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Biophysical Chemistry 112 (2004) 253–256

Biophysical Chemistry

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Crystal structure of the thrombin mutant D221A/D222K: the Asp222:Arg187 ion-pair stabilizes the fast form

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Received 15 June 2004; received in revised form 2 July 2004; accepted 2 July 2004
Available online 23 August 2004

Abstract

The thrombin mutant D221A/D222K (ARK) does not bind Na^+ and has interesting functional properties intermediate between those of the slow and fast forms of wild type. We solved the X-ray crystal structure of ARK bound at exosite I with a fragment of hirudin at 2.4-Å resolution. The structure shows a slight collapse of the 186 and 220 loops into the Na^+ binding site due to disruption of the Asp222:Arg187 ion-pair. The backbone O atoms of Arg221a and Lys224 are shifted into conformations that eliminate optimal interaction with Na^+. A paucity of solvent molecules in the Na^+ binding site is also noted, by analogy to what is seen in the structure of the slow form. These findings reinforce the crucial role of the Asp222:Arg187 ion-pair in stabilizing the fast form of thrombin.

Keywords: Thrombin; Allostery; Na^+ binding; Serine protease structure

Thrombin is an allosteric enzyme that exists in two conformations, slow and fast, that interconvert rapidly upon binding and dissociation of Na^+ [1]. The two conformations play distinct functional roles: the Na^+-bound fast form is responsible for the procoagulant, prothrombotic and signaling functions of the enzyme, whereas the Na^+-free slow form accounts for the anticoagulant role of the enzyme [2]. The structural basis of the slow→fast transition has recently been elucidated [3]. A cluster of residues in close proximity to the Na^+ site controls the energetics of cation recognition. Among these residues, Asp222 forms an ion-pair with Arg187 that latches the 220 loop onto the 186 loop and stabilizes the Na^+ binding environment. A naturally occurring mutant of thrombin where Arg187 is changed to Gln produces a phenotype with impaired thrombosis [4] due to reduced Na^+ binding, thereby confirming the importance of the Asp222:Arg187 ion-pair in stabilizing the fast form.

The thrombin mutant D221A/D222K (ARK) was prepared to mimic the sequence Asp-Arg-Lys found in coagulation factor Xa [5,6], which displays a reduced Na^+ affinity compared to thrombin [7]. ARK has functional properties intermediate to those of the slow and fast forms of wild type and does not discriminate among monovalent cations [5]. These properties, along with involvement of Asp222 in the mutation, have motivated the crystallization of ARK under conditions that could reveal the architecture of the water molecules embedding the Na^+ site and the primary specificity pocket of the enzyme.

1. Materials and methods

The thrombin mutant ARK was expressed and activated as previously described [5,6]. The mutant was concentrated to 3 mg/ml in 5 mM MES buffer, pH 6.0, 0.3 M NaCl and

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doi:10.1016/j.bpc.2004.07.027

was mixed with the 10-residue peptide hirugen (DFEEI-PEEYL) at a molar ratio of 1:10 and incubated at 4 °C overnight. Crystallization was achieved at 25 °C by vapor diffusion against a reservoir solution composed of 0.1 M sodium phosphate, pH 7.3, 26% PEG 8000. Equal volumes of the protein sample and reservoir solution (2 μl each) were initially mixed to prepare the hanging drops and small thin plate crystals were obtained after a few days. Larger diffraction quality crystals (~0.4×0.35×0.35 mm) were obtained after 10 days by introducing 10% CH_3CN as additive and using 20 μl sitting drops. X-ray diffraction intensity data were collected using a Rigaku RU 200 fine focus rotating anode generator operating at 50 kV and 100 mA with an R-AXIS II imaging plate detector. The crystal diffracted X-rays to about 2.2-Å resolution and belongs to the space group $C222_1$ with one molecule per asymmetric unit. The structure was solved by molecular replacement using the coordinates of the thrombin-PPACK complex [8] as search model and the program package CNS [9]. Due to the low data completeness in the resolution range of 2.4–2.2 Å, the structure of ARK is reported to a nominal 2.4-Å resolution. Crystallographic refinement was carried out by simulated annealing and conjugated-gradient minimization using CNS, and model building was performed with the program "O" [10]. Weak electron density was observed for the autolysis loop as well as for the N-terminal and C-terminal regions, thus models for these segments were not included in subsequent refinement steps. 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) >80 $Å^2$ were excluded from subsequent refinement. Structural comparisons were computed using LSQMAN [11]. The final refinement and model quality statistics are presented in Table 1. Coordinates of the ARK structure have been deposited to the Protein Data Bank with accession code 1TWX.

