Deconvolution of the Fluorescence Emission Spectrum of Human Antithrombin and Identification of the Tryptophan Residues That Are Responsive to Heparin Binding*

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Jennifer L. Meagher‡, Joseph M. Beechem§, Steven T. Olson¶, and Peter G. W. Gettins¶†

From the ‡Department of Biochemistry and Molecular Biology, College of Medicine and the ¶Center for Molecular Biology of Oral Diseases, College of Dentistry, University of Illinois at Chicago, Chicago, Illinois 60612 and the ‡Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee, 37232

Heparin causes an allosterically transmitted conformational change in the reactive center loop of antithrombin and a 40% enhancement of tryptophan fluorescence. We have expressed four human antithrombins containing single Trp → Phe mutations and determined that the fluorescence of antithrombin is a linear combination of the four tryptophans. The contributions to the spectrum of native antithrombin at 340 nm were 8% for Trp-49, 10% for Trp-189, 19% for Trp-225, and 65% for Trp-307. Trp-225 and Trp-307 accounted for the majority of the heparin-induced fluorescence enhancement, contributing 37 and 36%, respectively. Trp-49 and Trp-225 underwent spectral shifts of 15 nm to blue and 5 nm to red, respectively, in the antithrombin-heparin complex. The blue shift for Trp-49 is consistent with partial burial by contact with heparin, whereas the red shift for Trp-225 and large enhancement probably result from increased solvent access upon heparin-induced displacement of the contact residue Ser-380. The enhancement for Trp-307 may result from the heparin-induced movement of helix H seen in the crystal structure. The time-resolved fluorescence properties of individual tryptophans of wild-type antithrombin were also determined using the four variants and showed that Trp-225 and Trp-307 experienced the largest change in lifetime upon heparin binding, providing support for the steady-state fluorescence deconvolution.

Antithrombin is a member of the serpin family of proteinase inhibitors that requires the cofactor heparin to inhibit target proteinases of the blood coagulation cascade. Heparin binding induces a conformational change in antithrombin that is responsible for a 200–300-fold increase in rate of inhibition of factor Xa (1). This conformational change has been demonstrated by changes in UV (2), CD (2), NMR (3, 4), and fluorescence (5, 6) spectra of the protein following heparin binding. Of these methods, fluorescence has been the most extensively used to determine the thermodynamics and kinetics of heparin binding since the magnitude of the enhancement (40%) has provided the sensitivity necessary both to determine dissociation constants for antithrombin-heparin complexes that are in the nanomolar range and to follow relatively rapid heparin-induced conformational changes (7, 8).

Interpretation of such studies in structural terms has, however, been handicapped by not knowing which of the four tryptophans is(are) responsible for the fluorescence changes. An example is a fluorescence quenching study aimed at probing differential responsiveness of the tryptophans to heparin binding. This study was able to demonstrate two classes of tryptophans, one buried and one exposed, and to show that the buried class was responsive to heparin binding, but was unable to identify which tryptophans were involved (9). Non-fluorescence methods have also been used to examine the role of tryptophans in antithrombin. A 19F NMR study showed that two of the four tryptophans were very sensitive to heparin binding (10), but did not unambiguously identify the residues involved.

With the recent advances in the knowledge of the structure of antithrombin (12–16) and of the nature of the serpin inhibition mechanism, increasingly focused studies are being carried out to determine the details of the heparin activation mechanism. These include studies in which residues thought to be involved directly in heparin binding have been mutated (10, 17–23) and others in which the kinetics and thermodynamics of binding of heparin species of different lengths or compositions have been examined (1, 24–26). The four tryptophans in human antithrombin, Trp-49, Trp-189, Trp-225, and Trp-307, are well spread out (see Fig. 1) and hence have the potential to resolve heparin-induced structural changes in very different parts of the molecule. Trp-49 is toward the beginning of helix A and close to the heparin-binding site. Trp-189 is on the back face of helix F and in contact with the mechanistically critical β-sheet A. Trp-225 is at the end of strand 3 of β-sheet A and close to the side chain of Ser-380, which is the residue thought to be displaced from β-sheet A as part of the heparin-induced activation mechanism (27, 28). Trp-307 is on helix H. Each of these is between 15 and 35 Å from another tryptophan. It would be advantageous to determine the spectral properties and heparin responsiveness of each tryptophan to permit fuller exploitation of the information present in the fluorescence spectrum of antithrombin.

