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Transforming bivalent ligands into retractable enzyme inhibitors through polypeptide–protein interactions

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Abstract—The concept of bivalent polypeptides with controllable flexible linkers is demonstrated through the design of a new generation of 'antidote'-reversible inhibitors of thrombin. These molecules contain two binding moieties, each of which in isolation has only a moderate affinity of binding, which are linked together by a flexible peptide bridge. We show that activities of the potent bivalent inhibitors of thrombin can be reversed by the specific, but much weaker, binding of the linker moiety to protein 'antidotes'.

Multivalency is one extremely effective device nature has adopted to confer specific binding between biological molecules and molecular assemblies.[1] Multivalent molecular constructs can achieve high-affinity binding by linking together monovalent constituents that have only weak affinities when acting alone.[2,3] One other attractive feature of multivalent binding is that both the affinity and binding kinetics may be manipulated at will by the presence of constituent monovalent molecules.[2,4] As such, the multivalency concept has emerged as an increasingly successful strategy for the design of high-affinity ligands of enzymes.[5,6] and protein assemblies,[7–9] cell-surface receptors,[5,10–13] protein interaction surfaces,[3,9,14–17] and nano-patterned molecular arrays.[18,19] In most of the current applications, the linking bridges need to be optimized to achieve maximal binding affinity for the bivalent or polyvalent molecules.[7,10,14] We are interested in bivalent molecular designs that can couple binding affinity to an on/off-switch, in particular those that contain individual low-affinity and transient binding moieties. To achieve control of binding, a change (normally, decrease) in flexibility of the linker can be induced by an external trigger to disrupt the molecule's ability to bind in a bivalent high-affinity mode. Vice versa, removal of constraints imposed on the linker would restore the high-affinity binding of the freed bivalent ligand.

To demonstrate the feasibility of this approach, we have chosen human thrombin, an important target for pharmaceutical design, as a receptor for retractable bivalent inhibitors. The thrombin-inhibitory ligands were constructed using the same design scheme that led to bivalent antithrombotic polypeptides.[20–25] Moieties binding to the fibrinogen-binding exosite I (E) and the active site (A) of thrombin were formed by peptides $Hir^{55–65}$ and Bbs-Arg-(D-Pip), respectively,[20,25] where $Hir^{55–65}$ = Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln, Bbs =4-*tert*-butyl-benzenesulfonyl, and D-Pip = D-pipecolic acid (Fig. 1). The E- and A-monovalent 'heads' have compared affinities for thrombin, both in the vicinity of 0.5 μM[20,25] and therefore potentially produce bivalent inhibitors with an optimal difference between monovalent and bivalent modes of binding.

The binding heads were first connected by flexible polypeptide repeats[26] of varying lengths with a general formula of Gly-Ser-$(Gly-Ser)_n$-Gly (inhibitors **TI0**–**TI6**).[27] We found that the affinities[28] of the inhibitors **TI1**–**TI6** fall in the low- to sub-nanomolar range (Table 1), typical of clinically successful inhibitors from this family.[23,29–31] The poly Gly-Ser linkers were then replaced by two bioactive peptides, one of which binds specifically to an SH2 protein[32] and the second recognizes an antibody.[33] Linked molecules **TI7**–**TI9** still retained strong bivalent inhibitory activities toward thrombin

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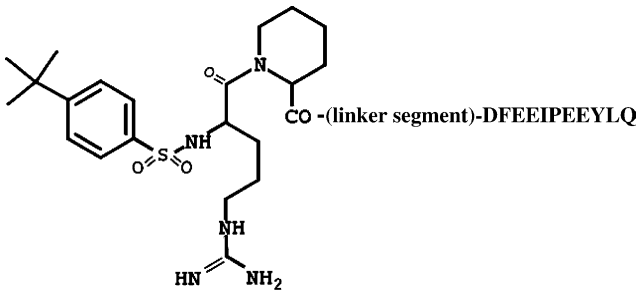

Figure 1. Chemical structure of a bivalent thrombin inhibitor[22,25] used to create retractable inhibitors of thrombin. The Bbs-Arg-(D-Pip) moiety resembles argatroban, a specific inhibitor of the thrombin active site in clinical uses as an anticoagulant.[29,30] The peptide moiety is derived from hirudin, a natural anticoagulant specific for binding to thrombin.[20,21]

Table 1. Inhibition constants K_i of thrombin inhibitors with a general formula of Bbs-Arg-(D-Pip)-Gly-(linker)-Gly-Hir$^{55–65}$ (Fig. 1)[a]

