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REVIEW

Role of A-Chain in Functioning of the Active Site of Human α-Thrombin

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Abstract—This review summarizes current data suggesting that A-chain of the human α-thrombin molecule plays a role of allosteric effector in catalytic reactions with various substrates. Special attention is paid to the relationship between A-chain structure and catalytic activity of thrombin. The existence of this relationship is based on studies of natural mutation of A-chain of the α-thrombin molecule. Use of molecular and essential dynamics confirmed the role of A-chain in changes of conformation and catalytic properties of this enzyme; these changes involve residues located in the specificity sites and some inserting loops. Current knowledge on structure and properties of thrombin can be used for the development of new antithrombin agents.

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Thrombin is known to be a polyfunctional enzyme involved into regulation of blood clotting and maintenance of blood fluidity; it exhibits procoagulant, anticoagulant, and fibrinolytic properties. Thrombin polyfunctionality associated with high selectivity with respect to cleavable bonds [1-3] is clearly attributed to intrinsic enzyme structure and functional groups exposed on the surface of the enzyme molecule. Basic studies on structure, functioning, and the role of the main structural elements of thrombin result in better understanding of their structure–function relationship and thus represent a basis for the development of antithrombin agents [4-13]. Study of the crystal structure of α-thrombin has shown that the surface of the thrombin molecule can be subdivided into several functional sites responsible for highly specific recognition of various substrates, inhibitors, and mediators [11-18].

Special attention is attracted to the relationship between A-chain structure and catalytic activity of thrombin as well as stability of this enzyme, and also conformational transition of functional groups of thrombin active site induced by Na^+ binding to the enzyme molecule. Study of this problem is very important in the light of new data suggesting the role of A-chain as an allosteric effector of α-thrombin during the catalytic process [2, 8, 10, 14-18].

On one hand, interest in the role of A-chain is associated with the search for a structural element of the thrombin molecule responsible for high selectivity of hydrolysis of physiological high molecular weight substrates catalyzed by thrombin; on the other hand, this interest is also related to studies of characteristic features of active site functioning.

It should be noted that not all serine proteases have an A-chain in their molecules. Very short A-chain (13 residues) of chymotrypsin does not exhibit particular functions, whereas trypsin lacks such a chain. However, for some blood clotting factors—kallikrein, factor XI, and plasmin—it has been demonstrated that their specific physiological activity is determined by additional binding sites located on the second, light non-catalytic chain [3, 19-24]. The A-chain is organized in a boomerang-like shape with multiple-turns and partially helical conformation, making a smooth contour of the part of B-chain positioned opposite the active site pocket responsible for primary binding [14, 15, 21]. The A-chain is linked to the B-chain via the Cys1–Cys122 disulfide bridge. Most

Abbreviations: BPTI) basic pancreatic thrombin inhibitor; ED) essential dynamics; MD) molecular dynamics; α-NAPAP) N-α-[2-naphthyl-sulfonyl-glycyl]-4-amidinophenylalanyl-piperidine; PAR1) protease-activated receptor 1; PPACK) D-Phe-Pro-Arg-chloromethylketone.

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other A–B interactions involve charged side chains; six of them form salt bridges and participate in interchain hydrogen bonds. Inner stabilization of the A-chain of thrombin is mainly achieved by polar bonds and formation of salt bridges. The role of the A-chain in the catalytic process has been investigated only in [25, 26]. Based on results of these studies, the authors concluded that A-chain is not essential for narrow specificity of thrombin. Authors of other studies [27, 28] came to the same conclusion, but they proposed that thrombin A-chain is required for normal functioning of the active site located in the B-chain. According to the spatial model of thrombin, the A-chain surrounds the B-chain globule nearly the active site. Positioning at the B-chain surface the A-chain may stabilize native conformation of the active site [3, 27, 28].

