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The serpins: nature's molecular mousetraps

JAMES A. HUNTINGTON AND ROBIN W. CARRELL

A special family of inhibitors, known as the serpins, has evolved an extraordinary mechanism to enable the control of the proteolytic pathways essential to life. The serpins undergo a profound change in conformation to entrap their target protease in an irreversible complex. The solving of the structure of this complex now completes a video depiction of the changes involved. The serpin, just like a mousetrap, is seen to change with a spring-like movement from an initial metastable state to a final hyperstable form. The structure shows how this conformational shift not only inhibits the protease but also destroys it. A bonus from these structural insights is the realisation that a number of diseases, as diverse as thrombosis, cirrhosis and dementia, all share a common mechanism arising from similar mutations of different serpins.

Introduction

The development of the ability of living cells to produce proteins has been accompanied by the evolution of mechanisms to break them down. Such proteolysis is required for the restructuring of the proteins of the developing cell, as well as to supply it with nutrient amino acids. To meet these needs a series of families of proteolytic enzymes have evolved, with a surprising number of them using precisely the same means of hydrolysing the peptide bonds that link the individual amino acids of each protein. The *serine proteases* are so called because they contain a serine in their active site, which in conjunction with an adjacent histidine and aspartate, can make a nucleophilic attack to hydrolyse the peptide bonds of the substrate protein. The active site cleft of the protease, in which this triad of catalytic amino acids is embedded, can be varied in shape and properties so as to limit access, and hence cleavage, to precisely defined sequences in

University of Cambridge, Department of Haematology, Wellcome Trust Centre for Molecular Mechanisms in Disease, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK

the substrate proteins. In this way, many of these *serine proteases* have developed specialised functions, not only as the digestive enzymes that breakdown foodstuffs but also as the highly specific proteases that trigger the essential pathways of life. Such pathways, with a sequential series of specialised proteases, are required for numbers of biological functions with the best known examples being the proteolytic cascades of the blood, including coagulation, fibrinolysis and immune activation[1].

Along with the evolution of the serine proteases there has been a parallel development of proteins that specifically inhibit them[2,3]. Organisms need to protect themselves against digestive attack. This is seen in the extreme in many of the foods we eat. Much of the bulk of beans, potatoes, and other vegetables is made up of a variety of inhibitors of serine proteases, as too is almost all of the white of eggs. This is one reason why we have to cook these foods, to denature the inhibitors and so allow the serine proteases of our digestive tract to break them down. Twenty different families of such inhibitors of serine proteases have independently evolved similar mechanisms of action. They each have an exposed reactive centre situated on a peptide loop that acts as an ideal fit and hence as a blocking agent, for the active site of the target protease (Figure 1a). Nineteen of these 20 families of protease inhibitors are widely distributed in plants and simpler species of life but just one family, *the serpins*, has become the predominant protease inhibitor in higher organisms and notably in man[4,5].

Serpins – inhibitors that change their shape

What differentiates the serpins from other families of protease inhibitors is their extraordinary ability to undergo a complete change in their shape. The serpins are also much larger than the other inhibitors, but they do share with them a reactive loop with a conformation matching the shape of the active site of the target serine protease. The difference is that whereas cleavage of the peptide loop in the other inhibitors leaves the loop in place so as to block the active site of the protease, in the serpins it results in the immediate displacement of the cleaved ends to opposite poles of the molecule (Figure 1).

This profound conformational change has now been well studied. The basis of the change became apparent in 1984 with the solving by Huber and colleagues[6] of the crystallographic structure of the most abundant protease inhibitor in human plasma, the serpin α_1-antitrypsin. This showed (Figure 1c) a well-ordered structure that is now known