Linker	Name	K_i (nM)
S-$(GS)_1$	**TI0**	36 ± 6.5
S-$(GS)_3$	**TI1**	0.5 ± 0.2
S-$(GS)_5$	**TI2**	0.6 ± 0.1
S-$(GS)_7$	**TI3**	1.3 ± 0.3
S-$(GS)_9$	**TI4**	2.0 ± 0.3
S-$(GS)_{11}$	**TI5**	4.6 ± 0.8
S-$(GS)_{13}$	**TI6**	6.7 ± 1.9
SPHYEKVS	**TI7**	1.0 ± 0.2
SPH(pY)EKVS	**TI8**	1.5 ± 0.4
EQKLISEEDL	**TI9**	66 ± 13

[a] The table lists amino acid sequences in one letter code. Bbs, D-Pip, pY, and Hir$^{55–65}$ stand for 4-*tert*-butyl-benzenesulfonyl, D-pipecolic acid, O-phosphorylated tyrosine, and Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln, respectively.

(Table 1). We show that these potent thrombin inhibitors are responsive to and can be neutralized by the respective protein 'antidotes.' Specifically, we demonstrate that the binding properties of two thrombin inhibitors, **TI8** and **TI9**, can be controlled by the linkers binding to the SH2 protein domain ($K_d \sim 0.25$ μM) and the antibody ($K_d \sim 0.5$ μM), respectively.[32,33]

Table 1 lists the inhibition constants K_i for the bivalent inhibitors measured in an amidolytic assay with the chromogenic substrate S-2238.[28] The K_i of the inhibitors **TI1**–**TI6** steadily grows with the increase of the linker length, as expected for bivalent ligands with flexible and non-interacting linker segments.[10,12] The inhibitor **TI0** has an elevated K_i, presumably because the short linker can span the geometric spacing between the two binding sites only in an extended conformation. In addition, the fully extended conformation of the Gly-Ser-Gly-Ser-Gly moiety may create unfavorable interactions with the thrombin surface. The potency of the inhibitor **TI8** is comparable with that of the peptides including Gly-Ser repeats and that of **TI7**. Inhibitor **TI7** has the same amino acid sequence as **TI8**, except that the tyrosine residue in the linker of **TI7** is not O-phosphorylated. The presence of the phosphorylated tyrosine is needed for the ability of the **TI8** linker to bind the SH2 protein,[32] but the phosphorylation itself has little effect on the inhibitory potency (Table 1). In all, it appears that the nature of the longer linkers contributes little to the inhibitory potency of **TI1**–**TI8** against thrombin. The bivalent inhibitor **TI9** is somewhat less potent than others, possibly due to a high abundance of negatively charged amino acid residues (Glu and Asp) in the linker, leading to electrostatic repulsions.[26]

The 'retraction' of thrombin inhibitors by linker-specific protein antidotes is demonstrated (Fig. 2) in the presence and absence of inhibitors **TI7**–**TI9** and corresponding linker-binding proteins.[34] Micromolar concentrations (several K_d values) of antidotes markedly reduced the potency of the inhibitors. In a control experiment, delay of clotting caused by the non-phosphorylated **TI7** (for presentation purposes, it was used with the clotting assay at a lower concentration than **TI8**, see legend of Fig. 2) is not altered by the SH2 protein

