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Biophysical Chemistry 121 (2006) 177–184

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

High resolution crystal structures of free thrombin in the presence of K^+ reveal the molecular basis of monovalent cation selectivity and an inactive slow form

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Received 22 November 2005; received in revised form 17 December 2005; accepted 17 December 2005
Available online 17 February 2006

Abstract

Structural biology has recently advanced our understanding of the molecular mechanisms of activation and selectivity in monovalent cation activated enzymes. Here we report a 1.9 Å resolution crystal structure of free thrombin, a Na^+ selective enzyme, in the presence of KCl. There are two molecules in the asymmetric unit, one with the cation site bound to K^+ and the other with this site free. The K^+-bound form shows key differences compared with the Na^+-bound structure that explain the different kinetics of activation. The cation-free form, on the other hand, assumes a conformation where the monovalent cation binding site is completely disordered, the S1 pocket is inaccessible to substrate and binding to exosite I is compromised by an unprecedented >20 Å shift in the position of the autolysis loop. This form, named S*, corresponds to the inactive Na^+-free slow form identified by early kinetic studies. A simple model of thrombin allostery that incorporates the contribution of S* is proposed.

Keywords: Thrombin; Allostery; Monovalent cation activated enzyme; Serine protease structure

1. Introduction

Thrombin is a Na^+-activated enzyme [1] that belongs to the vitamin K-dependent family of serine proteases involved in blood clotting [2]. As for other enzymes activated by monovalent cations [3], binding of Na^+ provides the driving force for substrate binding and catalysis by lowering the energies of complex formation in the ground and transition states. Early studies have documented that Na^+ activates thrombin better than the larger cation K^+ and the smaller cation Li^+ [1]. The activation is brought about by an allosteric equilibrium between the Na^+-free slow form with low substrate specificity, and the Na^+-bound fast form with high substrate specificity. Important details on this allosteric transition have emerged recently from extensive mutagenesis and structural analysis [4]. The monovalent cation (M^+) specificity of thrombin has also been redesigned and the enzyme has been converted into a K^+-specific protease [5]. In spite of these successes, the molecular basis of the M^+ preference of thrombin has remained elusive, as for many other M^+-activated enzymes.

The binding of Na^+ to thrombin is characterized by a $K_d = 14$ mM at 10 °C, which is about 10-fold lower than that of K^+ [5]. The difference in M^+ activation is primarily due to the acylation rate measured by k_{cat}, which is twice as fast in Na^+ [1] (see also Fig. 5). Other M^+-activated enzymes display similar marked preference [3]. Pyruvate kinase [6], the molecular chaperone Hsc70 [7] and dialkylglycine decarboxylase [8] have an absolute requirement for K^+, and Na^+ only provides minimal activation. Structures of these enzymes have recently been solved in the presence of K^+ and Na^+. Surprisingly, replacement of the essential K^+ with Na^+ in pyruvate kinase has resulted in no significant structural changes [9]. On the other hand, in the case of dialkylglycine dehydrogenase [8,10] and Hsc70 [11,12], replacement of the essential K^+ with Na^+ has revealed drastic changes in the geometry of coordination of the M^+ that propagate to neighbor residues involved in substrate recognition. The changes explain beautifully the differences in the kinetics of activation.

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doi:10.1016/j.bpc.2005.12.008

Table 1
Crystallographic data of the thrombin structure 2A0Q [14]

Data collection:	
Wavelength (Å)	0.90
Space group	$P2_12_12_1$
Unit cell dimensions (Å)	a=58.51
	b=69.74
	c=158.16
Resolution range (Å)	30.0–1.9
Observations	242,699
Unique observations	49,496
Completeness	94.6 (96.0)
R_{sym} (%)	8.0 (40.5)
$I/\sigma(I)$	16.9 (4.2)
Refinement:	
Resolution (Å)	30.0–1.9
$\|F\|/\sigma(\|F\|)$	>0
R_{cryst}, R_{free}	0.195, 0.240
Reflections (working/test)	45,143/2352
Protein atoms	4421
Solvent molecules	311
Rmsd bond lengths[a] (Å)	0.011
Rmsd angles[a] (°)	1.71
Rmsd B values (Å^2) (m.c./s.c.)[b]	2.4/3.3
<B> protein, molecule 1 (Å^2)	28.8
<B> protein, molecule 2 (Å^2)	38.6
<B> solvent (Å^2)	37.7
Ramachandran plot[c]*:*	
Most favored (%)	84.4
Additionally allowed (%)	15.4
Disallowed (%)	0.2

[a]Root-mean-squared deviation (Rmsd) from ideal bond lengths and angles and Rmsd in B-factors of bonded atoms. [b]m.c., main chain; s.c., side chain. [c]Calculated using PROCHECK [45].

