Antithrombin−Heparin Affinity Reduced by Fucosylation of Carbohydrate at Asparagine 155[†]

Louise Garone,[‡,§] Tim Edmunds,[‡] Eric Hanson,[‡] Richard Bernasconi,[‡] James A. Huntington,[∥] Jennifer L. Meagher,[∥] Bingqi Fan,[∥] and Peter G. W. Gettins*[,∥]

Department of Structural Protein Chemistry, Genzyme Corporation, One Mountain Road, Framingham, Massachusetts 01701-9322, and Department of Biochemistry, University of Illinois at Chicago, Chicago, Illinois 60612-4316

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ABSTRACT: The two human plasma antithrombin isoforms, α and *β*, differ in glycosylation at asparagine 135. Only the α form carries carbohydrate at this position and has lower affinity for heparin than the *β* form. We previously found additional heterogeneity in a recombinant N135Q antithrombin variant, evidenced by two isoforms with a 2-fold difference in heparin affinity [Turko, I. V., Fan, B., & Gettins, P. G. W. (1993) *FEBS Lett. 335,* 9−12]. To test whether this heterogeneity of heparin affinity results from specific glycosylation differences, we have determined the carbohydrate composition at the three remaining glycosylation sites, asparagine residues 96, 155, and 192, in each of the two N135Q isoforms, by a combination of peptide fragmentation and electrospray mass spectrometry. Patterns of glycosylation at residues 96 and 192 were similar for each isoform and showed the presence of mono-, bi-, and triantennary complex carbohydrate, as well as fucosylation of all types of chains. At position 155, however, there was a marked difference between the isoforms, with the form with lower heparin affinity being 97% fucosylated at this position, whereas the form with higher affinity for heparin was not fucosylated. Other differences in carbohydrate type showed no strong correlation between the two isoforms. We conclude that formation of the two heparin-affinity isoforms of N135Q antithrombin results from the specific difference in fucosylation at residue 155, which may result in different structural properties of the carbohydrate. Consistent with these findings was the elimination of heparin-affinity heterogeneity in a double N135Q-N155Q variant antithrombin. It is possible that fucosylation of antithrombin may occur *in vivo* as a means of modifying the physiological properties of the antithrombin through alteration of the amount of antithrombin bound to surface heparin-like species.

A complication in using overexpressed glycoproteins for structural and functional studies is heterogeneity in glycosylation and consequently possible differences in the properties of individual glycoforms. In a number of instances such heterogeneity has been shown to have little effect on the structure of the polypeptide but to have pronounced consequences *in vivo* on the clearance rates of the protein. There are other instances, however, where different glycoforms have markedly different properties even *in vitro*. Thus two glycoforms of plasminogen (Hayes & Castellino, 1979a,b) are activated at different rates (Takada & Takada, 1983), and two naturally occurring glycoforms of antithrombin have very different affinities for heparin, the allosteric activator of antithrombin as a proteinase inhibitor (Peterson & Blackburn, 1985; Brennan et al., 1987). Such differences in heparin affinity may also have great physiological significance, since it is expected that the most important inhibitory antithrombin species *in vivo* will be those that are bound to surface heparin-like molecules rather than those free in solution and not complexed with heparin (de Agostini et al., 1990). In addition, it has recently been shown that the presence of a single O-linked fucose residue altered the stability of a small proteinase inhibitor, *Pars intercerebralis* major peptide C (PMC-C), by ∼ 1 kcal mol^{-1}, as a result of specific hydrophobic and hydrogen-bond interactions between the fucose and the protein (Mer et al., 1996).

We are particularly interested in the effect of carbohydrate heterogeneity on the heparin affinity of recombinant human antithrombin produced by baby hamster kidney (BHK)[1] cells, since a major goal of our site-directed mutagenesis studies on antithrombin is to determine which residues are involved in heparin binding. Further alteration of the heparin affinity by particular carbohydrate patterns both complicates the analysis of the effects of the mutation and reduces the amount of an individual antithrombin glycoform produced in a given preparation. We have previously shown that wild-type human antithrombin produced by BHK cells occurs as multiple glycoforms, though with only three distinct affinities for heparin (Fan et al., 1993). Two of these isoforms carry four carbohydrate chains, whereas the third, and tightest heparin-binding isoform, appeared to carry only three carbohydrate chains. This is partly paralleled by the situation in human plasma, where α and *β* glycoforms exist, with the

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* Address correspondence to this author.

[‡] Genzyme Corp.

[§] Present address: Ares Advanced Technology, 280 Pond St., Randolph, MA 02368.

[∥] University of Illinois at Chicago.

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[1] Abbreviations: BHK, baby hamster kidney; N135Q, variant antithrombin with asparagine 135 changed to glutamine; N135Q-N155Q, antithrombin variant with both asparagines 135 and 155 changed to glutamine; L and H isoforms, lower- and higher-affinity forms, respectively, of recombinant N135Q antithrombin; K8, K15, and K18, the 8th, 15th, and 18th peptides, respectively, numbered from their position within the primary structure, of a lysyl endopeptidase digestion of human antithrombin.

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tighter heparin-binding isoform (β) lacking carbohydrate at residue 135 (Peterson & Blackburn, 1985; Brennan et al., 1987).

In an attempt to eliminate the heterogeneity in heparin affinity of BHK-derived recombinant human antithrombin, we have previously created an asparagine 135 → glutamine (N135Q) variant antithrombin (Turko et al., 1993). Although all of the resulting antithrombin carried carbohydrate only at the three remaining glycosylation sites, there was still heterogeneity in heparin affinity that resulted in two pools of antithrombin eluting from heparin−Sepharose, which were designated L and H glycoforms, signifying low (L) or high (H) affinity for heparin, respectively. To test whether a specific difference between the carbohydrate present in these two pools is responsible for this difference in heparin affinity, we subjected the two antithrombin isoforms to proteolytic digestion and characterized the individual carbohydrates attached to each glycopeptide by a combination of HPLC and electrospray mass spectrometry. We found that the carbohydrate at positions 96 and 192, though heterogeneous within a given heparin affinity glycoform, was very similar between the two antithrombin species. In contrast, the carbohydrate at position 155 showed 97% fucosylation of each type of carbohydrate chain in antithrombin from the lower heparin affinity pool and complete absence of fucosylation in antithrombin from the higher affinity pool. We predicted from this that a N155Q-N135Q double mutant antithrombin produced by BHK cells would be both homogeneous and of high affinity with respect to heparin binding. We tested this and found the prediction to be correct. We conclude that, in the absence of carbohydrate at position 135, glycosylation of the asparagine at position 155 in general and fucosylation in particular is a major determinant of heterogeneity in heparin affinity. This may be of importance *in vivo* as a means of further modulating the anticoagulant efficiency of surface-bound antithrombins. A practical consequence *in vitro* is that, by use of a double N135Q-N155Q cDNA as the base for future mutagenesis studies, we should be able to eliminate carbohydrate-derived heterogeneity in heparin affinity from the resulting variant antithrombins.

