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Structure of β-Antithrombin and the Effect of Glycosylation on Antithrombin's Heparin Affinity and Activity

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Antithrombin is a member of the serpin family of protease inhibitors and the major inhibitor of the blood coagulation cascade. It is unique amongst the serpins in that it circulates in a conformation that is inactive against its target proteases. Activation of antithrombin is brought about by a conformational change initiated upon binding heparin or heparan sulphate. Two isoforms exist in the circulation, α-antithrombin and β-antithrombin, which differ in the amount of glycosylation present on the polypeptide chain; β-antithrombin lacks the carbohydrate present at Asn135 in α-antithrombin. Of the two forms, β-antithrombin has the higher affinity for heparin and thus functions as the major inhibitor *in vivo* even though it is the less abundant form. The reason for the differences in heparin affinity between the α and β-forms have been shown to be due to the additional carbohydrate changing the rate of the conformational change. Here, we describe the most accurate structures of α-antithrombin and α-antithrombin + heparin pentasaccharide reported to date (2.6 Å and 2.9 Å resolution, respectively, both re-refinements using old data), and the structure of β-antithrombin (2.6 Å resolution). The new structures have a remarkable degree of ordered carbohydrate and include parts of the antithrombin chain not modeled before. The structures have allowed a detailed comparison of the conformational differences between the three. They show that the structural basis of the lower affinity for heparin of α-antithrombin over β-antithrombin is due to the conformational change that occurs upon heparin binding being sterically hindered by the presence of the additional bulky carbohydrate at Asn135.

Keywords: crystal structure; serpin; carbohydrate; glycosylation; heparin

Introduction

Antithrombin is an important regulator of the blood coagulation cascade, as shown by the high incidence of venous thrombosis among individuals with hereditary antithrombin deficiency.[1] It inhibits not only thrombin but also factors IXa, Xa, XIa and XIIa, circulating in blood in an inactive form and becoming active upon association with glycosaminoglycans. These include heparin (therapeutically) and heparan sulphate (physiologically).[2] The mechanism of heparin activation of antithrombin involves a change in conformation of the protein, the new conformation having a 300-fold higher affinity for the protease substrates.[3] This is the basis of the clinical use of low molecular mass heparin as an anticoagulant in the treatment and prevention of thromboembolic disease.[4]

Native antithrombin is found in a low heparin affinity form, α-antithrombin, and a higher heparin affinity form, β-antithrombin. The difference in dissociation constant between β-antithrombin and α-antithrombin is threefold for the pentasaccharide core of heparin, and over tenfold for full-length heparin.[5–7] Physiologically, β-antithrombin is thought to be more important than α-antithrombin for controlling thrombogenic events caused by injury to the vascular wall, even though it is present in plasma at only 5–10% the levels of

Abbreviations used: CDGS, carbohydrate-deficient glycoprotein syndrome.

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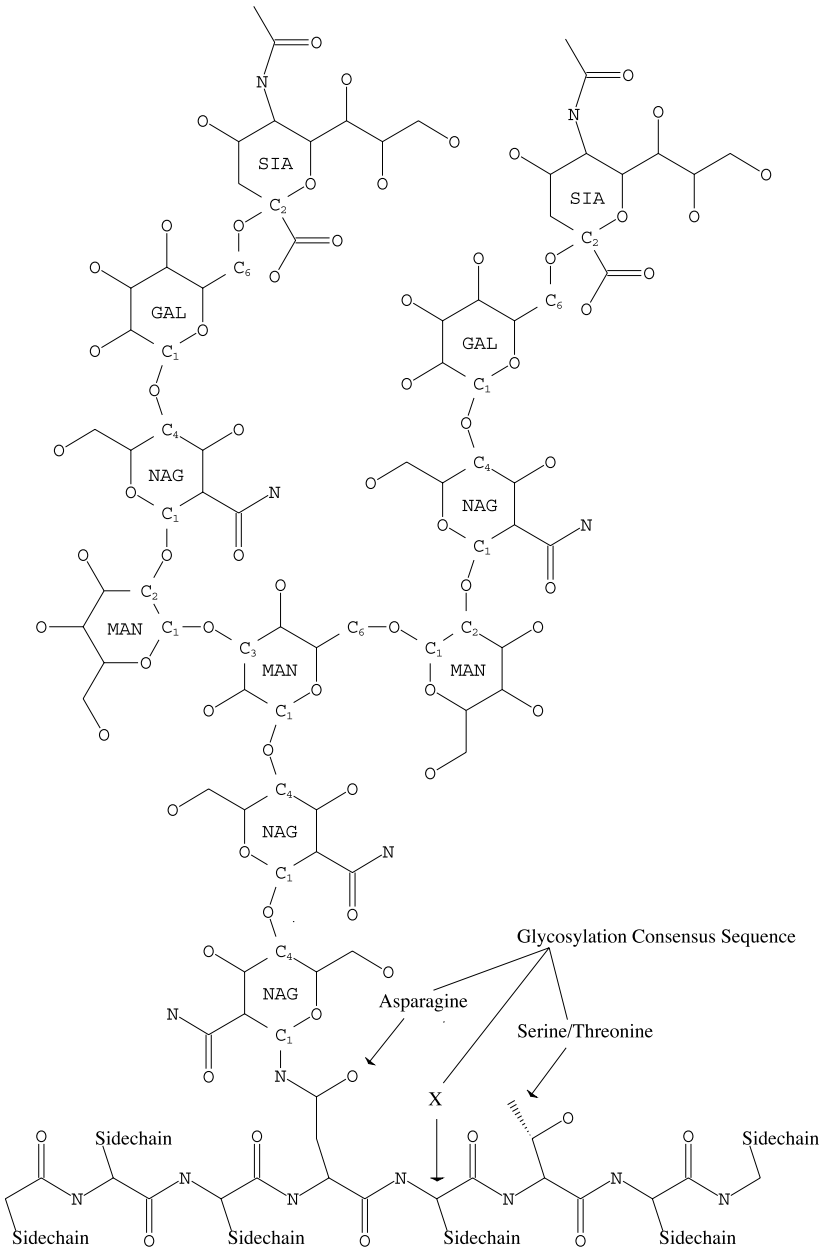