Much time passed before detailed intensive study of α-thrombin A-chain functioning and its effect of catalysis (mainly on manifestation of thrombin specificity with respect to substrates of various types) began. The initiating cause was identification of a natural mutant of α-thrombin with deletion of one of two neighboring lysine residues (Lys9/Lys10) in two unrelated Iranian patients with severe prothrombin deficiency and hemorrhagic diathesis. Prothrombin deficiency is an autosomal recessive disease characterized by two phenotypes: hypoprothrombinemia accompanied by low level of blood clotting activity and antigen (type I) and dysprothrombinemia with very low clotting activity but subnormal or normal level of antigens (type II) [22, 24, 29-35]. For specification of the role of human thrombin A-chain, a natural mutant (ΔK9), characterized by deletion of Lys9 was investigated.

Discovery of Na^+-induced transition of thrombin conformation from the slow into the fast form suggests the importance of balance between pro- and anticoagulant forms of thrombin in the body [22, 29, 32]. Based on results of calculations of energetic distribution for the lysine residue undergoing deletion in thrombin, it was demonstrated that allosteric binding of Na^+ with ΔK9 decreased on lysine deletion [22]. This is supported by the fact that deletion of Lys9 in the A-chain induces a slow-like conformation of the thrombin mutant (i.e. sensitivity to Na^+ is significantly lower in the mutant than in wild type (WT) thrombin). Indeed, results of hydrolysis of the substrate Tos-Gly-Pro-Arg-pNA demonstrated that K_m values for ΔK9 and WT thrombin were 3.62 and 5.50 μM, respectively, whereas k_{cat} values for ΔK9 and WT thrombin were 9.34 and 101 sec^{-1}, respectively. According to these data, putative conformational changes induced at the active site of thrombin by Lys9 deletion may more deeply influence intermediate stages of catalysis than substrate binding, which was more favorable in the case of the mutant than the WT enzyme [34, 35].

Studying the interactions of ΔK9 and WT thrombin with some substrates and receptors of thrombin (PAR1, protease-activated receptor 1; glycoprotein Ibα) it was found [22, 29, 34] that the k_{cat}/K_m values for the synthetic substrate D-Phe-Pip-Arg-pNA and fibrinopeptide A were 18 and 60 times lower for the ΔK9 mutant compared with WT thrombin. The k_{cat} value for the reaction of fibrinopeptide A hydrolysis reduced from 45.3 sec^{-1} (for WT thrombin) to 1.82 sec^{-1} (for ΔK9 mutant), whereas K_m values for these enzymes were 5.1 and 13 μM, respectively. It was found that ΔK9 weakly activated platelet PAR1 with EC_{50} value of 38.6 nM (EC_{50} is thrombin concentration required for 50% activation of platelets); for WT thrombin EC_{50} was 2.1 nM. For analysis of this phenomenon, the authors investigated hydrolysis of PAR1 peptide 38-60 *in vitro* and calculated kinetic parameters. It was found that there was a 116-fold decrease in k_{cat}/K_m value (from $1.74 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$ in the case of WT thrombin to $1.5 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ in the case of the thrombin mutant). This corresponds to the change of free activation energy by 2.8 kcal/mol. At the same time, binding of glycoprotein Ibα remained unchanged (the K_d values for WT and ΔK9 thrombin were 0.12 and 0.13 μM, respectively) [22]. Thus, weak platelet activation by the thrombin mutant is mainly associated with impairments of the interaction of thrombin with PAR1.

On hydrolysis of protein C by both forms of thrombin in the presence of 100 nM thrombomodulin, the k_{cat}/K_m value for the mutant thrombin was 30 times lower than for WT thrombin. However, K_d values for complexes thrombin–thrombomodulin were basically identical for both forms of thrombin [22, 29, 32].

