Mechanistic Studies of Affinity Modulation

Michael K. Rosen,‡ Christopher D. Amos,† and Thomas J. Wandless*,†

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305, and Cellular Biochemistry and Biophysics Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10021

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Abstract: A synthetic ligand for the protein FKBP12 was covalently linked to a peptide ligand (pYEEI) for the Fyn SH2 protein to create a bifunctional molecule called SLFpYEEI. This bifunctional molecule can simultaneously bind both proteins to form a trimeric complex. When SLFpYEEI is precomplexed with FKBP12, the peptide ligand binds 6-fold more weakly to the Fyn SH2 domain than SLFpYEEI alone. Isotope-edited NMR spectroscopy was used to investigate the molecular basis for the observed reduction in affinity. The results suggest that interactions between the pYEEI peptide and FKBP12 may play a significant role in diminishing the affinity of SLFpYEEI for the Fyn SH2 domain.

Introduction

Modulating the affinity of small molecule ligands for their protein targets is an important and widely practiced activity. Significant progress in rational ligand design has been made in recent years; however, most strategies rely on detailed structural knowledge of the protein−ligand complex, and this information is not always available.[1] Additionally, successful design features from one system are rarely generalizable to a different protein−ligand pair. Improving affinity has obvious utility for drug development. Diminishing affinity may also be useful for engineering selectivity between two similar proteins (e.g., protein kinases) when inhibition of only one target is desired.[2]

Disrupting an interaction is relatively easy by making structural modifications to the region of the ligand that directly contacts the target protein. However, in certain situations it would be useful to retain the target-binding properties of the ligand and modulate its affinity through a different molecular mechanism. The studies reported herein describe a conceptually new approach for controlling affinity that is dependent on the presence of a second protein. This strategy may prove to be of general utility for modulating protein−ligand interactions.

Two different protein ligands, SLF for FKBP and pYEEI for the Fyn SH2 domain, were covalently linked to generate a single bifunctional molecule capable of simultaneously binding two different proteins to form a trimeric complex.[3] The linker between the two ligands is sufficiently short to bring the two proteins into close proximity, and the net impact of protein−protein interactions on the stability of the trimeric complex will likely depend on the length and nature of the linker as well as the surfaces of the ligands and proteins. Pairwise protein−protein interactions may contribute either constructively or destructively to the overall thermodynamic stability of the trimeric complex.[4]

There are a variety of reasons to engineer diminished affinity without directly disrupting the protein−ligand interface. One might desire a bifunctional molecule that inhibits an essential enzyme, but is inert and nontoxic when bound to a presenter protein. Such a molecule would be expected to be selectively toxic to cells that lack the presenter protein while displaying no cytotoxic activity toward cells that possess the presenter protein. Molecules of this type would possess biological activity that is dependent on a particular cellular context, and this property may have general utility for treating diseases including bacterial infection and cancer.

For antibiotic activity, one would identify a protein that is widely expressed in human cells but lacking in microorganisms. The human presenter protein would be expected to detoxify a synthetic cytotoxic bifunctional molecule that otherwise displays antibiotic activity against bacteria that lack the protective presenter protein. In a similar way, differences in proteomic profiles of different human cell types might be leveraged to target one particular cell type for a desired pharmacological activity. Cancer cells that lack the p53 tumor suppressor protein might be targeted by cytotoxic bifunctional p53 ligands that would not display cytotoxic activity toward noncancerous cells that possess intact p53, which would be expected to detoxify the bifunctional molecules. The studies reported herein demonstrate that it is possible to design and synthesize molecules whose biological activity is dependent on a particular cellular context and that there are multiple molecular mechanisms by which this goal may be achieved.

Results and Discussion

SH2 domains are relatively small protein modules (∼100 residues) that bind to peptides containing phosphotyrosine residues.[5] These domains are found in a wide variety of proteins that are necessary for cellular signaling, and as a result, ligands directed against SH2 domains have been examined extensively

* To whom correspondence may be addressed. Phone: (650) 723−4005. E-mail: wandless@stanford.edu.
† Stanford University.
‡ Memorial Sloan Kettering Cancer Center.

