Heterogeneity of Recombinant Human Antithrombin III Expressed in Baby Hamster Kidney Cells

EFFECT OF GLYCOSYLATION DIFFERENCES ON HEPARIN BINDING AND STRUCTURE

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To determine the effects of differences in glycosylation on the structure and functional properties of recombinant human antithrombin (rHAT), we have characterized the properties of the recombinant protein overexpressed by baby hamster kidney cells. Three forms of rHAT, I–III, were isolated which differed in affinity for heparin. Form I had the lowest affinity and contained a high proportion of highly branched complex carbohydrate. Form II had higher affinity and contained both complex and high mannose-type chains. Form III had the highest affinity and was similar to form II in the type of carbohydrate present, but had a lower level of glycosylation, consistent with the absence of carbohydrate at one of the four glycosylation sites. 1H NMR spectra of plasma HAT and rHAT forms I–III suggested very similar protein structures for all forms. Heparin pentasaccharide produced almost identical NMR perturbation difference spectra. The only functional difference found was in the rates of inactivation of factor Xa. Forms II and III gave second order rate constants similar to that of plasma HAT, whereas form I gave a biphasic inhibition, with the first phase having a rate about four times that of the other forms. We conclude that carbohydrate heterogeneity does not alter the structure of the HAT polypeptide or the heparin-induced conformational change, but does affect the heparin affinity and can alter the rate of proteinase inhibition.

Human antithrombin (HAT) is a glycosylated 57,400-dalton plasma proteinase inhibitor that plays a critical role in regulation of serine proteinases involved in coagulation (1). It is a serpin (serine proteinase inhibitor) (2) and so is related to other serpins such as heparin cofactor II and α1-proteinase inhibitor. Both to address questions of mechanism, as well as to open up possible therapeutic applications, the ability to express rHAT in mammalian cells as a glycosylated protein represents a significant advance (3, 4). A major concern is, however, possible differences in glycosylation between the plasma and recombinant forms of the protein, and the effects that these might have on HAT function. Although such heterogeneity of glycosylation is not unusual in recombinant proteins, it is particularly important to be aware of this heterogeneity in HAT and of the effect it might have on heparin affinity, since heparin plays a crucial role as an activator of HAT. Indeed, two glycosylation isoforms of the protein have already been characterized in plasma that differ significantly in their affinity for heparin. These forms differ in the number of carbohydrate chains carried, with the higher affinity form having one less carbohydrate chain than the lower affinity form (5, 6). A recent study on rHAT has also demonstrated the presence of two forms of HAT with apparently different degrees of glycosylation and different affinities for heparin (7). However, even though the rHAT was derived from the same expression system as used here, no compositional difference was found for the two forms of the protein.

To be able to make valid comparisons between wild-type rHAT and an engineered variant, it is very important to understand the effects of variation in carbohydrate on the properties of the protein, so that alterations in heparin affinity are correctly ascribed. In addition, the structural basis for such differences in heparin affinity, i.e. whether it involves an altered mode of heparin binding or is a consequence only of steric and/or electrostatic interactions, needs to be determined if strategies to remove the heterogeneity through manipulation of the carbohydrate are to be justified. Finally, it is necessary to determine if there are differences in the pattern of glycosylation as a function of differences in cell growth conditions.

We have addressed the question of the effects of carbohydrate heterogeneity on the properties of rHAT by characterizing three distinct forms of the protein by affinity for heparin, inhibition of factor Xa, variation in type and degree of glycosylation, and by 1H NMR spectroscopy of native and heparin-complexed species. These isoforms were overexpressed in BHK cells (3) but differ in properties from two other BHK cell-secreted forms reported by others (7). We found differences in the type of carbohydrate chains attached to the protein and large variation in heparin affinity. Two of the three forms had second order rate constants for inhibition of factor Xa very similar to that of plasma HAT, whereas the
third showed a higher rate of inhibition and more complex kinetics. There was little or no alteration either in the structure of the protein or the mode of binding and activation by heparin. The carbohydrate appeared to have an effect on heparin binding primarily through steric interactions, which may also account for the altered kinetics of factor Xa inhibition by form I HAT.

EXPERIMENTAL PROCEDURES

Isolation of α and β Forms of Plasma Antithrombin—Plasma HAT's were isolated from pooled outdated human plasma obtained from the Vanderbilt Blood Bank. Plasma was treated with dextran sulfate and the supernatant applied to a heparin-Sepharose affinity column according to the directions of McKay (8), except that a wash with 0.7 M NaCl was used before elution of antithrombin with a 0.7→1.5 M NaCl gradient. The α form eluted at about 1.0 M NaCl, whereas the β form was obtained in a step elution from 1.5 M to 3.0 M NaCl. The pooled fractions were purified to homogeneity by DE52 ion exchange chromatography.

