Mechanism of Heparin Activation of Antithrombin

ROLE OF INDIVIDUAL RESIDUES OF THE PENTASACCHARIDE ACTIVATING SEQUENCE IN THE RECOGNITION OF NATIVE AND ACTIVATED STATES OF ANTITHROMBIN*

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To determine the role of individual saccharide residues of a specific heparin pentasaccharide, denoted DEFGH, in the allosteric activation of the serpin, antithrombin, we studied the effect of deleting pentasaccharide residues on this activation. Binding, spectroscopic, and kinetic analyses demonstrated that deletion of reducing-end residues G and H or nonreducing-end residue D produced variable losses in pentasaccharide binding energy of ~15–75% but did not affect the oligosaccharide’s ability to conformationally activate the serpin or to enhance the rate at which the serpin inhibited factor Xa. Rapid kinetic studies revealed that elimination of the reducing-end disaccharide marginally affected binding to the native low-heparin-affinity conformational state of antithrombin but greatly affected the conversion of the serpin to the activated high-heparin-affinity state, although the activated conformation was still favored. In contrast, removal of the nonreducing-end residue D drastically affected the initial low-heparin-affinity interaction so as to favor an alternative activation pathway wherein the oligosaccharide shifted a preexisting equilibrium between native and activated serpin conformations in favor of the activated state. These results demonstrate that the nonreducing-end residues of the pentasaccharide function both to recognize the native low-heparin-affinity conformation of antithrombin and to induce and stabilize the activated high-heparin-affinity conformation. Residues at the reducing-end, however, poorly recognize the native conformation and instead function primarily to bind and stabilize the activated antithrombin conformation. Together, these findings establish an important role of the heparin pentasaccharide sequence in preferential binding and stabilization of the activated conformational state of the serpin.

Antithrombin, a member of the serpin (serine proteinase inhibitor) superfamily of proteins, is the primary physiological inhibitor of blood coagulation proteinases, especially thrombin and factor Xa (for reviews, see Refs. 1 and 2). Antithrombin inhibits these proteinases by forming tight, possibly covalent complexes in which an exposed reactive center loop of the inhibitor is bound at the enzyme active site in the manner of a substrate. The rate of this inhibition is slow in comparison with most other serpin-proteinase reactions. However, the inhibition rate greatly increases up to several thousand-fold in the presence of the glycosaminoglycan, heparin (1, 2). This property of heparin is responsible for the widespread use of the polysaccharide as an anticoagulant drug.

The extent to which heparin accelerates antithrombin-proteinase reactions is dependent on a unique pentasaccharide sequence (DEFGH; see Fig. 1), present in about one-third of naturally occurring heparin chains (3–6). Heparin molecules containing this sequence bind antithrombin with high affinity and induce an activating conformational change in the inhibitor (7–10). Those polysaccharide chains that lack this sequence bind antithrombin with ~1000-fold lower affinity and only weakly activate the inhibitor (11). Whereas the antithrombin conformational change is necessary and sufficient for the inhibitor to accelerate the inactivation of factor Xa, it is not sufficient for accelerated thrombin inhibition (12). The latter acceleration additionally requires a longer heparin chain to bridge the proteinase and the inhibitor in a ternary complex (12–16).

The heparin binding site of antithrombin has been tentatively mapped to residues of helix A, helix D, and the N-terminal region of the inhibitor, which are contiguous in the x-ray structure (1, 2, 17–21). Structure-activity studies of variants of the pentasaccharide have additionally revealed key functional groups in the oligosaccharide responsible for tight binding to the inhibitor. Four anionic groups, namely two N-sulfates and two O-sulfates in the glucosamine residues D, F, and H of the pentasaccharide (Fig. 1) are critical for complex formation and are thought to make ionic interactions with basic residues in the heparin binding site of antithrombin (6, 14, 22, 23, 31, 41). Additionally, nonionic interactions make a considerable contribution (about 60% at physiological pH and ionic strength) to the total binding energy (12). The pentasaccharide binds to antithrombin in a two-step process, in which a low-affinity recognition complex is first formed, which then induces an activating conformational change in the inhibitor, leading to a high-affinity complex (10, 12). Structural and functional studies suggest that the activating conformational change in antithrombin involves structural changes in the heparin binding site, which transform the reactive center loop from a partially buried to a fully exposed conformation (17, 21,

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1 The DEFGH designation of the heparin pentasaccharide originates from its being a part of a larger decasaccharide fragment, designated ABCDEFGHJ, which was earlier isolated from heparin and later shown to contain the minimal pentasaccharide activating sequence (45).

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The mechanism of heparin activation of antithrombin is as yet ill-defined at the molecular level. Although residues in the pentasaccharide and in antithrombin that are critical for their high-affinity interaction are known, it has not been elucidated which of these residues are involved in the initial recognition and in the subsequent allosteric activation of the serpin. To assess the roles of individual pentasaccharide residues in the two-step activation mechanism, we have studied the effect of truncating pentasaccharide residues at either the reducing or the nonreducing-end (see Fig. 1) on the ability of the oligosaccharide to bind and induce the conformational change in antithrombin. These effects have been correlated with the ability of the truncated pentasaccharides to activate the inhibitor for rapid inhibition of factor Xa. In agreement with preliminary studies of antithrombin interactions with these oligosaccharides (38), the present study establishes that the nonreducing-end trisaccharide, DEF, of the pentasaccharide is capable of fully activating antithrombin and, consequently, that the reducing-end residues are not essential for this activation, although they stabilize the activated conformation. The study further demonstrates a critical role of the pentasaccharide sequence in preferential binding of the activated conformational state of the serpin, in keeping with the pentasaccharide functioning as a classical allosteric activator.

MATERIALS AND METHODS

Proteins—Human antithrombin was purified from outdated plasma as described previously (15, 27). 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 (28). Human factor Xa was prepared by purification of purified factor X, followed by purification on SBTI-agarose, as described previously (29). Factor Xa preparations were predominantly the α-form as judged by SDS-polyacrylamide gel electrophoresis and were >98% active by comparisons of active site and protein concentrations (29).

Oligosaccharides—The methyl glycosides of pentasaccharide and pentasaccharide variants were synthesized, and their structures were confirmed as described previously (30, 31, 42). A 2–10 mM solution of each variant was prepared based on weight in distilled, deionized water. Stoichiometric titrations of high concentrations of antithrombin (at least 10 times the $K_D$) with DEFGH, DEFGH, and DEFH* (Fig. 1), monitored by the endogenous fluorescence enhancement, showed fair agreement with the weight concentrations. The former concentrations were used for these tighter binding oligosaccharides, whereas concentrations based on weight were used for all other oligosaccharides.