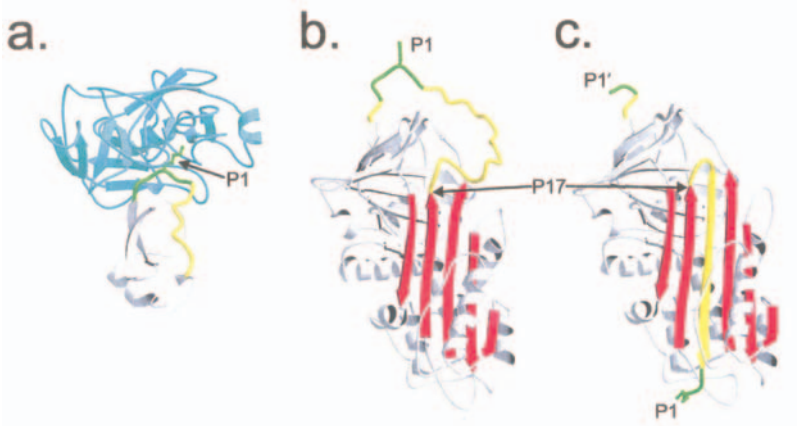

Figure 1. *Structures of serine protease inhibitors. The reactive centre loop of serine protease inhibitors is shown in yellow, with (in green) the region that fits into the active site cleft of the protease centred on the exposed sidechain of the reactive (P1) amino acid.* ***(a)*** *Shows how the common small inhibitors, such as BPTI, (below) fit into and block the active site of the protease (above in cyan).* ***(b)*** *The much larger serpins, shown on the same scale, retain the same blocking conformation but on a longer and flexible reactive loop.* ***(c)*** *cleavage of the loop at the P1–P1' reactive centre triggers a drastic change; the cleaved loop hinges at P17 to insert as a middle strand in the A-sheet (in red), converting it to the hyperstable, anti-parallel, form.*

to be shared in a quite precise way by all the serpins, with 9 helices and 3 β-sheets. One of these β-sheets – the A sheet – dominates the molecule. The surprise though, in this first structure of α_1-antitrypsin, was the insertion into the middle of the A sheet of an extra strand formed by the cleaved reactive centre loop converting the sheet to the more favourable anti-parallel configuration. The cleavage of the reactive centre was readily explicable, as this first successful crystallisation of a serpin had resulted from an attempt to crystallise the complex of α_1-antitrypsin with the protease chymotrypsinogen. But what revolutionised thinking about the serpin inhibitory mechanism was the unexpected conformational rearrangement that had taken place as a result of this cleavage. Although it would be another ten years before the structure of the intact inhibitor was solved (Figure 1b) it was clear that the reactive loop must initially be exposed, as in the other families of serine protease inhibitors. The unique difference is that on cleavage, the loop of the serpin then inserts into the A sheet, with the reactive centre residue, denoted as P1, being displaced by 70Å to the other pole of the molecule. This drastic move-

ment of the loop takes place round a hinge based on a conserved amino acid at position P17, 17 residues prior to the P1 reactive centre. Moreover, sequence alignments showed that the hinge formed by the adjacent residues P17–P10 has been homologously preserved in all serpins with inhibitory activity. Similarly all of these serpins, as with α_1-antitrypsin, were shown to undergo the same remarkable transition on cleavage of their reactive centre loops. The result is a transition of the molecule, from an initial metastable state with a melting point of 55°C, to the hyperstable state with a melting point greater than 120°.

It was clear from these and other findings that the serpins were comparable to molecular mousetraps, with an initial metastable form that converted, with a spring-like action, to a final hyperstable form upon attack by the target protease. This analogy was further strengthened by findings from studies of mutant forms of the serpins associated with disease. In particular, a variant of α_1-antitrypsin present in 4% of people of European descent[7], with a mutation of the P17 hinge amino acid, was shown to form long-chain polymers due to the linking of the reactive loop of one molecule to the opened A-sheet of the next[8]. Soon scores of examples of similar mutations causing a range of diseases, from thrombosis to dementia, were identified in other human serpins[9]. This has led to the concept of the syndrome of the hypersensitive mousetrap, so named because the causative mutations all predictably allow the premature triggering of the conformational change. This vulnerability of the conformational mechanism emphasised the overall puzzle as to why evolution had selected such a complicated approach to inhibiting proteases. Inhibition, after all, can be achieved by the much simpler blocking mechanism used by other families of serine protease inhibitors. To address this, Wright and Scarsdale[10] suggested that the movement of the reactive loop of the serpins was designed to carry the target protease with it, to the other end of the molecule. But why? What could be the advantage of such a displacement that would balance the obvious disadvantages of a conformationally unstable protein? The definitive answer to these questions has come from the recent crystal structure of the inhibitory serpin-protease complex[11]. The serpins really are molecular mousetraps!

