Significance of secondary structure predictions on the reactive center loop region of serpins: a model for the folding of serpins into a metastable state

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Abstract To address how serpins might fold so as to adopt the mechanistically required metastable conformation we have compared the predicted secondary structures of the reactive center loops (RCLs) of a large number of serpins with those of the equivalent regions of other non-serpin protein proteinase inhibitors. Whereas the RCLs of non-serpin inhibitors are predicted to be loop or β-strand, those of inhibitory serpins are strongly predicted to be α-helical. However, non-inhibitory serpins, which also adopt the metastable conformation, show no consistent preference for α-helix. We propose that the RCL primary structure plays little role in promoting the metastable serpin conformation. Instead we hypothesize that preference for the metastable state results from the incorporation of part of the RCL into β-sheet C, which as a consequence precludes incorporation of the RCL into β-sheet A to give the most stable conformation. Consequently the RCL must be exposed and by default will adopt the most stable conformation in this particular context, which is likely to be an α-helix irrespective of the primary structure. Thus the observed correlation between inhibitory properties in serpins and prediction of α-helix in the RCL may instead reflect a need for alanine residues between positions P12 and P9 for functioning as an inhibitor rather than a structural or mechanistic requirement for α-helix.

Key words: Metastable state; Protein folding; Reactive center loop; Secondary structure prediction; Serpin

1. Introduction

Proteins of the structurally homologous serpin (*ser*ine *p*roteinase *in*hibitor) family are of particular interest to protein biochemists both for their unusual functional properties and for their unusual folding and conformational properties. Thus serpins are mechanism-based, suicide substrate inhibitors that appear to form irreversible covalent complexes with target proteinase through a branched pathway mechanism that can lead either to stable complex formation or else to cleavage of the serpin as a substrate [2]. This is in marked contrast to the other common classes of protein inhibitors of serine proteinases, such as the Kunitz, Kazal, Bowman-Birk, or potato inhibitors, which form tight but reversible non-covalent complexes with their target proteinases [3]. Unlike almost all other proteins [4–8], serpins appear to fold in a kinetically trapped metastable conformation which under appropriate conditions can change to a more stable conformation.

There is now considerable structural information on the different conformations that serpins can adopt, based on X-ray structure determinations. The first serpin structure solved was of α_1-proteinase inhibitor. However, this was of a reactive center-cleaved form rather than of the native serpin [9]. This cleaved form is more stable than the native form as a result of insertion of the reactive center loop into β-sheet A, leading to the separation of the reactive center P1 and P1′ residues by ~70 Å. The structure of the intact form was speculated to be similar to the cleaved form except that the reactive center loop would be removed from β-sheet A and would adopt a conformation similar to that seen in Kazal-type inhibitors. The cleaved forms of α_1-antichymotrypsin [10], equine leukocyte elastase inhibitor [11], antithrombin [12] and a PAI-1 variant [13] all have the same type of reactive center loop-inserted structure. The first structure of an uncleaved serpin was that of ovalbumin, a non-inhibitory member of the family (Fig. 1). In this structure the reactive center adopts an α-helical conformation with no insertion of the reactive center loop into β-sheet A [14]. In contrast to the situation with the inhibitory serpins, ovalbumin does not undergo a conformational change upon cleavage [15] or have increased stability [16,17]. In spite of this difference the structure of native ovalbumin was considered to be a good model for a native inhibitory serpin. Subsequently the structures of a native α_1-antichymotrypsin variant [18] and of native α-proteinase inhibitor [19] gave support to the idea that the reactive center loop is in a helical conformation rather than the elongated 'canonical' conformation found in low molecular weight protein proteinase inhibitors and suggested to occur in serpins [20]. Recent structures of native antithrombin [21,22], in which the reactive center loop is partially inserted into β-sheet A, represent a special case where expulsion of the reactive center loop from β-sheet A must occur, as a result of heparin binding, to give the activated state [23]. Thus, the conformation of the serpin reactive center loop in both inhibitory and non-inhibitory serpins is likely to be helical, in a solvent exposed environment, with the P1–P1′ bond accessible to proteinase. In inhibitory serpins, cleavage within the reactive center loop may remove

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; LICI, Limulus coagulation inhibitor; α_2-M, α_2-macroglobulin; OM, ovomucoid; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; PEDF, pigment epithelium-derived factor; STI, soybean trypsin inhibitor; TFPI, tissue factor pathway inhibitor; UTMP, uterine milk proteins; strands of β-sheets A or C are designated s#A or s#C respectively, where # represents the strand number.

