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Antithrombin histidine variants ^{1}H NMR resonance assignments and functional properties

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Abstract Three variants of the 57.5 kDa human plasma proteinase inhibitor antithrombin, H1Q, H65C, and H120C, have been expressed in baby hamster kidney cells to permit assignment of the ^{1}H NMR resonances from the three histidines and evaluation of the role of these histidines in heparin binding. The NMR assignments have enabled more definitive interpretation of previous NMR-based studies of human antithrombin to be made. Although resonances of all three histidines are perturbed by heparin binding, only histidine 120 plays a significant role in the heparin binding site. The perturbations of resonances from histidines 1 and 65 indicate proximity to the heparin binding site and consequent sensitivity to the presence of heparin.

Key words: Antithrombin; Site-directed mutagenesis; NMR assignment; Histidine; Heparin binding site

1. Introduction

Antithrombin is a member of the serpin superfamily of proteins, which are high molecular weight inhibitors of serine proteinases that appear to undergo a major conformational change as a necessary part of the inhibition mechanism [1]. In addition, antithrombin is allosterically activated as an inhibitor of the blood coagulation proteinase factor X_a by the negatively charged polysaccharide heparin [2] through transmission of a conformational change to the reactive center P1 residue [3]. As a means of investigating the conformational changes that can occur in antithrombin, we have previously used ^{1}H NMR spectroscopy as a sensitive monitor of structural alterations [4–8]. Given the large size of antithrombin (57.5 kDa based on amino acid [9] and carbohydrate sequences [10]) we have been limited to one-dimensional NMR studies, but have made extensive use of NMR difference spectroscopy and examination of the imidazole resonances from the histidine residues, since these are extremely sensitive to environmental changes. Three of the five histidines in human antithrombin are significantly perturbed by heparin binding, with the pK_a of one histidine being increased by 0.32 pH units [4]. From consideration of the structure of native and cleaved antithrombins, we think that these are histidines 1, 65 and 120 [11–13]. However, interpretation of the one-dimensional NMR spectra in terms of specific structural alterations has been hampered by the lack of assignments for the histidine resonances. We describe here the preparation and characterization of histidine variants H1Q, H65C, and H120C, assignment of the NMR resonances from these three histidines, and interpretation of our earlier NMR data in light of these assignments.

2. Materials and methods

2.1. Site-directed mutagenesis and expression of recombinant human antithrombin

Mutagenesis was carried out on human antithrombin cDNA subcloned into mp19 M13 as described previously [3]. The antisense oligonucleotides used for the changes were 5′CAC AGG GCT CCC TTG ACA GGT CAC GCA3′, 5′T GGA ATC TGC CAG GCA CTG ATA GAA AGT GG3′, and 5′T GGC AAA GAA GCA GAT CTG ATC AGA TG3′ for the H1Q, H65C, and H120C mutations, respectively, in which the underlined triplet corresponds to the changed amino acid. Since it was considered possible that the alteration at position 120 would reduce the heparin affinity, thereby making protein isolation from heparin-Sepharose more difficult, antithrombin cDNA containing a second mutation, N135Q, was used as the starting template for preparation of the H120C variant. This results in the absence of carbohydrate from position 135 and a consequent increase in heparin affinity. However, the effects of this mutation have been well characterized in wild-type antithrombin, which thus permits identification of the specific contribution of the H→C change at position 120 to be made [14,15]. For the H65C and H1Q mutations, wild-type antithrombin cDNA was used as the template. Following mutagenesis, the presence of the mutation was confirmed by sequencing. The antithrombin cDNA was excised from M13 by digestion with *Sal*I and *Xba*I and ligated into the expression vector pMAStop digested with the same restriction endonucleases. Stably transfected BHK cells were prepared as previously described, and selected for resistance to methotrexate and neomycin [16].

2.2. Isolation of antithrombins

Recombinant antithrombin was isolated from serum-free growth medium of confluent BHK cells, and plasma antithrombin was isolated from pooled outdated human plasma, by heparin-Sepharose affinity chromatography, and, where necessary, by chromatography on G150 and DEAE, as described [14]. Protein concentrations were determined spectrophotometrically using $E^{0.1\%}_{280\,nm} = 0.65$ [17] and a molecular weight of 57.5 kDa. Different glycoforms of antithrombin are secreted by transfected BHK cells that differ in the type and extent of glycosylation and in affinity for heparin. The isoform designation of a particular fraction could be made from the mobility on SDS-PAGE and the lectin staining pattern [14].