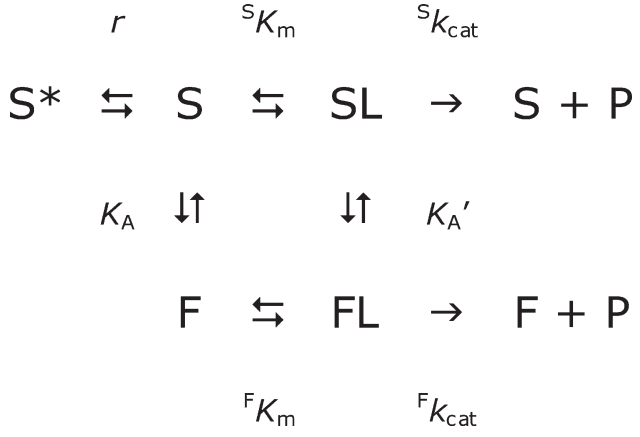
These results demonstrate that structural biology has the potential to unravel the molecular mechanism of M^+ activation and the origin of M^+ selectivity, two important aspects of M^+ binding to enzymes that cannot be resolved unequivocally by kinetic studies [13]. Here we report major aspects of the first structure of thrombin bound to K^+ [14] and compare it with the Na^+ bound form [4]. We also identify, as a serendipitous outcome, an inactive conformation of the slow form whose existence was predicted from kinetic studies of Na^+ binding almost ten years ago [15].

2. Materials and methods

The structure described in this paper has already been published as part of a study on the role of the RGD sequence of thrombin [14] and the coordinates have been released as 2A0Q. Due to the focus of our previous report and the space limits imposed on accelerated publications, the structure could not be discussed in terms of its relevance to M^+ specificity and activation, or in the context of thrombin allostery.

The R77aA mutant of thrombin was used as the key reagent for this structural study. This mutant prevents the autolytic cleavage at R77a in exosite I [16] and enables crystallization of thrombin free of inhibitors. Importantly, the R77aA mutation produces only a modest (<10-fold) perturbation of ligand binding to exosite I [17–19], and has no effect on the M^+ binding properties or the catalytic activity of the enzyme [4]. Therefore, the R77aA mutation does not introduce perturbations in the active site or the M^+ binding site that could bias the structure and complicate the interpretation of results potentially relevant to thrombin allostery. The protein was concentrated to 5.6 mg/ml in 50 mM choline chloride, 20 mM Tris, pH 7.4. Crystallization was achieved at 25 °C by vapor diffusion against 20% PEG 2000-monomethyl ether, 0.1 M Bis–tris, pH 6.6, and 0.4 M KCl. Equal volumes of the protein sample and reservoir solution were mixed (2 μl each) to prepare the sitting drops. Diffraction quality crystals (~0.3 × 0.06 × 0.06 mm) grew in one week and were cryoprotected in paratone oil prior to flash-freezing. Data were collected from a single crystal at the Advanced Photon Source (beamline 14-BM-C, Argonne National Laboratory) and processed using the HKL2000 package. Crystals were orthorhombic, in space group $P2_12_12_1$, and contained two molecules per asymmetric unit (Table 1). The structure was solved by molecular replacement using the coordinates 1SGI [4] and the program MOLREP from the CCP4 package [20]. Crystallographic refinement and model building were carried out with CNS [20] and the program XtalView [21]. Water molecules were added in the final stage of the refinement process. They were subject to visual inspection to check their positioning in electron density and allowed to refine freely. Water molecules with temperature factor (B-factor) higher than 70 Å^2 were excluded from subsequent refinement steps.

The allosteric model discussed in the text is based on the following kinetic scheme. The model is a simple extension of the Botts–Morales scheme for the action of a modifier on substrate hydrolysis [22]. It also incorporates the salient features of the kinetic model originally proposed by Lai, Di Cera and Shafer (LDS) to explain the kinetic pathway of the slow to fast conversion of thrombin [15]. In Scheme 1, thrombin is assumed to exist in two conformations in equilibrium, the M^+-free slow form S and the M^+-bound fast form F. Both forms bind and cleave a substrate L to generate the product P, but they do so with different values of K_m and k_{cat}. Consistent with the original proposal of the LDS model [15], the fast form is unique whereas



Scheme 1.

the slow form exists as an equilibrium of two species, S and S*, of which only S can bind M^+ or substrate. The value of r measures the ratio [S*]/[S]. K_A and K_A' are the association constants for M^+ binding to the S and SL forms. The structures of the four intermediates S, F, SL, and FL have been reported recently [4]. The structure of the second molecule in the asymmetric unit reported in 2A0Q [14] and discussed below is the first determination of the inactive slow form S*.