Production and Isolation of Recombinant Human Antithrombin—BHK cells (BHK-21, ATCC CCL10) were co-transfected with the plasmids pSV2hrf9 (9), containing the gene for dihydrodoxy reductase; PRM140 (10), containing the gene for Escherichia coli aminoglycoside 3'-phosphotransferase; and pMAAT (11), containing the gene for hAGS, a previously developed heparin binding peptide. Cells were cultured in 10-liter 2-liter roller bottles at 37 °C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, and containing 10 μm methotrexate and 400 μg ml⁻¹ of neomycin (G418) to maintain selection for transfected cells. Confluent cells were cycled every 24 h between the same medium and medium without fetal calf serum or drugs. Levels of HAT secretion were monitored by radial immunodiffusion and showed a consistent large increase from the first to third cycles, followed by an approximately constant secretion level of about 15 μg ml⁻¹. Since the level of HAT in the first cycle growth medium was very low, this medium was not saved. The growth medium from each of the remaining serum-free cycles was collected, concentrated by a 50% reduction of the protein. Data were fitted using nonlinear least squares analysis employing the program MINSQ.

Preparation of Samples for 1H NMR Spectroscopy—HAT samples for NMR analysis were prepared by five cycles of dialysis with D2O and lyophilization from 40% ethanol. Cells were harvested, washed with 1 liter of buffer containing 1.5 M NaCl and form I1 at 1.2 M NaCl, 100 mM NaCl, 100 μM EDTA, 10 μM β-mercaptoethanol, 0.4% Triton X-100 (w/v), and 0.2% SDS (w/v). Samples were heated to 90 °C for 2 min and then cooled to 37 °C prior to addition of 0.2 unit of N-glycosidase F for each 30 μg of HAT and reaction for 15 h at 37 °C. HAT samples were reacted with endoglycosidase H for 19 h at 37 °C under non-denaturing conditions in 20 mM sodium phosphate buffer at pH 5.6 containing 0.2 M NaCl, 100 μM EDTA, and 0.02% sodium azide. HAT samples were reacted with Vibrio cholerae neuraminidase under non-denaturing conditions for 20 h at 37 °C in 50 mM MES buffer, pH 6.5, containing 1 mM CaCl₂, 0.2% (w/v) polyethylene glycol, and 0.02% sodium azide.

Fluorescence Measurements—Fluorescence measurements were made on an SLM 8000 spectrofluorimeter, exciting at 280 nm and observing emission from tryptophan residues at 340 nm. A band pass of 4 nm was used for both excitation and emission. Samples were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 100 μM EDTA, and 0.1% polyethylene glycol. Dissociation constants for HAT-heparin complexes were determined by titration of heparin into HAT at protein concentrations between 20 nm and 1 μM, using the change in endogenous tryptophan fluorescence that accompanies the binding. For measurements at low concentrations of HAT, acrylic cuvettes were used to minimize surface binding of the protein. Data were fitted using nonlinear least squares analysis employing the program MINSQ.

RESULTS

Isolation of Isoforms of Recombinant Human Antithrombin—Three distinct peaks of immunoreactive HAT were isolated from the BHK cell growth medium, which differed significantly in their affinities for heparin-Sepharose™. Two forms were eluted by a linear salt gradient from 0.4 to 1.5 M NaCl: form I at 0.7 M NaCl and form II at 1.2 M NaCl. The highest affinity form, form II, was eluted as a sharp peak in the step wash with buffer containing 3.0 M NaCl. Using the same heparin-Sepharose™, α plasma HAT elutes at 1.0 M NaCl, and thus appears to have affinity for heparin-Sepharose™ between those of recombinant forms I and II. The relative amounts of forms I, II, and III of HAT were approximately 2:3:2, respectively. These three forms have been found in every isolation of wild-type rHAT we have carried out (10 to date). In addition, from isolations performed on as few as two cycles and as many as eight cycles of harvested medium, we have found that the three forms were present at early as well as late stages of secretion. Rechro-
matography of a mixture of the three forms on heparin-SepharoseTM, eluting with a linear salt gradient from 0.15 to 2.5 M NaCl, gave three well defined peaks (Fig. 1) with maxima corresponding to salt concentrations of 0.68 M (form I), 1.26 M (form II), and 2.08 M (form III). In contrast, the two BHK-derived rHAT species reported by Björk et al. (7) eluted at different salt concentrations (0.6 and 0.9 M).