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constraints on it, thereby allowing it to insert into β-sheet A. This can also occur in the intact serpin, with varying degrees of facility, as shown by the structures of latent PAI-1 [24] (Fig. 1B) and latent antithrombin [21,22] and by evidence for the existence of a latent α_1-proteinase inhibitor [25].

These structural data lead to the question of how, during the process of protein folding, the serpin adopts the less stable conformation with an exposed helical reactive center rather than the more stable loop-inserted conformation. In this paper we attempt to answer this question by examining the secondary structures predicted by the Rost and Sander protocol [26] for the reactive center loop regions of both serpins and non-serpin serine proteinase inhibitors. We found that the reactive center region of *inhibitory* serpins is in almost all cases strongly predicted to be α-helical. This is in contrast to the equivalent region not only of non-serpin serine proteinase inhibitors but also of non-inhibitory serpins. This suggests that the strong preference for α-helix correlates with the somewhat restricted property of inhibition in serpins rather than with the more general property of bringing about the metastable serpin fold. It thus appears that adoption of the exposed conformation of the reactive center loop does not depend on the primary structure of the reactive center region, but results from folding of the rest of the serpin such as to exclude the reactive center loop from β-sheet A. Our hypothesis is that the mechanism for such exclusion is the early formation of β-sheet C as a stable structure, which is mutually exclusive with formation of the more stable latent conformation of the serpin with the reactive center loop incorporated into β-sheet A.

2. Materials and methods

2.1. Secondary structure predictions

Secondary structure predictions used the neural training network method developed by Rost and Sander [26]. This method uses three levels of evaluation, with the third level being a winner-takes-all prediction of helix, strand or sheet secondary structure. For water soluble proteins this has been found to give prediction of secondary structure to an accuracy of better than 70% [26]. Sequences were submitted electronically to EMBL (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) [27]. The output included both the predicted secondary structure from winner-takes-all and probabilities for α-helix, β-strand, and neither α-helix nor β-strand.

3. Results

Both inhibitory and non-inhibitory serpins exist. Non-inhibitory serpins can be further divided into (i) those that undergo insertion of the reactive center loop into β-sheet A upon proteolysis and (ii) those that do not undergo such a cleavage-induced conformational change. Inhibitory serpins include antithrombin, plasminogen activator inhibitor 1, α_1-proteinase inhibitor, and α_1-antichymotrypsin. Non-inhibitory but conformationally labile serpins include corticosteroid binding globulin and thyroxine binding globulin [28], whereas non-inhibitory non-conformationally labile serpins include ovalbumin [17,29], angiotensinogen [29] and the recently discovered tumor suppressor serpin maspin [30]. Since the structural requirements for each class of serpin might depend on the functional properties of the serpin, we considered the secondary structure predictions for the reactive center loop region and flanking sequences separately for each type of serpin. In addition some serpins are incompletely characterized. A separate category of serpins of unknown properties was therefore also considered.

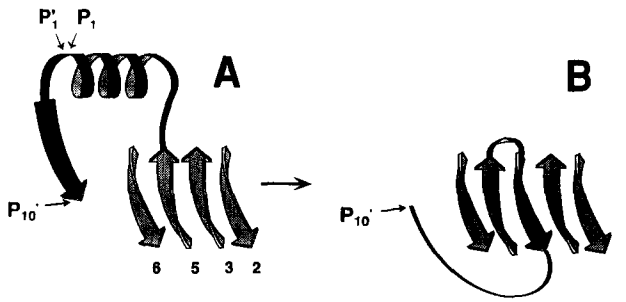

Fig. 1. Schematic ribbon representation of the four main strands of β-sheet A, strand 1 of β-sheet C, and the reactive center loop region in metastable and most stable conformations of serpins. A: Metstable conformation based on the structure of ovalbumin [14] in which the reactive center region is a two and a half turn α-helix, with the P1–P1′ bond at one end of the helix and each end of the helix attached through short stems to strands of β-sheets A (s5A) and C (s1C). B: Most stable conformation, based on the structure of latent PAI 1 [24]. Here the reactive center loop has fully inserted into β-sheet A. Since the polypeptide is still intact, this can only be accomodated by removal of strand 1C (residues P4′–P10′) from β-sheet C.