Table 1
Crystallographic data of the thrombin mutant D221A/D222K

Data collection	1TWX
Space group	$C222_1$
Unit cell dimensions (Å)	a=92.15
	b=80.01
	c=100.60
Resolution range (Å)	30.0–2.2
Observations	30,676
Unique observations	13,866
Completeness	72.0 (48.0)
R_{sym} (%)	6.8 (20)
$I/\sigma(I)$ (outermost shell)	(1.8)
Refinement	
Resolution (Å)	30.0–2.4
$\|F\|/\sigma(\|F\|)$	>0
R_{cryst}, R_{free}	0.205, 0.268
Reflections (working/test)	10,675/839
Protein atoms	2346
Solvent molecules	141
Rmsd bond lengths[a] (Å)	0.007
Rmsd angles[a] (deg.)	1.4
Rmsd B values (Å) (m.c./s.c.)[b]	1.6/2.1
⟨B⟩ protein ($Å^2$)	39
⟨B⟩ solvent ($Å^2$)	45
Ramachandran plot[c]	
Most favored (%)	82.8
Additionally allowed (%)	16.4
Disallowed (%)	0.0

[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 [15].

2. Results and discussion

The mutant ARK was crystallized in the presence of the exosite I inhibitor hirugen to enable a characterization of the active site environment in the absence of bound ligands. This strategy was first used for the wild type [12] and provided detailed information on the network of water molecules that embeds the Na^+ site, the primary specificity pocket and the active site region [13]. Information on the properties of this network is crucial to understand the molecular basis of thrombin allostery, as recently emerged from the structures of the slow and fast forms of thrombin [3]. Comparison of ARK and the hirugen-bound wild-type structure 1HAH [12] shows essentially the same overall conformation with the two structures featuring an r.m.s. deviation of only 0.5 Å. The autolysis loop of ARK is disordered from residues 148 to 149d, as typically found for thrombin structures at this resolution. The program WASP [14] returned no Na^+ specific valence >0.5 v.u. for the water molecules refined in the structure, suggesting the absence of bound Na^+. Closer examination of the Na^+ binding site shows no evidence of bound Na^+, even though ARK was crystallized in the presence of 0.1 M sodium phosphate and the protein was initially concentrated in 0.3 M NaCl. This result is consistent with the lack of monovalent cation sensitivity of the mutant [5].

Replacement of Asp221 with Ala and Asp222 with Lys abrogates the Asp222:Arg187 ion-pair that is critical for stabilization of the Na^+ binding site. The ion-pair is not present in the slow form and is only seen in the fast form [3]. Specifically, Asp222 is a residue of the allosteric core and its mutation to Ala compromise Na^+ binding >30-fold [3]. Mutation of Asp221 to Ala, on the other hand, has no effect on Na^+ binding but abrogates the transduction of this event into enhanced catalytic activity [3]. The double replacement in the ARK mutant therefore affects two critical residues of the 220 loop that are involved in Na^+ binding (Asp222) and allosteric transduction (Asp221). The crystal structure documents the extent of perturbation in this region. The side chain of Arg187 rotates approximately 50° relative to wild type due to lack of electrostatic coupling with Asp222. The loss of the ion-pair causes an inward

movement of the 186 loop, with a displacement of the backbone that is most visible (2.0 Å) at the level of Lys186d (Fig. 1). The 220 loop moves downward (Fig. 1) up to 0.6 Å relative to the backbone of the fast form, F [3]. As a consequence of this shift, the backbone O atoms of the Na^+-coordinating residues Arg221a and Lys224 move, respectively, 0.6 and 0.8 Å relative to the position in the F structure (Fig. 2). The changes produce an environment not suitable for Na^+ coordination.

The absence of bound Na^+ in the structure of ARK is also linked to a reduced number of solvent molecules in the region embedding the Na^+ site, the primary specificity pocket and the active site (Fig. 3). The well-organized network of water molecules seen in the fast form F of wild type [3] is not seen in the ARK mutant. The catalytic Ser195 also moves slightly away from its H-bonding partner His57 whose imidazole ring is also slightly rotated (the interatomic distance between Oγ of Ser195 and Nε2 of His57 is 3.2 Å), again reflecting a signature of the slow form S of wild type [3] (Fig. 3). Also notable is the conformation of the side chain of Glu192 that mimics that found in the S structure [3] (Fig. 3).

Taken together, these structural findings provide a rationale for the perturbed biochemical properties of the ARK mutant. The lack of Na^+ binding and allosteric transduction are a result of the abrogation of the Asp222:Arg187 ion-pair and of the anchoring role of the side chain of Asp221, a critical residue for thrombin allostery [3]. The mutation then causes a disorganization of the water network that connects the Na^+ site to the active site region in the Na^+ bound fast form, and induces changes in the orientation of Glu192 and Ser195 that mimic those found in the Na^+-free slow form [3]. The shift in the position of Glu192 seems to play a critical role in preserving activity toward protein C in the slow form through a reduction of the electrostatic clash with negatively charged residues at the P3 and P3′ positions of protein C. This explains the increased