The molecular mousetrap

The crystal structure of the final complex formed by a serpin with its target protease has long been regarded as the "Holy Grail' of its field[12]. It was clear that an understanding of the unique inhibitory

action of the serpins required the solving of the structure of the complex but repeated attempts over the last 20 years to crystallise it had been unsuccessful. The problem was that as soon as the serpin-protease complex is formed it becomes extremely susceptible to incidental proteolytic attack, giving heterogeneous products that counter attempts at crystallisation. To prevent such proteolysis we first added a surplus of the inhibitor α_1-antitrypsin to the protease trypsin, followed by a precise separation of the complex and then its crystallisation at refrigerated temperature. The crystals obtained gave X-ray diffraction at a resolution that showed interactions at atomic level as well as revealing the overall structure of the complex[11]. The result (Figure 2) immediately answers many of the questions relating to the function of the serpins but most significantly it fills in the missing frames that now complete a crystallographic video of the way these inhibitors change their shape and entrap their target proteases. We can now see the mousetrap in action (http://www-structmed.cimr.cam.ac.uk/serpins.html).

The consecutive structures of the video show how the protease takes the bait of the exposed reactive loop and initially forms a com-

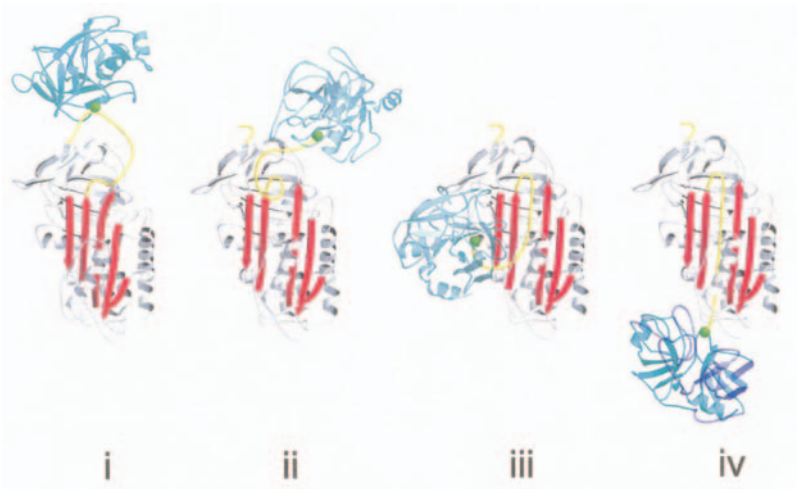

Figure 2. *Formation of the serpin-protease complex. The stepwise formation of the complex showing: (i) The initial docking of the protease and serpin. (ii–iii) The displacement of the protease through intermediate steps of reactive centre loop incorporation. The reactive centre of the serpin (green sphere) is covalently bound to the active site of the protease (cyan). (iv) The structure of the final complex shows the final position of the protease at the opposite pole of the serpin, and the consequent disruption of 40% of the protease (semi-transparent blue).*

plex identical to that formed by the other families of inhibitors (Figure 1a). In all of these inhibitors, including the serpins, the initial mechanism is the same, with the formation of a tight non-covalent complex followed by nucleophilic attack of the reactive centre (P1) carbonyl carbon of the inhibitor by the catalytic serine of the protease (Figure 3a). In the families of small protease inhibitors this process halts at various stages of the catalytic cycle, prior to the separation of the cleaved ends of the loop. Inherently though this complex is reversible, which ultimately results in release of active protease and intact inhibitor. In the serpins, as the structure shows, the reaction proceeds further. The formation of an ester bond between the active serine of the protease and the peptide carbonyl carbon of the P1 reactive centre amino acid of the serpin (Figure 3b) is accompanied by a separation of the cleaved ends of the loop (Figure 2iv). It is here that the special property of the serpins becomes evident. The cleavage of the loop that occurs with the ester

