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Proteolytically cleaved mutant antithrombin-Hamilton has high stability to denaturation characteristic of wild type inhibitor serpins

H. Tonie Wright[a,*], Morris A. Blajchman[b]

[a]*Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, VA 23298-0614, USA*
[b]*Department of Pathology, McMaster University, and the Canadian Red Cross Society, Hamilton, Ont., L8N-3Z5, Canada*

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Abstract

The serpin family of proteins consists primarily of proteinase inhibitors which form tight complexes with target proteinases. Inhibitor serpins are cleaved by proteinase and undergo a large conformational change in which the polypeptide segment terminating at the target reactive site, at which cleavage takes place, inserts itself as an additional strand, s4A, in the center of a preexisting β-sheet. This change in conformation increases the stability towards denaturation of the cleaved serpin relative to the native uncleaved form. Mutant serpins with single amino acid changes in the s4A strand have been identified, and in most cases these are proteinase substrates but not inhibitors. We have measured the stability to denaturation of one of these non-inhibitor substrate mutants, antithrombin-Hamilton, which has an Ala → Thr change at position P12 in strand s4A. We find that it undergoes the transformation to the more stable form which is observed for inhibitor serpins, from which we conclude that the Ala → Thr change in antithrombin-Hamilton does not prevent insertion of s4A into β-sheet A in the cleaved form.

Key words: Antithrombin; Serpin; Stability; β-sheet; Denaturation

1. Introduction

The serpins are a large family of proteins, most of which are inhibitors of serine proteinases [1–4]. They are distinct from the smaller proteinase inhibitors [5], such as basic pancreatic trypsin inhibitor, in being much larger and in having different mechanisms of inhibition. Both classes of inhibitors form tight complexes with target proteinases, but covalent serpin–proteinase complexes are stable, even under denaturing conditions. The mechanism of serpin inhibition has a slow, irreversible step with the characteristics of suicide substrate inhibition [6–9]. It has been inferred from crystal structures of postcleavage serpins that the reactive site segment of serpins undergoes a large change in position upon cleavage, from being exposed in the proteinase target site to being inserted as a new strand, s4A, in the preexisting β-sheet A [10,11]. This change is implicated in the inhibitor activity of the serpins, since the non-inhibitor serpin, ovalbumin, does not undergo the strand insertion upon proteinase attack to form plakalbumin [12,13]. This failure to insert in plakalbumin has been attributed to the size and charge of residue Arg-345[1] in strand s4A, located at the P14 position with respect to the cleavage site at P1. Insertion of s4A in plakalbumin into β-sheet A would require the insertion of the Arg-345 side chain into a hydrophobic interior pocket, which would be energetically very unfavorable. This failure of s4A to insert into β-sheet A in plakalbumin, and the fact that ovalbumin functions as a proteinase substrate instead of an inhibitor, led to the suggestion that strand insertion is a requirement for serpin inhibitor activity [12].

In addition to ovalbumin and plakalbumin, a number of serpin mutants at sites in strand s4A amino terminal to the cleavage site at P1, have been identified [14–26]. These point mutations occur primarily at positions P10 and P12, lack inhibitor activity, and usually but not always, are cleaved as proteinase substrates. Their lack of inhibitor activity has been ascribed to the same failure of s4A to insert into β-sheet A that characterizes ovalbumin: viz. altered size of an amino acid which must fit into the protein interior beneath β-sheet A upon strand insertion. However, the link between inhibitory activity and this strand insertion is still not completely understood.

Since these mutants offer a potential means of studying the relationship between strand insertion and proteinase inhibitor activity, we have determined whether cleavage of antithrombin-Hamilton by proteinase leads to the increase in stability to denaturation which is characteristic of insertion of s4A into β-sheet A in wild type serpins. We have measured the stability of intact antithrombin-Hamilton and its cleaved form and compared these with the stabilities of the corresponding wild type antithrombin. The results of these measurements are consistent with molecular model building studies which show that the P12 threonine of s4A can be accommodated in the strand inserted conformation of β-sheet A (Wright, H.T. and Scarsdale, J.N., submitted).

