Thrombosis as a conformational disease

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Conformational diseases are a newly recognized group of heterogeneous disorders resulting from the conformational instability of individual proteins. Such instability allows the formation of intermolecular linkages between β-sheets, to give protein aggregation and inclusion body formation. The serpin family of serine protease inhibitors provides the best-studied examples of the structural changes involved. Notably, mutations of α-1-antitrypsin result in its intracellular polymerization and accumulation in the liver leading eventually to cirrhosis. Here we consider how other conformational changes in another serpin, antithrombin, can cause its inactivation with consequent thrombosis. Thirteen different missense mutations in antithrombin are associated with either oligomer formation or with conversion of the active molecule into an inactive latent form. Each of these variant antithrombins is associated with an increased risk of thrombosis that typically occurs in an unexpectedly severe and sudden form. The trigger for this episodic thrombosis is believed to be the sudden conformational transition of the antithrombin with an accompanying loss of inhibitory activity. But what causes the transition? This is still unclear, though a likely contributor is the increased body temperature that occurs with infections hence the frequency of episodes associated with the urinary infections of pregnancy. The search for other causes is important, as the conformational perturbation of normal antithrombin is likely to be a contributory cause to the sporadic and apparently idiopathic occurrence of venous thrombosis.

Key words: thrombosis, heterogeneous disorders, proteins.

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The post-genomic era of the XXIst century has witnessed the development of technology that allows both the sequence and the expression of the whole genome to be analyzed. This technology will be of fundamental relevance in understanding the role of genetics in human disease and already the results being obtained are changing classical concepts in science. The complexity of the human being was confidently expected to require a genetic potential much higher than that carried by lower organisms. However, the original estimate for the human of 100,000 genes coding for 100,000 proteins has now been revised to less than 35,000 genes; a number similar to that present in simpler species such as the fruit fly and the mouse.[1] This result is not only surprising, but also raises a new question: how to explain the significant discrepancy between the number of genes and proteins: 35,000 and 100,000, respectively?[2] An explanation for this discrepancy is that single genes can encode multiple proteins. In eukaryotic cells, the variability of protein products encoded by a single gene is due to both alternative translation start sites and to alternative splicing. Hence, the same nucleotide sequence is able to give a range of proteins with different physiological functions. The final result of this strategy is a significant functional diversity together with genetic economy. Thus proteomics is excelling genomics. But, if the genome cannot be read in a single frame, it is questionable to simplify the study of proteins based only on their primary sequence. Furthermore, the functions arising from the same primary sequence of amino acids may vary according to the folding of the expressed protein. Many proteins are inherently able to change their conformation in a functionally relevant way in response to different conditions or signals. Practically all systems contain proteins that completely change their functional state (active, inactive, or even to a new function) in response to proteolysis, phosphorylation,

