Antithrombin Conformation and the Catalytic Role of Heparin

II. IS THE HEPARIN-INDUCED CONFORMATIONAL CHANGE IN ANTITHROMBIN REQUIRED FOR RAPID INACTIVATION OF THROMBIN?

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The role of antithrombin conformation in heparin-catalyzed inhibition of thrombin was investigated using antithrombins modified with the tryptophan reagent dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide (HNB). Affinity fractionation of HNB-labeled antithrombin (0.6-0.7 mol of HNB/mol of protein) on heparin-Sepharose using a linear salt gradient allowed separation of three singly labeled protein species and a fourth HNB-antithrombin species which co-eluted with unlabeled protein. Conformational alterations induced by heparin binding to each of the labeled antithrombins were assessed by spectroscopic techniques, including protein fluorescence, difference spectroscopy in the ultraviolet-visible range, and circular dichroism. Comparison of spectra of the labeled proteins in the presence and absence of added heparin indicated changes to occur in protein conformation at the sites of the bound HNB moieties and at aromatic amino acid residues within the protein matrix. These spectroscopic alterations mimicked changes induced by heparin in the native protein, but were reduced in magnitude. Rates of thrombin inactivation by the labeled antithrombins were measured over a wide range in both heparin concentration and inhibitor concentration to determine maximal rates of protease inactivation. The kinetic analysis indicated that each of these HNB-antithrombin derivatives, which undergo the heparin-induced changes to varying extents, can react with thrombin at the same maximal rate. Thus, this series of chemically modified antithrombin species demonstrated that the conformational change which is induced in antithrombin by heparin does not render the protein intrinsically more reactive toward thrombin.

When antithrombin binds to heparin, structural perturbations in the protein can be measured by a variety of spectroscopic techniques (1-6). These structural changes were initially used to support an early model for the mechanism of heparin catalysis proposed by Rosenberg and co-workers (7), in which heparin binding to the protease inhibitor induces a conformational change in antithrombin that renders the protein more reactive toward thrombin and other coagulation enzymes. Subsequent demonstration by chemical modification (8-10) and kinetic methods (11-14) that thrombin binding to heparin is required for the rapid, heparin-catalyzed reaction between antithrombin and thrombin has led to another model for the heparin effect, in which an intermediate ternary complex of both proteins bound to the heparin "template" results in an increased rate of reaction between the proteins by virtue of limiting their diffusion from three dimensions to a single dimension along the heparin chain.

Following the demonstration of Pomerantz and Owen (8) that a ternary complex between DIP-thrombin, antithrombin, and heparin could be formed in chromatography experiments, several investigators used kinetic methods to test whether a ternary intermediate was normally formed during heparin-promoted antithrombin-thrombin reactions. Griffith (11) has demonstrated that the heparin-catalyzed reaction between antithrombin and thrombin is saturable with respect to either protein, a kinetic pattern which can be formally likened to a two-substrate enzyme-catalyzed reaction. Neshem (12) has also used a kinetic approach to demonstrate that the observed reaction rates can be mathematically described over a wide range in heparin concentrations using equations which fit the ternary complex model. Recently, covalent heparin-antithrombin complexes have been used by Hoylaerts et al. (13) to further examine the heparin template model. In these studies, evidence is presented which indicates that the magnitude of the second-order rate constant for the thrombin-antithrombin reaction can be accounted for by diffusion of thrombin along the heparin chain to meet antithrombin after both proteins have formed a ternary complex with the heparin template. Additionally, Nesheim et al. (15) have shown that the specific activities of heparins with differing molecular weights correlate directly to the number of both antithrombin and thrombin molecules bound to heparin, as predicted by the ternary complex model.

