Switching cation-binding loops paves the way for redesigning allosteric activation

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An extensive number of enzymes found in both plants and animals are activated by monovalent cations (1, 2). Pioneering work in the 1940s and 1950s revealed a critical K^+ requirement for pyruvate kinase and the existence of Na^+ -dependent activation of β -galactosidase (3, 4). Enzymes requiring K^+ often can be activated by NH_4^+ and Rb^+ but have more difficulties accommodating the larger Cs^+ , and the smaller Na^+ or Li^+ . Enzymes requiring Na^+ typically have a preference for this cation over the smaller Li^+ and the larger K^+ , Rb^+ , and Cs^+ (1, 2). Efforts continue to assess exactly why an enzyme chooses one of these monovalent cations over another. Strategies that successfully change monovalent cation specificity do not automatically lead to preservation of any cation-dependent allosteric events (1, 5). In PNAS, Rana et al. (6) delve into this unresolved issue in their article, "Redesigning allosteric activation in an enzyme." Their study focuses on the serine protease thrombin, which is a Na^+ -dependent enzyme.

Activation by monovalent cations can be divided into two types (1, 2). With type I (cofactor-like) the cation is required for catalysis and functions by helping to anchor the substrate in the enzyme active site. Examples include members of the kinase and chaperone families. By contrast, with type II (allosteric) enzymes the monovalent cation is not required for catalysis but instead binds to an allosteric site and enhances enzyme activity. This approach can be further divided into the binding step and the transmission of conformational change to a more distant enzyme region. Certain enzymes involved in amino acid metabolism and in blood coagulation are examples of type II family members (1, 2).

Thrombin, a type II Na^+ -dependent enzyme, is a serine protease that targets several players in blood coagulation, anticoagulation, and platelet activation (7, 8). The enzyme uses insertion loops to limit substrate access to the active site cleft. In addition, thrombin contains two anion binding exosites (ABE-I and ABE-II) that it uses to promote interactions with selected proteins (9) (Fig. 1). Physiologically, thrombin is involved in catalyzing the conversion of fibrinogen ($\alpha\beta\gamma$)₂ into a fibrin blood clot, in activating coagulation enzymes (V, VIII, XI, and XIII), and in

Fig. 1. Highlighting important structural features of thrombin. Thrombin contains the standard serine protease catalytic triad (magenta), surface loops such as the β -insertion loop (maroon), and then anion binding exosites I (green) and II (blue) (ABE I, ABE II). The 186 (purple) and 220 (cyan) loops on thrombin accommodate a Na^+ ion. In the project by Rana et al. (6), these cation binding loops are replaced with those of the anticoagulant PC. Thrombin Protein Data Bank code 1SG8.

initiating platelet signaling events through activation of protease-activated receptors (PARs). Moreover, thrombin participates in anticoagulation by activating protein C (PC) in the presence of thrombomodulin (7, 8).

The clotting proteases thrombin, VIIa, IXa, Xa, and activated PC are all controlled by Na^+ -dependent events (1). Prior studies have demonstrated the importance of Na^+ in enhancing thrombin catalytic activity (10, 11). The location of the thrombin Na^+ binding site was successfully identified by X-ray crystallography (12, 13) and shown to involve the 186 and 220 loops of thrombin (Fig. 1). Additional biochemical and structural studies by the Di Cera group (14) have led to their proposals on the allosteric line of communication from the Na^+ site up to the active site.

Interestingly, redesigning type II monovalent cation activation can be quite challenging. Monovalent specificity and allosteric activation can be distinct properties and may not be linked. Earlier work by Di Cera and coworkers (5) demonstrated that thrombin binding specificity could be switched from Na^+ to K^+ , but a K^+ -activated enzyme could not be generated. A clear preference for Na^+ activation remained. The present research described in PNAS took a new approach.

For these thrombin studies (6), Rana et al. turn to the anticoagulant enzyme PC. With this anticoagulant, both Na^+ and K^+ contribute comparable enhancement to catalytic activity (5, 15). The 186 and the 220 loops of thrombin were replaced with the complementary ones for PC. As seen in Fig. 1, the 186 loop of PC is shorter than that of thrombin, and the 220 loop exhibits significant differences in residue character between the two enzymes.

After the thrombin-to-PC loop switches, changes to cation binding preference and to activation effects are observed with this reengineered thrombin (6). With such a chimera, Na^+ affinity decreases relative to wild-type thrombin, and K^+ affinity increases. This same thrombin chimera exhibits a significant reduction in catalytic activity. Substrates tested range from small synthetic peptides (FPR, FPK, and FPF) to larger physiological proteins (fibrinogen, PAR1, and PC). Interactions involving the critical substrate fibrinogen were the most hindered by the loop switches. The introduction of Na^+ or K^+ could, however, partially overcome reductions observed for the different substrates. Impressively, it was possible to generate a thrombin that bound K^+ stronger than Na^+ and also exhibited higher activity with this larger monovalent cation. A K^+ -activated thrombin species has now been designed (6).

Remarkable features are found by reviewing the k_{cat}/K_m values (substrate specificity) for the thrombin chimera against different substrates (6). Without any Na^+ or K^+ , the chimera exhibits comparable substrate specificity toward fibrinogen $\alpha\alpha$, PAR1, and PC. Addition of Na^+ or K^+ leads to improvements in k_{cat}/K_m for these key substrates, even for PC. The anticoagulant PC substrate is better known for its lesser reliance on Na^+ (7). The bulkier K^+ clearly dominates as the preferred monovalent cation for the thrombin substrates fibrinogen $\alpha\alpha$ and PAR1. Unexpectedly, the introduction of K^+ into the chimera environment yields an enzyme whose greatest substrate specificity was toward PAR1 (6).

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The thrombin-to-PC chimera designed by the Di Cera group still has room for improvement. It is valuable to point out that the catalytic activity of this enzyme is significantly reduced relative to wild type (6). Key determinants for monovalent cation specificity and activation must reside within the 186 and 220 loops of thrombin. Future mutagenesis studies will likely clarify the roles played by individual thrombin residues in controlling cation binding and in transmitting the allosteric effect up to the active site region. With this knowledge, the next generation of redesigned thrombin species can be prepared and tested.

Engineered versions of coagulation and anticoagulation enzymes have shown much promise for therapeutic purposes. Some mutants that have already been

designed to convert thrombin into an effective anticoagulant include E217K, W215A/E217A (WE), W215E, and Δ 146-149e (16–18). The most extensive in vivo testing has been done with the WE mutant. The collapse of the critical W215, E217 platform leads to a thrombin species that is a poor enzyme for procoagulant substrates. WE has been tested as an anticoagulant in nonhuman primates and demonstrated to have highly desirable properties associated with potency and efficacy (7, 19). Activated PC mutants are also being designed that exhibit greatly reduced anticoagulant and/or greatly reduced cytoprotective effects (20). Where the thrombin chimera reported by Rana et al. fits into this strategy is intriguing to consider. An analysis of the kinetic properties reveals that this

loop chimera has properties that map between those of the WE mutant and the Δ 146-149e mutant (missing the autolysis loop).

The highlighted PNAS article by Rana et al. reveals that a thrombin species has been created with the following characteristics: (i) tighter binding of K^+ over Na^+ , (ii) improved enzymatic activity in the presence of K^+ , (iii) enhanced reactivity toward PC in the presence of cations, and (iv) unexpected preference for the PAR1 substrate. The authors are well on their way to finding an alternative loop-cation pair that can fully support allosteric thrombin activation. This kind of valuable knowledge brings us one step closer to resolving the issue of how to systematically redesign allosteric activation of an enzyme.

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