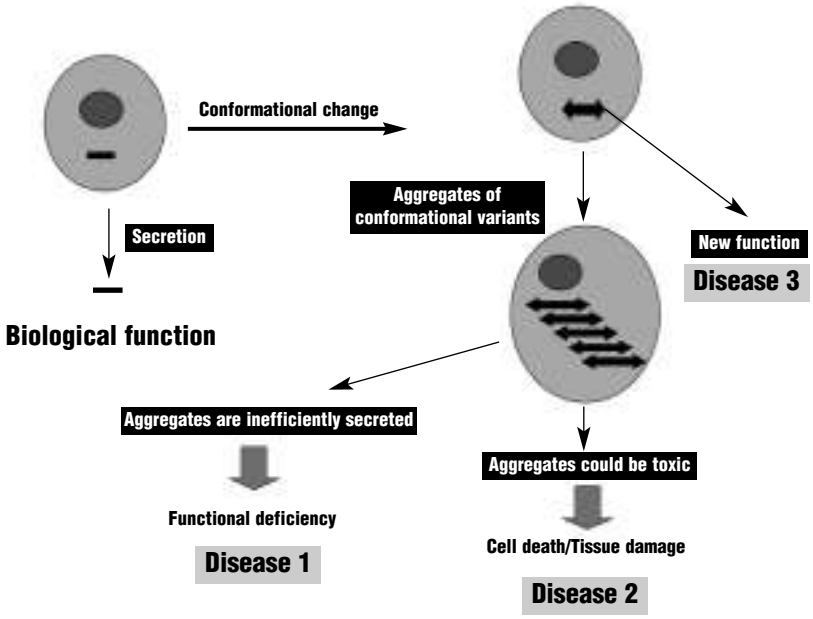

Figure 1: Schematic representation of aggregate formation of β-sheet-rich proteins and its possible pathological consequences. Abnormal conformations allow β-sheet interactions between molecules, forming aggregates inside the cell (oval). These variants (especially aggregates) are usually removed from the intracellular pathway followed by normal proteins, resulting in deficiency of the affected protein, which can be responsible for the development of one disease. In some cases, intracellular aggregates are very stable, being resistant to degradation. Their accumulation might be toxic and, after a long period of time, may result in cell death, causing a different late-onset disease. Finally, the abnormal conformation could lose the native function and acquire new functions that might be involved in a different disease. The thick line or arrows represent different conformational states of a protein.

de-phosphorylation, or binding to another protein. Such secondary modifications provide a successful way to regulate the function of a single protein, as they allow the activity of a protein to be controlled by switching it on or off, both in time and also in localization in cells or tissues. The main advantage of this strategy is its rapidity, since changes in the folding of proteins do not require synthesis of new proteins, and can be achieved instantly. Thus the complexity of a system does not necessarily require a large number of genes and proteins, since the tissue-specific requirements of higher organisms can be met by localized conformational changes.

This recent development in our understanding of the genome has also resulted in significant changes in our understanding of the molecular basis of diseases. In past decades, the recognition of the association of a gene with a disease was dependent on the finding of significant mutations causing either the deficiency or the inactivation of the encoded protein. As expected, mutations resulting in premature stop codons (nonsense mutations and frameshift mutations), and those affecting relevant functional domains readily explained a number of genetic disorders. However, it was difficult in many cases to explain the severe effect of single missense mutations that primarily affect neither the expression nor the function of proteins. Recently, however, an increasing number of papers provide evidence that such apparently minor mutations can modify the folding of the proteins to cause a decreased conformational stability that consequently results in disease.[3]

Conformational diseases

The conformation of proteins is maintained by the tight packing of their amino acid side-chains. The replacement of a single amino acid, subtle modifications of pH or temperature, or any other conditions able to modify the hydrogen-bond network of the protein, can be sufficient to cause a conformational change, especially in proteins with flexible structures. Recent results demonstrate that inappropriate changes in the conformation of an underlying protein often result in intermolecular linkage of the unstable protein. In such conformational diseases,[4] the molecular instability results in aberrant intermolecular linkages, in which single peptide strands become aligned to form highly stable β-sheets. The resulting β-linked structures have at least two different pathological consequences. The first is that because these aberrant β-linked proteins are unable to follow the usual intracellular pathways, their cellular localization and processing is disrupted. Thus, conformational transformation of the protein leading to β-linked aggregation causes a loss-of-function of the normal protein (Figure 1). A second consequence is that, once they have formed, these β-linked aggregates are quite stable, and hence are resistant to intracellular degradation, with a consequent accumulation that leads to formation of inclusion bodies. This accumulation of aggregated protein adversely affects the cell and can lead to its premature death, to give the long-term damage that characterizes the conformational dis-

Table 1. Main human diseases caused by aberrant linkage between β-sheets of conformationally unstable proteins.

Protein	*Disease*
Hemoglobin	Sickle cell anemia
	Unstable hemoglobin inclusion-body hemolysis
	Drug-induced inclusion body hemolysis
Prion protein	Creutzfeld-Jakob disease
	Bovine spongiform encephalopathy
	Gerstmann-Straussler-Scheinker disease
	Fatal familial insomnia
	Kuru
Glutamine repetitions	Huntington's disease
	Spinocerebelar ataxin
	Dentato-rubro-pallido-Luysian atrophy
	Machado-Joseph-atrophy
β-amyloid peptide	Alzheimer's disease
	Down's syndrome
	Familial Alzheimer's--amyloid precursor
	Presenilins 1&2
α-synuclein	Familial Parkinson's disease
Tau protein	Frontotemporal dementia (Pick disease)
Immunoglobulin light chain	Amyloidosis
	Systemic or nodular AL amyloidosis
Serum amyloid A protein	Reactive systemic amyloidosis
	Chronic inflammatory disease
Transthyretin	Senile systemic amyloidosis
	Familial amyloid neuropathy
	Familial cardiac amyloid
β2-microglobulin	Hemodialysis amyloidosis
	Prostatic amyloid
Apolipoprotein AI	Familial amyloid polyneuropathy
	Familial visceral amyloid
Cistatin C	Hereditary (Icelandic) visceral angiopathy
Lysozyme	Familial visceral amyloidosis
Serpins	Serpinopathies
1-antitrypsin	Emphysema/cirrhosis
C-1 inhibitor	Angioedema
Neuroserpin	Familial dementia
Antichymotrypsin	Chronic obstructive bronchitis
Antithrombin	Thrombosis