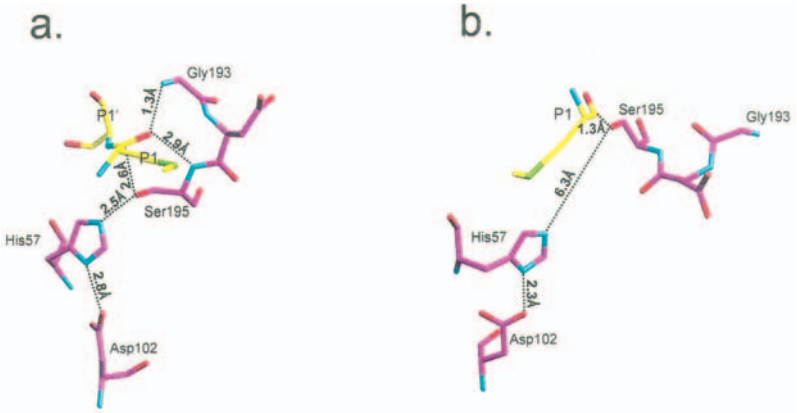

Figure 3. *The catalytic architecture of serine proteases.* ***(a)*** *A precise geometry of active site atoms is required for the hydrolysis of a peptide amide bond. The catalytic triad consisting of Asp 102, His 57 and Ser 195 potentiate the otherwise weak Oγ of Ser 195 as a nucleophile. The substrate P1 carbonyl carbon is positioned by a complementary fit into the active site cleft and by strong hydrogen bonding of the carbonyl oxygen with the mainchain amide hydrogens of the "oxyanion hole" composed of Ser 195 and Gly 193. Both the catalytic triad and oxyanion hole are critical for proteolysis.* ***(b)*** *The serpins arrest proteolysis before completion through a disruption of both the catalytic triad and the oxyanion hole. This is brought about by the plucking of Ser 195, along with the oxyanion hole, from its original position through its ester bond with the P1 carbonyl carbon and leverage applied through the clash of the protease and serpin in the final complex.*

linkage is accompanied in the serpins by a spectacular conformational rearrangement, with insertion of the cleaved loop into the A-sheet and a flinging displacement of the attached protease to the other end of the molecule. As the structure of the complex shows (Fig. 2iv), this forced displacement crushes the protease causing it to lose 40% of its ordered structure. It is well worth a trip to the web-site to see the video depiction – the analogy with a mousetrap is self-evident (http://www-structmed.cimr.cam.ac.uk/serpins.html). In effect the serpin, converted to a hyperstable form by the insertion of the extra strand in its A-sheet, acts as an anvil on which the less stable protease is distorted and smashed. But in order to see the full advantage of the conformational shift we have to look at the atomic detail of the complex.

Kill and overkill

To be effective a mousetrap must not only entrap its target but must also do so with irreversible lethality. The same is true of the serpin. The effectiveness of the serpins is due not just to the entrapment and gross distortion of the protease but rather to the lethal disruption of the active site of the protease that accompanies this distortion. The active serine of the protease, tethered by its ester linkage to the displaced loop of the serpin, is effectively pulled away from its position within the catalytic triad (Figure 3b). The critical factor in this lethal plucking action is the short length of the cleaved reactive loop, limited in all serpins to 17 amino acids (P17–P1)[13]. The consequence is an overlap of the two structures, which contributes to the crushing distortion of the protease, together with a plucking of the active serine away from its catalytic site (Figure 3). This disrupts the network of hydrogen bonds that makes up the catalytic architecture of the active site required for the hydrolysis of the ester linkage and hence for the release of the functional protease (Figure 3a). It is this irreversibility of binding of the protease that enables the serpins to provide the complete inhibition required for the control of proteolytic pathways such as coagulation. These pathways consist of series of different proteases, each one capable of amplifying the production of the next protease in the series. Thus the release of just one or two molecules of the coagulation protease factor Xa can rapidly result in the activation of tens of thousands of molecules of thrombin, with the resultant onset of potentially fatal thrombosis. To counter this the plasma serpin antithrombin, must rapidly and irreversibly inhibit any released factor Xa.

