SERPINS’ REACTIVE SITES LOOPS MOBILITY AND ITS FUNCTIONAL VALUE

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Summary. Protein inhibitors from the serpin family are important regulators of various metabolic processes. They differ significantly from most protein inhibitors of proteinases both in structure and in the mechanism of interaction with proteolytic enzymes. The loop of their reactive site is mobile, and the formed complex with enzymes is a covalent acyl-enzyme. Comparison of the properties of serpins both among themselves and with protein inhibitors of other families indicates the key role of the mobility of the loop of the reactive center in ensuring the selectivity of the inhibitors.

Keywords: hemostasis, serine proteinases, proteinase inhibitors, serpins.

Serine proteinases play an important role in the regulation of numerous physiological and pathophysiological processes. Limitation of excessive or dysfunctional proteolysis is an important link in this regulation. This restriction is mediated by the blocking of enzymes by protein inhibitors. As a rule, in the study of enzymatic processes, the main attention is paid to the state of enzymes. However, the properties of functional partners - substrates and inhibitors - are no less important. A similar approach in the case of serine proteinases allows us to distinguish three types of protein inhibitors, which differ in their mechanism of action.

The first type includes the superfamily of α-macroglobulins, which are involved in the regulation of various physiological and pathophysiological processes [1]. The key member of this family is α2-macroglobulin, a multifunctional blood protein that binds all four classes of proteolytic enzymes [2]. Its interaction with proteinases occurs outside the active center after cleavage of the so-called "bait site" (Fig. 1).
Fig. 1. Scheme of interaction of $\alpha_2$-macroglobulin with proteinase. Proteinases (red) captured by $\alpha_2$-macroglobulin retain the ability to interact with molecules of low molecular weight substrates (blue) [3].

Under these circumstances the enzyme loses its ability to hydrolyze large proteins, but retains it in relation to low molecular weight substrates [4,5]. That is, $\alpha_2$-macroglobulin is not so much an inhibitor as a restrictor, limiting the action of the corresponding enzymes [2].

The second type of protein inhibitors includes proteins that effectively block the active center of the enzyme due to the formation of a stable non-covalent Michaelis complex (L, Fig. 2).

$$E + I \leftrightarrow L \leftrightarrow C \leftrightarrow L^* \leftrightarrow E + I^*$$

Fig. 2. Scheme of interaction of protein inhibitors with proteolytic enzymes [6]. $E$ and $I$ - native enzyme and inhibitor, $I^*$ - inhibitor with a cleaved peptide bond of the reactive center, L and L* - intermediate non-covalent complexes containing native or cleaved inhibitor. Complex C is a covalent acyl-enzyme type adduct.

The non-covalent nature of the complexes determines a number of interesting properties. Inhibitors of this type are capable of effective interaction with anhydro-forms of enzymes, that is, with enzymes in which serine of the active center is converted to dehydroalanine. The stability of such complexes is comparable to complexes formed by active enzymes, while anhydro-forms are able to displace active enzymes from the complex [7,8]. Incubation of $\alpha_2$-macroglobulin - enzyme complexes with inhibitors of this forms leads to restoration of hydrolytic activity in relation to low molecular weight substrates [9]. Inhibitors of this type form more than two dozen families. Inhibitors of the same family have an extremely high degree of structural homology, while different families differ from each other [6]. In the process of formation of the complex, the active center of the enzyme interacts with the complementary site on the surface of the inhibitor - the reactive site (Fig. 3).

Fig. 3. Structural model of the complex of trypsin (red) with basic pancreatic trypsin inhibitor (green) [10].
Both in the enzyme and in the inhibitor approximately the same surfaces with a total area of about 1500 Å² come into close contact [11-13]. The six residues P3'...P3 (according to the Schechter-Berger nomenclature (Fig. 4) of the inhibitor account for 70 to 77% of the surface that is blocked during complex formation [11, 12].

Fig. 4. Placement of the polypeptide chain at the interaction with enzyme according to Schechter-Berger nomination [14]. The arrow indicates the peptide bond that is being cleaved.

