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CONFORMATION OF FK506 IN X-RAY STRUCTURES OF ITS COMPLEXES WITH HUMAN RECOMBINANT FKBP12 MUTANTS

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Abstract: In the X-ray structure of the FK506 complex with an FKBP12 double-mutant (R42K+H87V), the ligand is seen to adopt a conformation in its effector domain region that is distinctly altered compared to that found in the complex structure with native FKBP12. Nonetheless, molecular dynamics simulations indicate that the FK506 conformations seen in the native and mutant complex structures are energetically equivalent. Our observations suggest caution in the application of drug design strategies for calcineurin-mediated immunosuppressants that are based on mimicry of the FK506 conformation seen in the structure of the ligand complex with native FKBP12.

FK506, in complex with its 12kD M_r binding protein FKBP12, promotes immunosuppression through the inhibition of an intracellular calcium-dependent phosphatase, calcineurin (CN); CN inhibition subsequently leads to a down-regulation of transcription of IL-2 and other T-cell activation genes.[1] Homologous FKBP12-binding ligands such as rapamycin (which is itself an immunosuppressant by a different mechanism) can antagonize the CN inhibitory activity of FK506, however. These differences in activity have been attributed to differences in the chemical structures of FK506 and rapamycin, features that (in FK506) are thought to function as "effector domains"[1a] (Figure 1a) for CN-inhibition. Crystallographic studies of the respective FKBP12-ligand complexes have provided support for this model, showing that effector domains protrude from the surface of FKBP12 with distinct conformations that might well promote CN inhibition for FK506 while rejecting it for rapamycin.[2]

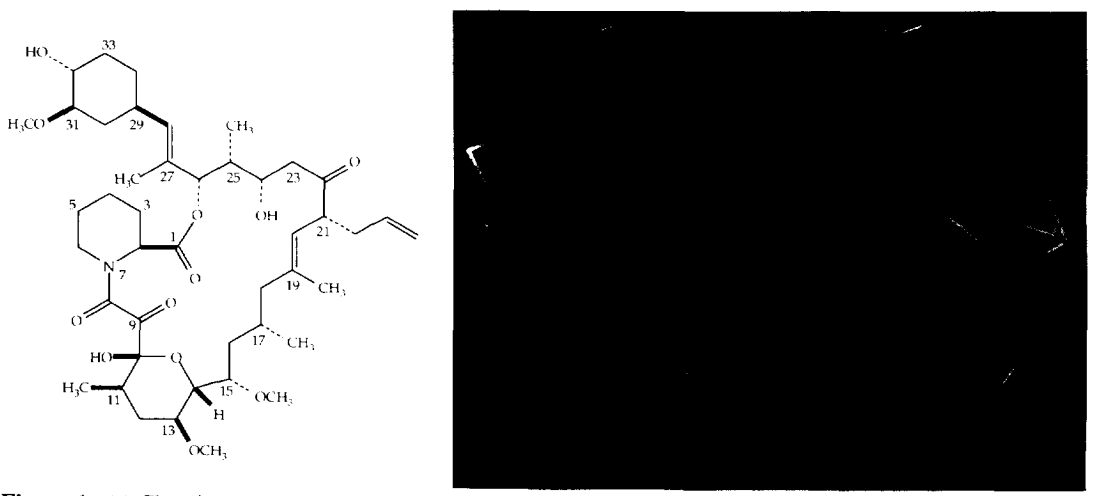

Figure 1. (a) Chemical structure of FK506. The effector domain includes the backbone atoms and substituents at C18 to C23 in the macrocycle, and C26 to C34 in the cyclohexyl ring. (b) Backbone α-carbon coordinates of native FKBP12 (in red) are taken from the structure of its FK506 complex. Superimposed are the corresponding coordinates from the structures of the R42K (light green), H87V (blue) and R42K+H87V (yellow) mutant FKBP12 complexes with FK506. The conformation of FKBP12 is not significantly perturbed in these complexes.