For each prediction a stretch of 40 amino acids was taken consisting of 25 residues preceding the P1–P1′ bond and 15 residues following this, i.e. from P25 to P15′. (The nomenclature system of Schechter and Berger [1], used to describe the subsites of interaction between a proteinase and its substrate, designates the residues on either side of the substrate scissile bond as P1 and P1′. Residues N-terminal to this are designated P2, P3 ... Pn and residues C-terminal are designated P2′, P3′ ... Pn′.) The alignments to determine the location of P1 have been previously reported by others and are based either on known inhibitory recognition sites or by optimization of sequence similarities between serpins [31]. The extension to P25 and to P15′ was used to provide significant stretches of the polypeptide outside the reactive center loop region itself (approximately P16–P3′) for comparison with the reactive center loop region.

3.1. Inhibitory serpins

Nineteen serpins known to be inhibitors of serine proteinases were considered. On the winner-takes-all basis of the Rost and Sander secondary structure prediction method [26], 16 out of 19 of these serpins resulted in predictions of α-helix within the reactive center loop region from about P14 to about P6, with non-consistent prediction of α-helix or β-sheet between P6 and P1 (Fig. 2). When the numerical probabilities for α-helix for these regions were examined (results not shown), the same 16 out of 19 serpins showed similarly shaped single peak profiles, though with different amplitudes at the maximum. The maximum probability for α-helix was in most cases centered on residues P11–P9. This probability fell to baseline in the region of the scissile P1–P1′ bond for most of these inhibitory serpins. The three exceptions to these findings were for a chimeric recombinant variant of α_1-antichymotrypsin of known structure [18], for a Limulus coagulation inhibitor (LICI) [32], and for heparin cofactor II, for none of which there was prediction of α-helix in the reactive center region. Instead β-strand was predicted for the region P12 to P2 (Fig. 2).