MATERIALS AND METHODS

Production and Isolation of N135Q Antithrombin Isoforms. Preparation of stably transfected BHK cells expressing the N135Q variant of human antithrombin was as described previously (Turko et al., 1993). Cells were grown to confluence in roller bottles and cycled between serum-free medium and growth medium containing fetal calf serum, as described (Fan et al., 1993). Antithrombin was isolated from the medium of serum-free cycles by affinity chromatography on heparin−Sepharose using a linear salt gradient as described (Fan et al., 1993). Antithrombin isoforms were rechromatographed on heparin−Sepharose, using only the central portion of each eluted form to eliminate any cross-contamination with the second isoform, and subsequently on DEAE-Sepharose to remove any free heparin. The two isoforms, defined by affinity for heparin, have been previously characterized by heparin affinity and are designated higher- (H) and lower- (L) affinity forms.

Preparation of N135Q-N155Q Variant Antithrombin. Site-directed mutagenesis to introduce the changes at amino acid positions 135 and 155 was carried out as previously described (Gettins et al., 1993) in M13mp19 on the subcloned 1.4-kb *Sal*I−*Xba*I fragment, excised from pMAAT3, that contains the entire antithrombin cDNA. The cDNA template already contained the N135Q mutation (Turko et al., 1993). The antisense oligonucleotide 5′ CTG GTA GGT CTC TTG GAA GGT AAG GGA 3′ was used to introduce the N → Q mutation at position 155 (glutamine anticodon underlined). The mutated *Sal*I−*Xba*I fragment was ligated into the expression vector pMAStop to create pMAAT3-N135Q-N155Q and cotransfected, with plasmids pRMH140 and pSV2dhfr, into BHK cells as described (Zettlmeissl et al., 1988). The presence of N → Q mutations at positions 135 and 155 was confirmed by dideoxy sequencing (Sanger et al., 1977) at two stages, (i) in the M13 phage and (ii) after ligation into pMAStop. Recombinant variant antithrombin was isolated from the serum-free cycles of stably transfected BHK cells by chromatography on heparin−Sepharose, as described above for the N135Q single-mutation variant.

Enzymatic Fragmentation of Antithrombin. Lysyl endopeptidase digestions of antithrombin species were carried out on 1−2-nmol aliquots of reduced and pyridylethylated protein. Samples were preincubated in 10 mM Tris and 4 M urea, pH 9.0, prior to addition of enzyme at a ratio of 1:15. Digestions were carried out for 2 h at 37 °C at a final urea concentration of 2 M. Reactions were stopped by the addition of 0.1% trifluoroacetic acid.

Carbohydrate Monosaccharide Compositional Analysis. Carbohydrate compositional analysis was carried out using a Dionex HPLC system with pulsed amperometric detection of monosaccharides according to the method of Hardy et al., (1988). Protein (98 μg, ~2 nmol), quantitated by BCA assay, was hydrolyzed in 2 M TFA for 2 h at 120 °C. Quantitation of fucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and mannose was by comparison to a standard curve for each sugar type. Sialic acid determination was carried out using the thiobarbituric acid method as modified by Powell and Hart (1986). Values reported are the average of triplicate determinations.

Carbohydrate Structural Analysis of Antithrombin Peptides. Peptides generated by lysyl endopeptidase digestion were separated on a Michrom BioResources UMA HPLC apparatus using a Michrom column (100 × 1 mm) packed with Vydac C8 resin. A gradient of 0−46% acetonitrile in 0.1% trifuoroacetic acid and a flow rate of 50 μL min^{-1} were used to elute the peptides. LC/MS analysis of the peptides was carried out directly on the eluate from the HPLC column using a Finnigan TSQ 700 instrument equipped with a Finnigan electrospray interface and operating in the single quadrupole mode. The eluate was introduced directly into the electrospray interface. LC/MS spectra were acquired at a scan rate of one every 3 s, over the *m*/*z* range of 200−4000. Peptide identification and matching was carried out using the Finnigan MAT PepMap program. For analysis of glycopeptides, the spectra were averaged over the glycopeptide peak to allow for slight differences in elution position of the different glycoforms. The oligosaccharide mass was obtained by subtraction of the theoretical peptide mass from the observed mass. Percentages of each glycoform were obtained from the integrated peak intensities and may result in some error due to differences in ionization efficiency. However, relative differences between the two antithrombin

isoforms or presence of a given, identified carbohydrate type are not in doubt. Peaks that had intensity less than 5% that of the most intense peak were not included in the analysis. This was an arbitrary cutoff but did not eliminate any identified glycopeptides and is not likely to bias the conclusions with regard to the role of fucosylation (see Results).

Determination of Heparin−Antithrombin Dissociation Constants. Dissociation constants (K_d) were determined at 25 °C and either *I* 0.15 or *I* 0.3 by titration of heparin into antithrombin (50 nM) and monitoring the change in endogenous tryptophan fluorescence at 340 nm with excitation at 280 nm. Measurements were made on an SLM8000 spectrofluorometer. Slits of 4 nm for excitation and 16 nm for emission were used. The buffer used was 20 mM sodium phosphate at pH 7.4, containing 0.1% PEG, 1 mM EDTA, and either 0.1 or 0.25 M NaCl to give *I* 0.15 or *I* 0.3 buffer, respectively. Titrations with heparin pentasaccharide were carried out at *I* 0.15. However, for titrations with full-length high-affinity heparin, the higher ionic strength was chosen to weaken the affinity sufficiently to allow more accurate determination of the dissociation constant to be made. Heparin stock solutions were of concentration such that no more than 5% volume change occurred during the titration. Fluorescence data, corrected for dilution and assuming a 1:1 stoichiometry for complex formation, were fitted by nonlinear least-squares analysis using the program MINSQ II (Micromath Scientific Software, Salt Lake City, UT). The errors given are the 95% confidence level from the least-squares analysis. Duplicate titrations were carried out and gave values that differed by no more than 20%.