The primary specificity of inhibitors of this species is determined by the nature of the amino acid residue located in the P1-position of the reactive center. As a rule, these residues correspond to the substrate specificity of enzymes, that is, for P1 positions of trypsin inhibitors arginine or lysine residues are characteristic, for chymotrypsin inhibitors - hydrophobic amino acid residues, and for elastase inhibitors - alanine residues. However, the primary specificity of protein inhibitors is not absolute and inhibitors of, for example, trypsin interact quite effectively with chymotrypsin and elastase [11]. The key feature of inhibitors of this type is the rigid fixation of the conformation of the reactive center, which is located in the so-called reactive site loop (RSL) - a relatively small area of the polypeptide chain, limited by one or two disulfide bonds. There is no structural homology between protein inhibitors from different families, but the conformations of their reactive centers are similar [6]. Violations of the conformation of the reactive center negatively affect the inhibitory properties of the protein. Thus, partial restoration of disulfide bonds leads to the destruction of inhibitory properties of the protein [15]. This is due to the violation of the condition critical for ensuring high affinity between the proteinase and the functionally determined bond. It consists in the synchronicity of the interaction of the binding S1 and allosteric S2' sub-sites of the enzyme with P1- and P2'-amino acid residues of the inhibitor placed in the proper conformation and corresponding to the ligand specificity [16]. For trypsin-like proteinases the ligand specificity of the allosteric site corresponds to positively charged and hydrophobic amino acid residues, and a decrease in the hydrophobicity of the substituent leads to a decrease in inhibitory properties, while a negatively charged substituent has no inhibitory properties [17]. The example of tissue factor pathway inhibitor (TFPI) is no less characteristic. Its structure consists of three domains that are homologues of Kunitz’s trypsin-kallikrein inhibitor. The reactive center of the first domain (K36↓A37I38) inactivates factor VIIa, the reactive center of the second (R107↓G108Y109) - factor Xa, and the third domain (R196↓A200N201) does not show an inhibitory effect [18]. (Hereinafter, P1-...P2' sequences of RSL of the mentioned inhibitors are given, where ↓ means a cleavable bond of the reactive center).

From the above follows the importance of fixing the reactive center of the
inhibitor in the "canonical" conformation optimal for interaction with the proteinase. However, these requirements may differ slightly among proteinases of the same type from different sources. Probably, this is precisely why protein inhibitors are able to distinguish between proteinases of the same species, but from different sources. Thus, chicken protein ovomucoid is an effective inhibitor of bovine trypsin, but it is ineffective against human trypsin [19]. The secretory inhibitor of the pancreas of a bull inhibits the activity of bovine and porcine trypsins, but is not effective against human trypsin [20]. It is also likely that due to this, the inhibitory parameters of cyclic polypeptides - analogues of RSL - differ in great diversity. If the inhibitory parameters of the cyclic 18-membered peptide - the model of the Barley inhibitory loop of the chymotrypsin inhibitor - are similar to the original protein (K$_{I}$2.8x10$^{-12}$ M and 2.9x10$^{-12}$ M, respectively) [21], then the constant of trypsin inhibition by the cyclic analogue of the inhibitory loop of the main pancreatic trypsin inhibitor is only 2x10$^{-6}$ M, which is several orders of magnitude weaker than that of the native inhibitor [22].

It should be emphasized that the functional connection of most of these inhibitors with the corresponding enzymes is more than doubtful [23,24]. Moreover, within the same family there are proteins that do not show any inhibitory effect at all [25]. This suggests that such proteins exhibit inhibitory properties due to the random and completely non-functional formation of fixed groups on their surface, which ensure synchronous interaction with the binding and allosteric regions of the enzyme.

The last considerations make it possible to explain the peculiarities of the inhibitory action of the third type of inhibitors belonging to the serpin family. This family is formed by an extensive group of homologous proteins involved in various physiological processes [26]. Protein inhibitors of the serpin family provide limitation of the hydrolytic and activation action of the protein-absorbing and fibrinolytic cascades of the hemostasis system [27]. Functional deficiency of certain serpins causes severe functional disorders [28,29]. Inhibitors of this family have a number of features. Thus, their common feature is the form of complexes with proteolytic enzymes. These are covalent acyl-enzymes (Fig. 5).

![Fig. 5. Scheme of complex formation between a proteinase and serpin inhibitor [26].](image-url)

Under physiological conditions, such complexes are quickly removed from the body [30]. Similar to the inhibitors of the previous type, the main part of the primary contact of the inhibitor with proteinases falls on the P$_4$-P$_3'$ area of the reactive
center [31]. The loops of the reactive centers of serpins are mobile, passing through the "canonical conformation" optimal for interaction with the active center of enzymes [32,33]. The cleavage in RSL both along the reactive center and outside it causes the loss of inhibitory properties [34,35]. Lengthening or shortening of RSL dramatically reduces the inhibitory properties of the protein [36-38]. Serpin complexes with anhydro-enzymes are unstable, and the synthetic polypeptide analog of the reactive center of the α1-inhibitor of proteinases does not show inhibitory properties [39,40].

Despite the belonging to the same family and pronounced structural homology, serpins differ significantly in their specificity of action. So, the most common representative of this family is the α1-inhibitor of proteinases. It effectively blocks trypsin, IIa, IXa, Xla, XIIa, activated protein C, neutrophil elastase and chymotrypsin [41]. This inhibitor contains methionine (M358↓S359I360) in the P1-position of the reactive center, which is not typical for either substrates or inhibitors of trypsin-like proteinases. At the same time, the mobility of the reactive center is quite limited, since the P3↓ and P4↓' positions are occupied by prolines [31,42]. It is likely that when in the "canonical conformation" insertion into the allosteric site of a ligand of the appropriate specificity leads to the "eroding" of the P1-specificity of the enzyme and the formation of the primary complex [43].

For most inhibitors of the serpin family, the process of forming a complex with the corresponding proteinase is not limited to the contact between the active center of the proteinase and the RSL of the inhibitor. Thus, the key enzyme of the fibrinolytic system, plasmin, has a pronounced multidomain structure (Fig. 6).