Interesting data have been obtained during studies of interaction of both forms of thrombin with physiological and synthetic inhibitors. These studies have been carried in the presence and absence of heparin. It was found that the rate of reaction during interaction of the mutant thrombin ΔK9 with antithrombin was 20 times lower compared with WT thrombin. In contrast to the rate of reaction catalyzed by ΔK9, the rate of reaction catalyzed by WT thrombin in the presence of 75 nM heparin was much higher ($1.3 \cdot 10^4$ and $6.8 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$, respectively). In the case of the ΔK9 mutant, such defective (heparin-independent) interaction suggests decreased formation of stable thrombin–antithrombin complex. However, the ΔK9 exhibited higher affinity to inhibitor, α-NAPAP (N-α-[2-naphthyl-sulfonyl-glycyl]-4-amidinophenylalanyl-piperidine) (K_i values of 0.66 and 2.1 nM for ΔK9 and WT thrombin, respectively). Binding of ΔK9 with basic pancreatic thrombin inhibitor (BPTI) was also characterized by lower K_d values compared with those for WT thrombin (40 and 109 μM, respectively) [22, 29].

These data suggest that loss of the A-chain Lys9 exerts a negative influence on the catalytic properties of thrombin. Apparently, conformational changes in the light chain can influence the active site cavity of this enzyme. The three-dimensional (3D) structure of a ΔK9 model studied by the method of molecular dynamics

(MD) was compared with the crystal structure of WT thrombin in geometrical and conformational properties. The goal of that study [22, 24, 29, 36-39] was to investigate the structure of both forms of thrombin, underlying differences in their functional properties. The mutant ΔK9 thrombin model was obtained in deionized aqueous solution [37-40]. It was shown that after 5 nsec the protein underwent conformational transition from the conformation typical for the fast form to more compact conformation of the slow form of thrombin. After 5 nsec, the decrease of protein surface exposed to water and rotation radius (R_q was defined as the main distance of atom collection from the center of their common mass) is considered as evidence for more compact structure of the slow form versus the more active fast form. These data are consistent with results of X-ray and crystallographic studies suggesting more "unfolded" conformation of the fast form compared with the slow form of thrombin [40-42]. The structure of the mutant protein was equilibrated with potential energy of the system after 5 nsec. Studies of time-dependence of carbon atom fluctuations in the ΔK9 polypeptide chain by MD have shown that thrombin and recognition binding sites are the most flexible loops including S2 and S3 (e.g. "aryl-binding" sites), fibrinogen recognition exosite, and heparin binding sites. Change of radius of carbon atom fluctuations as a function of time found for ΔK9 and WT thrombin confirms more compact conformation of ΔK9 [22, 37].

More detailed analysis of A-chain conformation has shown that deletion of Lys9 results in impairment of light chain structure accompanied by the increase of contact surface to 81 Å. This obviously occurs due to limited contacts responsible for stabilization of ion pairs by hydrogen bonds. These contacts stabilize the WT thrombin light chain in conformations characterized by lower contact area. The presence of "requested" contacts within the A-chain and also between A- and B-chains is determined by ionic interactions during the last 10 nsec simulation of the ΔK9 mutant. There is evidence that these ionic bonds are essential for stabilization of the WT thrombin A-chain in the boomerang-like conformation [22, 34]. It was then found that the distance between Lys14a–Asp14, Glu13–Arg14d (intrasalt bridges) and Asp1a–Arg206, Glu14e–Lys186d (intersalt bridges) were nearly optimal in the crystal structure, whereas other ionic interactions were unfavorable. Such changes in the electrostatic contacts may be the major reason for A-chain refolding in the ΔK9 mutant. According to results of X-ray analysis and MD studies, the Trp148 loop represents a putative site of high mobility, whereas conformation of the Na^+-binding site remains unchanged during simulation. There are conformational changes essential for the Trp60d loop (S2 pocket) [34, 38]. Amino acids appearing at S3 subsite (especially Trp215 and Ile174, located at the enzyme loops) exhibit strong fluctuations of carbon atoms during MD simulation. Significant fluctuations of ΔK9 carbon atoms at subsites S1 (Asp189), S2 (Trp60d and Tyr60a), and S3 (Trp215), significantly influencing the size of active site "cleft" of thrombin, suggest significant conformational changes of amino acid residues at these sites. Measurement of relative position of Asp189–Trp60d has shown that the distance between mass centers (22.4 Å) is somewhat higher in ΔK9 than that in WT thrombin (19.7 Å) measured by the X-ray method. Differences in positioning of aromatic amino acid side chains were more pronounced in the S2 subsite. For example, during the last simulation the distance between Trp60d and Tyr60a was two times longer in the mutant (8.2 Å) compared with WT thrombin (4.8 Å) [22, 32, 39].

