III LECCIÓN CONMEMORATIVA RICARDO CASTILLO

INTRODUCCIÓN

??? MARTÍNEZ BROTONS
¿¿¿Cargos???

Robin Carrell es profesor de la Universidad de Cambridge y director de uno de los grupos más competitivos del Cambridge Institute of Medical Research de Cambridge (Reino Unido). A lo largo de su extensa carrera profesional ha conseguido unir dos aspectos difíciles de conjuntar: clínica e investigación. Su formación como médico hematólogo le ha marcado en su posterior dedicación científica y ha permitido que todo su trabajo como investigador en aspectos tan básicos como la estructura cristalográfica nunca perdiera de vista el objetivo final de toda investigación biomédica que es la de aplicarla al ser humano y en concreto a la corrección de alteraciones que llevan al desarrollo de enfermedades. Su ejemplo es modelo de trabajo en la medicina del siglo XXI.

Desde un punto de vista cuantitativo, el Dr. Carrell ha publicado más de 250 artículos. Pero las cantidades no son buenos indicadores. Merece la pena destacar más la calidad de sus trabajos. Pocos son los que pueden presentarse con más de 20 trabajos publicados en revistas de la calidad básica de *Nature* o *Science*, 16 artículos en revistas punteras en medicina como *New England Journal of Medicine*, *Lancet* o *The Journal of Clinical Investigation*, y cientos de trabajos en revistas bioquímicas, estructurales, o hematológicas de alto factor impacto (*JBC, Blood, PNAS*, etc.). Como es obvio de tal calidad, muchos de sus trabajos han sido referencias claves en el campo en que ha trabajado.

El inicio de su carrera se centró en el estudio de la hemoglobina con el premio Nobel Max Perutz, para pasar posteriormente a trabajar en hemostasia. En los últimos 15 años, el profesor Carrell ha aportado datos de enorme importancia que permiten conocer las razones por las que las serpinas han sido las moléculas seleccionadas por la evolución para controlar rutas proteolíticas críticas para la vida, como la cascada de la coagulación. Sus aportaciones al conocimiento de la antitrombina, en especial, pero también del inhibidor del activador del plasminógeno tipo 1 (PAI-1) y el cofactor II de heparina, son cruciales. En sus trabajos demuestra la complejidad estructural de estas moléculas y su mecanismo de acción, y aporta nuevos conceptos como los de flexibilidad estructural, o cambios conformacionales implicados en mecanismos patológicos, que hoy son elementos de referencia en el campo de la hemostasia, la clínica trombótica (en especial el diseño de fármacos) y la biología estructural.

Todos estos méritos han sido reconocidos internacionalmente con multitud de premios y menciones, entre las que destacamos la reciente medalla de la Sociedad Internacional de Trombosis (ISTH) que recibió en París el año 2001.

El profesor Carrell ha conseguido marcar hitos en la historia de la coagulación, anticoagulación y trombosis, precisamente el título de la lección, con la que estoy seguro vamos a comprender mejor procesos complejos y cruciales para la vida del ser humano, pero que también son responsables de la patología tromboembólica, la principal causa de morbimortalidad en nuestra sociedad. En su conferencia también vamos a comprobar como toda la investigación básica tiene verdadera aplicación. Y sobre todo, vamos a disfrutar con la forma tan sencilla, visual y didáctica con la que el profesor Carrell explica mecanismos tan complejos como la coagulación, anticoagulación y trombosis.

El profesor Carrell reúne todas las cualidades que a uno le gustaría que le reconociesen públicamente. También destaca su faceta humana. Robin Carrell es un verdadero *English Gentleman*. Su atención, educación y trato humano deja huella en aquellos que lo hemos conocido personalmente. Merece la pena darse unos paseos por el Trinity College de Cambridge en su compañía para disfrutar de su enorme calidad personal y humana.

COAGULATION, ANTICOAGULATION AND THROMBOSIS: MOLECULAR MECHANISMS

R.W. Carrell
CIMR, University of Cambridge. Reino Unido.