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In its original form,[1a] the effector domain model focused attention on the bound conformation of FK506 as the principal mediator of FKBP12-dependent CN inhibition, minimizing the role of FKBP12 to that of a "presenter" of ligand functionality to calcineurin. This early model has retained its appeal as a workable approximation for the design of immunosuppressants based on CN inhibition,[3] because it is consistent with the reciprocal antagonism observed between FK506 and rapamycin, and because it can rationalize the observed loss of CN-inhibitory activity associated with minimal changes in the chemical structure of FK506.[3a] In addition, the model has demonstrated predictive value in ligand design, though only for antagonists of CN-inhibition.[4] Subsequent site-directed mutagenesis experiments have led to a broadened model[5] in which CN inhibition is mediated by an "effector surface" of protein residues and ligand features;[5c] proper reconstitution of this composite surface necessarily complicates the process of inhibitor design.[1f,2f,3d]

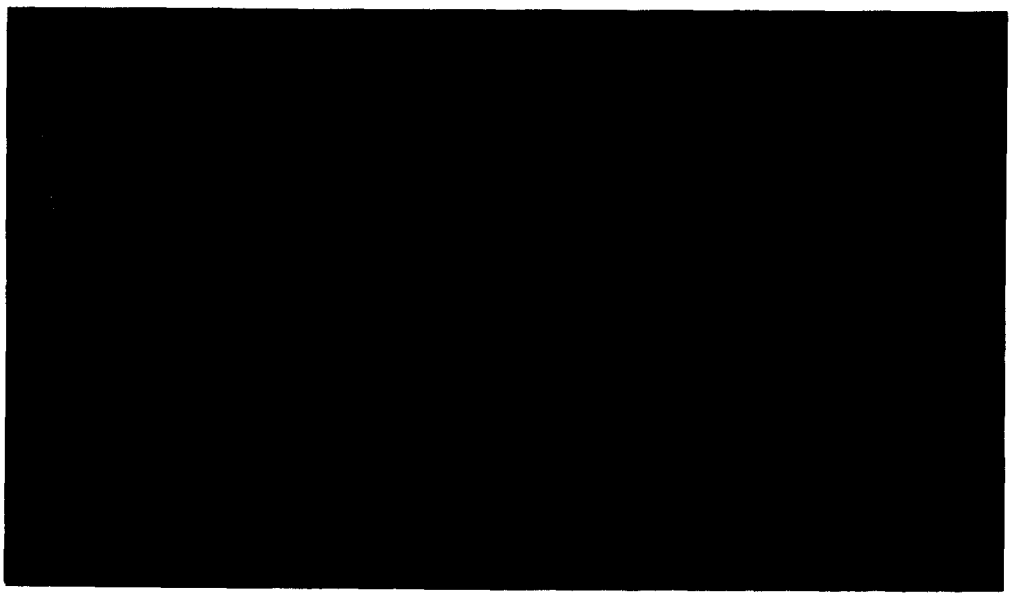

Figure 2. Overlap of native and R42K+H87V mutant FKBP12 complexes with FK506 (in red and yellow respectively). Coordinate rms deviations are given in Table 2. Superimposed is the electron density map of the double mutant complex (in blue), calculated with 2|Fo|-|Fc| coefficients. The R42K and H87V substitutions, other FKBP12 residues, and the FK506 ligand are identified in the figure. Apart from the altered conformation of FK506 (Figure 3; Table 3) and local perturbations around the mutated residues on the effector surface, the overall structure of the complexes is conserved.

In this paper, we examine the structural consequences of site-directed mutagenesis on the conformation of the FKBP12 protein, and in particular, on the conformation of FK506 itself. Crystallization, data collection and refinement statistics for the native and mutant complex structures under study are given in Table 1, along with rms differences *vs.* the native complex structure. All the complex structures were specifically solved[6] in the native complex crystal form in order to better rationalize the observed losses in CN inhibition in molecular terms. Ligand coordinate rms differences, average temperature factors, and biochemical data for the native and mutant FK506 complexes are given in Table 2.