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 the required pH. In the absence of added salt, the ionic strengths of buffers at pH 6.0 and 7.4 were 0.025 and 0.05, respectively. Sodium chloride was added to concentrations of 100, 250, 350, 500, 600, and 700 mM to achieve higher ionic strengths.

Spectroscopic Studies—Fluorescence emission spectra of antithrombin and its complexes with oligosaccharides were obtained at 25 °C in pH 6.0, I = 0.025 buffer with 1 μM antithrombin and at least 5 μM oligosaccharide. The spectra were recorded with an SLM 8000 spectrofluorometer in the ratio mode at 1-nm wavelength intervals with excitation at 280 nm (4-nm bandpass), an emission bandpass of 2 nm, and DEFGH as the excitation at 280 nm (4-nm bandpass), an emission bandpass of 2 nm, and DEFGH as the oligosaccharide. The spectra were recorded with an SLM 8000 spectrofluorometer, in the ratio mode at 1-nm wavelength intervals 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 bandpass. The fluorescence traces were acquired for about 10 half-lives and could be satisfactorily fit by a single exponential function for DEFGH, DEFGH*, DEF, and DEFGH* interactions, which provided the amplitude of the fluorescence change and the observed pseudo-first-order rate constant, $k_{obs}$. The traces for variant factor Xa phosphating with antithrombin could only be fit satisfactorily by a double exponential function that yielded two pseudo-first-order rate constants and two fluorescence amplitudes. Typically, 12–18 fluorescence traces were acquired for each set of concentrations and averaged.

Factor Xa Inhibition Studies—The accelerating effects of oligosaccharides on the kinetics of antithrombin inhibition of factor Xa were measured under pseudo-first-order conditions. A fixed 10 μM concentration of factor Xa was incubated with 2–12 μM antithrombin and oligosaccharide in pH 6.0 buffer in a 50-μL total reaction volume. The oligosaccharide concentration was typically less than 20 μM, except for EFGH* and FGH*, which were present in the 20–240 nM range, and at least three different oligosaccharide concentrations were examined. After incubation for various times, reactions were quenched with 950 μL of 100 μM Spectrozyme FXa in 20 mM sodium phosphate buffer, containing 100 mM sodium chloride, 0.1 mM EDTA, 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 ($k_{obs}$) at each oligosaccharide concentration was determined from the exponential decay of factor Xa activity (27).

The second-order rate constant for factor Xa inhibition by antithrombin was determined as described previously (10, 12). Pseudo-first-order conditions in which the oligosaccharide concentration was typically less than 20 nM, except for DEFH and DEFH* interactions with antithrombin, and [FGH]m represents the total concentration of FGH*. This equation is appropriate, because the condition, $[AT] < ([FGH]m - [FGH])$, together with the value of $K_{D,FGH}$ ensured that the free concentration of FGH* was approximated by the total oligosaccharide concentration.

Rapid Kinetic Studies—The rate of oligosaccharide binding to plasma antithrombin was measured in pH 6.0, I = 0.025 buffer at 25 °C in an Applied Photophysics stopped-flow fluorometer, as described previously (10, 12). Pseudo-first-order conditions in which the oligosaccharide and antithrombin molar concentrations were maintained at a ratio of at least 5:1 and in most cases ≥10:1 were employed. The interaction was monitored from the increase in intrinsic protein fluorescence with 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 bandpass. The fluorescence traces were acquired for about 10 half-lives and could be satisfactorily fit by a single exponential function for DEFGH, DEFGH*, DEF, and DEFGH* interactions, which provided the amplitude of the fluorescence change and the observed pseudo-first-order rate constant, $k_{obs}$. The traces for variant factor Xa phosphating with antithrombin could only be fit satisfactorily by a double exponential function that yielded two pseudo-first-order rate constants and two fluorescence amplitudes. Typically, 12–18 fluorescence traces were acquired for each set of concentrations and averaged.

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for antithrombin-oligosaccharide complexes were determined by titrating the oligosaccharide into a solution of antithrombin and monitoring the increase in intrinsic protein fluorescence accompanying the interaction, as described previously (9, 27). Antithrombin concentrations were in the range of 1–2 times the $K_D$, except for $K_D$ values >1 μM, in which the concentration was 10-fold the $K_D$. In the case of increasing oligosaccharide concentration was fit to the quadratic equilibrium binding equation, assuming a 1:1 binding stoichiometry (9, 27).

The nonionic and ionic contributions to the total pentasaccharide binding energy were resolved by analyzing the NaCl concentration dependence of the observed dissociation constant $K_{D,obs}$ according to the equation (12, 16, 33), $\log K_{D,obs} = \log K_{D,nonionic} + \log K_{D,ionic} - 2.303 \log [Na^+]$, where $K_{D,nonionic}$ is the salt-independent dissociation constant, $Z$ is the total number of charge-charge interactions involved in the association of the protein with heparin, and $Y$ is the fraction of monovalent cations bound per heparin ionic charge that are released upon protein binding. 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 after dividing by the value of 0.8 for $Y$ (16).

The accelerating effects of oligosaccharides alone a concentration of 10 μM and those for the complex formation by antithrombin oligosaccharide complexes ($k_{obs}$) were obtained by least-squares analysis of the linear dependence of $k_{obs}$ on antithrombin-oligosaccharide complex concentration according to the equation, $k_{obs} = k_{inact} \times ([AT] + k_{b} \times [H]) \times ([AT]/[K_D + [AT]])$, where [AT] and [H] are the total concentrations of antithrombin and heparin oligosaccharide, respectively, and $K_D$ is the dissociation constant of the complex (27). The expression $[H]_I$ is
mally affect pentasaccharide binding and activation of antithrombin in most cases (41, 42). For the one case in which a structural modification did significantly enhance pentasaccharide activity, namely the addition of 3-O-sulfate on saccharide H in EFGH, a reference pentasaccharide containing this structural modification (DEFGH') served as a control (42), thereby allowing the effect of the D residue to be determined.