2.3. Heparin-antithrombin dissociation constants

Heparin binding to antithrombin was monitored by the change in endogenous protein tryptophan fluorescence, and the resulting data fitted by non-linear least squares analysis using the program MINSQ II (Micromath Scientific Software, Salt Lake City, UT) to obtain the dissociation constant. Fluorescence measurements were made on a SPEX spectrofluorimeter, exciting at 295 nm and observing tryptophan emission at 335 nm. The buffer used was 20 mM sodium phosphate at pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000 and 0.1 M NaCl.

2.4. Factor X_a assay

Rates of inhibition of factor X_a by antithrombin species were meas-

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ured by discontinuous spectrophotometric assay of residual proteinase activity under pseudo-first-order conditions using spectrozyme-X_a chromogenic substrate. After incubation of antithrombin and factor X_a for a given time the reaction was terminated by dilution into 100 μM spectrozyme-X_a and residual factor X_a activity determined by monotoring the hydrolysis of substrate at 405 nm. Concentrations of factor X_a and antithrombin of 20 nM and 1 μM, respectively, were used for all measurements. All measurements were carried out at 25°C in 20 mM sodium phosphate at pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000 and 0.1 M NaCl.

2.5. NMR measurements

^{1}H NMR spectra were recorded at 500 MHz on a Bruker AMX500 spectrometer. A sweep width of 7500 Hz, pulse angle of 70°, and a selective low power solvent presaturation pulse of 0.5 s were employed. Each spectrum represents the average of 3000 scans. Samples were 0.1–0.15 mM in 20 mM sodium phosphate buffer at pH 7.0 in D_2O containing 150 mM NaCl, and were transferred to the D_2O buffer by 5 cycles of concentration and dilution in an Amicon ultrafiltration cell fitted with a PM30 membrane. pH 7.0 was chosen as a compromise between physiological pH for optimal stability and a very low pH to give the greatest separation of the histidine resonances.

2.6. Materials

Full-length high affinity heparin, M_r 9,000, was a generous gift from Dr. Steven Olson; human factor X_a, a generous gift from Dr. Paul Bock, Vanderbilt University, was prepared by affinity chromatography on soybean trypsin inhibitor-Sepharose [18]; plasmids pMAStop, pRMH140, and pSV2dhfr [16] were generous gifts from Dr. Gerd Zettlmeißl, Behringwerke, Marburg; 99.9 atom% D_2O was from Sigma (St. Louis, MO); Spectrozyme-X_a was from American Diagnostica (Greenwich, CT).

3. Results

3.1. Assignment of histidine C(2) ^{1}H resonances

The ^{1}H NMR spectrum of plasma antithrombin shows well-resolved signals from three of the five histidine residues in the protein (Fig. 1), each of which is sensitive to heparin binding in both native and cleaved states, as shown by previous studies [4–6]. The remaining two histidines give much broader resonances close to the central resonance [4]. Of the histidines in human antithrombin (at positions 1, 65, 120, 319, and 369), those at positions 319 and 369 are unlikely to give rise to the sharp heparin-sensitive resonances since these histidines are well removed from the heparin binding site, and heparin binding to cleaved antithrombin, which does not cause a long-range change in conformation [19], still perturbs the three narrow resonances. In contrast, the X-ray structure of native antithrombin [13] shows that histidines 1, 65 and 120 are close to the proposed location of the heparin binding site and are therefore likely to be sensitive to heparin binding in both native and cleaved states.

Positive assignments of the C(2) ^{1}H resonances from histidines 1 and 65 were made from their NMR spectra (Fig. 1 and Table 1), which showed that histidine 1 gives rise to the sharpest, central, resonance at 7.89 ppm, whereas histidine 65 gives rise to the broader, more downfield resonance at 8.15 ppm. Unfortunately a positive assignment of the histidine 120 resonance could not be obtained since H120C antithrombin proved to be unstable at the relatively high concentrations needed for NMR spectroscopy. This variant had a strong tendency to aggregate and denature, as judged by precipitation of the sample upon concentration in an Amicon ultrafiltration cell during transfer to D_2O buffer, as well as by the NMR spectrum of the trace amount of antithrombin that remained in solution, which had the appearance of a denatured form. This may be due to sulfide–disulfide exchange between the newly incorporated cysteine at position 120 and the nearby disulfide between cysteine 8 and cysteine 128, or to a critical stabilizing role played by histidine 120. By a process of elimination the resonance at 7.73 ppm was assigned to histidine 120. This assignment is entirely consistent with the properties of this histidine (see below).