Solution of the kinetic equations at steady state for Scheme 1 can be obtained using the results of a general theory [23], under the conditions of Michaelis–Menten kinetics that always apply to thrombin [1]. Michaelis–Menten kinetics also imply that binding and dissociation rates of M^+ are fast compared to those of substrate [15], so that the two independent parameters $s=k_{cat}/K_m$ and k_{cat} for Scheme 1 are given by [23]

$$s = \frac{{}^{S}(k_{cat}/K_m) + {}^{F}(k_{cat}/K_m)K_A x}{(1+r) + K_A x} \quad (1)$$

$$k_{cat} = \frac{{}^{S}k_{cat} + {}^{F}k_{cat}\,K_A' x}{1 + K_A' x}. \quad (2)$$

Only s may be influenced by the presence of S*, provided r is significant and $x=[M^+]$ is low compared to $1/K_A$. The value of r cannot be decoupled from measurements of s, because Eq. (1) can be rewritten in terms of only three independent parameters, namely: the specificity constant of the fast form, ${}^{F}(k_{cat}/K_m)$, that depends on the properties of F; the apparent specificity constant of the slow form, ${}^{S}(k_{cat}/K_m)/(1+r)$, that depends on the properties of S and the S–S* equilibrium; and the apparent equilibrium association constant for M^+ binding to the free enzyme, $K_A/(1+r)$, that depends on the binding properties of S and the S–S* equilibrium. The ratio α between the values of k_{cat}/K_m measured under saturating $[M^+]$ relative to $[M^+]=0$ is given by

$$\alpha = \frac{{}^{F}(k_{cat}/K_m)}{{}^{S}(k_{cat}/K_m)}(1+r). \quad (3)$$

This parameter measures the allosteric transduction of M^+ binding into enhanced catalytic activity [4,23] and is of particular relevance to our discussion. Scheme 1 can be extended to include an inactive fast form, F*, in equilibrium with S* and F. This would result in the addition of a parameter analogous to r to reflect the [F*]/[F] ratio. As for r, this parameter would group with the others to define only three independent parameters accessible to measurements of s and M^+ binding. In view of the lack of current functional evidence for the existence of F*, Scheme 1 will be used in our discussion (see Results).

Wild-type human thrombin was expressed, purified and titrated for activity as described [24]. The substrate H-D-Phe-Pro-Arg-*p*-nitroanilide (FPR) was used for measurements of the Michaelis–Menten parameters k_{cat} and $s=k_{cat}/K_m$ as a function of $[Na^+]$ and $[K^+]$ from direct integration of progress curves of substrate hydrolysis taking into account product inhibition [25]. Experimental conditions were: 50 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The $[M^+]$ was changed by addition of the chloride salt.

3. Results

In an effort to understand the molecular basis of thrombin preference for Na^+ vs K^+, the enzyme was crystallized free of any inhibitors in the presence of 400 mM KCl. The high concentration of K^+ was made necessary by its weak affinity (K_d=160 mM at 10 °C) toward thrombin [5]. Even at this high concentration, the M^+ binding site was expected to be only 70% bound, leaving significant room to the possibility of detecting thrombin in the M^+-free form. In a serendipitous outcome, crystals showed two molecules in the asymmetric unit, one bound to K^+ and the other K^+-free. In what follows, we will refer to the K^+-bound molecule as molecule 1, or the K^+ bound F form, to be compared directly to the Na^+ bound F form 1SG8 [4]. The K^+-free molecule will be referred to as molecule 2, or S* form (see Scheme 1).

3.1. The K^+ bound F form (molecule 1)

The overall structure of molecule 1 is similar to that of the Na^+ bound F form, with an r.m.s.d. of only 0.40 Å at the Cα atoms. K^+ is bound to the M^+ binding site to three backbone oxygens from Arg-221a, Lys-224 and Tyr-184a and four water molecules (Fig. 1). The coordination is similar to that found in K^+-activated enzymes [3] like tryptophanase [26] and tyrosine phenol lyase [27]. Table 2 offers a comparison between the O–K^+ and O–Na^+ distances in the F form of thrombin [4]. The O–K^+ distances are on the average 2.9 Å, in agreement with the expected value of 2.8 Å [28]. The valence at the K^+ peak is 1.2 valence units according to the program WASP [29]. There are five ligands common to Na^+ and K^+: the backbone oxygens of Arg-221a and Lys-224, and three water molecules. The fourth water molecule in the K^+ coordination shell replaces a water

