pressorium turgor was found to be essential for plant infection (*8*). When appressoria were incubated in solutions of increasing concentration, their ability to penetrate epidermal cell layers decreased with increasing external solute concentration. The high pressure of appressoria from these fungi is due in part to the melanin layer that forms an inner cell wall layer, especially in mature appressoria. Mutants of *Magnaporthe* and *Colletotrichum* species that fail to synthesize melanin are nonpathogenic and do not accumulate turgor (*7*). These studies strongly implicated turgor generation in plant infection, but direct evidence that appressoria produce enough force to puncture plant cuticles has been lacking until now.

Bechinger *et al.* (*4*) measure the force exerted by penetration pegs from *Colletotrichum graminicola*, a fungus producing similar melanized appressoria to *M. grisea* and the causal agent of anthracnose disease of corn (*9*). The technical difficulty of accomplishing this feat is difficult to overstate. Appressoria from *C. graminicola* are 7 to 9 μm in diameter, and the penetration peg breaching the plant cuticle is only 2 μm in diameter. The invasive force produced by the penetration peg was measured by growing appressoria on an optical waveguide composed of a polydimethylsiloxane membrane sandwiched between two thin films of aluminum. Light was focused on the underside of the waveguide through a prism and the reflected light detected by a charge-coupled device linked to an image processor and computer. The invasive force exerted by *C. graminicola* could be detected because the fungus deformed the waveguide as the penetration peg pushed against it. The resulting alteration in the thickness of the optical waveguide was detected by a change in the intensity of the reflected light. Penetration of the optical waveguide by appressorial penetration hyphae typically produced an indentation of 10 nm. The force necessary to produce this indentation was calibrated by comparison with the action of a glass capillary applying exactly defined forces against the waveguide. Bechinger *et al.* show that *C. graminicola* appressoria can exert an invasive force of 16.8 ± 3.2 micronewtons, a considerable force capable of breaching most plant cuticles. This force is consistent with the generation of enormous cellular turgor inferred by Howard *et al.* for *M. grisea* (*8*). The force exerted by *C. graminicola* increases quite rapidly (within 2 to 3 hours) after maturation of the appressorium and continues to be exerted for several hours thereafter, indicating that the force is not a sudden release of turgor but rather a sustained application of pressure by the penetration peg.

How such large forces are generated by appressoria of these fungal pathogens remains unknown, but two things are apparent. First, appressoria must adhere very tightly to the leaf surface, because if the force of adhesion did not exceed the force of penetration, the cell would simply lift away from the leaf. Second, appressoria of *C. graminicola* must accumulate large amounts of solute. Turgor pressure in appressoria appears to be the result of an influx of water into the cells, which develop within dew drops on the leaf surface. In *M. grisea*, the solute providing the osmotic potential for turgor generation is glycerol, which accumulates to concentrations in excess of 3 M (*10*). The glycerol is held in place, at least in part, by the thick melanin wall layer that prevents leaking of the small lipophilic molecule from the cell (*10*, *11*). The forces exerted by appressoria of *C. graminicola* suggest a turgor pressure of over 5 megapascals. This means that if a single solute accumulates in *C. graminicola*, it must do so to very high concentrations (as in *M. grisea*). Alternatively, the fungus would need to use a combination of solutes to produce the necessary turgor.

How the turgor is translated to physical force and applied at the penetration peg remains unknown (*11*). Localized cell wall dissolution and cytoskeletal reorientation play a role in the process (*7*, *12*), but the genetic and biochemical control of appressorium function is largely unstudied. The optical waveguide technique developed by Bechinger *et al.* (*4*) provides a wonderful opportunity to select mutants that are unable to produce functional appressoria and to identify and characterize the genetic components of this fascinating process.