Materials. Lysyl endoproteinase (E.C. 3.4.21.50) was purchased from Wako (Richmond, VA). High-affinity heparin of molecular weight 9000, prepared by affinity chromatography on antithrombin−agarose and containing 1.07 antithrombin binding sites/chain, determined by titration with antithrombin at a concentration well above the K_d, was a generous gift from Dr. Steven Olson, University of Illinois at Chicago. Synthetic high-affinity heparin pentasaccharide was a generous gift of Dr. Jean Choay (Sanofi Recherche). The plasmids pMAStop, pRMH140, and pSV2dhfr were generous gifts from Dr. Gerd Zettlmeissl, Behringwerke, Marburg, Germany. The plasmid pAT3 containing the wild-type antithrombin cDNA was a generous gift from Dr. Susan Bock, Temple University.

RESULTS

Characterization of Distinct H and L Glycoforms of Recombinant N135Q Antithrombin. Although we have previously documented the occurrence of distinct H and L isoforms of recombinant N135Q human antithrombin (Turko et al., 1993), we provide here further evidence for the nonarbitrary separation of recombinant N135Q antithrombin into these two pools, since the existence of distinct species, based on heparin affinity, is critical for the validity of any conclusions based on analysis of these isoforms. Purified H and L isoforms of N135Q antithrombin, when rechromatographed on a heparin−agarose affinity column, eluted at very different salt concentrations (Figure 1). Although there is overlap between the tails of the two peaks, this results from heterogeneity of the heparin on the affinity column, such that there is always some spreading of the peak of bound material, rather than from ranges of overlapping heparin affinities within a given pooled isoform. This is shown in two ways. First, if antithrombin from the center of the broad eluted peak of a given N135Q isoform is rechromatographed on the same heparin−agarose column, the same broadened shape is obtained for the eluted material. Second, heparin binding experiments carried out using well-defined heparin species give good fits to the experimental data that require use of only a single heparin affinity for each isoform. Thus, binding of the high-affinity heparin pentasaccharide gives distinct and well-defined K_d values of 5 and 42 nM for the H and L isoforms, respectively, at *I* 0.15 (Figure 2). Similar titrations at *I* 0.3 with higher affinity full-length heparin also give distinct and well-defined K_ds for the two isoforms (see below).

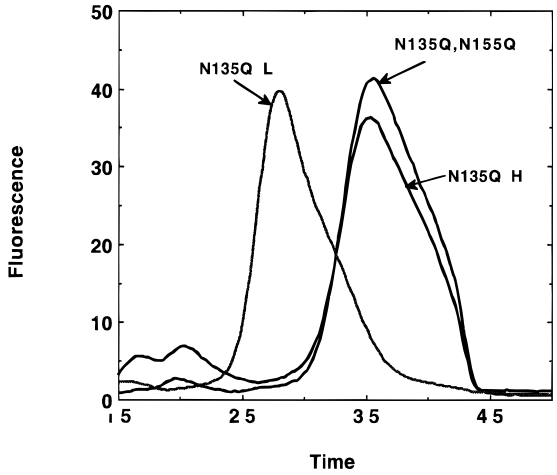

FIGURE 1: Elution profiles of N135Q-N155Q antithrombin and the L and H glycoforms of N135Q antithrombin from a heparin−Sepharose column. Ten micrograms of each form of antithrombin was applied at low ionic strength (0.15) and eluted with a linear NaCl gradient from *I* 0.15 to *I* 2.5. Antithrombin was detected in-line by tryptophan fluorescence emission. Samples were run under identical conditions with extensive reequilibration of the column between runs. The peak positions correspond to 1.3 M NaCl for L N135Q and 1.9 M NaCl for both H N135Q and N135Q-N155Q antithrombins.

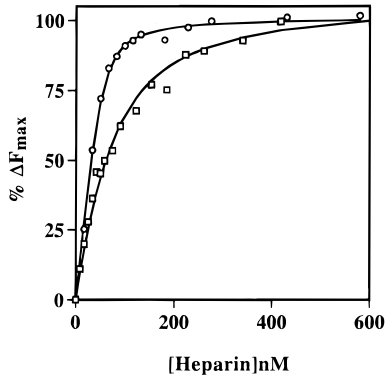

FIGURE 2: Distinct, monophasic binding curves for binding of heparin pentasaccharide to the H and L isoforms of N135Q recombinant antithrombin. Open circles, H isoform; open squares, L isoform. Binding of heparin was monitored by change in intrinsic protein fluorescence. Titrations were carried out at 50 nM antithrombin in *I* 0.15 buffer. The solid lines represent least-squares best fits of the data to a single dissociation constant for each isoform (K_d = 5 nM for H and 42 nM for L isoform).

Endoproteinase Fragmentation of N135Q Antithrombin Glycoforms. To determine if there were differences in glycosylation specific to particular carbohydrate attachment sites, individual glycopeptides were isolated and characterized from a lysyl endopeptidase digest of each form of

