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Transmission of Conformational Change from the Heparin Binding Site to the Reactive Center of Antithrombin†

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ABSTRACT: Heparin greatly increases the rates at which antithrombin inhibits target proteinases. An important part of this rate acceleration is a heparin-induced conformational change in antithrombin. To answer the question of whether or not this change is transmitted to the reactive center, we have prepared a recombinant P1 mutant of antithrombin, R393C, labeled the cysteine with nitrobenzofuran (NBD) fluorophore, and examined the perturbation of NBD fluorescence intensity as a function of bound sulfated oligosaccharide. Two high-affinity heparins, low-affinity heparin, and dextran sulfate were used. We found (i) that binding to antithrombin of all these oligosaccharides resulted in transmission of conformational change to P1 in the reactive center, (ii) that these oligosaccharides all gave enhancements of the rate of inhibition of factor Xa beyond any contribution from surface approximation, and (iii) that the degree of perturbation of P1 correlated with the enhancement of the rate of factor Xa inhibition that was not due to surface approximation.

Heparin, a linear, highly negatively charged, heterogeneous polysaccharide, acts as an anticoagulant *in vivo* through specific interactions with inhibitors of serine proteinases, most importantly with antithrombin. The anticoagulant properties result from a heparin-induced increase in the rates at which antithrombin inhibits target proteinases. The large rate enhancements for antithrombin inactivation of factor Xa or thrombin that occur upon binding of certain heparin species are accompanied by conformational changes in the antithrombin molecule (Nordenman et al., 1978; Olson & Shore, 1981; Stone et al., 1982; Gettins, 1987; Gettins et al., 1992). The nature of these changes and the specific roles they play in the acceleration of the rate of proteinase inhibition are, however, not known. In particular, it is not known whether the heparin-induced conformational changes include alteration of the structure of the reactive center region, the region involved in binding to the active site of the target proteinase. Since the reactive center and the heparin binding site are believed to be well separated (Mourey et al., 1990), such a heparin-induced alteration in reactive center structure would require long-range transmission of conformational change.

Antithrombin R393C,[1] a P1[2] variant, has been previously identified as a naturally occurring variant lacking thrombin inhibitory activity as a result of replacement of the specificity-determining arginine at position P1 by cysteine (Erdjument et al., 1987, 1988a,b; Ireland et al., 1991). Although this

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[1] Abbreviations: R393C, variant antithrombin in which arginine has been changed to cysteine at residue 393; NBD, nitrobenzofurazan; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BHK, baby hamster kidney.

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variant has not been well characterized because of its covalent, disulfide-mediated association with serum albumin, it appears to retain normal heparin binding, as judged by the salt concentration needed to elute it from heparin–Sepharose. We report here preparation of recombinant R393C human antithrombin, using a baby hamster kidney expression system (Zettlmeissl et al., 1988; Gettins et al., 1992), labeling of the cysteine with NBD, and examination of the perturbation of fluorescence intensity as a function of bound heparin or dextran sulfate. We found that oligosaccharide binding to antithrombin did result in transmission of conformational change to P1 in the reactive center and that the degree of perturbation of P1 correlated with the enhancement of the rate of factor Xa inhibition, which differed depending on the oligosaccharide used.