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PERSPECTIVES: BIOCHEMISTRY

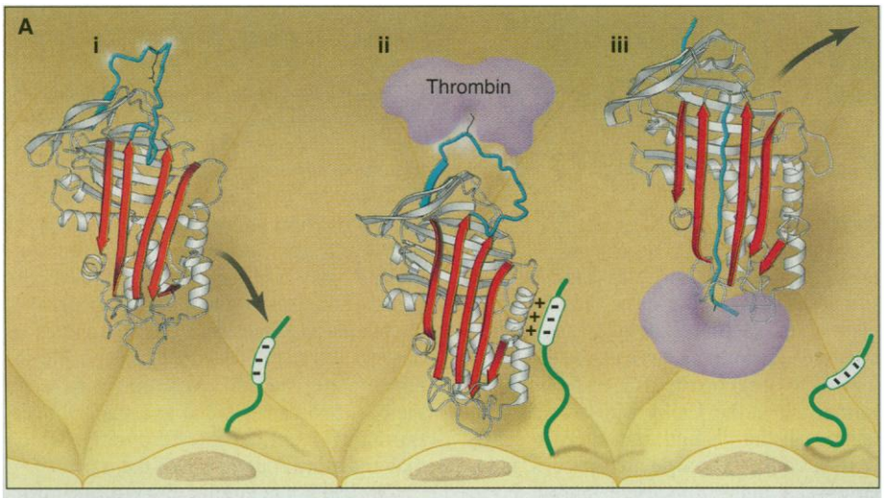
# How Serpins Are Shaping Up

**Robin W. Carrell**

Survival of the fittest is a rule that applies just as much to protein families as it does to organisms. The serpins are a protein family of highly adaptable serine protease inhibitors that have outmaneuvered their many relatives to become the predominant protease inhibitors in human plasma. Their success can be attributed to their ability to trap and irreversibly bind to their target proteases (see the figure) (*1*). Cleavage of the serpin's reactive loop by the protease triggers entrapment and inactivates the serpin. Furthermore, the conformational changes in the serpin that accompany this mousetrap-like action are now known to act as signals for a range of physiological responses (*2*). A striking example of this is reported by O'Reilly *et al.* (*3*) on page 1926 of this issue. They show that small conformational changes in the serpin antithrombin III can profoundly alter its activity—from that of an inhibitor of thrombin and other clotting proteases in plasma to an agent that blocks blood vessel formation (angiogenesis) and induces tumor regression.

Antithrombin differs from other serpins in that it is able to bind (through a cation site on its A and D helices) to heparin-like molecules attached to the cells that line blood vessels. Upon binding to these molecules antithrombin becomes immobilized and undergoes a series of small conformational changes resulting in exposure of the carboxyl-terminal loop (which contains the active site) and a dramatic increase in its affinity for thrombin and other coagulation proteases. Proteases that bind to the heparin-bound antithrombin cleave the exposed loop reversing the conformational changes at the heparin-binding site, with a consequent release of the protease-antithrombin complex into the circulation (see figure, panel A) (*4*). Exactly the same loss of heparin affinity occurs whenever antithrombin undergoes a transition from its active five-stranded structure to an inactive six-stranded form (*5*). This takes place not only when the serpin binds to its protease but also after cleavage of the loop by incidental proteolysis or as a result of a slow spontaneous conversion of antithrombin to its latent form present in trace amounts in

The author is in the Department of Haematology, Cambridge Institute for Medical Research, University of Cambridge, Hills Road, Cambridge CB2 2XY, UK. E-mail: rwc1000@cam.ac.uk




**The shape of things to come.** Antithrombin changes its function by folding its active-site loop (blue) in and out of the main β-pleated sheet of the molecule (red). (**A**) In the circulation: (i) Antithrombin is initially inactive with the loop partly inserted and the side chain of the active center obscured. (ii) Interaction with heparin (on the surface of vascular endothelial cells) tethers antithrombin to the wall of the microvasculature and induces a conformation change with an accompanying expulsion of the loop, which can then bind to the active site on thrombin (purple). (iii) Cleavage of the loop by thrombin allows its complete insertion as the middle sixth strand of the β sheet, with transport of thrombin to the opposite end of the molecule. The heparin-binding site reverts to a low-affinity conformation, and the antithrombin-thrombin complex is released into the circulation. (**B**) Conversion of antithrombin to the inactive six-stranded form can also occur as a result of cleavage of the loop by incidental proteolysis (Bi) to give a conformation as in (Aiii) or by induction of insertion of the intact loop to give locked L-forms, shown here as the fully inserted latent conformation (Bii).