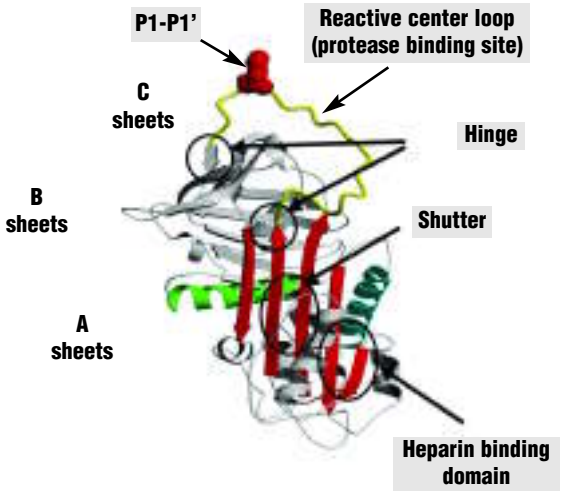

Figure 2. Schematic crystal structure of native antithrombin. α helix (ribbons) and β-sheets (arrows) are shown. The partially inserted reactive loop is also shown, pointing out the amino acids cut by the protease (P1-P1'). The mobility of this loop, which is able to insert inside the central β-sheet A forming a new β-sheet (s4A), is crucial for the efficient inhibitory anticoagulant function of antithrombin. Three regions have special relevance in the structural flexibility and function of antithrombin: A. The shutter region is centrally located and lies at the intersection of the major secondary structural elements of the β-sheet A. Along with the adjacent breach region, the shutter facilitates sheet opening and accepts the conserved hinge of the reactive center loop. B. The hinges of the reactive center. The structural relevance of shutter and hinges is emphasized by the high homology displayed in these regions by the family of serpins. C. The heparin-binding domain, specific to antithrombin and heparin co-factor II.

eases (Figure 1). The occurrence of cell death is cumulative and time-dependent, explaining why many conformational diseases have a slow onset and are developed at older ages. Finally, in some cases, the abnormal form may not only lose its native function but may achieve a new modified function that can be reflected in the development of atypically severe disease.

The term conformational diseases covers a group of heterogeneous disorders, which include the socially and medically relevant dementias of Alzheimer's disease, Parkinson's disease and the prion spongiform encephalopathies. However, the grouping is much more extensive than this and includes the amyloidoses, as well as familial emphysema and thrombosis, amongst many other disorders (Table 1). These diseases, although heterogeneous in origin, share common features in their pathological mechanisms with, in each case, the conformational change in an underlying protein leading to its intermolecular linkage and aggregation. As a consequence, lessons learned from the study of each individual disease have a relevance to the group as a whole and, in particular, to the development of the design of therapies to prevent the protein aggregation and accumulation.

The best studied examples of conformational diseases, in terms of the structural changes involved, are provided by the serpin family of **SER**ine **P**rotease **I**nhibitors.[5] More than 250 different serpins have now been identified in diverse organisms including viruses, mammals, plants, insects and most recently in bacteria.[6] All the members of the family share a tightly conserved structure with more than 30% sequence identity and a common framework tertiary structure.[7] Crystallographic structures of many serpins have now been solved and all are formed of some 400 residues folded into three β-sheets (A-C), and nine β-helices (A-I). A feature of each structure,