It should be noted that MD simulation revealed structural impairments in the S2 pocket. This is confirmed by experimental data on the threefold increase of ΔK9 affinity to BPTI. Studies have shown that the Trp60d loop is the main element preventing thrombin—BPTI interaction and promoting the increase of enzyme affinity [43]. This should be accompanied by the increase of "uptake radius" of the catalytic site, improvement of complement interaction between enzyme and substrate or inhibitor. However, it was found that conformational changes induced by mutation/deletion reduced complement substrate binding to the enzyme and k_{cat}/K_m value. This possibly occurs due to ΔK9-induced change of pK_a of His57 imidazole followed by influence of acylation/deacylation steps [22, 34, 41, 43].

Since the catalytic pocket of the thrombin active site contains the amino acid triad Asp102, His57, and Ser195, its structure has been investigated by X-ray (WT thrombin) or by simulation (ΔK9 mutant). Calculations of averaged distances between centers of mass atoms of these amino acids in ΔK9 and WT thrombin revealed distortion in geometry of the catalytic triad of amino acids. For example, in the case of ΔK9 and WT thrombin the distances between centers of mass of atoms of Ser195–$O^{\gamma[p]}$–His57–$N^{\varepsilon[p]}$ were 2.96 and 3.12, respectively, and for Asp102–O^{γ}–His57-ND they were 8.27 and 2.77, respectively. These results suggest the presence of hydrogen bond only between Ser195 and His57, whereas hydrogen bond between Asp102 and His57 exists only during a few nanoseconds of simulation and disappears on the last (10th) nanosecond of simulation when disruption of ΔK9 A-chain folding occurs [43]. Careful examination of the active site "cleft" has shown its clear geometric distortion in the ΔK9 model compared with crystal structure of Ser195. In the 3D structure of WT thrombin side chains of Asp189(S1), Trp60d(S2), Trp215(S3) as well as the catalytic triad, the amino acids are closely positioned in the parallel planes, whereas in the ΔK9 model (of the lowest energy) the two planes are almost quasiorthogonal. Alteration of positions of these amino acids in the catalytic pocket of ΔK9 may be the major reason for the sharp decrease of specificity constants during hydrolysis of both physiological and synthetic substrates.

Results of studies [22, 34, 39] demonstrate that deletion of Lys9 in thrombin causes effective changes of active site conformation, which is realized through a long chain of allosteric effects. It has been found that geometric change in the length of bonds and angles of thrombin polypeptide chain increases nucleophilic properties of Ser195–O^{γ} and changes relative geometry of Ser195–O^{γ} and carbonyl carbon of amide substrates. Optimal distance between Ser195–O^{γ} and carbonyl carbon and pyramidalization at the carbonyl carbon induced formation of intermediate tetrahedron formation due to nucleophilic–electrophilic interaction [34, 44-47].