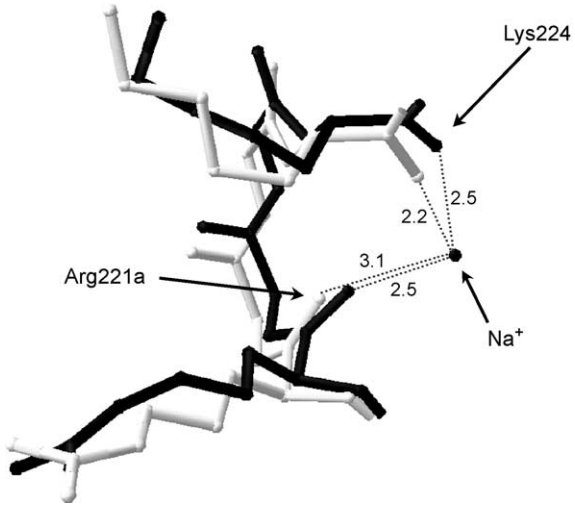

Fig. 2. View of the Na^+ binding site of ARK (gray) and F (black). Also shown in the F structure is the bound Na^+ which is not detected in ARK. Movement of the 220 loop in ARK results in the shift of the backbone O atoms of Arg221a and Lys224 that directly coordinate Na^+. The distances of the carbonyl oxygens shifted from 2.5 Å for both in F to 3.1 and 2.2 Å in ARK, respectively.

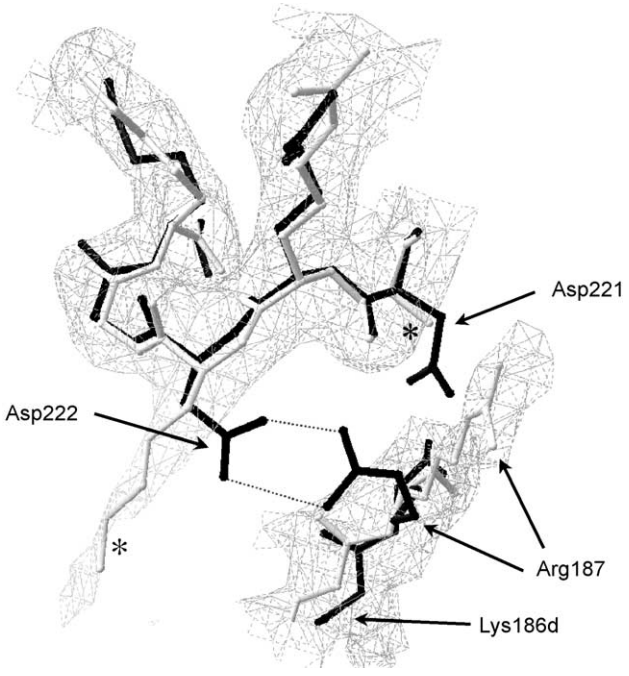

Fig. 1. Environment of the 220- and 186-loop region of the ARK (gray) and the fast form F (black) [3]. Mutated residues are indicated by asterisks. The side chain of Arg187 is shifted almost 50° relative to the position in the F structure due to lack of electrostatic coupling with Asp222. The backbone region at the level of Lys186d is clearly displaced, with an inward movement of about 2.0 Å. Also shown is the 2Fo-Fc electron density map of ARK, contoured at 0.8σ (gray).

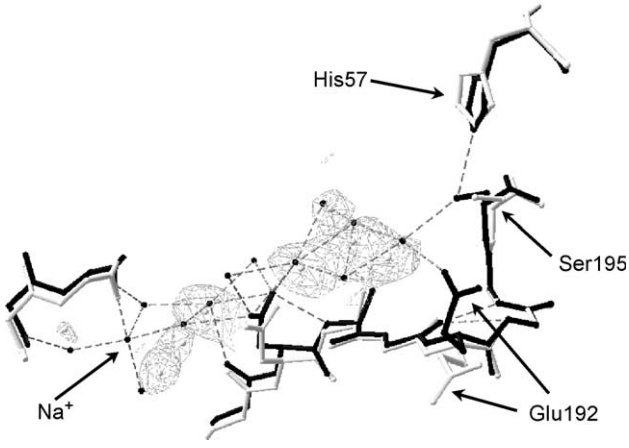

Fig. 3. View of the active site, primary specificity pocket and Na^+ binding site of the thrombin mutant ARK (gray) and the fast form F (black). The absence of bound Na^+ in ARK is linked to a reduction of the number of water molecules in the channel spanning from the Na^+ binding site to the catalytic region. The H-bonding network in F is shown as gray dashed lines. Also shown is the 2Fo-Fc electron density map of the ARK water molecules, contoured at 0.8σ (gray). A slight shift in the side chain of Ser195 moves it away from its H-bonding partner His57. The side chain of Glu192 is shifted away from the primary specificity pocket, and assumes a conformation similar to that of the slow form S.

anticoagulant activity of the mutant ARK [5] and lends further support to the mechanism of protein C activation by the slow form of thrombin [3].

Acknowledgments

This work was supported in part by NIH Research Grants HL49413, HL58141 and HL73813. A.O.P. is a recipient of a Fellowship from the American Heart Association.

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