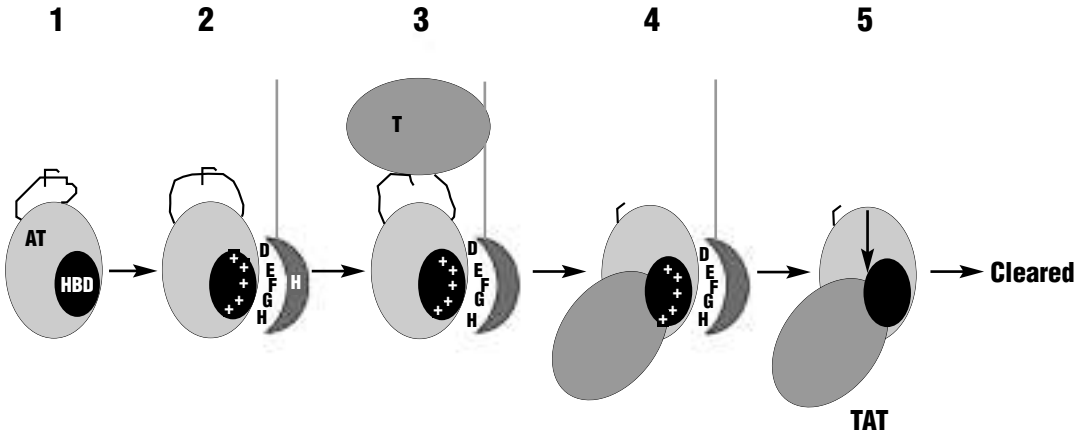

Figure 3. Conformational changes displayed by antithrombin (AT) during the suicide mechanism of inhibition of proteases. (i) The circulating antithrombin has the reactive loop partially inserted inside the molecule. This conformation has low inhibitory capacity. (ii) The binding of the essential pentasaccharide present in heparin and other glycosaminogycans (H) to the heparin-binding domain (HBD) results in a structural change in antithrombin that allows the release of the reactive loop. This process increases the inhibitory capacity of antithrombin up to 300-fold. (iii) Antithrombin binds thrombin (T) or another target protease by the reactive loop, which cuts it between P1-P1'. This proteolysis causes a significant conformational change in the antithrombin molecule. The cut reactive loop (P1-P15) is inserted between β-sheet sA3 and sA5, forming a new β-sheet (sA4). The consequences of these movements are crucial for protease and serpin. (iv) First, the insertion of the reactive loop also translocates the protease to the other pole of the serpin. The protease displays conformational changes that affect the reactive domain, losing proteolytic capacity. (v) Moreover, new interactions can be formed between protease and serpin allowing the formation of an almost irreversible complex (thrombin-antithrombin complex -TAT-) that is rapidly cleared from circulation. Moreover, the formation of the s4A also affects the heparin binding domain, releasing the heparin.

as depicted in Figure 2, is the presence at the top of the molecule of an exposed and mobile peptide loop containing the reactive center of the inhibitor. It is this loop that determines the specificity of inhibition and consequently the sequence of the loop varies from member to member of the family.[8] The flexible structure of this loop is essential for the inhibitory action, which leads to an irreversible destruction of the target protease. This occurs through a suicidal change in the serpin's conformation which disrupts and destroys the protease (Figure 3). However, this structural flexibility also makes serpins especially vulnerable to conformational changes. Therefore, even minor genetic modifications or subtle environmental changes can severely modify the flexibility of the molecule with consequent disadvantageous conformational change. Even small conformational changes of this type can have disastrous affects, due to the key role of serpins in regulating critical physiological processes, including coagulation, fibrinolysis, complement activation, angiogenesis, apoptosis, inflammation, as well as neoplasia and viral diseases (Table 1).[9]

Thrombosis as a conformational disease

An efficient hemostatic system requires a complex mechanism able to respond immediately to vascular damage with the quick release of thrombin. Thrombin is the final protease of the clotting cascade that directly generates the fibrin clot and activates platelets, in two parallel actions leading to the formation of the clot that prevents blood loss. In order to provide the rapid response needed to prevent hemorrhage, the hemostatic mechanism involves a cascade of proteolytic reactions leading to activation of proteases that rapidly amplify the response and generate ample amounts of thrombin. This rapid onset of coagulation is balanced by equivalent anticoagulation and fibrinolytic mechanisms that have to be equally rapid and efficient in their response. The key to maintaining optimal hemostasis is therefore provided by the range of serpins that control each of these series of proteolytic cascades, including heparin cofactor II, the plasminogen activator inhibitors (PAI-1 and 2), α2-antiplasmin and, especially, antithrombin.

Antithrombin is the most important endogenous anticoagulant, as demonstrated by the thrombotic risk associated with patients with heterozygous deficiency and the fact that homozygous deficiency is fatal.[10,11] Antithrombin has the typical three-dimensional structure of all the serpins, but displays two special features. The first is that the reactive center loop of circulating native antithrombin is partially inserted into the body of the molecule, at the same time obscuring the arginine (denoted P_1) that is the key amino acid determining the specificity of inhibition to thrombin and factor Xa. The second special feature of antithrombin is a site that binds a specific pentasaccharide present in heparin and other vascular glycosaminoglycans (Figure 2). The inhibitory