We have therefore prepared and characterized four variants of antithrombin containing single Trp → Phe mutations to deconvolute the fluorescence spectrum of human antithrombin into the contributions from each tryptophan and to determine which of the tryptophans have emission spectra that are sensitive to heparin binding. We found that the steady-state fluorescence of wild-type antithrombin, both in the absence and presence of heparin, consists of a linear combination of the fluorescence properties of the individual tryptophans. This lin-
ear behavior allowed the unambiguous deconvolution of the shape and relative intensities of the steady-state emission spectra, determination of the lifetimes of each individual tryptophan, and the effects of heparin binding on each of these parameters. Both approaches indicate that Trp-225 and Trp-307 are most responsive to heparin binding.

**EXPERIMENTAL PROCEDURES**

*Production and Isolation of Variant Forms of Antithrombin—* All of the variant antithrombins were created using human antithrombin cDNA on an N135Q background. The N135Q background reduced heterogeneous differences in glycosylation of the recombinant antithrombin by eliminating the glycosylation site at position 135 (29). The resulting antithrombin therefore resembles the β-form of plasma antithrombin, which also lacks carbohydrate at position 135 (30). The stoichiometric titration of 500 nM antithrombin with full-length high-affinity heparin as described previously (25), using single-stranded uracil-containing template and the antisense oligonucleotides 5'-GGA CAG TTC AAA GAC ACG CGG TGT-3', 5'-GAA CTT TGA CTT AAA CAG GCC CTT GA-3', and 5'-CAG TTC ATC CAG CAC-C-3', respectively (the underlined codons correspond to the mutation). The W189F variant was created using site-directed mutagenesis carried out in M13mp19 as described previously (25), using single-stranded uracil-containing template and the antisense oligonucleotides 5'-GTT GAC TTA TGA TTT GTT GAT GGC-3'. The resulting antithrombin therefore resembles the β-form of plasma antithrombin, which also lacks carbohydrate at position 135 (30). The W49F, W225F, and W307F variants were created by site-directed mutagenesis carried out in M13mp19 as described previously (25), using single-stranded uracil-containing template and the antisense oligonucleotides 5'-GGA CAG TTC AAA GAC ACG CGG TGT-3', 5'-GAA CTT TGA CTT AAA CAG GCC CTT GA-3', and 5'-CAG TTC ATC CAG CAC-C-3', respectively (the underlined codons correspond to the mutation). The W189F variant was created using the Altered Sites protocol (Promega), following the manufacturer's directions (42). Antisense oligonucleotides 5'-GTT GAC TTA TGA TTT GTT GAT GGC-3' were ligated into the expression vector pMAStop as described (25) to create the pMAAT-3'-F-N135Q expression vectors. The expression vectors, together with the selection plasmids pRHM1140 and pSV2dhfr, were then used to stably transfect baby hamster kidney cells as described previously (31). Transfected cells were selected by resistance to neomycin (Life Technologies, Inc.) and methotrexate (Sigma) as described previously (32). The variant antithrombins were isolated from serum-free cycles of medium collected from stably transfected baby hamster kidney cells grown to confluence in roller bottles. Purification was by affinity chromatography on heparin-Sepharose followed by anion-exchange chromatography on either DE52 (Whatman) or MonoQ (Amersham Pharmacia Biotech). Any polymeric antithrombin present was removed by further purification by size-exclusion chromatography using a Sephacryl G-150 matrix (Amersham Pharmacia Biotech). During cycling and purification, the level of antithrombin was monitored by radial immunodiffusion assay using commercially available plates containing sheep anti-human antithrombin antibody (Binding Site Ltd., Birmingham, United Kingdom).

*Determination of Concentration—* The extinction coefficient at 280 nm for the single tryptophan variants was calculated to be $E_{280}^{\text{em}} = 5.5$ by subtracting the absorbance contribution of an average protein tryptophan (30) from the wild-type antithrombin extinction coefficient of $E_{280}^{\text{em}} = 6.5$ (31). The concentration of the purified proteins was also checked using the BCA protein assay reagent kit (Pierce) following the manufacturer's ENHANCE Tube protocol. The concentrations were found to be in agreement with those determined using the calculated extinction coefficients.

Because of the importance of concentration for the fluorescence emission spectra, the absolute protein concentration was also determined by tryptophan fluorescence of denatured protein prior to recording the spectra (33). For the control antithrombin, different concentrations of antithrombin (25–100 nM) were incubated in 6 mM guanidine hydrochloride containing 10 mM dithiothreitol and 0.1 mM EDTA in 20 mM sodium phosphate buffer, pH 7.0. Samples were incubated for 60 min at 25 °C. The fluorescence spectra of the denatured samples were recorded on an SLM-AMINCO 8000 spectrofluorometer, exciting at 295 nm and measuring emission from 300 to 400 nm. Bandwidths were 4 nm for excitation and 16 nm for emission. The peak of the emission spectra was at 352 nm. The tryptophan fluorescence of the control showed a linear dependence on protein concentration. For the variant antithrombins, 100 nM antithrombin was incubated as described above, and the fluorescence emission spectra were recorded. The determined concentrations were in good agreement with those determined by absorbance at 280 nm, except for the W307F variant, which was slightly lower.

