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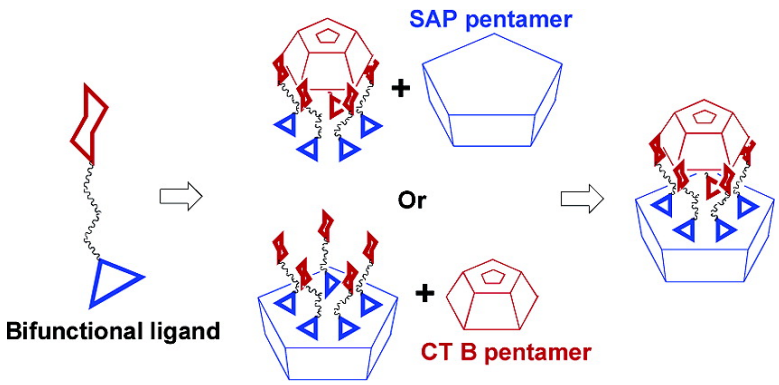

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Protein Heterodimerization through Ligand-Bridged Multivalent Pre-organization: Enhancing Ligand Binding toward Both Protein Targets

Jiyun Liu,[†] Zhongsheng Zhang, Xiaojian Tan, Wim G. J. Hol,[‡] Christophe L. M. J. Verlinde, and Erkang Fan*

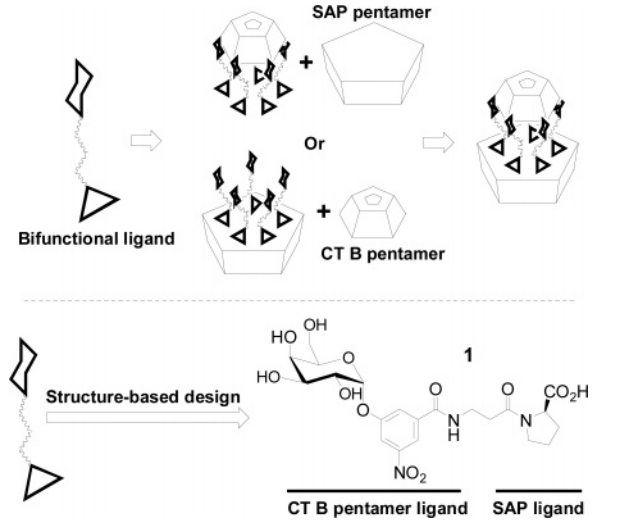
Biomolecular Structure Center, Department of Biochemistry, Department of Biological Structure, Department of Chemistry, and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195

Received October 11, 2004; E-mail: erkang@u.washington.edu

The design of high-affinity protein ligands is of fundamental interest in biomedical research. When a protein target possesses multiple identical or nonidentical binding sites, a multivalent approach may be taken to design multivalent ligands[1−7] that simultaneously occupy more than one site on the target protein. Often, one multivalent ligand and one multivalent protein are involved in such studies. Another area of ligand design concerns the so-called chemical inducer of dimerization, where one bifunctional ligand can bind two different proteins simultaneously and affect biological pathways.[8−16] In such cases, three entities are to be considered in the binding event: one bifunctional ligand and two separate and different target protein molecules. However, these interactions generally involve only a single binding site on each target protein. In this proof-of-principle report, we show that, using a structure-based approach, heterobifunctional ligands can be obtained to achieve dimerization of two different multivalent proteins. The ensuing pre-organized, multivalent binding event can enhance the binding of the bifunctional small molecule to both of its targets by several orders of magnitude.