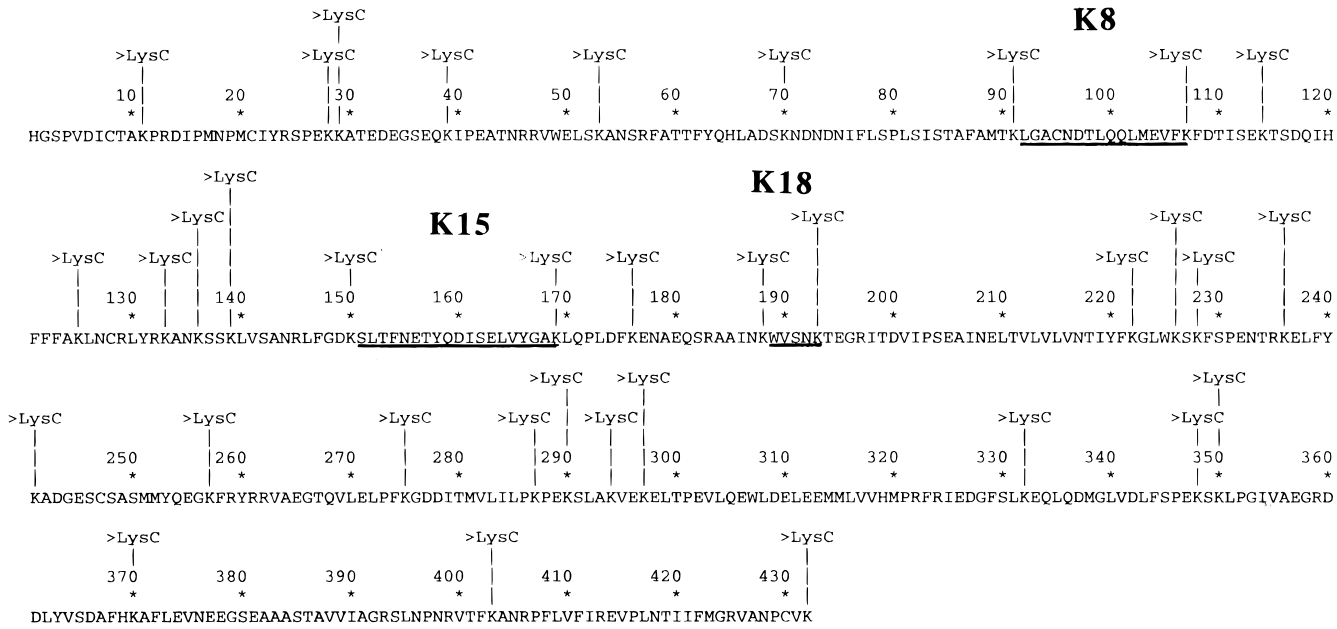

FIGURE 3: Lysyl endopeptidase cleavage map of human antithrombin, showing the expected cleavage into 35 peptides. The three glycopeptides K8, K15, and K18, containing asparagines 96, 155, and 192, respectively, are indicated.

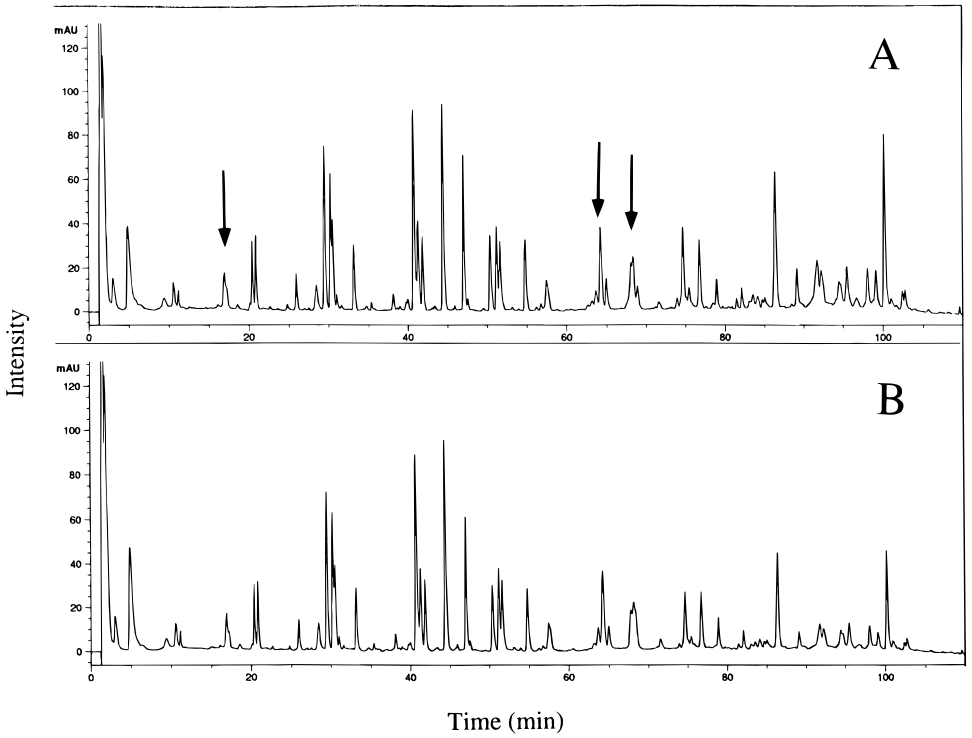

FIGURE 4: HPLC profiles of lysine endopeptidase C digest of L and H isoforms of N135Q recombinant human antithrombin. Peptides were detected by their absorbance at 215 nm. The three sets of glycopeptides are indicated by arrows. Glycopeptide K18, containing Asn192, is at 16.87 min; K8, containing Asn96, is at 64.2 min; and K15, containing, Asn155, is in the multiplet at ~68 min.

N135Q antithrombin. Lysyl endopeptidase cleaves after each lysine and is therefore expected to produce 35 peptides from proteolysis of human antithrombin (Figure 3). Similar peptide fragmentation patterns were obtained for digests of both H and L isoforms of N135Q antithrombin (Figure 4). Identification of the peptides was carried out by direct electrospray mass spectrometry of fractions from the HPLC column. All of the expected peptides were identified, except those of three or fewer residues. Peptides designated K8, K15, and K18, corresponding to the fragments LGAB^{96}**N**DTLQQLMEVFK, SLTF^{155}**N**ETYQDISELVY-GAK, and WVS^{192}**N**K, were identified as the glycopeptides containing asparagines 96, 155, and 192, respectively, for each of the two isoforms (Table 1).

A more detailed analysis of the electrospray mass spectra of the glycopeptides K8, K15, and K18 was performed to identify the types and relative amounts of oligosaccharide species present (Table 2). For both the L and H N135Q isoforms, position 192 showed the least heterogeneity by type of carbohydrate (Figure 5 and Table 2), with almost all being biantennary complex carbohydrate. Although position 96 showed greater heterogeneity, the carbohydrate was still all complex and showed a very similar distribution between mono-, bi-, and triantennary chains for each antithrombin

Table 1: Identification of Lysyl Endopeptidase-Derived Peptides of Antithrombin H and L Isoforms by Electrospray MS of HPLC C8 Fractions