The concentration of functional antithrombin was determined by stoichiometric titration of 500 nM antithrombin with full-length high-affinity heparin as described previously (34), using the well defined inflection point of the fluorescence at saturation with heparin. Since the inflection point was used rather than the absolute magnitude of the fluorescence enhancement, this method made no prior assumption about the size of heparin-induced fluorescence changes in the variants. The excitation wavelength was 280 nm, and the emission was recorded at 340 nm. Bandwidths of 4 nm for excitation and 2 nm for emission (kept constant for both recording spectra and making constant wavelength measurements) were used. Data were fitted to a single-site binding model equation by nonlinear least-squares analysis using the SCIENTIST program (MicroMath Scientific Software, Salt Lake City, UT). All experiments involving heparin were corrected for the presence of any inactive protein determined by this method.

*SDS-Polyacrylamide Gel Electrophoresis—* The ability of the variant antithrombins to form covalent complexes with proteinase was determined by incubation of antithrombin (5.8 μM) and thrombin (7.5 μM) in the absence of heparin (5.7 μM) for 30 min on ice. The reaction was then stopped by reaction with 0.5 mM phenylmethylsulfonyl fluoride for 5 min on ice. Electrophoresis of the reaction products on a 10% SDS-polyacrylamide gel was carried out as described previously (20). A covalent complex was evident from the appearance of a characteristic band at higher molecular weight than antithrombin or thrombin.

*Stoichiometry of Inhibition—* The stoichiometry of inhibition (defined as the moles of antithrombin required to inhibit 1 mol of target protein) was measured by incubating antithrombin at different concentrations (0–30 nM) with 20 nM factor Xa. The reactions were allowed to go to completion, and residual factor Xa activity was measured by diluting the reaction mixture 100-fold with 100 μM Spectrozyme Xa (American Diagnostica Inc., Greenwich, CT) and measuring the rate of substrate hydrolysis at 405 nm as described previously (20). The stoichiometry of inhibition was determined by plotting residual proteinase activity against the ratio of antithrombin to factor Xa. All reactions were carried out in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and 100 mM NaCl (i = 0.15).

*Determination of Antithrombin-Heparin Dissociation Constants—* Heparin binding studies were carried out by titration, using full-length high-affinity heparin as described previously (34). The binding of heparin to antithrombin was monitored by the change in endogenous tryptophan fluorescence, measured on a Spex Fluorolog 2 spectrofluorometer, exciting at 280 nm and observing emission at 340 nm. Dissociation constants were determined using 50 nM antithrombin and an excitation bandwidth of 1.7 nm and an emission bandwidth of 13.6 nm. Titrations were carried out at 25 °C in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol, and 100 mM NaCl. Data were fitted to a single-site binding model equation by nonlinear least-squares analysis using the SCIENTIST program.

*Measurement of Denaturation Temperatures by CD Spectroscopy—* Thermal unfolding of antithrombin was followed by monitoring the change in the CD signal as a function of temperature. CD spectra were recorded on a Jasco 710 spectropolarimeter in a 1-mm path length jacketed cell. The temperature was controlled by a NESLAB water bath. Antithrombin concentrations were 0.25–0.5 mg/mL in i = 0.15 buffer (20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% polyethylene glycol 8000, pH 7.4). A bandwidth of 2 nm, a step resolution of 0.5 °C, a response time of 16 s, and a scan rate of 0.5 °C/min were used. Data were plotted as the first derivative of the ellipticity at 220 nm against temperature to more clearly define the midpoint of the unfolding transition. $T_m$ values were reproducible to within 0.5 °C.

*Kinetic Assays—* The rates of inhibition of factor Xa by antithrombin in the presence and absence of heparin were measured under pseudo first-order conditions of 250 nM antithrombin, 5 nM factor Xa, and heparin concentrations of 0, 1, 2, 3, and 4 nM and were calculated as described previously (20). The reactions were carried out at 25 °C in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and 100 mM NaCl.

*Fluorescence Emission Spectra—* Fluorescence emission spectra were recorded prior to and following stoichiometric titration of antithrombin with full-length high-affinity heparin (see above). Bandwidths of 4 nm for excitation and 2 nm for emission were used. Spectra were recorded from 300 to 400 nm in 1-nm steps, with an integration time of 5 s. The antithrombin concentration was 500 nM. Full-length high-affinity heparin was added to a final concentration of 1.5 μM. This is sufficient to give complete saturation of each variant, given the tightness of heparin binding to function was determined.