Under the conditions previously used to study the antithrombin-pentasaccharide interaction (pH 7.4, I 0.15, 25 °C), binding of the tetrasaccharides but not the trisaccharides to antithrombin was measurable from the saturable enhancement in antithrombin fluorescence produced when the inhibitor was titrated with up to 100 μM oligosaccharide. Lowering the ionic strength to ≤0.05 or the pH of the buffer to 6.0 sufficiently enhanced the affinity of the oligosaccharides for antithrombin so that binding constants were measurable for all oligosaccharides under the same conditions. Table I summarizes the dissociation constants and maximal fluorescence changes measured for the binding of the truncated pentasaccharides to antithrombin under these various conditions.

At pH 7.4, I 0.15, 25 °C, tetrasaccharide DEFG* bound antithrombin with a 300-fold lower affinity than pentasaccharide DEFGH (ΔΔG° = 3.5 kcal/mol), in reasonable agreement with previous reports of a ~100-fold lower affinity of tetrasaccharide DEFG than DEFGH (23) and of the small contribution of the 2-O-sulfate of saccharide G missing in DEFG* (41). The decreased affinity of DEFG* for antithrombin is therefore mostly due to deletion of the reducing-end saccharide H. Tetrasaccharide EFGH* bound antithrombin with a 60,000-fold lower affinity than reference pentasaccharide DEFGH (ΔΔG° ~6 kcal/mol). That this loss in binding energy was due to deletion of the nonreducing-end D saccharide and not to the replacement of the N-sulfate of residue H with an O-sulfate in EFGH* was suggested by the finding that DEFGH* with an O-sulfate instead of an N-sulfate in residue H bound antithrombin with an affinity indistinguishable from DEFGH* (not shown). Previous studies similarly found a much greater contribution of residue D than residue H to pentasaccharide binding (6, 23).

The ~2.5 kcal/mol greater contribution of residue D than residue H to pentasaccharide binding energy was increased to ~4 kcal/mol when the ionic strength was lowered from I 0.15 to 0.05 at pH 7.4 and was further augmented to ~6.0 kcal/mol when both the pH and ionic strength were lowered to pH 6.0, I 0.025. The D residue interaction thus appears to be largely responsible for the increased pentasaccharide binding energy at lower pH. Contrasting the large effects of deleting residues D and H on the pentasaccharide binding energy, the further deletion of residues E and G produced relatively smaller losses in binding energy (Table I), again in agreement with previous studies (6, 23).

Binding to antithrombin of the oligosaccharides DEFG* and DEF lacking the reducing-end residues of the pentasaccharide enhanced the intrinsic protein fluorescence of the serpin to the same extent (~30%) as that of the reference pentasaccharides DEFGH and DEFGH under all conditions examined (Fig. 2). By contrast, binding of pentasaccharides missing the nonreducing-end residues induced different extents of fluorescence change than the reference pentasaccharides. Tetrasaccharide EFGH* thus consistently produced a larger fluorescence enhancement of ~45%, whereas trisaccharide FGH* induced a much reduced fluorescence enhancement of 7–14%. Similar results were obtained from the maximal fluorescence enhance-

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2 Structure-activity studies of the natural pentasaccharide, DEFGH, have shown that replacement of N-sulfates with O-sulfates or methylation of -OH groups minimally affects pentasaccharide binding to antithrombin or activation of the inhibitor toward factor Xa (41).
Dissociation constants and maximal fluorescence enhancements for antithrombin-heparin oligosaccharide interactions

Dissociation constants were measured at 25 °C in 20 mM sodium phosphate buffer containing 0.1 mM EDTA and 0.1% (w/v) PEG 8000 adjusted to the required pH. Sodium chloride was either absent (I 0.05 or 0.025) or was present at 100 mM (I 0.15). The enhancement in intrinsic fluorescence of antithrombin accompanying oligosaccharide binding was fit by the equilibrium binding equation to obtain $K_D$ and $\Delta F_{\max}$. See “Materials and Methods” for details. Reported errors represent the S.D. of at least two determinations.

| Oligosaccharide | pH 7.4, I 0.15 | pH 7.4, I 0.05 | pH 6.0, I 0.025 |
|-----------------|----------------|----------------|-----------------|
|                 | $K_D$ | $\Delta G^\ominus$ | $\Delta F_{\max}$ | $K_D$ | $\Delta G^\ominus$ | $\Delta F_{\max}$ | $K_D$ | $\Delta G^\ominus$ | $\Delta F_{\max}$ |
| DEFGH           | 5.0 ± 0.6 × 10^{-8} | 10.0 | 32 ± 3 | −6 × 10^{-10} | 13 | 28 ± 1 | −1 × 10^{-12} | 16 | 33 ± 2 |
| DEFH            | 1.7 ± 0.2 × 10^{-5} | 6.5 | 28 ± 1 | 1.8 ± 0.3 × 10^{-6} | 7.8 | 31 ± 3 | 3.4 ± 0.3 × 10^{-6} | 10.2 | 33 ± 3 |
| DEF             | ND              | 1.1 ± 0.4 × 10^{-5} | 5.7 | 28 ± 1 | 2.0 ± 0.6 × 10^{-6} | 7.8 | 32 ± 2 |
| FGH             | ND              | 1.2 ± 0.3 × 10^{-4} | 5.34 | −7′ | 1.1 ± 0.1 × 10^{-5} | 6.8 | 14 ± 1 |
| DEFGH           | −1 × 10^{-9}    | 12 | 30 ± 2 | −5 × 10^{-12} | −15 | ND | −1 × 10^{-14} | −19′ | ND |
| EFGH            | 6.1 ± 0.3 × 10^{-5} | 5.8 | 49 ± 1 | 2.8 ± 0.1 × 10^{-5} | 6.2 | 48 ± 1 | 6.9 ± 1.3 × 10^{-6} | 7.0 | 41 ± 4 |

$^a$ Obtained by extrapolation of the linear dependence of log $K_{D,ob}$ on log [Na+] using slope and intercept values given in the text for pentasaccharide DEFGH binding at pH 7.4 and 6.0 and values of 3.81 ± 0.13 and −5.77 ± 0.06 for pentasaccharide DEFGH binding at pH 7.4. $^b$ ND, not determined.

FIG. 2. Changes in the fluorescence emission spectrum of antithrombin induced by the binding of variant pentasaccharides. Spectra (average of two acquisitions) were recorded in pH 6.0, I 0.025 buffer at 25 °C, corrected for buffer background and for the emission due to free antithrombin, and then normalized to the same concentration based on measured dissociation constants in Table I. See “Materials and Methods” for further details.