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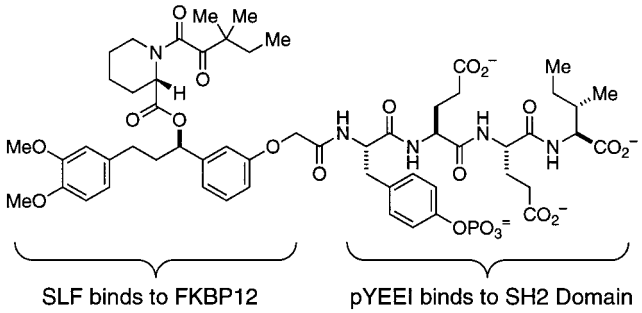

Figure 1. Structure of the bifunctional molecule, SLFpYEEI.

as potential therapeutic agents.[6] We chose the SH2 domain from a Src-family kinase, specifically the Fyn SH2 domain, as our representative target protein.[7]

The second protein, called the presenter protein, is FKBP12 (hereafter referred to as FKBP). Presenter proteins are ideally abundant cellular proteins that bind tightly to small organic ligands. Our strategy entails borrowing the presenter protein to modulate the affinity of a different ligand for its protein target.[3] Structural information is helpful when designing bifunctional molecules, and by all these criteria FKBP is well-suited to serve as a presenter protein. We chose a well-characterized ligand for FKBP known as SLF (K_d = 20 nM),[8] and we covalently linked SLF to the tetrapeptide SH2 ligand phosphotyrosyl-glutamyl-glutamyl-isoleucine (pYEEI) to create a bifunctional molecule called SLFpYEEI (Figure 1). In the absence of FKBP SLFpYEEI is designed to bind directly to the SH2 domain. However, when SLFpYEEI is complexed with FKBP the affinity of the pYEEI ligand for the SH2 domain is reduced.

The affinities between proteins and ligands have been measured using both isothermal titration calorimetry and a competition binding assay.[3] In the absence of FKBP SLFpYEEI binds to the Fyn SH2 domain with moderate affinity (K_d = 180 nM). When complexed with FKBP as a presenter protein, the bifunctional SLFpYEEI is a weaker ligand for the SH2 domain by a factor of nearly 6 (K_d = 1.0 μM). To gain a better understanding of the molecular basis for this decrease in affinity, we used NMR spectroscopy to directly examine the protein ligand complexes. Using ^{15}N-labeled FKBP, we monitored changes in the amide chemical shifts of FKBP upon addition of various ligands in ^{1}H/^{15}N HSQC spectra.[9] As chemical shift assignments and structures of FKBP (both free and bound to ligands) are known,[10,11] we could correlate spectral changes to perturbations of individual backbone amides upon formation of the different dimeric and trimeric complexes.

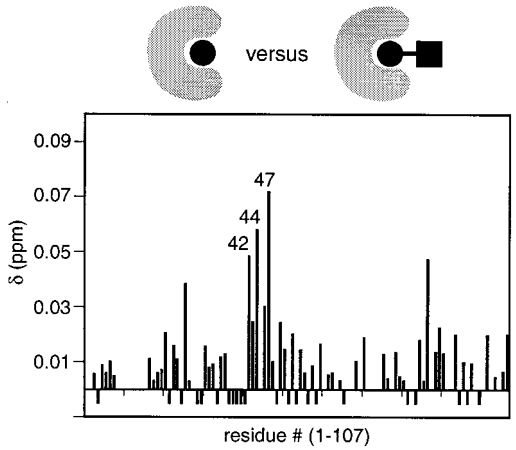

Figure 2. HSQC spectra of the FKBP−SLF complex and the FKBP−SLFpYEEI complex were compared. Chemical shift differences in backbone N−H signals ($\delta = [(\Delta^1H)^2 + (\Delta^{15}N/10)^2]^{1/2}$) are plotted on the *y*-axis versus residue number for FKBP. Arg 42, Lys 44, and Lys 47 are labeled, and unassigned residues are shown as negative values. FKBP is the gray crescent, SLF is the black circle, and pYEEI is the black square.

