

Mechanism of Heparin Activation of Antithrombin: Evidence for an Induced-Fit Model of Allosteric Activation Involving Two Interaction Subsites[†]

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ABSTRACT: The anticoagulant activation of the serpin antithrombin by heparin pentasaccharide DEFGH was previously shown to involve trisaccharide DEF first binding and inducing activation of the serpin, followed by disaccharide GH binding and stabilizing the activated state [Petitou et al. (1997) *Glycobiology* 7, 323–327; Desai et al. (1998) *J. Biol. Chem.* 273, 7478–7487]. In the present study, the role of conformational changes and charged residues of the GH disaccharide in the allosteric activation mechanism was investigated with variant pentasaccharides modified in the GH disaccharide. Perturbation of the conformational equilibrium of iduronate residue G through replacement of the nonessential 3-OH of this residue with –H resulted in parallel decreases in the fraction of residue G in the skew boat conformer (from 64 to 24%) and in the association constant for pentasaccharide binding to antithrombin [(2.6 ± 0.3)-fold], consistent with selective binding of the skew boat conformer to the serpin. Introduction of an additional sulfate group to the 3-OH of residue H flanking a putative charge cluster in the GH disaccharide greatly enhanced the affinity for the serpin by ~35-fold with only a small increase in the fraction of residue G in the skew boat conformation (from 64 to 85%). The salt dependence of binding, together with a recent X-ray structure of the antithrombin–pentasaccharide complex, suggested that the majority of the enhanced affinity of the latter pentasaccharide was due to direct electrostatic and hydrogen-bonding interactions of the H residue 3-O-sulfate with antithrombin. All variant pentasaccharides produced a normal enhancement of antithrombin fluorescence and normal acceleration of factor Xa inhibition by the serpin at saturating levels, indicating that conformational activation of antithrombin was not affected by the pentasaccharide modifications. Rapid kinetic studies were consistent with the altered affinities of the variant pentasaccharides resulting mostly from perturbed interactions of the reducing-end GH disaccharide with the activated antithrombin conformation and minimally to an altered binding of the nonreducing-end DEF trisaccharide to the native serpin conformation. Together, these results support a model in which the conformational flexibility of the G residue facilitates conversion to the skew boat conformer and thereby allows charged groups of the GH disaccharide to bind and stabilize the activated antithrombin conformation that is induced by the DEF trisaccharide.

Antithrombin is a serine proteinase inhibitor of the serpin superfamily which is essential for regulating the activity of blood clotting proteinases, its primary targets being thrombin and factor Xa (for reviews, see refs 1 and 2). Serpins inhibit their target proteinases by forming unusually stable complexes in which the proteinase active site is blocked by a substrate-like interaction with the serpin. Antithrombin is unusual among such inhibitors in requiring the glycosaminoglycan, heparin, as a cofactor. The reaction of antithrombin with its target enzymes is thus accelerated several-

thousand-fold by heparin to rates comparable to that of other serpin–proteinase reactions (3, 4). The acceleration requires the binding of a sequence-specific heparin pentasaccharide which induces an activating conformational change in the serpin (3, 5–9). This conformational change is sufficient to accelerate the inhibition of factor Xa through an allosteric mechanism, whereas acceleration of thrombin inhibition additionally requires the binding of the enzyme as well as the inhibitor to heparin in a ternary bridging complex (4).

Studies of the structure–activity relationships in the heparin pentasaccharide DEFGH (Figure 1) have established that four sulfate groups on glucosamine residues D, F, and H and two carboxylates on uronic acid residues E and G are essential for high-affinity binding and conformational activation of antithrombin (7, 10–12). Changes in the nature and positioning of the charged groups lead to loss of activity, indicating that the interaction is highly specific and not governed simply by electrostatic interactions (11, 12). The asymmetry of the charged groups together with molecular

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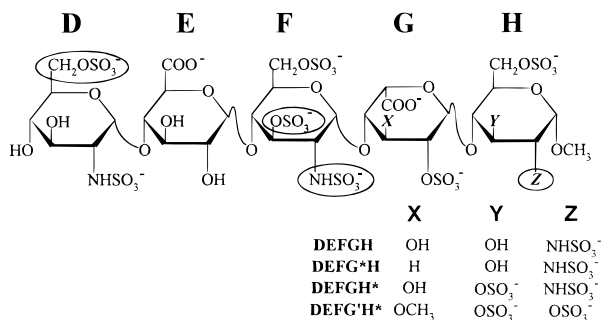


FIGURE 1: Structures of normal and variant pentasaccharides. Sulfate groups which are circled in the naturally occurring heparin pentasaccharide, DEFGH, are critical for tight binding to antithrombin (10, 12). Asterisks and primes denote modifications of residues G or H in substituents X, Y, or Z as indicated in pentasaccharides DEFG*H, DEFGH*, and DEFG'H*.

modeling studies of the antithrombin–pentasaccharide interaction have suggested a two-site model for binding of the pentasaccharide to antithrombin in which the charges on the nonreducing-end DEF trisaccharide bind to one site and the oppositely oriented charges on the reducing-end GH disaccharide bind to a second site (13, 14). The recent X-ray structure of a complex of antithrombin with a supersulfated pentasaccharide variant appears to confirm the model, the two interaction sites representing opposite ends of a cationic heparin binding groove in antithrombin formed by helix A, helix D, and the N-terminus (15).

Evidence has accumulated that the conformational flexibility of the single iduronic acid residue of the pentasaccharide also plays an important role in the interaction with antithrombin. NMR and molecular mechanics studies have shown that this conformational flexibility arises from the equilibrium interconversion of the iduronate residue between two low-energy conformational states, ¹C₄ or ²S₀ (16). This contrasts with the single ⁴C₁ conformation in which other pentasaccharide residues are rigidly held. The iduronate residue G of the pentasaccharide sequence also differs from other heparin iduronate residues in favoring the ²S₀ conformer, suggesting that this conformer may be required for productive binding to antithrombin (16, 17). Unfortunately, the electron density of the pentasaccharide in the antithrombin–pentasaccharide complex structure did not allow a precise definition of the conformation of the G residue (15). However, a model of the ²S₀ conformer established from solution NMR studies (17) provided a good fit to the electron density whereas a model of the ¹C₄ conformer did not fit well.