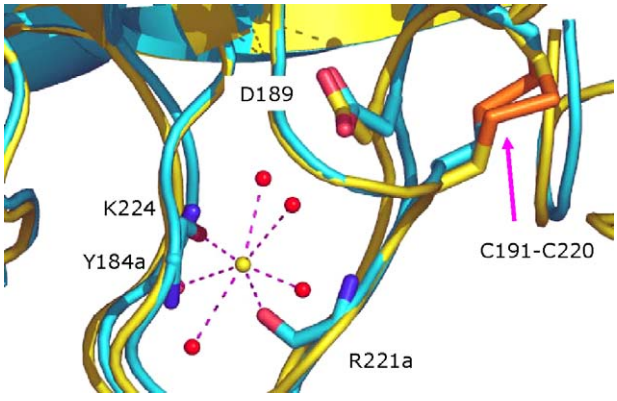


Fig. 1. The K^+ binding site of thrombin, according to molecule 1 of 2A0Q (CPK, with C atoms in cyan). K^+ maintains a close connection with Asp-189 in the S1 pocket, a crucial determinant of thrombin allostery [44], and is coordinated by seven ligands: three carbonyl oxygens from Tyr-184a, Arg-221a and Lys-224, and four water molecules. Note how the disulfide bond between Cys-191 and Cys-220 (arrow) rotates toward the S1 pocket in the K^+ structure, relative to the Na^+ bound F form 1SG8 (CPK with C atoms in yellow). H-bonds are depicted by broken lines (magenta).

Table 2
Coordination distances (in Å) for K^+ and Na^+ in thrombin

Ligand	K^+ bound F form	Na^+ bound F form
R221a O atom	2.74	2.51
K224 O atom	2.53	2.51
Y184a O atom	3.37	–
Water	3.04	2.52
Water	3.34	2.67
Water	2.55	2.89
Water	2.79	2.81
Average	2.91	2.65
Valence [a]	1.20	0.75

[a] In valence units, calculated using WASP [29].

molecule that bridges two coordinating water molecules in the Na^+ bound F form. Likewise, the backbone oxygen of Tyr-184a in the K^+ coordination shell does not participate in Na^+ coordination, but bridges two coordinating water molecules. The changes in coordination are caused by the larger ionic radius of K^+ (1.33 vs 0.97 Å) to which thrombin responds with a widening of the M^+-binding loop between Cys-220 and Tyr-225.

Movement of Cys-220 to accommodate the larger M^+ makes the disulfide bond with Cys-191 rotate towards the substrate-binding pocket (Fig. 1). The torsion angle χ_1 in Cys-220 changes from −61° in the Na^+ bound F form to −153° in molecule 1. The analogous changes in Cys-191 are from −157° to −50°. These changes propagate to residue Glu-192 and Gly-193 in the oxyanion hole (Fig. 2). The side chain of Glu-192 engages the catalytic residues His-57 and Ser-195 in H-bonding interactions, with the Oγ atom of Ser-195 H-bonding back to the backbone oxygen of Glu-192. In the Na^+ bound F form, the cross-talk between Glu-192 and Ser-195 is mediated by a water molecule [4]. The perturbation induced by K^+ binding culminates in a flip of the peptide bond between Glu-192 and Gly-193, whose backbone nitrogen defines the oxyanion hole together with Ser-195. The flip, along with the direct engagement of Ser-195 by Glu-192, should compromise the catalytic activity of the K^+ bound F form. However, access to the S1 pocket remains wide open in molecule 1. Therefore, the P1 Arg of substrate should be able to penetrate the pocket, engage the side chain of Asp-189, induce a rearrangement of Glu-192 and Gly-193 to restore the oxyanion hole and free Ser-195 for nucleophilic attack on the peptide bond. These substrate-induced changes would not be possible in an enzyme where access to the S1 pocket is blocked. This mechanism would predict the K_m to be similar to that of the Na^+ bound F form, but the k_{cat} to be lower due to the energy necessary to reorient Glu-192 and restore the oxyanion hole for formation of the transition state. Indeed, the difference in thrombin activation by K^+ and Na^+ is predominantly due to a higher k_{cat} in Na^+ [1] (see also Fig. 5). Hence, changes originating at the M^+ binding site upon K^+ binding, propagate via the Cys-191–Cys-220 disulfide bond to residues 192 and 193 in the oxyanion hole and explain the lower k_{cat} for substrate hydrolysis in K^+ relative to Na^+.