To emphasise the paramount requirement for irreversibility of inhibition, the conformational change meets this not only by disrup-

tion of the catalytic site but also by preparing the prompt destruction of the protease. The overlap of structures in the final complex together with the plucking action of the displaced loop perturb the structure of the protease to an extent that makes it susceptible to proteolytic cleavage. Normally, functional proteases are folded in such a way as to be highly resistant to proteolytic attack. However it had been previously observed that proteases in complex with a serpin were readily cleaved at multiple sites in their structure[14–16]. We can now see that each of these cleavages occurs in the portion of the protease molecule that is disordered in the complex. This disorder of the protease not only results directly from the formation of the complex but is further exacerbated by a breaking of the principal bond responsible for the conformational integrity of the active protease. Serine proteases are initially synthesised and secreted in an inactive and only partially-folded zymogen form. Their activation depends on an amino-terminal cleavage, with the new amino-terminus of the protease then forming a stabilising bond within the active site. This transformation, from zymogen to active protease results in a 20% increase in ordered structure[17]. However this process is reversed with the disruption of the active site that takes place on complexation with the serpin. The zymogen-activation salt-bridge is broken and the consequent loss of ordered structure greatly contributes to the overall unfolding of the protease. Thus the disruption of the catalytic triad of the protease, does not by itself ensure complete irreversibility, though it does slow the release of active protease to an extent that the half-life of the complex is measured in years rather than seconds. But well before such release occurs the unfolded protease will be irreversibly destroyed by incidental proteolysis.

Implications for health and disease

The significance of the special properties of the serpins becomes apparent when we study the *in vivo* environments in which they function. For example, although α_1-antitrypsin is an efficient inhibitor of the digestive protease trypsin, its main function in life is the inhibition of the enzymes released by inflammatory white cells. These neutrophil white cells secrete large amounts of elastase at sites of inflammation in order to break down surrounding connective tissue. To control this process the blood plasma contains a surplus of α_1-antitrypsin that can readily mop up any elastase released into the circulation. The neutrophils can function despite this surplus of plasma inhibitor because inflammation takes place in enclosed pockets of tissue rather than in the plasma itself. For example, if we

get a thorn in our thumb, the body responds by surrounding it with a myriad of neutrophils. The elastase and other proteases secreted by the neutrophils then dissolve the connective tissue directly surrounding the thorn. The liquefied tissue, filled with expired yellow-green pigmented neutrophils (pus!) can then be discharged along with the offending thorn. In all of this, α_1-antitrypsin has the key role of making sure that any elastase that diffuses to the margins of the healthy tissue is efficiently neutralised. Within the pocket of inflammation, clearance of formed serpin-protease complexes will occur very slowly if at all. However, the milieu of the pocket, with masses of released proteolytic enzymes, will ensure the prompt destruction of the complexed and disordered protease.

The pocket of inflammation formed by the neutrophils maintains its proteolytic milieu, despite being surrounded by high concentrations of inhibitor, due to a cunning molecular ploy. At the same time that the neutrophil secretes elastase it also releases a barrage of oxidative free radicals. Although these oxidative radicals are primarily bactericidal in function, evolution has adapted the reactive loop structures of a number of serpins so that they contain oxidation-sensitive amino acids. In this way, α_1-antitrypsin has a methionine at its reactive centre, which is readily oxidised and hence inactivated by free radicals produced by neutrophils[18]. Such free radicals are short lived but suffice to form an inhibitor-free radius around each neutrophil. As a consequence aggregations of neutrophils form foci of proteolysis but as their number decreases any protease or radicals released are overwhelmed by the high concentrations of α_1-antitrypsin. This understanding of the molecular function of α_1-antitrypsin as a tissue protectant explains why individuals with the common genetic deficiency of α_1-antitrypsin are liable to develop destructive lung disease in later life[7]. The lungs are formed of masses of small elastic sacs, which like a balloon, depend on their elasticity to maintain an in and out flow of air. In the absence of sufficient amounts of α_1-antitrypsin however, a slow and cumulative loss of lung elasticity takes place. This is greatly accelerated by situations which increase the exposure of the lungs to neutrophil elastase, most notably by tobacco smoking.