Rapid kinetic studies have supported a role for conformational flexibility in pentasaccharide binding to two subsites of antithrombin. Binding is a two-step process with an initial low heparin affinity interaction inducing a conformational change leading to a high-affinity interaction and inhibitor activation (4, 9). About half of the charge–charge interactions are made in the initial binding step while the remainder are made in the subsequent conformational activation step (4). Studies with truncated variants of the pentasaccharide have demonstrated that the rigid DEF unit binds in the first step whereas the flexible GH unit binds in the second step (18, 28), suggesting that the flexibility of the GH disaccharide may be involved in an induced-fit binding to the two proposed interaction sites of antithrombin.

In the present study, we have addressed the roles of the iduronate residue G conformation and of charged residues in the GH disaccharide in the dynamics of pentasaccharide binding and activation of antithrombin by comparing the binding of variant pentasaccharides in which the G residue conformation and the GH disaccharide charge cluster were altered. Our studies support a model in which the GH disaccharide charge cluster binds and stabilizes a secondary subsite, created in activated antithrombin by trisaccharide DEF binding, only when iduronate residue G adopts the ²S₀ conformation. These results suggest a critical role for conformational flexibility of the pentasaccharide in the dynamics of serpin activation in keeping with the structural changes which accompany formation of the antithrombin–pentasaccharide complex (15).

MATERIALS AND METHODS

Proteins. Human antithrombin was purified from outdated plasma as previously described (19). Molar concentrations of the inhibitor were calculated from absorbance measurements at 280 nm using a molar absorption coefficient of 37 700 M⁻¹ cm⁻¹ (20). Human factor Xa was prepared by activation of purified factor X, followed by purification on SBTI-agarose, as described previously (21). Factor Xa preparations were predominantly the α form as judged by SDS–PAGE and >90% active by comparisons of active site and protein concentrations (21).

Oligosaccharides. The methyl glycosides of normal and variant pentasaccharides were synthesized, and their structures confirmed as previously described (11, 14, 22, 29). A 1–2 mM solution of each pentasaccharide was prepared based on dry weight in distilled, deionized water. Stoichiometric titrations of high concentrations of antithrombin (>10 × K_D) with all pentasaccharides, monitored by the endogenous fluorescence enhancement (20), were in good agreement with the weight concentrations. The concentrations based on fluorescence titrations were used for all experiments.

NMR Spectroscopy. NMR spectra of pentasaccharides were obtained, and the relative conformer populations of the single iduronate residue were computed from observed interproton coupling constants, based on coupling constants determined for the iduronate residue in different conformations, as described by Ferro et al. (16). NOE experiments confirmed the validity of these computations (22).

Experimental Conditions. All experiments were conducted at 25 °C and in 20 mM sodium phosphate buffer, containing 0.1 mM EDTA and 0.1% (w/v) PEG 8000, adjusted to pH 7.4. In the absence of added salt, the ionic strength of the buffer was 0.05. Sodium chloride was added in increasing concentrations to achieve higher ionic strengths at the same pH.

Spectroscopic Studies. Fluorescence emission spectra of antithrombin and its complexes with pentasaccharides were obtained at 25 °C in pH 7.4, I 0.15 buffer with 0.8 μM antithrombin and 4.7–6.7 μM pentasaccharide. The spectra were recorded with an SLM 8000C spectrofluorometer in the ratio mode at 2 nm wavelength intervals with excitation at 280 nm (4 nm band-pass), an emission band-pass of 2 nm, and 10 s integrations of the fluorescence signal at each wavelength. Corrections for Raman bands and any back-

ground signal from the buffer were made by subtracting buffer spectra. Control experiments showed no significant fluorescence of the variant pentasaccharides alone at concentrations used in the formation of complexes. The corrected spectra consisted of signal from both the free (<10%) and bound antithrombin. The normalized fluorescence spectrum of each complex was calculated by subtracting the expected spectrum of free antithrombin, based on measured dissociation constants for complex formation, and scaling up the resultant spectrum to 1 μ M complex.

Equilibrium Binding Studies. Equilibrium dissociation constants for antithrombin–pentasaccharide interactions ($K_{D,obs}$) were measured by titrating pentasaccharide into a solution of antithrombin and monitoring the increase in intrinsic protein fluorescence accompanying the binding interaction, as previously described (8, 19). Antithrombin concentrations were in the range 0.5–2 $\times$ $K_{D,obs}$, except when $K_{D,obs}$ was greater than 1 μ M, in which case the protein concentration was $\sim$ 1 μ M. The increase in fluorescence signal with increasing pentasaccharide concentration was fit to the quadratic equilibrium binding equation using the predetermined 1:1 binding stoichiometry (4).

The nonionic and ionic contributions to antithrombin–pentasaccharide interactions were resolved by analyzing the NaCl concentration dependence of $K_{D,obs}$ for the interaction according to the equation (4, 23),

$$\log K_{D,obs} = \log K_{D,nonionic} + Z\Psi \log[Na^+] \quad (1)$$

where $K_{D,nonionic}$ is the dissociation constant at 1 M Na^+ , Z is the total number of charge–charge interactions involved in the association of the protein with heparin, and Ψ is the fraction of monovalent counterions bound per heparin ionic charge which are released upon protein binding. Ψ has a value of 0.8 (24). Least-squares analysis of the linear dependence of $\log K_{D,obs}$ on $\log[Na^+]$ yielded the nonionic component of the binding energy from the intercept, $\log K_{D,nonionic}$, and the value of Z from the slope.

Rapid Kinetic Studies. The rate of binding of pentasaccharides to antithrombin was measured in pH 7.4 buffer at 25 °C in an Applied Photophysics stopped-flow fluorometer, as previously described (4, 9). The NaCl concentration in the buffer was either 100 mM (I 0.15) or 250 mM (I 0.30). Pseudo-first-order conditions in which the pentasaccharide concentration was at least 5 times (typically 10 times) greater than the antithrombin concentration were employed. The interaction was monitored from the increase in intrinsic protein fluorescence with an excitation wavelength of 280 nm and an emission filter that transmitted light only at wavelengths above 310 nm. Excitation slits corresponded to an 8 nm band-pass. The fluorescence traces were acquired for at least 10 half-lives and could be satisfactorily fit by a single-exponential function for all variants, which provided the amplitude of the fluorescence change and the observed pseudo-first-order rate constant, k_{obs} . Typically 9–18 traces were individually analyzed at each concentration of pentasaccharide and the results averaged. For studies at low antithrombin concentrations (<25 nM), three to four fluorescence traces were typically averaged to improve the signal-to-noise ratio before nonlinear regression fitting by the exponential function. In these cases, the rate constant reported is the mean of three to four such averages.