The majority of signals (77 of 99 backbone amides) in the ^{1}H/^{15}N HSQC spectrum of the FKBP−SLF complex were assigned through comparison with published assignments of the FKBP−FK520 complex.[11a] We took a conservative approach and assigned only resonances that were nonoverlapping and possessed similar chemical shifts in both the FKBP−FK520 and FKBP−SLF complexes. The 22 resonances that were not assigned were present and accounted for in all spectra, but were clustered together in small groups that made assignment uncertain without further spectroscopic characterization. None of the 22 unassigned resonances experienced ligand-induced chemical shift perturbations.

These assignments formed the basis for understanding shifts induced by covalent attachment of the pYEEI peptide as well as subsequent binding of the Fyn SH2 domain to form the trimeric complex. Vectoral chemical shift differences between HSQC spectra of FKBP in complex with SLF and SLFpYEEI are shown in Figure 2. The most significant chemical shift changes occurred for residues 42, 44, and 47, which are part of a loop that is directly adjacent to the ligand binding site of FKBP. These changes suggest an interaction, either direct or indirect, between the pYEEI peptide and the 40s loop of FKBP.

The Fyn SH2 domain was titrated into the FKBP−SLFpYEEI complex in five aliquots to give a total of 0.3, 0.6, 0.9, 1.2, 1.5, and 2.5 equiv of SH2 domain relative to FKBP. An HSQC spectrum was obtained for each sample and the spectra were compared. The complexes are in fast exchange on the NMR time scale, so we were able to monitor individual chemical shift changes throughout titration of the Fyn SH2 domain to form the trimeric complex. A subset of FKBP resonances were observed to shift through the first three additions of SH2 domain but not thereafter, indicating rapid binding on the NMR time scale and 1:1:1 stoichiometry of the trimeric complex. Significant broadening of all FKBP resonances was also observed due to the increase in rotational correlation time upon formation of the trimeric complex (13 kDa to 25 kDa).

The trimeric complex was then disrupted by adding a 12-fold excess of monomeric tetrapeptide ligand (AcpYEEI), which provided the two dimeric complexes, FKBP−SLFpYEEI and SH2−AcpYEEI. As the FKBP−SLFpYEEI complex is regenerated upon addition of the AcpYEEI peptide the line widths of its resonances were significantly decreased, returning to values

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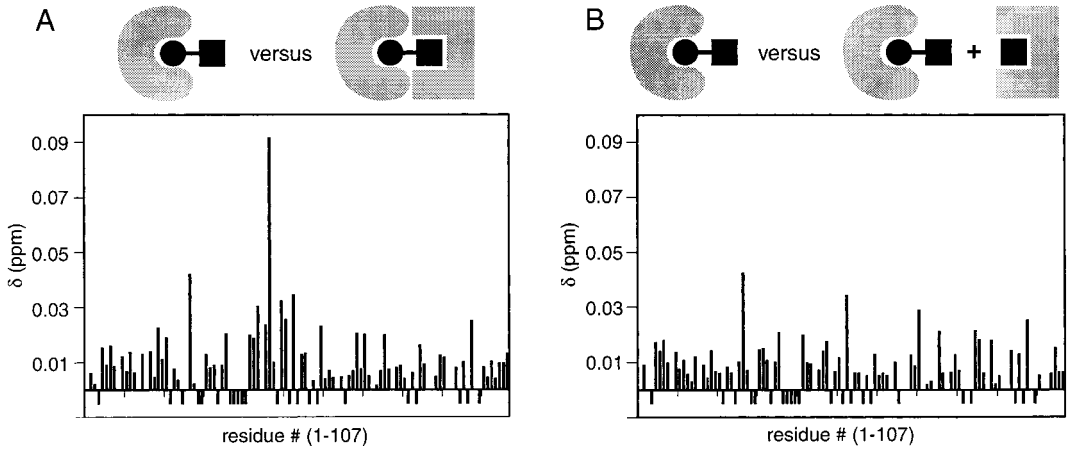