EXPERIMENTAL PROCEDURES

Mutagenesis of Human Antithrombin cDNA. Site-directed mutagenesis of human antithrombin cDNA to create the R393C variant was carried out in M13mp19 using single priming of a single-stranded uracil-containing template with the anti-sense oligonucleotide 5′ GTT GGG GTT TAG CGA ACA GCC AGC AAT CAC 3′ (the underlined triplet corresponds to the arginine to cysteine change) according to published procedures (Kunkel et al., 1985; Carter, 1985). The products of the *in vitro* extension and ligation reactions were used to transform *Escherichia coli* NK7085 (*ung*$^+$) cells. Plaques containing the desired mutation were identified by hybridization with the oligonucleotide used for mutagenesis. The change was confirmed by dideoxy sequencing of the single-strand phage (Sanger et al., 1977, 1980) using [^{32}P]ATP. The double-stranded replicative form of M13mp19 containing the mutagenized antithrombin cDNA was digested with restriction endonucleases *Sal*I and *Xba*I and the approximately 1430-bp fragment, covering the entire antithrombin coding region, ligated into the expression vector pMAStop (Zettlmeissl, 1990) cut with the same endonucleases. The ligation mixture was used to transform *E. coli* MC 1060. Positive colonies containing pMAAT3-R393C were identified by double digestion of isolated plasmid with *Sal*I and *Xba*I and observed release of a fragment of the expected size.

Preparation and Isolation of R393C Recombinant Antithrombin. BHK cells were transfected with pMAAT3-R393C and plasmids pRMH140 and pSV2dhfr, as described previously for wild-type antithrombin (Zettlmeissl et al., 1988). Stably transfected cells were selected by resistance to neomycin and to levels of methotrexate that were increased stepwise over a period of 3 weeks from 0.5 to 10 μM (Zettlmeissl et al., 1988). These cells were grown to confluence in 1.5-L roller bottles, and the medium was cycled between bovine serum containing and serum free, as described previously (Gettins et al., 1992). Medium from the serum-free cycles was collected and used for isolation of antithrombin. Expression, as judged by radial immunodiffusion assay, was between 10 and 20 μg mL^{-1}, which is comparable to expression levels obtained with wild-type antithrombin in the same cell system (Gettins et al., 1992; Fan et al., 1993). Antithrombin was isolated by affinity chromatography on heparin–Sepharose and size exclusion chromatography on Sephadex G150. Distinct fractions of antithrombin were obtained that differed in their affinity for heparin–Sepharose. These fractions had correspondence with the usual three fractions (designated I, II, and III) that we have found with wild-type recombinant antithrombin produced in BHK cells (Fan et al., 1993), as judged by the salt concentration at which each eluted from heparin–Sepharose. The distinct forms arise from differences in the type of attached carbohydrate. Form II most closely resembles the α form of plasma antithrombin and was the form of R393C recombinant antithrombin used in the present labeling studies. As discussed (Fan et al., 1993), forms I, II, and III appear to be distinct from the two forms characterized by Björk and co-workers (Björk et al., 1992), despite the fact that all five forms are derived from the same cell type. In addition, both monomeric and dimeric R393C antithrombin species were isolated. The dimeric species were disulfide cross-linked via the newly introduced cysteine at position 393. The monomeric species were presumed to be disulfide cross-linked to a low molecular weight thiol such as glutathione, since there was no detectable free SH in the preparation, as estimated by DTNB assay.

Fluorescent Labeling of R393C Recombinant Antithrombin. R393C antithrombin was specifically reduced at Cys393 immediately prior to labeling with fluorophore. Specific reduction of the disulfide involving Cys393 was effected by reaction with a stoichiometric amount of reduced thioredoxin (the thioredoxin was reduced immediately prior to use by reaction with a stoichiometric amount of dithiothreitol). Direct reduction with dithiothreitol was found to be too nonspecific and resulted in reduction of internal disulfides, as has been found previously for plasma antithrombin (Sun & Chang, 1989). Both monomeric and dimeric antithrombins were used for these labelings. The reduced antithrombin was then immediately reacted with iodoacetamido-NBD and the reaction mixture purified on heparin–Sepharose to remove not only excess labeling reagent and thioredoxin but also any antithrombin species in which an intramolecular disulfide had been reduced by the thioredoxin. This was possible since such undesired reduction results in a large reduction in heparin affinity (Björk & Fish, 1982). The labeling reactions were carried out in 50 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 2 mM EDTA and used a 10-fold molar excess of iodoacetamido-NBD. The stoichiometry of label incorporation was calculated from spectrophotometric measurement of the absorbance at 497 nm, using the extinction coefficient of 26 000 M^{-1} cm^{-1} (Trayer & Trayer, 1988). After purification of the labeled antithrombin on heparin–Sepharose, a value of 0.73 NBD labels per antithrombin was determined. The value of less than 1:1 resulted from coelution of labeled and unlabeled R393C antithrombin from the affinity column. This mixture was used without further purification, since the NBD fluorescence measurements reported only on the labeled species. Allowance was made for the presence of unlabeled R393C antithrombin in calculating affinities for heparin.