Results attained in equilibrium binding titrations (Table I). The wavelength of maximum emission intensity for antithrombin complexed with all oligosaccharides was very similar to that for antithrombin alone, in agreement with previous reports (9).

Origin of the pH-dependent Change in Pentasaccharide Binding Affinity—To determine whether the higher pentasaccharide affinity for antithrombin at pH 6.0 than at pH 7.4 was due to additional charge-charge interactions, the dissociation constant for the antithrombin-pentasaccharide interaction was measured as a function of the NaCl concentration both at pH 7.4 and 6.0. The log $K_{D, ob}$ increased linearly with increasing log [Na+] both at pH 7.4 and 6.0 (not shown), in accordance with the expected behavior of the protein-heparin interaction (12, 16, 33). The slopes of these lines indicated that 5.2 ± 0.1 and 4.0 ± 0.2 charge-charge interactions contributed to the binding at pH 6.0 and 7.4, respectively, whereas the intercepts gave values of −4.92 ± 0.05 and −4.58 ± 0.02 for log $K_{D, nonionic}$ at these respective pH values (see “Materials and Methods”). The values obtained at pH 7.4 are similar to those reported previously (12). These results indicate that the increase in pentasaccharide binding affinity when the pH is lowered from 7.4 to 6.0 results primarily from one additional ionic interaction at pH 6.0, which is presumably made by residue D.

Rapid Kinetic Studies of DEFGH, DEFGH, and DEF Binding to Antithrombin—The kinetics of binding of oligosaccharides DEFGH, DEFGH, and DEF to antithrombin was studied by continuously monitoring the protein fluorescence changes accompanying their binding by stopped-flow fluorimetry under pseudo-first-order conditions in pH 6.0 buffer. Binding was observable in the stopped-flow time frame as a monophasic exponential process in all cases. The dependence on oligosaccharide concentration of the observed pseudo-first-order rate constant (k$_{obs}$) for binding of these oligosaccharides to antithrombin is shown in Fig. 3. In all cases, a progressive saturation of k$_{obs}$ with increasing oligosaccharide concentration was observed, indicative of a two-step binding process, in which an initial binding of the oligosaccharide to antithrombin induces a subsequent conformational change in the inhibitor (induced-fit pathway in Scheme 1) (10, 12). The data of Fig. 3 were satisfactorily fit by the rectangular hyperbolic Equation 1, which characterizes the induced conformational change pathway (10).

$$k_{obs} = k_{-2} + \frac{k_{[I]} [H]}{[H] + K_1}$$  (Eq. 1)

In this equation, K$_1$ is the dissociation constant for the initial binding step, k$_{2}$ and k$_{-2}$ are the forward and reverse rate constants for the conformational change step, and [H]$_0$ is the initial heparin oligosaccharide concentration. Table II shows the results of nonlinear regression fitting of Fig. 3 data by Equation 1. The k$_{-2}$ values obtained from the fitted intercepts of these saturation curves were not distinguishable from 0 in the case of the intact pentasaccharide but were clearly nonzero for the truncated pentasaccharides, DEFGH and DEF, k$_{-2}$ for the DEFGH interaction was calculated from the kinetic constants, K$_1$ and k$_{2}$, and the dissociation constant, K$_{D, ob}$, from the relation, k$_{-2} = K_{D, ob} \times k_f/K_1$. For the truncated pentasaccharides where k$_{-2}$ was measurable, a comparison of calculated and measured values of K$_{D, ob}$ showed excellent agreement (Table II).

Comparison of the kinetic constants obtained for the pentasaccharide at pH 6.0, I 0.025 with those previously measured at pH 7.4, I 0.15 (12) indicated that the increase in pentasaccharide binding affinity at lower pH and ionic strength resulted primarily from an effect on the conformational change step and specifically to a ~1000-fold reduction in the rate constant for pentasaccharide binding.
reversal of the conformational activation step ($k_{-2}$). The affinity for the initial antithrombin-pentasaccharide recognition complex ($K_1$) was also enhanced about 20-fold by the change in conditions, whereas the forward rate constant for conformational activation, $k_2$, was not affected.

Tetrasaccharide DEFG* lacking the pentasaccharide reducing-end residue showed a modestly decreased affinity of the recognition complex of 5-fold and practically no effect on the rate of conformational activation. In contrast, the rate at which conformational activation was reversed substantially increased by more than 1000-fold. Further elimination of residue G from the reducing-end similarly only modestly affected the affinity of the initial recognition complex (~2-fold) or the rate of conformational activation but greatly increased the rate of reversal of the conformational activation step further ~20-fold. The primary effects of deleting reducing-end residues, G and H, on $k_{-2}$ resulted in a progressive destabilization of the conformational equilibrium for inhibitor activation, governed by the ratio $k_f/k_{-2}$. This ratio thus decreased from $\sim 7 \times 10^5$ for the intact pentasaccharide DEFGH to 190 for tetrasaccharide DEFG* and to 8 for trisaccharide DEF (Table II).

**Rapid Kinetic Studies of DEFGH**, EFGH*, and FGH Binding to Antithrombin—The observed pseudo-first-order rate constant, $k_{obs}$, for binding of pentasaccharide DEFGH to antithrombin at pH 6.0, I 0.025, 25 °C progressively increased to a limiting value with increasing concentration of oligosaccharide (Fig. 4A), similar to the pattern observed for binding of oligosaccharides DEFGH, DEFG*, and DEF to antithrombin. Fitting of this data by Equation 1 for the induced conformational change binding mechanism resulted in a $K_1$ value of 1.2 ± 0.4 μM, a $k_2$ value of 1210 ± 230 s⁻¹, and a $k_{-2}$ value indistinguishable from 0. $k_{-2}$ was estimated to be $\sim 10^{-6}$ s⁻¹ based on measured values of $K_1$ and $k_2$ and an approximated value for $K_{D,obs}$ (Table I). The $K_1$ and $k_2$ parameters for DEFGH* are very similar to those for heparin pentasaccharide, DEFGH, indicating that the greater affinity of DEFGH* than of DEFGH for antithrombin is due to a reduced $k_{-2}$ value. Both 3-O-sulfation of reducing end residue H and deletion of this residue therefore affect the same kinetic parameter. Replacement of the N-sulfate of residue H in DEFGH* with an O-sulfate had no effect on the binding kinetics (not shown).