Introduction

The last twenty years has seen the solving of the major molecular mechanisms that control coagulation and anticoagulation. It has long been realised[1,2] that these mechanisms can be modulated to meet the special requirements of tissues such as the microcirculation, which require principally anticoagulant activity, and the arterial circulation, which gives a high priority to coagulation and the prevention of haemorrhage. We can now see in structural detail how this modulation takes place, with the heparans and thrombodulin that line the microvasculature, switching antithrombin to an active inhibitory form, and converting thrombin from a coagulant protease to one that activates the anticoagulant protein C pathway. This modulation occurs through a series of often subtle changes in conformation which together given an extraordinary complexity of interactions. The advantage of such complexity is in the ability to finally balance the coagulation processes. But the disadvantage is the vulnerability of these intricate mechanisms to mutations and acquired changes. The consequence of these aberrations is thrombosis.

How antithrombin works

Antithrombin is a member of the serpin family of protease inhibitors. The serpins have evolved an extraordinary mechanism, comparable to that of a mousetrap, that allows complete inhibition of their target proteases[3-6]. Antithrombin circulates in a metastable state with its reactive centre, like the bait of the mousetrap, exposed exteriorly and containing a sequence that acts as a specific substrate or bait for coagulation proteases and, in particular, for factor Xa and thrombin (fig. 1A). As shown in figure 1B, cleavage of the reactive centre by the protease unleashes the spring formed by the reactive centre loop, with the loop moving on its proximal hinge to enter the main sheet of the molecule as its central strand. In doing so, the protease is displaced to the other end of the molecule with an accompanying destruction of some 40 % of the structure of the protease. This gives the total and effectively irreversible inhibition required to halt the proteolytic cascades that otherwise lead to thrombosis.

How heparin modulates anticoagulation

Antithrombin exists in the circulation in a relatively inactive form with the key arginine at its reactive centre being obscured by an internal orientation. It is only when antithrombin binds to the heparans of the microcirculation that the reactive loop changes conformation to fully expose the arginine and hence give the active inhibitory form (fig. 2A). The exposure of the reactive loop and the activation of antithrombin is a consequence of the binding of a precisely defined pentasaccharide fragment[7,8] present in both heparans and in therapeutic heparin preparations. The pentasaccharide, which is highly negatively charged, binds to a patch of positively charged arginines and lysines on the side of molecule as indicated in fig. 2B. This binding has two consequences. As well as activating antithrombin as an inhibitor of factor Xa, it also provides the attachment site for longer heparin molecules that can link to an exosite on thrombin and hence bridge and catalyze the thrombin-antithrombin complex (fig. 2B).

Hence it can be seen how the fractionated heparins can have selective functions. The low molecular weight/high affinity heparins will contain a relatively frequent presence of the critical pentasaccharide and hence will be particularly effective inhibitors of factor Xa. Whereas the higher molecular weight heparins will be less selective for factor Xa, but because they are long enough to bridge the complex they will be effective activators of the inhibition of thrombin.

Therapeutic heparin

Heparin does not naturally exist as such in the circulation. Therapeutic heparin is a highly sulphated and heterogeneous glycosaminoglycan derived from the intestine of pigs. It was previously used in unfractionated form with an average of 45 saccharides per chain. But only 1 in 3 of these chains contained the specific pentasaccharide. More recently heparin has been fragmented to yield low molecular weight/high affinity forms that range from 6 to 30 saccharide units. Although these are less efficient in bridging the thrombin antithrombin complex, they mostly contain the pentasaccharide se-

quence and hence give more effective inhibition of factor Xa.

Within the last two or three years, synthetic heparins[9] have become available for clinical use, with the natural pentasaccharide sequence giving an effective Xa inhibitor suitable for subcutaneous administration. This pentasaccharide (fondaparinux) has 100 % bioavailability and a half-life of 17 hours. In another form, the modification of the natural sequence of the pentasaccharide by the addition of an extra sulphate gives exceptionally strong binding to antithrombin and results, therapeutically, in a long-acting Xa inhibitor. Further synthetic heparins are likely to become available including longer chain forms that will inhibit thrombin as well as Xa. The special advantage of the synthetic heparins is their extraordinary specificity, with the expectation that they are unlikely to form the secondary interactions that result in platelet interactions with the risk of thrombocytopaenia.