Table 1: Conformer Populations of Pentasaccharides^a

saccharide	conformer population (%)			K_{CONF}
	² S ₀	¹ C ₄	⁴ C ₁	
DEFGH	64	36	0	1.8
DEFG*H	24	65	11	0.32
DEFGH*	85	15	0	5.7
DEFG'H*	94	6	0	16

^a Conformer populations were calculated from NMR spectra of pentasaccharides as previously described (16, 17, 22, 29).

Factor Xa Inhibition Studies. The accelerating effect of each pentasaccharide on the kinetics of antithrombin inhibition of factor Xa was measured under pseudo-first-order conditions similar to previous studies (25). A 5–10 nM concentration of factor Xa was incubated at 25 °C with 200–400 nM antithrombin and 0–10 nM pentasaccharide in pH 7.4 buffer containing 100 mM NaCl to give 50 μ L of total reaction volume. After incubation for various times, reactions were quenched with 950 μ L of 100 μ M Spectrozyme FXa in 20 mM sodium phosphate buffer containing 100 mM NaCl, 0.1 mM EDTA, and 0.1% (w/v) PEG 8000 at pH 7.4. The residual factor Xa activity was then measured from the initial rate of substrate hydrolysis at 405 nm. The observed pseudo-first-order rate constant (k_{obs}) for factor Xa inactivation at each pentasaccharide concentration (at least three concentrations were examined for each pentasaccharide) was determined by nonlinear regression fitting of the exponential decay of factor Xa activity. The second-order rate constant for factor Xa inhibition by antithrombin alone (k_{uncat}) and that for the inhibition by antithrombin–pentasaccharide complexes (k_H) were obtained by least-squares analysis of the linear dependence of k_{obs} on antithrombin–pentasaccharide complex concentration according to the equation

$$k_{obs} = k_{uncat}[AT]_0 + k_H[H]_0[AT]_0/([AT]_0 + K_D) \quad (2)$$

where $[AT]_0$ and $[H]_0$ are the initial concentrations of antithrombin and pentasaccharide variant, respectively, and K_D is the dissociation constant of the complex. The expression $[H]_0[AT]_0/([AT]_0 + K_D)$ in this equation represents the concentration of antithrombin–pentasaccharide complex, because under the experimental conditions, $[AT]_0 \approx [AT]_{free}$.

RESULTS

Conformer Populations of Iduronate Residue G in Normal and Variant Pentasaccharides. Table 1 reports the relative equilibrium proportions of the three conformational states of iduronate residue G, ¹C₄, ⁴C₁, and ²S₀, in the pentasaccharides studied, as computed from NMR spectroscopic analyses in the manner previously described (Figure 1) (14, 16, 22, 29). Also reported are conformational equilibrium constants, K_{conf} , defined as the ratio of skew boat (²S₀) to chair (¹C₄ and ⁴C₁) conformers, derived from these proportions. The iduronate residue G in DEFGH favored the skew-boat ²S₀ form over the chair ¹C₄ form in solution by 64 to 36% with no significant contribution of the ⁴C₁ chair conformer. Conversion of the 3-OH group of this residue to an H atom in DEFG*H resulted in the conformational preference changing in favor of the chair forms, ¹C₄ and ⁴C₁, with a corresponding reduction in the skew-boat ²S₀ form

Table 2: Thermodynamic Parameters for Antithrombin–Pentasaccharide Interactions^a

saccharide	$K_{D,obs}$ (nM)		ΔG° (kcal/mol)	ΔF_{max} (%)	Z	$K_{D,nonionic}$ (μ M)
	I 0.15	I 0.30	I 0.30	I 0.30		
DEFGH	50 $\pm$ 6	480 $\pm$ 24	8.6 $\pm$ 0.1	32 $\pm$ 3	4.0 $\pm$ 0.1	25.1 $\pm$ 0.5
DEFG*H	129 $\pm$ 15	1270 $\pm$ 100	8.0 $\pm$ 0.1	30 $\pm$ 2	4.1 $\pm$ 0.1	50.1 $\pm$ 0.5
DEFGH*	nd	14 $\pm$ 2	10.7 $\pm$ 0.1	31 $\pm$ 1	4.8 $\pm$ 0.2	1.6 $\pm$ 0.6
DEFG'H*	nd	13 $\pm$ 1	10.8 $\pm$ 0.1	33 $\pm$ 2	5.0 $\pm$ 0.1	1.6 $\pm$ 0.3

^a Observed dissociation constants ($K_{D,obs}$) and maximal fluorescence enhancements (ΔF_{max}) for antithrombin–pentasaccharide interactions were measured at pH 7.4, 25 °C at the indicated ionic strengths by fluorescence titrations of antithrombin with pentasaccharide as detailed in the Materials and Methods. The number of charge–charge interactions (Z) and nonionic contribution to $K_{D,obs}$ ($K_{D,nonionic}$) were determined by linear regression fitting of the dependence of $\log K_{D,obs}$ on $\log [Na^+]$ in Figure 2 by eq 1.

to 24%. By contrast, the introduction of a sulfate group at the 3-position of residue H in DEFGH* (14) or in DEFG'H* (29) increased the population of the ²S₀ conformer to 85 and 94%, respectively, at the expense of reducing the proportion of chair forms.

Fluorescence Spectroscopic and Equilibrium Binding Studies of Antithrombin–Pentasaccharide Interactions. Fluorescence emission spectra of antithrombin and its complexes with normal and variant heparin pentasaccharides revealed that all pentasaccharides enhanced the intrinsic fluorescence intensity of antithrombin to a similar extent (32 $\pm$ 2%, Table 2) and produced a small blue shift of $\sim$ 1 nm in the emission maximum. These fluorescence changes are characteristic of the activating conformational change in antithrombin (4, 8, 9), indicating that all pentasaccharides induced a similar or identical such conformational change.

Table 2 shows the dissociation equilibrium constants measured for antithrombin interactions with DEFGH and its variants at pH 7.4 and two ionic strengths (I 0.15 and I 0.30) using the fluorescence change to monitor complex formation (8). The observed dissociation constant ($K_{D,obs}$) measured at I 0.15 for DEFG*H (130 nM) was (2.6 $\pm$ 0.6)-fold greater than that for DEFGH (50 nM). The antithrombin affinities for DEFGH* and DEFG'H* could not be determined accurately at I 0.15 due to the much tighter interactions with these pentasaccharides ($K_D < 10$ nM) but were measurable at I 0.3. At the higher ionic strength, DEFGH* and DEFG'H* bound antithrombin with indistinguishable affinities which were (34 $\pm$ 7)-fold to (37 $\pm$ 5)-fold higher than that of DEFGH, corresponding to a 2 kcal/mol increase in binding free energy. DEFG*H under these conditions bound antithrombin with a (2.6 $\pm$ 0.3)-fold lower affinity than the natural pentasaccharide, a relative difference identical to that found at I 0.15 and corresponding to a 0.6 kcal/mol decrease in binding free energy.