Figure 1. Schematic diagram of the structure of the oligosaccharide linked to Asn96, Asn135, Asn155 and Asn192. The oligosaccharide is of the complex type, is bi-antennial, and has terminal sialic acid residues. Each oligosaccharide increases the molecular mass by 2204 Da or ~4% the molecular mass of the unglycosylated 464 amino acid residue protein (52,602 Da).[37,38]

α-antithrombin.[8] Indeed, thrombin inhibitory activity after injury of the aorta has been attributed to the β-antithrombin fraction of antithrombin alone.[9] β-Antithrombin is taken up from the circulation by the aortic wall and the major organs faster than for α-antithrombin,[8] and transendothelial passage and subendothelial absorption of β-antithrombin is faster than that of α-antithrombin, while subendothelium-bound β-antithrombin is desorbed by thrombin more readily than α-antithrombin. Antithrombin also inhibits the mitogenic activity of thrombin for smooth muscle cells. β-Antithrombin has an inhibitory capacity for the thrombin-induced proliferation of smooth muscle cells in the absence of heparin (possibly using glycosaminoglycans produced by smooth muscle cells as a cofactor), while α-antithrombin requires heparin co-factor.[10]

The two isoforms of antithrombin arise from differences in glycosylation. Antithrombin is modified by the addition of either three or four N-linked oligosaccharide side-chains of the bi-antennial complex type. Most of the chains (70%) are bisialylated but some (30%) lack one *N*-acetylglucosamine at the mannose-α1-3 or mannose-α1-6 branch (Figure 1). Enzymatic removal of the glycosylation drastically reduces the half-life of antithrombin *in vivo*.[11,12] The consensus motif for N-linked glycosylation is Asn-X-Ser/Thr, where X can be any amino acid except Pro and a β-turn conformation of the neighboring amino acid residues is preferred. Of the four glycosylation consensus motifs, three are of type Asn-X-Thr (at Asn96, Asn155 and Asn192) and one is of type Asn-X-Ser (at Asn135). All four are utilized even though only two (at Asn96 and Asn192) are located in β-turns. However,

Asn-X-Thr consensus motifs are utilized more efficiently than Asn-X-Ser consensus motifs.[13] This leads to partial glycosylation at Asn135 and consequently to two isoforms, α-antithrombin and β-antithrombin, with α-antithrombin having carbohydrate at Asn135 and β-antithrombin lacking carbohydrate at Asn135.[14] Although ~20% of antithrombin chains are not fully glycosylated, there is an even lower fraction of β-antithrombin in plasma (5–10%) because the rate of turnover of β-antithrombin from the circulation is about twice that of α-antithrombin.[15]

Antithrombin's inhibition of proteases involves a series of remarkable conformational changes.[16–18] The native conformation of the protein has three β-sheets, nine α-helices, and several long loops connecting the secondary structural elements. The structure is built around a central five-stranded β-sheet, called the A-sheet, which participates in all the conformational changes. The N-terminal end of the reactive center loop is trapped in the A-sheet and the P1 side-chain[19] is oriented towards the core of antithrombin rather than towards attacking proteases. As a result, antithrombin is trapped in an inactive conformation. During activation, heparin binds to antithrombin through a specific pentasaccharide fragment and initiates conformational changes including extension of helix D towards its C terminus. Transmission of these changes to the reactive center loop expels the hinge region from partial insertion in the A-sheet, and antithrombin becomes activated. Conformational changes continue once antithrombin locates a target protease, and is cleaved. Cleavage of serpins results in an ester bond linkage between the P1 residue of the reactive center loop of the serpin and the active site of the target protease. The reactive center loop rapidly becomes inserted as a sixth strand in the A-sheet, a process that also requires profound conformational changes in the rest of the protein and the induction of gross disorder in the protease.[20] In this way the protease is believed to be irreversibly trapped as a reaction intermediate covalently bound to antithrombin.[20]

Antithrombin can also undergo conversion to the latent conformation that mimics antithrombin in the cleaved state, as the intact reactive center loop is inserted into the A-sheet. The inhibitory and latent conformations of antithrombin readily co-crystallize as a dimer and have been well studied.[1,21]