3.2. The S* form (molecule 2)

Packing of the two molecules in the asymmetric unit is more extensive than that observed in the two molecules of the Na^+-bound F form in 1SG8 [4]. At the interface between the two molecules there is a second K^+ coordinated by the carbonyl oxygens of Gly-142 and Ile-16 of molecule 2, Arg-221a of molecule 1, and four water molecules [14]. This K^+ site is obviously not functional, as it would not be present in solution where thrombin exists as a monomer [1,30,31]. The packing interactions result in a very different conformation of the autolysis loop 142–149 (Fig. 3), although one cannot discount the alternative possibility that the intrinsic flexibility of the loop may be at the origin of the more extensive packing interactions. In the SL form of thrombin, the entire autolysis loop is visible [4]. The backbone nitrogen of Asn-143 H-bonds to the

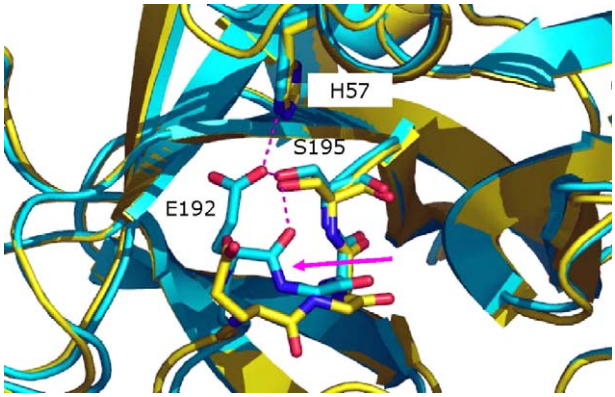


Fig. 2. Perturbation of the active site and oxyanion hole in the K^+ bound F form (CPK, with C atoms in cyan), relative to the Na^+ bound F form 1SG8 (CPK with C atoms in yellow). The perturbation, started at the disulfide bond between Cys-191 and Cys-220 (Fig. 1), propagates to residues 192–193 and causes a shift in the side chain of Glu-192 and a flip of the peptide bond with Gly-193 (arrow). As a result of these changes, Glu-192 becomes engaged in H-bonding interactions with the active site residues His-57 and Ser-195, and the backbone nitrogen of Gly-193 defining the oxyanion hole with the backbone nitrogen of Ser-195 assumes a nonfunctional configuration. H-bonds are depicted by broken lines (magenta).

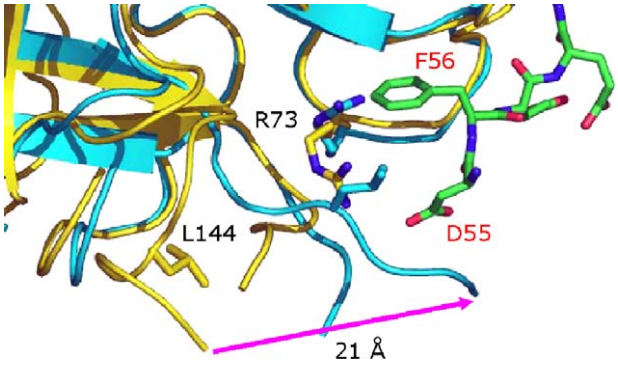


Fig. 3. Conformational change of the autolysis loop of S* thrombin, according to molecule 2 of 2A0Q (CPK, with C atoms in cyan). The structure is compared with the 1HAH structure of thrombin (CPK with C atoms in yellow) bound to the exosite I inhibitor hirugen (CPK with C atoms in green, residues labeled in red). The proximal segment of the autolysis loop moves 21 Å away toward exosite I (arrow drawn at Thr-147). The shift causes Leu-144 to move 10 Å causing a displacement of Arg-73 in exosite I. The displacement likely abrogates hirugen binding to exosite I by removing an important ionic interaction with Asp-55 [19] and producing a steric clash with Phe-56.

backbone oxygen of Glu-192. There is a salt bridge between Glu-146 and Arg-221a, that contributes to the integrity of the M^+ binding site, and a H-bond between the carbonyl oxygen of Glu-146 and the backbone nitrogen of Cys-220. The β structure of the autolysis loop that contacts the 220 loop and Glu-192 is at an approximate right angle to the β structure formed by residues 132–140 and 155–163 in the core of the molecule. The vertex of this right angle is at the space between residues Trp-141, whose side chain is flipped, and Ser-153. This right angle appears to be conserved in all thrombin structures, even those where the autolysis loop is partially disordered, and enables the autolysis loop to contact and stabilize the 220 loop defining the M^+ binding site. In molecule 1, the entire segment from Asn-143 to Pro-152 is disordered, but in molecule 2 residues 143–147 and 151–152 are visible in the electron density maps and the right angle is not present [14]. The entire loop is dislodged up to 21 Å and swings toward exosite I in a conformation never before observed in thrombin structures (Fig. 3). A notable consequence of the repositioning of the autolysis loop is the interference with ligand binding to exosite I. When the structure 1HAH of thrombin bound to hirugen [32] is superimposed to molecule 2, it becomes obvious that the inhibitor would not be able to bind to exosite I. In molecule 2, the sharp rearrangement of the autolysis loop moves Leu-144 > 10 Å toward exosite I, where it displaces the side chain of Arg-73. These changes would cause Leu-144 to come 2.6 Å from the side chain of Asp-55 of hirugen and would bring Arg-73 within 1.9 Å of the phenyl ring of Phe-56 of hirugen. The abrogation of the ionic interaction between Arg-73 of thrombin and Asp-55 of hirudin causes a 100-fold drop in affinity [19]. A similar drop in hirugen already modest affinity (K_i = 1 μM), combined with the steric clash with Phe-56, is likely to abrogate binding of the inhibitor to exosite I.