peptide[a]	name[b]	mass[c]		
		L isoform	H isoform	theoretical
HGSPVDIBTAK	K1	1232.3	1234.4	1232.44
PRDIPMNPMBIYRSPEK	K2	2152.6	2152.7	2152.61
K	K3	n.d.	n.d.	146.2
ATEDEGSEQK	K4	1093.1	1092.7	1093.08
IPEATNRRVWELSK	K5	1699.0	1699.2	1698.95
ANSRFATTFYQHLADSK	K6	1957.3	1957.2	1957.14
NDNDNIFLSPLSISTAFAMTK	K7	2299.8	2299.2	2299.6
LGABNDTLQQLMEVFK+$N_4H_5F_1S_1$	K8(1)[d]	3977.1	3976.8	3976.18
LGABNDTLQQLMEVFK+$N_4H_5F_1S_2$	K8(2)	4268.1	4268.1	4267.44
FDTISEK	K9	838.9	838.9	838.92
TSDQIHFFFAK	K10	1340.5	1340.5	1340.51
LNBRLYRK	K11	1170.2	1170.2	1170.46
AQK	K12	n.d.	345.0	345.41
SSK	K13	n.d.	320.1	320.35
LVSANRLFGDK	K14	1219.3	1219.4	1219.42
SLTFNETYQDISELVYGAK+$N_4H_5F_1S_1$	K15(1)	4239.9	n.p.	4239.29
SLTFNETYQDISELVYGAK+$N_4H_5F_1S_2$	K15(2)	4530.9	n.p.	4530.55
SLTFNETYQDISELVYGAK+$N_4H_5S_1$	K15(1)	n.p.	4093.5	4093.15
SLTFNETYQDISELVYGAK+$N_4H_5S_2$	K15(2)	n.p.	4384.5	4384.41
LQPLDFK	K16	859.9	859.9	860.03
ENAEQSRAAINK	K17	1330.5	1330.4	1330.43
WVSNK+$N_4H_5F_1S_1$	K18(1)	2694.3	2693.8	2693.63
WVSNK+$N_4H_5F_1S_2$	K18(2)	2985.6	2985.2	2984.89
TEGRITDVIPSEAINELTVLVLVNTIYFK	K19	3249.4	3249.5	3248.78
GLWK	K20	502.2	502.3	502.62
SK	K21	n.d.	n.d.	233.28
FSPENTRK	K22	977.8	977.9	978.08
ELFYK	K23	698.6	698.4	698.83
ADGESBSASMMYQEGK	K24	1799.3	1799.1	1799
FRYRRVAEGTQVLELPFK	K25	2209.7	2209.7	2209.59
GDDITMVLILPK	K26	1314.6	1313.6	1314.62
PEK	K27	n.d.	n.d.	372.43
SLAK	K28	417.0	417.1	417.51
VEK	K29	374.0	374	374.45
ELTPEVLQEWLDELEEMMLVVHMPRFRIEDGFSLK	K30	4261.6	4261	4260.98
EQLQDMGLVDLFSPEK	K31	1849.4	1849.4	1849.1
SK	K32	n.d.	n.d.	233.28
LPGIVAEGRDDLYVSDAFHK	K33	2202.7	2202.7	2202.46
AFLEVNEEGSEAAASTAVVIAGRSLNPNRVTFK	K34	3449.4	3449.5	3448.85
ANRPFLVFIREVPLNTIIFMGRVANPBVK	K35	3421.8	3421.8	3421.2

[a] Standard single-letter abbreviations are used for all normal amino acids. B represents derivatized cysteine. The following abbreviations are used for sugars: N, *N*-acetylhexosamine, H, hexose; F, fucose; S, sialic acid. [b] The sequence of peptides corresponds to the position in the primary structure of human antithrombin (see Figure 3). [c] n.d., not detected because of small size; n.p., not present. [d] The separate glycoforms designated (1) and (2) for peptides K8, K15, and K18 represent major forms present.

fraction. In marked contrast, the carbohydrate at position 155 showed much greater heterogeneity, with large differences between the L and H isoforms. The L form was all complex, with most being biantennary and lesser amounts mono- and triantennary. The H form showed a roughly even mixture of biantennary complex, oligomannose, and hybrid chains.

The extent and site specificity of sialylation and fucosylation were also compared. This assessment is simplest for positions 96 and 192, since the carbohydrate at each of these positions is of the same type (complex) in both antithrombin isoforms. Similar extents of sialylation were present in each isoform at a given site, though the degree of modification was lower at position 192 than at position 96, with less fully sialylated species for the Asn192 carbohydrate (Figure 6). Fucosylation at positions 96 and 192 in both isoforms was close to 100%. For position 155 only 55% of the chains in the H isoform and 81% of the chains in the L isoform were sialylated. The only complete difference between the two isoforms was the degree of fucosylation at position 155. Whereas the chains at this position in the L isoform were 97% fucosylated, those in the H isoform completely lacked fucose side chains (Figure 7).

Although the analysis of the electrospray mass spectrum was confined to those peaks with an intensity >5% that of the largest peak, this is unlikely to result in a biased conclusion with respect to the glycoform differences in the types of carbohydrate present for two reasons. First, in the L glycoform, analysis of the K15 glycopeptides indicated no fucose present, whereas analysis of both the K8 and K18 peptides under the same conditions showed 100% of chains with fucose. Fucosylation *per se* therefore neither favors nor disfavors presence in the peaks of >5% intensity. Second, in comparing the carbohydrate types of the K15 glycopeptides of the H and L isoforms, the analysis used was capable of demonstrating very significant differences.

Table 2: Percentage Glycoform Distribution for H and L Forms of N135Q Recombinant Antithrombin from Electrospray Mass Spectrometric Analysis of Peptides K8, K15, and K18

		L isoform[c]			H isoform[c]		
		N96	N155	N192	N96	N155	N192
High-Mannose							
N_2H_5	1217.50					13.2	
N_2H_6	1379.24					14.9	
N_2H_7	1541.38					3.4	
Complex							
monoantennary							
N_4H_4F	1404.30		8.7				
N_3H_4S	1549.42					3.1	
N_3H_4FS	1695.56	2.5	5.3				
N_4H_4FS	1898.76			2.2			2.2
biantennary							
N_4H_5F	1769.64		9.5	5.8			5.7
N_5H_4F	1810.70			4.6			3.8
N_4H_5S	1914.76					10.2	
N_4H_5FS	2060.90	14.8	36.4	38.7	13.9		33.4
N_4H_5FSg	2076.90			7.3			
N_5H_4FS	2101.96	2.2		16.3			6.7
N_4H_5Sg	2117.96						2.5
$N_4H_5S_2$	2206.02		3.1			18.1	
$N_4H_5FS_2$	2352.16	37.3	28.9	25.2	42.9		43.3
N_4H_5FSSg	2368.16	3.4			2.8		2.4
N_5H_5SSg	2425.22	2.5			2.5		
triantennary							
N_5H_6FS	2426.24	3.0			2.4		
N_8H_4FS	2711.56		2.4				
$N_5H_6FS_2$	2717.50	14.4			11.7		
$N_6H_5FS_2$	2758.56	3.3					
$N_6H_6FSg_2$	2952.70		5.3				
$N_5H_6FS_3$	3008.76	14.0			21.3		
$N_6H_6S_3$	3065.82	2.6			2.7		
Hybrid							
N_3H_5	1420.30					4.3	
N_3H_6	1582.44					8.3	
N_3H_5S	1711.56					9.7	
N_3H_6S	1889.70					14.6	

[a] Abbreviations: N, *N*-acetylhexosamine; H, hexose; F, fucose; S, sialic acid (*N*-acetylneuraminic acid); Sg, *N*-glycolylneuraminic acid. Types of carbohydrate were identified by mass and by comparison with a database of all possible compositions. Independent compositional analyses have confirmed the validity of this analytical approach. [b] Oligosaccharide masses were calculated from the observed mass of the glycopeptide by subtraction of the mass of the peptide. [c] One hundred percent represents all identified glycopeptides with an intensity >5% that of the most abundant peak in the electrospray spectrum.