**Dissociation Constants for Heparin-Antithrombin Complexes**—To quantitate the differences in affinity of the three forms of rHAT for heparin, and also to examine the effect of glycosylation differences on binding of full-length heparin versus heparin pentasaccharide, dissociation constants were determined for heparin-HAT complexes using the enhancement of endogenous tryptophan fluorescence that accompanies heparin binding. Data are presented in Table I at physiological ionic strength (I 0.15) and at I 0.3. Dissociation constants at both ionic strengths and for both heparin species are in the order form I > α plasma > form II > form III, which is as expected from the heparin-SepharoseTM elution positions. About a 20-fold increase in dissociation constant was found for both full length and pentasaccharide heparin, at I 0.15, between form III and form I, representing a ΔΔG of 1.8 kcal mol⁻¹. There are again differences between these three forms and the two BHK-derived forms examined by Björk et al. (7), for which dissociation constants for the HAT-HAH complex of 175 nM and 22 nM were reported. These values are, however, consistent with the reported salt concentrations for elution from heparin-SepharoseTM, which indicate the former would be eluted before our form I, and the latter after form I but before α plasma HAT and form II.

For all three of the present forms at each ionic strength, the relative affinities of full length heparin and heparin pentasaccharide maintain the approximately 5-fold difference in Kd observed for α plasma HAT. However, for a given heparin species and a given ionic strength, the magnitude of the relative affinities of form I:plasma:form II:form III do not bear a linear relationship to the salt concentration at which they elute from heparin-SepharoseTM. Thus the relative affinities of full length heparin for form I, plasma, form II, and form III HATs at I 0.15 are 1:4:9:17, whereas the relative salt concentrations for elution of the four proteins are 1:1.5:1.9:3.1.

**Rate of Inhibition of Factor Xa**—The second order rate constants for inhibition of factor Xa were determined for rHAT forms I–III under pseudo first-order conditions by assay of residual factor Xa activity (20). The values are given in Table II together with values for α plasma HAT determined here and reported previously. Good agreement was found between the published value for plasma HAT and the value determined here. Forms II and III also gave values that were similar to that of α plasma HAT. In contrast, form I gave a biphase semi-log plot of residual factor Xa activity. Fitting these data to a bi-exponential inhibition gave two rate constants; one four times faster than, and one about the same as, the rate constants for forms II and III. The addition of polybrene did not alter the biphase nature of form I inhibition or reduce the rates of inhibition.

**Carbohydrate Composition of Antithrombin Species**—To investigate the source of the variation in affinity of the different recombinant forms of HAT, the nature and extent of glycosylation of the protein were examined. Fig. 2 shows an SDS gel of α and β plasma HAT and rHAT forms I–III. Each of the three recombinant species showed a spread of mobilities on the gel, indicative of microheterogeneity. In contrast, both α and β plasma HATs gave relatively sharp bands (Fig. 2, lanes 1, 2, and 6). There was also a difference in median mobility for the three rHATs in the order I > II > III, with form I migrating similarly to α plasma HAT, form III migrating similarly to β plasma HAT, and form II having intermediate mobility. Comparison of these bands with those shown by Björk et al. (7) for their two fractions revealed differences, with their fraction I appearing to be less mobile than the

**TABLE I**

<table>
<thead>
<tr>
<th>Antithrombin</th>
<th>0.15 I</th>
<th>0.3 I</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAH Pentasaccharide</td>
<td>HAH Pentasaccharide</td>
<td></td>
</tr>
<tr>
<td>Form I</td>
<td>40 ± 20</td>
<td>220 ± 20</td>
</tr>
<tr>
<td>α Plasma</td>
<td>10 ± 1</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>Form II</td>
<td>4.5 ± 1</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>Form III</td>
<td>2.3b</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

* ND, not determined due to weakness of binding.

* Determined by extrapolation from values determined at 0.5, 0.4, and 0.3 I.

**FIG. 2**

SDS-polyacrylamide gel of HAT species run under reducing conditions. Lanes 1 and 6, plasma β HAT; lane 2, plasma α HAT; lane 3, form I rHAT; lane 4, form II rHAT; lane 5, form III rHAT; lane 7, 66- and 43-kDa molecular mass standards.
present form I and their fraction II having a much greater band width than any of the three forms examined here. Following reaction with N-glycosidase F, which is capable of removing all types of N-linked carbohydrate, all four forms of HAT (α plasma and forms I–III) migrated identically, with a higher mobility consistent with the molecular weight of 49,000 expected for the deglycosylated protein (Fig. 3, lanes 6–9).