We demonstrate this concept with two protein pentamers, the B pentamer of cholera toxin (CTB), a member of the AB_5 toxin family that is well-studied for multivalent ligand design,[5] and human serum amyloid P component (SAP).[17] We were initially inspired by a crystal structure of SAP, in which two pentamers of SAP are arranged in a "face-to-face" fashion, bridged by noncovalent $\pi-\pi$ stacking of five copies of SAP's monovalent ligand dAMP.[18] It appeared from the structure that a bivalent version of a SAP ligand should promote the formation of a pentavalent complex, with two copies of SAP and five copies of a symmetric bivalent ligand. We reasoned that if a heterobivalent ligand with proper design were made with one ligand for CTB and one for SAP then, in solution, binding of the heterobivalent ligand to either protein (CTB or SAP) will form a complex that is presenting a multivalent scaffold ready to bind the other target protein (Scheme 1). This noncovalent, multivalent pre-organization effect may significantly enhance the affinity of the heterobivalent ligand for both targets. Such an approach will allow the use of one ligand to target two multimeric proteins even if only monovalent ligands with modest affinities are available. Alternatively, when a high-affinity monovalent ligand is available for one multimeric protein, it can then be used as a template to target other multimeric proteins that only have low-affinity monovalent ligands. During our investigation, Pepys and co-workers recently reported a homobivalent ligand for the dimerization of two SAP pentamers and observed greatly enhanced inhibitory potency against SAP-related function.[19] This result was another impetus for our current study on heterobivalent ligands.

Scheme 1. Ligand-Induced Multivalent Dimerization of SAP:CTB



The design of bifunctional ligand **1** was guided by the crystal structures of CTB and SAP binding to their respective ligands: a CTB:MNPG derivative complex[20] (MNPG: *m*-nitrophenyl-α-D-galactopyranoside) and a SAP:D-proline derivative complex.[19] Because the coordinates of the SAP:D-Pro derivative complex were not deposited in the Protein Database, a model was generated with the FLO/QXP software[21] by docking *N*-acylated D-Pro to the SAP structure from the dAMP complex.[18] Then, the five-fold axes of the CTB and SAP pentamers were superposed, CTB was fixed and SAP translated along the five-fold axis and rotated around it such that the distance between the ligands was minimized while avoiding van der Waals clashes between the proteins. It soon became clear that an ethylene linker was sufficient to reach the simplified SAP ligand from the MNPG moiety of the CTB ligand.

After synthesis of **1** on solid support (see Supporting Information), we first used dynamic light scattering (DLS) to probe the solution binding behavior of **1** to CTB and SAP. The experiment was carried out at 2 μM protein concentration (as protein pentamer) to generate detectable signal from only solution species equal or larger than the SAP pentamer with our instrument. This reduced complications of using DLS to measure various components in solution, because the signal from CTB alone is not observable under these experimental conditions due to its smaller size. As shown in Table 1, the formation of a CTB:**1**:SAP ternary complex is clearly supported by the DLS data. Either SAP alone (at 1−4 μM) or the mixture of SAP + CTB (at 2 μM each per pentamer) produced a signal corresponding to the SAP pentamer. Adding **1** into the SAP + CTB solution produced a larger solution species that was consistent with a CTB:**1**:SAP ternary complex. No significant higher random aggregates were observed in those experiments.

[†] Department of Chemistry.
[‡] Howard Hughes Medical Institute.

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Table 1. DLS Evidence of CTB:**1**:SAP Ternary Complex[a]

condition	hydrodyn. radius (nm)	polydisp. (%)	MW (by DLS) (kD)	expctd. MW (kD)
(A): SAP (1−4 μM)	5.0 (0.2)	26	145	127
(B): SAP + CTB (2 μM each)	5.2 (0.1)	17	155	127
(C): condition B + **1** (200 μM)	5.7 (0.3)	23	201	185
(D): condition B + **1** (400 μM)	5.9 (0.2)	30	212	185

[a] The measurements were carried out in a buffer as reported[19] to ensure that SAP was stable in pentameric form in solution.[22] Reported values are averages of 4−6 independent trials (standard error in parentheses).