* Determination of Fluorescence Lifetimes—* Time-resolved fluorescence measurements were performed with a Coherent Antares neodymium/YAG laser. The output of this laser was frequency-doubled and used to synchronously pump a dual-dye jet laser (Coherent 702) using rhodamine 6G for excitation of tryptophan at 295 nm. Output pulses from the dye laser were utilized at 4 MHz and had a pulse width of ~1 ps. Time-resolved detection utilized time-correlated single-photon
counting with a Hamamatsu microchannel plate detector (Model R2809U), high frequency 50X amplifiers (Philips Scientific Model 774), constant fraction discriminators (Tennelec Model 454), time-to-amplitude converters (Tennelec Model 862), and pulse-height analysis analog-to-digital converters (Nucleus PCA-II). The instrument response was typically 40–50 ps. The collimated fluorescence emission was passed through a Hoya 320-nm cut-off filter for tryptophan emission and Glan-Thompson polarizers, before being focused onto the entrance slit of a Spex 0.22-m emission monochromator. Tryptophan emission was measured at both 330 and 350 nm. A half-wave plate in the excitation beam was utilized to rotate the excitation polarization to horizontal for the determination of the polarization bias (g factor) of the detection instrumentation.

Data Analysis of Fluorescence Lifetimes—Time-correlated fluorescence intensity decay data, obtained for both horizontal (I_H) and vertical (I_V) polarizer orientations, were used to calculate the total intensity (S(t)) using Equation 1,

\[ S(t) = g I_V + 2I_H \]  

(Eq. 1)

where g is the polarization bias of the detection instrument. Total intensity decays for wild-type and all four variant antithrombins could be adequately represented by three lifetimes, described by Equation 2,

\[ S(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3} \]  

(Eq. 2)

where \( \tau \) is the amplitude (wavelength-dependent) of the emission lifetime (wavelength-dependent). Global analysis of the 330 and 350 nm fluorescence decay data was performed using the Globals Unlimited software package (35). The decay curves of the individual tryptophan residues (obtained by subtraction of steady-state weighted decay curves for variant antithrombin from that of wild-type antithrombin) were similarly analyzed and fitted to give either one or two lifetimes for tryptophan decay, depending on the tryptophan. Interpretation of the time-resolved results was performed in such a way as to be essentially independent of the form of the fitting function utilized. Appropriately subtracted and normalized decay curves for each tryptophan in the absence and presence of heparin were overlapped and determined to either be identical or significantly different, within the error defined by the photon counting noise of the data.

Materials—Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Life Technologies, Inc. Human α-factor Xa was prepared from purified factor X as described previously (25). α-Thrombin was prepared from prothrombin, isolated from outdated human plasma by the method of Miletich et al. (36), by reaction with snake venom as described (37). The plasmids pMA5Stop, pRKH1140, and pVSv26hrf (32) were gifts from Dr. Gerd Zettelmeißl (Behringwerke, Marburg, Germany). Full-length high affinity heparin (Mn 9000) was prepared by fractionation of heparin first by size-exclusion chromatography and then by antithrombin affinity chromatography as described (34). Heparin concentrations used are of antithrombin-binding sites determined by stoichiometric titration with plasma antithrombin of known active concentration (34).

RESULTS

Functional Properties of Variants—The replacement of tryptophan by phenylalanine in the four antithrombin variants examined, W49F, W189F, W225F, and W307F, was found to be conservative with respect to the ability of the variant antithrombin to inhibit proteinase by the serpin suicide substrate mechanism. Thus, all four variants formed SDS-stable covalent complexes with proteinase (gel not shown), which is a hallmark of the serpin inhibition mechanism. The stoichiometries of inhibition of factor Xa in the absence of heparin were found to be similar to that of the control antithrombin (Table 1), indicating that the mutations had not significantly affected the relative efficiencies of the inhibitory and substrate branches of the pathway. To determine if the mutations had adversely perturbed the heparin-binding site or the energetics of the heparin-induced conformational change, the dissociation constants for the antithrombin-heparin complex were also measured. Measurements were made at physiological ionic strength, so that the \( K_d \) values could be used to determine degree of heparin saturation of antithrombin in other experiments carried out under these conditions. Since heparin binding was very tight for all of the variants, this led to relatively large errors in the determined values. Nevertheless, the \( K_d \) values clearly showed that none of the mutations had reduced the affinity of the heparin-binding site (Table 1).