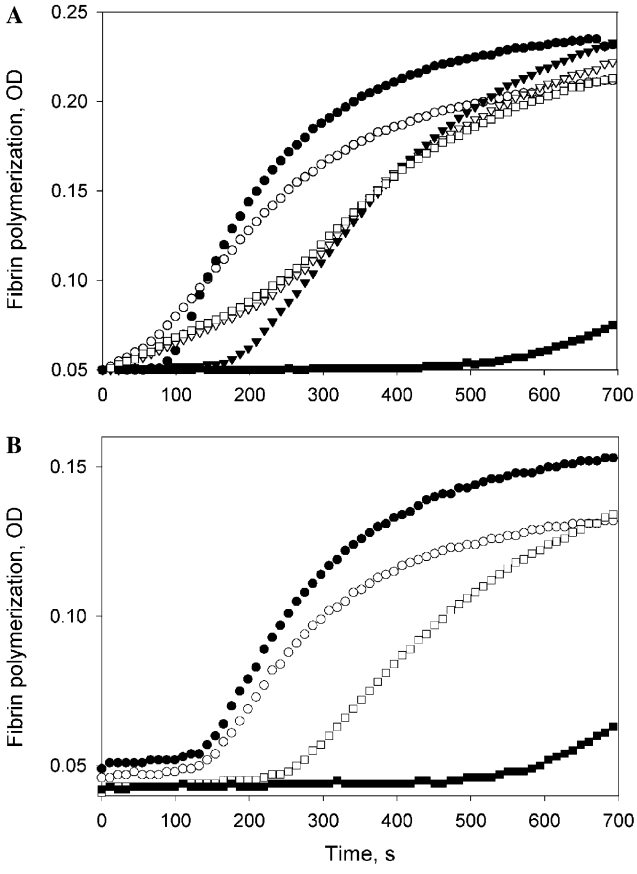

Figure 2. 'Retraction' of bivalent inhibitors by linker-specific protein antidotes in a fibrinogen clotting assay. Clotting curves were recorded (A) in the presence of 1 nM **TI7** (▽,▼), or 4 nM **TI8** (□,■), or absence of inhibitors (○,●). Addition of 3 μM SH2 protein domain had little effect on the clotting time in the absence of inhibitors (○) and the potency of **TI7** (▽), but significantly reduced the potency of **TI8** (□), (B) in the presence (□,■) or absence (○,●) of 150 nM **TI9**. Addition of ~1.2 μM anti-c-myc antibody 9E10 (Sigma) only slightly slowed clotting of free thrombin (○), but reversed the inhibitory effect of **TI9** (□).

domain, consistent with the loss of SH2 binding in the absence of phosphorylation.

The dose-dependency of the inhibitor 'retraction' by SH2 is in agreement with the expected affinity of SH2 to the linker moiety of **TI8** (Fig. 3). Various concentrations of the SH2 protein were added to the clotting assays while the concentration of **TI8** was maintained constant at 4 nM. Clotting times determined from the resulting family of clotting curves were fitted to a model of competitive binding of thrombin and SH2 with the **TI8** molecule.[34] Using $K_i = 1.5 \pm 0.4$ nM (Table 1), we obtained $K_d^{SH2} = 0.5 \pm 0.1\mu M$, which is reasonably close to the dissociation constant of 0.23 ± 0.05 μM determined previously for the binding of the isolated linker peptide with SH2.[32] As such, linker-mediated control of **TI8** can be represented by a simple thermodynamic cycle shown in Figure 4. In an ideal situation, SH2 binding may rigidify the linker moiety preventing the **TI8** molecule from having bivalent interactions with thrombin. Further details of the underlying molecular events will be resolved by future structural and binding studies.

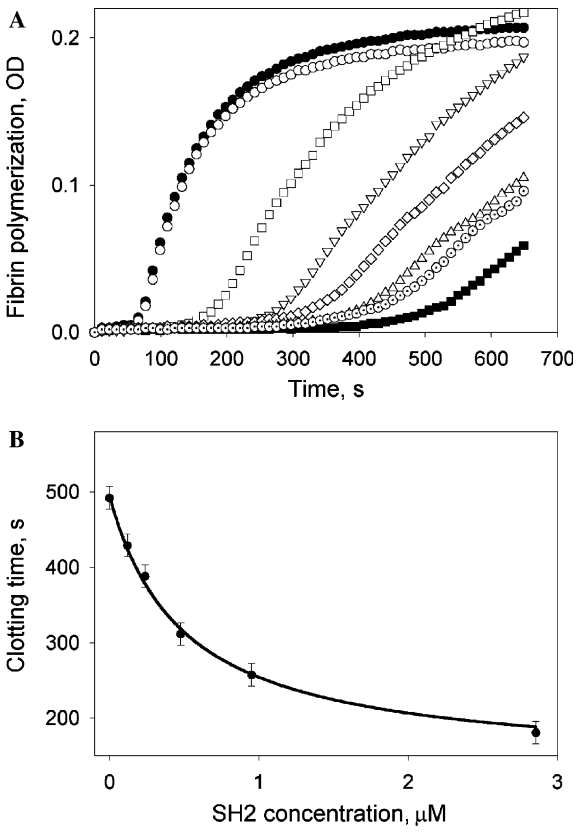

Figure 3. Dose-dependent SH2-induced activation of inhibited thrombin by the inhibitor **TI8**. (A) Clotting curves were recorded in the presence (■,⊙,△,♦,▽,□) or absence (○,●) of 4 nM **TI8**, and in the presence of various concentrations of SH2 protein domain [0 μM SH2 (■,●); 0.12 μM SH2 (⊙); 0.24 μM SH2 (△); 0.48 μM SH2 (♦); 0.95 μM SH2 (▽); and 2.86 μM SH2 (□,○)]; other conditions were as in Figure 2, (B) measured clotting times are plotted against the concentrations of added SH2; solid line represents the best fit of the model of competitive interaction of **TI8** with thrombin and with SH2 to the experimental points.

