase in the alternative pathway, is converted by factor D to two fragments (102,103). The larger fragment, Bb, M_r of 60 kDa, possesses the active site of C3 convertase, C3bBb. Factor D is an enzyme consisting of a single polypeptide chain that is involved in the assembly of the C3 convertase in the alternative pathway (104). Factor D cleaves factor B, its only-known substrate, in the context of the biomolecular complex C3bB. It has been proposed that binding to C3b induces a conformational change in factor B, thus allowing it to fit into the substrate-binding site of factor D.

There are few reports about effects of heparin on factors B and D. One of these, by Kazatchkine and coworkers (42) showed that fluid phase heparin inhibited

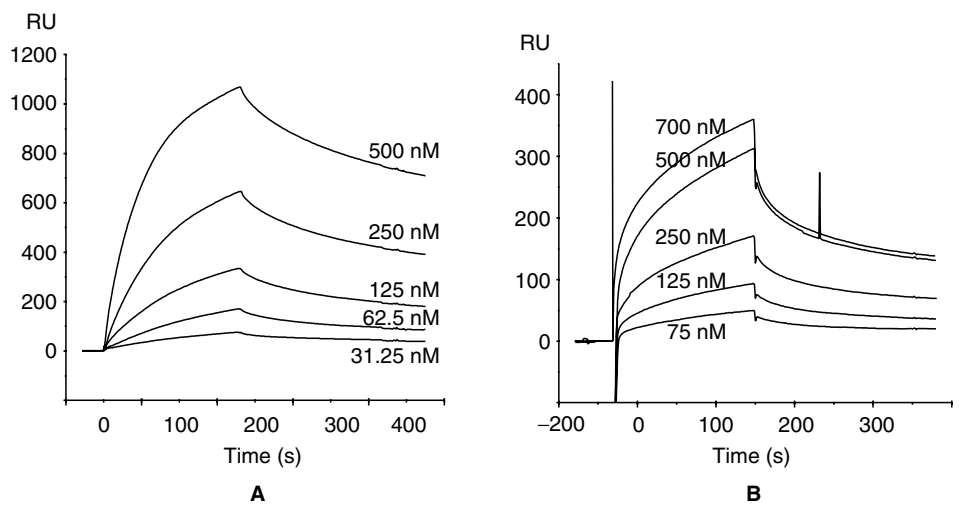


Figure 17 SPR sensorgrams of interaction between biotinylated heparin immobilized on a streptavidin (SA) chip with (A) factor I at 12.5, 50, 75, and 100 nM; (B) factor H at 75, 125, 250, 500, and 700 nM.

C3 convertase C3bBb formation by preventing the interaction of cell-bound C3b binding with factor B in the presence and absence of factor D. They found this effect of heparin was concentration-dependent and required the presence of sulfo groups, because no inhibition effect was observed in the presence of completely (*N*- and *O*-) desulfonated heparin (42). They also demonstrated that this inhibition effect resulted from the binding of heparin to the cell-bound C3b and masking the factor B binding in C3b. Furthermore, they suggested that heparin does not inactivate factor B, or directly affect its binding affinity (105), but instead inhibits consumption of factor B by factor D in a fluid phase.

To study the binding of heparin and factor B, our group performed kinetic analysis using SPR (Fig. 18A) (49) and obtained kinetic constants for this binding: k_{on} ($M^{-1}s^{-1}$) $= 2.58 \times 10^5$, k_{off} (s^{-1}) $= 2.77 \times 10^{-4}$, K_d (M) $= 2.1 \times 10^{-9}$ (Table 2).

C. Heparin Regulation of Properdin (Factor P)

Properdin has an M_r of 224 kDa and is composed of four identical polypeptide chains held together noncovalently. Properdin functions as an enhancing regulator and stabilizes the C3 convertase during the activation of the alternative pathway (106,107). The native properdin (nP) then becomes activated properdin (aP), which retards C3bBb decay, thus enhancing C3 cleavage.

