

ABSTRACT: Molecular dynamics (MD) and free energy perturbation (FEP) methods are used to study the binding and mechanism of isomerization of a tetrapeptide (AcAAPFNMe) by FK506 binding protein (FKBP). Detailed structures are predicted for the complexes of FKBP with the peptide in both ground-state and transition-state forms. The results support a mechanism of catalysis by distortion, where a large number of nonbonded interactions act together to stabilize preferentially the twisted transition state. The two most important groups for the catalysis are suggested to be Trp⁵⁹ and Asp³⁷, but several other groups are identified as directly or indirectly involved in the binding and catalysis. However, the structural results do not support the notion that the keto oxygen of the immunosuppressive agents FK506 and rapamycin mimics the oxygen for the twisted peptide bond in the FKBP-transition-state complex.

The isomerization of peptidic bonds is a difficult process with free energy barriers typically being 15–20 kcal/mol (Drankenberg & Forsen, 1971; Larive & Rabenstein, 1993). Most peptides correspond to secondary amides and are found exclusively in the *trans* conformation, which is about 2.5 kcal/mol more stable than the *cis* (Drankenberg & Forsen, 1971). However, the tertiary nitrogen in prolyl residues allows the coexistence of *cis* and *trans* isomers in proteins, which may require interconversion during protein folding. Considering the difficulty of this reaction, and the need for efficient folding mechanisms *in vivo*, the existence of enzymes which catalyze the isomerization of prolylpeptides (peptidylprolyl isomerases (PPIs)) is not surprising. PPIs were first characterized by Fischer and co-workers (Fischer et al., 1984), and since then different studies have demonstrated that these enzymes catalyze the *cis* ↔ *trans* isomerism of prolylpeptides during the folding of various proteins (Fischer & Bang 1985, Bächinger, 1987; Lang et al., 1987; Jackson & Fersht, 1991).

The interest in two PPIs, FKBP (FK506 binding protein) and cyclophilin, increased dramatically after the discovery that they are receptors for the immunosuppressant agents FK506 (Harding et al., 1989) and cyclosporin (Fischer et al., 1989a; Takahashi, et al., 1989). Particularly, FKBP was found to be the receptor for a family of immunosuppressant drugs which includes FK506, rapamycin, and ascomycin. It was demonstrated that the binding of the drugs to the protein yields complexes which inhibit T-cell activation (see Rosen & Schreiber, 1992).

FKBP is a small, and highly conserved cytosolic protein, which was isolated and purified by Schreiber and co-workers (Harding et al., 1989), who also cloned the protein (Standaert et al., 1990), and determined the 3-D structure in solution (Michnick et al., 1991) and in the crystal (van Duyne et al., 1991). Actually, 3-D structures of the protein as well as of the protein bound to FK506 (van Duyne et al., 1991a), rapamycin (van Duyne et al., 1991b), and ascomycin (Meadows et al., 1993) are available. The protein's structure features

a five-stranded antiparallel β sheet and a short amphipathic α helix, which partially covers one face of the β sheet. The active site, defined as the region where the protein binds the drugs, is located between the α helix and the β sheet in a hydrophobic pocket formed by the side chains of Trp⁵⁹, Tyr²⁶, Phe⁴⁶, Phe³⁶, Phe⁹⁹, Ile⁵⁶, Tyr⁸², and Val⁵⁵.

Both FK506 and rapamycin bind to FKBP as surrogates of prolylpeptides inhibiting the PPI activity (Schreiber, 1991; Rosen & Schreiber, 1992). The low K_i and the presence of a C9-keto oxygen orthogonal to the pipercolinyl ring suggest that both drugs interact with FKBP as mimics of the transition state for *cis* ↔ *trans* isomerization. The fact that cyclosporin, a potent immunosuppressant drug, was a powerful inhibitor of the PPI activity of cyclophilin led to the speculation that the immunosuppressant effects of these drugs were associated with their inhibition of PPI activity (see Rosen & Schreiber, 1992). This attractive hypothesis was ruled out by two observations: (i) rapamycin and FK506 have very different mechanisms for immunosuppressant activity, although their inhibition of PPI activity is similar (Bierer et al., 1990a), and (ii) the analog 506BD is a strong inhibitor of FKBP but is not an immunosuppressant (Bierer et al., 1990b). Accordingly, the relationship, if any, between the PPI and immunophilin activities of FKBP and cyclophilin remains to be determined.