Although the evidence supporting the ternary complex model for heparin-catalyzed thrombin inactivation by antithrombin is convincing, the role of the heparin-induced conformational change to the overall rate enhancement has been debated (7, 13, 16). Limitation of protein diffusion to one dimension along the heparin chain sufficient to account for the large increase in the antithrombin-thrombin reaction rate in the presence of heparin, or does the conformational change...
which occurs upon heparin binding to antithrombin contribute directly to the enhancement in the rate of thrombin inactivation? Addressing the question of the relative contribution of the heparin-induced structural change in antithrombin to the overall catalytic rate enhancement was a major objective in this work.

Each of the spectroscopic methods which have been used as evidence for the putative heparin-induced conformational change indicates perturbation of tryptophan residues in antithrombin (1-6). These spectroscopic data prompted Blackburn and co-workers (17, 18) and other investigators (19, 20) to employ chemical modification of tryptophan to examine the structural requirements for heparin interaction with the antithrombin III molecule. The results of the modification of antithrombin III with dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide by Blackburn and Sibley (17) showed specific HNB incorporation into approximately 1 tryptophan residue, identified as residue 49 (18). Incorporation of HNB into Trp-49 blocks heparin binding to antithrombin and thus abolishes the heparin-enhanced rate of inactivation of thrombin.

Secondary sites of tryptophan modification were apparent upon reaction of antithrombin III with the hydroxynitrobenzyl reagent (17); more than 1 tryptophan clearly reacts at high concentrations of reagent. These results prompted a detailed analysis of antithrombin derivatives containing labeled tryptophan residues in order to provide additional information concerning the mechanism of heparin binding and protease inactivation by antithrombin. This study entails separation and characterization of four HNB-antithrombin species isolated by affinity chromatography on heparin-Sepharose. In addition to isolation of antithrombin modified at tryptophan 49, which is devoid of heparin binding, other HNB-labeled derivatives were prepared which exhibit heparin affinity which is diminished compared to the affinity of native antithrombin. The objectives of this study were to characterize these derivatives according to: 1) heparin-binding properties, as measured by several spectral and conformational probes, and 2) kinetic properties with respect to thrombin inactivation in the presence and absence of added heparin. Preparation of antithrombin derivatives which have measurable, although weaker, affinity for heparin provided a novel approach to answering mechanistic questions concerning the heparin-induced conformational change and its contribution to acceleration of the reaction between antithrombin and thrombin.

EXPERIMENTAL PROCEDURES

RESULTS

Pseudo-first-order Kinetics of Thrombin Inactivation by the HNB-Antithrombin Derivatives—Following fractionation and characterization of the four HNB-antithrombin derivatives according to their altered heparin-binding properties, the modified antithrombins were used in kinetic studies designed to probe the mechanism of heparin catalysis of the antithrombin-thrombin reaction. Titration of the rates of reaction of thrombin with native antithrombin III and the four HNB-antithrombin derivatives over a wide range in heparin concentrations is shown in Fig. 6. All of the antithrombin species examined, with the exception of Derivative 1, exhibit increased rates of reaction that reach relatively broad maxima with increasing heparin until the rates begin to decrease at high levels of heparin. Derivative 1 shows a slight increase in reaction rate with thrombin upon the addition of heparin, but the reaction rate does not change significantly between 0.4 and 18 μM heparin concentrations. Maximal rate constants measured for Derivatives 1-4 are 30, 150, 380, and 600 × 10⁻⁴ s⁻¹, respectively. The maximum rate constant observed with native antithrombin III is 580 × 10⁻⁴ s⁻¹. The maximum rate of thrombin inactivation by Derivative 2 is only about one-fourth of the maximum rate achieved with Derivative 4, and Derivative 3 reaches a maximal rate of thrombin inactivation which is intermediate between rates exhibited by the other two derivatives.

In the absence of heparin, the four HNB-antithrombin derivatives inactivate thrombin at approximately the same rate as does unmodified antithrombin. Calculated rate constants in the absence of heparin are 2.7, 2.3, 3.5, 3.2, and 3.0 × 10⁻⁴ s⁻¹ for Derivatives 1, 2, 3, 4, and native antithrombin, respectively. The maximal rate increase for native protein with heparin thus represents a 200-fold increase over the reaction rate in the absence of heparin.