CREDIT: DEPICTIONS BY T. DAFFORN FROM CRYSTAL STRUCTURES DETERMINED IN THE DEPARTMENT OF HAEMATOLOGY, UNIVERSITY OF CAMBRIDGE. ILLUSTRATION: K. SUTLIFF

normal plasma (see figure, panel B) (*6*). In vitro, antithrombin is rapidly converted by mild stress into a range of six-stranded locked "L-forms," principally the latent conformation and its intermediates, together with dimers and oligomers formed by the insertion of the reactive loop of one molecule into the β sheet of the next. The important point in support of the O'Reilly findings is that these diverse forms share precisely superimposable conformational changes that all result in the loss of heparin affinity and the release of antithrombin.

How could such changes in conformation at the heparin-binding site affect angiogenesis and tumor growth? A partial answer comes from recent studies of another serpin, the plasminogen activator inhibitor, PAI-1. This plasma serpin has a binding site for vitronectin (a protein found in the extracellular matrix) that resembles the heparin-binding site of antithrombin. Lawrence and others (*2*, *7*) have shown that when PAI-1 binds to vitronectin it blocks another site on vitronectin from binding to integrins, which are adhesion molecules present on mobile cells such as tumor cells and cells involved in the formation of new blood vessels. Hence, high levels of PAI-1 impair tumor cell adhesion and result in an increased risk of the release of tumor cells into the circulation (metastasis) (*8*). In contrast, low or absent levels of PAI-1 inhibit the adhesion of cells involved in angiogenesis, which is essential for tumor growth and invasion (*9*). Of particular relevance is the dependence of these effects on the conformation of PAI-1. As with heparin and antithrombin, vitronectin only binds to the active conformation of PAI-1, and there is a 1000-fold decrease in affinity for PAI-1's cleaved, latent, or other six-stranded conformations. In this way, PAI-1 provides a precept for how conformational changes in other plasma serpins could alter their functions. This precept is not exceptional. Much earlier (*10*) cleaved $\alpha_1$-antitrypsin was shown to activate leukocyte mobility, and more recently (*11*) cleavage of an elastase inhibitor was shown to convert the molecule into an active deoxyribonuclease.

The O'Reilly study, with its demonstration of a remarkable suppression of tumor growth by inactivated conformations of antithrombin, provides yet a further example. The loss of heparin affinity resulting from inactivation of antithrombin is not a passive process because the binding site is not lost but changes shape and becomes available for binding to other receptors on cell surfaces or in the extracellular matrix. Such molecular crosstalk (*12*) is known to occur between other spent coagulation factors and the adhesion receptors that control vascular remodeling. This is true also of PAI-1, where the conformational loss of affinity for vitronectin is matched by an increased affinity for other cell surface receptors (*7*, *8*). It is therefore not unexpected that antithrombin, once it has completed its job of inhibiting clotting, should take on a new function as a modulator of angiogenesis and hence of tumor growth. The surprise finding is the magnitude of the effect. The O'Reilly report is a provocative pilot study that leaves many questions unanswered. How could the production of such small amounts of modified antithrombin suppress a tumor on the contralateral flank of an animal? What are the precise cellular interactions of modified antithrombin? Importantly, as the authors ask, will these modified forms have clinical efficacy? The good news is that there should be no hindrance to human therapeutic trials for infusions of L-antithrombin, as such material has been used unwittingly in transfusion medicine for more than 10 years. At least one manufacturer of antithrombin concentrates has, in a final pasteurization step, been converting up to 40% of the antithrombin into latent and other L-forms (*13*). A controlled trial of transfusions of multigram quantities of these concentrates showed no adverse effects. Frustratingly, patients with malignancies were excluded from the trial!

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