For deeper understanding of intramolecular mechanisms responsible for functional defects in thrombin, the effects of pH on catalytic activity and thrombin inhibition, Na^+ binding with this enzyme as well as stability of thrombin and also characteristics of stabilized interactions between A- and B-chains of thrombin have been investigated [48]. The pH dependence (in the range of pH from 5.5 to 10) of amidase activity of ΔK9 and WT thrombin was investigated by means of earlier described experimental and theoretical approaches [48-51]. It was found that protons caused a similar influenced on amidase activity of both thrombin forms, although the pK_a values of ionized functional groups involved in catalysis significantly differed. There was marked increase of pK_a value in the first ionized group, which was 7.3 in the ΔK9 mutant and 6.86 in WT thrombin. Since this group belongs to the side chain of His57, we suggest that deletion of Lys9 causes allosteric effect on equilibrium of protonation of active site His57 and increases affinity of both forms of thrombin to protons [48-51]. The second pK_a value is referred to the N-terminal group Ile16 [51, 52]. At three levels of protonation, it was found that k_{cat} and k_{cat}/K_m values are decreased mainly due to the decrease of k_{cat} value, related to acylation rate. On the contrary, the decrease of K_m value in the case of ΔK9 corresponds to better substrate positioning at the catalytic pocket of the unprotonated form of the mutant thrombin. Interestingly, there is a reverse proportional dependence between $\log(k_{cat}/K_m)$ values of WT and ΔK9 thrombin forms with His57, protonated in the presence of 0.15 M NaCl, and corresponding values of His57 [22, 48, 52].

These data suggest disruption of intermediate tetrahedral conformation, including partial cleavage of a carbon–nitrogen bond stabilized by hydrogen bonding with His57 imidazole nitrogen (N^{ε}). Imidazole behaved as an ordinary acid and this facilitated amine pushing out of the intermediate tetrahedron. This conclusion is consistent with data obtained during studies of protons during amide substrate hydrolysis by α-thrombin [48, 52]. Thus, reduction of the acylation rate for human α-thrombin is associated with disruption of the intermediate tetrahedron, providing different affinity for protons of the His57 amide. During enzyme interaction of thrombin with its inhibitor, NAPAP, the pK_a values were somewhat higher than for interaction of WT thrombin with substrate D-Phe-Pip-Arg-pNA. Changes in pK_a values for catalytic His57 in the thrombin mutant suggest that deletion of Lys9 exerts an allosteric influence on conformational state of corresponding domains of catalytic B-chain.

For explanation of mechanisms of these effects, binding of Na^+ with thrombin and conformational stability of mutant and WT thrombin have been investigated [48, 52]. Binding of both forms of thrombin with Na^+ is characterized by increase of fluorescence at 342 nm. True fluorescence of the mutant thrombin was 20% lower compared with WT thrombin (changes of fluorescence by +18 and +9% for WT thrombin and ΔK9, respectively). This suggests that the Na^+-binding loop of the mutant thrombin still retains significant affinity for this cation, but conformational transitions determining these interactions are less pronounced compared with WT thrombin. It is possible that in the mutant thrombin Na^+ binding should differ from conformational transitions that occur in WT thrombin. This means that conformational transitions induced in the B-chain by deletion of Lys9 are unique and differ from the fast- and the slow-forms of thrombin [37, 40, 42, 53].

Subsequent studies employed analysis of essential dynamics for investigation of separate sites of ΔK9 and WT thrombin; this required for identification of transitions characteristics for their folding that would differ from described local fluctuations [48, 54]. It was demonstrated that the A-chain and Na^+-binding site exhibited higher mobility in WT thrombin than in the ΔK9 mutant. Significant conformational transition occurs after ~5 nsec in WT thrombin, and after ~10 nsec in ΔK9.

The transition of the Na^+-binding site was found to be associated with transition of such important thrombin active center subsites as specificity subsite S3 (Trp215–Ile174), the Trp60 loop, disulfide bond Cys168–Cys182, and the exosite for fibrinogen binding; these sites were detected after 10 nsec. X-Ray studies have demonstrated that the disulfide bond formed by Cys168–Cys182 is subjected to spatial transition after binding with Na^+ [40, 48]. It was also found that the distance between Cys182 sulfur atom and Tyr225 Cβ reduces by 1 Å in WT thrombin bound with Na^+; this causes conformational changes of the catalytic pocket of this enzyme and increases its activity. These results are consistent with behavior of WT thrombin, whereas MD analysis has shown that binding of Na^+ causes a twofold reduction of this distance in the ΔK9 mutant. This resulted in the decrease of conformational mobility of the Cys168–Cys182 disulfide bond during interaction of ΔK9 with Na^+ [40, 48].