The rate constants for the inhibition of factor Xa were determined both in the absence and presence of heparin (Table 1). The basal rates of inhibition of factor Xa by the W49F and W189F antithrombin variants were similar to that of the control antithrombin. The basal rates of inhibition for the W225F and W307F antithrombin variants were ~3-fold higher. This was not due to contamination with heparin since the rates were determined in the presence of Polybrene. Since it is thought that antithrombin exists in two conformations, one low activity conformation in which the P14 residue (Ser-380; P14 is the position 14 residues N-terminal of the scissile bond, which is defined as the bond between the P1 and P1* residues) is inserted into β-sheet A with its side chain in contact with Trp-225 (Fig. 1) and one fully activated conformation in which the P14 side has been expelled from the β-sheet, these higher rates may reflect a small shift in the equilibrium toward the activated conformer. This would be readily understandable for the W225F variant. Such a shift would not compromise the present deconvolution analysis since it would necessitate less than a 1% increase in population of the active conformer to account for the increase in rate observed. The reason for a possible shift in conformer population for the W307F variant is less obvious and, if such a shift is present, would suggest a more subtle linkage to the reactive center loop. Heparin increased the rate of inhibition of factor Xa by all four variants. However, the rate for the W307F variant was ~3-fold lower than for the control antithrombin, whereas the other three variants were more similar to the control (Table 1). This again suggests a linkage between Trp-307 and the reactive center loop conformation and hence the activity of the heparin-bound state.

Stability of Variants—The denaturation temperature of each variant was examined by CD spectroscopy, monitoring the change in ellipticity at 220 nm. Although all four variants were less stable than the control antithrombin (Table 1), this was by a maximum of 4.2 °C. There appeared to be a correlation between the degree of burial of the indole side chain and the reduction in stability upon changing tryptophan to phenylalanine, such that the more buried the residue, the lower the reduction in stability upon changing tryptophan to phenylalanine, such that the more buried the residue, the lower the unfolding temperature. Thus, Trp-225 and Trp-307 are most buried, Trp-189 is less buried, and Trp-49 is completely exposed (Fig. 2). Given the possibly additive effects of such mutations on stability, this suggests that direct determination of the properties of individual tryptophan by using multiple tryptophan-to-phenylalanine replacements may cause too great a reduction in protein stability to make working with such variants advisable. This point has also been made by others (38, 39).

Deconvolution of Tryptophan Emission Spectra—Steady-state tryptophan emission spectra were recorded for each of the

![](image_url)
Trp → Phe variants. Each variant gave an emission spectrum with lower fluorescence intensity than wild-type antithrombin (data not shown). To determine if each tryptophan contributed independently to the emission spectrum, a comparison was made between the sum of the emission spectra of the four variants with that of wild-type antithrombin. If each tryptophan has emission properties that are independent of the other tryptophans, the wild-type spectrum should be identical to one-third of the sum of the spectra of individual tryptophan variants. The near identity of these two spectra (Fig. 3A) indicated that each tryptophan emits independently of the other three. This is perhaps not surprising given that the inter-tryptophan separations are 15–35 Å. The contribution of each tryptophan to the spectrum of wild-type antithrombin could therefore be obtained by subtraction of the spectrum of the appropriate single substitution variant from that of wild-type antithrombin (Fig. 3B). Trp-307 was found to be responsible for the majority of the initial fluorescence intensity, accounting for 63%. Trp-49, Trp-189, and Trp-225 contributed to much lesser extents of 8, 10, and 19%, respectively. The most blue-shifted tryptophan was Trp-225 (λ\text{max} = 330 nm), followed by Trp-307 (λ\text{max} = 337 nm). Trp-49 and Trp-189 had emission maxima at 356 and 345 nm, respectively, consistent with more solvent-accessible environments.

Spectra were also recorded in the presence of a saturating amount of full-length high affinity heparin. The emission spectrum of the wild-type antithrombin-heparin complex was very similar to one-third of the sum of the spectra of the single substitution variants (Fig. 4A), although slightly higher in intensity. The slightly higher intensity of the control spectrum may result from one or more of the variants not undergoing the full conformational change of wild-type antithrombin. This might be the case for W307F antithrombin, which shows a lower rate of inhibition of factor Xa in the presence of heparin (Table I). The results therefore again show that the tryptophans have emission properties independent of one another in the heparin-bound conformation. Deconvolution was thus possible by appropriate pair-wise subtraction of heparin-complexed variant spectra from heparin-complexed control spectra (Fig. 4B).