The mechanisms for the PPI action of the immunophilins are also obscure. Fischer and co-workers (Fischer et al., 1989b) proposed a mechanism based on the formation of covalent adducts between the peptide and protein. Kofron et al. (1991) suggested an alternative mechanism in which the protonation of the proline nitrogen would facilitate the isomerization process. Other authors (Albers et al., 1990; Harrison & Stein, 1990; Park et al., 1992; Rosen & Schreiber, 1992) have suggested a mechanism based on the preferential stabilization of the transition state by nonbonded interactions (catalysis by distortion). However, the lack of a structure for a complex between an immunophilin and a peptide has limited the experimental characterization of the rotamase mechanism.

In this paper, we present a molecular dynamics (MD) study on the mechanism of peptide isomerization catalyzed by FKBP. Kinetic measurements have been made for *cis* → *trans* isomerization of tetrapeptides (Fischer et al., 1989; Albers et

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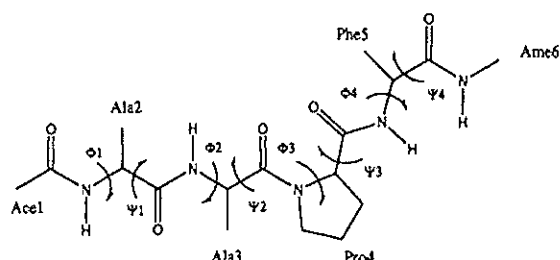


FIGURE 1: Structure and nomenclature of the peptide used in the simulations.

al., 1990; Harrison & Stein, 1990; Kofron et al., 1991; Harrison & Stein, 1992; Park et al., 1992). Consequently, structures for the complexes of a tetrapeptide with a *cis* peptide bond and of its transition state were sought and obtained here. Support for the model includes free energy results for mutation of the P1 residue. Specific interactions responsible for selective stabilization of the transition state are identified. The model clarifies in detail the mechanism of rotamase action and is open to experimental testing. The binding insights should also be useful in the design of high-affinity ligands for FKBP with potential immunosuppressive activity.

COMPUTATIONAL METHODS

A peptide with the sequences Ace-Ala-Ala-Pro-Phe-Ame, where Ace (acetate) and Ame (methylamide) are the N- and C-terminal groups, was used to mimic the natural substrate. Figure 1 shows the structure and related nomenclature; in the following, plain numbering is used for the peptide residues and superscript numbering is used for protein residues. This sequence was selected because it resembles closely the synthetic peptides (Suc-Ala-X-Pro-Phe-pNA) used to measure the activity of PPIs (Fischer et al., 1989; Albers et al., 1990; Harrison & Stein, 1990; Kofron et al., 1991; Park et al., 1992). The peptide was built in both *cis* and *twisted* conformations around the Ala3-Pro4 peptidic bond. The peptide in the *cis* conformation is used as a model of the substrate, and the *twisted* conformation, as a model for the transition state.

The molecular dynamics calculations were carried out with the AMBER program, version 4.0 (Pearlman et al., 1991). The AMBER/OPLS force field (Weiner et al., 1984; Jorgensen & Tirado-Rives, 1988; Jorgensen & Severance, 1990) was used to describe both the peptide and the protein interactions; the only modifications (see Table I) were for the peptidic bond Ala3-Pro4, where the torsional barrier and improper torsion at nitrogen were removed (in both planar and *twisted* conformations), and the bond lengths and angles at N(Pro4) were assigned to average values for planar and *twisted* amides (Duffy et al., 1992). The stretching and bending parameters related to N(Pro4) were reduced in order to allow the peptide to accommodate easily to the protein environment. Four constraints were used to guarantee that the Ala3-Pro4 peptidic bond stayed in the *cis* or *twisted* conformation. The same force constants were used for both conformers in order to make comparable the Hamiltonians for the *cis* and *twisted* peptides (Table I). The contribution of these constraints to the total energy was not included in any of the simulations.

