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Carbohydrate isoforms of antithrombin variant N135Q with different heparin affinities

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We have changed one of the carbohydrate-bearing asparagine residues of human antithrombin to glutamine by site-directed mutagenesis and expressed the variant antithrombin, N135Q, in baby hamster kidney cells. Two isoforms were secreted, both of which had higher affinity for heparin than human plasma α antithrombin. Both forms had normal inhibitory activity toward factor X_a and showed normal heparin acceleration of proteinase inhibition. The mutation resulted in a higher production of the very high affinity form from about 30% to 60% of the total secreted antithrombin. This form should be the most useful for comparison of the effects of other mutations on heparin binding and proteinase inhibition.

Antithrombin; Site-directed mutagenesis; Variant; Carbohydrate; Glycosylation; Heparin affinity

1. INTRODUCTION

Antithrombin is the principal inhibitor of the key blood coagulation serine proteinases, thrombin and factor X_a [1]. The rates at which it inhibits these proteinases can be greatly accelerated by binding to heparin at a distinct site on the inhibitor. Although the role of heparin appears to be different for inhibition of each of these proteinases, involving predominantly conformational change in the case of factor X_a inhibition [2], but a ternary complex, template mechanism in the case of thrombin inhibition [3], both types of inhibition require binding of a specific heparin pentasaccharide [4] to the heparin binding site of antithrombin. Binding of heparin species containing this pentasaccharide to antithrombin results in a conformational change in the reactive center loop [5] where the inhibitor interacts with the target proteinase.

In the absence of an X-ray crystallographic structure of either the antithrombin–heparin complex or antithrombin alone, site-directed mutagenesis in combination with characterization of the structural and functional properties of the variant provides a useful means of evaluating the contribution of individual residues to heparin binding and activation. This approach is also greatly aided by the availability of an X-ray structure of a proteolytically cleaved form of bovine antithrombin, which, although having a heparin binding site different from native antithrombin [6], serves as a useful starting point for identifying potentially important heparin-binding residues.

Since one of the main criteria for evaluating an amino acid substitution is the effect it has on heparin affinity, care must be taken with glycosylated recombinant antithrombins that equivalent carbohydrate isoforms are compared, since differences in the number and composition of carbohydrate chains per se affect heparin affinity [7–11]. We have shown that glycosylated recombinant human antithrombin (HAT) expressed in baby hamster kidney (BHK) cells was composed of three isoforms with different heparin affinities, that resulted from differences in carbohydrate content and composition [11]. Both to reduce the number of isoforms, thereby obtaining more antithrombin of one type from a given isolation, and to increase the affinity of the antithrombin, so that subsequent reductions caused by critical heparin binding site mutations do not effectively abolish heparin binding, we have prepared a variant antithrombin, N135Q, to mimic the high affinity β plasma form of HAT that lacks carbohydrate at residue 135. We describe here the properties of the BHK-derived N135Q variant HAT and show that most of the original objectives were achieved by this mutation.

2. MATERIALS AND METHODS

2.1. *Isolation of α and β forms of HAT*

α and β plasma HATs were isolated from pooled outdated human plasma obtained from the Vanderbilt Blood Bank by affinity chromatography on heparin–Sepharose as previously described [11]. Ion-exchange chromatography on DE 52 was used to remove any contaminating heparin. Concentrations of both plasma and recombinant HATs were determined spectrophotometrically using $E^{1\%}_{280\,nm} = 6.5$ [12].

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Abbreviations: BHK, baby hamster kidney; HAT, human antithrombin; N135Q, variant of antithrombin in which Asp-135 has been changed to glutamine; forms I, II, and III represent differently glycosylated forms of wild-type recombinant human antithrombin with low, intermediate, and high affinities for heparin; HAH, high affinity heparin.

2.2. *Production and isolation of recombinant HAT*

Site-directed mutagenesis of HAT cDNA was carried out in M13mp19 as described previously [5] using a single-stranded uracil-containing template and the anti-sense oligonucleotide 5′ CTT GGA GGA TTT CTG GGC TTT TCG ATA 3′ (the underlined triplet corresponds to the asparagine-to-glutamine change). The mutated antithrombin cDNA was ligated into pMAStop, as described [5], to generate plasmid pMAAT3-N135Q. BHK cells were transfected with plasmids pMAAT3-N135Q, pRMH140, and pSV2dhfr and stable transfectants selected by resistance to neomycin and methotrexate, as described previously [13]. Antithrombin was isolated from serum-free growth medium of confluent cells as described [11].