Resolution of Ionic and Nonionic Contributions to Antithrombin–Pentasaccharide Interactions. Figure 2 shows an analysis of the salt dependence of $K_{D,obs}$ for the interaction of antithrombin with DEFGH and its variants at pH 7.4 and 25 °C. $\log K_{D,obs}$ was a linear function of $\log [Na^+]$ for all pentasaccharides, in keeping with previous findings with DEFGH and with the expected behavior of the protein–heparin interaction (4, 23, 26). Linear regression analysis of the data for each pentasaccharide according to eq 1 indicated the number of ion-pair interactions (Z) from the slopes and the nonionic contribution to $K_{D,obs}$ ($K_{D,nonionic}$) from the intercepts (see Materials and Methods). These results are reported in Table 2. DEFG*H made the same number of ion-pair interactions with antithrombin as DEFGH, i.e., four, whereas one additional ion-pair was made between

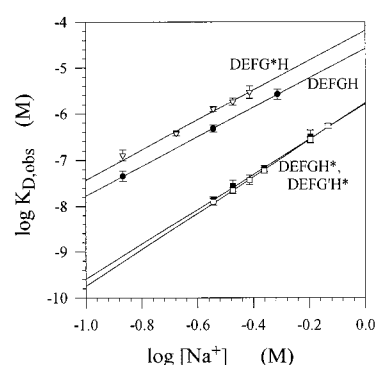


FIGURE 2: Salt dependence of the binding of pentasaccharides to antithrombin. Plots of $\log K_{D,obs}$ vs $\log [Na^+]$ are shown for antithrombin interactions with pentasaccharides DEFGH (●), DEFG*H (▽), DEFGH* (■), and DEFG'H* (□). $K_{D,obs}$ was measured as a function of the NaCl concentration at pH 7.4 and 25 °C by fluorescence titrations of antithrombin with pentasaccharide as described in the Materials and Methods. Error bars represent $\pm$ SE. The solid lines show linear least-squares fits of the data according to eq 1.

DEFGH* or DEFG'H* and the inhibitor. The latter accounted for about one-quarter ($\sim$ 0.5 kcal/mol) of the increased binding free energy of DEFGH* and DEFG'H* relative to DEFGH at I 0.3.¹ The remainder of the changes in $K_{D,obs}$ for the variant pentasaccharide interactions resulted from changes in $K_{D,nonionic}$. The nonionic component of the binding affinity for DEFGH* and DEFG'H* interactions with antithrombin was thus increased 16-fold relative to that for DEFGH, representing 1.5 kcal/mol of the increase in binding free energy. Similarly, the change in DEFG*H-binding affinity relative to that of DEFGH was completely accounted for by a change in the nonionic component of the binding free energy.

Rapid Kinetics of Antithrombin–Pentasaccharide Interactions. The kinetics of pentasaccharide–antithrombin interactions were analyzed by continuously monitoring the protein fluorescence changes accompanying binding by stopped-flow fluorimetry under pseudo-first-order conditions as in previous studies (4, 9). Figure 3 shows that the pseudo-first-order binding rate constant (k_{obs}) increased in a saturable manner

¹ Since $\Delta\Delta G^\circ$ at 25 °C is given by $1.364 \text{ kcal/mol} \times (\log K_{D,obs} - \log K'_{D,obs})$ where $K_{D,obs}$ and $K'_{D,obs}$ are observed dissociation constants for normal and variant pentasaccharides, respectively, and $\log K_{D,obs}$ is given by eq 1, $\Delta\Delta G^\circ$ is equal to $1.364 \text{ kcal/mol} \times (\log K_{D,nonionic} - \log K'_{D,nonionic}) + 1.364 \text{ kcal/mol} \times (Z\psi - Z'\psi) \times \log [Na^+]$ where the first and second terms of the sum represent the nonionic and ionic contributions to $\Delta\Delta G^\circ$, respectively. The ionic contribution to $\Delta\Delta G^\circ$ at a given salt concentration can therefore be calculated from $1.364 \text{ kcal/mol} \times \log [Na^+]^{\Delta Z\psi}$ where ΔZ is the difference in the number of charge pairs for normal and variant pentasaccharides.

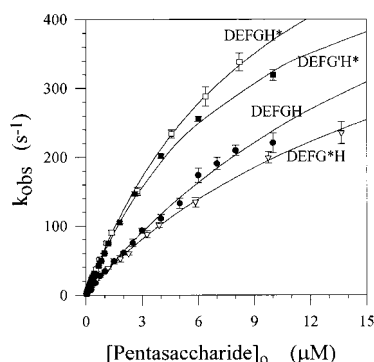


FIGURE 3: Rapid kinetics of pentasaccharide binding to antithrombin. Shown is the dependence on pentasaccharide concentration of the pseudo-first-order rate constants (k_{obs}) for the binding to antithrombin of pentasaccharides DEFGH (●), DEFG'H (▽), DEFGH* (□), and DEFG'H* (■). k_{obs} values were measured by monitoring intrinsic antithrombin fluorescence changes accompanying pentasaccharide binding by stopped-flow fluorimetry at 25 °C in pH 7.4, I 0.15 buffer, as detailed in the Materials and Methods. Solid lines represent nonlinear least-squares fits of the data by eq 3 which describes the induced conformational change binding mechanism of Scheme 1.

with increasing pentasaccharide concentration for all pentasaccharides, similar to previous findings with pentasaccharide DEFGH (4). These results indicated that all pentasaccharides bound and activated antithrombin by the same two-step process depicted in Scheme 1.