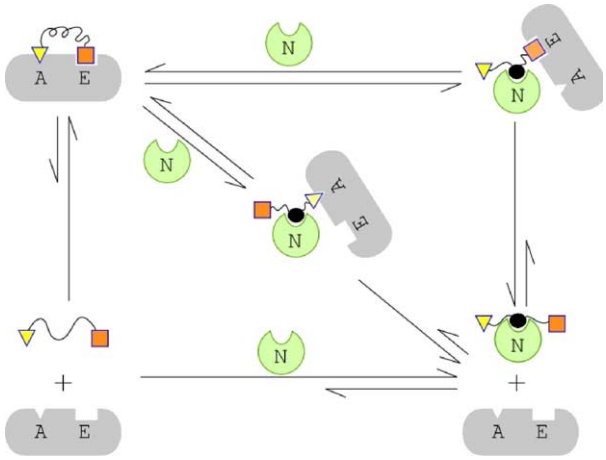

Figure 4. A proposed thermodynamic cycle for linker-mediated control of bivalent ligands. Upon binding of a linker-specific well-structured protein (labeled by 'N'), the flexible linker generally adopts a well-defined conformation required for the interaction, which prevents the ligand from acting in a bivalent fashion.

Retractable or tweezer-like artificial receptors have recently been designed in the area of host–guest chemistry.[35] To control specific complexation of some of these receptors, their shapes are changed by means of ion-binding induced rotation around a limited number of chemical bonds.[35c] In our design, control of binding is realized through interactions of a flexible polypeptide linker to a linker-specific well-structured protein. Since the structural specifics of the flexible linker appear less crucial for the bivalent and bridged mode of association, the linkers and the pairs of binding 'heads' are in principle interchangeable independently of one another, thus allowing for potentially numerous ways to control the inhibitory activities. In the current study, we translated SH2–peptide and antibody–peptide interactions into the activation of inhibited thrombin. As such, retractable inhibitors can be envisioned for other enzymes in biological pathways such as within the blood-clotting cascades and cell-signaling networks. The ability to control molecular binding and inhibitory action may have a number of applications in biotechnology and medicine. For example, retractable ligands can be developed into affinity purification systems complete with agents to release the targeted proteins or enzymes. The new generation of retractable thrombin inhibitors may also represent an approach to antidote-reversible anticoagulant therapy, complementary to arising nucleotide aptamer-based therapeutics.[36]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.08.085.

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28. Human α-thrombin was supplied by Haematologic Technologies Inc (VT, USA). The stock solution of human α-thrombin was 12.6 mg/ml in concentration with a specific activity of 3300 NIH units/mg. Amidolytic activities of thrombin were followed at 25 °C using eight inhibitor concentrations and three to five concentrations of the chromogenic substrate S-2238 (Chromogenix) as described in Refs. 20,23. Inhibition constants were extracted from the Lineweaver–Burk equation using weighted linear regression. Errors in K_i determination were estimated by use of Monte-Carlo sampling with 1–3% variance of the experimental points.
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$$T_M \sim 1/[E_{free}]; [E_{free}] = [E_0] \times K_i/(K_i + [I_{free}]);$$
$$[I_{free}] = [I_0] \times K_d^{SH2}/(K_d^{SH2} + [SH2]);$$
$$T_M \sim (K_d^{SH2} \times ([I_0] + K_i) + K_i \times [SH2])/([E_0] \times K_i \times (K_d^{SH2} + [SH2]));$$

where T_M is the measured clotting time, $[E_{free}]$ and $[I_{free}]$ are the concentrations of free thrombin and free **TI8** in solution, $[E_0]$, $[I_0]$, and [SH2] are the total concentrations of thrombin, **TI8**, and SH2 in solution, and K_i and K_d^{SH2} are bivalent inhibition constants of **TI8** and the dissociation constant of the SH2–**TI8** complex, respectively. This model neglects monovalent modes of **TI8** binding to thrombin, and the resulting equations are derived for the case of $[SH2] \gg [I_0] \gg [E_0]$, and $[S] \ll K_M$, where [S] is the total concentration of bovine fibrinogen and K_M is the Michaelis constant for thrombin and fibrinogen. These assumptions are fully justified by the binding properties and the experimental conditions used for the assays.
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