COMMENTARY

Slow thrombin in solution

James A. HUNTINGTON[1]

University of Cambridge, Department of Haematology, Division of Structural Medicine, Thrombosis Research Unit, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, U.K.

As a tool for understanding biological mechanisms, X-ray crystallography possesses unparalleled power to enlighten, resolve controversy and shift a field of study on to a secure new paradigm. Thanks largely to developments in crystallographic methods, the technique has become accessible to the general biochemist and we have thus witnessed an exponential increase in the number of protein structures deposited every year. It is now commonplace for several structures to be published of the same protein under different crystallization conditions, sometimes resulting in conflicting mechanistic interpretations. Such a controversy has arisen over thrombin's conformational response to Na^+ co-ordination, and in this issue of *Biochemical Journal*, De Filippis and colleagues put the two structural models of thrombin allostery to the test by returning to the techniques of solution biochemistry.

Key words: allostery, conformation, mechanism, sodium, structure, thrombin.

Since the first structure of thrombin was published in 1989 by Bode et al. [1], over 120 thrombin structures have been deposited in the Protein Data Bank and untold numbers have been withheld from deposition for commercial reasons. The great interest in thrombin is justified by its central role in blood coagulation, highlighted by Ken Mann in his recent review [2] entitled "Thrombin: can't live without it; probably die from it". Thrombin is the final protease in the blood coagulation cascade, whose manifold activities include: cleavage of fibrinogen to form the fibrin clot, activation of platelets through cleavage of PARs (protease-activated receptors), stabilization of fibrin clots through activation of Factor XIII, and stimulation of its own formation through activation of Factors V, VIII and XI. When bound to the endothelial cell-surface receptor TM (thrombomodulin), thrombin can also serve an anticoagulant function by cleavage activation of protein C. Activated protein C is another serine protease that shuts down thrombin formation by inactivating Factors Va and VIIIa, critical cofactors for the prothrombinase and Xase complexes respectively. Crystallography and mutagenesis studies have built a reasonably complete picture of how thrombin utilizes unique structural features to recognize its multiple substrates (for a recent review, see [3]), and it has become clear that competition for thrombin exosites is involved in directing thrombin activity [4]. However, the role of thrombin allostery remains obscure.

There are two distinct versions of thrombin allostery, one related to TM binding and one to Na^+ co-ordination, but both involve changes in the conformation of the active-site cleft, causing a switching in preferred substrate. Although the structure of thrombin bound to TM did not show any active-site conformational change [5], the fact that an active-site inhibitor was used makes it difficult to firmly conclude that no conformational change would otherwise be observed. Indeed, recent reports suggest that thrombin active-site interactions play a central role in improved recognition of protein C when thrombin is bound to TM [6]. Since TM binds with high affinity to the exosite primarily responsible for recognition of prothrombotic substrates, the anticoagulant mechanism of TM binding may thus be to block prothrombotic substrates while facilitating recognition of protein C by allosterically altering the properties of thrombin's active-site cleft.

The hypothesis of Na^+ allostery is less clear, but in a nutshell it contends that thrombin exists in two forms, slow and fast, depending on Na^+ co-ordination. The slow form (Na^+-free) is a compromised enzyme that cleaves substrates inefficiently, but upon complexation with TM recovers full catalytic activity. Thus the specificity index of slow thrombin favours cleavage of protein C, and Na^+ binding serves to activate thrombin towards its many procoagulant substrates, such as fibrinogen and PARs. Of course, the Na^+ co-ordination state must be variable for this to be a regulatory mechanism of any physiological relevance, and the reported K_d value of thrombin for Na^+ is accordingly in the range of the plasma concentration of Na^+.

The effect of Na^+ on the activity of thrombin was first noted by Orthner and Kosow in 1980 [7]. In this and many subsequent studies, spectroscopic techniques were employed to decipher thrombin's conformational response to Na^+ binding, but until recently only the structure of 'fast' thrombin was known (i.e. Na^+ co-ordinated or cofactor, substrate or inhibitor bound). Several structures claiming to be of the slow form have now been published, leading to two distinct hypotheses of the thrombin conformational change induced by Na^+ binding. The first purported structure of slow thrombin was published in 2002 by Di Cera and co-workers [8]. The structure was surprising in that, in spite of the wealth of biochemical data suggesting a large-scale conformational change, the only observed difference from fast thrombin was a small backbone and side-chain shift in the active site. The authors noted a 1 Å ($\equiv$0.1 nm) movement of the main chain segment of the active-site loop (residues 190–192 using chymotrypsin template numbering), and a 60° rotation of the side chain of Ser-195. The slow kinetic behaviour of Na^+-free thrombin was explained by non-optimal hydrogen bonding in the active site. This low-resolution structure (2.8 Å) was closely followed by a high-resolution structure of the same crystal form (2.3 Å) [9]. Although no other conformational changes were observed, the water channel connecting the Na^+-binding site to the active site was better resolved, resulting in the hypothesis that

[1] email jah52@cam.ac.uk

a reordering of a solvent network was the mechanism of active-site perturbation.

