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CHEMICAL INDUCERS OF DIMERIZATION: THE ATOMIC STRUCTURE OF FKBP12-FK1012A-FKBP12

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Abstract: A ligand that simultaneously binds two proteins must have two high affinity protein binding domains joined in a fashion that facilitates, or at least does not prevent, protein–protein interaction. Designing such ligands is challenging, and a high resolution X-ray structure of FKBP12-FK1012A-FKBP12 illustrates the subtleties of one successful design.

Ligand-induced dimerization is the key signaling event for many extracellular receptors.[1] A particularly well studied case is human growth hormone (22 kDa), which uses two different faces to bring together two identical human growth hormone receptors and initiate a cytoplasmic signal transduction cascade.[2] Organic chemists, recognizing the opportunity to control biological processes via protein dimerization, have created small cell permeable dimerizers called CIDs (chemical inducers of dimerization) that can be used in conjunction with specially designed protein receptors to control cellular responses.[3]

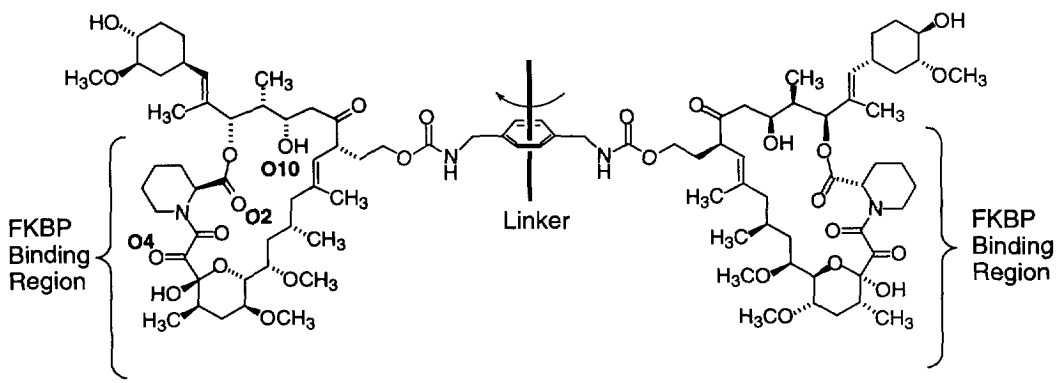

Figure 1. The structure of FK1012A.

The most widely used CID, FK1012A (Fig. 1), uses two binding regions of FK506 to link the FK506 binding protein (FKBP) domains of fusion proteins.[3] In the original demonstrations of FK1012A's applicability, an artificial intracellular receptor was formed from a myristolylated ζ domain from the T cell receptor followed by three FKBP12 domains.[3] FK1012A-induced dimerization of the fusion protein initiates a signaling cascade that results in the expression of a reporter gene.[3] More recently, a related system using FK1012A-induced dimerization of a fusion protein containing an intracellular fragment of the erythropoietin receptor and three FKBP12s allowed cells normally dependent on interleukin 3 to proliferate in its absence.[4] Further advances in

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applying CID technology to biological systems could benefit from a detailed structural understanding of FK1012A-induced dimerization, and the high resolution X-ray crystal structure[5] reported here provides some interesting insights.

Figure 2. The FK1012A-induced aggregate of FKBP12. The view on the left is down the molecular twofold axis, and the view on the right is perpendicular to the twofold.

The structure of the aggregate formed by crystallizing a 1:2 mixture of FK1012A and FKBP12—an equimolar ratio of binding regions and binding pockets—is shown in Figure 2. In addition to the anticipated FK1012A-induced FKBP12 dimer, two additional FKBP12s with unliganded binding pockets wrap tightly around the linker region of FK1012A. These unliganded FKBP12s resemble a pair of hands grasping a barbell formed by an FK1012A bar and FKBP12 weights. The overall complex has a twofold axis of symmetry running perpendicular to the aromatic ring of the linker and relating the two liganded (L-FKBP12) and unliganded (U-FKBP12) proteins. The length of the extended linker separating the L-FKBP12s is 18 Å, and the linker runs through a tunnel formed by the U-FKBP12s. The two U-FKBP12s have their hydrophobic binding pockets against hydrophobic regions of L-FKBP12s and the FK1012A linker. The two U-FKBP12s also interact strongly with each other forming nine hydrogen bonds between symmetry-related U-FKBP12s and only two hydrogen bonds to L-FKBP12. The overall topology of the FKBP12–FK1012A complex is virtually identical to that originally reported FKBP12–FK506 complex.[6] FKBP12 has a five stranded β-sheet wrapping around a short α helix with a hydrophobic binding pocket between the sheet and helix. The rms deviation between FKBP12–FK1012A and FKBP12–FK506 is only 0.89 Å for all atoms. The only major structural differences are in the binding pocket, the 50s loop, and the linker region.