The large conformational changes involved in the binding of heparin to antithrombin mean that the binding is not a simple one-step reaction, but rather it is broken into two-steps: the association of heparin and antithrombin, followed by conversion of antithrombin to the active conformation. β-Antithrombin's higher affinity for heparin arises not from stronger initial binding to heparin but from faster conversion of β-antithrombin to the active protease-binding conformation. Rapid-kinetic studies have shown that the dissociation equilibrium constant for the first step (K_1) is very similar for the α and β-isoforms but the equilibrium constant for the conformational change induced by heparin in the second step (K_2) is substantially lower for β-antithrombin.[5] The oligosaccharide at Asn135 does not interfere with the initial, weak binding of heparin to α-antithrombin, and indeed in the structure of α-antithrombin there is no indication that the Asn135-linked oligosaccharide interacts with the heparin-binding site. Instead, the carbohydrate side-chain at Asn135 reduces the heparin affinity of α-antithrombin primarily by altering the heparin-induced conformational change. This may take one of two possible forms. Either the attached oligosaccharide decreases conformational flexibility at Asn135 and destabilizes the activated relative to the native conformation, or there is a structural difference between the protein component of α and β-antithrombin such that β-antithrombin has a structure intermediate between the active and native α-antithrombin conformations.

To investigate the structural basis for the activity of β-antithrombin, we have solved the crystal structure of β-antithrombin at 2.6 Å resolution, and in order to compare this structure with equivalent quality structures of α-antithrombin with[18] and without[22] the heparin pentasaccharide, further refinement of these structures was carried out. We here provide a molecular insight for the effect of incomplete glycosylation of Asn135 and demonstrate that the higher heparin affinity of β-antithrombin is not due to an intermediate-active conformation but to a facilitation of the heparin-induced conformational change.

Results

The asymmetric unit of the β-antithrombin crystal structure contained an antithrombin dimer formed by two molecules of β-antithrombin, one in the inhibitory and the other in the latent conformation, and the crystal form was the same as all non-heparin bound antithrombin structures to date (1ant, 2ant, 1dzg and 1dzh). Latent-inhibitory antithrombin dimers are not an artifact of crystallization: the dimer occurs *in vivo*, although it is likely to be rapidly eliminated from the circulation.[21] In latent β-antithrombin a phosphate ion was modeled near Arg145, Lys169 and Lys193 because the density in this region was stronger than that expected for a water molecule, phosphate was present in the crystallization conditions, and the chemical environment of the site was consistent with that of a phosphate ion, although the close proximity to sites of carbohydrate attachment raises the possibility that the density was due to a partially ordered sugar moiety instead. Two glycerol molecules were also included in the models, glycerol being present in the cryoprotectant. The final overall *R*-factor/*R*-free of β-antithrombin was 19.1%/24.2% for data in the range 20.0–2.6 Å.

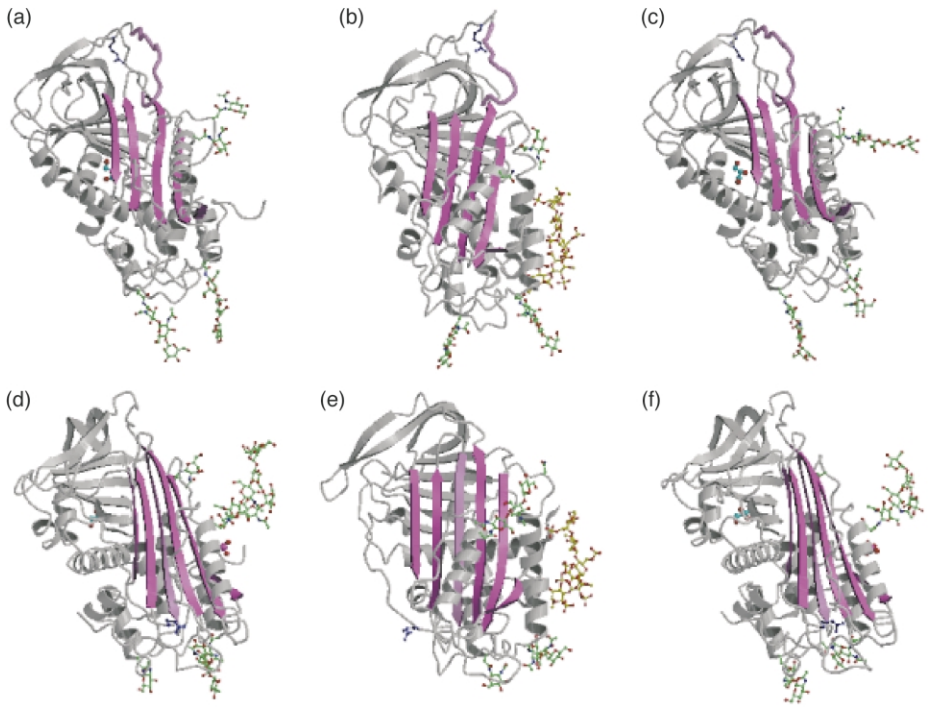

Figure 2. Structures of (a) inhibitory α-antithrombin, (b) inhibitory α-antithrombin + pentasaccharide, (c) inhibitory β-antithrombin, (d) latent α-antithrombin, (e) latent α-antithrombin + pentasaccharide, (f) latent β-antithrombin. The serpin is shown in ribbon representation with the A-sheet highlighted in magenta and the inserted strand shown in light purple. The P1 arginine residue is shown in blue. The glycerol molecules bound to inhibitory and latent α and β-antithrombin are shown with carbon atoms in cyan. The phosphate ion bound to latent α and β-antithrombin is shown with phosphorus atoms in magenta. The Figure was produced with BOBSCRIPT.[39,40]

Refinement using old data[18,22] of α-antithrombin and α-antithrombin bound to heparin pentasaccharide using Refmac5[23,24] with the addition of CNS[25] calculated partial structure factors for bulk solvent correction greatly improved the electron density and brought the structures to a quality similar to that of β-antithrombin. We attribute these improvements to recent advances in crystallographic refinement methods.