The finding of similar types of quantities of carbohydrate in the K8 and K18 glycopeptides between the H and L isoforms is therefore unlikely to be due to an insensitivity or bias of the analysis against the presence of other types of carbohydrate chain.

Monosaccharide Composition Analysis of Wild-Type Antithrombin Isoforms. Given this correlation between the presence of fucose on carbohydrate chains at position 155 and a reduction in the affinity of the antithrombin for heparin, it would be expected that there should also be differences in fucosylation between the three wild-type antithrombin heparin-affinity isoforms. These three isoforms have been designated I, II, and III (Fan et al., 1993) and have affinities for heparin in the order I < II < III. The expectation is that forms I and II, which both carry carbohydrate chains at positions 96, 135, 155, and 192, should differ in the number of fucose residues present, with one more fucose in form I than in form II, corresponding to fucosylation of the carbohydrate at position 155 in form I but not in form II.

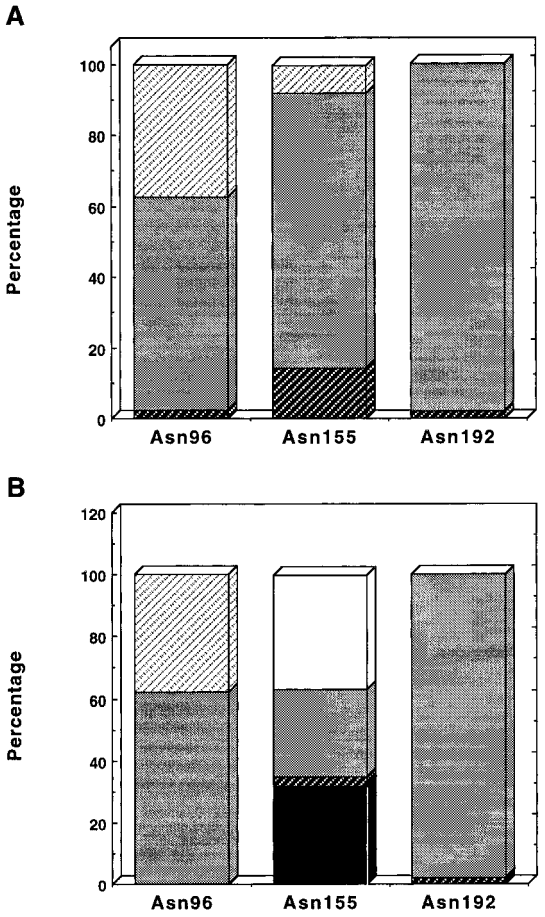

FIGURE 5: Site-specific distribution of carbohydrate chains by type in L and H isoforms of N135Q recombinant antithrombin based on data given in Table 2. (A) L N135Q glycoform; (B) H N135Q glycoform. Light hatching, monoantennary complex carbohydrate; gray, biantennary complex carbohydrate; heavy hatching, triantennary complex carbohydrate; black, high mannose; white, hydrid carbohydrate.

Similarly form III, which probably lacks carbohydrate at position 135 and has heparin affinity similar to the H isoform of the recombinant N135Q variant, should have fucose residues only at positions 96 and 192. Although we did not perform the same detailed peptide mapping and mass spectrometric analysis of these wild-type isoforms as for the N135Q isoforms, we did determine the total monosaccharide composition of the carbohydrate present. As predicted, differences were found for the number of fucose residues present. The number of residues per antithrombin molecule declined from about 4 in form I to about 3 in form II and to about 2 in form III (Table 3). Other changes were also found that corresponded to the previously found general decrease in the extent of glycosylation from form I to form II to form III (Fan et al., 1993) (Table 3). These changes were smaller on a percentage basis, however, than the decrease in fucosylation.

Properties of N135Q-N155Q Recombinant Antithrombin. In contrast to the two isoforms of recombinant antithrombin obtained by heparin−Sepharose affinity purification of medium from BHK cells expressing the single-mutation N135Q variant, only a single isoform of antithrombin was obtained from the medium of cells expressing the double-mutation N135Q-N155Q variant. This sample is expected to carry carbohydrate chains only at positions 96 and 192. In keeping with this, the protein migrated faster on SDS−

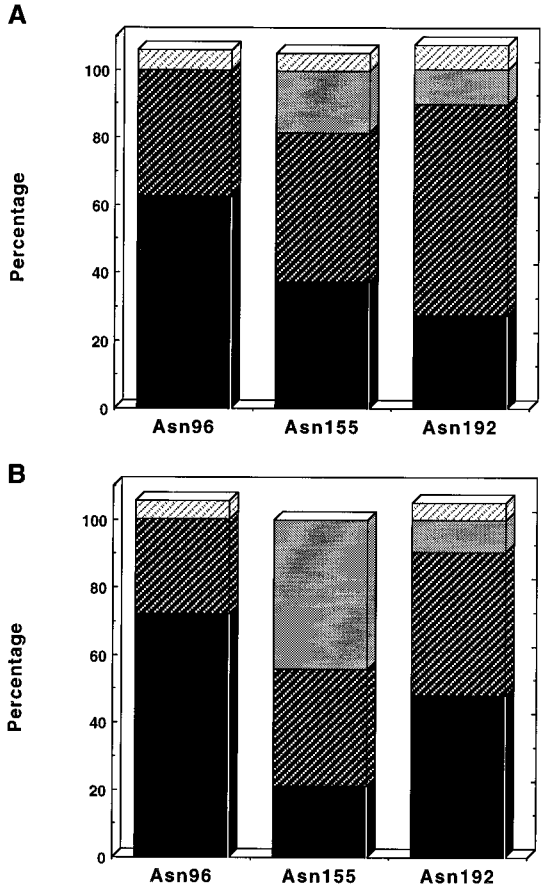

FIGURE 6: Site-specific distribution of sialic acid residues in L and H isoforms of N135Q recombinant antithrombin based on data given in Table 2. (A) L N135Q glycoform; (B) H N135Q glycoform. Black, completely sialylated; heavy hatching, partially sialylated; gray, asialo; light hatching, contains one or more *N*-glycolylneuraminic acid residues. One hundred percent represents all those carbohydrate chains that have the potential to be sialylated. Some sites have total sialylation greater than 100%. This results from inclusion of N-glycolylneuraminic acid as a separate category as well as in degree of sialylation.