Standard OPLS Lennard-Jones parameters were used for the *cis* and *twisted* structures (Jorgensen & Tirado-Rives, 1988; Jorgensen & Severance, 1990). Standard OPLS charges for amides were also used to describe the planar structure (Jorgensen & Tirado-Rives, 1988), while the charges for the

Table I: Force Field Parameters for the Ala3-Pro4 Peptide Bond*

bond or angle	equil. value	force constant
C-NW	1.39	100
C-NW-CH	115.0	5
C-NW-CQ	115.0	5
CH-NW-CQ	114.0	5
dihedral angle	equil. value	force constant
Ca(A3)-C(A3)-N(P4)-Ca(P4)	120/0	200
O(A3)-C(A3)-N(P4)-Ca(P4)	-60/180	200
C(A3)-N(P4)-CD(P4)-Ca(P4)	-120/180	100
Ca(A3)-C(A3)-O(A3)-N(P4)	180/180	100
atom	q (cis)	q (Twisted)
Ca(A3)	0.200	0.340
C(A3)	0.500	0.437
O(A3)	-0.500	-0.457
N(P4)	-0.570	-0.570
Ca(P4), CD(P4)	0.285	0.225

* The atom type NW corresponds to N(Pro4) of the *twisted* conformer; other atom types are from the standard AMBER/OPLS force-field (Weiner et al., 1984; Jorgensen & Tirado-Rives, 1988). Distances are in Å, angles in deg, charges in electrons, and force constants in kcal/mol Å², or kcal/mol rad². The dihedral parameters are used as constraints to keep the *cis* or *twisted* conformation. Standard AMBER torsional parameters for the Ala3-Pro4 bond are removed.

twisted amide bond were derived from recent calculations on *twisted* dimethylacetamide (see Table I and Duffy et al. (1992)). These charges correctly reproduce properties of amides in solution, including free energies of hydration (Jorgensen & Tirado-Rives, 1988; Duffy et al., 1992).

The crystal structure of FKBP bound to rapamycin (van Duyn et al., 1991b) was used as a starting point for the calculations. First, the peptide in the *twisted* form was overlaid on rapamycin. The conformation of the peptide was modified by rotation around single bonds to obtain the best fit with rapamycin and to reproduce well the environment of analogous hydrogen bond donor and acceptor groups. Once a suitable conformation of the peptide was obtained, the drug was removed, yielding a protein-peptide complex in which the peptide not only reproduces the general shape of rapamycin but also has a similar pattern of hydrogen bonds with the binding pocket.

The protein-peptide model was solvated with a sphere of 23-Å radius containing TIP3P water (Jorgensen et al., 1983) centered on the center of mass of the peptide. This led, after removal of water molecules with unreasonably short contacts, to a solvation sphere with 1002 water molecules. A soft harmonic term with a force constant of 1.5 kcal/(mol Å²) was used to retrieve water molecules drifting beyond the boundary of the sphere. The resulting system is shown in Figure 2, where it is clear that the sphere of water provides extensive hydration of the binding site for the MD calculations. The solvated system was then divided into two regions: (i) an inner part, which includes the peptide, the water, and all residues which have at least one atom closer than 12 Å to any atom of the peptide, and (ii) an outer part, which contains the rest of the protein. The first part includes 78 residues of the protein and was free to move in all minimizations and MD simulations, while the outer part with 25 residues was kept rigid in all calculations. This initial system was then prepared through 2000 steps of energy minimization (500 where the peptide and the protein were kept rigid, 500 where only the peptide was fixed, and 1000 where the entire system was minimized), and 40 ps of MD for heating from $T = 198-298$ K and equilibration. The final equilibrated structure was used to run 500 ps of constant temperature MD at 298 K. The