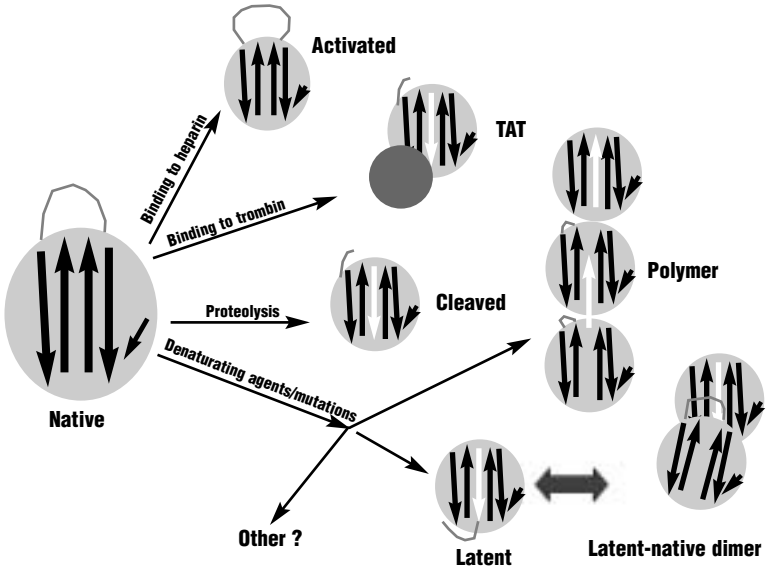

Figure 4. Conformational changes of native antithrombin observed spontaneously or in response to different conditions or mutations. The circulating native antithrombin has the reactive loop partially inserted and five β-sheets A (five-stranded conformation). Binding of heparin allows complete release of the reactive loop, resulting in a five-stranded conformation with increased inhibitory function. Proteolytic rupture of the reactive loop by target or non-target proteases, results in the insertion of the reactive loop, forming a cleaved six-stranded structure (alone or complexed with the protease). The insertion of an intact reactive loop inside the molecule also generates a six-stranded structure that is called latent antithrombin. This structure forms dimers by a C-sheet linkage with a native molecule. Finally, the insertion of the intact reactive loop in the central β-sheet A of other molecules, allows formation of polymers.

activity of antithrombin requires a flexible structure. The binding of this DEFGH pentasaccharide to antithrombin triggers a minor, but functionally relevant change in conformation of the protein that allows the reactive center loop to be completely released (Figure 3). The new conformation has a 300-fold higher affinity for the coagulation protease, factor Xa.[12] The binding of the protease to the reactive loop results in its cleavage between the P1 arginine and the adjacent P1' serine. In doing so, the protease is linked by a covalent bond to the cleaved loop which moves from its external exposed position to form a new central β-sheet s4A in the main β-sheet, transforming the five-stranded molecule into a six-stranded structure. The protease, tethered by its bonding to the loop, is in this way translocated to the other pole of the serpin. These movements have direct structural and functional consequences that affect both the protease and the serpin.

The protease suffers a gross loss of ordered structure, with total disruption of its active site, to give irreversible inhibition of the protease. The heparin-binding site of the antithrombin is modified, returning to a low heparin affinity conformation that allows the release of the heparin.

The movement of the reactive loop of the serpin and the translocation of the protease allow new interactions between the protease and antithrombin leading to the formation of a stable thrombin-antithrombin complex (TAT). The anticoagulant function of antithrombin is completely fulfilled as the TAT complexes are quickly removed from circulation (Figure 3). However, the movement of the reactive center loop and the central opening of β-sheet A, although essential for inhibitory activity, also renders the molecule sensitive to mutations or other change resulting in its dysfunction (Figure 4). The integration of the reactive loop inside the molecule can also result from its proteolytic cleavage by non-target proteases such as elastase. This change in the cleaved antithrombin, from a five-stranded stressed state to a six-stranded relaxed-state, is accompanied by an extraordinary increase in thermal stability and a loss in heparin affinity.

It has been shown that a similar A-sheet transition from a five-stranded to a hyperstable six-stranded form can occur in serpins even if the reactive loop is intact. Thus, the uncleaved reactive loop of antithrombin can be inserted into its own β-sheet A, to form what is called the latent structure.

Alternatively, the opening of β-sheet A of one molecule can allow the entry of the uncleaved reactive loop of another molecule to form a dimer, which then extends into long chains of polymers. As with the latent form, polymeric antithrombins also have a six-stranded structure with an accompanying low affinity for heparin.