2.3. *Characterization of carbohydrate species on antithrombin*

The types of carbohydrate present on the two isoforms of N135Q recombinant HAT were identified by use of a glycan differentiation kit (Boehringer-Mannheim) following the manufacturer's directions, as described [11].

2.4. *Antithrombin assay*

The level of antithrombin secretion from BHK cells at various stages of the transfection and production procedures was monitored by radial immunodiffusion, using prepared plates containing sheep anti-HAT antibody (The Binding Site Ltd., Birmingham, UK).

2.5. *Fluorescence measurements*

Fluorescence measurements were made on a SPEX spectrofluorimeter, exciting at 295 nm and observing tryptophan emission at 335 nm. Heparin binding experiments were carried out as previously described [11], and the resulting data fitted by non-linear least squares analysis using the program, MINSQ II (Micromath Scientific Software, Salt Lake City, UT). Because of the high affinity of the two recombinant antithrombin isoforms for heparin, K_d values were determined at 0.3 I to lower the affinity and allow more accurate dissociation constants to be obtained. The buffer used was 20 mM sodium phosphate at pH 7.4 containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000 and 0.25 M NaCl.

2.6. *Factor X_a assay*

Rates of inhibition of factor X_a by antithrombin species were measured under pseudo-first order conditions. Second-order rate constants for the heparin-catalyzed inhibition of factor X_a were determined in the presence of catalytic concentrations of heparin as previously described [14]. Concentrations of factor X_a and antithrombin of 20 nM and 1 μM were used for all measurements.

2.7. *Materials*

Spectrozyme X_a was from American Diagnostica, Greenwich, CT, Dulbecco's modified Eagle's medium, fetal calf serum and neomycin were from Gibco; and methotrexate was from Sigma Chemical Co., St. Louis, MO. Thrombin was prepared from outdated human plasma by the method of Miletich et al. [15] as described previously [14]. Human factor X_a, a gift from Dr. Paul Bock, Vanderbilt University, was prepared by affinity chromatography on soybean trypsin inhibitor–Sepharose [16]. M_r 9,000 high affinity heparin (HAH) was a gift from Dr. Steven Olson, Henry Ford Hospital, Detroit, and contained one high affinity binding site for antithrombin. Synthetic heparin pentasaccharide [4] was a gift from Dr. Jean Choay, Centre Choay. Plasmid pAT3c [17] was a gift from Dr. Susan Bock, Temple University. Plasmids pMAStop, pRMH140, and pSV2dhfr [13] were gifts from Dr. Gerd Zettlmeißl, Behringwerke, Marburg.

3. RESULTS

3.1. *Isolation of recombinant HAT isoforms*

Two isoforms of recombinant N135Q HAT were isolated from the growth medium of stably transfected BHK cells. One form was eluted from heparin–Sepharose in the linear gradient at 1.2 M NaCl and the other was eluted in the 3 M NaCl step [11]. The proportion of lower-to-higher affinity forms was about 40%:60%. Re-chromatography of a mixture of the two forms gave two distinct peaks (Fig. 1). Conductivity meter measurements showed that the two forms eluted at 1.28 and 1.91 M NaCl for the lower and higher affinity forms, respectively. Because of the high heparin affinity of these two forms, determination of the K_d for heparin–antithrombin complexes was made at I 0.3 rather than physiological ionic strength (I 0.15), to enable a more accurate estimate of K_d to be made. The K_ds of the low affinity form for both pentasaccharide and HAH were lower than for α plasma antithrombin, consistent with its elution at a higher salt concentration than the plasma species, whereas the high affinity form had an affinity similar to that of the β plasma species (Table I).

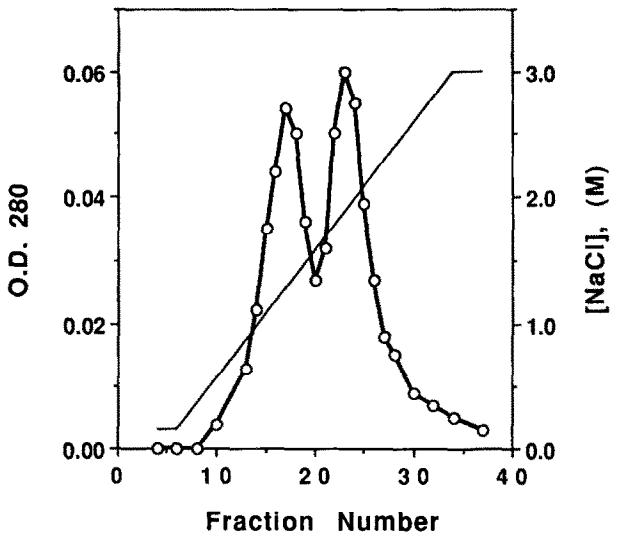

Fig. 1. Elution profile of the two isoforms of recombinant human N135Q variant antithrombin from heparin–Sepharose. About 0.5 mg of each purified isoform was recombined and applied to the column equilibrated in 0.15 I buffer. The antithrombin species were eluted by a linear salt gradient in the same buffer at pH 7.5. The salt concentrations indicated were determined from conductivity measurements on individual fractions.