Of the four tryptophans, Trp-307 was again the single largest contributor to the spectrum and was responsible for 38% of the total fluorescence enhancement upon heparin binding (based on integrated area), although with little change in emission maximum (Fig. 5 and Table II). Trp-225 underwent the largest individual change, a 1.2-fold increase (38% of the total fluorescence enhancement) and red shift of 5 nm (Fig. 5). Trp-49 showed only a slight increase in integrated intensity, but underwent a very large blue shift of 15 nm upon heparin.

**Fig. 1.** Structure of human antithrombin showing the location of the four tryptophans, based on the crystal structure of Schreuder et al. (15). Also shown is Ser-380, the P14 residue that is initially present inserted into β-sheet A, but is expelled by the heparin-induced conformational change. The reactive center loop is indicated at the top of the molecule. Note that the two-dimensional representation makes Trp-307 and Trp-49 appear closer than they are.

**Fig. 2.** Environment of each of the four tryptophans of human antithrombin, based on the crystal structure (15). Tryptophan is shown in cyan; acidic residues are in red; hydrophobic residues are in yellow; and other residues are in white. P14 (Ser-380) is not visible in this view of Trp-225.

**Fig. 3.** Fluorescence emission spectra of wild-type and variant antithrombins. The independent behavior of tryptophans in antithrombin and deconvolution of the spectrum in the absence of heparin are demonstrated. A, the solid line is the spectrum of the control antithrombin, and the dashed line represents one-third of the sum of the spectra of individual tryptophan variants. B, shown is deconvolution of the spectrum of antithrombin into contributions of individual tryptophan residues. An antithrombin concentration of 500 nM was used. WT, wild-type antithrombin.
binding (Fig. 5), consistent with burial of some or all of the indole ring. Comparatively, Trp-189 was relatively insensitive to heparin binding (Fig. 5), contributing only 10% to the total fluorescence enhancement. Tryptophans 225 and 307 are therefore the most sensitive to heparin binding, contributing 76% of the observed fluorescence enhancement at 340 nm.

Fluorescence Lifetime Measurements—The fluorescence decay of the four variants in the absence and presence of heparin was measured using pulsed excitation at 295 nm, measuring emission at both 330 and 350 nm. For the control antithrombin and each of the single Trp → Phe variants, the best fit of the decay curves, both in the absence and presence of heparin, was obtained using three separate lifetimes, with short (0.19–0.27 ns), intermediate (1.47–1.73 ns), and long (5.13–6.90 ns) values. A clear result from this empirical analysis was that there was no simple one-to-one association of any of the three lifetimes with any single tryptophan residue.

Using the linearity condition determined from the steady-state spectral decomposition, the steady-state weighted subtraction of the time-resolved fluorescence decay curves of each variant from that of wild-type antithrombin was performed to resolve the shape of the decay curves of each individual tryptophan. These time-resolved subtraction spectra of individual tryptophans were best fitted by either one or two lifetimes (\( \tau_{\text{short}} \) and \( \tau_{\text{long}} \)). Whereas the time-resolved subtraction procedure is not robust enough to accurately determine small changes from the short lifetime component (200–300-ps term, 5% intensity contribution), the dominant medium and long lifetime component shapes are easily resolved using this technique. The fluorescence decay curve shapes of Trp-49 and Trp-189 in the presence and absence of heparin are absolutely overlapping (to within 5%). Clear lifetime differences upon heparin binding were observed only for Trp-225 and Trp-307 (Table III). This result is consistent with the steady-state spectral decomposition, reaffirming the observed independent behavior of the individual tryptophans in this protein.

**DISCUSSION**

By creating four single Trp → Phe variants, we have been able to deconvolute the steady-state tryptophan emission spectrum of human antithrombin into the contributions from each of the four tryptophans, both in the absence and presence of...
one of the solvent-accessible tryptophans underwent a blue shift upon heparin binding, consistent with what was found for Trp-49.

The present findings also allow a reasonable interpretation of the earlier $^{19}$F NMR study (10), which showed that two of four 6-fluorotryptophans gave changes in $^{19}$F chemical shift upon heparin binding. One was identified as Trp-49, consistent with our present finding that the environment of Trp-49 is perturbed upon heparin binding, even though it does not give a major fluorescence enhancement. This leaves Trp-225 and Trp-307 as the possible candidates for the second $^{19}$F NMR-sensitive tryptophan. From examination of the structure of antithrombin and the environment of each of the tryptophans, it seems that only Trp-307 could reasonably have the far upfield-shifted $^{19}$F chemical shift seen for the second heparin-sensitive tryptophan, being very close to three acidic residues that might give such a shift (Fig. 2), whereas Trp-225 is more likely to give rise to one of the downfield $^{19}$F resonances, which is consistent with a more buried environment (Fig. 2). It should be noted that these two different methods, tryptophan fluorescence and $^{19}$F NMR, report different pairs of tryptophans as being responsive to heparin binding. From fluorescence enhancement, one finds that Trp-225 and Trp-307 are most perturbed, whereas from $^{19}$F NMR, it appears that Trp-49 and Trp-307 are most perturbed. Based on knowledge of the heparin-binding site and the mechanism of heparin activation, neither of these pairs would be considered most likely to be responsive to heparin binding. This emphasizes that circumspection must be used in trying to interpret spectral changes in structural terms without a definitive assignment and highlights the usefulness of the present approach in making such assignments.