Factor Xa Assay. Rates of plasma antithrombin inhibition of factor Xa were measured under pseudo-first-order conditions (10–20 nM antithrombin and 1–2 nM factor Xa) in the presence of saturating (oligo)saccharide (15–60 nM pentasaccharide or high-affinity heparin and 25 or 50 μM low-affinity heparin or dextran sulfate). Rate enhancements were determined as the ratio of the second-order rate constants for the accelerated reaction relative to those for the unaccelerated reaction. Measurements were made in a low ionic strength buffer (20 mM sodium phosphate, 0.1 mM EDTA, 0.1% PEG, pH 7.4).

[2] The nomenclature system of Schechter and Berger (1967), used to describe the subsites of interaction between a proteinase and its substrate, designates the residues on either side of the substrate scissile bond as P1 and P1′. Residues N-terminal to this are designated P2, P3, ..., P*n*, and residues C-terminal are designated P2′, P3′, ..., P*n*′.

Table I: Characterization of Binding of Heparin and Dextran Sulfate to NBD–R393C and Plasma Antithrombins[a]

(oligo)saccharide	ΔF_{NBD} (%)[b]	ΔF_{Trp} (%)	K_D (nM)	rate enhancement of factor Xa inhibition[c]
dextran sulfate	22 ± 2	18 ± 1	3100 ± 200	120 ± 10 (45)
low-affinity heparin	42 ± 2	15 ± 1	5300 ± 400	240 ± 10 (90)
high-affinity heparin	95 ± 5	39 ± 2	2.7 ± 0.7	320 ± 10 (120)
heparin pentasaccharide	96 ± 5	38 ± 1	4.4 ± 0.6	120 ● 10 (120)

[a] All measurements were made in low ionic strength buffer (20 mM sodium phosphate, 0.1 mM EDTA, 0.1% PEG, pH 7.4) to increase the affinity of the weaker binding oligosaccharides for antithrombin and, thus, to enable saturation of the binding site on antithrombin to be achieved at accessible concentrations of these oligosaccharides. [b] Enhancements of NBD fluorescence (ΔF_{NBD}) are given as the maximum value obtained upon saturation and were determined by titration of (oligo)saccharide into NBD-labeled R393C recombinant antithrombin and monitoring the NBD emission intensity at 550 nm with excitation at 480 nm. [c] Numbers in parentheses are rate enhancements corrected for surface approximation contributions, as described in the text.

Determination of Antithrombin–Saccharide Dissociation Constants. Dissociation constants (K_d) were determined by titration of (oligo)saccharides into 1 μM or 10 nM plasma antithrombin and monitoring the endogenous tryptophan fluorescence at 340 nm with excitation at 280 nm. Data, corrected for dilution, were fitted by linear least squares analysis using the program MINSQ II (Micromath Scientific Software, Salt Lake City, UT).

Fluorescence Measurements. Fluorescence measurements were made on an SLM8000 fluorometer. NBD fluorescence was monitored at 550 nm with excitation at 480 nm. Slits of 8 and 4 nm were used for emission and excitation, respectively.