The overall *R*-factor/*R*-free for α-antithrombin improved from 21.7%/29.9% for data in the range 5.0–2.6 Å[22] to 19.1%/23.5% for data in the range 20.0–2.6 Å. The improvement arose mainly from: addition of 20 sugar moieties (seven to chain I and 13 to chain L); removal of seven residues (I1-2, I26-29 and I432); addition of 21 residues including eight residues of the reactive center loop in the latent conformation (I38-42, L3-6, L13, L27-28, L396-404); moving the C^{α} position by more than 0.5 Å for 121 residues, 58 in chain L (within the regions L7-25, L43-46, L74, L97, L111-118, L133-138, L199, L204-205, L238-249, L276-277, L288-292, L356-363, L395, L405-408 and L430-431), and 63 in chain I (within the regions I3-25, I43-47, I110-118, I128-138, I199, I243-247, I253-256, I355-359, I382-386 and I392); the addition of a phosphate ion (in the same position as that added to β-antithrombin); and the addition of a glycerol molecule (in the same position as that added to β-antithrombin; Figure 2). Changes were also made in the conformation of the side-chains and whether or not side-chains were included in the model, and the modelling of the bound water molecules: 70 water molecules were included in the final model. The root-mean-square deviations (after least-squares superposition of the models) for C^{α} atoms and all atoms in both the newly refined and old structures were 1.6 Å and 2.0 Å for the I chain, and 1.0 Å and 1.6 Å for the L chain.[26]

The overall *R*-factor/*R*-free for α-antithrombin bound to heparin pentasaccharide improved from 20.3%/28.7% for data in the range 6.0–2.9 Å[18] to 19.9%/25.2% for data in the range 20.0–2.9 Å. The improvement arose mainly from: addition of nine sugar moieties (three to chain I and six to chain L); removal of four residues (I2-5) from the model; addition of 12 residues (I26-28, I37-38, L26-27, L32-38); and moving the C^{α} position by more than 0.5 Å for 62 residues, 27 in chain L (within the regions L5-25, L39-40, L133-135, L177-179, L357-360, L396-397 and L404) and 35 in chain I (within the regions I6-25, I40, I135-138, I175-183, I204, I245, I265, I302, I307, I356-361 and I381-386). Changes were also made in the conformation of the side-chains and whether or not side-chains were included in the model, and the modelling of the bound water molecules: 14 water molecules were included in the final α-antithrombin + pentasaccharide structure. The root-mean-square deviations (after least-squares superposition of the models) for C^{α} atoms and all atoms in both the newly refined and old structures were 0.5 Å and

Table 1. Data processing and refinement statistics

	α-Antithrombin	α-Antithrombin + heparin pentasaccharide	β-Antithrombin
A. *Data processing and refinement*			
PDB entry	1e05	1e03	1e04
Replaces PDB entry	1ant, 2ant	1azx	–
Space group	*P*21	*P*21	*P*21
Cell dimensions			
a,b,c (Å)	61.4, 98.3, 90.4	70.5, 87.1, 97.4	62.1, 99.3, 88.6
β (deg.)	103	109	102
Resolution (Å)	20.00/2.6	20.00/2.9	20.00/2.6
$\langle I/s(I)\rangle$	($\langle F/s(F)\rangle = 29.2$)	5.2	8.3
Completeness (%)	72	94.2	94.5
Multiplicity	1.8	2.5	2.5
R_{merge} (%)[a]	4.8	12.4	8.3
Reflections in working/free set[b]	23,397/1351	22,206/1180	25,938/1351
R-factor/*R*-free (%)[c]	20.7/25.8	19.9/25.2	20.5/24.3
r.m.s deviation of bonds/angles from ideality	1°/0.03 Å	1°/0.03 Å	1°/0.02 Å
B. *Model Latent*(*chain L*)			
C^{α} residues	Ser3-Lys28, Glu42-Lys432	Val5-Glu27, Glu32-Val431	Val5-Glu27, Glu42-Val431
Carbohydrate at Asn96[d]	NAG801	NAG801	NAG801-NAG802
(1) Branch 1[e]	(1) None	(1) None	(1) None
(2) Branch 2[f]	(2) None	(2) None	(2) None
Carbohydrate at Asn135	NAG821	None	Not applicable
(1) Branch 1	(1) None	(1) None	
(2) Branch 2	(2) None	(2) None	
Carbohydrate at Asn155	NAG841-NAG842-MAN843	NAG841-NAG842-MAN843	NAG841-NAG842
(1) Branch 1	(1) None	(1) None	(1) None
(2) Branch 2	(2) MAN844	(2) None	(2) None
Carbohydrate at Asn192	NAG861-NAG862-MAN863	NAG861-NAG862-NAG863	NAG861-NAG862-MAN863
(1) Branch 1	(1) MAN864-NAG865-GAL866	(1) MAN864	(1) MAN864
(2) Branch 2	(2) MAN868-MAN869	(2) None	(2) MAN868
C. *Inhibitory*(*chain I*)			
C^{α} residues	Ser3-Ser25, Gln38-Val431	Asp6-Lys28, Glu37-Val431	Val5-Cys21, Arg46-Val431
Carbohydrate at Asn96	NAG801-NAG802-MAN803	NAG801-NAG802	NAG801-NAG802-MAN803
Carbohydrate at Asn135	NAG821	NAG821	Not applicable
Carbohydrate at Asn155	NAG841-NAG842-MAN843	NAG841-NAG842-MAN843	NAG841-NAG842
Carbohydrate at Asn192	NAG861	None	NAG861-NAG862-MAN863
D. *Non-protein*			
Glycerol	2	0	2
Water	70	14	84
Phosphate ion	1	0	1
Pentasaccharide	0	2	0