FIGURE 2: FKBP-peptide complex inside the 23-Å sphere of water used in the MD simulations.

integration step was 0.002 ps, and the coordinates were stored every 0.2 ps. SHAKE (Ryckaert et al., 1977) was used to maintain all bonds lengths at their equilibrium values. A nonbonded spherical cutoff of 8 Å and a residue-based nonbonded list, updated every 25 integration steps, were used.

The structure at $t = 100$ ps was used to generate the *cis* peptide-protein complex. The change between *twisted* and *cis* peptides was done in six steps with the angles of the constraints and the charges gradually modified from *twisted* to *cis* values (Table 1). After each change, the peptide alone was optimized *in vacuo* for 100 cycles; harmonic constraints were used to guarantee that most of the conformational changes would occur mainly in the Ala3-Pro4 area. This simple procedure mimics a fast change from *twisted* to *cis*, and we found it to be superior to other methods such as stepwise rotation for the full protein-peptide system and simulated annealing.

The *cis* peptide was then placed inside the binding pocket of the solvated protein occupying the same location as the *twisted* conformer. The system was optimized, heated, and equilibrated during 40 ps using a procedure analogous to that described above. A 500-ps MD trajectory was then run for the *cis* system. It should be noted that the *cis* and *twisted* systems contain the same number of atoms, identical definition of the hydration shell, identical partitioning between the inner and outer regions, and, except for the charges, the same Hamiltonian.

Free energy calculations were used to predict the difference in isomerization rate between two peptides, Ace-Ala-Ala-Pro-Phe-Ame and Ace-Ala-Gly-Pro-Phe-Ame, for comparison with experimental data on the corresponding Suc-Ala-X-Pro-Phe-pNA peptides (Albers et al., 1990; Harrison & Stein, 1992; Park et al., 1992). The thermodynamic cycle in Figure 3 was utilized to obtain the change in free energies of activation. The free energy changes for the Ala3 → Gly3 mutations was computed by statistical perturbation theory according to eq 1 (Zwanzig, 1954). These mutations were started with the structures and velocities of the *cis* and *twisted* systems at the end of the MD runs. The calculations were done in 41 double-

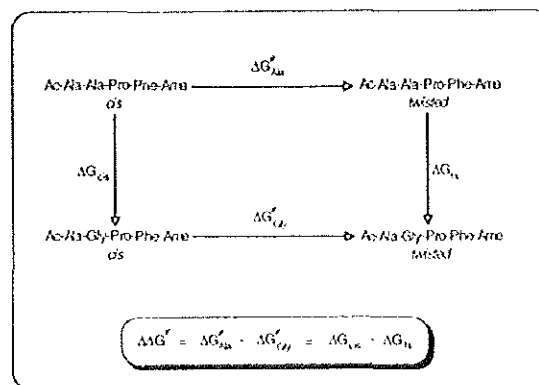


FIGURE 3: Thermodynamic cycle used to determine the $\Delta\Delta G^\ddagger$ of isomerization between the Ala and Gly peptides. Dark lines are the peptide and light lines are the rapamycin molecule.