The H87V and R42K single-mutant complexes, and the R42K+H87V double-mutant complex exhibit a 4-fold, 180-fold and 680-fold reduction in CN inhibitory activity respectively. Nonetheless, full peptidyl prolyl

	Native	H87V	R42K	R42K+H87V
$P4_22_12$ unit cell; a, c (Å)[a]	58.39, 55.76	58.45, 55.91	58.31, 55.93	58.36, 56.04
resolution (Å)	6.0 - 1.5	6.0 - 1.6	6.0 - 1.5	6.0 - 1.6
# of observations	76344	43114	43803	66733
% of reflections with $I > 2\sigma(I)$	81.4	84.8	88.1	88.5
R-factor (%)	16.6	19.2	18.7	18.7
# of water molecules observed	85	74	83	65
rms bond length error (Å)[b]	0.016	0.018	0.016	0.020
rms bond angle error (deg)[b]	2.74	3.16	2.85	3.35

Table 1. Summary of crystallographic data for native and mutant FKBP12-FK506 complex structures. [a] in this space group, the a and b unit cell dimensions are equal. [b] root mean square (rms) error *vs.* idealized geometry.

isomerase (PPIase) activity and FK506-mediated PPIase inhibition are retained (Table 2), as might be expected, given the conserved structure of FKBP12[2] seen in Figure 1. What was surprising in the structure of the double-mutant complex, however, was our observation of an alternate conformation for the bound FK506 (Figure 3), in which the macrocyclic backbone of the ligand is shifted by 3.5Å at position C17 in the effector domain of the ligand (Figure 1), and as much as 5.5Å for the C17-methyl substituent (Table 3). Such a large conformational change was not foreshadowed in the ligand conformations seen in the individual R42K and H87V mutant complexes. In those structures, the conformation of FK506 closely resembles that seen in the native complex structure (Table 2), though differences in the ligand temperature factors are evident (Figure 4).

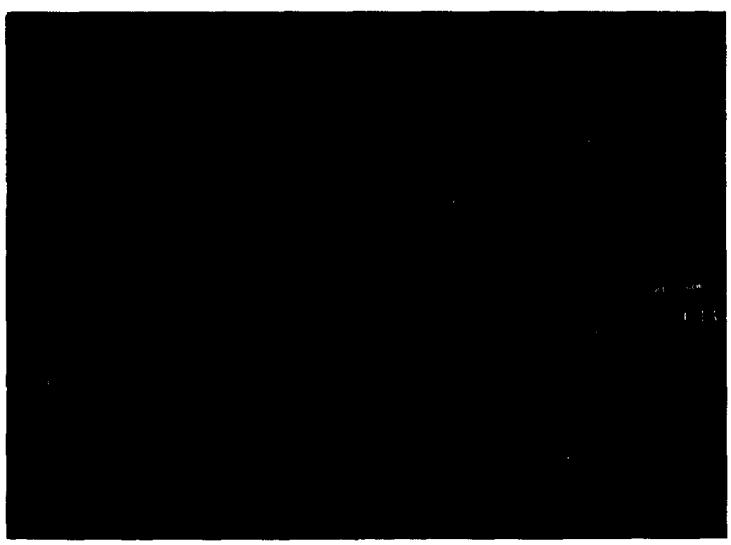

Figure 3. Comparison of the FK506 conformations observed in the native (red) and R42K+H87V mutant (yellow) FKBP12 complexes; mutant complex electron density is in blue as in Figure 2. The macrolide backbone of FK506 in the effector domain region is significantly distorted with respect to its conformation in the native FKBP12 complex, as detailed in Table 3 below. Key elements, corresponding to the chemical structure of FK506 in Figure 1 include **C9**, **C13**, **C21**, **C24**, the pipecolinyl ring (**Pip**; C3-N7) and the cyclohexyl ring at the so-called "northwest" region of the molecule (**NW**; C29-C34).

The mutant and native complex structures reported here share the same crystal packing arrangement.[2] Within those crystals, the ligand can participate in intermolecular contacts that might well limit the number of allowed conformations accessible to FK506. In the double-mutant complex, however, substitutions at R42K and H87V may sufficiently relax those contacts to allow for the observed alternate conformation of FK506 (Figure 3).

To further characterize the two ligand conformations, molecular dynamics simulations were carried out on the native and double-mutant complex structures. For the double-mutant complex, the local minimum corresponding to the altered conformation was found to have energies comparable to those obtained for the ligand conformation in the native complex.[7] In all cases, the final simulation-averaged ligand structure for the native and mutant FKBP12 complexes resembled the conformation input at the start of the simulation, with averaged potential energies approximately 3 kcal/mole in favor of the native FK506 conformation. Given that the simulations were run *in vacuo*, and given inaccuracies in the force field, one can conclude that both conformations are of reasonable energy. Further confirmation of the observed alternate conformation of FK506 was provided by an NMR study in solution of the ligand[8] structure in the double-mutant complex; interestingly, the native conformer was not populated in these studies.