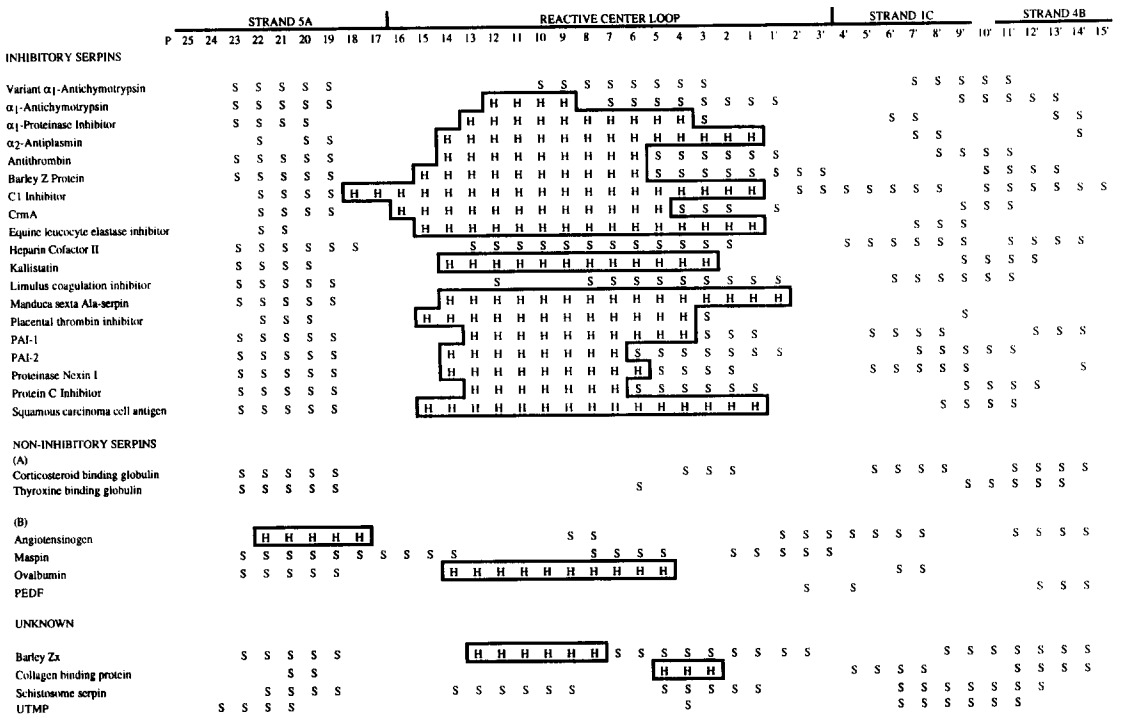

Fig. 2. Secondary structure predictions (winner-takes-all) for all serpins examined. The letters S and H signify prediction for β-strand and α-helix respectively. Blank spaces signify no prediction for β-strand or α-helix. The sequences used and the alignments for determining the position of the P1–P1′ residues were from the literature.

3.2. Loop-insertable non-inhibitory serpins

Neither of the two naturally occurring loop-insertable non-inhibitory serpins, corticosteroid binding globulin and thyroxine binding globulin, gave a prediction of α-helix within the reactive center loop and each gave only a small prediction of β-strand towards the C-terminal end of the reactive center loop (Fig. 2).

3.3. Non-loop-insertable non-inhibitory serpins

Of the non-loop-insertable non-inhibitory serpins, neither angiotensinogen, maspin, nor PEDF gave any prediction of α-helix within the reactive center region, whereas ovalbumin gave prediction of α-helix from P14 to P5 (Fig. 2). On average the α-helix prediction showed a very different distribution within the 40 residue region from that for the inhibitory serpins (not shown).

3.4. Serpins of uncertain properties

Different predictions were found for members of this group. UTMP [33,34] and the schistosome serpin [35] were not predicted to contain helix within the reactive center region, whereas barley Zx [36] was predicted to be α-helical between P13 and P8 and collagen binding protein [37] was predicted to have a short stretch of helix from P5 to P3 (Fig. 2).

3.5. Reactive center region primary structures

To determine whether the prediction of α-helix in the N-terminal region of the reactive center region of inhibitory serpins resulted from conservation of particular amino acids in this region rather than from more varied but nevertheless helix-promoting residues, we compared the primary structures in this region for the serpins considered in Fig. 2 (Fig. 3). As has been noted by others [13,38], there is a very high tendency for inhibitory serpins to have the sequence Ala-Ala-Ala-Ala, or a variation in which any replacements were by small uncharged side chains such as serine, glycine or threonine, whereas in the non-inhibitory serpins there was no such pattern. Equally striking was that this conservation was restricted to the N-terminal portion of the reactive center region, which is the part that is expected to undergo loop insertion during formation of stable complex with proteinase, leaving the remaining residues (P9–P3′) exposed or possibly in contact with subsites on the proteinase.

3.6. Secondary structure prediction for serpins outside the reactive center region

As a control for the accuracy of the predictive algorithm when applied to serpins, we used the same protocol to predict the secondary structure for the complete sequence of two serpins of known structure, ovalbumin and PAI-1 [14,24]. Generally excellent agreement was found *outside the reactive center region* between the observed and predicted secondary structure for each of these serpins, with successful prediction of all of the regions of α-helix and β-sheet, though with some errors in the secondary structure boundaries (results not shown). It is of note that the stretch of β-sheet (s5A), which immediately precedes the reactive center, is well predicted for all except angiotensinogen. This is in contrast to the failure to predict the more stable β-strand for the reactive center region of PAI-1 (see Fig. 2), which is the structure seen in both cleaved and latent forms. Thus the Rost and Sander protocol gives valid results for the majority of the sequence, but not for the reactive center, for which primary structure clearly can not be the sole determinant of secondary structure.