To identify the type of carbohydrate chains present in each of the forms of HAT, SDS-polyacrylamide gels were run, and the protein bands transferred to nitrocellulose for binding of glycan-specific lectins as described under “Experimental Procedures.” The results are shown in Fig. 4 for binding of five separate lectins with specificities for terminal 2→6-linked sialic acid (Fig. 4A), terminal 2→3-linked sialic acid (Fig. 4B), terminal mannose (Fig. 4C), terminal galactose-β(1→4)-N-acetylgalactosamine (Fig. 4D) (present in N-linked complex and hybrid glycans), and terminal galactose-β(1→3)-N-acetylgalactosamine (present in O-linked glycans) (Fig. 4E). Plasma HAT gave an intense band in Fig. 4A, indicating the presence of terminal 2→6-linked sialic acid, but gave no significant intensity for any of the other four lectins. Although the positive band in Fig. 4A was expected, based on the reported carbohydrate composition (17), the absence of terminal galactose-β(1→4)-N-acetylgalactosamine was surprising, given the reported presence of 15% of branches lacking sialic acid and thus terminated by galactose-β(1→4)-N-acetylgalactosamine. This is, however, in accord with a report by Franzén et al. (21) who found only sialic acid-terminated carbohydrate chains in antithrombin. Form I rHAT contained significant amounts of terminal galactose-β(1→4)-N-acetylgalactosamine (Fig. 4D) and of terminal 2→3-linked sialic acid (Fig. 4B), but only a trace of high mannose glycans (Fig. 4C) which was indistinguishable from background, and no terminal 2→6-linked sialic acid (Fig. 4A) or O-linked carbohydrate (Fig. 4E). Form II rHAT contained less terminal 2→3-linked sialic acid and terminal galactose-β(1→4)-N-acetylgalactosamine than for form I, whereas there was a greater abundance of high mannose-type carbohydrate (Fig. 4C). Form III rHAT also differed from forms I and II in the relative amounts of the three types of carbohydrate, with a lesser amount of terminal 2→3-linked sialic acid (Fig. 4B) and an amount of high mannose comparable to that of form II and greater than that of form I, and only a trace of terminal galactose-β(1→4)-N-acetylgalactosamine (Fig. 4D). The composition reported by Björk et al. (7) for their two forms of HAT was 100% complex carbohydrate, with 50% bi-, 37% tri-, and 12% tetra-antennary chains.

The positions of the bands in Fig. 4 indicate that the distribution of different types of carbohydrate in a given form of rHAT may not be uniform. Thus, the bands for terminal galactose-β(1→4)-N-acetylgalactosamine (Fig. 4D, lane 3) and high mannose in form II (Fig. 4C, lane 3) occur toward the bottom of the total protein band, whereas the band for terminal 2→3-linked sialic acid (Fig. 4B, lane 3) occurs toward the top of the protein band. To investigate this further, separate digests of the four forms of HAT were carried out with endoglycosidase H and neuraminidase, and the samples run on an SDS gel. If the distribution of different carbohydrate chains is uniform, the endoglycosidases should have little effect on the diffuseness of the Coomassie-stained protein band, whereas if the distribution is nonuniform, removal of one type of carbohydrate would be expected to affect only part of the protein band. Duplicate SDS gels were run for each experiment, with the second being transferred to nitrocellulose and probed with the appropriate lectin to check for removal of each type of carbohydrate chain in each case. Complete removal of high mannose and hybrid chains by endoglycosidase H and of sialic acid residues by neuraminidase occurred, as evidenced by staining of the unreacted sample, but absence of stain for the digested material (data not shown). The SDS gel of the neuraminidase digest is shown in Fig. 5a. The compact band for plasma HAT (lane 1) remained compact but increased its mobility after digestion (lane 5) with neuraminidase. Each of the three rHATs not only increased in mobility upon neuraminidase treatment, but also gave a less diffuse band, suggesting an initial nonuniform distribution of sialic acid within the protein band.

![Fig. 4. Detection of different types of carbohydrate moiety in HAT species by means of specific lectin binding.](image-url)

The procedure are given under “Experimental Procedures.” Lanes 1–4 represent plasma, form I, form II, and form III HATs, respectively. Lane 5 in panel E is asialofetuin run as a positive control. A, detection of (2→6)-linked sialic acid; B, detection of (2→3)-linked sialic acid; C, detection of terminal mannose; D, detection of terminal galactose-β(1→4)-N-acetylgalactosamine; E, detection of terminal galactose-β(1→3)-N-acetylgalactosamine, the arrow indicates the position of the HAT species.

![Fig. 3. Polyacrylamide gel of HAT species before and after digestion with N-glycosidase F run under denaturing conditions.](image-url)
shows the effect of endoglycosidase H treatment on the four HAT species. No effect was seen for plasma and form I HATs, which contain, at most, traces of high mannose or hybrid carbohydrate. Form II HAT became more diffuse as a result of endoglycosidase H treatment, while form III split into two distinct bands, one of which was more mobile than in the untreated protein and one of which appeared to be unaffected by the digestion.