We can now better interpret heparin-induced fluorescence changes in antithrombin in structural terms. The large blue shift of Trp-49 upon heparin binding is understandable as arising from proximity to heparin reducing solvent access to the tryptophan ring and possibly burial by heparin. This might also account for the previously unexpected finding of a large shift of Trp-49 upon heparin binding, consistent with what was found for Trp-49.

Simple empirical exponential analysis of the fluorescence lifetimes of wild-type antithrombin and the three tryptophan-containing mutants revealed that there was no simple way to associate a particular decay component of a multi-exponential model with a particular tryptophan residue. However, since strict linearity was observed in the steady-state fluorescence emission, time-resolved subtractive “deconvolution” could be performed to yield a reasonably detailed description of the fluorescence decay shape of individual lifetimes for each tryptophan. Such analysis revealed that Trp-225 and Trp-307 are affected by heparin binding, whereas Trp-49 and Trp-189 are relatively invariant (Table III). This therefore provided independent support for the conclusions based on deconvolution of the steady-state spectra.

The successful deconvolution now allows us to identify which tryptophans were placed in the “solvent-exposed” and “buried” categories in an earlier fluorescence study (9). Trp-225 and Trp-307, the tryptophans that are responsible for the heparin-induced fluorescence enhancement, must be the buried tryptophans, whereas Trp-49 and Trp-189 must be the solvent-accessible class. This is also consistent with the earlier finding that one of the solvent-accessible tryptophans underwent a blue shift upon heparin binding, consistent with what was found for Trp-49.

### Table II

| Tryptophan | −Heparin | +Heparin | Contribution to enhancement |
|------------|----------|----------|-----------------------------|
|            | $\lambda_{\text{max}}$ | Total | $\lambda_{\text{max}}$ | Total | At 340 nm | Integrated area |
| Trp-49     | 356      | 8        | 341                     | 12   | 18        | 14             |
| Trp-189    | 345      | 10       | 345                     | 9    | 8         | 10             |
| Trp-225    | 330      | 19       | 335                     | 26   | 37        | 38             |
| Trp-307    | 337      | 63       | 340                     | 52   | 36        | 38             |

* Percentage total is at 340 nm.
* Integrated area is from 300 to 400 nm.

### Table III

| Tryptophan | $\tau$ | $\tau_{\text{short}}$ | $\tau_{\text{long}}$ | Long | Effect of heparin on $\tau$ |
|------------|--------|------------------------|-----------------------|------|-----------------------------|
|            | ns     | ns                     | ns                    |      |                             |
| Trp-49     | 2      | 0.8                    | 3.2                   | 50   | None                        |
| Trp-189    | 3.2    | 1.9                    | 6.4                   | 35–50| None                        |
| Trp-225    | 2.3    | 2.3                    | 0                     |      | Transition to biexponential decay of 1.3 and 4.7 ns, $\langle \tau \rangle = 2.62$ ns |
| Trp-307    | 330 nm | 2.6                    | 1.2                   | 6.6  | 25 Shift of wavelength-dependent decay to wavelength-independent 350 nm pattern |
| Trp-307    | 350 nm | 3.4                    | 1.2                   | 6.6  | 40                           |

* Mean lifetime, defined as $\langle \tau \rangle = \sum \sigma \tau \Sigma \alpha$, where $\alpha$ is the amplitude of the particular lifetime.

hemin. This deconvolution showed that Trp-307 contributes the most to the spectrum of native antithrombin and is one of two tryptophans that account for most of the enhancement upon heparin binding. The other major contributor to the enhancement is Trp-225, which is also the second largest contributor to the emission spectrum of native antithrombin and undergoes the largest percentage change in emission intensity upon heparin binding as well as a 5-nm red shift. The perturbation of Trp-49 is mostly a 15-nm blue shift, with only a small change in intensity, whereas Trp-189 is negligibly affected by heparin binding.