	STRAND 5A									REACTIVE CENTER LOOP													
	P	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
INHIBITORY SERPINS																										
Variant α_1-antichymotrypsin		H	K	A	V	L	D	V	F	E	E	G	T	E	A	S	G	T	T	A	V	K	I	I	P	M
α_1-Antichymotrypsin		H	K	A	V	L	D	V	F	E	E	G	T	E	A	S	A	A	T	A	V	K	I	T	L	L
α_1-Proteinase Inhibitor		H	K	A	V	L	T	I	D	E	K	G	T	E	A	A	G	A	M	F	L	E	A	I	P	M
α_2-Antiplasmin		H	Q	S	T	L	E	L	S	E	V	G	V	E	A	A	A	A	T	S	I	A	M	S	R	M
Antithrombin		H	K	A	F	L	E	V	N	E	E	G	S	E	A	A	A	S	T	A	V	V	I	A	G	R
Barley Z Protein		H	K	S	F	V	E	V	N	E	E	G	T	E	A	G	A	A	T	V	A	M	G	V	A	M
C1 Inhibitor		H	Q	T	V	L	E	L	T	E	T	G	V	E	A	A	A	A	S	A	I	S	V	A		R
CrmA		H	K	T	Y	I	D	V	N	E	E	Y	T	E	A	A	A	A	T	C	A	L	V	A		D
Equine leucocyte elastase inhibitor		H	K	S	F	V	D	L	N	E	E	G	T	E	A	A	A	A	T	A	G	T	I	M	L	A
Heparin Cofactor II		H	Q	G	T	I	T	V	N	E	E	G	T	Q	A	T	T	V	T	T	V	G	F	M	P	L
Kallistatin		H	K	A	T	L	D	V	D	E	A	G	T	E	A	A	A	A	T	T	F	A	I	K	F	F
Limulus coagulation inhibitor		H	R	T	L	I	E	V	N	E	E	G	T	E	A	S	G	I	S	S	V	V	A	G	V	R
Manduca sexta Ala-serpin		Q	K	A	F	I	E	V	N	E	E	G	A	E	A	A	A	A	N	A	F	G	I	V	P	A
Placental thrombin inhibitor		H	K	S	F	V	E	V	N	E	E	G	T	E	A	A	A	A	T	A	A	I	M	M	M	R
PAI-1		Q	K	V	K	I	E	V	N	E	S	G	T	V	A	S	S	S	T	A	V	I	V	S	A	R
PAI-2		H	Q	A	M	V	D	V	N	E	E	G	T	E	A	A	A	G	T	G	G	V	M	T	G	R
Proteinase Nexin I		Q	K	A	K	I	E	V	S	E	D	G	T	K	A	S	A	A	T	T	A	I	L	I	A	R
Protein C Inhibitor		H	K	A	V	V	E	V	D	E	S	G	T	R	A	A	A	A	T	G	T	I	F	T	F	R
Squamous carcinoma cell antigen		H	K	A	F	V	E	V	T	E	E	G	A	E	A	A	A	A	T	A	V	V	G	F	G	S
NON-INHIBITORY SERPINS																										
(A)																										
Corticosteroid binding globulin		H	K	A	V	L	Q	L	N	E	E	G	V	D	T	A	G	S	T	G	V	T	L	N	L	T
Thyroxine binding globulin		H	K	A	V	L	H	I	G	E	K	G	T	E	A	A	A	V	P	E	V	E	L	S	D	Q
(B)																										
Angiotensinogen		L	N	S	I	F	F	E	L	E	A	D	E	R	E	P	T	E	S	T	Q	Q	L	N	K	P
Maspin		S	N	V	I	H	K	V	C	L	E	I	T	E	D	G	G	D	S	I	E	V	P	G	A	R
Ovalbumin		H	A	A	H	A	E	I	N	E	A	G	R	E	V	V	G	S	A	E	A	G	V	D	A	A
PEDF		H	R	A	G	F	E	W	N	E	D	G	A	G	T	T	P	S	P	G	L	Q	P	A	H	L
UNKNOWN																										
Barley Zx		H	K	S	F	V	E	V	N	E	E	G	T	E	A	A	A	R	T	A	R	V	V	T	L	R
Collagen binding protein		H	A	T	A	F	E	W	D	T	E	G	N	P	F	D	Q	D	I	Y	G	R	E	E	L	R
Schistosome serpin		Q	R	N	V	L	K	L	N	E	S	G	I	E	A	T	T	V	T	S	P	I	F	V	P	F
UTMP		E	I	E	L	S	E	H	A	L	T	T	D	A	A	K	D	K	D	N	L	L	K	V	P	A

Fig. 3. Alignment of the primary structures of the serpins considered in Fig. 2. The gaps in the sequence for C1 inhibitor and crmA are to optimize alignment of the sequence while still identifying P1 correctly. The sequences used and the alignments for determining the position of the P1–P1′ residues were from the literature.