The above results of the effect of endoglycosidase H and neuraminidase treatment, together with the apparent non-uniform distribution of different types of carbohydrate for a given form of rHAT (Fig. 6), suggest segregation of the different types of carbohydrate, i.e., a given HAT molecule has all or most of its four carbohydrate chains of the same type. To further investigate this, a polyacrylamide gel of the four HAT species was run under nondenaturing conditions. This is shown in Fig. 6. The three rHATs gave a spread of bands, with a generally lower mobility for form III than for form II or form I, while α plasma HAT showed a single band and had the highest mobility. Discrete bands could be seen within the total spread for the recombinant proteins presumably reflecting different numbers of sialic acid residues. A second, similar gel was run, transferred to nitrocellulose, and probed with lectins specific for each of the types of carbohydrate shown above to be present. It was found that terminal mannose, shown above to be present in forms II and III, was restricted to the top third of the band, while sialic acid was confined to the bottom third of the band (data not shown). Highly branched complex carbohydrate, present in forms I and II, was present in the middle region of the protein bands.

Effect of Carbohydrate Variation on 1H NMR Spectra of Plasma and Recombinant Human Antithrombins—As an independent means of examining the differences in glycosylation of the four forms of HAT, their 1H NMR spectra were compared in the regions dominated by resonances from the carbohydrate moieties. Although these regions also contain resonances from the protein, the protein contribution is constant, so that differences should be due principally to variation in the type and extent of glycosylation. Fig. 7 shows the region from 3.4 to 4.5 ppm, which contains most of the resonances from the nonanomeric ring hydrogens of each of the constituent sugars. Although it is not possible to interpret these poorly resolved one-dimensional spectra in detailed structural terms, two significant conclusions can be drawn. One is that there is a major difference in appearance between the spectra of plasma HAT and those of the other three species, with much lesser differences in shape between the spectra of the three recombinant proteins. The second conclusion is that the overall intensity in the form III spectrum is less than that in the spectrum of form II (note that the percentage difference is greater than at first appears, since protein contributes a constant amount to each spectrum). The same conclusions hold for the N-acetyl methyl group resonances (Fig. 8). Plasma HAT, which contains (2→3)-linked sialic acid rather than the (2→6)-linked sialic acid present in the recombinant proteins, gave a very different spectrum from the recombinant proteins. In addition, the overall resonance intensity declined from the α plasma form to form I to form II to form III. Since both N-acetylglucosamine and N-acetyleneuraminic (sialic) acid residues have N-acetyl methyl resonances in this region, the differences, while indicating a reduction in the number of N-acetyl groups, cannot be unambiguously interpreted in terms of relative reductions of N-acetylglucosamine and N-acetyleneuraminic acid residues.

The change from (2→6)- to (2→3)-linkage for the sialic acid residues in recombinant compared to plasma HAT is also manifested in another region of the spectrum (not shown). Resonances occurred at 1.80 and 2.76 ppm in the spectrum of plasma HAT (the chemical shifts expected for the H3 axial and equatorial protons of the (2→6)-linked sialic acid residues (22)), which were missing from the spectra of forms I to III of rHAT. Instead, new resonances were visible at 1.72 and 2.67 ppm, which are the chemical shifts for the equivalent protons in (2→3)-linked sialic acid residues (22).

Structural Comparison of Plasma and Recombinant Human Antithrombin from their 1H NMR Spectra—Since individual resonances are not resolved for most of the protons in the 1H NMR spectrum of HAT, and since the large size of the protein precludes use of homonuclear two-dimensional NMR methods, comparison of the spectra of the four HATs must be based on the appearance of distinctive features in the one-dimensional spectrum that depend on the tertiary structure of the molecule. While such spectra do not enable resonance assignments to be made, the sensitivity of many resonances...
Glycosylation Variation in Recombinant Antithrombin

**Fig. 7.** $^1$H NMR spectra of HAT species in the region containing ring hydrogen resonances. From left to right, spectra are from α plasma, form I, form II, and form III HAT. The base line is shown below each spectrum. Each division on the chemical shift scale represents 0.1 ppm.

**Fig. 8.** Comparison of the N-acetyl methyl region of the 500 MHz $^1$H NMR spectra of the four forms of HAT. From left to right, spectra are from α plasma, form I, form II, and form III HAT. The vertical scale is the same as in Fig. 7, but the horizontal scale is expanded 2-fold for clarity. It should be noted that protein contributes significantly in this region. Each division on the chemical shift scale represents 0.05 ppm.

