Hypothesis

The F-helix of serpins plays an essential, active role in the proteinase inhibition mechanism

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Abstract Proteinase inhibition by serpins requires a 70 Å translocation of the proteinase, circumvention of the blocking helix F, and a crushing of the proteinase to render it catalytically incompetent. I propose that temporary displacement of the F-helix during proteinase transit, and its subsequent return after complete passage of the proteinase, not only allows the proteinase to reach its final location, but provides an absolutely essential coupling mechanism for making the final proteinase crushing step energetically favorable. The F-helix is therefore not a passive impediment to proteinase translocation, but a critical, active element in permitting the serpin inhibition mechanism to operate successfully.

Key words: Serpin mechanism; Proteinase inhibition; High energy intermediate; Serpin; Thermodynamic coupling

1. Introduction

Serpins inhibit serine proteinases by a remarkable conformational change-based mechanism in which the proteinase, having initiated cleavage of the scissile bond between residues P1 and P1′ of the serpin reactive center loop, is translocated 70 Å from one pole of the serpin to the other [2–5] (Fig. 1A,E). Translocation results from the existence of a covalent acyl linkage between the proteinase active site serine and the P1 residue carbonyl, and the full insertion of the reactive center loop into β-sheet A of the serpin. The reactive center loop must be sufficiently long to permit movement of the proteinase completely to the 'bottom' of the serpin, but short enough that in this position there is sufficient tension between the proteinase and the serpin that the latter is crushed against the former [6]. This results in major rearrangements within the active site of the proteinase, such that the proteinase is no longer effective as an enzyme and the acyl enzyme intermediate is kinetically stabilized against deacylation [4,7–9].

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Abbreviations: P, P2, etc., designation of residues in the reactive center loop, using the nomenclature of Schechter and Berger [1] in which the scissile bond is between residues P1 and P1′, residues N-terminal to this are designated P2, P3, etc. and those C-terminal P2′, P3′, etc.

It has long been recognized that the cleaved loop-inserted form of serpins has extraordinarily high stability compared with the native state ($\Delta G^1+\Delta G^2$ in Fig. 2) [10,11]. It has further been shown that enthalpy changes for reactive center loop cleavage and insertion are much more favorable than for formation of a covalent serpin–proteinase complex [12]. The difference in ΔH has been attributed to unfavorable ΔH needed for distortion of the proteinase. The implication is that there is an analogous difference in ΔG that reflects the energy needed to distort the proteinase (ΔG^3 in Fig. 2). Accordingly, it is assumed that the energy needed to crush the proteinase derives from the favorable energy of insertion of the reactive center loop into β-sheet A.

2. Problems with the current mechanism

One unrecognized problem with this proposal of where the energy for proteinase crushing comes from, is the sequence of the steps involved. Since proteinase crushing is envisioned to occur at the end of insertion of the reactive center loop into β-sheet A, during which any favorable ΔG derived from loop insertion has already been expended, the penultimate conformation of the complex in which the proteinase has yet to be distorted (state F in Fig. 2) would be more stable than the final state (state E in Fig. 2) in which the proteinase has been crushed and hence kinetically inhibited. The equilibrium between these states would thus greatly favor the penultimate one, in which the proteinase was undistorted and hence catalytically functional. This is experimentally clearly not the case.

The second problem with the mechanism is how the reactive center loop manages to burrow underneath helix F, which overlies the lower part of β-sheet A in both native and complex structures (Fig. 1A,E) and how the proteinase manages to pass 'through' helix F, which lies directly in its path of translocation.

3. Hypothesis

I propose a simple solution to both mechanistic problems. Whereas the reactive center loop can insert into β-sheet A and move the proteinase without steric hindrance up to the point at which the proteinase encounters helix F (structure B in Fig. 1), further loop insertion and proteinase movement is impeded by the obstructing F-helix. To displace this would require energy. I propose that just such progressive displacement of helix F occurs as the loop inserts towards completeness and