Measurements of the rate of reaction between the HNB-antithrombin derivatives with thrombin in the presence of heparin were also performed using a different approach: the reaction was modeled as a two-substrate enzyme-catalyzed reaction in which heparin is the catalyst and antithrombin and thrombin represent the two substrates. This approach had been previously used (11) to indicate the saturability of the heparin-promoted rate of reaction upon titration with either of the two protein substrates, providing evidence for the formation of a ternary intermediate as a necessary step in overall heparin catalysis of the protease-inhibitor reaction. In this case, the concentrations of thrombin and heparin were fixed, and the kinetic rate was titrated with each of the antithrombin derivatives, which had been established to bind heparin with varying degrees of heparin-associated changes.
in conformation. Inclusion of the tarombin substrate Chromozym-TH (K_m = 3 μM) during the entire course of reaction with antithrombin serves to attenuate the reaction such that, at all protein concentrations used, the rate of reaction in the absence of the catalyst, heparin, is negligible.

The results of titration of the kinetics of the heparin-antithrombin-thrombin reaction with each of the antithrombins are shown in Fig. 7. As expected, Derivative 4, being a mixture of a small amount of HNB-labeled protein and a larger fraction of unlabeled antithrombin, exhibited kinetics which were saturable with antithrombin and which did not differ from those observed with native protein. More important, Derivatives 2 and 3 also exhibit a saturation-type kinetic pattern, with maximal rates of reaction which are identical to that seen with native antithrombin III. It is apparent that, although these derivatives bind heparin to some degree more weakly than antithrombin and without the same structural alterations apparent with native protein, the maximal heparin-catalyzed rate of thrombin inactivation observed with these derivatives equals that of unlabeled antithrombin. Table III summarizes the observed maximal rate constants and the apparent K_m values, characteristic of each of the HNB-antithrombin derivatives, which were determined by experimentally manipulating the ternary system using the two substrate-enzyme model.

As seen in Fig. 7, the reaction rate observed with HNB-antithrombin Derivative 1 never reaches the maximal rate observed with the other proteins, but the rate also does not appear to have saturated over the protein concentrations used in this assay. Lack of saturation with HNB-antithrombin Derivative 1 is probably attributable to the extremely weak heparin affinity exhibited by this protein. It cannot be discounted that the increase in rate at high concentrations of Derivative 1 might be attributable to contamination with a small amount of native-like material. Even if only 1% contaminating native material were present, the rate of reaction observed with 4000 nM total “Derivative 1” would be approximately the rate observed. Karp et al. (33) have suggested that unfractined HNB-labeled antithrombin binds to heparin with an affinity that is only about one order of magnitude weaker than that of native antithrombin. Furthermore, these authors contend that Derivative 1 inactivates thrombin in the presence of heparin at a rate that is intrinsically slower than that of unmodified antithrombin because of its inability to undergo the heparin-associated conformational change. In an experiment designed to measure the heparin affinity of Derivative 1 by kinetic methods, Derivative 1 was added in concentrations of 600 nM to a reaction mixture including 15 to 1500 nM native antithrombin, 2 nM thrombin, 0.15 mM Chromozym, and 15 nM heparin. The heparin-promoted antithrombin-thrombin reaction was unaffected by addition of Derivative 1, indicating that Derivative 1 does not compete with native antithrombin for binding of heparin.