Transformation of native antithrombin to the monomeric latent structure takes place spontaneously under physiological conditions, with some 3% of the total circulating antithrombin changing to the latent form each day.[13,14] Polymers, however, do not spontaneously form in the plasma but only appear under severe denaturing conditions such as high temperatures (60°C) or low pH (less than 6.5).[15] As will be shown, the transformations to the latent and polymeric forms are facilitated by missense mutations that affect relevant mobile regions of the antithrombin molecule.[13] Such transformations of native antithrombin to a cleaved, latent or polymeric con-

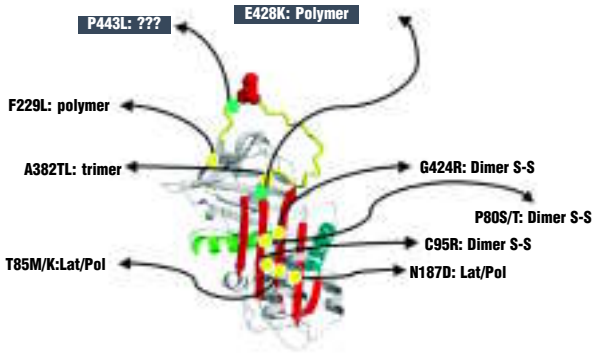

Figure 5. Conformational mutations and the structural consequence identified in antithrombin (white) and heparin cofactor II (gray). The P443L affecting the heparin cofactor II should have a strong conformational effect as the variant is retained inside the cell associated with the GRP78/BiP chaperone, but no oligomers have been identified. S-S: disulfide linked; Lat: latent; Pol: polymer.

formation have direct functional consequences, as each of these forms is inactive. They are not able to bind proteases, and hence there is a loss of the anticoagulant capacity of the native molecule. Therefore, mutations or other conditions that favor spontaneous transformation of antithrombin to its latent or polymeric forms will predictably result in an increased risk of thrombosis.

Conformational mutations in antithrombin and other hemostatic serpins

The world-wide genetic analysis of patients with congenital deficiency of antithrombin has identified some 130 different mutations affecting the gene encoding this protein.[11] Many of these mutations have an obvious effect that explains the associated phenotype. For example, non-sense mutations and insertions or deletions causing a frameshift explain the associated type I deficiency. Moreover, missense mutations affecting the heparin binding site or the reactive center disturb these functional domains, explaining the associated type II deficiency. However, recent attention has focused on a group of mutations that are characteristically associated with particularly severe and episodic thrombotic disease. Some 40 different missense mutations have now been found to result in a severe type I deficiency often associated with pleiotropic consequences. The study of these variants raised the likelihood that the unusually severe disease resulted from an aberrant conformational change rather than just the loss of activity (Figure 5). These deductions were strengthened by the finding that the mutations occurred in the mobile regions of antithrombin, mainly in the hinges of the reactive center loop, or in the region involved in the shutter-like opening of the main β-sheet of the molecule required for insertion of the reactive loop into the sheet. The modification of just a single amino acid in these sensitive regions can disturb the network of interactions in the whole molecule, resulting in overall conformational changes that can affect all the functions of the molecule, including its inhibitory activity and its heparin binding affinity. But most importantly, such conformational changes can result in a loss of stability and facilitate the formation of intermolecular linkages. The significance of this group of mutations is emphasized by their repeated identification in different members of the serpin family, being associated in each case with the late-onset of pathology that characterizes conformational diseases. For example, mutations in the hinge or shutter region of both α1-antitrypsin and neuroserpin are associated with the formation of polymers. The polymerization of each of these serpins apparently occurs by the same mechanism, with the opening of the β-sheet of one molecule allowing the insertion of the reactive loop of the next to give loop-sheet linkages. These polymers are retained inside the cells, causing plasma deficiency and also cell damage due to the toxicity of aggregates, with resultant liver cirrhosis and dementia, respectively.[9] Although such loop-sheet polymers are the best characterized consequence of conformational instability within the serpins, the range of β-interlinkages is more extensive than this one form.[16] In particular, we showed that P80S and G424R mutations affecting the shutter region of antithrombin, identified in two Spanish families with severe deficiency of this anticoagulant, resulted in the formation of dimers with both non-covalent and disulfide linkages of two intact molecules of the mutant antithrombin.[17] In contrast to the polymers formed with homologous mutations identified in α1-antrypsin or neuroserpin, the mutated closed-dimer identified with these variant antithrombins is not only hyperstable and has no inhibitory activity, but also, unlike standard loop-sheet dimers, does not undergo transition to polymers. The results with these unusual mutants indicate that the variant accumulates intracellularly in the hepatocytes, as recently supported by studies with antithrombin Morioka. In this variant, the mutation (C95R) causes the loss of one of the three disulfide bonds of antithrombin and results in a dimeric form with an intermolecular disulfide bond.[18] However, even if these mutant dimeric antithrombins accumulate within the hepatocyte, the much slower overall rate of synthesis of antithrombin as compared to that of α1-antitrypsin or neuroserpin, explains the absence of cellular damage due to the retention of antithrombin dimers. Thus, the prime pathological

consequence is an effective lack of secretion, which results in an apparent type I deficiency, with a severe loss of circulating anticoagulant activity and associated increased thrombotic risk.