[a] $R_{merge} = \sum_{hkl}\sum i|I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum i\langle I_{hkl}\rangle$.
[b] The selection of reflections for the free set was same for all three structures.
[c] *R*-factor $= \sum_{hkl}||F_{obs}| - |F_{calc}||/\sum_{hkl}|F_{obs}|$.
[d] NAG, *N*-acetyl-D-glucosamine; MAN, α-D-mannose; GAL α-D-galactose.
[e] Branch 1, mannose-α1-3.
[f] Branch 2, mannose-α1-6.

1.0 Å for the I chain, and 0.4 Å and 0.9 Å for the L chain.

The amount of oligosaccharide that could be modeled into electron density at each of the glycosylation sites in α-antithrombin and β-antithrombin is listed in Table 1. A remarkable amount of oligosaccharide could be modeled at Asn192 in the latent molecules (Figure 3), where the oligosaccharide formed part of a crystal packing interface. Electron density features in the vicinity of Asn155 in the inhibitory α and β-antithrombin molecules may also have been due to sugar residues, but the features were discontinuous and oligosaccharide could not be built into the density while maintaining the correct stereochemistry. Electron density for the three longest carbohydrate chains, those at AsnI96, AsnL155 and AsnL192 are shown in Figure 3.

Discussion

Kinetic binding studies have shown that the differences in heparin affinity between α-antithrombin and β-antithrombin arise in the second step of the two-step mechanism of antithrombin-heparin binding.[5] The carbohydrate at Asn135 does not hinder the first step, the initial docking

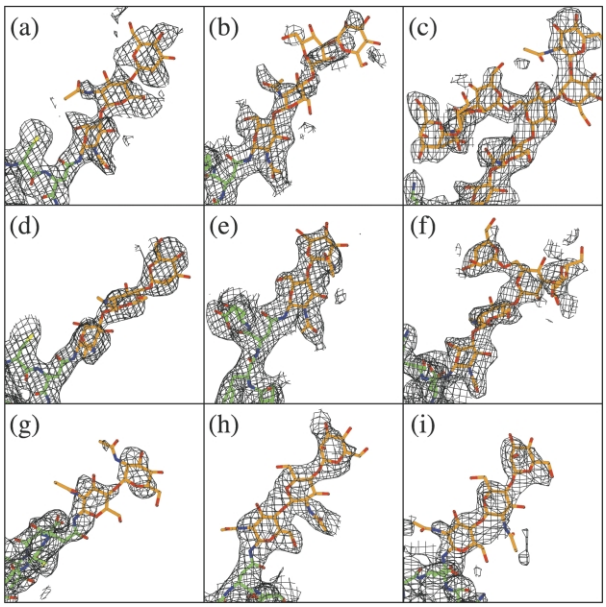

Figure 3. Electron density for the longest carbohydrate chains. Carbohydrate shown attached at (a) α-antithrombin I96, (b) α-antithrombin L155, (c) α-antithrombin L192, (d) β-antithrombin I96, (e) β-antithrombin L155, (f) β-antithrombin L192, (g) α-antithrombin + pentasaccharide I96, (h) α-antithrombin + pentasaccharide L155, (i) α-antithrombin + pentasaccharide L192. The electron density is shown contoured at 0.12 electrons/Å^3 for α-antithrombin, at 0.15 electrons/Å^3 for β-antithrombin and at 0.18 electrons/Å^3 for α-antithrombin + pentasaccharide. The Figure was produced with BOBSCRIPT.[39,40]

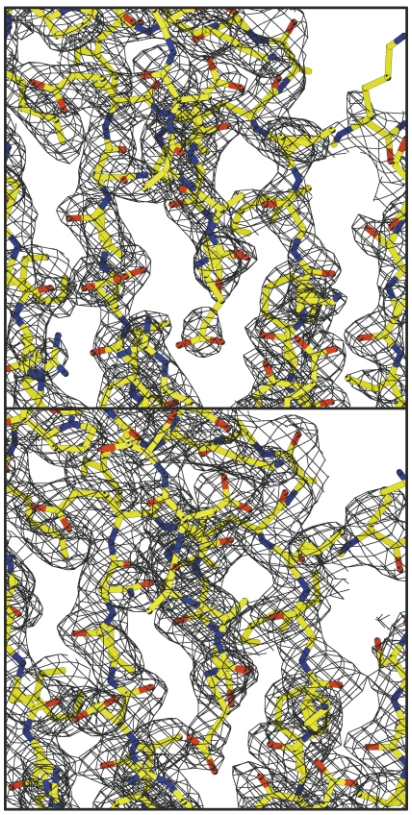

Figure 4. Electron density for the hinge region of α-antithrombin (top) and β-antithrombin (bottom). There are no significant differences between the two structures in this region. Electron density is shown contoured at 0.2 electrons/Å^3. The Figure was produced with BOBSCRIPT.[39,40]

of heparin to antithrombin, but hinders the second, conformational activation step. The underlying reason for the difference has been the subject of speculation as the effect may be either direct or indirect. A direct inhibition would involve the carbohydrate inhibiting the reaction by sterically hindering the conformational change, whereas an indirect effect would arise through the lack of carbohydrate allowing β-antithrombin to take up a structure intermediate between that of α-antithrombin and α-antithrombin + heparin, so that it was capable of switching to the active conformation more rapidly.