To through-space interactions turns the spectra into useful fingerprints for discerning differences in the tertiary structure of the four proteins. Such through-space perturbations in chemical shift may occur for any resonance. However, they are most readily seen in regions of the spectrum in which amino acids in small peptides do not normally resonate (23, 24), since these regions would be devoid of resonances in the absence of defined tertiary structure. This also makes these regions most sensitive to alterations in structure. One such region is upfield from the random coil position of methyl resonances of valine, leucine, and isoleucine, i.e. upfield of 0.9 ppm, and contains resonances from methyl groups that experience upfield shifts due to proximity to the side chains of aromatic residues (24). There were four distinct features in all four HAT spectra; at -0.55, -0.4, -0.1, and 0.05 ppm (spectra not shown). The two most upfield shifted peaks must be perturbed from the unfolded state chemical shifts by at least 1.4 and 1.3 ppm, respectively. We estimated that each resonance represents a single methyl group resonance. Since these resonances are so strongly upfield shifted, they are likely to be very sensitive to differences in structure that alter the location and orientation of nearby residues, and are in fact perturbed by heparin binding (see below). In all four forms of HAT these two peaks are clearly discernible at the same chemical shifts, suggesting very similar structures in the vicinity of the side chains that give rise to these resonances. The other two features represent the overlapping resonances of many upfield shifted resonances. Here the perturbations from the unfolded state are approximately 1.0 ppm for the next most upfield shifted peak and 0.8 ppm for the fourth feature. As with the first two peaks, the other two features are very similar for the four forms of HAT.

**Fig. 9.** Comparison of the downfield region of the $^1$H NMR spectra of α plasma and rHATs. a, 72 μM form I rHAT; b, 151 μM form II rHAT; c, 96 μM form III rHAT; d, 140 μM plasma HAT. Spectra were recorded at 303 K and represent the average of 3000 free induction decays. Spectra are normalized based on the overall spectral envelope in regions where only protein resonances occur.
7.50 and 6.86 ppm (23, 24). Resonances between 6.80 and 6.00 ppm usually arise from aromatic amino acid side chains that are upfield shifted as a result of the tertiary structure bringing other aromatic side chains into close proximity. It can be estimated from the peak area that 56 out of a total of 200 aromatic side chain proton resonances in HAT occur in this region. All of the features in the spectrum of α plasma HAT in this region (Fig. 9a) are closely reproduced in the spectra of the forms II and III of rHAT. Form I, however, does show small differences in the shape of resonances at 6.50 and 6.75 ppm.

Resonances between 6.80 and 7.50 ppm arise from aromatic side chains with chemical shifts that may or may not have been perturbed from those of the free amino acids, as well as from unexchanged amide protons. Plasma HAT and rHAT's II and III gave almost indistinguishable spectra, with the exception of the sharp resonance at 7.07 ppm in plasma and form III species, which was less pronounced in form II. Form I, while generally similar, showed several differences; at 7.00, 7.07, 7.25, and 7.45 ppm. Although not all of these resonances can be assigned with confidence, the resonance at 7.07 ppm is from the C(4) proton of histidine 1, while the difference at 7.45 ppm may arise from perturbation of tryptophan 49 (25).

Below 7.5 ppm are three classes of resonance. The first comprises C(2) proton resonances from histidine side chains. These can be exquisitely sensitive to differences in environment, since their chemical shifts in the physiological pH region are determined both by the local magnetic field and the pKs of the imidazole side chain, both of which can be affected by proximity to other groups. In HAT there are five histidine residues, all of which have titratable side chains (19). The C(2) proton resonances occur in three peaks at 7.75, 7.84, and 7.90 ppm at this pH. The most downfield and most upfield of these three resonances represent individual histidine residues, while the larger middle peak at 7.84 ppm, represents protons from the remaining three histidines (19), including histidine 1. Very small chemical shift differences of 0.001-0.002 ppm occur between the forms, which may result from small structural differences, very small changes in pKs, or very slight pH differences. A decrease in amplitude for the middle resonance in forms II and III compared to plasma and form I parallels the difference seen at 7.07 ppm for the C(4) proton resonance of the same histidine. The second class of resonances comprises those from downfield shifted aromatic side chains, while the third contains amide nitrogen-hydrogen resonances. Amide resonances in small peptides occur from small structural differences, very slight pH differences. A decrease in amplitude for the middle resonance in forms II and III compared to plasma and form I parallels the difference seen at 7.07 ppm for the C(4) proton resonance of the same histidine.