FK1012A binds to L-FKBP12 in the same orientation and conformation as FK506 binds to FKBP12; the rms deviation between ligand atoms in the binding pocket is only 0.36 Å. Trp59 forms the bottom of the hydrophobic binding pocket, and in all previously published structures of FKBP12 complexes the indole NH points directly at the center of the aromatic ring of Phe99 (Fig. 3). In L-FKBP12, the side chain of Trp59 flips, and while it generally occupies the same region of space, its NH points at the center of the Phe48 aromatic ring

(Fig. 3). Trp59, Phe99, and Phe48 are all conserved residues in FKBP12's binding pocket. The 50s loop, so-called because most of the residues have sequence numbers in the 50s, has played a prominent role in previous discussions of the interaction of FKBP12 with FK506 and putative peptide ligands.[6,7] In the FKBP12–FK506 complex it forms a water mediated type II reverse turn from Met49 O (i) to Lys42 NH (i+3) (Fig. 3). This water molecule is tetrahedrally coordinated to the main chain O of Glu54 and the side chain carboxyl of Glu60. Since Glu60 also hydrogen bonds to Gly51 N, it may play a crucial role in defining the loop conformation. In the L-FKBP12–FK1012A structure, the conformation of the 50s loop is radically changed, but despite these changes, it is still preserves the β strand interaction with the ligand. Met49 through Gln53 form an α helical turn with Met49 O (i) hydrogen bonded directly to Gln53 NH (i+3) (Fig. 3). The bridging water slides over to make only three contacts. The most dramatic change between the two structures in the 50s loop is Gln53 and Gln54, which switch positions with movements of up to 10 Å. The side chain of Glu54 now projects into solvent while Gln53 projects back towards the protein and forms a hydrogen bond to ligand O10 and Met49 NH. Even with this dramatic motion of the side chains, Glu54 O and Ile56 N maintain their β strand interactions with O10 and O2 of ligand.

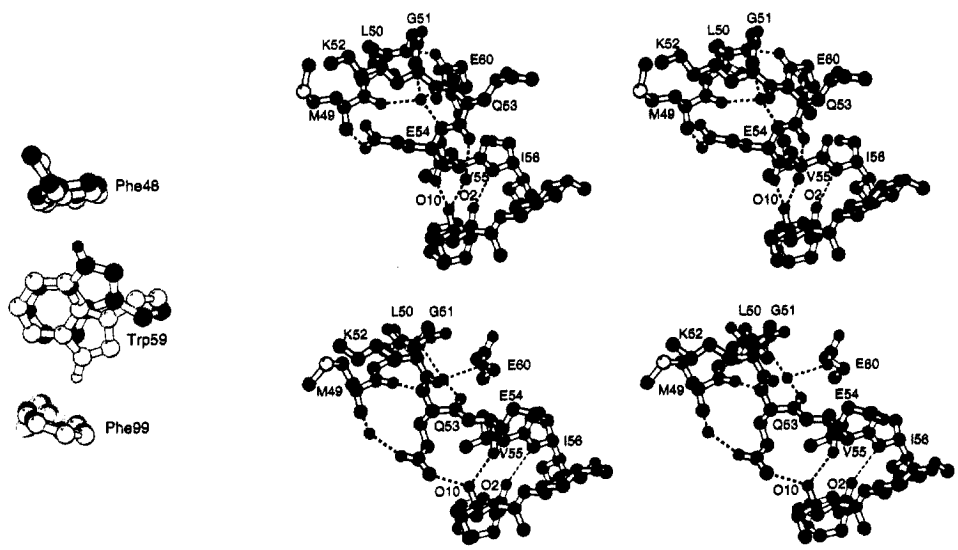

Figure 3. On the left is the Trp59 region in the FKBP12–FK506 complex (yellow) and the FKBP12–FK1012A complex (blue). On the right are stereo views of the 50s loop in FKBP12–FK506 (top) and FKBP12–FK1012A (bottom).