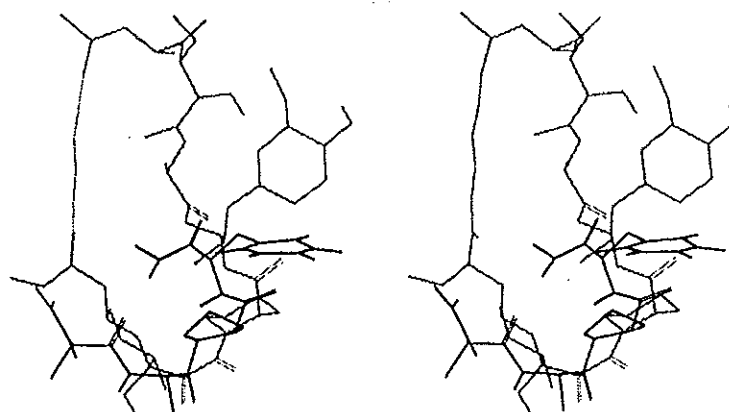
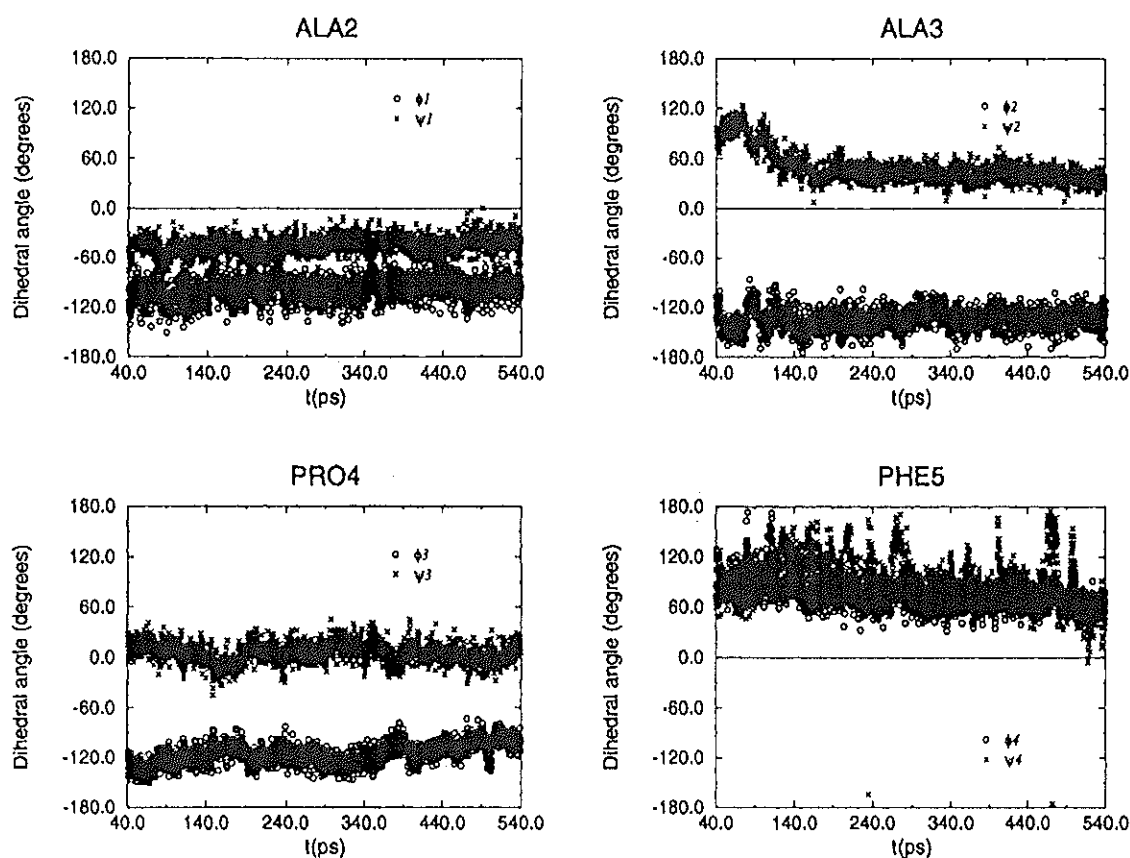
$$\Delta G = \sum_{\lambda_i=0}^{\lambda_i=1-\Delta\lambda} -kT \ln \left(\exp[-(V_{\lambda_i+\Delta\lambda} - V_{\lambda_i})/kT] \right)_{\lambda_i} \quad (1)$$