The two N135Q isoforms migrated on SDS-PAGE with the same mobility as the β plasma form of antithrombin but slightly lower mobility than form III of the recombinant wild-type antithrombin (Fig. 2, lanes 4, 5 and 7). It was known for the β plasma form, and believed to be the case for the recombinant wild-type form III, that they carried only three carbohydrate chains. These three antithrombins showed higher mobility than the two forms of recombinant wild-type antithrombin and the α plasma form, which carry four carbohydrate chains (Fig. 2, lanes 1, 2 and 6).

3.2. *Carbohydrate composition of HAT isoforms*

We suspected that the reason for the existence of two

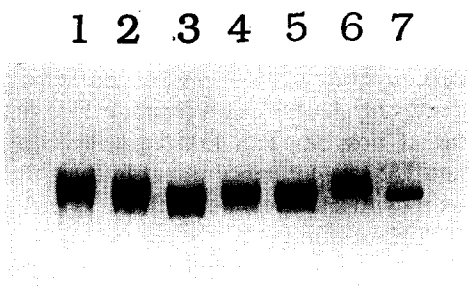

Fig. 2. SDS-polyacrylamide gel (10%) of different human antithrombin species, stained with Coomassie brilliant blue. Lanes 1–3, wild-type antithrombin fractions I, II and III [11] respectively; lanes 4 and 5, recombinant N135Q variant lower and higher affinity forms, respectively; lanes 6 and 7, α and β plasma forms, respectively.

isoforms of N135Q antithrombin was differences in the type of carbohydrate chains present in each form. Specific lectin binding was used to characterize the carbohydrate in each form and confirmed the existence of differences between them (Fig. 3). The higher affinity form showed the presence of terminal (2 → 3)-linked sialic acid, trace amounts of terminal mannose, but no terminal galactose. In contrast, the lower affinity form showed the presence of high amounts of both terminal galactose and terminal (2 → 3)-linked sialic acid, but no high mannose chains, consistent with the presence of some tri- and tetra-antennary complex carbohydrate chains [18]. No O-linked carbohydrate was detectable in any of the forms of plasma or recombinant antithrombin, as shown by failure to stain with *Datura stramonium* agglutinin lectin before or after removal of N-linked carbohydrate with *N*-glycosidase F (data not shown). Data on wild-type forms I, II and III, which have previously been reported [11], are shown for comparison. There is no direct parallel between these three forms and the high and low affinity forms of the N135Q variant, although form II wild-type, which has higher heparin affinity than form I wild-type, has a lower amount of highly branched complex carbohydrates than form I. This is similar to the relationship between high and low affinity N135Q species. The relative amounts of high mannose differ, however, between the wild-type forms and the N135Q variant forms.

3.3. *Interaction of HAT isoforms with proteinases*

To examine the functional properties of the two N135Q isoforms, the ability to form complexes with proteinase, and the rates of proteinase inactivation were examined. Upon incubation with stoichiometric amounts of thrombin or factor X_a, both species gave high molecular weight complex bands on SDS-polyacrylamide gels (not shown). Rates of inhibition of factor X_a were obtained for both isoforms that were similar to that of α plasma antithrombin (Table I). For comparison, the rate of factor X_a inhibition was also determined for β plasma antithrombin, since this has not been reported previously. It was also similar to that for the α plasma species. Enhancements of the rates of proteinase inhibition by HAH for both N135Q isoforms were similar to those of plasma antithrombin species (Table I).

4. DISCUSSION

We have shown that replacement of Asp-135 by glutamine, preventing attachment of carbohydrate at the fourth glycosylation site of antithrombin, resulted in a decrease in the number of isoforms of recombinant antithrombin from three to two. These two forms were able to inhibit factor X_a at rates very similar to those of both α and β forms of plasma antithrombin. In addition, HAH gave similar rate enhancements to those found for the plasma antithrombins. The only significant difference in properties was in affinity for heparin.