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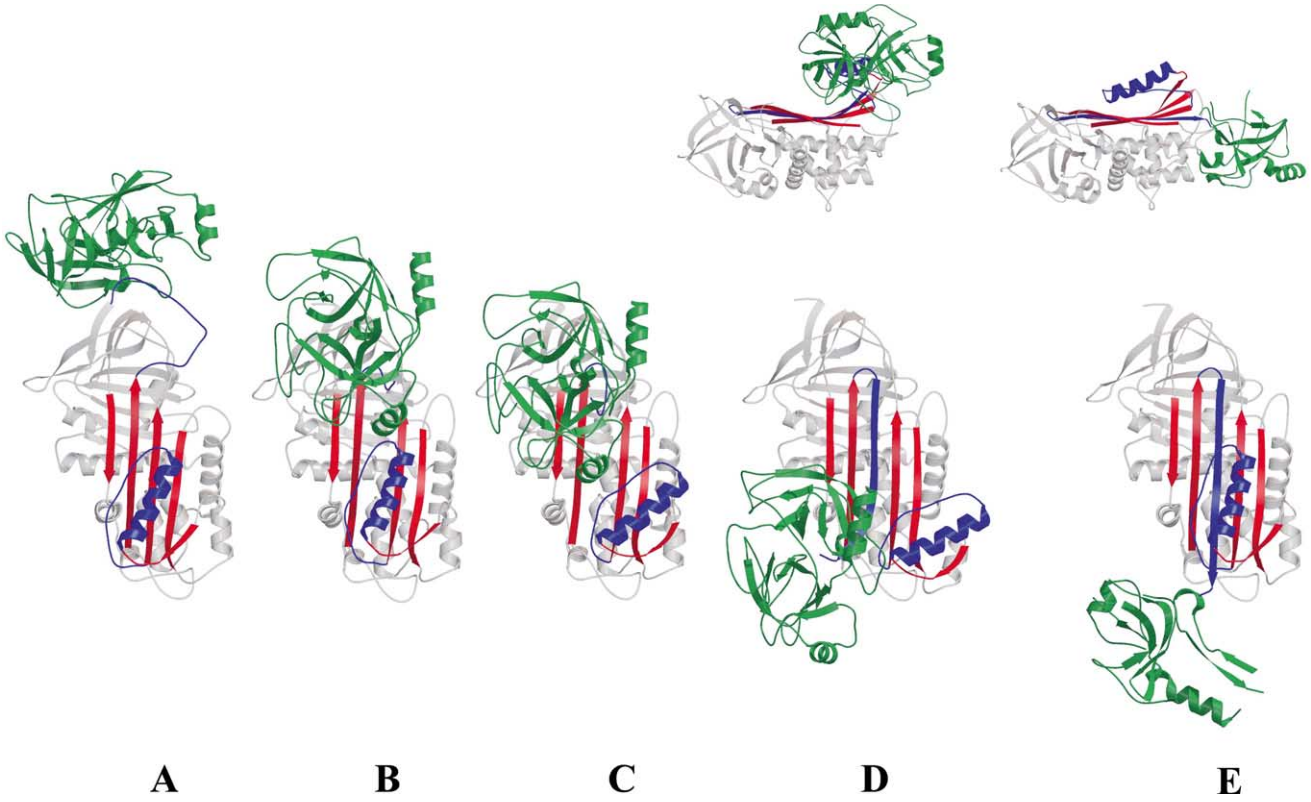

Fig. 1. Schematic of the structures involved in complex formation. A: Complex at the point of cleavage of the reactive center loop and prior to loop insertion. B: Complex after the first few reactive center loop residues have inserted and just as the proteinase reaches helix F. C: Partial displacement of helix F (in blue) needed to permit continued progress of the proteinase (in green) towards the bottom of the serpin. D: Full displacement of helix F with removal of the P1 side chain from the proteinase S1 pocket. Above panel D is a sideways view of structure D to show that the proteinase is still 'above' the plane of β-sheet A and otherwise not distorted. E: Final complex in which the F-helix has returned and the proteinase has been distorted through compression against the bottom of the serpin, and consequently been fully inactivated. Above panel E is a sideways view of the structure to show the movement of the proteinase below the plane of β-sheet A and against the bottom of the serpin.

the proteinase moves towards the bottom of β-sheet A (states C to D in Fig. 2). The energy required to displace the helix (ΔG^4)[1] comes from each successive loop residue that inserts into β-sheet A and is provided in step with the gradual displacement of the helix. Once the proteinase has passed to the very end of β-sheet A, with the reactive center loop fully inserted into the sheet (state D in Fig. 2), it can then access the bottom of the serpin and its crushed conformation (state E in Fig. 2). Since this final movement of the proteinase also allows helix F to return to its position across β-sheet A, the two processes of proteinase crushing and helix return are not only conformationally coupled, but can occur with a favorable energy of $\Delta G^4 - \Delta G^3$ (Fig. 2). This simple model thus proposes that the displaced F-helix acts as a spring to store the energy of loop insertion until it is needed, so the final step is viewed, under normal circumstances, as a single energetically favorable concerted step between states D and E, with no intermediates.