Scheme 1



According to Scheme 1, the pentasaccharide makes an initial low-affinity interaction with antithrombin ($\text{AT} \cdot \text{H}$), characterized by dissociation constant K_1 , which then induces a conformational change in the inhibitor ($\text{AT}^* \cdot \text{H}$) leading to high heparin affinity and activation of the serpin, with forward and reverse rate constants of k_2 and k_{-2} (4, 9). The dependence of k_{obs} on heparin concentration for this scheme should be given by the equation

$$k_{\text{obs}} = k_{-2} + \frac{k_2[\text{H}]_0}{K_1 + [\text{H}]_0} \quad (3)$$

Figure 3 shows that the kinetic data for each pentasaccharide was adequately fit by eq 3 and Table 3 reports the kinetic parameters obtained by this fitting. The dissociation constant for the initial binding interaction (K_1) was the same for DEFGH and DEFG'H while 1.4–2-fold lower values were obtained for DEFGH* and DEFG'H*, respectively. The

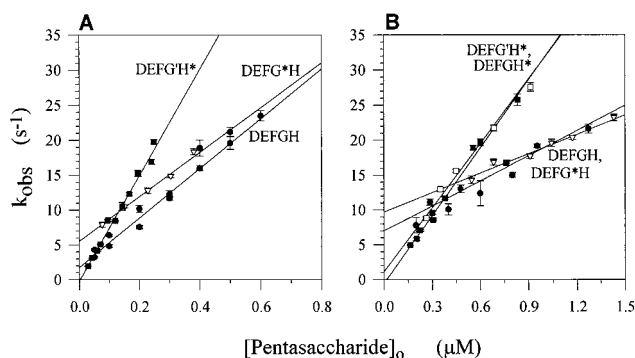


FIGURE 4: Determination of overall association and dissociation rate constants for pentasaccharide binding to antithrombin. Shown is the saccharide concentration dependence of k_{obs} for the binding to antithrombin of pentasaccharides DEFGH (●), DEFG'H (▽), DEFGH* (□), and DEFG'H* (■) at pH 7.4, 25 °C in I 0.15 (A) and I 0.30 (B) buffers. k_{obs} was measured as in Figure 3. Solid lines represent linear least-squares fits of the data by eq 4. Error bars represent $\pm$ SE.

k_2 values for the conformational activation step for all variant pentasaccharides were nearly the same and equal to that for pentasaccharide DEFGH ($700 \pm 120 \text{ s}^{-1}$). Except for pentasaccharide DEFG'H, the k_{-2} values obtained from the ordinate intercepts were not distinguishable from zero.

Figure 4 shows k_{obs} values measured in the pentasaccharide concentration range where $[\text{H}]_0 \ll K_1$ at I 0.15 and I 0.3 in order to more accurately determine k_{-2} . Under these conditions, eq 3 simplifies to the linear equation

$$k_{\text{obs}} \approx k_{-2} + \frac{k_2[\text{H}]_0}{K_1} = k_{\text{off}} + k_{\text{on}}[\text{H}]_0 \quad (4)$$

in which the slope and ordinate intercept represent the overall association and dissociation rate constants, $k_{\text{on}} = k_2/K_1$ and $k_{\text{off}} = k_{-2}$, respectively, for the interaction (4). Over the concentration range examined, k_{obs} showed a linear dependence upon pentasaccharide concentration for all pentasaccharides in both I 0.15 (Figure 4A) or I 0.30 (Figure 4B) buffers. k_{on} and k_{off} values obtained from linear regression fits of these data are provided in Table 3. k_{on} values in all cases agreed with those calculated from the ratio of k_2/K_1 within experimental error and were more precisely determined than k_{off} . (Table 3). k_{on} for DEFG'H was slightly reduced (1.1–1.3-fold) relative to DEFGH at the two ionic strengths, suggesting that increases in k_{off} accounted for the major part of the 2.6-fold reduction in affinity of this pentasaccharide. This conclusion was supported by the (1.4 ± 0.3) – (3 ± 0.7) -fold greater k_{off} values determined for DEFG'H than for DEFGH, given the larger experimental error in k_{off} . k_{on} values for pentasaccharide DEFGH* at I

Table 3: Kinetic Parameters for Antithrombin–Pentasaccharide Interactions

saccharide	K_1 (μM), I 0.15	k_2 (s^{-1}), I 0.15	k_{on} (k_2/K_1) ($\mu\text{M}^{-1} \text{s}^{-1}$)		k_{off} (k_{-2}) (s^{-1})		K_D calculated ^b (nM)	
			I 0.15	I 0.30	I 0.15	I 0.30	I 0.15	I 0.30
DEFGH	20 ± 4	700 ± 120	35.5 ± 2.1	12.0 ± 1.4	1.8 ± 0.3	7.0 ± 1.1	51 ± 12	580 ± 160
DEFG'H	20 ± 4	580 ± 90	31.9 ± 1.8	9.2 ± 0.8	5.5 ± 0.4	9.8 ± 0.8	172 ± 22	1060 ± 180
DEFGH*	14 ± 6	800 ± 200	84.8 ± 2.6	32.4 ± 1.6	-0.5 ± 0.1	-0.5 ± 0.4		
DEFG'H*	10 ± 1	730 ± 40	nd	30.7 ± 2.5	nd	1.1 ± 0.9		36 ± 32

^a Kinetic parameters for antithrombin–pentasaccharide interactions at pH 7.4, 25 °C and the indicated ionic strengths were determined by nonlinear regression fitting of kinetic data of Figure 3 by eq 3 or by linear regression fitting of data of Figure 4 by eq 4. Errors represent $\pm$ SE. nd = not determined. ^b Calculated from the ratio $k_{\text{off}}/k_{\text{on}}$.

Table 4: Accelerating Effect of Pentasaccharides on the Second-Order Rate Constant for Antithrombin Inhibition of Factor Xa^a

saccharide	k_H ($M^{-1} s^{-1}$)
DEFGH	$(6.5 \pm 0.2) \times 10^5$
DEFG*H	$(5.8 \pm 0.3) \times 10^5$
DEFGH*	$(7.2 \pm 0.5) \times 10^5$
DEFG'H*	$(6.0 \pm 0.5) \times 10^5$

^a Second-order rate constants for pentasaccharide-catalyzed (k_H) reactions of antithrombin with factor Xa were measured at pH 7.4, 25 °C, I 0.15 as described in the Materials and Methods. Errors represent $\pm$ SE.

0.15 and 0.3 and for DEFG'H* at I 0.3 were 2.3–2.7-fold greater than those for DEFGH at the two ionic strengths, indicating that the 34–37-fold enhanced affinities measurable for these pentasaccharides at I 0.3 and even greater differential affinities expected at I 0.15 from the salt dependence of the interactions (Figure 1) resulted mostly from decreases in k_{off} . This expectation was born out by the much lower values of k_{off} observed for DEFGH* and DEFG'H* relative to DEFGH at I 0.3, which were indistinguishable within the experimental error from the $\sim$ 15-fold anticipated lowering of k_{off} . The agreement between direct measurements of $K_{D,obs}$ and values calculated from the ratio k_{off}/k_{on} , in cases where both kinetic parameters were well determined (Tables 2 and 3), supported the conclusion that the greater affinities of DEFGH* and DEFG'H* and lower affinity of DEFG*H for antithrombin relative to DEFGH were due mostly to altered k_{off} values and less to changes in k_{on} values.