**DISCUSSION**

The tryptophan-specific reagent dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide, an environment-sensitive spectral probe, was employed to gain further insight into the role of tryptophan residues in effective heparin binding and protease inactivation by antithrombin III. Incorporation of the tryptophan-specific reagent into the protein yielded derivatives with distinguishable chromatographic properties and allowed affinity fractionation of HNB-modified antithrombin on heparin-Sepharose into four species which eluted from the column at differing ionic strengths. The HNB-labeled antithrombins were designated Derivatives 1-4, according to their order of elution from the heparin affinity column. Further studies showed the differences in chromatographic behavior of the antithrombin derivatives to be reflected in protein function, including the heparin-induced changes in spectral properties and apparent binding constants for heparin estimated from the kinetics of heparin-promoted thrombin inactivation.

The bulk of the modified protein corresponds to the previously characterized HNB-antithrombin species which is labeled at tryptophan-49 (18). Of the remaining three HNB-labeled derivatives, which were isolated upon application of a linear salt gradient to the affinity column, two appear to contain a single HNB group incorporated per protein chain. The fourth antithrombin species to elute from the heparin column consists of a mixture of about 20% HNB-labeled protein, with native-like antithrombin comprising the remaining 80%. For the most part, the physical properties of Derivative 4 thus represent properties of native antithrombin III. As such, the singly labeled antithrombin species, Derivatives 1-3, are the proteins which were extensively characterized in this study according to heparin binding behavior and thrombin inactivation kinetics.

**Heparin Binding by the HNB-Antithrombin Derivatives**—The heparin-induced conformational change of antithrombin can be measured by several spectroscopic changes which can be attributed in part to perturbation of tryptophan residues.
The change in the HNB-antithrombin derivatives was examined by fluorescence, UV spectroscopy, and near-UV circular dichroism. With each of the spectral techniques used, it was apparent that the singly modified antithrombin derivatives exhibit spectral signals which are shifted in magnitude compared to those observed with native antithrombin III. The smaller fluorescence, circular dichroism, and absorption differences observed with the derivatives are presumably indicative of altered or reduced conformational changes which occur in these proteins upon heparin binding. The heparin-induced changes observed with the HNB-antithrombin derivatives vary from virtually undetectable, with Derivative 1, to near native-like, with Derivative 4. Derivatives 2 and 3 exhibit changes of intermediate magnitude, but the heparin-induced changes observed with Derivative 2 are consistently smaller than those observed with Derivative 3. Thus, modified antithrombins have been prepared which respond to heparin with conformational changes which clearly differ, as measured with any of several conformational probes.

**Thrombin Inactivation by the HNB-Antithrombin Species**

These HNB-antithrombin derivatives which have been shown to differ from native antithrombin in heparin-binding properties have provided a unique approach to answering mechanistic questions concerning the nature of interaction between heparin, antithrombin, and thrombin. Does approximation of antithrombin and thrombin via an intermediate ternary complex between heparin and the two proteins account for the overall rate enhancement of heparin-catalyzed inactivation of thrombin? Alternatively, does the heparin-induced conformational change render antithrombin intrinsically more reactive with thrombin? The reduction in the extent of the change in conformation which occurs in the HNB-antithrombin species provided proteins which could be employed to answer this question using a kinetic approach. First, the rate of heparin-accelerated reaction between the antithrombin species and thrombin was measured as a fraction of heparin concentration. With each of the antithrombin derivatives, as heparin was initially increased, the pseudo-first-order rates of inactivation of thrombin by antithrombin also increased and approached a maximum. Further increases in heparin concentration were associated with a pseudo-plateau and reaction rates decreased at higher levels of heparin.