PAGE than the three-carbohydrate chain N135Q H isoform, which in turn migrated faster than the four-carbohydrate chain wild-type form II (not shown). To better define the salt concentration at which the N135Q-N155Q antithrombin eluted from heparin−Sepharose relative to the H and L N135Q isoforms, some of each antithrombin was rechromatographed on a small column of heparin−Sepharose and the salt concentration for elution was determined by conductivity measurements of the fractions. The N135Q-N155Q antithrombin eluted as a single peak at a salt concentration for the peak maximum of about 1.9 M. This compares with salt concentrations of 1.3 and 1.9 M for the L and H isoforms, respectively, of N135Q antithrombin. The K_D for dissociation of full-length high-affinity heparin from complex with this double variant, determined by following the enhancement of endogenous tryptopan fluorescence upon binding heparin, was 3 ± 2 nM at I 0.3. This compares with a value of 9 ± 2 nM for the H isoform and 20 ± 2 nM for the L isoform measured under the same ionic strength conditions (I 0.3) (Turko et al., 1993). The difference in K_D of the N135Q-N155Q and H N135Q, despite similar salt concentrations for elution, may reflect a nonionic effect of the N155 carbohydrate on the heparin affinity.

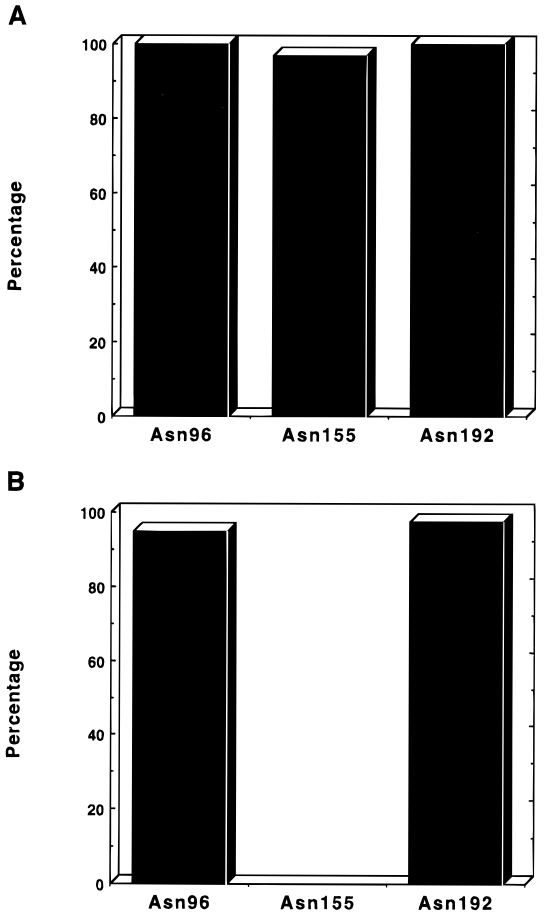

FIGURE 7: Site-specific distribution of fucose residues in the L and H isoforms of N135Q recombinant antithrombin based on data given in Table 2. (A) L N135Q glycoform; (B) H N135Q glycoform.

Table 3: Monosaccharide Composition of Recombinant Wild-Type Human Antithrombin Isoforms I, II, and III

	monosaccharide composition[a] (mol/mol of antithrombin)			
antithrombin	fucose	GlcNAc	galactose	mannose
I	3.8 ± 0.1	14.0 ± 0.8	8.0 ± 0.16	11.5 ± 0.5
II	2.7 ± 0.1	11.5 ± 0.6	6.9 ± 1.4	10.7 ± 0.6
III	1.7 ± 0.02	8.3 ± 0.2	4.5 ± 0.3	8.5 ± 0.1

[a] The values given are the mean ± the range of triplicate determinations.

DISCUSSION

From a detailed analysis of the site specificity of glycosylation of the H and L isoforms of recombinant human N135Q variant antithrombin, we found a strong correlation between the presence of fucose in the carbohydrate at position 155 and a reduction in heparin affinity. This correlation suggests that this difference in fucosylation is the basis for the existence of two isoforms of N135Q antithrombin, as defined by affinity for heparin. Although other glycosylation differences were present, none of them represented a clear distinction between the two isoforms and thus could not be the basis for determining whether the heparin affinity was high or low. Thus, although all of the carbohydrate at position 155 was of the complex type in the L isoform, there was still a major amount of complex carbohydrate (31%) at this position in the H isoform

(Figure 6). At other positions the differences between the H and L isoforms were even less pronounced (Figures 5 and 6).

Fucosylation as a Potential Modulator of Antithrombin Activity in Vivo. The binding of antithrombin to heparin results in very large increases in the rates at which target proteinases of the blood coagulation pathways are inhibited (Olson & Björk, 1994). *In vivo*, heparin and heparin-like glycosaminoglycans are found in the vascular endothelium rather than freely circulating in the blood stream. Thus the antithrombin species that are the most effective at inhibiting thrombin or factor Xa are most likely to be those that are surface-bound to heparin or heparin-like species. Anything that alters the interaction of antithrombin with these heparin-like glycosaminoglycans could therefore have consequences for the antithrombotic potential of the antithrombin. In the case of β-antithrombin, which lacks carbohydrate at position 135, it has been shown that there is preferential association of the β-form with the aortic wall (Witmer & Hatton, 1991). In contrast, a variant of antithrombin isolated from a patient with pulmonary embolism was shown to have reduced heparin affinity as a result of carrying an extra carbohydrate chain at position 7, which was a consequence of an Ile → Asn mutation at that position (Brennan et al., 1988). Alteration in the carbohydrate content and structure of antithrombin can thus have consequences *in vivo*. Although neither report on the structure of the carbohydrate of human plasma α-antithrombin shows the presence of fucose (Franzén et al., 1980; Mizuochi et al., 1980), core fucose has been demonstrated in other normal blood plasma proteins such as factor VIII (Hironaka et al., 1992) and may occur in antithrombin under altered physiological conditions where the levels of fucosyltransferases are elevated. An example of the latter is in various types of cancer. Thus, it has been shown in patients with hepatocellular carcinoma that there are 2-fold higher levels of α-6-fucosyltransferase activity (Hutchinson et al., 1991) and that this correlates with a specific change in the nature of the carbohydrate on their α_1-proteinase inhibitor, a serpin related to antithrombin, from nonfucosylated to core-fucosylated complex chains (Saitoh et al., 1993). A similar increase in fucosylation of plasma α_1-proteinase inhibitor has been reported in patients with breast and ovarian cancers (Goodarzi & Turner, 1995).