The absence of K^+ in the M^+ binding site destabilizes the region causing complete disorder of the 221–224 residues. As a consequence, the RGD sequence 187–189 becomes almost fully exposed to the solvent [14]. There is a significant change in the disulfide bond between Cys-191 and Cys-220, which contributes to the disorder of the 220 loop along with the massive dislodgement of the autolysis loop. In the aryl binding site next to the catalytic triad the side chain of Trp-215 flips 192° relative to the position found typically in thrombin structures [4]. The flip is apparently stabilized by a H-bond with Glu-217 (Fig. 4). The flip could not occur without creating an unacceptable collision between the side chain of Trp-215 and the backbone, so the backbone starting at Gly-216 changes conformation. This causes residues 216–220 to collapse into the M^+ binding site and also to occlude access to the S1 pocket. The distance between the backbone nitrogens of Gly-216 and Glu-192 defines the diameter of the cylindrical cavity leading to the S1 pocket. This distance is 8.4 Å in the S form, but drops to 4.7 Å in molecule 2. Hence, molecule 2 portrays thrombin in a conformation that cannot bind M^+, substrate and inhibitors at exosite I.

The first and only investigation of the kinetic pathway of the Na^+ induced slow to fast conversion of thrombin was carried out by Lai et al. [15] almost ten years ago and led to the formulation of the LDS model. The LDS model states that the

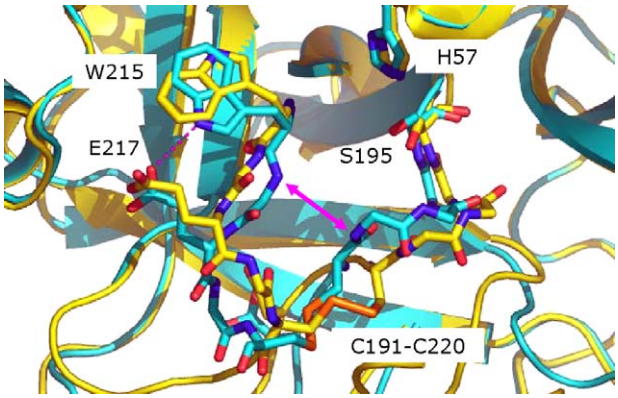


Fig. 4. Conformational change of the active site region in the S* form (CPK, with C atoms in cyan), relative to the S form 1SGI (CPK with C atoms in yellow). The side chain of Trp-215 flips 192° and is stabilized by a H-bond with the side chain of Glu-217. This change causes a collapse of the 216–220 backbone into the M^+ binding site. Perturbations of the disulfide bond between Cys-191 and Cys-220 propagate to residues 192–193. These changes and the perturbation around Trp-215 shrink the aperture between the backbone nitrogens of Gly-216 and Glu-192 (arrow) from 8.4 Å in the S form to 4.7 Å in molecule 2, thereby occluding access to the S1 pocket. H-bonds are depicted by broken lines (magenta).

Na^+ free slow form of thrombin is an equilibrium mixture of two conformers, E_{S1} and E_{S2}, with Na^+ binding selectively to E_{S2}. The Na^+ bound fast form, on the other hand, is unique. Interestingly, in their original study Lai et al. also reported that an active site inhibitor and the exosite I inhibitor hirugen bound selectively to E_{S2}, and concluded that E_{S1} is a thrombin form unable to bind at the Na^+ binding site, the active site and exosite I. The structures of E_{S2} and of the Na^+ bound fast form corresponding to S and F in Scheme 1 have been solved recently [4]. The structure of the S* form of thrombin (molecule 2) reported here is a representation of the E_{S1} form in the LDS model [15]. Its M^+ binding site is completely disordered, the S1 pocket is occluded and a massive shift of the autolysis loop compromises binding of ligands to exosite I.