There is an inverse proportional dependence between fluorescence intensity and mobility and also exposure of some Trp residues (60d, 96, 148, 207, and 215) to solvent. For example, the residues Trp207 and Trp29 located at the border of the A- and B-chains and interacting with Arg137 via a structure including three

water molecules possessing low mobility are characterized by fluorescence representing 35 and 9% of total fluorescence of WT thrombin [55]. Using MD simulation, the ratio of solvent area (water) to solvent accessible surface (SAS) was calculated for the above-mentioned amino acids. The surface of ΔK9 Trp207 exposed to the solvent it nearly three times higher (SAS = 24.9 Å) than in WT thrombin (SAS = 9.3 Å). A similar tendency was also found for Trp29 (SAS = 9.6 and 14.1 Å in WT thrombin and ΔK9, respectively), whereas in the case of other Trp residues (60d, 96, 148, and 215) the difference is not so dramatic [40, 48, 56-58]. Thus, results of the ΔK9 mutant fluorescence in dependence on Na^+ concentration suggest low mobility Na^+-binding site, higher exposure to solvent, and lower flexibility of side groups of some Trp residues.

The study of stability of the two forms of α-thrombin by means of various concentrations of urea showed higher lability of ΔK9 versus WT thrombin [48-57]. It should be noted that 6 M urea caused total denaturation of both forms of thrombin. Clinically lower phenotype ΔK9 expressed *in vivo* is explained by higher sensitivity of the mutant to denaturation by urea; this may be consequence of intracellular precipitation or increased enzyme degradation [22, 34, 47].

In series of experiments, the stability of the two forms of the enzyme has been investigated by means of more complex mode of denaturation related to impairments in disulfide bonds [48, 56-59]. The A- and B-chains of thrombin molecule are covalently linked by one interchain disulfide bond (Cys1–Cys122), whereas B-chain is also stabilized by three intrachain disulfide bonds (Cys42–Cys58, Cys168–Cys182, Cys191–Cys220). During disulfide bond cleavage, urea disrupts noncovalent interactions between the two chains and increases sensitivity of disulfide bonds to β-mercaptoethanol. However, low concentrations of this reagent cleave disulfide bonds and redistribute them depending on conformational changes induced by denaturation.

Denaturation in the presence of 6 M urea and 0.2 mM β-mercaptoethanol for 180 min released A-chain, whereas the native forms of both enzymes disappeared. The rate constants for A-chain release were $1.69 \cdot 10^{-2}$ and $2.96 \cdot 10^{-2}$ min^{-1} for WT thrombin and ΔK9, respectively. Loss of the intact enzyme (A- and B-chains) is characterized by first order reaction constants of $1.70 \cdot 10^{-2}$ and $3.01 \cdot 10^{-2}$ min^{-1} for WT thrombin and ΔK9, respectively. The lag phase required for appearance of stable isomer was shorter for ΔK9 (20 min) than for WT thrombin (33 min) [42, 48].