A second hypothesis, published by my group, was based initially on the structure of active-site variant S195A [10], followed by that of a recombinant slow-variant E217K [11] (1.8 Å and 2.5 Å respectively). The structure of S195A thrombin was almost identical to that of normal fast thrombin, save for the orientation of a few critical side chains. Residues previously shown to move in response to Na^+ co-ordination, Trp-60d and Trp-215, were in new positions, which blocked the portion of the active-site cleft that binds bulky hydrophobic substrate residues (aryl-binding pocket). Three other residues which stack against the tryptophans were also observed in new conformations: the disulphide bond between Cys-168 and Cys-182 was flipped, and Phe-227 was rotated. The effect was an orthogonal arrangement of aromatic side chains in S195A thrombin, compared with parallel stacking of the same side chains in fast thrombin. We proposed that the partially obscured active site explained the poor catalytic properties of slow thrombin, and that the five side chains formed an allosteric switch which sensed Na^+ co-ordination. The structure of E217K thrombin supported our interpretation, with the allosteric switch found once again in the slow conformation, and additionally revealed how the disruption of the Na^+-binding site blocks of the rest of the active-site cleft, disrupts the oxyanion hole and alters the hydrogen bonding of the catalytic triad.

Thus two sets of structures have resulted in two distinct hypotheses of the structural features explaining the slow kinetic behaviour of Na^+-free thrombin: the first involves a subtle change in the active-site loop, with no obstruction of the substrate-binding cleft; the second involves a conformational rearrangement which blocks substrate access to the active-site cleft. Because crystal contacts are a necessary 'evil' in protein crystallography, it is always possible that the observed structure may not represent the predominant solution conformation. Therefore all crystal structures must be judged against existing biochemical data, and in some cases should be taken as hypotheses that require further testing by solution techniques. The controversy surrounding the conformation of slow thrombin is a prime example of this.

The timely work by De Filippis and colleagues, reported in this issue of *Biochemical Journal* [12], elegantly addresses the two conflicting models of Na^+ allostery. The authors put thrombin through a 'battery' of spectroscopic tests in the presence of several salts. Far-UV CD spectra in 200 mM NaCl, KCl, LiCl and ChCl (choline chloride) revealed a signal which decreases specifically with Na^+ binding. As none of the structures suggest a change in either secondary or tertiary structural composition of thrombin in response to Na^+ binding, the authors interpret the change in signal as reflecting an alteration in the environment of clusters of aromatic residues and disulphide bonds. This argument is strengthened by a CD signal which is greater than that expected for the helical content of thrombin, reflecting a large contribution from aromatic residues. Since three aromatic clusters help form the S2 and S3 substrate-binding pockets, they conclude that it is likely that the far-UV CD spectral changes correspond to conformational changes in this region. This conclusion is supported by the near-UV CD spectra, which show an increase in the asymmetry in the local environments of aromatic side chains and disulphide bonds in the presence of Na^+. Similarly, an 18 % intrinsic fluorescence enhancement is observed exclusively for Na^+, which suggests changes in the environment of aromatic residues (tyrosine and tryptophan particularly) and a rigidification of the whole molecule. A previous study has shown that the fluorescence enhancement is abrogated for the W215F variant, whereas all other properties are unaffected [13], strengthening the claim that Trp-215 moves upon Na^+ co-ordination. Rigidification is also observed by limited proteolysis, with particular respect to the 148-loop (also known as the γ-loop). These data strongly support a model of thrombin allostery which involves a reordering of aromatic residues and disulphide bonds, leading to a more rigid conformation of thrombin when co-ordinated to Na^+.

In order to assess the effect of Na^+ binding on the conformation of the active-site cleft (what thrombin allostery is all about), the authors make a clever use of an unconventional probe which binds to the active site in an unusual fashion, but still engages the primary specificity pockets of thrombin. The very-N-terminus of hirudin binds in the active site such that residues Val-Ser-Tyr roughly occupy the positions of Pro, Arg and D-Phe, respectively, of the inhibitor PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethylketone). The 44 residues C-terminal to Val-Ser-Tyr also make contacts in and around the active-site cleft, and serve, in this instance, to ensure that the mutations are unlikely to affect binding positions. The most telling result is the preferential loss of affinity for the Y3A variant towards binding of the fast form, indicating that the slow form has a more constricted aryl-binding pocket (the region which interacts with D-phenylalanine of PPACK). Such a constriction is easily explained by the two structures of 'slow' thrombin, which show a blocking of the aryl-binding pocket with Trp-215. Another interesting finding was that the effect of the triple mutation Ala-Arg-Ala was additive for fast thrombin, but deviated significantly from additivity for thrombin in the absence of Na^+. This was explained by the authors as evidence of structural plasticity (i.e. flexibility) of slow thrombin, suggesting that conformational change is required to bind the N-terminal hirudin fragment in the absence, but not the presence, of Na^+. This is consistent with a study on the rapid kinetics of Na^+ binding, which revealed a multistate equilibrium in the absence of Na^+ with a significant fraction of thrombin in a conformation that was incapable of binding either to Na^+ or to active-site inhibitors [14]. Binding of the N-terminal hirudin fragment to this form would similarly be prohibited in the absence of conformational change, so that the apparent co-operativity may be explained by the extensive interactions outside the active-site cleft; of particular relevance are the interactions between the hirudin fragment and the Na^+-binding residues Arg-221a and Lys-224 (including Glu-217).

In conclusion, where protein crystallography has led to conflicting mechanisms of thrombin allostery, De Filippis and co-workers [12] have gone back to the spectroscopic techniques used originally to characterize the conformational change, and have successfully distinguished between the models. Certainly, more thrombin structures will be published and some will claim to represent the slow form. However, any such structure will have to answer to the careful biochemical studies conducted over the years, as well as those described in the current manuscript. It is also clear that interpretation of the structures will need to account for possible crystallization artefacts and the likelihood that the two-state, slow–fast model is a gross simplification of the solution equilibrium of multiple thrombin conformers.

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