

## **Studies of serpins unfold at a feverish pace.**

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Research Article

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Serine proteases play key roles in a very wide variety of important biological processes (e.g., development, cell migration, wound healing, blood clotting, fibrinolysis, ovulation, trophoblast implantation, tumor invasion and metastasis, neural outgrowth, etc.). A detailed understanding of the biochemistry and the regulation of these enzymes, therefore, is of fundamental importance and, in specific cases, may facilitate the design of improved therapeutic agents. During many of the critical biological events enumerated above, an appropriate stimulus (e.g., exposure of blood to the subendothelium after vascular injury) triggers the local generation of specific proteolytic activity. Frequently, the developing proteolytic activity is restricted to the original microenvironment by the presence of high concentrations of specific inhibitors in the surrounding tissues. In these situations, then, the delicate balance between zymogens, activated proteases, and specific protease inhibitors can influence profoundly the biological effects of a given stimulus. Disruption of this equilibrium often precipitates catastrophic consequences.

The recent rapid accumulation of important new information regarding serine proteases and serine protease inhibitors (serpins) may presage an impending golden age for the study of these proteins and promises to yield new understanding of the molecular details of their function in both health and disease. For example, recent structure/function studies of tissue type plasminogen activator (t-PA) and its cognate serpin plasminogen activator inhibitor type 1 (PAI-1) have provided new insights into the coevolution of specificity between serine proteases and serpins as well as regulation of the activity of chymotrypsin-like enzymes by zymogen activation (1, 2). Additional studies have demonstrated that site directed mutagenesis can be used to simultaneously alter several properties of t-PA and thereby create promising new candidates for improved thrombolytic agents (3). Fascinating studies of variants of trypsin and  $\alpha$ -lytic protease have supplied new comprehension of subtle but critical structural determinants of the specificity of chymotrypsin-like enzymes (4, 5). Moreover, the recent development of robust new techniques that allow expeditious examination of the substrate specificity of proteases is certain to accelerate further the already rapid rate of advancement in this area (6, 7).

Even judged against this background of extraordinary progress, recent developments in the study of serpins have been impressive. In stark contrast to "standard inhibitors" of serine proteases, the region of a serpin that fits into the active site of a protease (i.e., the reactive center loop) appears to be mobile and therefore able to adopt several distinct conformations. The first detailed description of one of these conformations was provided in 1984 by Loebermann and co-workers (8) when they solved the structure of a form of  $\alpha$ 1-protease inhibitor whose reactive center loop had been proteolytically cleaved. This structure provided dramatic evidence that, at least after proteolytic cleavage, the reactive center loop possessed striking conformational flexibility. In this cleaved molecule the P1 and P1' residues (i.e., the two amino acids initially forming the scissile

bond) were located  $\sim 70$  Å apart. During this large conformational rearrangement after cleavage, the two free segments of the reactive center loop were incorporated into relatively inaccessible locations as additional strands (strand 4A and 1C) of separate beta sheet structures in the protein. The remarkable alteration in conformation and the concomitant formation of a large number of new, favorable interactions by the free strands of the reactive center loop provide an explanation for the earlier observation that, unlike standard inhibitors, cleaved serpins were no longer active as protease inhibitors and were more stable than the intact, inhibitory molecule.

8 yr after the pioneering work of Loebermann and co-workers, Mottonen, Goldsmith, and their collaborators presented another landmark study (9). These investigators solved the structure of "latent" PAI-1, thereby providing a detailed description of a second distinct serpin conformation. Surprisingly, as in the cleaved conformation, the reactive center loop of the intact latent conformation was inserted into beta sheet A to form a new strand 4A. Conformational flexibility of the reactive center loop, therefore, clearly was not dependent upon proteolytic cleavage.

In the last year, three groups of investigators have prepared crystals from solutions containing intact, active serpins and reported the corresponding new structures (10–12). As expected from the previously described structures of standard inhibitors and the noninhibitory serpin ovalbumin, the reactive center loop of these molecules is anchored by two short stalks and extends away from the core of the molecule into solution to occupy a very accessible location. Subtle differences among these three structures, especially concerning whether any residues of the reactive center loop are inserted into beta sheet A, and the participation of the reactive center loop in crystal contacts in two of the structures have fueled continuing controversy regarding fine details of the active serpin conformation. Moreover, the observation that, in all three structures, the reactive center loop is not in an optimal conformation for interaction with a protease suggests that these structures may not be identical to the "docking conformation" of an active serpin in solution. Nevertheless, these important structures raise extremely interesting, well-defined questions regarding both structure and function of serpins as well as provide new insights regarding the active conformation. Two of these structures may also provide insight into yet another conformation that can be adopted by serpins both in vitro and in vivo, the serpin polymer.

In this issue of *The Journal*, Bruce and his co-workers (13) provide a noteworthy demonstration that some of these new insights can explain the molecular basis of specific human diseases. These investigators present a very interesting study of a novel variant (Rouen-VI) of antithrombin isolated from a 40-yr-old propositus who had experienced three severe thrombotic episodes. Initial assays of the patient's plasma indicated that the concentration of antithrombin antigen and activity were within the normal range. However, upon transport of the plasma from Rouen to Cambridge and storage for 1 wk at 4°C, the antithrombin activity in this plasma declined to a value  $\sim 50$ –60% of normal. Moreover, amplification and sequencing of the patient's antithrombin genes revealed that the patient was heterozygous at this locus, containing one normal allele and one allele that carried the previously unreported mutation

Asn 187→Asp. Bruce and colleagues then purified these two forms of antithrombin and demonstrated that, although both inhibited thrombin at similar rates, the Rouen-VI variant was significantly less stable than wild-type antithrombin when challenged either with mild denaturants or elevated temperature. For example, the purified Rouen-VI variant was completely inactivated by incubation at 41°C for 20 h while the wild-type protein retained ~90% of its activity. Further analysis suggested a mechanism for this inactivation: at elevated temperatures antithrombin Rouen-VI spontaneously converts into the “latent” conformation and also forms polymers.

Based upon their careful biochemical characterization of antithrombin Rouen-VI, Bruce and co-workers made the provocative suggestion that thrombotic episodes in this patient might be precipitated by pyrexia. Subsequent examination of the medical records of the proband and her father confirmed that five of six severe thrombotic episodes experienced by these individuals were, in fact, associated with an incidental pyrexial infection. The authors of this manuscript deserve congratulations for an intriguing conclusion to an elegant study.

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