The linker of FK1012A interacts with the U-FKBP12s (Figs. 1 and 2). The U-FKBP12s form a hydrophobic 20 × 13 × 8 Å tunnel, and the linker runs through the tunnel. The tunnel is larger than the linker, and in spite of the hydrophobic environment, the two sides separated by 13 Å are coated with ordered water molecules. One of the water coated sides is made up of the polymethylene chain of Arg57, which forms a

hydrogen bond with Val 55 on the symmetry related Val55. The two 'dry' sides of the tunnel are composed of hydrophobic atoms in the side chains of Tyr82 and Glu79, which has its carboxyl turned away. There are no ligand-protein hydrogen bonds in the linker region, and the high thermal parameters of the linker atoms suggests few stabilizing interactions.

The U-FKBP12 molecule provides an unusual opportunity to examine an unliganded FKBP12 binding pocket via X-ray diffraction. FKBP12 seems to undergo only minor structural changes upon binding, and in agreement with that, the rms deviation of FKBP12–FK506 and U-FKBP12 is only 0.55 Å. The main changes occur in the surface loops connecting secondary structural elements and, not surprisingly, in the binding pocket. The 40s loop, Ser39-Pro45, is a break in β-5. This loop has the same conformation first noted in the FKBP12–FK506 complex with Arg42 reaching into the binding pocket to form a hydrogen bonded triad with Tyr26 and Asp37.[6] Since this orientation has also been noted for Arg42 in the FKBP12–FK506-calcineurin complex,[8] it appears that ligand binding is not necessary for its correct orientation. Asp41 and Gln43 are less well defined in the unliganded structure and point away from the protein into solvent. The 80s loop, Ser77-Thr96, connects β-2 to β-3 and serves as a flap to partially cover the binding pocket. NMR studies suggest that this loop in the absence of bound ligand is highly mobile in solution.[6] In U-FKBP12 the 80s loop is not constrained by any crystal contacts. In comparison with the FKBP12–FK506 structure, the main chain shifts by a little over 1 Å at Pro88 due to a change from one envelope conformation to another, and the His87 side chain's imidazole is rotated by 130°. His87 and Ile90 are in van der Waals contact with FK506 in the FKBP12–FK506 structure and represent favorable hydrophobic interactions. In addition, Gly89 and Ile90 are a crucial part of the calcineurin binding surface in the FKBP12–FK506-calcineurin complex.[8] In U-FKBP12, these residues retain a conformation similar to the FKBP12–FK506 complex.[6] Thus the composite surface presented to calcineurin has important contributions from protein elements that can occupy their binding conformations in the absence of a bound ligand.

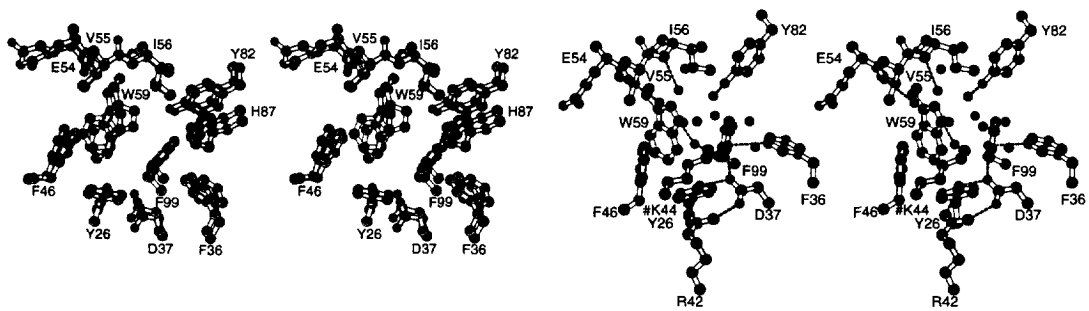

Figure 4. The binding pocket of U-FKBP12. The left hand stereo pair shows the changes in active site residues between FKBP12–FK506 (black atoms) and U-FKBP12 (green atoms). The right hand stereo pair shows the twelve well-ordered water molecules in the binding pocket.