Implications for Wild-Type Antithrombin and Other Glycosylation Variants. The results presented on both the wild-type antithrombin isoforms and the single N135Q-N155Q isoform are consistent with the effect of fucosylation being general. Thus for wild-type antithrombin, which occurs in three isoforms, two of which carry carbohydrate at all four potential glycosylation sites (forms I and II) and one of which carries only three of the four carbohydrate chains, there is close to an integral decrease in the total number of fucose residues per antithrombin molecule (Table 3) in the series I to II to III. This is what is predicted if I and II differ in heparin affinity as a result of fucosylation of the Asn155 carbohydrate in I but not in II, and if II and III (neither fucosylated at the Asn155 carbohydrate) differ in heparin affinity only as a result of the absence of carbohydrate at Asn135 in III but not in II (paralleling the difference between the plasma β and α forms, respectively). In contrast, other differences, such as the presence or absence of sialic acid, should not be important in determining the heparin affinity. In keeping with this, it has been shown that enzymatic removal of sialic acid does not affect the heparin affinity of antithrombin (Björk et al., 1992). However, complete removal of carbohydrate at position 155, such as in the N135Q-N155Q double variant, should remove the opportunity to modify the heparin affinity by fucosylation at Asn155 and thus result in only a single isoform, as was found.

Specific Effects of Carbohydrate on Heparin Affinity. Carbohydrate chains on antithrombin thus appear, at best, to have a neutral effect on the affinity for heparin and, at worst, to cause a reduction in affinity. At position 135 the presence of carbohydrate results in a 13-fold reduction in heparin affinity in plasma antithrombin (Turko et al., 1993), whereas the presence of carbohydrate at position 155 causes about a 3-fold reduction in heparin affinity, as judged by comparing the dissociation constants of the heparin complexes with the N135Q H isoform and the N135Q-N155Q double variant. Fucosylation of the Asn155 carbohydrate, however, can cause an approximately 2-fold additional reduction in heparin affinity (Turko et al., 1993). Both types of modification of heparin affinity are likely to result from reduction in accessibility of heparin to its binding site rather than from structural changes in the polypeptide portion of antithrombin, since it has been shown that differences in glycosylation do not affect the conformationally sensitive ^{1}H NMR spectrum of antithrombin isoforms outside of the carbohydrate-dominated regions of the spectrum (Fan et al., 1993). In the case of fucosylation the effect may result from an alteration in the preferred orientation of the whole carbohydrate chain or in its mobility as a result of the presence of fucose on the *N*-acetylglucosamine that is directly attached to the protein asparagine. This explanation is supported by a recent study which showed that the presence of a core fucose residue rigidified the antennae and favored an extended conformation of an isolated biantennary complex carbohydrate chain (Stubbs et al., 1996). Another recent study on the effect of an O-linked fucose residue on the stability of the protein to which it was attached showed an increase in stability resulting from specific fucose−protein interactions (Mer et al., 1996).

Site-Specific Differences in Glycosylation in Antithrombin. Separate factors must be considered in attempting to explain (i) the extent of glycosylation at a particular site in antithrombin and (ii) the extent of processing of a carbohydrate chain once it is attached. The presence or absence of carbohydrate at a potential glycosylation site is most likely determined by local primary structure rather than the ultimate local tertiary structure, since transfer of the carbohydrate chain occurs cotranslationally. In contrast, processing of the initially attached $Glc_3Man_9GlcNAc_2$ chain occurs posttranslationally in the Golgi after the polypeptide has folded. Here transferase accessibility, determined by the local tertiary structure, may be more important.

Studies on the rate of glycosylation of hexapeptides with the sequence Tyr-Asn-Gly-X-Ser-Val, in which X was varied, have shown that asparagine could be glycosylated when X was threonine, serine, or cysteine, with rates in the order threonine > serine > cysteine (Bause & Legler, 1981). The tripeptides covering the glycosylation sites at positions 96, 135, 155, and 192 in human antithrombin are Asn-Asp-Thr, Asn-Lys-Ser, Asn-Glu-Thr, and Asn-Lys-Thr (Chandra et al., 1983). This provides an explanation for the lower frequency with which asparagine 135 is glycosylated, since

this is the only asparagine of the four with serine rather than threonine in the third position. This has been confirmed by mutation of S137 to threonine, which resulted in complete glycosylation of Asn135 (Picard et al., 1995). The lower glycosylation of Asn135 leads to the 10% of plasma antithrombin (β form) that carries carbohydrate chains only at positions 96, 155, and 192 (Peterson & Blackburn, 1985) and probably accounts for the approximately 20% of BHK-derived recombinant antithrombin that carries only three carbohydrate chains (form III) (Fan et al., 1993).

As a newly synthesized glycoprotein traverses the rough endoplasmic reticulum and is then passed to the Golgi stack, processing of the carbohydrate occurs that changes the type of chain from high-mannose to hybrid to complex and can determine the degree of chain branching, of sialylation, and of fucosylation (Kornfeld & Kornfeld, 1985). From the distributions of different carbohydrates at asparagines 96, 155, and 192, it appears that there is a processing hierarchy 96 > 192 > 155. Thus the Asn96 carbohydrate is exclusively of the complex type, is completely fucosylated, and has a high proportion of triantennary chains (Table 2). The Asn192 carbohydrate is also exclusively of the complex type and is also almost completely fucosylated (97%) but contains no triantennary chains. Instead the complex chains are almost exclusively biantennary (98%). The degree of sialylation also follows this trend, with a higher percentage being completely sialylated in the Asn96 carbohydrate chains (66% averaged between H and L isoforms) than in the Asn192 chains (36% averaged between H and L isoforms). The Asn155 carbohydrate is much less completely processed. Only this site carries detectable amounts of high-mannose and hybrid chains (16% and 18%, respectively, averaged between the H and L isoforms). The remaining carbohydrate is complex, shows the lowest degree of sialylation, and as pointed out above, is completely nonfucosylated.

A final point is that the different distributions of carbohydrate types at Asn155 between the H and L isoforms is understandable in terms of the influence of fucosylation in determining whether heparin affinity is H(igh) or L(ow). Thus, high-mannose and hybrid chains are not usually fucosylated (Kornfeld & Kornfeld, 1985) and, when present at Asn155, should result in H isoform antithrombin. In those antithrombin molecules that carry complex carbohydrate at Asn155, those that are nonfucosylated should belong to the H isoform, whereas those that are fucosylated will belong to the L isoform. Fractionation of the total recombinant antithrombin pool by affinity for heparin−Sepharose thus results in a separation by type of carbohydrate at position 155 (high-mannose, hybrid, and complex for the H isoform vs 100% complex for the L isoform) solely as a result of whether or not the chains are fucosylated.

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