Thrombin: a coagulant and an anticoagulant

Few molecules have been better structurally studied than that of thrombin[10,11]. As summarised by Huntington and Baglin[12], the structures show how evolution has adapted the molecule to give interactions that alter and modulate its proteolytic function. The active site of thrombin is buried in a valley formed by two flanking loops (60-Loop and gamma-Loop in fig. 3A). These loops limit access to the active site and hence determine the specificity of cleavage by thrombin. Also shown in figure 3A are the anion-binding exosites I and II. Exosite I binds fibrinogen and is responsible for the sequestration of thrombin in fibrin clots. This binding site is also competed for by thrombomodulin, which as well as blocking the binding fibrinogen switches the cleavage preference of thrombin to the anticoagulant protein C. Exosite II has a key inhibitory role in providing the site that heparin binds to in the bridging complex with antithrombin (fig. 2C). Inevitably the story is even more complicated than this. For example, exosite I is competed for not only by fibrinogen and thrombomodulin, but also by the protease activated receptors (PARs) of the platelets. In this way, thrombin specificity is affected by multiple interactions, depending on its tissue location and to what it is bound.

Thrombin inhibitors

The principal inhibitor of thrombin in the plasma is antithrombin. Therapeutically, however, there is now much interest in specific inhibitors, several of which are derived from blood sucking parasites and bats. A number of peptide inhibitors have individually evolved that bind to thrombin and block its activity as illustrated diagrammatically in figure 3B. Examples are hirudin, a 65-residue polypetide from the medicinal leech, that reacts with both the reac-

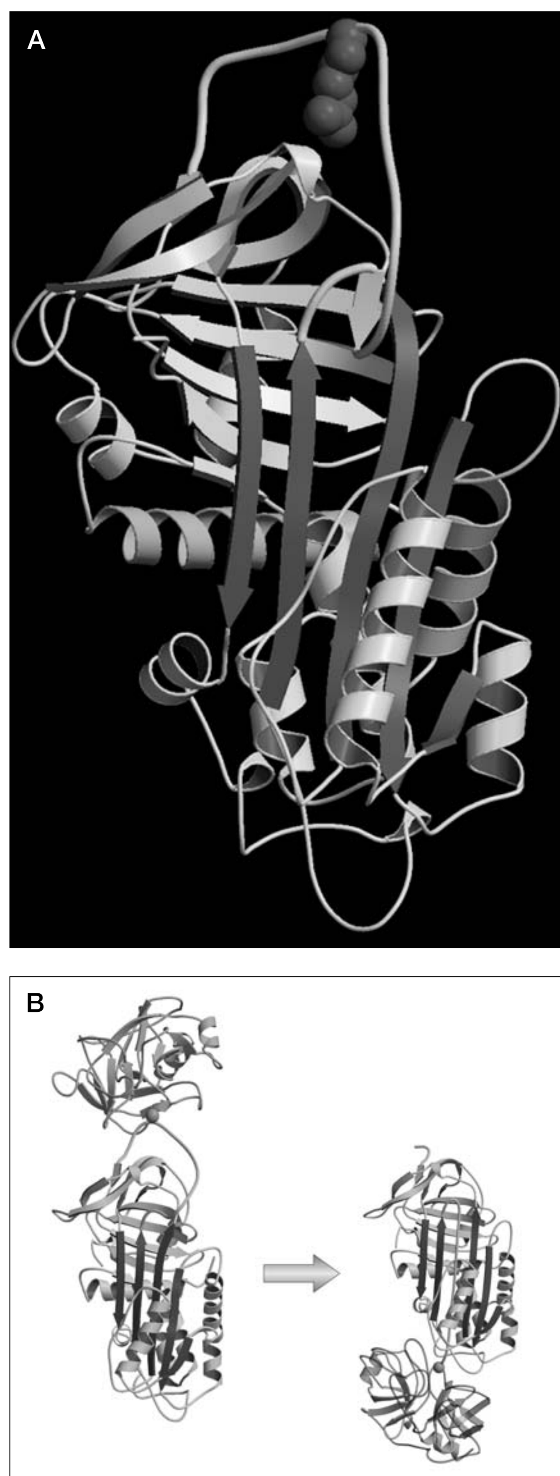

Figure 1. A) Antithrombin circulates with its reactive centre arginine (at top) obscured by its internal orientation. The reactive loop is partially inserted into the main sheet of the molecule with the strands of the sheet being held together by the encircled shutter region that triggers the opening of the sheet. B) Inhibition occurs when the protease cleaves the reactive centre with (on the right) the insertion of the loop into the main sheet and displacement of the protease to the other end of the inhibitor with consequent loss, by the protease, of ordered structure (shaded).