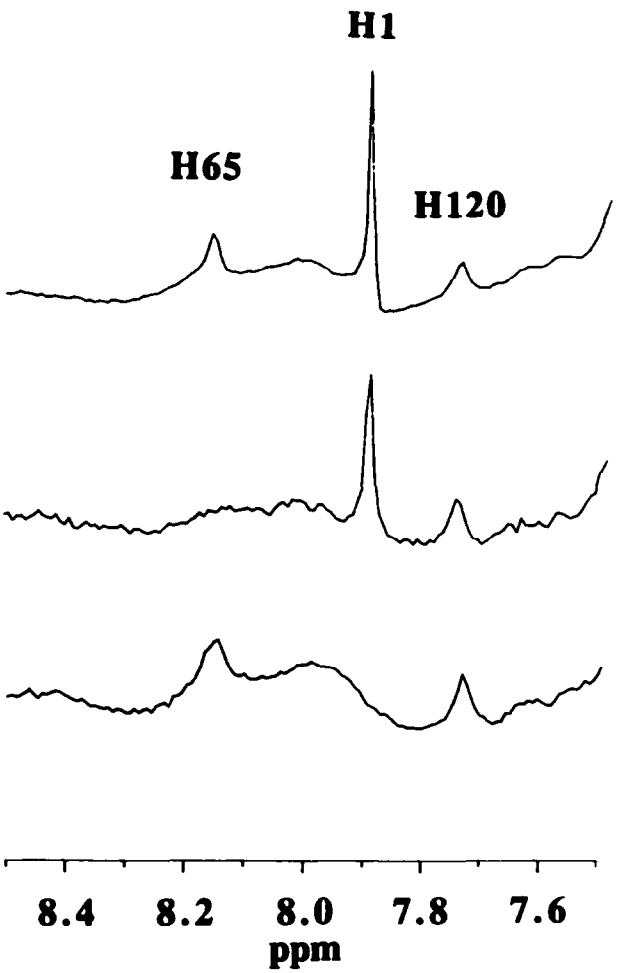

Fig. 1. C(2) ^{1}H resonances from histidines 1, 65 and 120 in the 500 MHz NMR spectrum of plasma and recombinant variant human antithrombins. (Top) Plasma antithrombin, with assignments indicated for the three histidines; (middle) H65C variant antithrombin, which lacks the resonance at 8.14 ppm from histidine 65; (bottom) H1Q variant antithrombin, which lacks the resonance at 7.89 ppm from histidine 1.

A similar instability has recently been reported for another heparin-binding site variant antithrombin, R129Q [20], which was predominantly in the conformationally altered locked conformation [21,22] analogous to the latent form of plasminogen activator inhibitor 1 [23]. Since the transition to the locked form involves insertion of the reactive center loop into β-sheet A of antithrombin, this again suggests a structural linkage between the reactive center loop region and the heparin binding site of antithrombin, such as has been shown by other means [3,24].

3.2. Antithrombin-heparin dissociation constants

Heparin–antithrombin dissociation constants were determined for the form II glycoforms of H1Q and H65C variant antithrombins, which most closely resembles the dominant

Table 1
NMR characterization of histidines 1, 65 and 120

Histidine	$\Delta\nu_{1/2}$ (Hz)[a]	σ (ppm)[a]	$\Delta\sigma$ (HAH)[b]	$\Delta\sigma$(psacc)[b]	$\Delta\sigma$(cleaved)[c]	pK_a^d	ΔpK_a^d	Effect of guanidine[e]
1	3	7.89	+ 0.042	+ 0.031	+ 0.041	5.93	+ 0.07	Smooth change
65	13	8.15	− 0.017	− 0.087	− 0.087	6.90	0	Opposite sign changes at low and high concns.
120	15	7.73	+ 0.091	+ 0.018	+ 0.028	5.13	+ 0.32	Discontinuous change

[a] Half height linewidth ($\Delta\nu_{1/2}$) and chemical shift (σ) are from spectrum shown in Fig. 1, with a sample pH of 7.0.
[b] Change in chemical shift ($\Delta\sigma$) upon binding full-length heparin (HAH) or pentasaccharide at pH 6.5, taken from [7].
[c] Change in chemical shift ($\Delta\sigma$) at pH 6.35 upon cleavage of native antithrombin in the reactive center loop by thrombin, taken from [6].
[d] pK_a, and change in pK_a of histidine upon binding full-length, unfractionated, heparin, taken from [4].
[e] Behavior of histidine C2 resonance upon titration with guanidine hydrochloride at low concentrations. The abrupt change reported for histidine 120 occurs at a guanidine concentration of 0.6 M, with smooth changes below and above the transition. Taken from [5].