Accelerating Effects of Pentasaccharides on Antithrombin Inhibition of Factor Xa. Table 4 reports the second-order rate constants for factor Xa inhibition by antithrombin–pentasaccharide complexes (k_H) at pH 7.4, I 0.15, and 25 °C. These values were determined as in previous studies by monitoring the exponential decay of factor Xa activity under pseudo-first-order conditions as a function of the pentasaccharide concentration and at an antithrombin concentration sufficient to saturate the pentasaccharides (18, 19). k_H values for all variant pentasaccharides were similar and equivalent to the value observed with pentasaccharide DEFGH, i.e., $\sim 6 \times 10^5 M^{-1} s^{-1}$. Comparison with k_{uncat} values measured directly in the absence of pentasaccharide [$(2.3 \pm 0.3) \times 10^3 M^{-1} s^{-1}$] or by extrapolation of the dependence of k_{obs} on pentasaccharide concentration to zero concentration [$(2.1\text{--}2.7) \times 10^3 M^{-1} s^{-1}$] indicated that all pentasaccharides produced a similar $\sim$ 300-fold enhancement in the rate of factor Xa inactivation by antithrombin.

DISCUSSION

Previous studies have shown that the single iduronate residue of the antithrombin-activating sequence in heparin is unusual in favoring the 2S_0 conformational state over the 1C_4 and 4C_1 states more commonly adopted by such residues (16). This sequence-dependent change in the conformational preference of the iduronate residue has suggested that adoption of the 2S_0 conformational state may be required for the pentasaccharide to productively bind and activate antithrombin (16, 17). In the present study, we have tested this hypothesis by modulating the conformer populations of residue G, based on previous observations that the nature of substituents within or adjacent to an iduronate residue alters

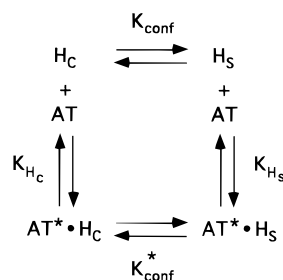
its solution conformational preference (16). Our studies have further addressed the proposed role of an asymmetric charge cluster in the GH disaccharide in binding to a secondary site in antithrombin induced by inhibitor activation (12). The existence of this charge cluster was previously suggested from the observation that an additional sulfate placed within the cluster at the 3-OH of residue H enhances pentasaccharide activity (14). However, this study did not show to what extent the enhanced activity resulted from effects on the conformation of iduronate residue G or from direct interactions of the 3-O-sulfate with antithrombin. It has also been unclear whether pentasaccharide binding to or activation of antithrombin is affected by this pentasaccharide modification (12).

The substitution of C-3 –OH groups of pentasaccharide residues G and H with a hydrogen atom or sulfate group was not expected to abolish any hydrogen-bonding interactions of the –OH group with antithrombin, since structure–activity studies have shown that only charged carboxylates and sulfates and not –OH groups participate in pentasaccharide binding (12). Our observation that pentasaccharides DEFGH* and DEFG'H* whose 3-OH of residue G was either methylated or free have indistinguishable antithrombin-binding properties confirmed this expectation. Modifications of C-3-OH groups of residues G and H further did not change the mode of pentasaccharide activation of antithrombin, as judged from the ability of the variant pentasaccharides to induce tryptophan fluorescence enhancements in antithrombin and accelerated rates of factor Xa inhibition by the inhibitor, which were indicative of normal conformational activation of the serpin by the pentasaccharides. Such findings are consistent with our previous observation that the nonreducing-end trisaccharide fragment DEF, which was common to all the pentasaccharides studied, is sufficient to induce the activating conformational change in antithrombin, although with a substantially decreased affinity for antithrombin (18, 28).

Rapid kinetic studies showed that the changes in pentasaccharide affinity for antithrombin produced by modifications of the structure of pentasaccharide residues G and H were minimally due to changes in the initial low-affinity interaction with the native antithrombin conformation but mostly arose from alterations in the subsequently induced high-affinity interaction with the activated antithrombin conformation. The latter alterations resulted mainly from changes in the rate constant for conversion of the high heparin affinity conformation back to the low heparin affinity conformation (k_{-2} in Scheme 1). Similar primary effects on k_{-2} were previously found when pentasaccharide residue H or both residues G and H were deleted (18). Such observations indicated that the flexible reducing end residues G and H do not significantly bind antithrombin in the initial binding step and instead bind the inhibitor with high affinity only after its conformational activation. Our current findings therefore lend support to our previous conclusion that the flexible reducing-end pentasaccharide residues G and H function primarily to preferentially bind and stabilize the activated conformational state of antithrombin induced by the rigid nonreducing-end residues DEF (18, 28).

To determine how changes in the preference for the 2S_0 conformer of the pentasaccharide would be expected to quantitatively affect pentasaccharide binding affinity for

Scheme 2



antithrombin, a general model for differential binding of the skew boat and chair conformers was considered (Scheme 2):

In this model, H_C and H_S represent heparin pentasaccharide with iduronate residue G in either the chair conformations, 1C_4 or 4C_1 , or in the skew-boat conformation, 2S_0 , respectively, K_{conf} and K_{conf}^* are the conformational equilibrium constants for free and bound pentasaccharides, and K_{H_C} and K_{H_S} are intrinsic association constants for the binding of pentasaccharide conformers to antithrombin. The designations, $\text{AT}^* \cdot H_C$ and $\text{AT}^* \cdot H_S$, for the antithrombin–pentasaccharide complexes, signify the ability of either pentasaccharide conformer to activate antithrombin given the sufficiency of the common DEF fragment of the pentasaccharide conformers to effect such activation (18). The observed association constant for pentasaccharide binding to antithrombin, K_{obs} , for this model is given by

$$K_{\text{obs}} = \frac{[\text{AT}^* \cdot H_C] + [\text{AT}^* \cdot H_S]}{[\text{AT}][H_C] + [H_S]} \quad (5)$$

Expansion and rearrangement of this equation yields

$$K_{\text{obs}} = \left(\frac{[\text{AT}][H_C]}{[\text{AT}^* \cdot H_C]} + \frac{[\text{AT}][H_S]}{[\text{AT}^* \cdot H_S]} \right)^{-1} + \left(\frac{[\text{AT}][H_C]}{[\text{AT}^* \cdot H_S]} + \frac{[\text{AT}][H_S]}{[\text{AT}^* \cdot H_C]} \right)^{-1} \quad (6)$$