3.7. Non-serpin inhibitors

Two distinct types of non-serpin inhibitors were examined, lock-and-key inhibitors and the α_2-macroglobulin family which inhibit by physical sequestration.

Because of the smallness of some of the lock-and-key inhibitors, which include proteins of the Kunitz, Kazal, Bowman-Birk and potato inhibitor families, there were not always 25 residues N-terminal to the reactive center bond available for analysis. We therefore considered a smaller stretch of polypeptide that could be analyzed for all members of these classes of non-serpin inhibitors, which represented 30 residues from P15 to P15′. Of 16 such regions examined none gave any prediction of α-helix in the region corresponding to the predicted α-helical region of the reactive center loop of the serpins (Fig. 4).

For the α-macroglobulins analyzed, the stretch of 30 residues was taken from the bait region and was chosen to start at the end of the region of highly conserved sequence (residue 670 in the case of human α_2-macroglobulin). This 30 residue stretch was centered approximately on the primary proteinase cleavage sites within the bait region and so corresponded approximately to the region examined for the other non-serpin inhibitors. In the region containing proteolytic cleavage sites and immediately N-terminal to this there was no prediction of α-helix, but instead a strong prediction of β-strand (Fig. 4).

4. Discussion

Prior to this study, a plausible mechanism for the adoption of the metastable state by serpins was that the reactive center loop adopts a helical conformation at an early stage during folding and is consequently prevented form integrating into β-sheet A. However, for such a mechanism to hold it must apply generally to serpins that adopt the metastable state, irrespective of whether or not they are inhibitory. Although in this study most inhibitory serpins did show preference for α-helix, the extent of this helix was variable and three inhibitory serpins showed no prediction for helix at all. Moreover, both loop-insertable and non loop-insertable non-inhibitory serpins showed little or no prediction for α-helix. We therefore conclude that adoption of an α-helical conformation during folding can not be the basis for formation of the metastable state. Instead, there must be factors outside the reactive center loop that determine the preference for adoption of the metastable state.

In determining which regions of the serpin core are most likely to influence the exclusion of the reactive center region from β-sheet A, it is useful to contrast the structures of native ovalbumin and latent PAI 1, shown schematically in Fig. 1A,B, since these are likely to represent the alternative metastable and most stable types of structure respectively for a