Stopped-flow traces of the binding to antithrombin of tetrasaccharide EFGH* truncated at the nonreducing-end at pH 6.0 showed two striking differences from the kinetics of binding of pentasaccharides DEFGH and DEFGH* and of oligosaccharides DEFG* and DEF truncated at the reducing-end (Fig. 4B). First, individual traces were not fit satisfactorily by a single exponential function but instead required a double exponential function to achieve a good fit. Second, $k_{obs}$ for the fast phase I fluorescence changes accompanying the binding interaction by stopped-flow fluorimetry at 25 °C in pH 6.0, I 0.025 buffer, as detailed in "Materials and Methods." Solid lines represent nonlinear least-squares fits of the data by Equation 1, which describes the induced conformational change binding mechanism (Scheme I).
progressively decreased rather than increased with increasing EFGH concentration, whereas \( k_{obs} \) for the slow phase II remained practically independent of the oligosaccharide concentration (Fig. 4C). That the biphasic changes in fluorescence indeed reflected the binding of tetrasaccharides EFGH to antithrombin was confirmed by the observation that the fluorescence amplitudes for each phase showed parallel saturable increases with EFGH concentration (Fig. 4D), which could be fit by similar \( K_D \) values of 6.0 ± 0.8 \( \mu M \) for phase I, 9.7 ± 1.1 \( \mu M \) for phase II, and 7.2 ± 0.7 \( \mu M \) for the sum of the two phases, values that were indistinguishable from the \( K_D \) measured by equilibrium binding titrations (Table I).

The hyperbolic decrease in the phase I \( k_{obs} \) with increasing EFGH concentration indicated that EFGH was binding and activating antithrombin in this phase by an alternative two-step pathway, in which oligosaccharide binding occurred only subsequent to the activation of the inhibitor in a pre-equilibrium conformational change step (Scheme 1, pre-equilibrium pathway) (34, 35). In keeping with such a mechanism, the \( \Delta F_{max} \) of −30% associated with this phase was similar to that produced by pentasaccharide activation of antithrombin. The appearance of the additional kinetic phase II indicated that the two-step conformational activation of antithrombin in phase I was followed by a further conformational change in the inhibitor in phase II, occurring with a rate constant of −1.5 s\(^{-1}\). Phases I and II data were analyzed by Equation 2 for a ligand-induced protein conformational change proceeding through the pre-equilibrium pathway (34, 35).

\[
k_{obs} = k_3 + \frac{k_2 K_3}{[H] + K_4} \quad \text{(Eq. 2)}
\]

In this equation, \( k_3 \) is the rate constant for pre-equilibrium conformational activation of antithrombin in the absence of heparin, \( k_{-3} \) is the reverse rate constant for this activation, and \( K_4 \) is the dissociation constant for oligosaccharide binding to the activated antithrombin formed in the pre-equilibrium step (Scheme 1). Fitting of the data in Fig. 4C by this equation yielded a limiting \( k_{obs} \) at high oligosaccharide concentration, corresponding to \( k_3 \), of 6.4 ± 0.5 s\(^{-1}\). Large errors associated with measurements of \( k_{obs} \) at lower oligosaccharide concentrations, due to smaller fluorescence changes, resulted in an indeterminate value of the ordinate intercept and a consequent inability to accurately determine \( k_3 \) and \( K_4 \) parameters.

No time-dependent changes in fluorescence were observable for the binding of trisaccharide FGH\(^+\) to antithrombin in pH 6.0 buffer despite the use of oligosaccharide concentrations, resulting in significant complex formation. However, an increase in fluorescence was evident immediately after mixing antithrombin with the trisaccharide, as shown by comparison with the baseline fluorescence established by mixing antithrombin with buffer. These results indicated that binding of FGH\(^+\) to antithrombin was complete within the 1.5-ms dead time of the stopped-flow instrument.

**Competitive Binding of FGH\(^+\) and DEFG\(^*\) to Antithrombin**—To determine whether FGH\(^+\) bound to the same site on antithrombin as the pentasaccharide, competitive binding experiments were performed. Tetrasaccharide DEFG\(^*\) was used as the competitor because of the much greater affinity of DEFG for antithrombin than of FGH\(^+\) at pH 6.0. Equilibrium binding of DEFG\(^*\) to antithrombin in the absence and presence of fixed concentrations of FGH\(^+\) at −1 and −4 times the \( K_D \) for the FGH\(^+\)-antithrombin interaction was monitored from the −20% greater increase in inhibitor fluorescence produced by DEFG\(^*\) than by FGH\(^+\) binding (Table I). The apparent dissociation constant for DEFG\(^*\) binding to antithrombin increased from 34 ± 3 nM in the absence of FGH\(^+\) to values of 89 ± 6 and 181 ± 13 nM in the presence of FGH\(^+\) concentrations of 13.3 and 43.3 \( \mu M \), respectively. These measured \( K_D \) values were indistinguishable from the calculated \( K_D \) values of 75 ± 10 and 167 ± 20 nM that were expected if FGH\(^+\) was acting as a competitive inhibitor of DEFG\(^*\) binding to antithrombin (see “Materials and Methods”).

**Accelerating Effects of Truncated Pentasaccharides on Factor Xa Inhibition by Antithrombin**—The second-order rate constant for the uncatalyzed inhibition of factor Xa by antithrombin (\( k_{cat} \)) was reduced by −15-fold at pH 6.0 from that at pH 7.4 (Table III), consistent with the increased levels of catalytically inactive serine proteinase resulting from protonation of histidine 57 of the catalytic triad (36). Second-order rate constants for the accelerated inhibition of factor Xa by antithrombin-oligosaccharide complexes (\( k_H \)) at pH 6.0 were evaluated from the slopes of the linear dependence of the pseudo-first-order inhibition rate constant on the concentration of antithrombin-oligosaccharide complex (see “Materials and Methods”) and are presented in Table III. Antithrombin complexes with DEFGH, DEFG\(^*\), and DEF showed indistinguishable \( k_H \) values of 4–5 \( \times 10^4 \) M\(^{-1}\) s\(^{-1}\), representing about 300-fold rate enhancements, indicating that deletion of reducing-end residues G and H did not affect the ability of the oligosaccharides to activate antithrombin. Similar \( k_H \) values for antithrombin complexes with DEF and DEFGH were also found at pH 7.4 (Table III). Complexes of DEFGH\(^+\) and EFGH\(^+\) with antithrombin resulted in \( k_H \) values similar to that of the antithrombin-DEFGH complex, indicating no effect of H residue 3-O-sulfation or D residue deletion on antithrombin activation. In contrast, binding to antithrombin of trisaccharide FGH\(^+\) significantly reduced \( k_H \) to −10% of the pentasaccharide accelerated rate constant, indicating that removal of both residues D and E from the nonreducing-end greatly compromised the ability to activate antithrombin.
DISCUSSION