4. Discussion

The structures of the two molecules of thrombin in the asymmetric unit reported in this study provide important new information on two basic aspects of thrombin function: the molecular basis of M^+ selectivity and the nature of the slow form. The first aspect has been the subject of intense structural investigation in recent years for a number of enzymes activated by M^+ [3]. With the K^+-bound structure reported here, thrombin joins a group of selected enzymes for which the M^+ binding site has been characterized in terms of Na^+ and K^+. Crystallographic results on these enzymes have varied in their elucidation of the kinetics of activation. In the case of Trp synthase, the changes between the Na^+-bound and K^+-bound structures are very significant [33], but surprisingly there is no difference in the kinetics of activation [34]. In pyruvate kinase, the first enzyme to be identified to require a M^+ [6], replacement of K^+ with Na^+ results in no structural changes [9], although the enzyme is practically inactive without K^+ [6]. However, in the case of dialkylglycine dehydrogenase [8,10] and Hsc70 [11,12],

replacement of the essential K^+ with Na^+ changes drastically the geometry of coordination and perturbs residues that control binding of substrate. Likewise, in the case of thrombin, the replacement of Na^+ with K^+ elicits changes in the coordination geometry that propagate long-range to compromise the architecture of the oxyanion hole. The changes explain the higher k_{cat} measured in the presence of Na^+.

The second aspect has also enjoyed substantial attention recently. The slow form of thrombin was originally defined as the Na^+-free form of the enzyme [1]. However, the term "slow" has recently been adopted to define any structure of "inactive" thrombin, whether Na^+-free [35] or Na^+-bound [36], and whether mutant [35,36] or wild-type [37]. Such "inactive" forms of thrombin have been proposed to play a crucial role in the molecular mechanism of thrombin allostery [36,37]. Specifically, the activating effect of Na^+ has recently been explained by assuming that thrombin exists in an equilibrium between two forms, active and inactive, with Na^+ pulling the equilibrium toward the active form [37]. Existing functional data easily refute such proposal. The inactive–active equilibrium model is a special case of Scheme 1, where S and F are renamed "active" and S* "inactive". The assumption of a single active form in equilibrium with the inactive form [37] implies ${}^S(k_{cat}/K_m)={}^F(k_{cat}/K_m)$ and ${}^Sk_{cat}={}^Fk_{cat}$ in Scheme 1, which yields the following expressions for s, k_{cat} and α (see Eqs. (1)–(3))

$$s = {}^S(k_{cat}/K_m)\frac{1+K_Ax}{(1+r)+K_Ax} = {}^F(k_{cat}/K_m)\frac{1+K_Ax}{(1+r)+K_Ax} \quad (4)$$

$$k_{cat} = {}^Sk_{cat} = {}^Fk_{cat} \quad (5)$$

$$\alpha = 1+r. \quad (6)$$

The inactive–active equilibrium model predicts a k_{cat} independent of $[Na^+]$ and a value of $\alpha=1+r$ that, under specified solution conditions, depends solely on the S–S* equilibrium. Because this equilibrium is by definition independent of the presence of substrate or M^+, it follows that the value of α, under the same solution conditions, must be the same for all thrombin substrates and M^+ used. Contrary to such predictions, k_{cat} changes drastically with $[Na^+]$ [1,38,39] and the value of α changes two orders of magnitude under the same solution conditions, depending on the physiologic [40] or synthetic [41] substrate used. The data in Fig. 5 depicts the effect of Na^+ and K^+ on the cleavage of FPR by thrombin and illustrates further the inconsistencies of the inactive–active equilibrium model [37]. In both cases, the k_{cat} is strongly dependent on $[M^+]$ and the ratio α between the values of s at $[M^+]=\infty$ and $[M^+]=0$ also depends on the M^+. On the other hand, the data obey beautifully the predictions of Scheme 1 and Eqs. (1)–(3).

It has been known since 1992 [1] that thrombin is allosterically activated by Na^+ and that the mechanism of activation requires an equilibrium between two active forms, slow (S) and fast (F), that differ in their Michaelis–Menten parameters. All existing functional data on thrombin support this mechanism. In 1997, Lai et al. [15] have proved the existence of an inactive slow form in equilibrium with the active S form. The inactive slow form discovered by Lai et al. does not bind M^+, substrate or inhibitors at the active site or exosite I [15]. The structure of S* presented here has all the requisites of the inactive slow discovered by Lai et al. The recent structure of inactive wild-type thrombin in the Na^+-free state [37] resembles the structure of S* in the conformation of the 220-loop and the conformation of Trp-215. However, this structure has exosite I available for binding and is therefore neither a representation of the inactive slow form discovered by Lai et al. [15], nor a representation of the active slow form S. Our model of thrombin allostery in Scheme 1 provides a coherent synthesis between existing structural and functional data. In our model, we retain for clarity the terms "slow" and "fast" as originally defined by Wells and Di Cera [1], i.e., slow=Na^+-free and fast=Na^+-