Materials. Dextran sulfate was an M_r 8000 species from Sigma; low- and high-affinity heparins were prepared from M_r 8000 or 9000 heparins by affinity chromatography on antithrombin–agarose (Olson, 1988). High-affinity sequences were not detectable in the low-affinity heparin (<0.001%), as determined by the lack of exchange of added fluorescein-labeled high-affinity heparin into the low-affinity heparin after antithrombin affinity chromatography.[3] Heparin pentasaccharide was a synthetic species having high affinity and high anti-factor Xa activity (Sinaÿ et al., 1984). Thioredoxin and DTNB were purchased from Sigma (St. Louis, MO). Iodoacetamido-NBD was from Molecular Probes (Eugene, OR).

RESULTS AND DISCUSSION

The R393C variant of antithrombin is not capable of inhibiting thrombin or factor Xa, as a result of the change in the P1 specificity-determining residue. Since the affinity of antithrombin for heparin is very sensitive to changes in the structure of the protein [e.g., Björk and Fish (1982) and Sun and Chang (1989)], the affinity of the variant for heparin was measured as an indicator of structural alterations resulting from the mutation. The dissociation constant for the R393C antithrombin–heparin complex was determined using the change in tryptophan fluorescence that accompanies such binding. The K_d (10 ± 2 nM) and tryptophan fluorescence enhancement (40 ± 2%) at physiological ionic strength were indistinguishable from wild-type antithrombin, suggesting that the variant protein retains an intact heparin binding site and undergoes the normal heparin-induced conformational change.

Binding of high-affinity heparin resulted in a 95% enhancement of the NBD fluorescence of R393C–NBD (Table I). The enhancement was saturable and reached a maximum at a stoichiometry of 1 mol of heparin/mol of antithrombin (Figure 1). An identical fluorescence enhancement was observed when the much shorter synthetic heparin pentasaccharide (Sinaÿ et al., 1984) that represents the minimum sequence needed to give the full heparin-induced conformational change was bound (Table I). When a low-affinity heparin species was bound, which was of comparable length but lacked the specific pentasaccharide sequence, only a 42% maximal enhancement of NBD fluorescence was observed (Table I). Dextran sulfate gave an even smaller 22% increase in NBD fluorescence at saturation (Table I). Displacement of bound heparin by dextran sulfate and consequent reversal of the NBD fluorescence enhancement toward the limiting value found with dextran sulfate alone (data not shown) demonstrated that dextran sulfate bound at the same site as the three heparin species.

For valid comparison of the rate enhancements obtained with the different polysaccharides, it must be noted that two factors can contribute: (i) conformational change and (ii) surface approximation (Olson et al., 1992). The latter results from simultaneous binding of factor Xa and antithrombin to the same polysaccharide molecule but is only possible for species which are long enough (dextran sulfate, low-affinity heparin, and high-affinity heparin). Heparin pentasaccharide gives rate enhancement only as a result of conformational change, since it is too short for a surface approximation contribution. Thus the 2.7-fold difference in rate enhancement between pentasaccharide and high-affinity heparin represents the contribution of surface approximation for the latter species (Olson et al., 1992). Taking this difference into account (Table I), we see that the changes in NBD fluorescence for full-length saccharides correlate with the rate enhancements of plasma antithrombin inhibition of factor Xa produced by the same polysaccharides and, to a lesser extent, with the saccharide-induced enhancements of endogenous tryptophan fluorescence (Table I). Thus high-affinity heparin gave large enhancements of factor Xa inhibition and NBD fluorescence. Low-affinity heparin gave smaller enhancements of factor Xa inhibition and of NBD fluorescence, and dextran sulfate gave even lower enhancements of factor Xa inhibition and of NBD fluorescence (Table I). The changes in endogenous tryptophan fluorescence showed similar, but not exactly parallel, rankings. Pentasaccharide and high-affinity heparin gave similar large enhancements, whereas low-affinity heparin and dextran sulfate gave similar but much smaller enhancements (Table I).