We can now better interpret heparin-induced fluorescence changes in antithrombin in structural terms. The large blue shift of Trp-49 upon heparin binding is understandable as arising from proximity to heparin reducing solvent access to the tryptophan ring and possibly burial by heparin. This might also account for the previously unexpected finding of a large shift of Trp-49 upon heparin binding, consistent with what was found for Trp-49.

The present findings also allow a reasonable interpretation of the earlier $^{19}$F NMR study (10), which showed that two of four 6-fluorotryptophans gave changes in $^{19}$F chemical shift upon heparin binding. One was identified as Trp-49, consistent with our present finding that the environment of Trp-49 is perturbed upon heparin binding, even though it does not give a major fluorescence enhancement. This leaves Trp-225 and Trp-307 as the possible candidates for the second $^{19}$F NMR-sensitive tryptophan. From examination of the structure of antithrombin and the environment of each of the tryptophans, it seems that only Trp-307 could reasonably have the far upfield-shifted $^{19}$F chemical shift seen for the second heparin-sensitive tryptophan, being very close to three acidic residues that might give such a shift (Fig. 2), whereas Trp-225 is more likely to give rise to one of the downfield $^{19}$F resonances, which is consistent with a more buried environment (Fig. 2). It should be noted that these two different methods, tryptophan fluorescence and $^{19}$F NMR, report different pairs of tryptophans as being responsive to heparin binding. From fluorescence enhancement, one finds that Trp-225 and Trp-307 are most perturbed, whereas from $^{19}$F NMR, it appears that Trp-49 and Trp-307 are most perturbed. Based on knowledge of the heparin-binding site and the mechanism of heparin activation, neither of these pairs would be considered most likely to be responsive to heparin binding. This emphasizes that circumspection must be used in trying to interpret spectral changes in structural terms without a definitive assignment and highlights the usefulness of the present approach in making such assignments.

We can now better interpret heparin-induced fluorescence changes in antithrombin in structural terms. The large blue shift of Trp-49 upon heparin binding is understandable as arising from proximity to heparin reducing solvent access to the tryptophan ring and possibly burial by heparin. This might also account for the previously unexpected finding of a large reduction in heparin affinity for a W49K antithrombin variant (10). The insensitivity of Trp-189 is explicable in terms of its position on the back side of helix F, in contact with the lower portion of $\beta$-sheet A (Fig. 1) and distant from both the heparin-binding site and the reactive center loop (Fig. 1). The large enhancement of Trp-225 fluorescence probably results from removal of Ser-380 (P14) from contact with the indole ring of Trp-225 as a result of displacement of P14 from $\beta$-sheet A (12, 13, 27) and may also be the origin of the change in fluorescence decay shape observed upon heparin binding. The direct involvement of Trp-225 in expulsion of P14 and the key role of this expulsion in heparin activation must also account for the large perturbation in basal rate of factor Xa inhibition for this variant, which probably reflects a shift in the equilibrium between the P14-inserted and P14-expelled conformations in favor of the latter activated conformation (27, 28). The somewhat surprising finding was the large enhancement in fluorescence of Trp-307, located on helix H. Although this tryptophan lies on an axis, orthogonal to the plane of $\beta$-sheet A, that passes through P14, the separation between P14 and tryptophan 307 is 22Å at the closest approach. It therefore appears that perturbation of Trp-307 is indirect. In a recent review of serpin conformations (40), it was pointed out that there are rearrangements of several of the helices of antithrombin upon heparin binding, including helix H. There thus appears to be a structural linkage, not only between the heparin-binding site and the reactive center loop, but also with other regions of antithrombin that are indirectly perturbed by the principal functional changes in $\beta$-sheet A. This structural linkage may explain why the mutation from Trp to Phe at position 307 has the
largest effect of the four Trp→Phe mutations on the basal rate of factor Xa inhibition and on the heparin-accelerated rate of factor Xa inhibition. Heparin binding therefore appears to affect large portions of the antithrombin molecule, including regions not directly implicated in heparin binding or change in conformation of the reactive center loop.

Finally, the modest change in tryptophan fluorescence of antithrombin upon formation of the reactive center loop-cleaved form (8% enhancement) (7) is also now understandable. Reactive center loop-cleaved antithrombin has the reactive center loop fully inserted into \( \beta \)-sheet A as an extra strand (41). This places Ser-380 in the same location as in native antithrombin, i.e., in contact with Trp-225, but different from the heparin-bound state, where it has been expelled from \( \beta \)-sheet A and is solvent-exposed. The fluorescence of Trp-225 in cleaved antithrombin should therefore be very similar to that in native antithrombin. Similarly, there should be no conformational perturbation of Trp-307 since Ser-380 is still inserted into \( \beta \)-sheet A.

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