wide sampling windows of 5 ps each (2 ps of equilibration and 3 ps of averaging) for a total of 205 ps for each mutation. The entire peptide was considered as the perturbed group in the mutation; not only the intergroup interactions, but also all the intragroup nonbonded interactions (including 1-4) were considered in the evaluation of the free energy changes. We had determined in pilot calculations that the explicit inclusion of bonded contributions in the free energy calculation led to increased noise in the final free energies, but did not alter significantly the final results. The averaging in eq 1 was performed by MD simulations at constant $T = 298$ K, where T is the temperature, k is Boltzmann's constant, λ is the coupling parameter, which controls the evolution of the Ala → Gly mutation, and V_λ is the potential energy of the system.

A similar simulation was performed to determine whether or not the structure of the *cis* peptide was able to explain experimental binding data (Parker et al., 1992). Thus, the difference in free energy of binding between Ala and Gly (cis)peptides ($\Delta\Delta G_{\text{bind}}$) was determined as the difference between the free energy changes in the mutation Ala → Gly in water ($\Delta G_{\text{mut}}^{\text{water}}$), and in the protein complex ($\Delta G_{\text{mut}}^{\text{FKBP}}$). In order to compute $\Delta G_{\text{mut}}^{\text{water}}$ the Ala (cis)peptide in the conformation adopted inside FKBP at the end of the MD was introduced into a cubic box (edge ≈ 30 Å) of TIP3P water. The closest water molecules were removed to yield to a system with 807 water molecules. The system was minimized for 3000 cycles (2000 with the peptide fixed and 1000 with the entire system free). The system was heated from 198 to 298 K during 5 ps of NVT MD (during the first 3 ps the water was allowed to move), and equilibrated during 20 ps of NPT MD ($T = 298$ K; $P = 1$ atm). The free energy change for the Ala → Gly mutation was computed using 41 double-wide sampling windows of 2.5 ps (1.25 ps of equilibration and 1.25 ps of averaging) for a total of 102.5 ps. Periodic boundary conditions were used in all the simulations. All the remaining technical details of the simulations were identical to those noted above for the peptide-protein complexes.

RESULTS

Transition-State Structure. The structure of the twisted transition state model, which was obtained after the fitting, optimization, and equilibration process resembles the general

FIGURE 4: Superposition of the structures of bound rapamycin and the *twisted* peptide after the equilibration.FIGURE 5: Dihedral angle histories for the *twisted* peptide.

structure of rapamycin, as shown in Figure 4. The main chain of both molecules overlaps well, and the H-bond donors and acceptors are also in proximal positions. In particular, O(Ala3) is placed very close to the keto oxygen of rapamycin, and the Pro ring shares the space of the pipercolinyl ring.

Analysis of the 500 ps trajectory shows flexibility in the peptide, especially for the side chain of Phe5 and in the Ala3-Pro4 region. The histories for the main chain dihedral angles shown in Figure 5 reveal that most movements are short range oscillations, which do not lead to dramatic changes in the structure. However, there is also a systematic movement

occurring for the Ala3-Pro4 region in the 80-120 ps period, which leads to a significant change in the relative orientation of the peptide inside the protein, as shown in Figure 6. The conformation of the 60 and 540 ps structures are similar, as noted in Figures 5 and 6 with a general "turn" topology stabilized by one or two intrachain hydrogen-bonds. The largest differences between the 60 ps and 540 ps structures are in the positioning of the proline ring and Ala3-Pro4 with respect to the binding site (see Figures 6 and 7). The reduction in ψ for Ala3 after 60 ps secures the hydrogen bonds for the turn structure and leads to better positioning of the Ala3