The correlation between enhancements of NBD fluorescence and of factor Xa inhibition rates indicates that the structural alterations that occur when an activating oligosaccharide binds to antithrombin involve a conformational change that is sensed at the P1 position of the reactive center loop. Given the expected exposed location of the P1 residue, based (i) on its ability to form disulfide cross-links to other proteins [present results and Erdjument et al. (1987, 1988a,b)] and (ii) on the structure of ovalbumin (Stein et al., 1990) (the only serpin for which a native structure is available), it is likely that this

[3] V. J. Streusand and S. T. Olson, unpublished material.

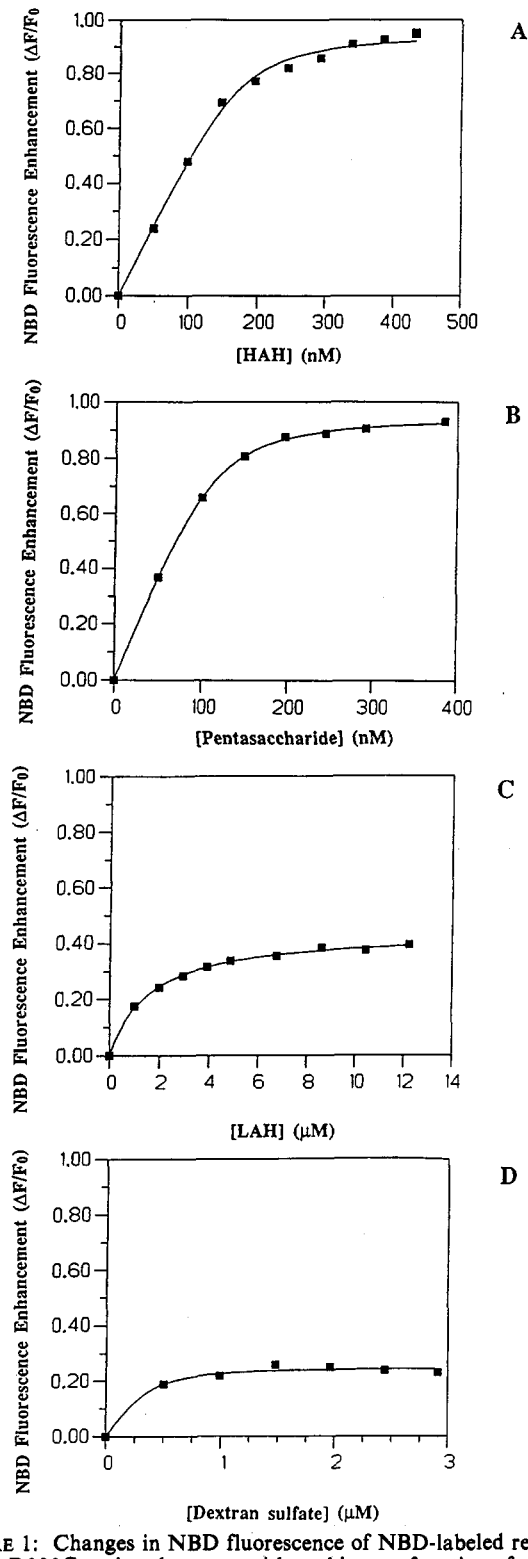

FIGURE 1: Changes in NBD fluorescence of NBD-labeled recombinant R393C variant human antithrombin as a function of added heparin or dextran sulfate. The solid lines are the least squares best fits for binding with a stoichiometry of 1:1. Panels: (A) titration of high-affinity heparin into 0.19 μM antithrombin; (B) titration of heparin pentasaccharide into 0.11 μM antithrombin; (C) titration of low-affinity heparin into 0.18 μM antithrombin; (D) titration of dextran sulfate into 0.36 μM antithrombin.

change in conformation involves P1 directly. The tryptophan fluorescence enhancements also change roughly in step with the enhancements of rates of factor Xa inhibition, though the proportionality is not the same as for the NBD fluorescence enhancements. This is not too surprising, since it has been found that enhancement of tryptophan fluorescence is not the best indicator of the activating conformational change in antithrombin that results in enhancement of the rate of factor Xa inhibition (Atha et al., 1985; Gettins et al., 1992).