	Native	**H87V**	**R42K**	**R42K+H87V**
FKBP12 rms diff *vs.* native (Å)[a]	---	0.277	0.146	0.351
FK506 rms diff *vs.* native (Å)[b]	---	0.593	0.139	1.275
Avg. FK506 temperature factors[c] ($Å^2$)	12.3	19.6	15.3	20.6
Calcineurin Ki (nM)[d]	5.5 (1.8)	23 (6)	590 (200)	3700 (1200)
FK506 Ki (nM)[d]	0.6 (0.2)	0.8 (0.1)	0.6 (0.2)	2.1 (0.5)
PPIase specific activity ($sec^{-1}\ \mu M^{-1}$)[d]	4.3 (0.4)	4.1 (0.5)	3.8 (0.3)	4.4 (0.4)

Table 2. Structural and biochemical[5a] characteristics of the native and mutant FKBP12-FK506 complexes. [a] coordinate root mean square (rms) differences *vs.* the corresponding native FKBP12 α-carbon atoms; to put these values in perspective, Becker *et al.*[2d] report a corresponding rms difference of 0.012Å between the α-carbon coordinates of their structure and that reported by van Duyne *et al.*[2a] [b] rms differences between corresponding FK506 macrolide backbone atoms in the two complexes. [c] temperature factors are a measure of structural disorder and/or mobility. [d] estimated error for these measurements[5a] are given in parentheses.

It is difficult to attribute with any confidence the relative contribution of ligand *vs.* protein conformational differences, to the 680-fold reduction in CN-inhibitory activity seen with the FKBP12 double-mutant complex (Table 2). On the one hand, even minor changes in the chemical structure of FK506,[3a] are known to dramatically affect CN-inhibition and immunosuppression. The X-ray structure of native FKBP12 in complex with L-685,818 (an 18-hydroxy, 21-ethyl analog of FK506)[2d] has shown that a loss of CN-inhibitory activity can be brought about by structural changes that are quite subtle.[9] On the other hand, a plausible case can also be made for the assignment of those losses to changes in the protein structure.[10] Support for this latter view is provided by the structure[11] of a triple-mutant FKBP13 complex with FK506 that is equipotent with the native FKBP12 complex in inhibiting CN.[5c] In that structure, the effector domain was seen to be disordered in the same region of the ligand (Figure 3) that adopts an altered conformation in the FKBP12 double-mutant complex. In contrast, the conformation of FK506 in the native FKBP13 complex structure[12] overlaps that of the native FKBP12 complex, even though the former is inactive as a CN inhibitor at >5μM concentration.

FK506 Atom ID	C14	C15	C15-O	C15-Me	C16	C17	C17-Me	C18	C19	C19-Me	C20	C21
Distance (Å)	0.31	0.77	2.57	3.80	2.32	3.51	5.46	2.74	1.58	2.03	0.87	0.35

Table 3. Coordinate differences between equivalent atoms in the conformations of FK506 (Figure 3) observed in the native and mutant R42K+H87V mutant FKBP12 complex structures.

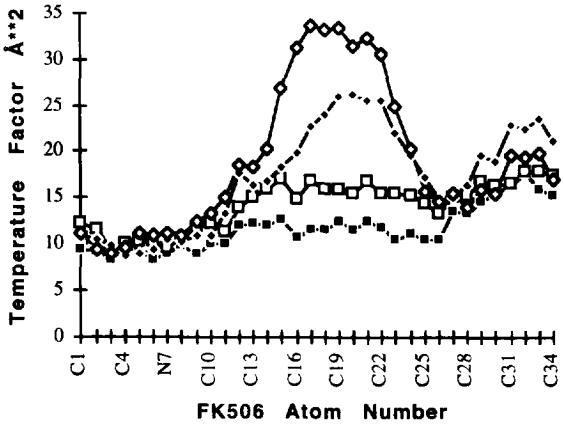

Figure 4. X-ray temperature factor data for the backbone atoms of the FK506 macrocycle, numbered as in Figure 1. A temperature factor increase is observed in the effector domain region of FK506 for the R42K+H87V (open diamonds) and H87V (filled diamonds) mutant complexes. At the same time, the temperature factors for the native (filled squares) and R42K (open squares) remain low and uniform across the ligand. Higher temperature factors are a measure of increased disorder in a structure, and often correlate with structural mobility. Interestingly, these temperature factor data do not correlate with the observed losses in CN-inhibitory activity for the mutant FKBP12 complexes (Table 2).