Comparison of β-antithrombin with α-antithrombin reveals that they are similar with respect to reactive center loop pre-insertion (Figure 4), partially opened A sheet and the internal orientation of the P1 arginine residue; however, the region of the greatest interest for comparing the structures is the loop at the C terminus of helix D (Figure 5). This loop contains Asn135 and undergoes a major conformational change (from coil to helix) in response to the binding of heparin, so would be an obvious candidate site for the presence of any conformational features in β-antithrombin intermediate between those of α-antithrombin and antithrombin + heparin. The conformation of the residues in this loop varies across all inhibitory antithrombin structures, and is different again in the β-antithrombin structure. The loop is poorly packed to the rest of the protein and electron density in this region was very weak for all structures. High temperature factors in this region indicate that the loop is flexible and/or takes up a number of slightly different conformations. However, the conformation of the loop in β-antithrombin was much more similar to that of α-antithrombin (no helix D extension) than to that of α-antithrombin + heparin pentasaccharide (helix D extension). Differences in the region of the C terminus of helix D therefore do not reveal an intermediate structure.

Other regions that undergo conformational change are also potential sites of intermediate structure. The greatest difference between α-antithrombin and β-antithrombin occurs at the N terminus of helix A, where the helix is extended in α-antithrombin (Figure 5). Helix A elongation was also observed in the structure of α-antithrombin with bound pentasaccharide, where the change appeared in both the latent and inhibitory molecules. Since this feature is present regardless of the presence of the pentasaccharide, it is unlikely that helix A elongation is involved in the heparin-induced conformational change. Instead, it is likely that differences in the conformation of