The most interesting comparison between L- and U-FKBP12 is the binding pocket itself, which changes in a subtle but interesting fashion. The conserved aromatic residues—Tyr26, Phe36, Phe46, Trp59, Tyr82 and Phe99—shift slightly, less than 1 Å, from positions in the FKBP12–FK506 structure (Fig. 4). The indole ring of Trp59 is rotated by 70° to point the indole NH into the middle of the binding pocket. This simple rotation helps fill in the binding pocket and reduce its hydrophobicity. Overall, the amount of accessible hydrophobic surface area decreases by almost 400 Å^2. The binding pocket is filled by twelve well-ordered water molecules and a lysine side chain from a symmetry related protein (Fig. 4). Generally water molecules in hydrophobic areas of proteins are difficult to detect with X-ray diffraction because they are disordered and contribute little to the diffraction signal.[9] Neutron diffraction studies of trypsin have also revealed water molecules in a small hydrophobic pocket.[9] The residues that form hydrogen bonds to FK506—Asp37, Glu54, Ile56 and Tyr82—all make hydrogen bonds to five of the waters. One water forms a hydrogen bond bridge from lysine and the Trp59 side chain (Fig. 4). All of the other water molecules, except one, either participate in a hydrogen bonding network with each other or interact with the lysine residue. This exceptional water is tetrahedrally coordinated by forming hydrogen bonds with the carboxyl group of Asp37, another water, and by making C-H···O interactions with the ε-hydrogens of Phe36 and Phe99. This water occupies the same position, within 0.5 Å, as the C9 carbonyl oxygen (O10) of FK506 bound to FKBP12. The tightly bound waters in the U-FKBP12 binding pocket organize it in essentially the conformation needed to bind FK506 or rapamycin.

A closing observation concerns the relevance of the crystalline tetrameric aggregate to FK1012A's activity as a CID, and there is evidence that the tetrameric structure is not merely an artifact of crystallization. First, FK1012B, where the *para*-xylene of the linker has been replaced by a hexamethylene chain, forms an isomorphic structure even though the linkers are of different length. This isomorphism suggests that interactions between the proteins, not interactions with the linker, determine the composition of the aggregate. Also, the crystallizing solutions has 1:2 FK1012A:FKBP12 stoichiometry (i.e., there is one binding domain (Kd roughly 0.2 nM) for every FKBP12 binding pocket) and the aggregate forms in preference to the dimer. A model now exists where FK1012A-induced dimerization involves dual roles for FKBP12 domains—a primary one involving binding the L-FKBP12 and a secondary one involving the binding of U-FKBP12 to the linker.

This study, which revealed the conformational mobility of Trp59, the well ordered waters in an 'empty' hydrophobic binding pocket, and the aggregation of FKBP12, illustrated the challenges of designing CIDs. Both the supposedly familiar elements of structure-based drug design, such as filling a binding pocket, as well as the unfamiliar elements involving bringing macromolecular surfaces together in a defined fashion[10] gave unexpected results. Designing more effective CIDs will be a challenging and rewarding task.

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5. A 10 mg/mL solution of FK1012A in MeOH was added in a 1:2 molar ratio to a 10 mg/mL solution of FKBP12 in 10 mM Tris pH 8.2. The sample was gently mixed and allowed to incubate overnight to ensure complete binding. Crystals were grown using the hanging drop method with 0.5 mL reservoirs consisting of 5.1 M sodium formate and 0.1 M sodium acetate pH 4.6. The drops consisted of 3 μL of protein and 3 μL of reservoir solution. Crystals of 0.5 mm^3 grew within a week and belong to space group $C222_1$ with a = 133.59(2), b = 40.51(2), c = 93.00(2) Å. The asymmetric unit consists of one half of an FKBP12–FK1012A–FKBP12 dimer and one unliganded FKBP12. Intensity data were collected on a SDMS Mark II area and a rotating anode copper source and gave 15896 unique reflections (91% completeness, Rsym 4.6%). The structure was solved by molecular replacement techniques. Rigid body refinement (X-PLOR, 10–3.5 Å data with $|Fo| > 2\sigma$) produced an initial R of 38.6%. Simulated annealing with slow cooling and conjugate gradient positional refinement to 3.0 Å resolution reduced the R to 21.9%. Maps ($2|Fo|-|Fc|$ and $|Fo|-Fc|$) were used to manually adjust the model. After further postional refinement and adjustment to 2.5 Å resolution, the ligand was fit unambiguously into 3σ density in the $|Fo|-|Fc|$ map. The final model contains half an FKBP12–FK1012A–FKBP12 complex, an unliganded FKBP12, and 176 water molecules and has R of 17.3% for all $|Fo| > 2\sigma$ data in the resolution range 8–2.0 Å. The rms deviations from ideality are 0.01 Å and 2.8° for bond lengths and bond angles, respectively. Data have been deposited with the Protein Data Bank.
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