The role that sulfated glycoconjugates exert on properdin function has been studied (76,84,108). Heparin proteoglycan or GAG from rat peritoneal mast cells and heparin inhibits the formation of C3 convertase in the presence or absence of properdin. This capacity of GAG depends largely on whether the convertase is

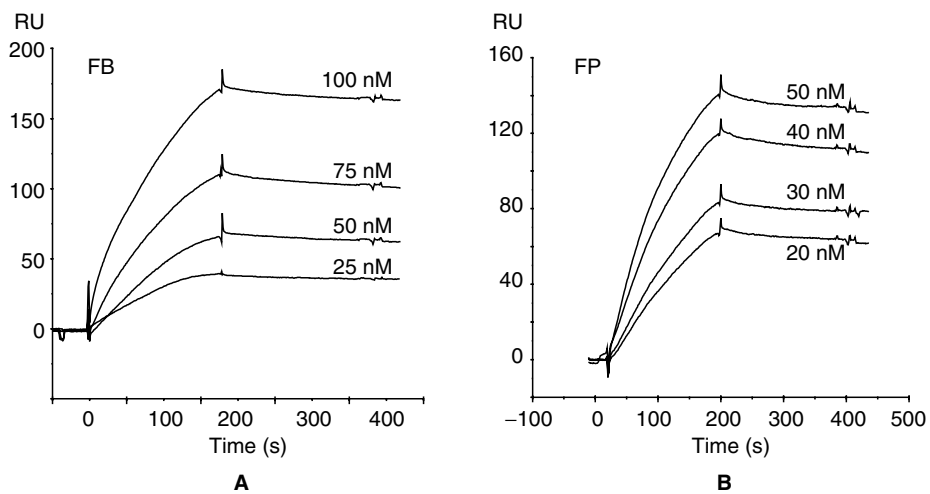


Figure 18 SPR sensorgrams of interaction between factor B (A) at 12.5, 50, 75, and 100 nM; factor P (B) at 20, 30, 40, and 50 nM, with biotinylated heparin immobilized on a poly(ethylene glycol) (PEG)-based chip.

destabilized, or stabilized with nP or aP. Heparin has no effect on the decay of preformed convertase either stabilized or destabilized or with nP or aP (107). Weiler and Linhardt (63) found that much higher concentrations of heparin could achieve accelerating decay of the properdin-stabilized convertase than that was needed to prevent its formation.

The interaction between properdin and carbohydrate was not clearly described until Holt et al. (109) examined the binding of nP and aP to glycoconjugates. Their report showed that both forms of properdin specifically binds sulfatide (Gal (3-SO_4)$\beta 1 \rightarrow 1'$ ceramide, probe to study glycolipid dynamics in model membranes), and some natural or synthetic carbohydrate polymers. Dextran sulfate ($M_r \sim 5$ kDa) had the greatest affinity for aP, followed by dextran sulfate ($M_r \sim 500$ kDa), fucoidan and heparin showing the lowest affinity. They also found that aP bound to sulfatide with higher affinity than nP, and the binding of nP to sulfatide was not prevented by dextran sulfate. The possible reason could be that the polymerization of nP to the activated form aP requires a conformational change that may convert a low-affinity binding site to high-affinity binding sites.

The kinetic examination of the interaction between heparin and factor P has been recently completed by our group using SPR (49). The sensorgram of this binding is shown in Fig. 18B and the kinetic constants are k_{on} ($M^{-1}s^{-1}$) = 3.84×10^4, k_{off} (s^{-1}) = 8.74×10^{-5}, K_d (M) = 2.68×10^{-8} (Table 2).

D. Heparin Regulation of Factor J

Factor J is a cationic glycoprotein and inhibits both the classical and the alternative pathways (110). In the classical pathway, factor J inhibits C1 activity (111), and in the alternative pathway, Factor J prevents the generation of the fluid-phase and cell-bound C3 convertase and accelerates the decay of preformed convertase by directly interacting with C3b and Bb to disrupt C3bBb formation (107,112).

It has been reported that factor J binds heparin strongly (39), and its inhibitory activity in the alternative pathway can be regulated by heparin. A concentration-dependent increase in inhibition of convertase generation has been reported (112). Factor J is capable of neutralizing heparin's inhibitory activity through the mechanism of charge neutralization. Hence, the relative concentrations of factor J and heparin could well control the balance between activation and inhibition of complement. No qualitative binding constants or binding kinetics for the factor J-heparin interaction has been reported.