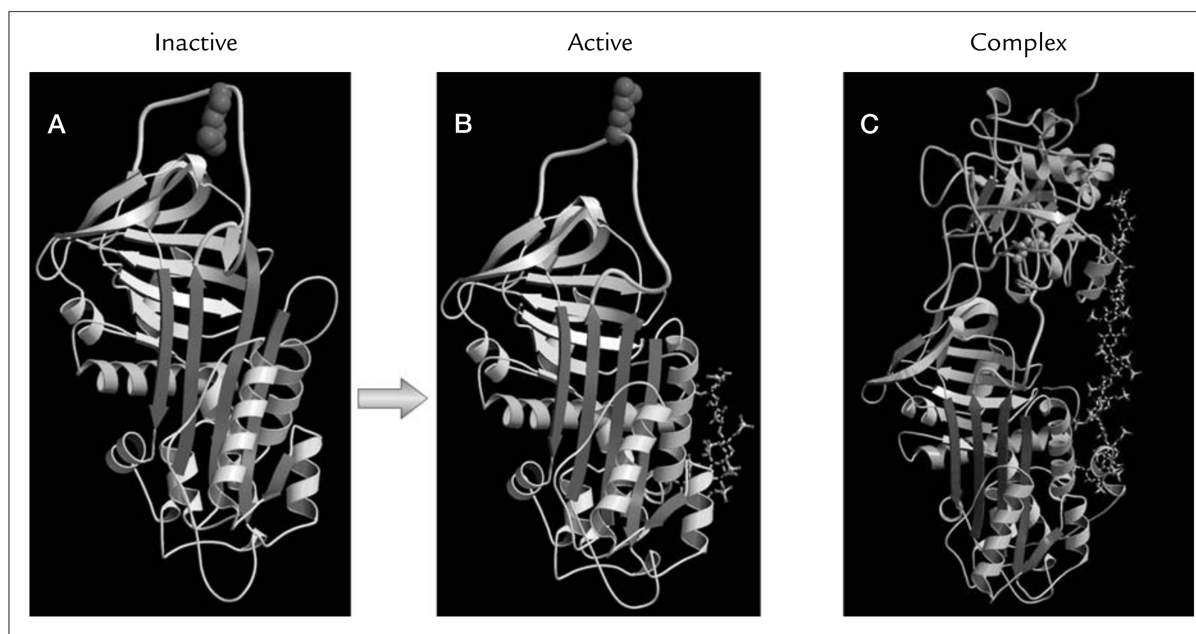

Figure 2. Heparin activation of antithrombin. (A) The obscured reactive centre arginine is revealed when (B) the heparin pentasaccharide on the right binds to antithrombin and expels the reactive loop. (C) The complex of thrombin with antithrombin is induced and stabilised by the bridging of longer chain heparins with exosite II on thrombin.

tive site and exosite I. From this, various recombinant modified hirulogs have been developed, such as hirugen. The availability of crystallographic structures has also allowed the design of synthetic peptides, such as PPACK, that specifically block the active site. From these derivatives have been developed, including melagatran, a small inhibitor of thrombin, available in an orally available prodrug form as ximelagatran. Similarly, an arginine-based compound, argatroban, is now in use for the treatment and prevention of heparin induced thrombocytopaenia.