Figure 3. Chemical shift differences in backbone N−H signals (δ as in Figure 2) are plotted on the *y*-axis versus residue number for hFKBP12. (A) Changes observed by forming the trimeric complex upon addition of 2.5 equiv of the Fyn SH2 domain to the FKBP−SLFpYEEI complex. (B) Changes observed by comparing the original FKBP−SLFpYEEI complex with the same complex after formation and disruption of the trimeric complex. FKBP is the gray crescent, SLF is the black circle, pYEEI is the black square, and the Fyn SH2 domain is the gray rectangle.

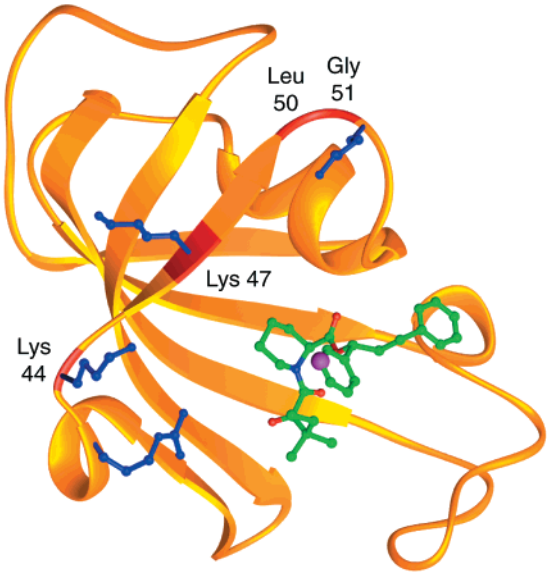

Figure 4. Ribbon drawing (ref 16) of FKBP bound to a close structural analogue of SLF (ref 8, PDB: 1FKG). The backbone is shown in gold, and the four residues that shift upon formation of the trimeric complex (Lys 44, Lys 47, Leu 50, and Gly 51) are shown in red. A purple sphere on SLF represents the point of attachment of the pYEEI peptide. The basic side chains (Arg 42, Lys 44, Lys 47, Lys 52) that are close to the pYEEI attachment site are shown in blue but are not labeled for clarity.

comparable to those observed in the original FKBP−SLFpYEEI dimeric complex. In addition, several of the resonances that shifted upon addition of the SH2 domain returned to their initial positions. Vectoral differences between the FKBP−SLFpYEEI and FKBP−SLFpYEEI−SH2 spectra as well as between the FKBP−SLFpYEEI and the FKBP−SLFpYEEI + SH2−AcpY-EEI spectra are plotted in Figure 3, spectra A and B, respectively. Resonances that show greater than 0.025 ppm differences in Figure 3A and less than 0.015 ppm differences in Figure 3B are indicative of changes brought about by formation of the trimeric complex which are subsequently reversed upon disruption of the trimeric complex. These four residues (Lys 44, Lys 47, Leu 50, and Gly 51) are highlighted in red on the FKBP structure shown in Figure 4.

The small number of significant chemical shift perturbations upon formation of the trimeric complex suggests that the two ligands comprising SLFpYEEI are linked by a tether that is sufficiently long for the two proteins to avoid directly interacting with each other in the trimeric complex. The three residues that are most affected by covalent attachment of pYEEI to the SLF ligand are Arg 42, Lys 44, and Lys 47, which possess positively charged side chains under these conditions (pH 6.5). The FKBP 40s loop is located adjacent to the site of attachment of pYEEI to SLF as shown in Figure 4. The observed chemical shift perturbations of backbone amide resonances suggest that the negatively charged pYEEI ligand interacts with the positively charged 40s loop of FKBP.[12]

An attractive interaction between pYEEI and the FKBP 40s loop, whether direct or indirect, may be responsible for the 6-fold reduction in affinity of FKBP−SLFpYEEI for the Fyn SH2 domain. Alternatively, the presence of FKBP may induce conformational changes in the pYEEI peptide that perturb the binding environment between the pYEEI ligand and the SH2 domain,[13] although such influences on the peptide ligand would not be observed by the NMR experiments described here. Further high-resolution studies of the trimeric complex will likely reveal the specific interactions that are responsible for affinity modulation and may facilitate the design of bifunctional molecules that possess greater selectivity.