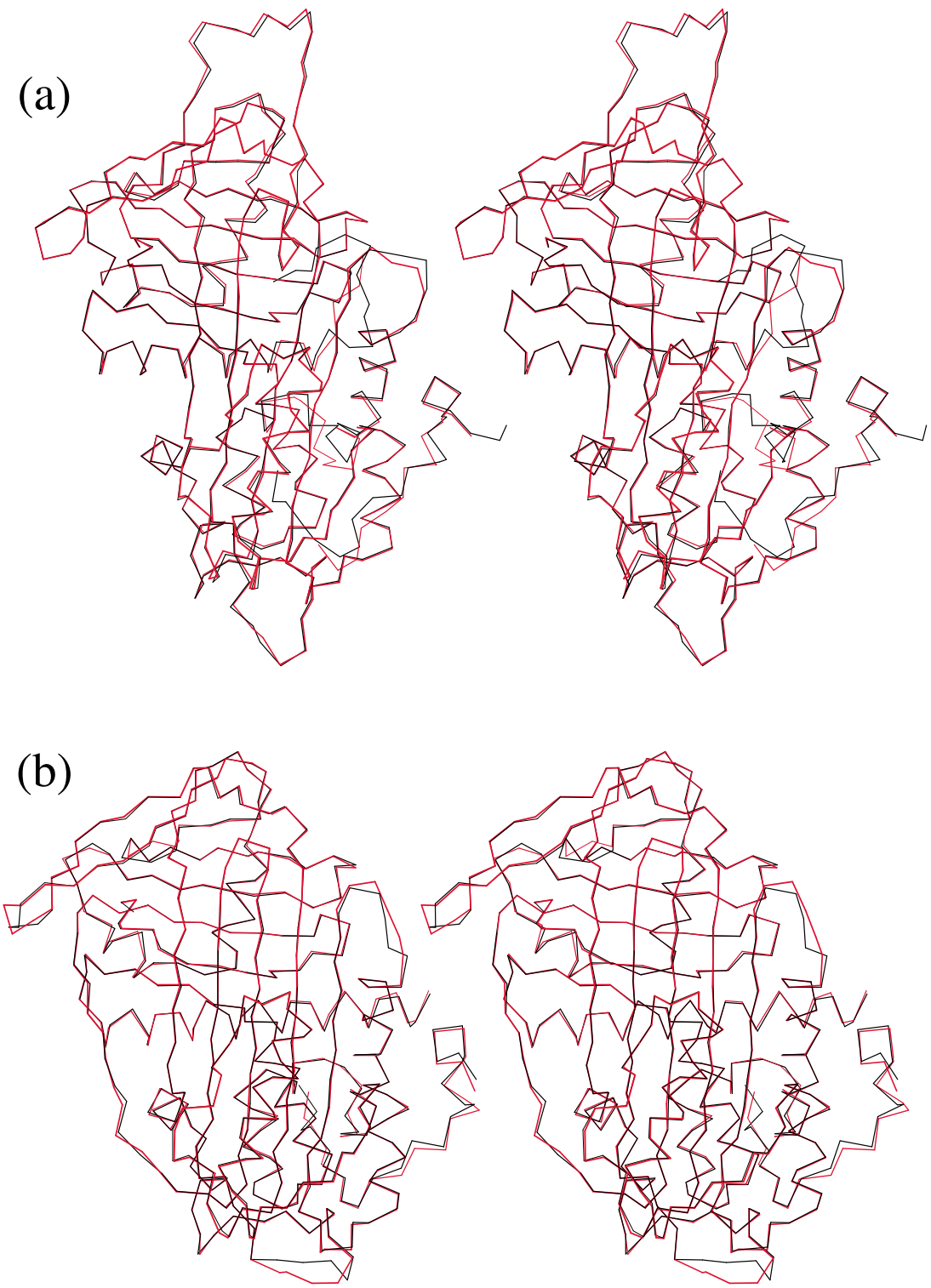

Figure 5. Stereo comparison of the structures of α-antithrombin (black) and β-antithrombin (red), in (a) inhibitory and (b) latent conformations. The Figure was produced with BOBSCRIPT.[39,40]

these regions represent different crystal packing interactions for flexible regions of the protein. The disordered oligosaccharide at Asn135 and the disordered loop at the N terminus of helix A attempt to fill the same solvent region in the crystal. Crowding will limit the volume available to the protein chain in the case were there is oligosaccharide present (in the case of α-antithrombin), causing the protein chain at the end of helix A to be constricted to an interface at the edge of the solvent region, thus becoming ordered and visible in the electron density. Some regions of the N terminus have moved significantly from the earlier refinements and some residues added to the structures

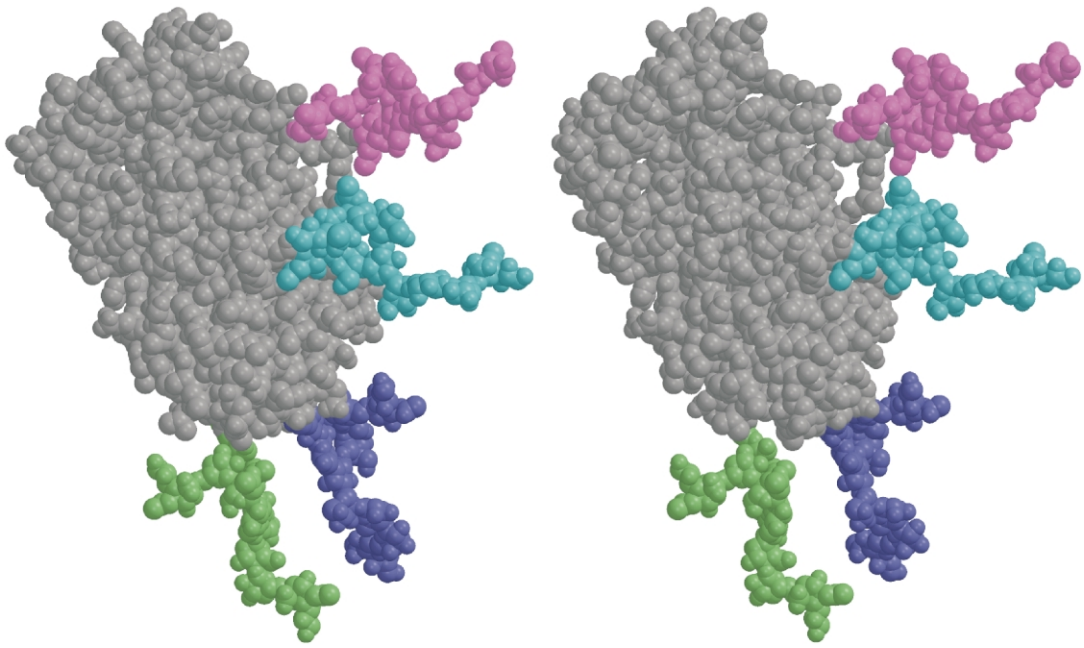

Figure 6. Stereo space-filling representation of α-antithrombin with the complete oligosaccharaide modeled at each glycosylation site. Antithrombin is shown in approximately the same orientation as used in Figure 2. The conformation of the carbohydrate at each position is modeled on the structure of the Asn192-linked carbohydrate, for which only the two terminal sialic acid residues are missing. These have been added to the model in a stereochemically allowed position. Carbohydrate at Asn96 shown in green, at Asn135 shown in magenta, at Asn155 shown in purple, and at Asn192 in cyan. The Figure was produced with BOBSCRIPT.[39,40]

due to better electron density, but none of the residues are those directly involved in heparin binding.

Taken together, the antithrombin structures presented here reveal that the conformation of β-antithrombin is not an intermediate between those of α-antithrombin and α-antithrombin + heparin, and therefore that the lower affinity for heparin can be attributed to the effects of conformational inhibition.