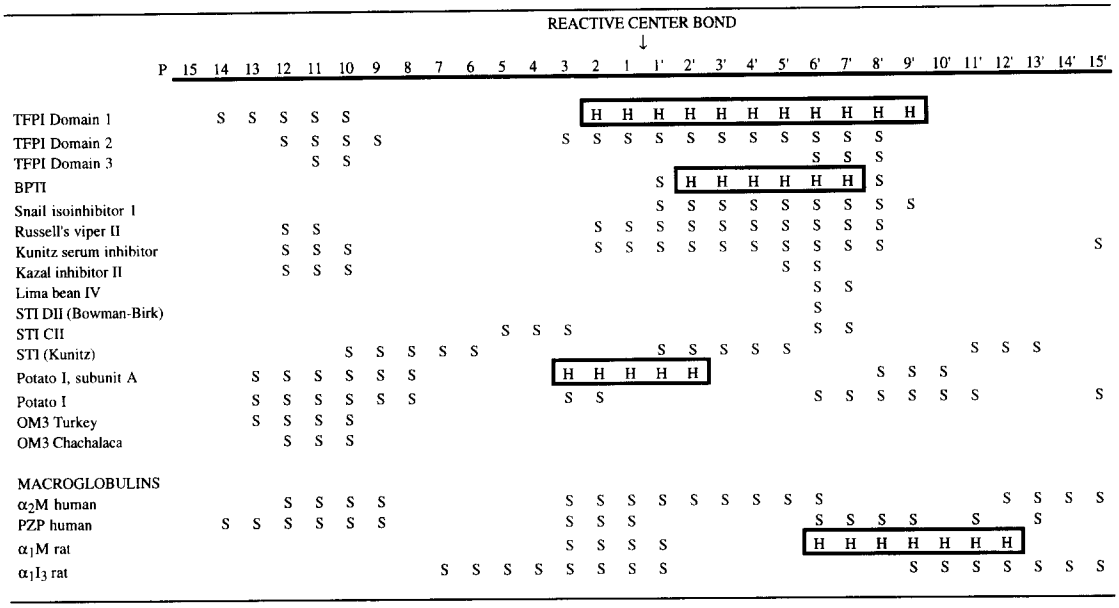

Fig. 4. Secondary structure predictions for non-serpin protein proteinase inhibitors of serine proteinases.

given serpin. To convert the metastable structure into the stable structure requires not only insertion of the reactive center loop into β-sheet A, but also removal of strand 1 from β-sheet C to provide a 'return' from the now distant P end of the inserted reactive center region to the top of the molecule. We therefore hypothesize that early formation and stabilization of β-sheet C with incorporation of strand 1C and consequently formation of β-sheet A without strand 4 inhibits subsequent incorporation of the reactive center region into sheet A. This is therefore the mechanism that ensures folding of the serpin in the metastable state. Since the reactive center region is attached at each end to a strand of a β-sheet (s5A and s1C), thereby physically anchoring the start and end of the region, such exclusion of the region from β-sheet A during folding would, by default, ensure an exposed environment for it. This might then occur for all serpins and be independent of the primary structure of the reactive center region and of the inhibitory properties of the serpin.

That helix has indeed been found for the native structures of ovalbumin, an α_1-antichymotrypsin variant, and α_1-proteinase inhibitor may represent adoption of a context-dependent conformation [39,40] that results from the constraints imposed by the rest of the protein and the typical presence of some hydrophobic residues in the region from P9 to P1 rather than from the specific sequence of the loop (Fig. 3). Such a conformation might also serve to stabilize the active exposed conformation of the serpin by requiring helix unfolding to occur prior to loop insertion, thereby raising the energy barrier for insertion and reducing the tendency to convert spontaneously to a latent conformation.

Indirect support for the idea that formation of β-sheet strand 1C is a critical determinant of serpin metastable folding comes from two studies on PAI-1. Although both studies deal with the already folded protein, they both illustrate the importance of β-sheet C in the stability of the serpin. In one study it was shown that the native-to-latent transition, which is facile in PAI-1, could be slowed down by mutations in and around strands 3C and 4C. It was proposed that, in forming the latent conformation, strand 1C must pass through a 'gate region' formed by strands 3C and 4C and that the mutations reduced the mobility of this region, thereby stabilizing strand 1C and hence the metastable state [41]. In the other study the crystal structures of a cleaved PAI-1 variant and latent PAI-1 were compared. It was found that to convert the native structure into the latent conformation strand 1C must pass though a gateway formed by two surface loops. Loop 1 joins strands 3C and 4C and loop 2 connects strand 3B and helix G. Passage of strand 1C through this gateway might be initiated in PAI-1 by electrostatic repulsion caused by residues of the same charge at the C-terminal end of strand 1C and within loops 1 and 2. It was proposed that, since other serpins do not have the appropriate residues for such charge repulsion they do not spontaneously adopt a latent conformation [13]. Thus β-sheet C and adjacent structures may be critical in preventing conversion of the metastable serpin into its more stable latent conformation. Our present studies indicate that this region might also be critical during the *folding* of the nascent polypeptide.

If a part of the serpin outside the reactive center region itself (i.e. β-sheet C) ensures that the reactive center loop is exposed, why then should there be the correlation between the secondary structure of the reactive center region and the inhibitory properties of the serpin that was found? From comparison of the primary structures of serpins in this region (Fig. 3) it is clear that the prediction of α-helix in the N-terminal part of the reactive center region of inhibitory serpins arises from the presence of a tetrad of small residues, mostly alanines, between P12 and P9. When such a sequence is not present the serpin in almost all cases is non-inhibitory. The prediction of α-helix is thus likely to be a consequence of the mechanistic need for these particular residues to be present rather than from a need, on mechanistic grounds, for helix in this region.

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