The ternary complex model which has been proposed to account for the mechanism of the heparin effect qualitatively explains this kinetic pattern in the presence of heparin, a pattern which cannot readily be rationalized otherwise. At low concentrations of heparin, with the two proteins both in excess over the catalyst, the binding sites for antithrombin and thrombin on the heparin chains remain saturated as heparin concentration is increased. Under these conditions, the reaction rate increases linearly as heparin concentration increases. At the opposite extreme of high concentrations of heparin, with heparin in excess over the proteins, further increases in heparin concentration yield a decrease in reaction rate as thrombin and antithrombin are sequestered on separate heparin chains. At intermediate heparin concentrations the reaction rate will reach a maximum. The optimal heparin concentration at this maximum will correspond to conditions which give the highest probability of simultaneous occupancy of the thrombin- and antithrombin-binding sites on the maximum number of individual heparin chains. As Nesheim (12) showed, this optimal heparin concentration will be dependent on the concentrations of thrombin and antithrombin. Additionally, however, the heparin concentration which corresponds to maximal rate of reaction will necessarily be related to the strengths of binding of both antithrombin and thrombin to the mucopolysaccharide. The dependence of the heparin-antithrombin-thrombin reaction kinetics upon heparin thus is characterized by: 1) increasing reaction rates at subsaturating levels of heparin, 2) a “plateau” in rate at higher concentrations of heparin, corresponding to optimal saturation of heparin with both reactants, and 3) a decrease in rate as heparin is increased to levels at which thrombin and antithrombin are bound to separate heparin chains.

The rate, \( v \), of the heparin-catalyzed reaction can be mathematically described using the equation derived by Nesheim (12) for the ternary-complex mechanism:

\[
v = k \cdot f_h \cdot f_t \cdot H_t
\]

in which \( f_h \) and \( f_t \) represent the fractional saturation of heparin with antithrombin and thrombin, respectively, \( k \) is the rate constant, and \( H_t \) represents total heparin concentration. This equation can be expressed in terms of total antithrombin concentration, \( A \), and total thrombin concentration, \( T \), as:

\[
v = k \cdot b_i \cdot b_h \cdot b_t \cdot H_t
\]

in which \( b_i \) and \( b_h \) represent fractional saturation of the proteins, antithrombin and thrombin, respectively, with heparin. In the present study, computer modeling was performed to demonstrate that the maximal rate observed in the plateau region depends upon affinity of heparin exhibited by antithrombin and thrombin. Equation 2 was used to determine the kinetic patterns which would be predicted by the template model for the heparin dependence of the antithrombin-thrombin reaction using antithrombins with altered heparin-binding properties (Fig. 8). For this purpose, rates of reaction for fixed concentrations of thrombin and antithrombin were calculated over a wide range in heparin concentration. The curves generated show the characteristic dependence upon heparin concentration described above.

The curves shown in the upper panel of Fig. 8 differ in one aspect: each represents the kinetic pattern predicted for antithrombins which differ in heparin-binding affinity. As heparin affinity decreases, the maximum rate attainable in the system decreases. Additionally, the corresponding heparin concentration at maximum rate changes, increasing as the affinity of antithrombin for heparin decreases. Thus, as heparin binding becomes weaker, higher concentrations of heparin are required to bind optimal amounts of antithrombin. Computer modeling of the reaction clearly shows that the maximum rate of reaction possible between protease and inhibitor is lowered as heparin affinity for the inhibitor is lowered.

The lower panel of Fig. 8 shows the predicted kinetic behavior which results if the heparin affinity of both proteins is held constant and only the intrinsic rate constant is varied. As shown in the figure, computer modeling shows that lowering of the rate constant results in a decrease in maximum rate, but the position of the peak in rate, which represents the heparin concentration at which single chains of heparin are optimally saturated with both proteins, does not vary. If a heparin-induced allosteric change were responsible for increased reactivity with thrombin, the data shown in this panel indicate that antithrombins which differ in the extent of the allosteric change will also differ in the maximum rate at which the inhibitor plus heparin can inactivate thrombin.