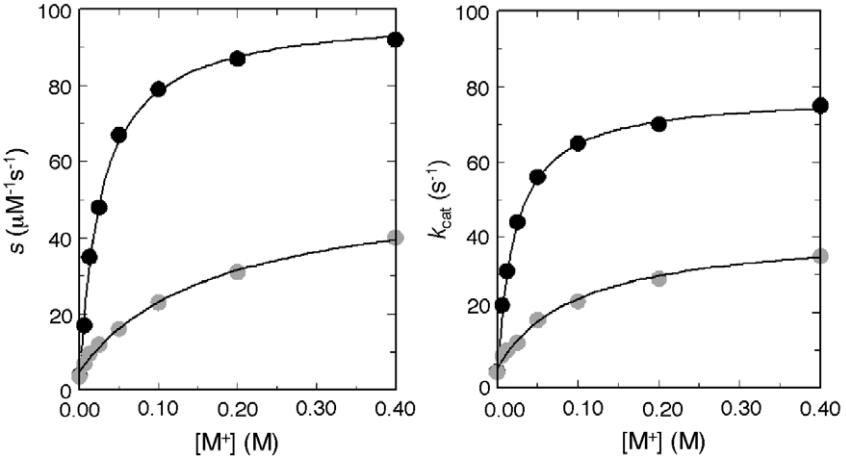


Fig. 5. M^+ dependence of the kinetic constants $s=k_{cat}/K_m$ (left) and k_{cat} (right) for FPR hydrolysis by thrombin in the presence of Na^+ (●) or K^+ (●). Experimental conditions are: 50 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The $[M^+]$ was changed by addition of the chloride salt. Curves were drawn according to Eqs. (1) and (2) in the text, with best-fit parameter values: (data at left) ${}^S(k_{cat}/K_m)/(1+r)=2.3\pm0.1$ μM^{-1} s^{-1}, ${}^F(k_{cat}/K_m)=99\pm3$ μM^{-1} s^{-1}, $K_A/(1+r)=38\pm1$ M^{-1} (●); ${}^S(k_{cat}/K_m)/(1+r)=2.5\pm0.1$ μM^{-1} s^{-1}, ${}^F(k_{cat}/K_m)=54\pm2$ μM^{-1} s^{-1}, $K_A/(1+r)=5.8\pm0.2$ M^{-1} (●); (data at right) ${}^Sk_{cat}=4.7\pm0.2$ s^{-1}, ${}^Fk_{cat}=78\pm2$ s^{-1}, $K_A'=45\pm2$ M^{-1} (●); ${}^Sk_{cat}=5.4\pm0.2$ s^{-1}, ${}^Fk_{cat}=42\pm1$ s^{-1}, $K_A'=10\pm1$ M^{-1} (●). The values of α (see Eq. (3)) for the curves at left are 43 ± 2 in the presence of Na^+ and 22 ± 1 in the presence of K^+.

bound. The slow form exists as a mixture of two forms in equilibrium, S and S*. The fast form F is unique. These forms correspond to E_{S2} (S), E_{S1} (S*) and E_F (F) in the LDS model [15]. The structures of these forms are 1SGI for S, 1SG8 for F and molecule 2 of 2A0Q for S*. The S and F forms cleave substrate with different values of k_{cat}/K_m and k_{cat}. The differences between the Michaelis–Menten constants of the S and F forms account for most of the Na^+ effect on thrombin, whereas the S–S* equilibrium makes only a small contribution. In fact, using the value of $r=[S^*]/[S]=0.14$ measured by Lai et al. [15] at 37 °C, and knowing that thrombin is 60% bound to Na^+ at this temperature [5,42], one can calculate that the inactive S* form in vivo is at most 5% of the total.

Although the physiologic importance of the S* form is minuscule compared to that of the active S and F forms of thrombin, the potential mechanistic significance of S* is noteworthy. All recent structures of inactive wild-type [37] and mutant [35,43] thrombins share one or more features with the structure of S* reported here and can be considered members of an "ensemble" of conformations generated by fluctuations around the dominant conformation S*. Under the effect of mutations, or under conditions mirroring the packing interactions or non-ideality of crystal growth, members of this inactive "S* ensemble" could become significantly populated. Inactive conformations of thrombin explain the anticoagulant properties of the E217K [35] and W215A/E217A [43] mutants, as well as the RGD-dependent adhesive properties of the enzyme [14]. Substantial structural evidence now exists about the plasticity of the thrombin fold and its tendency to assume inactive conformations in the absence of ligands [14,35–37,43]. On the other hand, functional evidence of the existence of inactive conformations of thrombin is limited to one kinetic study published by Lai et al. almost ten years ago [15]. Progress in this area must therefore await the results from new studies of thrombin in solution.

Acknowledgments

This work was supported by NIH research grants HL49413, HL58141, HL73813.

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