Substitution into eq 6 of the expressions for the equilibrium constants K_{conf} , K_{H_C} , and K_{H_S} given by eqs 7–9,

$$K_{\text{conf}} = \frac{[H_S]}{[H_C]} \quad (7)$$

$$K_{H_C} = \frac{[\text{AT}^* \cdot H_C]}{[\text{AT}][H_C]} \quad (8)$$

$$K_{H_S} = \frac{[\text{AT}^* \cdot H_S]}{[\text{AT}][H_S]} \quad (9)$$

yields an equation for the dependence of K_{obs} on K_{conf} , K_{H_C} , and K_{H_S} :

$$K_{\text{obs}} = K_{H_C} \frac{1}{1 + K_{\text{conf}}} + K_{H_S} \frac{K_{\text{conf}}}{1 + K_{\text{conf}}} \quad (10)$$

In this equation, the ratios $1/(1 + K_{\text{conf}})$ and $K_{\text{conf}}/(1 + K_{\text{conf}})$ represent the fraction of pentasaccharide in chair and skew boat conformations, respectively. Equation 10 indicates

that K_{obs} is simply the sum of the intrinsic association constants for the binding of each pentasaccharide conformer to antithrombin weighted by the fraction of pentasaccharide in each conformation. If $K_{H_C} \ll K_{H_S}$, the left-hand term of the sum will be small relative to the right-hand term and K_{obs} will be approximately proportional to the fraction of pentasaccharide in the 2S_0 conformational state. This relation will hold for pentasaccharides whose K_{conf} differs due to changes in pentasaccharide structure, provided such changes affect only the equilibrium between pentasaccharide conformers and not the interactions of these conformers with antithrombin, i.e., as long as K_{H_C} and K_{H_S} are not affected.

This condition is likely to be true for DEFG*H in which the G residue 3-OH was replaced with –H, since the present and past studies indicate that the 3-OH group does not participate in pentasaccharide binding (12). Comparison of K_{obs} and $K_{\text{conf}}/(1 + K_{\text{conf}})$ for pentasaccharides DEFGH and DEFG*H reveals a good proportional relationship between these equilibrium parameters. A 2.7-fold decrease in the fraction of pentasaccharide in the 2S_0 conformation for pentasaccharide DEFG*H relative to DEFGH thus produced a (2.6 ± 0.3) -fold decrease in K_{obs} . Such a proportional relationship is in accord with eq 10 for the condition $K_{H_C} \ll K_{H_S}$ and suggests a value for K_{H_S} of $3.1 \times 10^7 \text{ M}^{-1}$. These data therefore support a model in which antithrombin preferentially binds to the 2S_0 pentasaccharide conformer over the two chair conformers and suggest that the magnitude of this binding preference is considerable. The 2S_0 pentasaccharide conformer would be expected to bind the serpin with a ~ 1000 -fold greater affinity than the chair conformers if the reducing-end GH disaccharide bound antithrombin only when residue G was in the 2S_0 conformational state, based on the binding-energy contribution previously measured for the GH disaccharide (18). The preferential binding of the 2S_0 pentasaccharide conformer is supported by the recent report that a variant pentasaccharide whose iduronate was fixed in the 1C_4 conformation has a very weak activity in accelerating antithrombin inhibition of factor Xa (27). The prospect of synthesizing additional pentasaccharide variants such as DEFG*H to further test the preferential binding model appears limited by the difficulty in altering just the iduronate conformational equilibrium without affecting direct pentasaccharide interactions with antithrombin.

This was certainly the case for pentasaccharides DEFGH* and DEFG'H* in which perturbation of direct interactions with antithrombin rather than effects on the iduronate conformational equilibrium appeared to be the primary basis for the observed changes in affinity. Thus, eq 10 for the preferential binding model of Scheme 2 suggests that the 1.3–1.5-fold increase in the fraction of 2S_0 conformer produced by 3-O-sulfation of residue H in DEFGH* and DEFG'H* should produce at most a 1.3–1.5-fold increase in K_{obs} . The observed ~ 35 -fold greater affinity of DEFGH* and DEFG'H* than of DEFGH for antithrombin therefore cannot be attributed to the increased preference of residue G for the skew boat conformation. On the basis of modeling studies of the antithrombin–pentasaccharide interaction, placement of the additional sulfate group in a negative charge cluster in the GH disaccharide was expected to increase the number of direct charge–charge interactions with a positively charged second site in antithrombin (12, 13). Studies of the salt dependence of the interaction of pentasaccharides

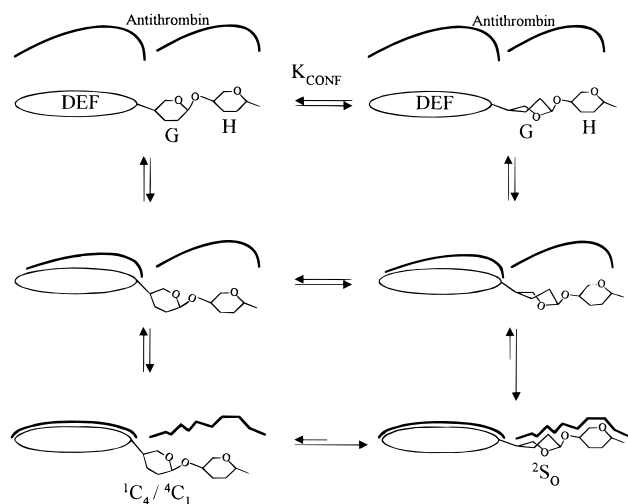


FIGURE 5: Proposed model for preferential binding to antithrombin of the 2S_0 conformer of the iduronate residue G in heparin pentasaccharide DEFGH. The rigid nonreducing-end trisaccharide, DEF, initially recognizes and binds the native antithrombin conformation with the unbound reducing-end GH disaccharide in either chair ($^1C_4/^4C_1$) or skew boat (2S_0) conformations. This induces the activating conformational change in antithrombin which enhances the complementarity of the serpin with DEF and produces a complementary site for GH when iduronate residue G is in the 2S_0 conformational state. The 2S_0 conformer of GH then binds and stabilizes the activated antithrombin conformation. The unbound GH disaccharide in the 1C_4 conformational state is also induced into the 2S_0 state due to the preferential binding of the latter conformer to activated antithrombin.