Apart from elucidating the structural basis for the differences between α and β-antithrombin, the new antithrombin structures also contain a remarkable amount of modeled carbohydrate. Carbohydrate adds considerable bulk to the protein (Figure 6). Several glycosylation variants have been produced with marked effects on antithrombin activity. Antithrombin variant Ser137Thr produces an α-antithrombin-low heparin affinity-like isoform exclusively and antithrombin variant Ser137Ala produces a β-antithrombin-high heparin affinity-like isoform exclusively.[5] Mutation of Thr to Ser in the third positions of the glycosylation consensus sequences at Asn96 and Asn192 produces additional glycoforms.[27] Glycosylation variants have also been found to be implicated in antithrombin deficiency and disease states.[28]

An antithrombin glycosylation variant similar to β-antithrombin, caused by the mutation Asn135Thr, has been isolated from an asymptomatic heterozygous individual with levels of antithrombin reduced to 70% normal (borderline type I antithrombin deficiency). Although activity was increased to resemble that of β-antithrombin, the individual was antithrombin-deficient because the variant had lower rates of translation and export compared to those of the wild-type.[29]

Antithrombin variant Ile7Asn (Rouen III) was isolated from an individual with type II antithrombin deficiency who suffered pulmonary embolism during a period of heparin treatment. The mutation introduced a fifth glycosylation consensus motif (Asn-X-Thr) that was partially utilized for glycosylation.[14] The close proximity of this site to the heparin-binding region is likely to inhibit the binding of heparin, but the reduced activity may also arise from inhibition of conformational change as activation of the wild-type causes the N terminus, including residue 7, to change secondary structure from coil to helix. Ile7 moves approximately 5 Å and rotates through approximately 90°, from a surface-exposed position to a partially buried position, and so glycosylation at this site is likely to inhibit the burial of the residue. Indeed, the unglycosylated fraction of the variant protein also has reduced affinity, suggesting that the mutation alone inhibits conformational change.

Antithrombin variant Leu(−10)Pro, isolated from a heterozygous patient with venous thrombotic disease, is a glycosylation variant that causes type I antithrombin deficiency. The variant lacked a functional signal peptide (residues −32 to −1) because residue −10 was not hydrophobic. The

variant was not recognized for glycosylation in the endoplasmic reticulum and the 50% reduction in levels of secreted antithrombin were consistent with absence from the plasma of antithrombin derived from the mutant allele.[12] The activity of the unglycosylated protein was not investigated, but recombinant proteins lacking any one of the four glycosylation sites have increased heparin binding affinities.[5,30]

A different type of glycosylation variant of antithrombin arises in the carbohydrate-deficient glycoprotein syndrome (CDGS). The CDGS are a family of autosomal recessive diseases with multiple organ manifestations. The syndrome causes partial or total lack of terminal sialic acids in the N-linked glycans of secretry glycoproteins, and affects coagulopathy. Antithrombin isoforms lacking one to eight of eight possible terminal sialic acid residues are found with variable intensity and frequency, and levels are reduced to ~50% of the normal lower limit.[31–33] The deficiency is presumably due to faster clearance of the variant, as neuraminidase treatment of both plasma and recombinant antithrombin has shown that removal of sialic acid does not affect activity[7,34] but substantially reduces the plasma half-life.[11]

Post-translational modification by glycosylation not only aids protein solubility and inhibits proteolysis, it can also modify *in vivo* function. This often overlooked property of glycosylation is particularly evident in antithrombin, where the α and β-isoforms, which only differ structurally in the presence or absence of carbohydrate at Asn135, have different *in vivo* localization and activity. The conformational change required to expel the hinge region and induce anticoagulant activity lends itself to regulation by glycosylation because it involves large movements of side-chains and backbone positions. It is this conformational change that is the target of drug design and the more accurate structures described here should greatly aid in this effort.

Materials and Methods

Protein purification

β-Antithrombin was purified from human plasma as described.[35] Briefly, the supernatant from a dextran sulphate/calcium chloride precipitation was applied to a heparin-Sepharose affinity column (Pharmacia) and α and β-antithrombin separated with a NaCl gradient: α and β-antithrombin eluted at ~0.8 M and ~1.3 M NaCl, respectively. Anion-exchange chromatography (HiTrap-Q, Pharmacia) was employed for further purification of β-antithrombin.

Crystallization

β-Antithrombin at 20 mg/ml in 20 mM Tris–HCl (pH 7.4) was crystallized in 16% (w/v) PEG 4000, 50 mM Na/KPO_4 (pH 6.7). Half of the native β-antithrombin converted to latent β-antithrombin during the course of the crystallization.

Data collection

X-ray diffraction data were collected from a single crystal on a MAR Research image plate detector using synchrotron radiation at a wavelength of 1.488 Å (Station 7.2 at Daresbury SRS, UK). The crystal was cryoprotected in 25% MPD, 20% PEG 4000 50 mM Na/KPO_4 (pH 6.7) for about five seconds and then flash-cooled in a liquid nitrogen stream. Data were collected at 100 K to 2.6 Å resolution and were scaled and merged with SCALA, CCP4 and other programs in the CCP4 suite. The free *R* flags (defining the selection of reflections for the free *R* set) were transferred from the α-antithrombin data set.[22] Data statistics are given in Table 1.

Structure determination

Initial phases of the crystal structure of latent/active β-antithrombin dimer were obtained by molecular replacement with AMoRe[24,36] using the 2.6 Å resolution structures of latent and active α-antithrombin (from PDB entry 2ANT) as a search model. Iterative rounds of rebuilding and refinement were conducted using the programs O[26] and Refmac5[23,24] (with bulk solvent correction calculated using CNS[25] and added to the Refmac5 calculated structure factors as partial structure factor terms). The structures of α-antithrombin and α-antithrombin bound to heparin pentasaccharide were further refined using the same methods to an equivalent resolution. Statistics of the refined models are given in Table 1.

Protein Data Bank atomic co-ordinates

The structures discussed here have been deposited with the Protein Data Bank with accession codes 1e03 for α-antithrombin + heparin, 1e04 for β-antithrombin and 1e05 for α-antithrombin.

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