The stabilizing interaction between A- and B-chains has been investigated for both forms of thrombin [14, 48]. Stabilization within the A-chain and between A- and B-chains occurs mainly via salt bridges and hydrogen bonds including charged side chains. The A-chain is fixed by five electrostatic interactions of side chains located in three separate clusters (D1a-K9, K14a-D14-R4-E8, and E13-R14d). Besides covalent disulfide bonding between Cys1 and Cys122, there are seven salt bridges located in five clusters (D1a-R206, E8-K202-E14c, D14-R137, K135-E14-K186d, and K14a-E23) that bind A- and B-chains. Electrostatic and hydrogen bonds stabilizing intrachain binding of A- and B-chains have been determined by the MD simulation; it was found that the number of these bonds is 10% higher in WT thrombin than in ΔK9. In this case, higher flexibility of the A-chain determined by numerous contacts during electrostatic or hydrogen bonding between A- and B-chains of WT thrombin is consistent with slower release of light chain under mild reduction of disulfide bonds [22, 48].

It is known that different interactions, especially ionic and hydrogen bonds between A- and B-chains of thrombin, stabilize the right orientation of two clefts in the catalytic B-chain of this enzyme [19, 48, 49]. Deletion of Lys9 may cause rearrangement of this ionic network. For example, in WT thrombin Asp14 effectively binds to Arg137. In the ΔK9 mutant, this bond should be strongly altered because Asp14 preferentially interacts with Lys202 as a result of deletion of Lys9; in WT thrombin Lys202 electrostatically binds to Glu14c [19, 21, 22, 48]. Disruption of the Asp14–Arg137 salt bridge may influence the environment containing two Trp residues (Trp207 and Trp29). It is suggested that even slight changes in polarity and/or flexibility of Trp residues may significantly influence fluorescence of ΔK9 [48, 55, 56], and a hydrophobic cluster located after Arg137 contains side chains of Phe181, Phe199, Phe227, and Tyr228, which are located near the active site of thrombin. They may be destabilized and this determines efficiency of the influence on the pocket of primary thrombin specificity. Such consideration is also supported by results of X-ray studies of crystal structure of this enzyme [40]. Conformational changes of Trp215 determined by Na^+ binding may also exert effective influence; it is possible that these changes are responsible for reduction of fraction change of fluorescence in the Na^+-bound ΔK9 conformer. Other evidences supporting conformational changes induced by deletion of Lys9 in the A-chain and in subsites of the mutant enzyme are also possible [36, 48, 50].

Taking into consideration these denaturation experiments, it has been concluded that in the ΔK9 mutant A-chain refolding should influence the structure of the whole thrombin molecule by reducing interchain A–B contacts. Using the MD method changes of the boomerang-like shape of the A-chain into a handle-like shape have been found [48, 49]. Kinetic and other studies demonstrated reduction of A-chain flexibility, which results in impairments of the interchain contacts during electrostatic and hydrogen bonding in the mutant ΔK9 molecule. These A-chain refolding changes should be allosterically transmitted to the active site cleft and

induce changes in geometry and protonation rate of catalytic residues of α-thrombin and also reduction of allosteric effects in the case of binding of enzyme with Na^+ [22, 48, 49].

Based on the above-mentioned data, one may conclude that the A-chain of thrombin plays a role of effector and that this enzyme exhibits a unique conformational flexibility. Data on the role of structure and conformation of the A-chain on catalytic activity of thrombin may be used in pharmacology for design of new antithrombin agents [38, 47-50, 60-65]. These data are also important for the development of new inhibitors that can be used for correction of impairments of blood clotting.

Mecozzi et al. [60] quantitatively determined cation–π-complexes for 17 various aromatic compounds; based on these data, they predicted binding ability for new cation–π-systems of aromatic rings with enzymes; this is very important for the development of new pharmacological preparations. It has been noted that the cation-binding complexes contain such amino acids as Phe, Tyr, Trp (Trp is used most frequently). Trp is a "suitable" amino acid for the cation–π-binding site; this is confirmed by results of studies of its electrostatic potential surface and binding energy [65-70]. It has been shown that Tyr is better than Phe as its hydroxyl group is also involved in correct orientation of the aromatic moiety and therefore the cation–π interaction is well directed and occurs on the electrostatic potential surface rather than at the margin of the aromatic ring during its contact with cation [60, 61].