*Corresponding author.

[1] Sequence numbering and nomenclature of the secondary structural elements is that of Huber and Carrell [11].

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2. Materials and methods

2.1. Isolation and purification of antithrombins

Antithrombin was obtained from the propositus as a 50:50 mixture of wild type and antithrombin-Hamilton, and purified by heparin–Sepharose chromatography. Wild-type antithrombin was separated from cleaved antithrombin-Hamilton by thrombin–Sepharose chromatography [23]. The antithrombin mixture purified by heparin–Sepharose chromatography in 0.15 M NaCl, 0.02 M Tris, pH 7.5 (Tris-buffered saline, TBS) was suspended with an equal volume of thrombin–Sepharose beads, washed with the same buffer, and incubated for 60 min. The beads were then pelleted by brief centrifugation and the supernatant containing cleaved antithrombin-Hamilton removed. Measurements described here were made with the 50:50 mixture of uncleaved wild type antithrombin and antithrombin-Hamilton and with homogeneous cleaved antithrombin-Hamilton purified as described.

Wild type antithrombin was purified by heparin–Sepharose chromatography and was homogeneous on SDS polyacrylamide gel. Cleaved wild type antithrombin was prepared by incubation with pancreatic elastase in TBS for 1 h under the conditions described by Olson [25]. Free heparin was removed by addition of a 10-fold excess (by weight) of protamine sulfate followed by centrifugation for 30 min at 16,000 × *g* in a microfuge. This sample was desalted on a Biogel P6DG column and concentrated by pressure dialysis. The sample was a single band and identified as cleaved by its slower mobility on a 9% non-reducing SDS PAGE gel.

2.2. Denaturation profiles of antithrombins

The stability to denaturation of the intact and cleaved forms of antithrombin was measured by monitoring the red shift of the intrinsic fluorescence as a function of guanidine hydrochloride concentration on a Shimadzu RF5000U spectrophotofluorometer [29]. Samples of antithrombin in TBS were diluted with water and with concentrated TBS and 8 M guanidine hydrochloride solution in water to give a final sample volume of 350 μl of 0.02 M Tris, 0.15 M NaCl, pH 7.5 at a concentration of 50 or 100 μg/ml of antithrombin in varying concentrations of guanidine hydrochloride. After equilibration at 25°C, samples were excited at a wavelength of 280 mμ and the λ_{max} for emission determined from a scan between 300 mμ and 360 mμ. Data were expressed as a surrogate fractional concentration of native antithrombin in the expression:

$$F = \frac{\lambda_{denatured} - \lambda_{obs}}{\lambda_{denatured} - \lambda_{native}}$$

measured as a function of guanidine hydrochloride concentration.

3. Results and discussion

The denaturation curves for intact and cleaved antithrombin and antithrombin-Hamilton show that the cleaved forms of both the wild-type and mutant antithrombin are highly stabilized to denaturation relative to their intact forms (Fig. 1). This is consistent with insertion into β-sheet A of strand s4A, liberated from the reactive site region upon proteolysis, in the cleaved antithrombin-Hamilton mutant.

Increased stability to denaturation of the inhibitor serpins, and lack of such stabilization in non-inhibitor serpins, following proteolytic cleavage has been observed a number of times [29–34] and has been ascribed to the insertion into β-sheet A of the reactive site strand s4A liberated by peptide bond cleavage of the reactive site. The mutant serpins with single amino acid changes in this s4A strand in the P10 to P14 residues, by analogy to the case of ovalbumin/plakalbumin and angiotensinogen [35], have been thought to be unable to insert s4A into β-sheet A because of the substitution of an oversize or polar amino acid for the wild-type one [20]. This would be consistent with the observation from crystal structures that the even numbered residues (e.g. P10, P12 and P14), at which almost all the mutations are observed to occur, are oriented with their amino acid side chains facing inward to the hydrophobic interior of the molecule and are thus constrained in size and polarity if strand insertion is to occur.