The predicted behavior shown in the two panels in Fig. 8 is reminiscent of the kinetic pattern observed with the HNB-antithrombin derivatives, which exhibit heparin-binding properties that differ from those of native antithrombin. The maximum rate achieved with the singly labeled antithrombin
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Fig. 8. Upper panel, prediction of the heparin-accelerated rate of reaction between antithrombin and thrombin for a series of antithrombins with weakened heparin affinities. Curves showing reaction rate patterns were calculated for concentrations of 10 nM thrombin and 100 nM antithrombin, using a heparin dissociation constant of $1 \times 10^{-7}$ M for thrombin and $1 \times 10^{-5}$ M (curve A), $5 \times 10^{-5}$ M (curve B), $1 \times 10^{-4}$ M (curve C), and $1 \times 10^{-3}$ M (curve D) for antithrombin. The values estimated for the binding affinities represent realistic estimates, and the protein concentrations used in the modeling studies were chosen arbitrarily to conform to pseudo-first-order conditions. Lower panel, predictions of the heparin-accelerated rates of reaction between antithrombin and thrombin with variations in the rate constant for reaction. This panel shows the effect of varying the rate constant, $k$, in Equation 3. The value of $k$ (curve A) was varied by division of $k$ by 2 (curve B), 4 (curve C), and 8 (curve D). Antithrombin was fixed at a concentration of $1 \times 10^{-7}$ M, and thrombin was fixed at a concentration of $1 \times 10^{-7}$ M, with a heparin $K_d$ of $1 \times 10^{-5}$ M.

derivatives is lower than for native antithrombin, increasing in the order Derivative 1 < Derivative 2 < Derivative 3. Since the maximum rate of thrombin inactivation observed is predicted to be dependent upon both affinity for heparin and the intrinsic reaction rate for the antithrombin-thrombin reaction within the ternary complex, it is not clear from these data alone whether the reduced heparin affinity or an altered conformational change in these proteins is responsible for lower rates of reactivity with thrombin.

The modeling studies demonstrate the need for another experimental approach to distinguish between the effects of heparin binding affinity and heparin-induced allosteric effects in antithrombin on reaction kinetics. To determine whether 1) lowered heparin affinities or 2) altered reactivity with protease was indicated by the results of the kinetic heparin titration, another approach was taken to look at the kinetics of the HNB-antithrombin species. The apparent heparin-binding constants and the maximum rates of reaction of each of the HNB-labeled antithrombins were estimated by modeling the heparin-catalyzed reaction between antithrombin and thrombin as a two-substrate enzyme-catalyzed reaction (11). Using this model, the hyperbolic dependence of the reaction rate on antithrombin yielded the apparent $K_m$ of the "enzyme," heparin, for each of the antithrombin species and the apparent $V_{max}$ for the reaction. The apparent $K_m$ values measured for the HNB-antithrombin derivatives were clearly weaker than that of native antithrombin, with binding affinities increasing in the order Derivative 1 < Derivative 2 < Derivative 3 < Derivative 4. Thus, the weaker apparent heparin-binding constants seen for Derivatives 1–3 were consistent with the lower maximal rates of reaction observed upon heparin titration of the inhibitory rates of each of the derivatives.

More noteworthy is the maximal rate of reaction observed with native antithrombin and each of the HNB-antithrombin derivatives. As antithrombin concentration is increased, all of the antithrombins reach the same maximal rate of reaction, regardless of the magnitude of the heparin-induced conformational change observed in each of the modified antithrombin derivatives. If the full native-like heparin-promoted isomerization were required to cause the protease-binding site of antithrombin to assume a more reactive conformation, as originally purported by Rosenberg and Damus (7), the rates of reaction of the HNB-antithrombin derivatives would be determined and limited by the conformational changes which occur in each of them to lesser extents than in unlabeled antithrombin. Thus, our data indicate that the heparin-induced change in antithrombin conformation does not render the protein intrinsically more reactive with thrombin.

These data are in strong support of the study of Olson et al. (16) which indicate that the conformational change which occurs in antithrombin upon interaction with heparin serves to increase protein affinity for the heparin chain, allowing for rapid reaction with thrombin bound to the same heparin chain. The $K_m$ of heparin measured for each of the labeled antithrombins was observed to vary directly with the extent of a conformational change measured by any of several spectroscopic approaches. In contrast, an effect of the magnitude of the conformational change measured with the antithrombin derivatives on $V_{max}$ was not detectable in these studies.