Two refinements of the hypothesis must be considered likely to occur in most natural serpin–proteinase interactions. The first is that, given the limited length of the reactive center loop, it is unlikely that the subsite contacts between P residues of the loop and S subsites can be maintained as the loop fully inserts into the β-sheet. Of these, the most important is the P1 interaction with the S1 pocket of the proteinase. It is known that, in the final complex, the P1 side chain no longer is in this pocket [4,9]. Thus, this energetically unfavorable removal (ΔG^5 of Fig. 2) represents part of the total energy of proteinase crushing (ΔG^3 of Fig. 2). If it has already occurred in state D as a consequence of full loop insertion into β-sheet A, this would increase the energy difference between states D and E by ΔG^5 and increase the relative population of the inactive final conformation (state E). Such removal of the side chain might also cause some distortion of the immediately adjacent reactive site residues of the proteinase and would represent an additional contribution to ΔG^5. The second refinement considers possible favorable interactions between the proteinase and the bottom of the serpin in the final state. This is only likely to occur for a serpin–proteinase pair that represents co-evolved serpin and physiological target proteinase. Such favorable complementarity (ΔG^6 in Fig. 2) would add to the ΔG of inhibition ($\Delta G^{\text{inhibition}}$) by this amount and further shift the equilibrium between states D and E in favor of the inactivated E state.

[1] Note that the free energy of displacing the F-helix will probably differ from that for the return of the F-helix, since β-sheet A, which underlies the helix, has expanded following reactive center loop insertion. The difference may be positive or negative depending on the change in contacts.