We have elucidated the role of individual saccharide residues of a specific heparin pentasaccharide in the allosteric activation of the serpin, antithrombin, by determining the effects of deleting pentasaccharide residues on this activation. These effects had to be analyzed at a lower ionic strength and pH than was used in past studies to reliably measure the affinities and rates of interaction of the truncated pentasaccharides with antithrombin. Such conditions did not appear to alter the activation mechanism, since the interaction of the pentasaccharide with antithrombin under these conditions was characterized by the same two-step binding process, fluorescence enhancement, and acceleration of factor Xa inhibition previously found at physiological ionic strength and pH (10, 12). Notably, the binding energy contribution of residue D was more dominant under these experimental conditions due to an additional electrostatic interaction of residue D with antithrombin. The origin of this additional electrostatic binding energy is presently unclear, since histidine residues close to the heparin binding site do not appear to participate in ionic interactions with heparin at pH 6 (37).

While both reducing and nonreducing-end residues contribute to pentasaccharide binding energy, our results show that these residues are not absolutely essential to activate antithrombin for rapid inhibition of factor Xa. Thus, all truncated pentasaccharides, with the exception of oligosaccharide FGH−, which appears to bind antithrombin nonproductively (see below), were capable of inducing the tryptophan fluorescence enhancement and acceleration of factor Xa inhibition observed in the full-length pentasaccharide (10, 12).

FIG. 4. Effect of truncating nonreducing-end residue D on the kinetics of pentasaccharide binding to antithrombin. Panel A shows the oligosaccharide concentration dependence of pseudo-first-order rate constants (k_{obs}) for the binding of pentasaccharide DEFGH− to antithrombin at 25 °C in pH 6.0, I 0.025 buffer, measured as in Fig. 3. The solid line is the fit of the data by Equation 1 for the induced conformational change binding model of Scheme 1. Panel B shows selected stopped-flow traces of the binding of tetrasaccharide EFGH− to antithrombin under the same conditions. The concentrations of oligosaccharide are indicated. Antithrombin concentrations were 0.3 μM (lower traces) and 0.45 μM (upper trace). The solid lines are fits of the data by a double-exponential function. Panel C shows the variation of the two pseudo-first-order rate constants obtained from the double-exponential fits of kinetic data for the antithrombin-EFGH− interaction as a function of the EFGH− concentration. The solid line represents the fit of the data by Equation 2 for the pre-equilibrium conformational change binding mechanism in Scheme 1. Panel D shows the increase in amplitudes for the two kinetic phases with increasing EFGH− concentration at 0.3 μM antithrombin. Error bars represent ± S.E. The solid lines depict the fit of the data by the quadratic equilibrium binding equation (27).
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TABLE III
Accelerating effect of heparin oligosaccharides on the second-order rate constant for antithrombin inhibition of factor Xa

Second-order rate constants for uncatalyzed ($k_{\text{uncat}}$) and oligosaccharide-catalyzed ($k_H$) reactions of 2 nM antithrombin with 10 nM factor Xa in pH 6 buffer at 25°C (unless otherwise indicated) were measured from the linear dependence of $k_{\text{obs}}$ on heparin oligosaccharide concentration, corrected for the extent of oligosaccharide saturation by antithrombin, as detailed under “Materials and Methods.” All errors represent ± 2 S.E.

| Oligosaccharide Condition | $k_{\text{uncat}}$ | $k_H$ | Acceleration (k$_H$/k$_{\text{uncat}}$) |
|---------------------------|---------------------|-------|-------------------------------------|
| DEFH$^a$ pH 7.4, I 0.15   | 2.3 ± 0.1 × 10$^{-3}$ | 6.1 ± 0.3 × 10$^{-3}$ | 270 ± 25 |
| DEFH pH 6.0, I 0.025      | 1.5 ± 0.1 × 10$^{-3}$ | 5.0 ± 0.2 × 10$^{-3}$ | 330 ± 44 |
| DEFH$^a$ pH 6.0, I 0.025  | 1.5 ± 0.1 × 10$^{-3}$ | 4.3 ± 0.4 × 10$^{-3}$ | 280 ± 40 |
| DEF pH 6.0, I 0.025       | 1.4 ± 0.1 × 10$^{-3}$ | 4.5 ± 0.2 × 10$^{-3}$ | 320 ± 35 |
| DEF$^a$ pH 7.4, I 0.05    | 2.6 ± 0.2 × 10$^{-3}$ | 5.3 ± 1.2 × 10$^{-3}$ | 290 ± 60 |
| FGH$^a$ pH 6.0, I 0.025   | 1.5 ± 0.1 × 10$^{-3}$ | 5.2 ± 0.6 × 10$^{-3}$ | 36 ± 6 |
| DEFH$^a$ pH 6.0, I 0.025  | 1.5 ± 0.2 × 10$^{-3}$ | 5.6 ± 0.6 × 10$^{-3}$ | 370 ± 90 |
| EFGH$^{a*}$ pH 6.0, I 0.025 | 1.4 ± 0.1 × 10$^{-3}$ | 3.9 ± 0.2 × 10$^{-3}$ | 280 ± 30 |

$^a$ Obtained from the intercept of plots of $k_{\text{obs}}$ versus oligosaccharide concentration. An independent measurement of the uncatalyzed reaction in pH 6 buffer yielded a value of 1.7 ± 0.1 × 10$^{-3}$ s$^{-1}$.

$^b$ Data taken from previous work (12).

$^c$ The antithrombin concentration was 0.4 μM, and oligosaccharide concentrations ranged from 40 to 160 nM.

$^d$ Indistinguishable values of 2.0 ± 0.2 × 10$^{-3}$ M$^{-1}$ s$^{-1}$ for $k_{\text{uncat}}$ and 5.7 ± 0.3 × 10$^{-3}$ M$^{-1}$ s$^{-1}$ for $k_H$ were obtained using 12 μM antithrombin.

$^{*}$ Indistinguishable values of 1.5 ± 0.2 × 10$^{-3}$ M$^{-1}$ s$^{-1}$ for $k_{\text{uncat}}$ and 4.0 ± 0.3 × 10$^{-3}$ M$^{-1}$ s$^{-1}$ for $k_H$ were obtained using 5.6 μM antithrombin.