The effector domain model has guided drug design efforts in CN-based immunosuppression towards linear and macrocyclic ligands that might track the conformation of FK506 seen in the native complex structure;[3] and failure to develop a viable lead compound in this area has often been attributed to a failure in mimicry of the FK506 effector domain.[3] The discovery of an alternate conformation for the ligand in the double-mutant complex structure suggest that this overall design strategy should be exercised with caution, and that other more synthetically accessible platforms may yet be devised to project competent CN-inhibitory elements beyond the surface of FKBP12. Nor can one now dismiss the possibility of a ligand conformational rearrangement as part of the mechanism of CN inhibition. Ultimately, structure-based drug design in this area may require a direct examination of the structure of the FKBP12-FK506 complex with calcineurin itself.

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6. Native and mutant human recombinant FKBP12 protein was prepared as described (2f,5a,13). Native and mutant FKBP12-FK506 complexes were prepared and crystals were grown essentially as described (2a). Native crystals were used to seed the mutant complex crystallization experiments, and all the species reported here crystallized isomorphously in the native crystal form, as shown in Table 1. Diffraction data were collected on an X1000 multi-wire area detector (Siemens Analytical Instruments; Madison, WI) or on an R-Axis II image palte area detector (Rigaku/MSC; Woodlands, TX). Data collection and processing used software provided by the manufacturers. All data were collected at room temperature. The reported structure (2a) of native FKBP12 in complex with FK506 (Brookhaven Protein Data Bank (14a); entry 1FKF) was used directly as an initial model for the crystallographic refinement of the mutant and native protein complexes. Refinement was by simulated annealing using the X-PLOR program package (14b). Mutated amino acids were initially refined as alanine, and the actual mutant side chains were introduced as refinement progressed. Water molecules were positioned in the model with the aid of a peak search program (14c). The program QUANTA (Molecular Simulations; Burlington, MA) was used to examine electron density maps and protein models, and for the superposition of structures and the calculation of the coordiante rms differences reported (Table 1).
7. Molecular mechanics and molecular dynamics (MD) calculations were run using the *AMBER* program (15a). Force field parameters for FKBP12 were from Weiner *et al.* (15b). FK506 parameters were as described (15c). Four 500 psec MD simulations were run, varying the experimentally observed conformation of the bound FK506 (native bound conformation *vs.* R42K+H87V mutant bound conformation), and of the FKBP12 protein (native *vs.* mutant). In each case, the protein was fixed at the observed X-ray conformation, with a small harmonic positional restraint (force constant = 1.0 kcal/mole) imposed on the ligand dicarbonyl to keep FK506 in the active site.
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9. Reported rms differences are 0.011Å between the FK506 and L-685,818 ligands (3a) and 0.014Å between the corresponding Cα coordiantes of native FKBP12 (2d); both are near the operational limits of the data.
10. Bound FK506 in the respective single-mutant complexes adopts the conformation seen in the native complex, with coordinate rms differences *vs.* the native complex structure of 0.139Å and 0.539Å for the R42K and H87V mutant complexes respectively, compared to 1.275Å for the double-mutant complex. In addition, temperature factors for bound FK506 are distinctly higher (particularly in the effector domain region) for the double mutant and H87V complexes than for the native and R42K complexes, whose values are low and uniform across the ligand structure (Figure 4). These observations fail to correlate with the corresponding losses in CN-inhibitory activity seen in Table 2, an observation that is consistent with both FK506 conformations being competent with respect to CN-inhibition. In turn, the physical separation between the R42 and H87 loci in the structure of the complexes (Figure 2), the generally additive character of the electron density maps of the single-mutant complexes as seen in the double mutant complex (data not shown), as well as the multiplicative relation between CN-inhibitory Ki's for the mutant complexes (Table 2), all suggest that for these mutants, the loss of CN-inhibitory activity may be dominated by protein-related *vs.* ligand-related effects.
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16. Coordinates for the structures reported here will be deposited with the Brookhaven Protein Data Bank.

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