Experimental Methods

Preparation of the Reagents. The SLFpYEEI bifunctional molecule was synthesized as described in ref 3. ^{15}N-labeled FKBP12 was prepared by growing *E. coli* harboring the FKBP12 expression plasmid in M9 minimal media using ^{15}N-ammonium chloride as the sole nitrogen source.[10a] The Fyn SH2 domain (residues 143−252, SIQA-GMPR) was subcloned into a pET22 expression vector, expressed in *E. coli* BL21-(DE3) cells, and purified as described.[7] All protein samples were buffer-exchanged and stored in NMR buffer, which consists of 50 mM phosphate, 100 mM NaCl at pH 6.5 with 10% D_2O. All spectra were acquired at 30 °C.

Titration To Form the FKBP12−SLFpYEEI−Fyn SH2 Trimeric Complex. A sample of ^{15}N-FKBP12 (500 μL, 700 μM) in NMR buffer was treated with 1.0 equiv of SLFpYEEI to form the FKBP12−SLFpYEEI dimeric complex. ^{1}H/^{15}N HSQC spectra of both free FKBP12 and the FKBP12−SLFpYEEI complex were acquired (see below). The Fyn SH2 domain (197 μL of a 570 μM stock, 0.3 equiv) was added to the FKBP12−SLFpYEEI sample, and an HSQC spectrum

(12) In an extended conformation of SLFpYEEI, the distance from the C-terminal carboxylate to the point of attachment to SLF is 17.0 Å. The side chain nitrogens of residues 42, 44, and 47 are located 8.1, 11.3, and 9.8 Å from the point of attachment to SLF, respectively.

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was acquired. A second aliquot of Fyn SH2 domain (197 μL of a 570 μM stock, 0.3 equiv) was added to generate a 900-μL sample containing 0.6 molar equiv of the Fyn SH2 domain relative to the FKBP12−SLFpYEEI complex. The sample was removed from the NMR tube and transferred to a Centri-Plus concentrator, and an additional aliquot of the SH2 domain (197 μL of a 570 μM stock, 0.3 equiv) was added to bring the total Fyn SH2 level to 0.9 equiv. The sample was concentrated to 500 μL and transferred back to the NMR tube, and an HSQC spectrum was acquired. This process was repeated as necessary to raise the concentration of the Fyn SH2 domain to 1.2, 1.5, then 2.5 molar equiv (relative to FKBP12), while maintaining the volume of the NMR sample below 1 mL. HSQC spectra were acquired and analyzed for every sample of the titration.

Addition of Ac-pYEEI To Disrupt the Trimeric Complex. The trimeric complex comprised of ^{15}N-FKBP12−SLFpYEEI−SH2 domain was prepared as described above with a stoichiometry of 1:1:2.5, respectively. The peptide Ac-pYEEI is a high-affinity ligand for the Fyn SH2 domain, and an aliquot of this peptide (55 μL of a 58 mg/mL stock solution in NMR buffer) was added to the 500-μL sample containing the trimeric complex. The amount of Ac-pYEEI was present in 5-fold excess relative to the SH2 domain. Care was taken to standardize the pH of all solutions that were used.

^{1}H/^{15}N-HSQC spectra. NMR spectra were recorded on a Varian Inova 600 MHz spectrometer equipped with a pulsed field gradient unit, using enhanced sensitivity pulsed field gradient pulse sequences.[14] Spectra were processed and analyzed with nmrPipe/nmrDraw and nmrview software packages, respectively.[15]

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