These results can be interpreted with respect to the model shown in Fig. 9. Following the binding of heparin to antithrombin, the antithrombin molecule can undergo a conformational change indicated by the conversion of A-H to A*-H. Since the conformational change stabilizes the antithrombin-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig9}
\caption{A model for the heparin-promoted inactivation of thrombin by antithrombin. The cycle of the heparin-antithrombin-thrombin reaction is shown to depict binding of both proteins to heparin to form a ternary intermediate, followed by reaction between antithrombin and thrombin, and release of heparin from the inactive complex. A represents antithrombin, with A* representing antithrombin which has undergone the heparin-induced conformational change. T represents thrombin, and H represents heparin. A1 represents the thrombin-cleaved form of antithrombin.}
\end{figure}

$^3$ The $V_{max}$ could not be estimated using our experimental conditions for Derivative 1 (see "Results" section).
heparin complex significantly, decreasing the dissociation constant by approximately two orders of magnitude (16). A*-H represents the predominant form of the native protein bound to heparin. On the other hand, the ability of the protein to undergo the conformational change is impaired in each of these chemically modified HNB-antithrombins. Nevertheless, the maximal rate of reaction with thrombin is not decreased with these antithrombin species. Thus, in terms of the model in Fig. 9, thrombin reacts equally well with A-H or A*-H, and the reaction is limited only by formation of the heparin-antithrombin-thrombin complex. The ability of the proteins to form this ternary complex is a function of their affinities for heparin. Following reaction of thrombin and antithrombin on the heparin template, a conformational change occurs in the heparin-binding region of antithrombin which triggers release of heparin from the complex (25), allowing heparin to continue in the catalytic cycle. Olson and Shore (34) have recently shown that the rate-limiting step in the heparin-catalyzed reaction is formation of the thrombin-antithrombin complex, which occurs simultaneously with the conformational change in antithrombin which leads to weaker binding of heparin to the complex.

The approach used in the present study, which utilized antithrombin derivatives with limited heparin-induced conformational changes and weaker overall heparin affinity, readily indicated that the conformational change does not lead to more efficient reactivity with protease, as was initially suggested by Rosenberg and Damus (7). The heparin enhancement of the rate of the antithrombin-thrombin reaction can therefore be attributed to approximation of the two proteins on the heparin chain with nominal contribution from an allosteric change rendering antithrombin more highly reactive with protease.

REFERENCES
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The results from several experiments are presented here, including kinetic measurements and conformational changes. The use of thrombin and antithrombin complexes with heparin-Sepharose has allowed for the study of conformational changes in the antithrombin molecule. The intrinsic fluorescence of antithrombin has been used as a probe to study these changes. Additionally, the effect of heparin on the intrinsic fluorescence of antithrombin has been measured.

**Table I. HNB-Labeling and Recoveries of Modified Antithrombin Derivatives**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>HNB/mol protein</th>
<th>Efficiency (%)</th>
<th>Recovered %</th>
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<tr>
<td>1</td>
<td>1.0 - 1.1</td>
<td>0.15</td>
<td>50 - 60</td>
</tr>
<tr>
<td>2</td>
<td>0.9 - 1.2</td>
<td>0.3</td>
<td>5 - 10</td>
</tr>
<tr>
<td>3</td>
<td>0.5 - 0.6</td>
<td>0.5</td>
<td>50 - 60</td>
</tr>
<tr>
<td>4</td>
<td>0.8 - 0.9</td>
<td>0.3</td>
<td>50 - 60</td>
</tr>
<tr>
<td>5</td>
<td>0.9 - 1.0</td>
<td>0.3</td>
<td>50 - 60</td>
</tr>
</tbody>
</table>