Enhancement associated with antithrombin activation and of maximally accelerating antithrombin inhibition of factor Xa at saturation. Previous assertions that residues D and H are essential for full conformational activation of the serpin (39) were based on indirect measurements of the rate of factor Xa inactivation at a single concentration of inhibitor-oligosaccharide complex. In the present study, direct measurements of factor Xa inactivation over a wide range of oligosaccharide concentrations approaching inhibitor saturation clearly show that deletion of residues D or H alone or G and H together has no significant effect on pentasaccharide activation of antithrombin.

Rapid kinetic studies allowed us to distinguish whether defects in pentasaccharide binding to antithrombin were due to a reduced ability of the pentasaccharide to bind the native low-heparin-affinity state of antithrombin or to induce the inhibitor into the activated high-heparin-affinity state (Scheme 1, induced-fit pathway) (10, 12). Deletion of reducing-end residue H or both G and H from the pentasaccharide only modestly affected binding to the low-heparin-affinity state of the serpin but greatly impaired conformational activation to the high-heparin-affinity-state. This impairment was due to increases in the rate constant for reversal of the conformational activation step ($k_{-2}$ in Scheme 1), which destabilized the activated conformation relative to the native conformation by as much as ~100,000-fold (Table II). Such findings suggest that the binding energy of residues G and H is utilized to form interactions mostly or exclusively with the activated antithrombin conformation. The loss of such interactions would thus be expected to increase the rate constant for reversal of the conformational change, since this rate constant should be inversely related to the number of interactions that must be disrupted in going from the activated conformation back to the native conformation. Although loss of residues G and H greatly destabilized the activated antithrombin conformation, the conformational equilibrium constant, $k_{2}/k_{-2}$, remained large enough (>8) to still favor the activated conformation (>89%). This explains why oligosaccharides lacking residue H or both G and H appeared to fully activate antithrombin for accelerated factor Xa inhibition when present at saturating levels. While residues G and H of the pentasaccharide are thus not required for conformational activation, they are nevertheless critical for stabilizing the activated conformation induced by residues D, E, and F.

Contrasting with these effects of deleting pentasaccharide reducing-end residues, deletion of the nonreducing-end residue D resulted in an altered mechanism of oligosaccharide binding and activation of antithrombin. This altered binding mecha-
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FIG. 5. Proposed model for allosteric activation of antithrombin by pentasaccharide DEFGH (A) and by tetrasaccharide EFGH (B). A, the nonreducing-end residues, D, E, and F, first form a low-affinity recognition complex with partially complementary sites in unactivated antithrombin. The inhibitor then undergoes a conformational change in this complex, which induces a full complementary fit of the trisaccharide with the activated conformation and generates complementary sites for binding reducing-end saccharides G and H. The reducing-end residues are then able to bind and lock the inhibitor in the activated state. B, the deletion of saccharide D reduces the affinity of the nonreducing-end saccharides, E and F, for native antithrombin sufficiently so that binding to the small fraction of activated antithrombin in pre-equilibrium with the native inhibitor becomes the preferred mode of binding. Because EFGH best complements the activated conformation, binding of EFGH pulls the native inhibitor into the activated state. A subsequent further conformational change induced in antithrombin by EFGH (see “Discussion”) is not depicted in this model.

must be binding to antithrombin by the induced conformational change pathway. This could happen if FGH" bound nonproductively to the DEF sites of native antithrombin involved in recognizing the pentasaccharide. Binding of FGH" in the pentasaccharide site was confirmed from the observation that the trisaccharide competes with tetrasaccharide DEFGH for binding to this site. Nonproductive binding of FGH" to the DEF interaction sites of native antithrombin would be compatible with the glucosamine/hexuronic acid specificity of the DEF sites. Such nonproductive binding to unactivated antithrombin would be favored because of the sizable binding energy available from the D and possibly also the F interaction sites (6, 23) in the unactivated conformation together with the unfavorable energy requirement for activating antithrombin to allow productive binding. Moreover, such nonproductive binding would explain the reduced ability of FGH" to activate antithrombin, as judged both from a decreased protein fluorescence enhancement (11) and lower enhancement of the rate of factor Xa inhibition (about 10% of that of the pentasaccharase). The extent of this reduced activation is reminiscent of the reduced activating effect of low-affinity heparin that lacks the pentasaccharase activating sequence (11). The more favorable, nonproductive mode of binding of FGH" to antithrombin may also explain why so little binding energy is lost when residue E is deleted as compared with the deletion of residue D.

Together, the results of the present and past studies favor the model for pentasaccharide binding and activation of antithrombin depicted in Fig. 5. In this model, binding of the rigid nonreducing end residues D, E, and F (40) occurs first to the native antithrombin conformation, with residue D making a primary contribution to the binding energy, although residues E and F may also contribute. Residues G and H make very weak or no interactions with this conformation. Both modeling studies (43) and a preliminary x-ray structure of the antithrombin-pentasaccharase complex (44) suggest that this initial binding of residues D, E, and F is at the C-terminal end of helix D with the reducing-end oriented toward the N terminus of this helix. Residues D, E, and F then induce antithrombin to undergo an activating conformational change in which the inhibitor reactive center loop is exposed to allow rapid inhibition of factor Xa (21, 25). The activated conformation produces a complementary fit of saccharides D, E, and F in the heparin binding site, which enhances their binding to the activated conformation and thereby stabilizes this conformation. In particular, more favorable interactions of the unique 3-O-sulfate of residue F in the activated conformation may provide the driving force for this conformational change, since loss of the 3-O-sulfate mostly abolishes conformational activation (39). However, increased interactions of residues D and E with the activated antithrombin conformation are also likely, given the substantial binding energy resulting from the interaction of residue D and the decreased activation resulting from nonproductive binding of oligosaccharide FGHH in the DEF interaction sites.

Further stabilization of the activated conformation results from the generation of additional complementary sites of interaction for the more flexible reducing-end residues G and H in this conformation (40). Conformational changes in the reducing-end saccharides, in particular the conformationally flexible idurionate residue G, may be required to align the charges in these residues for an optimal fit with the activated conformation (40). According to our model, the pentasaccharase functions as a classical allosteric modifier by preferentially binding and stabilizing the activated antithrombin conformation. Unique to this model are the different roles of nonreducing and reducing-end pentasaccharide residues in recognizing the native low-heparin-affinity state and in preferentially binding and stabilizing the activated high-heparin-affinity state.