Effect of Binding the Synthetic High Affinity Heparin Pentasaccharide to Antithrombin Species—1H NMR spectra can be also used to obtain comparative structural information on plasma and recombinant HATs through examination of the spectral perturbations resulting from heparin binding. NMR difference spectra are shown in Figs. 10 and 11 for heparin pentasaccharide binding. Fig. 10 shows the region downfield from the water resonance, whereas Fig. 11 shows the upfield-shifted methyl region. The four sets of difference spectra are almost identical, though with minor differences in forms I and II, corresponding to resonances from histidine 1. The difference spectra consist of both positive and negative peaks, representing the finishing and starting chemical shifts respectively of perturbed resonances. For such difference spectra to be the same (with the minor exceptions mentioned), the heparin pentasaccharide must not only perturb the same residues, but in the same way in each case. Significantly, the negative resonance at 6.80 ppm, which is only present in heparin-HAT difference spectra involving binding of activating heparin species (26), and which arises from a tyrosine residue, is present in all four spectra.

**DISCUSSION**

We have presented evidence for the existence of three distinct forms of rHAT derived from stably transfected BHK cells, based upon well defined differences in affinity for both heparin-Sepharose™ and free heparin (Fig. 1 and Table I). The properties of these species appear to differ, however, from those of two other forms of rHAT derived from BHK cells (7), suggesting a total of five distinct forms. Differences in growth conditions or in the population of transfected cells may influence the degree of carbohydrate processing and thus the forms of HAT that are secreted. This contrasts with only two forms known to exist in human plasma (α and β), which differ in affinity for heparin (5, 6). The lower affinity form (β) is carried by the higher affinity form (α) results from the additional carbohydrate in form I partially at asparagine 135. Comparison of both the 1H NMR spectra (Fig. 9) and H NMR difference spectra upon heparin binding (Figs. 10 and 11) suggests that the protein portions of these recombinant molecules are structurally similar to plasma HAT and that they bind heparin at the same site and with the same structural alterations. There is, however, a functional difference between form I rHAT and the other two rHATs, shown in the difference in second order rates of inhibition of factor Xa. Forms II and III inhibit factor Xa at the same rate as α plasma HAT, whereas form I gave a biphasic inhibition, with the faster rate being about four times that of forms II and III (Table I). We have no explanation for the biphasic nature of the inhibition. The higher rate may result from the additional carbohydrate in form I partially mimicking the larger rate enhancements that result when heparin binds to HAT.

A study of the related serpin α1-proteinase inhibitor, comparing glycosylated plasma and unglycosylated recombinant forms, also concluded that the carbohydrate did not alter the protein structure (27). The structural differences between α plasma HAT and recombinant forms I, II, and III appear to be restricted to the nature and amount of carbohydrate chains, as well as to small perturbations to the most sensitive surface amino acid side chains, i.e. histidines. Such differences in glycosylation between plasma and rHATs have been noted for rHAT expressed in Chinese hamster ovary cells (28) and BHK cells (7). The significance of the present study is that it examines three forms of rHAT and demonstrates clear differences in carbohydrate composition for each form. Parallel NMR studies provided information on the relative amounts of carbohydrate and allowed low resolution comparison of the protein fold of the three species. Although glycosylation affects heparin affinity, it does not appear to affect the structure of the protein.

In the earlier study on CHO-derived rHAT, it was noted that there was considerable microheterogeneity, which results in diffuseness of the protein band on SDS gels, and which was attributed to the presence of different types of carbohydrate chain, including bi-, tri-, and tetra-antennary complex

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2 B. Fan, B. Crews, and P. Gettins, unpublished results.
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FIG. 10. Downfield region 'H NMR difference spectra of α plasma and recombinant HATs showing the effects of binding the high affinity heparin pentasaccharide at a slight molar excess (10% excess). The spectra were generated by subtraction of the spectrum of the heparin-HAT complex from the unliganded spectrum. Positive peaks thus represent perturbed resonances in native HAT, while negative peaks are new resonance positions in the complex. a, form I rHAT; b, form II rHAT; c, form III rHAT; d, α plasma HAT. The HAT samples were those used to obtain the spectra in Fig. 9 and are at pH 7.35. The concentrations are as given in the legend to Fig. 9.

chains (28). In the present study this carbohydrate heterogeneity did not lead to a continuous spectrum of affinities for heparin, but to three quite well defined species, based on heparin affinity. Nevertheless, this fractionation did not greatly reduce the microheterogeneity of the individual forms, so that forms I and II gave bands on SDS-PAGE that were about as diffuse as unfractionated HAT, though form III gave a somewhat more focused band (Fig. 2).