DEFGH* and DEFG'H* with antithrombin indeed showed that the 3-O-sulfate of DEFGH* and DEFG'H* does make an additional electrostatic interaction with the inhibitor compared to pentasaccharides DEFGH and DEFG'H lacking this 3-O-sulfate. While the additional electrostatic interaction accounted for only about a quarter of the enhanced binding energy ($\Delta\Delta G^\circ$) of DEFGH* and DEFG'H* relative to DEFGH at I 0.3, the electrostatic component of the binding energy deduced from the salt dependence of binding only includes charge–charge interactions which are exchangeable with monovalent salt ions and excludes potential hydrogen-bonding interactions of the sulfate group (23). The recently reported X-ray structure of antithrombin complexed with a similar supersulfated pentasaccharide suggests that hydrogen bonds do make a significant contribution to the H residue 3-O-sulfate interaction (15). We therefore conclude that the enhanced binding affinity of the supersulfated pentasaccharides for antithrombin arises mostly from ionic and hydrogen-bonding interactions of the H residue 3-O-sulfate with a second GH disaccharide binding site of the serpin and only slightly from the increased preference of residue G for the 2S_0 conformational state.

Together, our present and past results (18, 28) therefore suggest that the conformational flexibility of iduronate residue G facilitates pentasaccharide binding and activation of antithrombin through the induced-fit binding model depicted in Figure 5. In this model, the nonreducing-end DEF unit recognizes and binds with low affinity to the native antithrombin conformation with iduronate residue G either in the 1C_4 or 2S_0 conformational state. This binding induces the activating conformational change in antithrombin which enhances the complementarity of DEF for the serpin and

creates a complementary second site for binding the 2S_0 conformer of the GH disaccharide charge cluster. The structure of the antithrombin–pentasaccharide complex localizes the DEF binding site to one end of a groove bounded by the C-terminus of helix D and extending toward the N-terminus of this helix, whereas the GH binding site is created at the other end of the helix D groove by the junction of helix A with an induced helix P (15). Once the GH binding site is formed by activation of the serpin, the 2S_0 conformer of the disaccharide binds and thereby shifts any remaining unbound disaccharide in the 1C_4 conformation into the 2S_0 conformation, whereupon it also binds, yielding an overall high-affinity pentasaccharide interaction. According to this model, the flexibility of the GH unit facilitates conversion of residue G to the 2S_0 productive binding conformer once the rigid DEF unit has bound and activated the serpin, thereby allowing the GH unit to bind and stabilize the activated serpin conformation.

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REFERENCES

1. Gettins, P. G. W., Patston, P. A., and Olson, S. T. (1996) *Serpins: Structure, Function and Biology*, R. G. Landes, Austin, TX.
2. Björk, I., and Olson, S. T. (1997) in *Chemistry and Biology of Serpins* (Church, F. C., Cunningham, D. D., Ginsburg, D., Hoffman, M., Stone, S. R., and Tollefsen, D. M., Eds.) pp 17–33, Plenum Press, New York.
3. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) *J. Biol. Chem.* 255, 10081–10090.
4. Olson, S. T., Björk, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) *J. Biol. Chem.* 267, 12528–12538.
5. Thunberg, L., Bäckström, G., and Lindahl, U. (1982) *Carbohydr. Res.* 100, 393–410.
6. Choay, J., Petitou, M., Lormeau, J. C., Sinaï, P., Casu, B., and Gatti, G. (1983) *Biochem. Biophys. Res. Commun.* 116, 492–499.
7. Atha, D. H., Lormeau, J. C., Petitou, M., Rosenberg, R. D., and Choay, J. (1985) *Biochemistry* 24, 6723–6729.
8. Olson, S. T., and Shore, J. D. (1981) *J. Biol. Chem.* 256, 11065–11072.
9. Olson, S. T., Srinivasan, K. R., Björk, I., and Shore, J. D. (1981) *J. Biol. Chem.* 256, 11073–11079.
10. Björk, I., and Lindahl, U. (1982) *Mol. Cell. Biochem.* 48, 161–182.
11. Petitou, M., and van Boeckel, C. A. A. (1992) in *Progress in the Chemistry of Organic Natural Products* (Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., and Tamm, C., Eds.) pp 143–210, Springer-Verlag, New York.
12. van Boeckel, C. A. A. and Petitou, M. (1993) *Angew. Chem., Int. Ed. Engl.* 32, 1671–1690.
13. Grootenhuis, P. D. J., and van Boeckel, C. A. A. (1991) *J. Am. Chem. Soc.* 113, 2743–2747.
14. van Boeckel, C. A. A., Beetz, T., and van Aelst, S. F. (1988) *Tetrahedron Lett.* 29, 803–806.
15. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) *Proc. Natl. Acad. Sci., U.S.A.* 94, 14683–14688.
16. Ferro, D. R., Provasoli, A., Ragazzi, M., Torri, G., Casu, B., Gatti, G., Jacquinet, J. C., Sinaï, P., Petitou, M., and Choay, J. (1986) *J. Am. Chem. Soc.* 108, 6773–6778.

17. Ragazzi, M., Ferro, D. R., Perly, B., Sinay, P., Petitou, M., and Choay, J. (1990) *Carbohydr. Res.* 195, 169–185.
18. Desai, U. R., Petitou, M., Björk, I., and Olson, S. T. (1998) *J. Biol. Chem.* 273, 7478–7487.
19. Olson, S. T., Björk, I., and Shore, J. D. (1993) *Methods Enzymol.* 222, 525–560.
20. Nordenman, B., Nyström, C., and Björk, I. (1977) *Eur. J. Biochem.* 78, 195–203.
21. Bock, P. E., Craig, P. A., Olson, S. T., and Singh, P. (1989) *Arch. Biochem. Biophys.* 273, 375–388.
22. Lei, P. S., Duchaussoy, P., Sizun, P., Mallet, J. M., Petitou, M., and Sinay, P. (1998) *Bioorg. Med. Chem.* (Submitted for publication).
23. Mascotti, D. P., and Lohman, T. M. (1995) *Biochemistry* 34, 2908–2915.
24. Olson, S. T., Halvorson, H. R., and Björk, I. (1991) *J. Biol. Chem.* 266, 6342–6352.
25. Björk, I., Ylinenjärvi, K., Olson, S. T., Hermentin, P., Conradt, H. S., and Zettlmeissl, G. (1992) *Biochem. J.* 286, 793–800.
26. Olson, S. T., and Björk, I. (1991) *J. Biol. Chem.* 266, 6353–6364.
27. Sakairi, N., Basten, J., van der Marel, G. A., van Boeckel, C. A. A., and van Boom, J. H. (1996) *Chem. Eur. J.* 2, 1007–1013.
28. Petitou, M., Barzu, J., Herault, J.-P., and Herbert, J.-M. (1997) *Glycobiology* 7, 323–327.
29. Petitou, M., Jaurand, G., Derrien, M., Duchaussoy, P., and Choay, J. (1991) *Biorg. Med. Chem. Lett.* 2, 95–98.

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