There is also a second, rather perplexing, group of mutations which have a milder conformational effect but are associated with even more severe disease consequences. Characteristically these mutations result in a change in stability that does not greatly effect their expression such that the variants are classified as being of type II. Also, because they are often secreted in a normally folded and active form, there are not necessarily any gross changes in functional activity. It is only subsequent to the secretion of these variants in the plasma that major conformational changes occur with consequent pleiotropic changes and the onset of what is often severe episodic thrombosis. Examples are the A382T mutation allowing formation of circulating trimers[19] and other mutations that facilitate the formation of polymers[20-22] or transition to the latent form.[21,22] All of these aberrational forms of antithrombin (dimers, trimers, polymers, as well as the latent form) have reactive loops that are buried by insertion into their own or another molecule. Hence, all are inactive with loss of the anticoagulant properties of the native conformation, and with a resultant increased thrombotic risk. Thrombosis in these patients often occurs at a relatively early age as life-threatening or recurrently severe episodes. Thus, the severity of these phenotypes is greater than would be expected for a mere loss of function or inefficiency of secretion. It seems then, that conformational mutations of antithrombin may have an additional pathological effect that increases the severity and risk of venous thrombosis. In the case of mutations that facilitate the latent transition there is an additional loss of anticoagulant activity, as the abnormal latent molecule then forms a dimer with a normal native molecule[23] causing an additional loss of activity (Figure 4). This propagated loss of activity is compounded by the preference of the latent molecule to dimerize with the most active anticoagulant form of antithrombin, the α-antithrombin glycoform.

There is also increasing evidence that the inactive conformational forms of the plasma serpins in general, and of antithrombin in particular, have potent messenger functions. Recently, it has been demonstrated that the latent conformation, as well as causing a loss of anticoagulant activity, also gains a function, as a significant anti-angiogenic agent.[24] Similarly, circulating polymerized α1-antitrypsin induces chemotaxis,[25] and proteolytically cleaved variants of antitrypsin display pro-inflammatory properties.[26] Again in a way that is not understood, the putative gain-of-function of these conformational variants may be exacerbated by specific situations such as pregnancy or infection, frequently observed to be associated with thrombosis in patients with some of these antithrombin variants.[17,20-22] The hemostatic consequences of such conformational mutations are not just restricted to antithrombin. Two interesting examples have been recently described in heparin co-factor II, another anticoagulant serpin that also inhibits thrombin. In one variant, a missense mutation (P443L) in the reactive loop of heparin co-factor II results in a heterozygous deficiency.[27] This appears to have key structural consequences as the mutant variant is retained inside the cell associated with the GRP78/BiP chaperone, one element of the quality control of the cell.[27] Another conformational mutation of heparin co-factor II has been identified in a family with homozygous deficiency.[28] This missense change (E428K) affects a conserved glutamate at the critical hinge of a mobile peptide loop, 17 residues distant (P17) from the reactive center at P1.[29] Interestingly, the same P17 mutation of a glutamate to a lysine is also responsible for the archetypal example of a conformational disease, the deficiency of the plasma serpin α1-antitrypsin (commonly present in people of European descent). The consequence of this mutation in α1-antitrypsin is an instability of folding such that the reactive loop of one molecule can insert into a β-sheet of another to give sequentially the formation of long bead-like polymers of the abnormal 1-antitrypsin. The combination of the misfolding and the intracellular accumulation of polymers results in grossly reduced secretion of the protein and hence in the plasma deficiency.[30] Recently, it was demonstrated that a disease in the fruit fly *Drosophila* was due to the same P17 Lys mutation in a serpin that controls the fly's immune response.[31] Evidence that the same mechanism of polymerization occurs in all these variants is provided by engineered expression in *E. coli* of mutant heparin co-factor II, with either a P17 Lys or Ala substitution (Corral *et al.*, *unpublished results*). The absence, with the P17 Lys mutant of heparin co-factor II, of any apparent effects on liver function is not surprising. Heparin cofactor II, like antithrombin, is synthesized in the liver to concentrations reaching only a fraction of those of α1-antitrypsin. So it is not surprising that the misfolding and accumulation of heparin co-factor II in hepatocytes has no apparent effect on liver function. The conformational consequences of this mutation in heparin co-factor II seem to be restricted to the grossly reduced secretion of the protein and hence its deficiency in plasma. The surprising finding, compatible with that of others, is the absence of any evident significant pathological consequences of the plasma deficiency of heparin co-factor II, an observation that is supported by a recent mice knockout model.[32] However, heparin co-factor II deficiency might increase thrombotic risk when combined with other thrombophilic defects.[29,33] The conclusion of all this is