α plasma form in glycosylation pattern, and for the highest affinity isoform of H120C, which most closely resembles the minor plasma β form and wild-type recombinant isoform III. The heparin affinities of the H1Q and H65C variants were very similar to that of wild-type antithrombin, indicating that neither histidine is directly involved in heparin binding (Table 2). In contrast, the affinity of the H120C variant was significantly reduced compared to wild-type isoform III, indicating that the histidine plays a role in heparin binding (Table 2). The weakening in affinity corresponds to a $\Delta\Delta G$ of about 2.4 kcal·mol^{-1}. It should be noted that, at the low concentrations of H120C antithrombin used for these binding experiments, the protein behaves as a monomer, as judged by its elution position from a Sepharose G150 sizing column used in purification.

3.3. Rate of inhibition of factor X_a

Rates of inhibition of factor X_a by each of the three variant antithrombins were determined and were found to be very similar to those of wild-type and of plasma antithrombins (Table 2), indicating no structural defect in the reactive center region as a result of any of the amino acid substitutions.

4. Discussion

By making histidine substitution variants of human antithrombin it has been possible to make definitive assignments of the resonances from two of the three histidines the NMR signals of which are strongly perturbed by heparin binding, and a probable assignment of the third, and also to determine the importance of these residues in heparin binding and proteinase inhibition. Having made these assignments it was then possible to make a residue-specific attribution of previously observed properties of these histidine residues. This includes the pK_a of each histidine in the absence and presence of heparin, the chemical shift perturbation of each histidine resonance upon binding different heparin species and upon cleavage of the reactive center peptide bond, and the behavior of these resonances upon addition of low concentrations of guanidine hydrochloride (Table 1). The properties and roles of each of the three histidines is discussed in turn.

Table 2
Kinetic and thermodynamic properties of antithrombin species

Antithrombin	K_D (nM)[a]	ΔG (kcal·mol^{-1})	k_{app} (mM^{-1}·s^{-1})[b]
H1Q	9 ± 4	11.04	2.3
H65C	5.5 ± 1	11.34	4.2
Wild-type form II	4.5 ± 1	11.45	2.6
H120C	130 ± 20	9.45	2.5
Wild-type form III	2.3[c]	11.85	3.6

[a] Dissociation constant for antithrombin–high affinity heparin complex determined from the enhancement of endogenous tryptophan fluorescence of antithrombin upon binding heparin.
[b] Apparent second order rate constant for inhibition of factor X_a by antithrombin, determined by discontinuous assay of residual factor X_a activity.
[c] Determined by extrapolation from values determined at I 0.3, 0.4 and 0.5 [14].

4.1. Histidine 1

Replacement of histidine 1 by the approximately isosteric glutamine resulted in no alteration in the affinity of antithrombin for heparin or of antithrombin's ability to inhibit proteinase. The residue is thus not involved in either heparin binding or proteinase inhibition. The very small change in pK_a upon binding heparin (Table 1) is consistent with lack of involvement in heparin binding. However, this histidine senses binding of full-length and pentasaccharide high affinity heparin, and of lower affinity heparin species [4,7,8], as well as the conformational change that occurs upon cleavage of the reactive center loop and insertion of the reactive center into β-sheet A, as judged by the change in chemical shift of the imidazole NMR resonances (Table 1). In cleaved antithrombin, the perturbation of the histidine 1 chemical shift upon binding heparin is about the same as in native antithrombin [6]. These properties suggest that histidine 1 is close to the principal region of the heparin binding site but is not in contact with the heparin chain.

Other things being equal, an N-terminal histidine should have a lower pK_a than that of an internal histidine residue. Based upon this expectation, we had tentatively identified the histidine with the pK_a of 5.13 as histidine 1 [4]. We have now shown that this is incorrect and that histidine 1 has a pK_a of 5.93, which, though normal for an internal histidine, is abnormally high for an N-terminal histidine. This suggests that the imidazole side chain of histidine 1 or the free α-amino group is involved in an ionic interaction to account for the abnormally high pK_a. Since the linewidth of the C(2) NMR resonance of this histidine is extremely narrow (Table 1), indicating relatively unhindered rotation of the imidazole side chain, it is more likely that the terminal amino group is involved in an ionic interaction. It appears that this interaction is unaffected upon binding heparin or upon reactive center loop cleavage, as judged by the minimal changes in pK_a and linewidth.