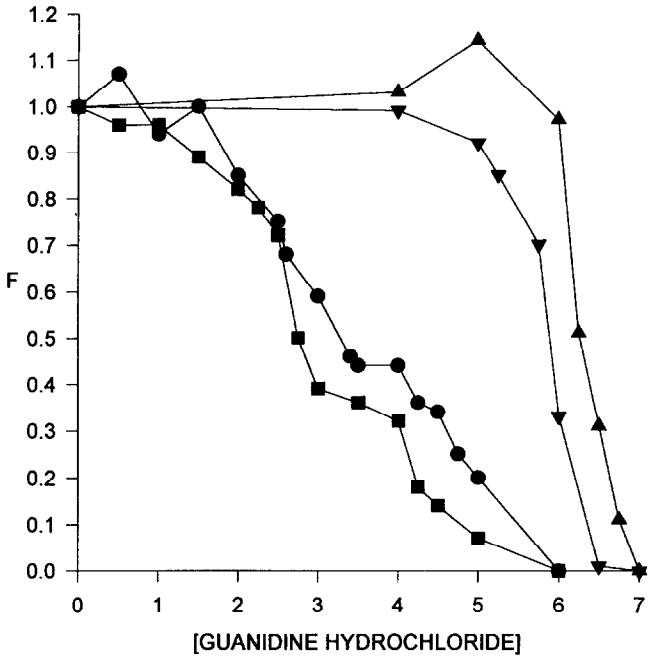

Fig. 1. Denaturation transition curves of antithrombin wild type and antithrombin-Hamilton measured as a function of guanidine hydrochloride concentration. The parameter *F*, defined in the text, is the red shift of the emission maximum expressed as a fraction of the red shift for fully denatured antithrombin. (□ = uncleaved wild type; ● = uncleaved mutant; ▽ = cleaved wild type; △ = cleaved mutant).

It has also been shown for α_1-proteinase inhibitor that uptake of exogenous free peptides of varying length, with sequences of the α_1-proteinase inhibitor s4A strand, affect the inhibitor activity of the serpin [21]. Peptides of length and sequence corresponding to P1–P12, P1–P14, P8–P14 and P4–P14 all abolish the inhibitor activity of α_1-proteinase inhibitor and transform it into a trypsin substrate. Peptides truncated at the amino termal end corresponding to P1–P8 up to P1–P11 retain significant inhibitor activity, but the amounts of stable complex formed with several proteinases relative to native inhibitor are reduced in the presence of these peptides.

The observation described here of increased stability to denaturation of the antithrombin-Hamilton mutant is consistent with insertion of s4A into β-sheet A. This is further supported by model building studies of this mutant which indicate that the threonine residue can be

accommodated in the site occupied by alanine in the wild type (Wright, H.T. and Scarsdale, J.N., submitted). This model even suggests a possible increase in stability of the strand-inserted form of antithrombin-Hamilton relative to the wild type, based on the utilization of more hydrogen bond donor and acceptor groups in buried positions under s4A, and we do observe a small increase in stability of cleaved antithrombin-Hamilton relative to the wild type (Fig. 1).

The multiple serpin mutants in the P10–P14 residues of s4A, and the structure of the non-inhibitor, ovalbumin, are consistent with an important role for s4A insertion in serpin inhibitor activity. The peptide insertion studies of Schulze et al. [21] also imply that failure of the P12–P14 segment s4A to insert is sufficient to abolish inhibitor activity. However, the results on antithrombin-Hamilton reported here do not support the converse proposition that s4A insertion in P12–P14 is sufficient to confer inhibitor activity on a serpin. We suggest that the kinetics of strand insertion may also play a role in determining whether a serpin functions as an inhibitor. Investigation of this possibility will require a different type of experiment than the equilibrium and static crystal studies done thus far.

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