Conclusions: Aberrations and thrombosis

As summarised above, knowledge of the structural changes involved in the control of coagulation has opened new prospects for therapy. Clinically, the new understandings also provide insights into the aberrations that result in thromboembolic disorders. This is readily demonstrated with respect to antithrombin. As expected, mutations that directly effect the inhibitory activity of antithrombin, such as mutations at the active centre, result in familial thromboembolism. Mutations at the proximal hinge of the reactive loop, or of the shutter that triggers the conformational change (fig. 1A), slow the entry of the loop and diminish the inhibitory activity of the antithrombin. But this slowing also allows the main sheet of the molecule to open and, as a consequence, the loop of another molecule to insert to give intermolecular linkage with a variety of disadvantageous consequences[13,14]. Alternatively, mutations in the distal hinge of the molecule allow the insertion of the whole intact reactive loop to give the irreversible and inactive latent form. This form preferentially binds the most active isoform of antithrombin in the plasma, β-antithrombin, with a consequent vulnerability to severe episodic thromboembolism. Mutations at the heparin binding site cause a mild predisposition to thrombosis in the heterozygote due to the lack of activation as an inhibitor of Xa and to the failure to form the bridging complex with heparin that accompanies the inhibition of thrombin. Such mutations usually result in a low affinity for heparin but unexpectedly, an even more severe disease can result from mutations that increase the affinity for heparin. These are frequently accompanied by a diminution of inhibitory activity with the seriousness of the consequences being due to the preferential binding of the mutant antithrombin to the sites on the vasculature that are normally occupied by the highly active β antithrombin. These are just some of the changes that result in disease. The reassuring development is that we can now understand in detail how each of these dysfunctions occur. The overall conclusion is that – as with all complex mechanisms – everything that can go wrong, will go wrong!

Acknowledgement

We thank J.A. Huntington and T. Baglin for their help and for permission to reproduce figure 3 from reference 12.