The C2 resonance shows a smooth change in chemical shift upon addition of guanidine, resulting simply from ionic strength effects (Table 1). No discontinuity is seen, such as

occurs with histidine 120, suggesting that the guanidine-induced conformational change does not affect this histidine.

4.2. Histidine 65

As with histidine 1, this histidine appears not to be involved in heparin binding or proteinase inhibition, as judged by dissociation and inhibition rate constants and by the lack of pK_a change upon binding heparin, but again has ^1H NMR resonances that are sensitive to heparin binding and to cleavage in the reactive center loop (Table 1). The location of this histidine on the A helix, the end of which abuts the proposed heparin binding site [13], is consistent with this sensitivity to heparin binding without direct involvement in heparin binding. The sensitivity to cleavage in the reactive center loop suggests that the subsequent conformational change significantly affects the A helix, which is located a considerable distance from the reactive center loop. Guanidine has opposite effects on the C2 resonance above and below 0.4 M (Table 1), suggesting sensitivity to a guanidine-induced conformational change.

4.3. Histidine 120

Based on the reduction in affinity of antithrombin for heparin upon replacement of histidine 120 by cysteine, which corresponds to a $\Delta\Delta G$ of about 2.4 kcal·mol^{-1}, it appears that this histidine plays an important role either directly in binding to heparin or indirectly in maintaining the correct conformation of the heparin binding site. Since the pK_a of this residue is very low in native antithrombin and is only raised by 0.32 pH units upon binding heparin, resulting in the imidazole side chain being unionized at physiological pH even in the heparin–antithrombin complex, it is unlikely that a charged interaction between histidine 120 and heparin occurs. In addition it has been shown that, although the chemical shift of this residue is markedly affected by full-length heparin, it is only slightly affected by high affinity pentasaccharide, suggesting that it is located at the end of the pentasaccharide binding site [7]. This implies a lack of direct involvement in binding the critical pentasaccharide heparin sequence. Since the difference in affinity of heparin pentasaccharide and full-length heparin corresponds to only a single additional ionic interaction [2], which is not likely to be supplied by histidine 120 for the reason given, the chemical shift and pK_a perturbation of histidine 120 by full-length heparin is likely to be an indirect effect. This suggests that the role of histidine 120 in determining heparin affinity is to maintain a particular conformation for the heparin binding site. This is consistent with the effect that low concentrations of guanidine have on the histidine 120 resonance in native but not in cleaved antithrombin [5]. Thus in native antithrombin the structural transition that occurs upon addition of guanidine to 0.6 M, which results in a reduction in heparin affinity and which is thought to involve partial insertion of the reactive center loop into β-sheet A, causes an abrupt change in the chemical shift of the histidine 120 resonance, indicating a linked change in the heparin binding site. In contrast, no such abrupt chemical shift change is seen upon adding guanidine to cleaved antithrombin. Since cleaved antithrombin already has the complete reactive center loop inserted into β-sheet A, no further structural transition involving the reactive center loop is possible and consequently there can be no conformational change transmitted to the heparin binding region.

To interpret the increase in pK_a of histidine 120 upon binding heparin, it is first necessary to understand why the pK_a of this histidine is depressed from that of a normal histidine. In the X-ray structure of cleaved bovine antithrombin, histidine 120 appears to be on the outer face of the D helix, in close proximity to the positive charges of arginines 46 and 47 and lysine 125 (human numbering) [11]. These residues are all ones proposed to be involved in direct electrostatic interactions with heparin pentasaccharide. Thus the low pK_a of histidine 120 probably arises from the cluster of nearby positive charges, and the increase in pK_a upon heparin binding arises from the partial neutralization of these charges by interaction with the negatively charged heparin.

4.4. Conclusion

NMR studies on H1Q and H65C variant antithrombins have provided definitive assignments for two of the three heparin-perturbable histidines in antithrombin. The assignment of the histidine 120 resonance is more tentative, but consistent with the available data. Using the histidine resonance assignments, older NMR data have been interpreted more definitively. We concluded that the binding site of the high affinity heparin pentasaccharide ends close to histidine 120, and that this histidine is involved in maintaining the correct conformation of the heparin binding site but is not directly involved in binding heparin. Histidines 1 and 65 are close to, but not in, the heparin binding site and report on the binding of the highly charged heparin molecule. Both histidines 65 and 120 are sensitive to the guanidine-induced conformational change that is thought to involve the reactive center loop region. The instability of the H120C variant and the proposal that histidine 120 plays a role in maintaining the conformation of the heparin binding site are consistent with one another and reminiscent of a recent report on an arginine 129 variant of antithrombin which also showed instability [20].

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