Table 2. IC_{50} for Inhibition of CTB Binding to Surface Receptor[a]

condition	IC_{50} (μM)	enhancements due to SAP
varying MNPG, No SAP	1050 (110)	na
varying MNPG, [SAP] = 0.1 μM	1150 (300)	none
varying **1**, No SAP	620 (130)	na
varying **1**, [SAP] = 0.1 nM	270 (60)	2-fold
varying **1**, [SAP] = 1.0 nM	49 (13)	13-fold
varying **1**, [SAP] = 10.0 nM	3.6 (1.0)	170-fold
varying **1**, [SAP] = 0.1 μM	1.4 (0.3)	440-fold
varying **1**, [SAP] = 0.4 μM	0.98 (0.18)	630-fold

[a] IC_{50} values are the average of at least three independent experiments, with standard error reported in parentheses.

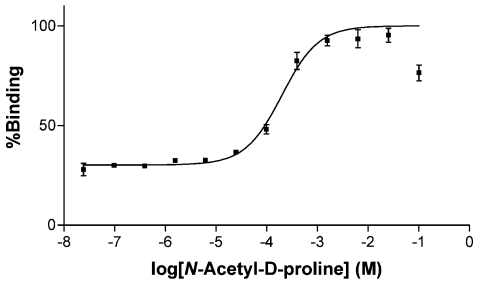

Figure 1. Enhanced inhibition due to the CTB:**1**:SAP ternary complex is diminished by a competitive SAP ligand, *N*-acetyl-D-proline.

Next, we investigated if ligand binding can be enhanced through the CTB:**1**:SAP ternary complex at a range of protein concentrations typically found under physiological conditions, using a well-established competitive inhibition assay for blocking surface receptor binding of CTB (see Supporting Information).[23]

Table 2 shows a summary of the IC_{50} measurements on inhibition of CTB surface receptor binding for the monovalent ligand MNPG and the heterobifunctional ligand **1**. The presence of SAP has little effect on the potency of MNPG. In contrast, almost 3 orders of magnitude of gain in IC_{50} of **1** can be achieved in the presence of SAP. It is also remarkable that, even at low to subnanomolar concentrations of SAP (CTB at 100 pM), the enhancement is still substantial. This is very significant for the future translation of results from this model study to other multivalent protein targets under physiological conditions.

Affinity enhancement of **1** toward SAP in the ternary complex can be estimated from the CTB inhibition assay by varying SAP, while fixing **1** at 10 μM. In this case, an apparent IC_{50} at 4.5 nM of SAP was obtained. Because the K_d of *N*-acetyl-D-proline (equivalent to the SAP-binding portion of **1**) to SAP is 15 μM,[19] this means that at 10 μM of **1** and 100 pM of CTB, the CTB:**1**:SAP ternary complex is still formed considerably at a SAP concentration ~3000-fold lower than the K_d of **1** toward SAP.

Figure 1 shows that the enhancing effect of SAP on the potency of ligand **1** against CTB can be modulated with a competitive monovalent ligand of SAP. The experiment was carried out at 10 μM of **1** and 10 nM of SAP. Under this condition, there is about 30% CTB binding to its surface receptor remaining. In the presence of increasing amounts of *N*-acetyl-D-proline, the degree of inhibition of CTB binding to surface receptors by **1** with SAP was decreased, and eventually CTB binding to surface receptors was restored completely because **1** by itself at 10 μM should not exhibit any significant inhibition. This result again indicates that enhancement of the potency of bifunctional ligand **1** against CTB is due to the formation of CTB:**1**:SAP ternary complex in solution.

In summary, we have shown that heterobifunctional ligands can be designed to promote heterodimerization of two protein targets in a multivalent fashion. Up to 3 orders of magnitude in affinity enhancement is observed in our current example. Significant affinity enhancement is achievable under typical physiological concentrations of proteins. The full scope of application of this bifunctional ligand-promoted multivalent protein dimerization is currently under investigation.

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Supporting Information Available: Synthesis and assay details. This material is available free of charge via the Internet at http://pubs.acs.org.

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