![Fig. 6. Amino acid sequence of human plasminogen. A - heavy and B - light chains of plasmin; K1, K2, K3, K4, K5 - the first, second, third, fourth and fifth kringles, respectively [44].](image)

In addition to its natural partner - plasmin – serpin inhibitor α2-antiplasmin is capable for inhibiting trypsin and chymotrypsin. The features of the immediate surroundings of its reactive center (M374S375R376↓M377S378) are noteworthy. If plasmin and trypsin cleave the bond R376↓M377, then chymotrypsin cleaves, depending on the temperature, M374S375 or M377S378. [45]. In the first (functional) case,
The reactive center of the inhibitor contains arginine in the P₁-position, and serine in the P₂'-position. At first, such a composition seems doubtful for the reactive center of a protein inhibitor. Shifting the reading by one residue gives the sequence M↓SI, which is analogous to the reactive center of the α₁-inhibitor of proteinases. However, unlike the α₁-inhibitor of proteinases, limited oxidation of α₂-antiplasmin does not affect the inhibitory properties [46]. Both the proteolytic part of plasmin (B-chain) and non-proteolytic kringle structures show a high level of autonomy. The primary interaction of plasmin with α₂-antiplasmin is mediated by binding sites located in the kringle structures of the enzyme molecule. However, the separated proteolytic part of plasmin (so-called micro-plasmin, K₅₃₀Pm) is also subject to α₂-antiplasmin inhibition, albeit at a significantly reduced rate. These data, as well as studies of the interaction of α₂-antiplasmin with plasmin derivatives with a blocked active center, suggest the presence of an additional site of interaction with the inhibitor in the catalytic domain [47]. Such a multicenter interaction ensures the specificity and effectiveness of blocking plasmin with its functional inhibitor.

Most serpins are characterized by a narrow focus of blocking certain trypsin-like enzymes of the hemostatic system. A significant number of additional components - complex formation modulators - are involved in their interaction with the corresponding enzymes. Thus, the interaction of many serpins with functionally determined proteinases is mediated by the formation of a ternary complex with the participation of glycosaminoglycans, in particular, heparin (Fig. 7).

![Fig. 7. Modulation by a cofactor of complex formation between serpin and proteinase [26].](image)

The latter plays the role of a bridge between the enzyme and the inhibitor and an allosteric effector of both the enzyme and the inhibitor. A classic example of such modulation is the formation of a ternary complex between thrombin, antithrombin III (R₃⁹₃↓S₃⁹₄L₃⁹₅) and heparin. The same complex formation was shown for the interaction of protein C inhibitor (R₄₄₄↓T₄₄₅L₄₄₆) with activated protein C and thrombin, plasminogen activator inhibitor-1 (R₃₄₆↓M₃₄₇-A₃₄₈) with thrombin, protease nekhin 1 and thrombin. Heparin accelerates inhibition of thrombin by heparin cofactor II, inhibition of factors IXa, Xa by antithrombin III [26,29]. The complex of the protein Z-dependent proteinase inhibitor PZPI and vitamin K interacts with heparin to inhibit factors IXa, Xa and Xla. The complexation of this inhibitor with the
lipid membrane and heparin enhances the binding of factors IXa and Xla [42]. Reactive centers of inhibitors of plasminogen activators of type 1 and type 2 contain alanine and glycine residues in P₂'-positions (R₃₄₆↓M₃₄₇A₃₄₈ and R₃₈₁↓T₃₈₂G₃₈₃, respectively). At the same time, according to the specificity of action, both tissue plasminogen activator and urokinase-like one are fully subject to the S₁-S₂'-rule [48], cleaving the activation site of plasminogen at the bond R₅₆₁↓V₅₆₂V₅₆₃ (Fig. 6). However, these highly specific inhibitors are involved in a long list of intermolecular interactions involving various protein and non-protein components [49,50]. All this indicates multi-link allosteric modulation of reactive centers of inhibitors in the process of complex formation.

Summarizing the given data, several assumptions can be made regarding the mechanisms of action of inhibitors from the serpin family and the role of the mobility of the loop of the reactive center in ensuring the selectivity of their action. The traditional consideration of enzymatic processes from the point of view of the state of the enzyme alone is not informative. In the case of functionally determined interactions, the state of the target protein plays an equally important role. The pivotal factor is bringing the conformation of the site of functional interaction into the optimal conformation. In this process, additional areas of intermolecular interaction are involved as an enzyme, an inhibitor, and additional protein and non-protein factors. Without this, the protein does not exhibit an inhibitory effect, or its inhibitory properties are extremely weak. All this plays an important role in ensuring the action of inhibitors and the regulation of the processes mediated by them.

In the context of the discussed topic, one cannot fail to mention the fact that α₂-macroglobulin and α₁-proteinase inhibitor were discovered in our country [51] by the outstanding biochemist-enzymologist Kuzma Mykytovych Veremeyenko, the centenary of whose birth we should celebrate this year.

References:


