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Structural coupling between FKBP12 and buried water

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Abstract

Globular proteins often contain structurally well-resolved internal water molecules. Previously, we reported results from a molecular dynamics study that suggested that a buried water (Wat3) may play a role in modulating the structure of the FK506 binding protein-12 (FKBP12) 1. In particular, simulations suggested that disrupting a hydrogen bond to Wat3 by mutating E60 to either A or Q would cause a structural perturbation involving the distant W59 side chain, which rotates to a new conformation in response to the mutation. This effectively remodels the ligand binding pocket, as the side chain in the new conformation is likely to clash with bound FK506. To test if the protein structure is in effect modulated by the binding of a buried water in the distance, we determined high resolution (0.92 – 1.29 Å) structures of wild type FKBP12 and its two mutants (E60A, E60Q) by x-ray crystallography. The structures of mutant FKBP12 show that the ligand-binding pocket is indeed remodeled as predicted by the substitution at position 60, even though the water molecule does not directly interact with any of the amino acids of the binding pocket. Thus, these structures support the view that buried water molecules constitute an integral, noncovalent component of the protein structure. Additionally, this study provides an example in which predictions from molecular dynamics simulations are experimentally validated with atomic precision, thus showing that the structural features of protein-water interactions can be reliably modeled at a molecular level.

Keywords

buried water; protein-water interaction; FKBP12; molecular dynamics simulation

2 Introduction

Water is intimately involved in modulating protein stability, structure and function. The analysis of protein-water interaction is difficult as it must include the multiple roles of water as a bulk solvent as well as the possibility that it may contribute to the finer details in the makeup of protein molecules. While the principal outcome of protein folding is desolvation

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of hydrophobic surface and partitioning of hydrophobic residues in the core, folding to compact structures necessitates the burial of polar main chain nitrogens and oxygens in the core as well. To satisfy the hydrogen bonding needs of these polar atoms, proteins form extensive secondary structure, α-helices and β-sheets, in which most of the buried polar atoms participate in stable intramolecular hydrogen bonds. On the other hand, the hydrogen bonding needs of main chain polar atoms not involved in secondary structures, as well as those of buried polar side chain atoms, are often satisfied by buried water molecules 1–5. As these water molecules provide energetically favorable interactions, they are usually structurally well-resolved 5,6. The details of the interaction between buried water molecules and protein atoms are important to model the protein core, and they have been examined using a variety of techniques, including mutational, biochemical, spectroscopic and structural studies as well as computer simulation and database studies 1,7–15. These studies have suggested that internal water molecules are important structural components and likely play roles in flexibility, folding, and stability of a protein 13,16–18.

As proteins fold, they often create internal cavities, also called “packing defects.” Biochemical studies have shown that interval cavities destabilize the structure by an amount that varies with the cavity size 19. Some of the larger cavities may also be occupied by water molecules that contribute to structural stability by making favorable interactions with the protein, e.g. hydrogen bonds and van der Waals contacts 20. The enthalpic contribution of a bound water molecule toward stability has been estimated to be approximately −2 kcal/mol 13. Yet, there are also entropic costs associated with trapping water molecules, which result in a loss of approximately 2 kcal/mol in the free energy of folding 21. On average, therefore, internal waters appear be considered energy-neutral, which may explain the abundance of trapped water molecules in protein structures.

Although thermodynamic studies are highly informative and important to understand the energetics of protein-water interaction, they typically do not provide direct information on the structural roles of buried waters. For example, it is difficult to infer from biochemical studies what exact roles, if any, bound water molecules play in shaping the protein core. If the water molecules are important structurally, it should be possible to find examples that showcase structural coupling between them. However, high resolution structures of mutant proteins have shown that the response of buried water molecules to structural perturbations in the protein is not always predictable 22. Highlighting the variable nature of protein-water interactions, interior water molecules may increase or decrease structural flexibility depending on the situation 23–25. Together, these examples suggest that the structural coupling between protein and buried water may be difficult to unambiguously prove or model with atomic precision.

We have previously reported the results of molecular dynamics (MD) studies of FKBP12 (Fig. 1a). The protein belongs to a class of peptidyl prolyl isomerases that catalyze the cis-trans isomeration of proline, and is also the target of the immunosuppressant FK506 1. The high resolution structure of FKBP12 shows a buried solvent molecule Wat3 (which was referred to as Wat137 in Ref. 1) that makes three main chain hydrogen bonds to the 50s loop (residues 49 to 56—nomenclature by Liang et al 26) and one side chain hydrogen bond to E60 in the helix. Wat3 is well-resolved in FKBP12 and is also conserved in other FKBP12-related proteins including FKBP25 and FKBP52, suggesting that the molecule may be indispensable to this family of proteins 26,27. To that end, our molecular dynamics simulations have implicated that Wat3 may play a role in modulating the shape of the ligand-binding pocket, despite its peripheral location. A critical observation that led to this conclusion was a series of simulation studies with FKBP12 mutants, in which a single residue, E60, that is hydrogen bonded to Wat3 had been mutated to another residue. The mutation was intended to perturb the hydrogen bonding pattern around Wat3 through

selective removal of a single water hydrogen bond and to see what effects such a mutation may have on the overall protein structure. Our simulated structures showed that these mutants undergo a structural rearrangement around Wat3, which then propagates to the distant ligand-binding pocket (Fig. 9 in Ref. 1). Since then, we have determined the structures of wild type FKBP12 and two single point mutants, E60A and E60Q, to test the accuracy of our prediction. The results from the study, presented below, validate many of the predicted features. These structures provide a direct example that a buried water can play a role in defining the protein core. A comparison of the wild type and mutant structures also helps understand the mechanism by which a water hydrogen bond to E60 contributes to the integrity of the ligand binding pocket. Finally, by providing an experimental confirmation of MD predictions, we demonstrate that the current state of simulation has reached a level where the detailed protein-water interactions can be modeled accurately.

3 Results

To elucidate the broader role of Wat3 in establishing structure, we determined the structures of FKBP12 and mutants in which E60 has been mutated to A or Q. Globally, the overall folds of the mutants are similar to the previously determined wild type structure 28, and each still comprises a five-stranded anti-parallel β sheet wrapped around a short α helix with a right-handed twist (Figure 1a). The structure of wild type FKBP12 was re-determined under the identical conditions to facilitate comparison with the mutant structures. The structures are high resolution (see Figure 1b and Table 1), which is particularly important in order to unambiguously identify bound water molecules. The overall structures of the mutants are similar to that of native FKBP12 with an rmsd of 0.27 – 0.33 Å for all Cα atoms. The largest differences are found in solvent-exposed side chains, residues of the 50s loop and the N-terminal end of the helix.

Wild type FKBP12 has one buried water molecule, where buried water is defined as a water molecule with zero solvent accessible surface area. This water molecule, Wat3, was initially chosen for the study because we concluded based on a statistical analysis that a hydrogen bond to a main chain atom is more important than a similar hydrogen bond to a side chain atom, and Wat3 in the FKBP12 structure 1FKF makes three main chain hydrogen bonds to M49, K52, and E54, as well as a side chain hydrogen bond with E60. The multiple hydrogen bonds to main chain atoms suggest a possible structural role, while side chain hydrogen bond allows a potential manipulation of the interaction through site directed mutagenesis. In particular, we can introduce a conservative mutation at E60 to subtly modulate the coupling between Wat3 and FKBP12, or introduce a nonconservative mutation to disrupt the hydrogen bonding network to a greater extent. We experimentally tested one mutant of each kind. In particular, we introduced the E60Q mutation to substitute a carboxylate oxygen with an amide nitrogen, thus converting a hydrogen bond acceptor to a hydrogen bond donor (Figure 2). Surprisingly, the structure of E60Q mutant shows that Wat3 no longer hydrogen bonds with the Q60 side chain directly. Rather, the side chain of Q60 hydrogen bonds exclusively with the main chain atoms of G51 and K52, while Wat3 continues to maintain two main chain hydrogen bonds. One consequence of the main chain hydrogen bonds involving the Q60 side chain is that the positions of K52 carbonyl oxygen and the backbone amide of Q53 are inverted. As a result, the main chain conformation around K52 and Q53 is substantially different from that of wild type, just as it was predicted by the former MD studies (Fig. 2b and 1).

Similarly, the E60A mutation disrupts the hydrogen bonding network around Wat3 by decoupling the molecule from the residue at position 60. The missing side chain of E60 creates a void that is filled by an extra water molecule Wat8, which occupies the same position as one of the E60 side chain oxygens and appears to be involved in a water-water

hydrogen bond with Wat3 that mimics the E60 – Wat3 interaction (Figure 2a). A water cluster comprising two water molecules was also observed during the entire duration of the simulation (data not shown). In contrast to the E60Q mutant, the conformation of the 50s loop remains similar to that of wild type, suggesting that the presence of an amide group in Q60 was responsible for the main chain remodeling within the 50s loop.

The targeted disruption of a hydrogen bond to Wat3 leads to structural adjustments in the E60A and E60Q mutants beyond its immediate neighbors. This is seen in the wild type structure, in which the interaction between Wat3 and E60 appears to disrupt an intrahelical backbone hydrogen bond between E60 CO and A64 NH. In the native structure, these atoms are separated by 3.22 – 3.42 Å with the potential hydrogen bond at 28 – 58° out of the amide bond plane, disfavoring a hydrogen bond between the two residues. In contrast, the corresponding interatomic distances are 2.92 and 2.96 Å in the E60A and E60Q mutants, respectively, leading to the formation of an intrahelical hydrogen bond in each structure. Hence, one of the consequences of the hydrogen bond between Wat3 and the E60 Oε2 seems a displacement of the residue E60 out of alignment with the helical axis. The disruption of a main chain hydrogen bond in a helix should be energetically unfavorable without a compensating effect. In the wild type structure, the interaction involving Wat3 appears to be contributing to the occurrence of a helical kink by generating a lateral pull. While the side chain of Q60 still forms hydrogen bonds with distant polar atoms, including G51 NH, K52 CO and V55 CO, its Cα has shifted by 1.5 –1.66 Å with respect to the E60 Cα (Figure 3). This movement brings the main chain carbonyl oxygens of Q60 within hydrogen bonding distance of A64 NH. Similarly, the Cα atom of A60 is shifted by 1.64 – 1.74 Å from E60 Cα (Figure 3), thus resulting in a stable intrahelical hydrogen bond with A64. Additionally, these shifts are highly localized. For example, the Cα atoms of residues 58 and 62 in the E60A are each only displaced by 0.38 and 0.47 Å, respectively, from their positions in the native structure. Taken together, these structural details suggest that the main effect of the side chain hydrogen bond involving Wat3 and the E60 is to induce a highly focused adjustment at individual atomic positions that produces an observable structural shift.

As the N-terminus of the helix is displaced toward the center of the protein as a result of the formation of an intrahelical hydrogen bond (Figure 3), the W59 side chain located on opposite face of the helix is pushed against other hydrophobic side chains in the interior. Since the core of FKBP12 is tightly packed with large and small hydrophobic residues, the lateral movement of W59 creates a steric clash with other buried side chains, especially F99. The steric repulsion between the two residues probably explains why the indole ring of W59 is rotated in both mutants (Figure 4) and the phenyl ring of F99 is rotated by approximately 15°. W59 forms part of the basin of the hydrophobic ligand binding pocket, which requires a specific conformation of the side chain indole ring. The rotation of W59 side chain around its Cβ-Cγ bond to avoid a steric clash with F99 places the indole within the ligand binding pocket, partially occluding the space that is normally occupied by a ligand. The FK506-bound structure of FKBP12 suggests that modeling FK506 in the E60Q or E60A mutants would result in FK506 severely overlapping with the W59 side chain in the new conformation (Figure 5).

4 Discussion

Ordered water molecules found in protein structures may serve a variety of critical functions. For example, water molecules are used in some enzymatic reactions to define substrate specificity and facilitate catalysis [29–32]; buried solvent molecules in the core contribute to thermal stability by filling internal cavities; and water molecules at the molecular interface facilitate ligand-receptor or protein-protein interaction by increasing the

complementarity of the contacting surfaces 33–38. The latter role of bound solvent molecules has given rise to the notion of water as structural glue, which aptly describes its ability to form multiple hydrogen bonds with both interacting molecules. Statistical analyses of high resolution protein crystal structures have shown that internal water molecules are not evenly distributed but rather clustered around regions without secondary structure, suggesting that trapped water molecules may be important to satisfy the hydrogen bonding needs of buried polar atoms 1,5,39.

While former studies have shown the structural and functional significance of ordered water molecules, evidence demonstrating direct structural coupling between protein and a trapped solvent molecule is lacking. The current study provides evidence that an interior water molecule may contribute to the structural integrity of a protein by forming multiple hydrogen bonds with protein atoms. To that end, Wat3 in FKBP12 participates in a network of interactions that is important for the proper construction of the ligand binding pocket located > 8 Å away, and disrupting the network results in a remodeling of the binding pocket (Figure 5b). The mechanism by which a single protein-water hydrogen bond dictates the structural details of a distant part of the protein was not clear in the previous MD simulations. The high resolution structures described in this paper show that the water exerts its influence by laterally shifting the position of one helix residue, i.e. E60, by hydrogen bonding with its side chain. This is seen most clearly by comparing the wild type and mutant structures, which shows that the loss of the hydrogen bond in E60A and E60Q moves the helix by 1.1 – 1.8 Å perpendicular to its helical axis, forcing the W59 side chain to adopt an alternate conformation to avoid steric clash with F99. W59 in the new conformation would be incompatible with ligand binding, although the side chain may easily rotate back to the original conformation in the presence of a ligand to accommodate ligand binding. Elucidating the functional implication of the side chain conformation will require a future binding study.

FKBP12 was selected in part because the protein has a fully buried water molecule and we can easily investigate the significance of directional protein-water interaction through a targeted substitution at E60. Additionally, the residue 60 position is largely solvent accessible, which minimizes a potential complication arising from introducing a mutation in the protein core. Considering that Wat3 can easily rotate within its crystallographic location, we did not think the E60Q substitution would result in any significant structural change. Yet, the structural rearrangement in E60Q within the 50s loop bespeaks a different story. The modulation of a single hydrogen bond induces an unexpected rearrangement in the local structure, in which the residues 52 and 53 main chain dihedrals transition from a left handed α-helical conformation to an extended conformation (Figure 2b). The fact that a similar rearrangement is not observed in the 50s loop for E60A is likely due to the presence of a second highly coordinated water molecule that acts as a surrogate for one of the terminal oxygens of E60. The conservation of Wat3 in FKBP12 and other related proteins suggests that a conserved buried water molecule may be used to coordinate side chains and to stabilize the target global configuration. The elucidation of the role of Wat3 in FKBP12 thus demonstrates that a water molecule may be important for the structural integrity of a protein as well as its thermal stability.

A previous database study has reported a prevalence of buried water molecules around residues without secondary structure 1. One such water molecule, Wat3 forms part of a network of interacting residues that include the 50s loop, the residues within the N-terminal half of the helix (e.g. W59 and E60), and V55 and F99, whose conformations change in response to the rotation of the W59 side chain. The high level of coupling between Wat3 and protein is also substantiated by its low B-factor of 7.7 $Å^2$, which is lower than the average value of B-factors for protein atoms, 8.0 $Å^2$ or for all water molecules, 19.3 $Å^2$. The “depth”

of the network, spanning several “layers” of residues around the water, also suggests that the relationship of this water to the protein is qualitatively different from that of water molecules whose principal contribution is thermostabilization. The manner by which Wat3 impose conformational constraints on structure is reminiscent of some metal ions, e.g. Ca^{2+} and Zn^{2+}, that are known to play important structural roles through strong, noncovalent, directional interactions with neighboring polar atoms 40,41.

While a strategically positioned internal solvent molecule may qualitatively change the structural and functional properties of the protein, it is often difficult to verify these predictions experimentally. To circumvent this challenge, we combined simulation studies of FKBP12 with structure determination. The crystal structures of wild type and mutant FKBP12 confirm the predicted structural role of Wat3 with atomic precision, including the modulation of the W59 side chain dihedral angle. The close agreement between simulation and structure hints that the currently available modeling software accurately captures the essentially features of protein-water interaction. Therefore, at least in some limited applications, further elaboration of the model, e.g. incorporating explicit polarization effects, may not be necessary to obtain quantitatively accurate results. Our simulations also predicted that Wat3 escapes into the solvent within the 12 nsec simulations, which is in agreement with the estimated values of residence time for highly dynamic water molecules. But the lost water molecule is rapidly replaced by another water molecule, suggesting that the occupation of the site is thermodynamically stable.

In addition to the surprising accuracy of the predictions, there are also discrepancies between prediction and observation. Notably, long-range hydrogen bond between G58 NH and Y80 CO was predicted to be lost for E60A but was intact in the experimental structure. Likewise, K52 and Q53 were seen to undergo conformational changes during one of two independent simulations of E60A, but the crystal structure shows they retain the conformation seen in wild type. These latter discrepancies, however, may be due to limited total computational time rather than methodology 42, and longer and multiple independent simulations might have provided a more accurate picture of the structural impact of bound water in the E60A mutant.

A predictive understanding water-mediated long-range interaction is particularly important for efforts in protein folding and design. A modified Hamiltonian with an explicit term to account for protein-water interaction has been shown to improve the accuracy of structure prediction 43. Structural waters have also been modeled using solvated rotamers with some success 44. While the agreement between simulation and experiment reported herein is encouraging, this success also does not yet tell us how to incorporate water molecules systematically during protein design. For example, two proteins related to FKBP12 (PDB codes 1FD9 and 1PBK) do not contain a buried solvent molecule, although the ligand binding pocket in each still resembles that of FKBP12 26,45. In each structure, however, the identity and arrangement of the residues in the 50s loop and the helix have been changed to result in a helix with a missing main chain hydrogen bond between residues equivalent to E60 and A64. Therefore, nature seems to have found two independent solutions to achieve the same structural goals—one with a bound structural water and the other without. Once we understand the protein-water interaction better, we may hope to replicate some of these successes in the laboratory and predictably design protein molecules in which a bound water molecule plays a prescribed role. The complementary use of molecular dynamics simulations and crystallographic studies discussed in this paper presents a small step towards achieving this goal.

5 Methods

5.1 Molecular simulations

The details of the simulations were described in a previous publication 1. Briefly, NAMD2 46 with the CHARMM (v.27) force field 47 was used to simulate wild type and E60 mutants of FKBP12 for 12 ns in a solvent box with periodic boundary conditions under constant pressure (1 atm) and temperature (300 K). The net charge of the system was reduced to zero by addition of sodium and chloride ions to the final ionic strength of 100 mM, and the electrostatic interactions were implemented using the particle mesh Ewald sum. The TIP3P model of water was used in the simulations.

5.2 Protein expression and purification

Wild type, E60A and E60Q fused to GST were expressed and purified from *E. coli* BL21 (DE3) (Novagen). Cells from 250 ml of culture were lysed using 40 ml B-per (Pierce) in the presence of 1 mM PMSF, and 3 ml of glutathione resin (Clontech) were added to the supernatant at 4 °C for 2 hrs. After the unbound fraction has been washed away FKBP12 was cleaved from GST by adding 15 μl AcTEV (Invitrogen) over three days at 22 ^{o}C directly to the resin. The eluate was collected and further purified on a size-exclusion column (Superdex 75). Finally, the appropriate fractions, in 100 mM sodium chloride and 50 mM Tris pH 8.0, were pooled and concentrated to ~ 15 mg/ml using Amicon centricon (3 kDa cutoff).

5.3 Structure determination

Crystals of wild type and mutant FKBP were grown using the hanging drop vapor diffusion method with 1.9 – 2.1 M sodium maleonate pH 7.0 (Hampton Research) and 50 mM DMSO in the reservoir. An equal amount of protein solution and reservoir were mixed on the cover slip and crystals appeared after 2 – 4 weeks. The crystals were gradually transferred to 3.0 M sodium maleonate pH 7.0 and equilibrated overnight to remove DMSO, and were frozen in liquid nitrogen for storage and data collection. Datasets were collected at Atomic Light Source, Beamline 8.2.2 (Table 1) and processed using HKL2000 48. The three datasets were highly isomorphous with each other and had the unit cell in the space group $P2_1$. The structure of wild type FKBP was solved by molecular replacement using the DMSO-bound wild type structure 49 in CCP4 Molrep50. The E60A and E60Q structures were calculated using wild type phases and models were adjusted using COOT 51. The structures were refined in Refmac/CCP452 and SHELXL3. Statistics of the final refined structures are shown in Table 1. Coordinates have been deposited in the Protein Data Bank under accession codes 2PPO (WT), 2PPP (E60Q) and 2PPN (E60A).

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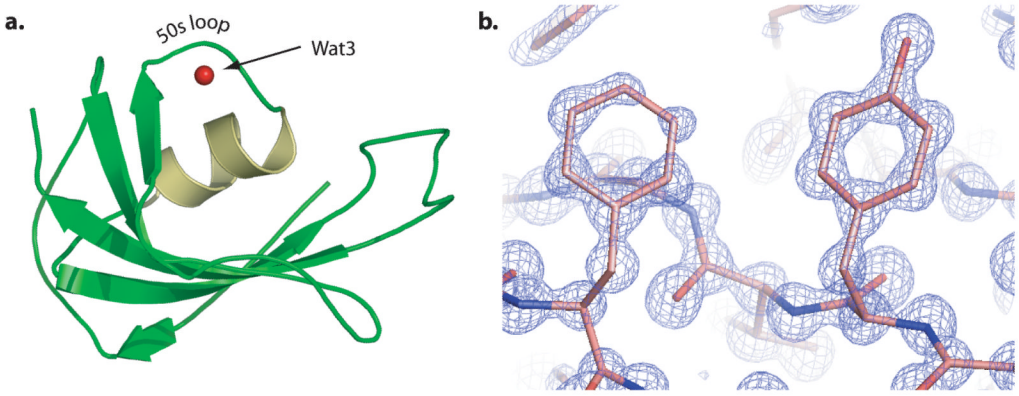

Figure 1.
a. FKBP12 is comprised of a five-stranded anti-parallel β sheet wrapped around a short a helix. The 50s loop hydrogen bonded to the conserved structural water Wat3 is highlighted.
b. The side chains are superimposed on the electron density at 2.5σ.

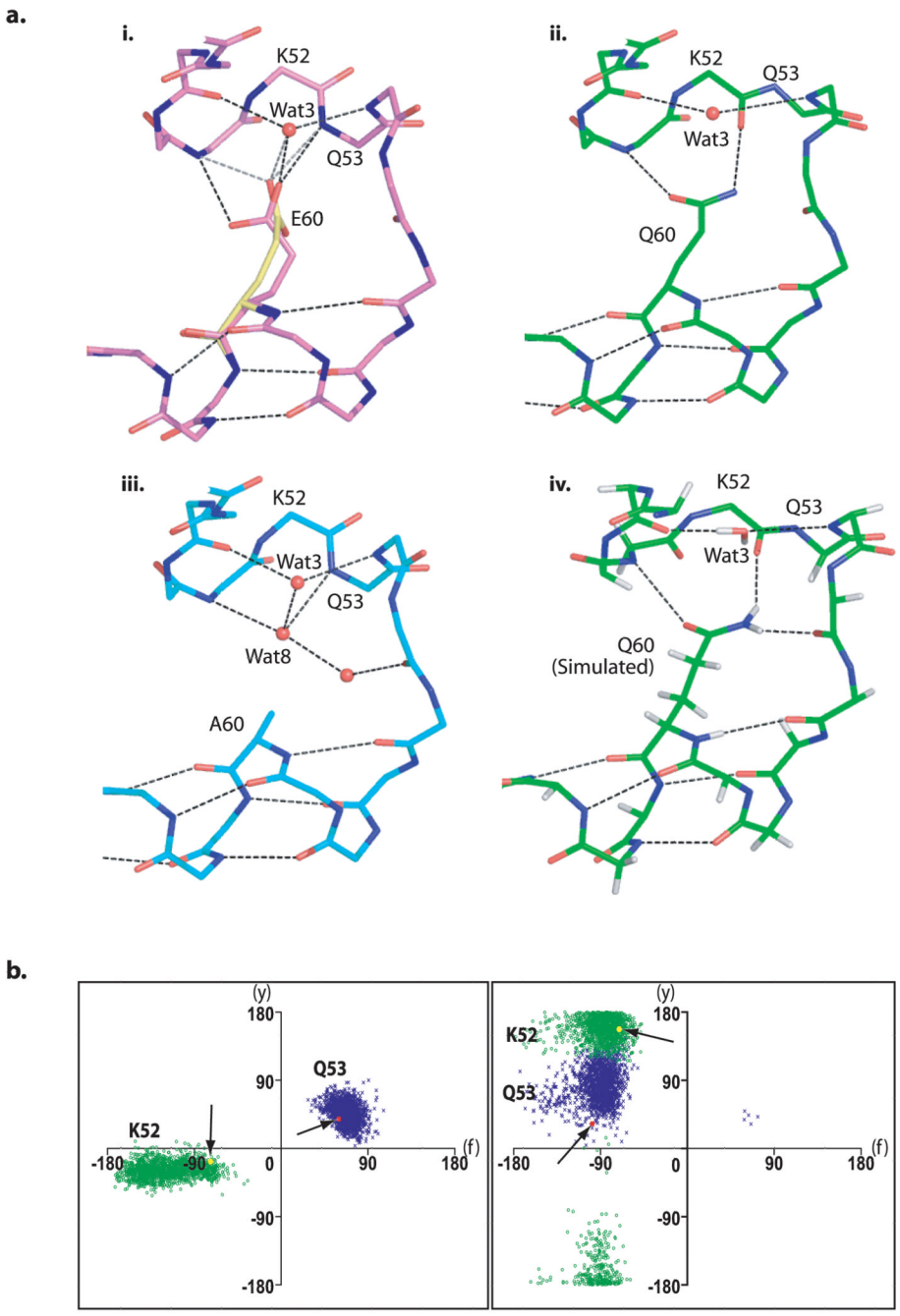

Figure 2.

a. The hydrogen bonding network around Wat3 in i) wild type, ii) E60Q, iii) E60A, and iv) simulated structure of E60Q. E60 adopts two alternate conformations in wild type, which differ from each other by a rotation about one of the terminal oxygens.The coordinates of Oε1 are approximately the same in both conformations, thus permitting it to form a stable hydrogen bond with Wat3. The second “B” conformation is shown in yellow.

b. Ramachandran plots for K52 (green) and Q53 (blue) from simulations of wild type (left) and E60Q (right) structures. The yellow (K52) and red (Q53) dots (also indicated by arrows) correspond to the dihedral angles observed in the final crystal structures.

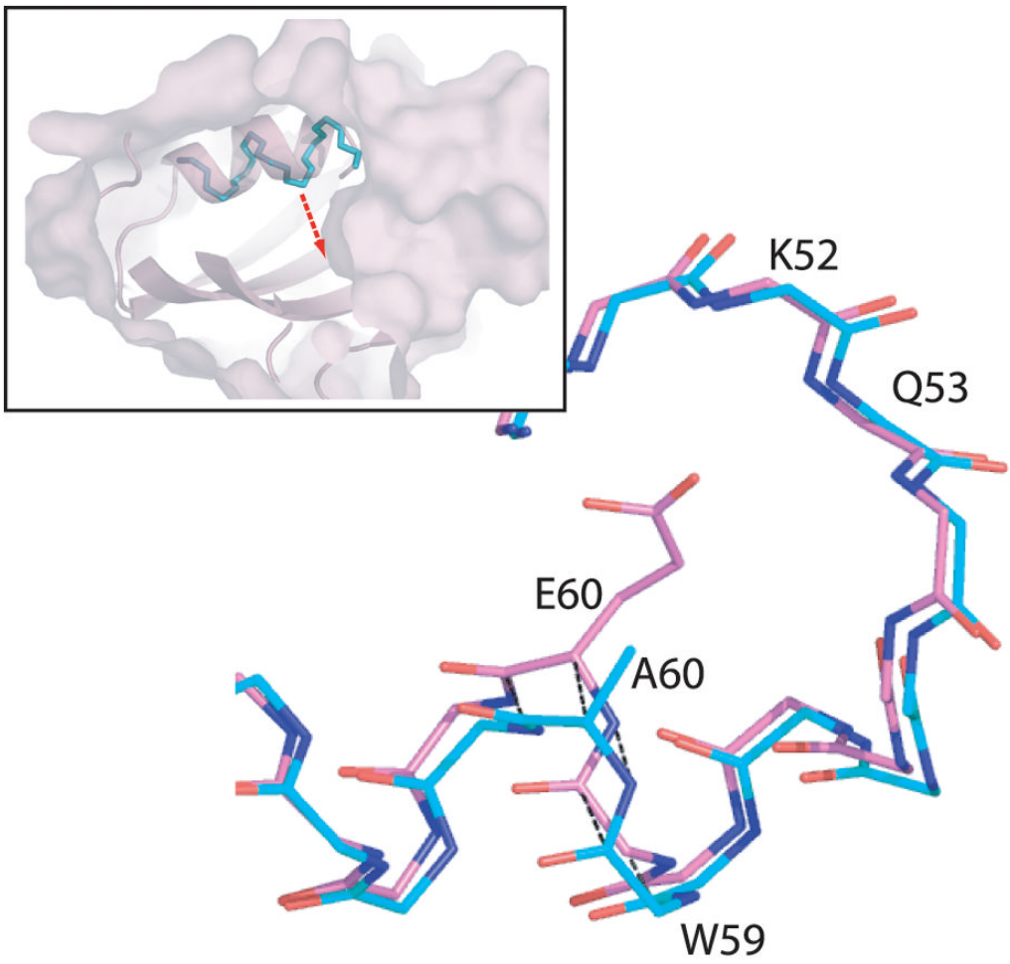

Figure 3.
The N-terminus of the helix moves toward the center of the protein, with the main chain atoms from W59 and A60 of the mutant moving by 1.28 – 1.74 Å (dotted lines). (Inset) The Cα trace of the E60A mutant (cyan) is shown against the surface rendering of wild type FKBP12 (purple), which is partially cut away to reveal the protein core.

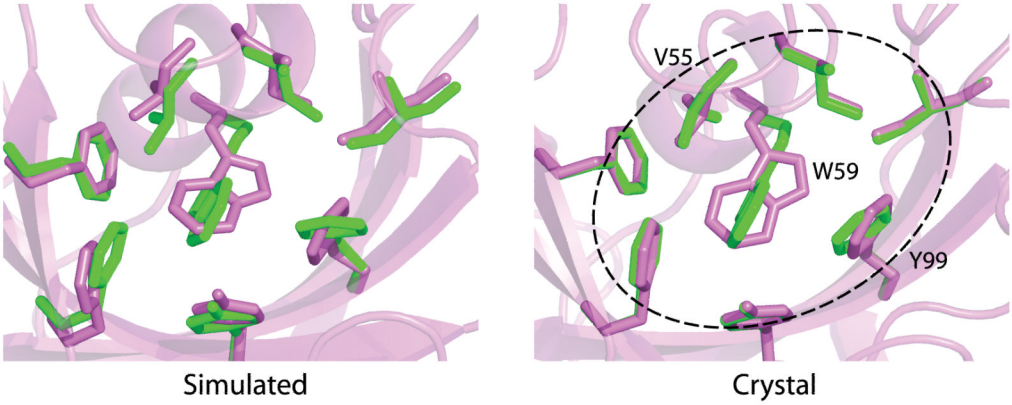

Figure 4.
(a) Simulated and (b) crystal structures of the ligand binding pocket of wild type (purple) and E60Q mutant (green). The ligand binding pocket is outlined in oval and corresponds to a view from outside. The side chain of W59 rotates to a new conformation to avoid steric clash with F99. The conformations of V55 and F99 also change to optimize van der Waals contacts with W59 in the new conformation.

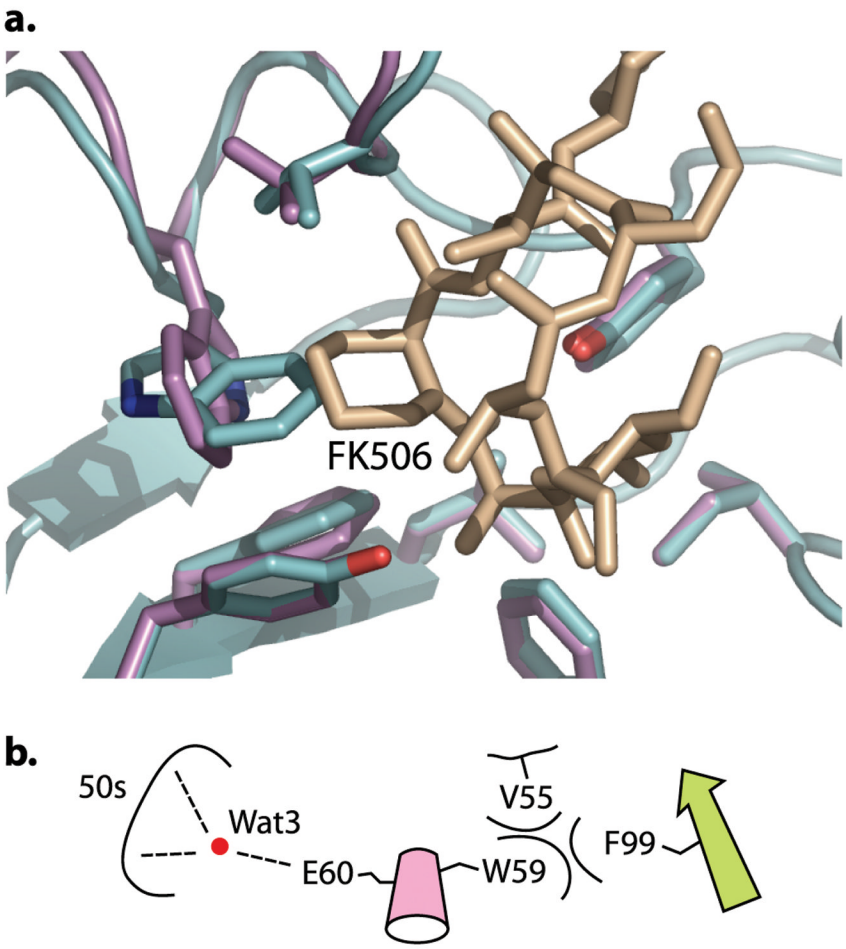

Figure 5.
a. FK506 (from PDB 1FKF) modeled in the ligand binding pocket of wild type (violet) and E60Q (cyan) FKBP12. The rotation of W59 leads to a conformation that creates steric clash between its side chain and the docked FK506.
b. The network of interacting amino acids and Wat3 in FKBP12.

Table 1

A summary of data collection and final statistics

Data collection statistics			
	Native	**E60Q**	**E60A**
Resolution (Å)	50 – 0.92	50 – 0.97	25 – 1.29
Wavelength (Å)	0.98	1.04	0.98
Intensity/σ (last shell)	8.9 (5.5)	24.7 (2.2)	46.2 (2.75)
Redundancy (last shell)	3.3 (2.4)	3.3 (2.4)	3.5 (1.9)
Completeness (last shell) (%)	98.4 (96.4)	89.5 (80.4)	92.5 (85.9)
Total number of reflections	527524	666749	790335
Unique reflections	71227	62089	29420
R_{sym} (last shell)	0.079 (0.18)	0.053 (0.32)	0.092 (0.280)
Unit cell dimensions in space group P21 (Å,°)	*a*=28.76, *b*=62.7, *c*=32.28, β=113.66 *a*=28.6, *b*=62.52, *c*=32.25, β=114.13		*a*=28.79, *b*=62.56, *c*=32.42, β=113.7
Final statistics			
	Native	**E60Q**	**E60A**
Resolution (Å)	0.92	0.97	1.29
r.m.s. deviation bond lengths (Å)	0.015	0.030	0.012
r.m.s. deviation bond angles (°)	2.40	2.50	2.25
Number of water molecules	118	125	137
No. of residues in multiple conformations	15	8	5
R/R_{free}	16.85/19.89	14.38/17.54	13.29/18.16