The similarity in the effects of long-chain high-affinity heparin and heparin pentasaccharide in causing the same perturbation in NBD fluorescence, taken together with the same changes produced in the endogenous tryptophan fluorescence and the same conformational contribution to the rate enhancements of factor Xa inhibition, supports a model for antithrombin–heparin interaction in which the pentasaccharide causes the full conformational change that contributes to enhancement of proteinase inhibition. This is the same conclusion reached by Olson et al. (1992) from resolution of the contributions of conformational change and bridging to enhancement of the rates of proteinase inhibition. In contrast, it has been proposed on the basis of early CD data that high-affinity heparin causes additional conformational changes in antithrombin than does the pentasaccharide alone and that these additional changes result in some or all of the higher rate enhancements typically observed for proteinase inhibition (Stone et al., 1982). Neither the findings of Olson et al. (1992), which also included a CD comparison of the effects of pentasaccharide and high-affinity heparin binding to antithrombin, nor the present results are consistent with the model of Stone et al. (1982).

The lower, but nevertheless substantial enhancements of NBD fluorescence and of rate of factor Xa inhibition upon binding low-affinity heparin or dextran sulfate in saturating amount are particularly interesting, since it has been assumed that neither species (i) produces an activating conformational change in antithrombin or (ii) increases the rate of factor Xa inhibition. We have now shown that both low-affinity heparin and dextran sulfate cause significant rate enhancements of factor Xa inhibition, most of which cannot be attributed to surface approximation effects. Since surface approximation effects are distinguished by their marked dependence on salt concentration (Olson et al., 1992), the observation that the maximum rate enhancements of these polysaccharides at saturation are only weakly dependent on salt[3] is consistent with the antithrombin conformational change being mostly responsible for the polysaccharide rate enhancements. It seems likely that low-affinity heparin and dextran sulfate both change the conformation of antithrombin at the P1 residue.

There are two main alternative explanations for the different changes reported in the reactive center region with the different saccharides used. One is that each saccharide results in alteration of the reactive center loop of every antithrombin molecule to which it is bound, giving a homogeneous antithrombin–saccharide structure but a structure that depends on the particular saccharide forming the complex. The other explanation is that the antithrombin–saccharide species are in equilibrium between two conformations, one resembling the native structure and one resembling the structure induced by binding of high-affinity heparin. Different saccharides, all at concentrations sufficient to saturate the saccharide binding site of antithrombin, could shift the equilibrium between the two antithrombin–saccharide conformations. It has been shown that heparin binding to antithrombin occurs as a two-step process, with initial binding involving electrostatic interactions and no conformational change, followed by a slower step that involves a change in conformation (Olson et al., 1981). Different saccharides might significantly alter this equilibrium constant, without necessarily altering the initial electrostatic binding. Indeed, this has been observed for pentasaccharide and high-affinity heparin, for which the main

difference in affinity for antithrombin arises from differences in the equilibrium constant (K) for the second-step conformational change, being 2600 for high-affinity heparin and 780 for pentasaccharide (Olson et al., 1992). Although this difference in K does not result in a large percentage change in the population of the two conformers, a change in the equilibrium constant to a value close to 1 would result in a large change in populations. Such a large change in the second-step equilibrium constant is quite compatible with the large differences in overall affinity found for low-affinity heparin or dextran sulfate compared with high-affinity heparin or heparin pentasaccharide (Table I).

Although we cannot yet distinguish between these possibilities, the present findings imply a continuum of rate enhancements for different heparins, or similar species, rather than an all-or-nothing phenomenon triggered by the minimal pentasaccharide. Indeed, it may be possible to synthesize small heparinoids capable of giving even greater rate enhancements than the minimal pentasaccharide, which are also more selective for antithrombin and consequently less likely to produce unwanted side effects when used clinically.

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