Table 2. Conformational mutations identified in antithrombin (AT) and heparin co-factor II (HCII): phenotype and clinical consequences.

	Mutation	*Deficiency*	*Carriers*	*Thrombosis*	*Age of onset*	*Recurrence*	*Infection*	*Pregnancy*	*Reference*
AT									
	P80S	Type I	15	53%	18	Yes	Yes	Yes	17
	G424R	Type I	21	62%	30	Yes	Yes	Yes	17, 35
	P80T	Type I	2	100%	NR	NR	NR	NR	36
	C95R	Type I	8	12.5%	<42	Yes	NR	No	18, 37
	A382T	Type II	6	50%	18	Yes	NR	Yes	19
	F229L	Type II	5 (1 +/+)	40%	19	No	Yes	No	20
	N187D	Type II	2	100%	24	Yes	Yes	Yes	21
	T85M	Type II	4	75%	42	Yes	NR	NR	22
	T85K	Type II	2	50%	10	No	Yes	NR	22
HC II									
	P443L	Type I	7	2 arterial thrombosis	68	No	No	No	27
	E428K	Type I	15 (2 +/+)	1 venous thrombosis*	22	Yes	No	No	29

Age of onset: mean age of the first thrombotic episode in all carriers. Infection or pregnancy associated with the onset. NR: not reported. +/+ Homozygous subjects.
**This patient also carried a stop mutation affecting the antithrombin gene.*

that it is likely that the secretion of conformationally unstable serpins into the plasma has pathological consequences due to mechanisms that are as yet ill understood.[34] Table 2 summarizes the phenotypic and clinical consequences of all conformational variants identified in antithrombin and heparin co-factor II.[10,17-22,27,29,35-37]

Conformational changes caused by other factors

The perplexing feature of the second group of unstable antithrombin variants is their often sudden transition to a conformationally inactive form with the onset of severe thrombosis. The external factors that trigger such inactivating transitions are not well understood. The transition could reflect an interaction with other prothombotic factors such as factor V Leiden, or prothrombin 20210A. One factor that is significant is body temperature, because even the slight increases from 37 to 40°C that occurs in patients with fever are sufficient to trigger the conformational transition *in vitro* and presumably *in vivo*. Interestingly, there are several cases of the onset of severe vena caval thrombosis in males, often as young adults, in conjunction with infectious pneumonia and fever. The onset of thrombosis in females, however, seems predominantly to occur during pregnancy and in association with urinary infections. In both these examples in males and females, the increase in body temperature will undoubtedly exacerbate pathological thrombosis. However, there are clearly other factors involved as well. For example, it is not only pregnancy and infection that predispose to thrombosis in females, but also the raised estrogen levels that result from standard contraceptive pill usage. Why and how this happens is still unknown.

Conclusions

A new pathological mechanism resulting from the conformational instability of hemostatic serpins has been discovered to be involved in thrombosis. We reviewed several cases of unexpectedly severe and sudden thrombosis associated with variant antithrombins whose defects in function relate to the instability of the protein and the tendency to aggregate, especially at the elevated body temperature accompanying infection. Unfortunately, these variants are difficult to detect in plasma by simple laboratory tests. However, the missense mutations responsible for these variants affect key structural regions of the serpin. Two practical conclusions may be drawn from this scenario. First, the simple phenotypic measurement of antithrombin activity performed in regular clinical laboratories should be complemented by a genetic analysis, which may help to identify conformational variants with a strong thrombotic risk. Second, infection might increase the risk of thrombosis, not only in patients with these unstable variants, but probably in all patients, especially with a deficiency of antithrombin. Finally, it is important to search for other factors able to cause conformational changes of normal antithrombin in order to identify new risk factors for thrombosis.

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