All the binding data of heparin with complement proteins are best analyzed in light of the relative concentrations that these proteins are found within the serum (Table 1). The dissociation constant K_d represents the ratio of free complement components in equilibrium with complement protein–heparin complex (a smaller K_d indicates high affinity). The ratio of K_d (nM)/serum concentration (nM) gives a dimensionless value (Table 3). For a given K_d, the higher the protein concentration, the higher the percentage of serum complement protein is bound to heparin, or heparan sulfate. For example, the K_d of heparin–C4 binding is 60.8 nM and the reported serum concentration is 2047 nM giving a ratio of 0.03. The ratio of

Table 3 Serum Concentrations of Selected Complement Proteins and Their Relationship to K_d

Complement protein	Conc. in serum (μg/ml)	Conc. in serum nM	K_d (nM)/Serum conc. (nM)	[AB]/[A] + [AB] at 0.1 U/ml heparin	[AB]/[A] + [AB] at 1.0 U/ml heparin
C1	<17	<89	>0.31	0.406–1	0.944–1
C2	15–20	130–173	1.85–2.46	0.104–0.11	0.58–0.60
C3	1500	8333	0.004	0.0066	0.066
C4	430	2047	0.03	0.03	0.26
C5	75	394	0.06	0.13	0.90
C6	60	468	0.07	0.11	0.84
C7	60	495	0.03	0.11	0.89
C8	80	490	0.35	0.08	0.60
C9	58	734	0.53	0.05	0.16
C1INH	262.5	2500	0.01	0.02	0.22
B	120–300	1290–3225	0.0006–0.0016	0.02–0.04	0.17–0.43
P	20	90	0.30	0.41	0.95
I	34	386	0.09	0.13	0.86
H	470	3133	0.096	0.016	0.16

The concentration of complement protein bound to heparin [AB] is divided by the total (free and bound) concentration of complement protein ([A] + [AB]) at two concentrations 0.1 U/ml (55 nM) and 1.0 U/ml (550 nM).

K_d/serum concentration for the 14 complement proteins studied mostly ranged from 0.01 to 0.53 (Table 3). Thus, these data suggest as much as a 50-fold difference in the fraction of heparin-bound complement proteins in the serum. Neither the soluble concentration of endogenous heparin (or heparan sulfate) in human blood, nor its concentration on the endothelium, is known. Thus, we decided to consider the effect, on complement, of exogenous heparin, administered at two standard therapeutic doses.

Heparin is normally administered at doses designed to result in plasma concentrations ranging from 0.1 to 1.0 U/ml corresponding to 55–550 nM, based on a specific activity of 150 U/mg and an average molecule weight of 12,000 Da. From these two concentrations and the K_d/serum concentration value, the fraction of bound complement protein ($[AB]/([A] + [AB])$) was calculated and is presented in Table 3. Among all the complement proteins examined, factor P binds to heparin most strongly. Low-dose heparin results in 41% of the heparin bound and that value increases to 95% bound at a plasma–heparin concentration of 1 U/ml. In contrast, even at a 1 U/ml heparin concentration, we calculate $< 26\%$ of C3, C4, C9, C1INH, and factor H are bound to heparin, among which C3 binds to heparin with the smallest proportion. These values only represent estimated values as they do not take into account competition for heparin with other heparin-binding proteins in the circulation. Despite this complication, it is clear that some complement proteins are certainly present primarily in their bound state while others in their unbound state in heparinized patients.

The studies reported here are the first to demonstrate the quantitative information in the form of affinity constants for some complex formation using a surface plasmon resonance biosensor instead of conventional solid-phase assays. The major advantage of biosensor data, compared to other measurement of macromolecular interaction, is that the formation and breakdown of complexes can be monitored in real time, which offers the possibility to determine the interaction mechanism and kinetic rate constants associated with a binding event. The data presented in this report represent the essential first step for understanding how heparin and heparan sulfate regulate multiple steps in the complement system including ones in both the classical and alternative pathways. Furthermore, these data provide insights at the molecular level required for the design of new therapeutic approaches for regulating complement activation.

V. Conclusions

Heparin and the structurally similar heparan sulfate regulate multiple steps in the complement system including ones in both the classical and alternative pathways. Quantitative data in the form of association rates, dissociation rates, and affinity constants for complex formation are provided for many of these interactions. Based on the serum concentration of complement proteins, an understanding of the relative impact of clinical doses of heparin has been assessed. These data should provide some insight into the design of therapeutic approaches to regulate complement activation.

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