Based on previous results for the two carbohydrate isoforms, α and β, of plasma antithrombin, in which the absence of the fourth carbohydrate chain from position 135 in the β form resulted in a large increase in affinity for heparin, we had hoped that the N135Q mutation would result in elimination of all of the heparin binding heterogeneity present in recombinant BHK-expressed antithrombin [11] and consequently the production of only one form of recombinant antithrombin. The failure to completely eliminate such heterogeneity upon removal of the Asp-135 carbohydrate appears to result from differences in size of the remaining three carbohydrate chains. The higher affinity form, bearing three

Table I

Heparin binding and proteinase inhibitory properties of N135Q antithrombin isoforms compared to those of plasma antithrombin isoforms

Antithrombin	Antithrombin–heparin dissociation constant, K_d, (nM)		Rate of factor X_a inhibition	
	Pentasaccharide	HAH	k_{uncat} ($mM^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)[b]
α Plasma	500 ± 50[a]	93 ± 12[a]	2.3[c]	1.3[c]
β Plasma	36 ± 3	7 ± 2	4.0	1.5
N135Q (low affinity)	220 ± 20	20 ± 2	2.1	1.4
N135Q (high affinity)	20 ± 2	9 ± 2	4.2	1.5

[a] From Fan et al. 1993 [8].
[b] k_{cat}/K_m values determined in the presence of HAH.
[c] From Olson et al. 1992 [13].

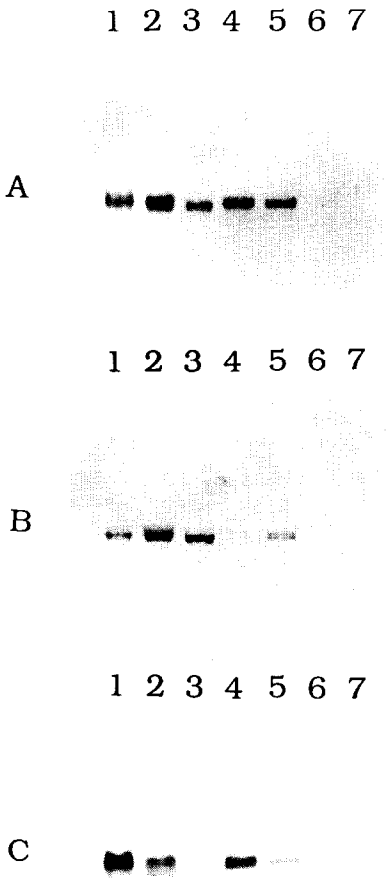

Fig. 3. Detection of different types of carbohydrate chain by lectin binding. (A) Staining for the presence of $\alpha(2 \rightarrow 3)$-linked sialic acid; (B) staining for the presence of terminal mannose, indicative of high mannose chains; (C) staining for the presence of terminal Galβ-$(1 \rightarrow 4)$GlcNAc, indicative of tri- and tetra-antennary complex carbohydrate chains.

bi-antennary complex carbohydrate chains, evidenced by the presence of sialic acid but no terminal galactose [18] and only a trace of high mannose, had properties similar to β plasma antithrombin, which also bears three bi-antennary complex carbohydrate chains. The second isoform, although also carrying only three carbohydrate chains, had reduced heparin affinity as a result of the increased bulk of the carbohydrate, which appeared to include some tri- and tetra-antennary chains, evidenced by the presence of significant amounts of terminal galactose [18]. This is similar but not identical to what we found for recombinant wild-type antithrombin expressed in the same BHK cell system [11], where two of the three isoforms carried four carbohydrate chains, but differed in affinity for heparin as a result of the relative amounts of highly branched carbohydrate chains in the lowest affinity form I species compared to the higher affinity form II species. At the time we characterized the three wild-type isoforms it was not possible to say whether the effect of the bulk of the carbohydrate chains was exerted at only one of the four sites of glycosylation (residue 135) or at more than one. It now appears that carbohydrate at sites other than 135 can influence the affinity of the protein for heparin, with the effect being that less carbohydrate results in higher affinity.

In conclusion, we have reduced, but not eliminated, the heterogeneity of recombinant antithrombin by eliminating one of the four carbohydrate chains. The failure to remove all heterogeneity indicates that carbohydrate chain(s) in addition to 135 affect the affinity for heparin negatively. The study again highlights the critical importance of characterizing the number and size of carbohydrate chains in any glycosylated recombinant antithrombin before valid comparisons can be made between a wild-type and a variant species.

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