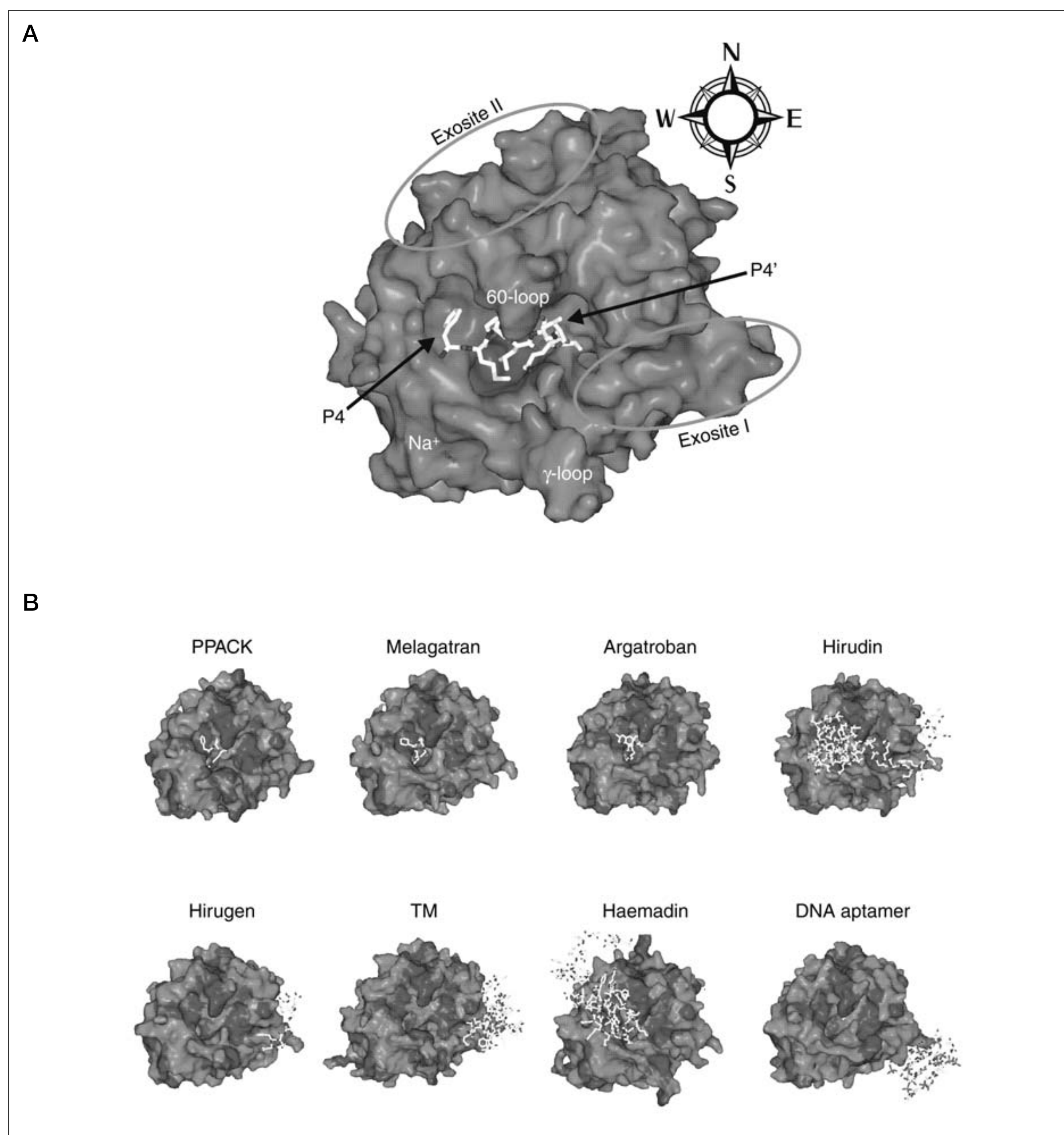

Figure 3. Thrombin inhibition. A) Shows the standard orientation of thrombin indicated by the compass above and showing the occupied active centre guarded on each side by the marked loops. The exosites indicate the principal anion binding sites involved in the interaction of thrombin with other modifiers and tissue components. B) A diagrammatic indication of the way that a range of small molecules block the active site and/or binding sites on thrombin. A detailed description of this figure is given by Huntington and Baglin in TRENDS in Pharmacological Sciences[12], with whose permission this figure is reproduced.

References

1. Olson ST, Björk I. Regulation of thrombin by antithrombin and heparin cofactor II. En: Berliner LJ, editor. New York: Thrombin, Structure and Function, 1992; p. 159-217.
2. Marcum JA, McKenney JB, Galli SJ, Jackman RW, Rosenberg RD. Acceleration of thrombin-antithrombin complex formation in rat hindquarters via heparin-like molecules bound to the endothelium. J Clin Invest 1986;74:341-50.
3. Schreuder HA, De Boer B, Dijkema R, Mulders J, Theunissen HJM, Grootenhuis PDJ, Hol WGJ. The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. Nature Struct Biol 1994;1:48-54.
4. Carrell RW, Stein PE, Fermi G, Wardell MR. Biological implications of a 3Å structure of dimeric antithrombin. Structure 1994;2:257-70.
5. Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. The anticoagulant activation of antithrombin by heparin. Proc Natl Acad Sci USA 1997;94:14683-8.
6. Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. Nature 2000;407:923-6.
7. Choay J, Petitou M, Lormeau JC, Sinay P, Casu B, Gatti G. Structure activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity, Biochem Biophys Res Commun 1983;116:492-9.

8. Lindahl U, Thunberg L, Backstrom G, Riesenfeld J, Nordling K, Bjork I. Extension and structural variability of the antithrombin binding sequence in heparin. J Biol Chem 1984;259:12368-76.
9. Petitou M, Herault JP, Bernat A, Driguez PA, Duchaussoy P, Lormeau JC, Herbert JM. Synthesis of thrombin-inhibiting heparin mimetics without side effects. Nature 1999;398:417-22.
10. Bode W, Mayr I, Baumann U, Huber R, Stone SR, Hofsteenge J. The refined 1.9 A crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. EMBO J 1989;8:3467-75.
11. Stubbs MT, Bode W. The clot thickens: clues provided by thrombin structure. Trends Biochem Sci 1995;20:23-8.
12. Huntington JA, Baglin TP. Targeting thrombin – rational drug design from natural mechanisms. TIPS 2003;24:589-95.
13. Corral J, Huntington JA, González-Conejero R, Mushunje A, Navarro M, Marco P, et al. Mutations in the shutter region of antithrombin result in formation of disulfide-linked dimers and severe venous thrombosis. J Thromb Haemost 2004;2:931-9.
14. Carrell R, Corral J. What can Drosophila tell us about serpins, thrombosis and dementia? Bioessays 2004;26:1-5.