The major distinction between forms I, II, and III that correlates with affinity for heparin appears to be the amount of carbohydrate present, rather than the type. Thus, although the intensities of carbohydrate NMR resonances (Figs. 7 and 8) showed a clear trend from form I (most) to form III (least) and different median mobilities were seen by SDS-PAGE (Figs. 2 and 5), the glycan detection experiments (Fig. 4) demonstrated the presence of the same type of carbohydrate in more than one form of HAT, e.g. both forms II and III contain high mannose and bi-antennary complex carbohydrate chains (Fig. 4). The mobility difference on SDS-PAGE (Fig. 2) is, however, sufficient to suggest different numbers of carbohydrate chains (four for form II and three for form III). Forms I and II both contain highly branched complex carbohydrate (Fig. 4), but differ in that form I contains this type almost exclusively, while form II contains only a small proportion of this type of chain. This again results in a difference in median mobility on SDS gels. From a native gel run under non-denaturing conditions (Fig. 6), as well as the effects that neuraminidase or endoglycosidase H treatment has on the diffuseness of the protein band on SDS-PAGE (Fig. 5), it appears that the different types of carbohydrate present within each form of HAT are not randomly distributed between the four possible sites of glycosylation on a given HAT molecule, but rather are segregated, resulting in molecules which contain mostly one type of carbohydrate. These results are consistent with the number of carbohydrate chains present on a given molecule of HAT and the bulk of the chains determining affinity for heparin and thus designation as form I, II, or III. Form I seems predominantly to contain four highly branched carbohydrate chains, resulting in the lowest affinity for heparin. Form II has a mixture of molecules containing four high mannose chains, four bi-antennary complex chains, and a smaller number of molecules containing mixed chains, including some highly branched chains. The total bulk of carbohydrate is less than for form I molecules and results in higher affinity for heparin. Form III appears to differ from form II primarily in the amount of carbohydrate rather than type. Its mobility is similar to that of β plasma HAT (Fig. 2) and may indicate a loss of one carbohydrate chain, analogous to the difference between α and β plasma HATs. While the present studies do not unambiguously demonstrate this difference for forms II and III, it seems likely, since differential degrees of glycosylation are thought to result from structural differences around sites of carbohydrate attachment (29). Since Asn-135 is less frequently glycosylated in human plasma than the other three asparagines, this is also likely to be the case whenever the same protein is expressed, even in cells from other species.

Having examined the nature of the differences between forms I, II, and III of rHAT, it is interesting to correlate these differences with the dissociation constants for both full length
heparin and pentasaccharide, as well as with the ionic strength-dependence of the dissociation constants. The 5-fold reduction in affinity seen with plasma HAT for binding pentasaccharide compared with full length heparin at 0.15 (Table II), and maintained at 0.3, also seems to hold for the three forms of HAT, suggesting that the additional charge interaction for full length heparin compared with pentasaccharide (30) is unaffected by variation in carbohydrate, and that heparin binds in a similar manner in all four forms. Since form II appears to be most closely similar to α plasma HAT, both in affinity and in the apparent size of the carbohydrate chains, it serves as the most appropriate base for comparing the effects of carbohydrate variation. At 0.15, the effect of replacing the bi-antennary carbohydrate with tri- and tetra-antennary chains, to give form I, is to reduce heparin affinity approximately 10-fold. Probable removal of one chain, to give form III, results in an increase in affinity of 2-fold (Table II). The greater difference in affinity between forms I and II than between forms II and III suggests a critical crowding in the vicinity of the heparin binding site. Removal of a chain from a noncritically crowded HAT (II → III) has less effect than increasing crowding (II → I). It is interesting that a variant HAT containing a fifth, presumably bi-antennary, carbohydrate chain at residue 7 has greatly reduced heparin affinity (31). The ionic strength dependence also shows a variation with the HAT species. Thus both α plasma and form II HATs show an approximately 10-fold reduction in affinity for both pentasaccharide and full length heparin upon increasing the ionic strength from 0.15 to 0.3.

However, for both forms I and III, the ionic strength dependence is much less, resulting in only a 4-fold reduction in affinity for the same change in ionic strength (Table II). These results suggest the importance of non-charge effects in determining the alteration in binding affinity resulting from deviations from the type of carbohydrate present in plasma α HAT or form II rHAT.

The demonstration of the importance of carbohydrate in determining the affinity of HAT for heparin, though not the mechanism of interaction with it, emphasizes the care that needs to be taken in future functional and structural studies on HAT that will use recombinant variant HATs. It is essential that comparisons, either to wild-type, to plasma, or to other variant HATs, be made between corresponding glycosylated forms. The present studies characterizing forms I, II, and III provide criteria of gel mobility, relative elution position on heparin-Sepharose" 13, and carbohydrate composition and distribution, that should permit classification of a variant HAT species according to the form I–III designation used here.

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REFERENCES