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REFERENCES
1. Olson, S. T., and Björk, I. (1994) Semin. Thromb. Hemostasis 20, 373–409
2. Gettins, P. G. W., Patston, P. A., and Olson, S. T. (1996) Serpins: Structure, Function and Biology, R. G. Landes, Austin, TX
3. Choay, J., Lormeau, J. C., Petitou, M., Sinay, P., and Fareed, J. (1981) Ann. N. Y. Acad. Sci. 370, 640–649
4. Thumberg, L., Backstrom, G., and Lindahl, U. (1982) Carbohydr. Res. 106, 383–410
5. Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B., and Gatti, G. (1983) Biochem. Biophys. Res. Commun. 116, 492–499
6. Althay, D. H., Stephens, A. W., and Rosenberg, R. B. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1030–1034
7. Nordenman, B., Danielsson, Å., and Björk, I. (1978) Eur. J. Biochem. 90, 1–6
8. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. B. (1986) Biochemistry 25, 1006–1011
9. Olson, S. T., and Shore, J. D. (1981) J. Biol. Chem. 256, 11065–11072
10. Olson, S. T., Srivivasan, K. R., Björk, I., and Shore, J. D. (1981) J. Biol. Chem. 256, 11073–11079
11. Streusand, V. J., Björk, I., Gettins, P. G. W., Petitou, M., and Olson, S. T. (1995) J. Biol. Chem. 270, 9043–9051
12. Olson, S. T., Björk, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) J. Biol. Chem. 267, 12528–12538
13. Olson, S. T., and Shore, J. D. (1982) J. Biol. Chem. 257, 14891–14895
14. Lindahl, U., Thumberg, L., Backstrom, G., Riesenfeld, J., Nordling, K., and Björk, I. (1984) J. Biol. Chem. 259, 12568–12579
15. Olson, S. T. (1988) J. Biol. Chem. 263, 1698–1708
16. Olson, S. T., and Shore, J. D. (1991) J. Biol. Chem. 266, 6353–6364
17. Jorgensen, M., Stein, P. E., Permi, G., and Wardell, M. R. (1994) Structure 2, 257–270
18. Morrey, L., Samama, J. P., Delarue, M., Petitou, M., Choay, J., and Moras, D. (1993) J. Mol. Biol. 232, 233–241
19. Bae, J. H., Desai, U. R., Perumal, A., Caldwell, E. O., Weiler, J., and Linhardt, R. J. (1994) Biochim. Biophys. Acta 121, 121–129
20. Grootenhuis, P. D. J., and van Boeckel, C. A. A. (1991) J. Am. Chem. Soc. 113, 743–747
21. van Boeckel, C. A. A., Grootenhuis, P. D. J., and Visser, A. (1994) Nat. Struct. Biol. 1, 423–425
22. Björk, I., and Lindahl, U. (1982) Mol. Cell. Biochem. 48, 161–182
23. Althay, D. H., Lormeau, J. C., Petitou, M., Rosenberg, R. D., and Choay, J. (1985) Biochemistry 24, 6723–6729
24. Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J., and Hol, W. G. J. (1994) Nat. Struct. Biol. 1, 48–54
25. Huntington, J. A., Olson, S. T., Fan, B., and Gettins, P. G. W. (1996) Biochemistry 35, 8486–8503
26. Gettins, P. G. W., Fan, B., Crews, B. C., Turko, I. V., Olson, S. T., and
Mechanism of Heparin Activation of Antithrombin

27. Olson, S. T., Björk, I., and Shore, J. D. (1993) Methods Enzymol. 222, 525–560
28. Nordenman, B., Nyström, C., and Björk, I. (1977) Eur. J. Biochem. 78, 195–203
29. Bock, P. E., Craig, P. A., Olson, S. T., and Singh, P. (1989) Arch. Biochem. Biophys. 273, 375–388
30. Petitou, M., Duchaussoy, P., Lederman, I., Choay, J., Jacquinet, J. C., Sinay, P., and Torri, G. (1987) Carbohydr. Res. 167, 67–75
31. 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 Tanum, C., eds) pp. 143–210, Springer-Verlag New York Inc., New York
32. Nordenman, B., and Björk, I. (1981) Biochim. Biophys. Acta 672, 227–238
33. Mascotti, D. P., and Lehman, T. M. (1995) Biochemistry 34, 2908–2915
34. Fersht, A. (1985) Enzyme Structure and Mechanism, 2nd Ed., pp. 139–144, W. H. Freeman, New York
35. Lindahl, P., Raub-Segall, E., Olson, S. T., and Björk, I. (1991) Biochem. J. 276, 387–394
36. Fersht, A. R., and Renard, M. (1974) Biochemistry 13, 1416–1426
37. Fan, B., Turko, I. V., and Gettins, P. G. W. (1994) FEBS Lett. 354, 84–88
38. Petitou, M., Barzu, J., Herault, J.-P., and Herbert, J.-M. (1997) Glycobiology 7, 323–327
39. Atha, D. H., Lermek, J. C., Petitou, M., Rosenberg, R. D., and Choay, J. (1987) Biochemistry 26, 6454–6461
40. Ragazzi, M., Ferro, D. R., Perly, B., Sinay, P., Petitou, M., and Choay, J. (1990) Carbohydr. Res. 195, 169–185
41. van Boeckel, C. A. A., and Petitou, M. (1993) Angew. Chem. Int. Ed. Engl. 32, 1671–1690
42. van Boeckel, C. A. A., Beetz, T., and van Aelst, S. F. (1988) Tetrahedron Lett. 29, 863–866
43. Grootenhuis, P. D. J., Westerdhuin, P., Meuleman, D., Petitou, M., and van Boeckel, C. A. A. (1995) Nat. Struct. Biol. 2, 736–739
44. Carrell, R. W., Skinner, R., Jin, L., and Abrahams, J. P. (1997) Thromb. Haemostasis 77, 516–519
45. Choay, J. (1989) Ann. N. Y. Acad. Sci. 556, 61–74
Mechanism of Heparin Activation of Antithrombin: ROLE OF INDIVIDUAL RESIDUES OF THE PENTASACCHARIDE ACTIVATING SEQUENCE IN THE RECOGNITION OF NATIVE AND ACTIVATED STATES OF ANTITHROMBIN

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