There is another reason why the ability of the serpins to change their shape has made them the predominant protease inhibitors in higher organisms. Evolution has been able to adapt this change in shape so as to modulate the activity of individual serpins. For example, antithrombin[19], the principal inhibitor of coagulation, circulates in the blood in a relatively inactive form, with its reactive centre loop partially inserted into the A-sheet of the molecule[20,21]. It is not until

the antithrombin binds to the cells lining the capillaries and small vessels that it becomes fully activated and takes up its role as a protector of the microcirculation against thrombosis. Similar mechanisms and molecular ploys are now being revealed in the other serpins of the blood, with the movement of the loop in and out of the molecule allowing control of when and where inhibitory activity is switched on or off. This illustrates how a complicated and mobile molecular mechanism may inherently have advantages for the survival of a species, in that such complexity readily allows the evolution of the modulation mechanisms needed to adapt to changing environments and challenges. The disadvantage however, as with all moving mechanisms, is the critical specifications of the hinges and slides required for this movement. In the serpins, even minor changes in these critical hinge regions are sufficient to destabilise the molecule and trigger conformational change, with disastrous consequences. Thus in antithrombin, mutations that allow the premature triggering of the change from the metastable active form to the hyperstable inactive form, cause the onset of thrombosis[9].

The same destabilising hinge-mutations in α_1-antitrypsin have quite different but equally disadvantageous effects[22]. The resulting premature opening of the A-sheet of α_1-antitrypsin allows the insertion into it of a loop from another molecule to give, sequentially, the formation of long-chain polymers[8]. These polymers accumulate at the site of synthesis of α_1-antitrypsin, in the cells of the liver. The result is the gradual loss of these cells, with over a period of years the onset of liver cirrhosis[23,24]. Thus those unfortunate Europeans who homozygously inherit genes with mutations in the hinge regions of α_1-antitrypsin find themselves at double jeopardy. The accumulation of the polymerised α_1-antitrypsin in their liver leads to cirrhosis and the accompanying deficiency of the inhibitor in the blood makes them susceptible to the chronic destructive lung disease emphysema. Other manifestations of such hypersensitive triggering of the serpin molecular mousetraps include the familial dementias resulting from the polymerisation of brain-specific serpins[25], and the severe allergic responses due to dysfunction of the serpin that controls the onset of the immune response[26]. These examples illustrate how we can now see and understand the way in which the same mutations, at the same sites, in different serpins, result in a range of diverse diseases. The important practical corollary is that we can now also begin to plan strategies of treatment equally applicable to all the diseases. The requirement is to be able to lock the serpin molecule in a fixed and hence stable conformation. This is readily attainable *in vitro*, but the challenge is to deliver such therapies in a targeted and effective way in life.

Conclusion

The solving of questions in biology is akin to the completion of a large and difficult jig-saw puzzle. Bit by bit patterns appear, but the overall picture often only becomes clear with the fitting of just a few final pieces. We have tried to show here how the crystal structure of the serpin-protease complex has brought into context previous disparate findings in a way that now completes an overall understanding of the functions of the serpin family of protease inhibitors. The combination of structural and functional studies is a powerful investigative approach. This is particularly so in medical research where a structure provides a centrepiece not only for functional studies, but also for the input of clues provided by natural mutations and by the diseases that result from them. The end result as reported here is rewarding. It reflects the work of all in our group in Structural Medicine, in which biochemists and cell biologists work side by side with structural biologists in problem driven research. It is a formula we can recommend to others.

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