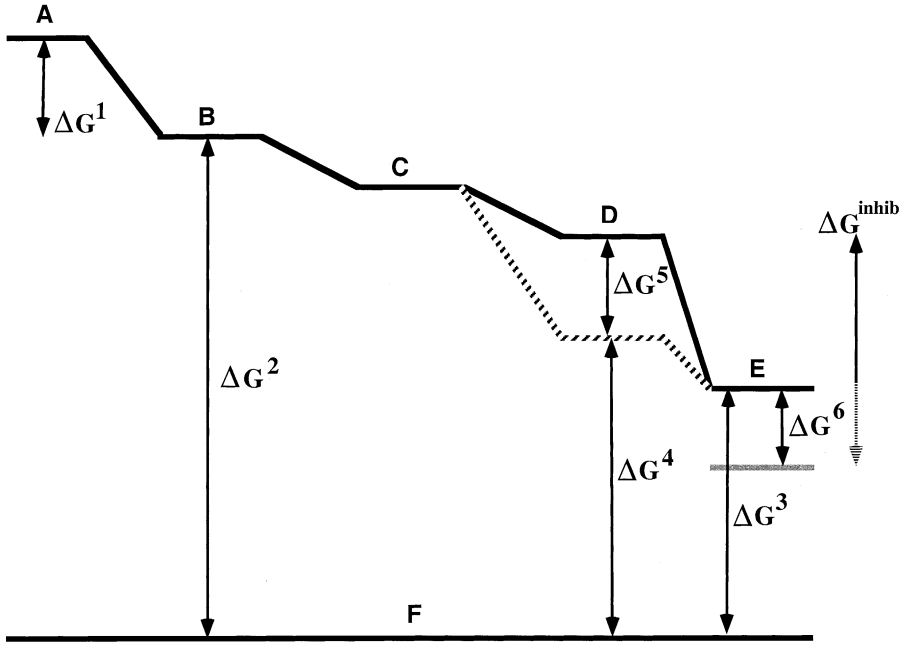

Fig. 2. Energy diagram for the proposed coupling mechanism that allows the energy derived from insertion of the reactive center loop to be 'stored' in a displaced F-helix until needed at the final step to distort the proteinase and ensure that the equilibrium between penultimate and final states greatly favors the latter. The energy derived from loop insertion can be considered to be in two parts, that involving insertion up to about P9 and involving no need to move helix F (ΔG^1) and that for insertion of P8 to P1 (ΔG^2). The energy to displace helix F is ΔG^4 (for simplicity, the ΔG^4 shown is for *return* of helix F: see footnote 1), while the energy to pull the P1 side chain from the S1 pocket of the proteinase is ΔG^5, so the energy of loop insertion that is 'stored' is ΔG^4 if P1 is not pulled out and $\Delta G^4+\Delta G^5$ if it is pulled out. ΔG^3 is the total energy required to distort the proteinase, including extraction of the P1 side chain. ΔG^6 is the additional stabilization afforded the final complex if there are specific favorable serpin–proteinase interactions in that state. The energy of stabilizing the final inactive complex (state **E**) over the penultimate complex (state **D**), $\Delta G^{\text{inhibition}}$, is thus $(\Delta G^4+\Delta G^5)-(\Delta G^3-\Delta G^6)$, where ΔG^6 is most likely to be dependent on the specificity of the serpin–proteinase pair. The baseline is designated as state F, and corresponds to an imaginary structure in which the reactive center loop has fully inserted without any distortion occurring to the proteinase. Note that energy levels shown are arbitrary and would be dependent on the actual serpin–proteinase pair.

4. Predicted consequences and consistency with published findings

1. The first consequence of the hypothesis is that the 'final' serpin–proteinase complex represents an equilibrium between states D and E of Fig. 2. State E is that found in the X-ray crystal structure and is expected to be catalytically inactive. Deacylation from this state would only occur by uncatalyzed base hydrolysis. Conformation D would represent a state in which the proteinase had partial activity resulting from the alignment of the acyl intermediate and the catalytic histidine being unfavorably altered as a result of removal of the P1 side chain from the S1 pocket, and with other changes in the active site resulting from this side chain extraction. Here deacylation would be enzyme-catalyzed, but at a reduced rate. Two recent studies are entirely consistent with this prediction. The first examined the pH dependence of deacylation of serpin–proteinase complexes for several serpin–proteinase pairs [13]. It was shown that there is exactly the predicted equilibrium between two states, a dominant one in which deacylation is independent of His-57 of the proteinase catalytic triad and depends only on uncatalyzed base-mediated hydrolysis, and a minor one in which there is His-57-dependent catalyzed deacylation, but at a rate that, while three orders of magnitude faster than the uncatalyzed rate, is orders of magnitude lower than fully functional proteinase. The occupancy of the two states at pH 7.2 calculated from activity measurements was 0.2% for partially active state D, and 99.8% for the inactive state E. While this represents very effective proteinase inhibition, it involves only a modest energy difference ($\Delta G \approx 3.7$ kcal mol^{-1}) between the two states. This is far less than the energy release (admittedly ΔH) measured for loop insertion during covalent complex formation, which was 20–31 kcal mol^{-1} depending on the serpin–proteinase pair [12].
   The second study was of solvent isotope effects and pH dependence of deacylation of various α_1-antichymotrypsin:neutrophil elastase pairs, in which different mutations were introduced into the reactive center loop that affected the kinetic stability (i.e. rate of deacylation) of the final complex [14]. Some complexes were quite unstable, with half-lives for deacylation of minutes, whereas others had long-term stability. According to my hypothesis, this would represent very different positions for the equilibrium between states D and E. In accord with this, it was found that the kinetically unstable complexes (expected to be predominantly D state) deacylated in a manner that was dependent on the pK_a of the catalytic histidine, whereas the stable complexes (predominantly E state) deacylated in a manner independent of the histidine pK_a.
2. A second prediction is that, for a given serpin that is able to inhibit a range of proteinases, rates of deacylation should be lower for complexes with physiological cognate

proteinases than for complexes with non-cognate proteinases. One of the two studies discussed above addressed this by comparing rates of deacylation of antithrombin complexes with thrombin and factor Xa (both cognate) and with trypsin (non-cognate). The latter deacylated much more rapidly than either of the former [13].