**Table II. Spectroscopic Properties of HNB-antithrombin Derivatives**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>λmax (nm)</th>
<th>λem (nm)</th>
<th>λexc (nm)</th>
<th>Extinction (ε)</th>
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<td>5</td>
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<td>450</td>
<td>310</td>
<td>2400</td>
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</table>

**Figure 1. Separation of HNB-antithrombin species by heparin affinity chromatography.** A column (1.5 cm x 15 cm) was packed with heparin-Sepharose equilibrated with 0.1 M sodium phosphate, pH 7.2. The protein solution was applied to the column and eluted with a linear gradient of 0.1 M NaCl in 0.1 M NaHCO3. The HNB-labeled antithrombin was eluted at a salt concentration of 0.1 M NaCl.

**Figure 2. Titration of antithrombin III with heparin.** Aliquots of high-affinity heparin (2.5 x 10^-5 M) were added to 1.5 ml protein samples (100 µg/ml). The fluorescence for Derivatives 1, 2, 3, and 4 was measured at 430 nm and 450 nm, respectively. Increase in absorbance was calculated from the peak and the 410 nm trough. The increase in absorbance is expressed as a percentage of the initial fluorescence (F0/F).

**Figure 3. Heparin-induced changes in the ultraviolet-visible spectra of the antithrombin- decreases in absorbance upon the binding of heparin to antithrombin.** The ultraviolet-visible spectra of antithrombin were recorded in the presence of increasing concentrations of heparin. The changes in absorbance are expressed as a percentage of the initial absorbance.

**Figure 4. Intrinsic fluorescence of antithrombin.** The intrinsic fluorescence of antithrombin was measured in the presence of increasing concentrations of heparin. The changes in intrinsic fluorescence are expressed as a percentage of the initial intrinsic fluorescence.

**Figure 5. Difference spectra of HNB-antithrombin derivatives.** The difference spectra of HNB-antithrombin derivatives were recorded in the presence of increasing concentrations of heparin. The changes in absorbance are expressed as a percentage of the initial absorbance.

**Figure 6. The heparin-induced changes in the intrinsic fluorescence of antithrombin.** The intrinsic fluorescence of antithrombin was measured in the presence of increasing concentrations of heparin. The changes in intrinsic fluorescence are expressed as a percentage of the initial intrinsic fluorescence.

**Figure 7. The heparin-induced changes in the intrinsic fluorescence of antithrombin.** The intrinsic fluorescence of antithrombin was measured in the presence of increasing concentrations of heparin. The changes in intrinsic fluorescence are expressed as a percentage of the initial intrinsic fluorescence.

**Figure 8. The heparin-induced changes in the intrinsic fluorescence of antithrombin.** The intrinsic fluorescence of antithrombin was measured in the presence of increasing concentrations of heparin. The changes in intrinsic fluorescence are expressed as a percentage of the initial intrinsic fluorescence.

**Figure 9. The heparin-induced changes in the intrinsic fluorescence of antithrombin.** The intrinsic fluorescence of antithrombin was measured in the presence of increasing concentrations of heparin. The changes in intrinsic fluorescence are expressed as a percentage of the initial intrinsic fluorescence.
Heparin-induced changes in circular dichroic spectra of the HNB-antithrombin derivatives. The CD spectra recorded in the visible region of circular dichroism at room temperature in the presence of 0.15 M KCl. The spectrum for derivative 1 is representative of the spectrum observed with unmodified antithrombin III. This spectrum is characterized by the presence of the characteristic CD bands of antithrombin. The pair of minima at approximately 270 and 210 nm which arise from tryptophans which are "shielded" to the aqueous environment. The pair of minima at 260 and 280 nm which can be attributed to the 0-0 and 0-1 transitions of phenylalanines and tyrosines. The spectrum for derivative 3 is characteristic of the heparin-induced changes in antithrombin conformation which can be to detected heparin-induced conformational changes.