There are reports on synthesis of *N*-terminal fragment (1-47) of hirudin containing non-coded amino acids and exhibiting antithrombin activity similar to that of intact hirudin [64, 65]. The effect of systemic substitution of Tyr3 in this inhibitor for non-coded amino acids or insertion of various groups into the *para*-position of this residue (e.g. fluorine, iodine, nitro group, etc.) was studied. Surprisingly, the substitution Tyr3→Ala resulted in disappearance in affinity of allosteric forms of thrombin, whereas a larger chain (β-naphthylalanine) improved binding with the fast form of thrombin. Many studies [38, 58, 60, 61] investigated affinity of the fast form of thrombin for analogs of hirudin fragment 1-47; it depends on hydrophobicity of the side chain of an amino acid located at the 3rd position. Structural analyses of the peptide—thrombin complexes have demonstrated that iodine atom in *para*-Phe and charged nitrogen atom in *para*-aminomethyl-Phe productively interact with the aromatic moiety of Trp215 (subsite 3) and equilibrate their binding desolvation free energy. Based on the experimental data, it was concluded that "non-traditional" hydrogen bonds X–H···π and cation–π-interactions may play a key role in protein structure, stability, and ligand recognition. Calculations have shown that among many combinations of cation binding to aromatic moiety the NH_4^+, K^+ bond with indole is the most energetically favorable [63-65]. These predictions have been confirmed during study of hydrogen bonding X–H···π, employing 529 protein structures; the results indicate that Trp indole moiety is the most frequent hydrogen π-acceptor and Lys–Trp is the most frequent donor–acceptor pair [64-70].

Thus, relative changes in affinity of the fast form of thrombin for analogs of hirudin (1-47) may be well determined by desolvation effects, but they also underline the importance of hidden interactions in recognition of hirudin–thrombin complex [41, 58, 64, 65]. It has also been demonstrated that hirudin–thrombin interaction is destabilized in the presence of negative charges at the *para*-position of phenol ring as in the case of *para*-NO_2-Phe, where oxygen atoms are characterized by strong electron density. In all cases, the inhibitor–thrombin interactions are characterized by the presence of electrostatic interactions with electron π-system of Trp215, which are favorable for *para*-aminomethyl-Phe or unfavorable for NO_2-Phe.

The third position of the hirudin molecule plays the key role for modulation forces of hirudin–thrombin interaction; this position is responsible for preferential binding of this inhibitor with the procoagulant form of the enzyme. The presence of small amino acids (like Ala) at the third position decreases affinity of the fast form of thrombin for hirudin by 65 times, but insignificantly influences binding with the slow form of this enzyme. On the contrary, substitution of Tyr3 for massive *para*-Nal increased affinity to the fast form of thrombin. These results determine the direction of studies for the development of new thrombin inhibitors providing an increase of anticoagulant properties of this enzyme.

In conclusion, allosteric regulation of catalytic activity is mainly typical for enzymes possessing complex subunit structure. Thus, the assumption of allosteric effects for the relatively small protein requires assumption of both existence of effector sites distantly located from the active site and high conformational mobility of some segments of the polypeptide chain of the enzyme [9, 71-74].

Thus, in this work we have analyzed allosteric regulation of thrombin activity by its A-chain. Recent studies of the mutant thrombin characterized by lack of Lys9 in its A-chain confirm the effect of A-chain on conformation and catalytic activity of B-chain of thrombin. Conformational changes have been investigated by kinetic and spectroscopic methods and also by MD simulation and essential dynamics of the enzyme molecule; this resulted in identification of changes within the "aryl-binding" site of the active center of thrombin, especially in the Trp60d loop (S2 subsite); transition of Trp215 (S3 subsite); geometric distortion of the catalytic triad site. This determines limitation of substrate access to the active site cleft (which is closed by the large Trp148 residue) and a sharp decrease of catalytic activity of the mutant form of thrombin.

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