3. Whereas the 'final' serpin–proteinase complex is predicted to be an equilibrium between states D and E, there is the possibility of temporary buildup of earlier intermediates, most notably state B, which represents a complex in which the proteinase has yet to displace helix F and which might therefore be quickly generated following initiation of loop insertion into β-sheet A. However, such partial insertion is viewed as occurring only after formation of the acyl intermediate and hence removal of the constraints on the reactive center loop rather than as a pre-existing equilibrium with significant population of the partially inserted state. In keeping with this the two known structures of non-covalent serpin–proteinase complexes, one examined by X-ray crystallography and one by nuclear magnetic resonance, show no evidence of a significantly populated partially inserted state [15,16].
4. The serpin inhibition mechanism is a branched pathway with the two outcomes being successful completion of the cleavage path and successful formation of the kinetically trapped covalent complex. Relative proportions of each outcome depend on the relative rate constants for each of these branches. My hypothesis predicts that in serpins where there is a particularly strong favorable interaction between the underside of helix F and β-sheet A, there is likely to be a high activation energy for helix F displacement and accordingly a very slow rate constant for the inhibitory branch of the pathway. Non-inhibitory serpins are therefore more likely than inhibitory serpins to have such strong helix F-β–sheet A interactions. Very nice support for this came from the X-ray structure of a reactive center loop variant of ovalbumin [17]. Reactive center loop residues of wild-type ovalbumin that slow down loop insertion near the hinge point [18,19] were replaced with favorable ones and the P1 residue changed to arginine so that trypsin could be used as the reacting proteinase. The variant was still completely non-inhibitory, with the only product being P1–P1′-cleaved ovalbumin, confirming an extremely slow rate for the inhibitory pathway. Nevertheless, the X-ray structure revealed that the reactive center loop had inserted completely into β-sheet A. Based on the hypothesis of helix F displacement, it would then be expected that there would be exceptionally strong interactions involving helix F and β-sheet A, which was indeed found to be the case.
5. The converse of point 4 is the predicted effect of weak helix F-β–sheet A interactions. Native, active serpins represent metastable conformations that can convert to inactive, so-called latent, conformations by insertion of the reactive center loop into β-sheet A and extraction of strand 1 from β-sheet C. Such insertion would, like covalent complex formation with proteinase, require temporary movement of helix F. There should therefore be a correlation between the tendency to convert to a latent conformation and the strength of the helix F-β–sheet A interactions. PAI-1 is the serpin most susceptible to conversion to the latent conformation. Attempts by random mutagenesis to retard this conversion produced variants with up to ~100-fold slower rates of conversion [20]. The best variant examined contained four mutations, two of which were located in the loop connecting helix F to β-sheet A, which would need to be moved as part of displacing helix F.
6. There are two final examples of previously puzzling experimental observation that can now be rationalized by the proposed hypothesis. The first is the alteration in kinetic stability of serpin–proteinase complexes as a result of mutations in the reactive center loop between P2 and P7 [14,21–23]. If the only conformation that contributed to the final state of the serpin–proteinase complex were the completely inactive one found in the X-ray structure, it would be hard to explain the influence of mutations in the P2 to P7 region on rates of deacylation of the final complex. However, residues within this stretch come into contact with the underside of the F-helix once they are inserted into β-sheet A in the complex. Accordingly the energy released when the F-helix returns to its position overlying β-sheet A (ΔG^4 in Fig. 2) will be influenced by the nature of the residues in this strand of the sheet that had previously been the exposed reactive center loop. In turn this will affect the magnitude of $\Delta G^{\text{inhibition}}$ and hence the fraction of the final complex that has the more active D conformation (Fig. 1). This readily accounts for the various studies showing large changes in kinetic stability of complexes between the same serpin and proteinase as a function of such reactive center loop mutations.

   The second puzzling observation was in a study on a monoclonal antibody that bound tightly to covalent complexes of antithrombin, with different proteinases, but that did not bind to either native or cleaved forms of the serpin [24]. The epitope was found to be a pentapeptide from strand s4A, which would be expected to be obscured under helix F and the connecting strand in both native and cleaved states, and also in a covalent complex that resembled that found in the X-ray structure of α_1PI and trypsin. Accordingly, the authors favored a different complex structure involving only partial loop insertion, with the implication that the F-helix was partially displaced. The present model readily explains not only how the antibody could bind to covalent complexes without the need for a new 'final' structure, but also to not bind to cleaved serpin. Thus, since the energy difference between states D (epitope exposed) and E (epitope hidden) for complexes is only a few kcal mol^{-1} (see above) the antibody would recognize and bind to state D and pull the equilibrium over to that state, using part of the intrinsic epitope affinity. In contrast, for cleaved serpin the energy difference between the normal cleaved state and one in which the F-helix is displaced and the epitope exposed would be very much higher (ΔG^4 in Fig. 2), and is less able to form significant amounts of antibody–serpin complex.

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