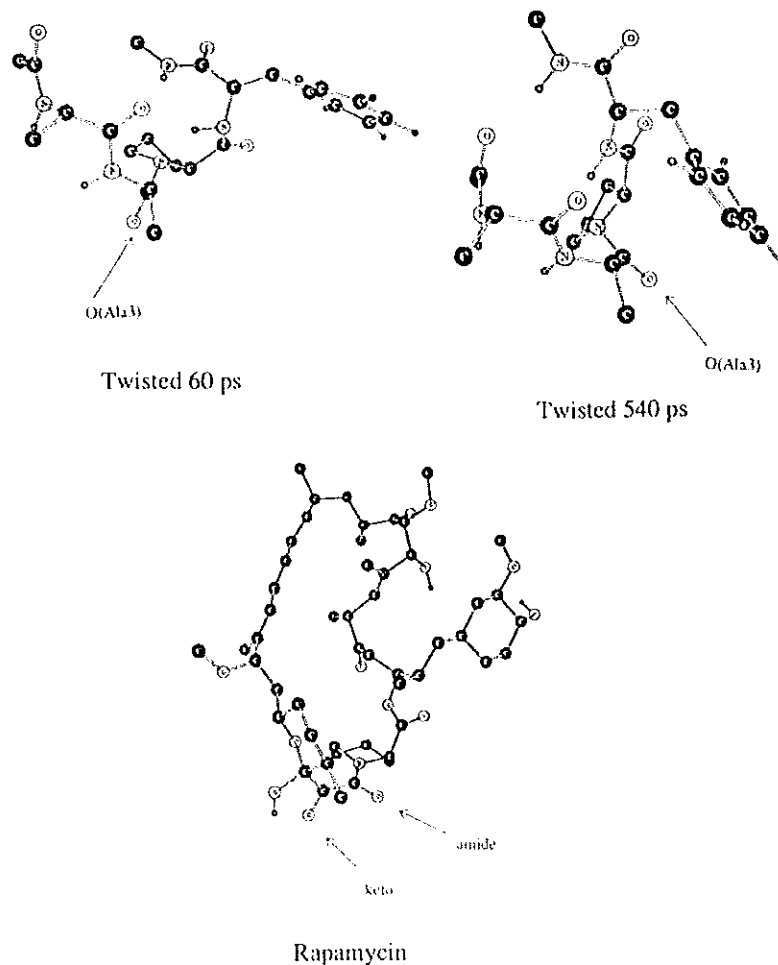


FIGURE 6: Representation of the structure adopted inside the binding site by rapamycin, and the *twisted* peptide at 60 and 540 ps. A common reference axis was used to orient the molecules.

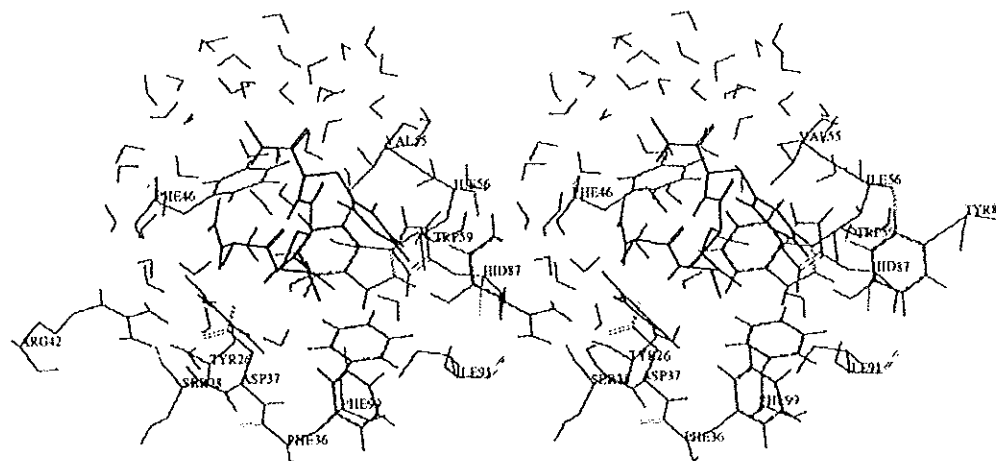


FIGURE 7: The *twisted* peptide bound to the active site of FKBP, carbonyl group with respect to the Asp³⁷ side chain (see below).

The distances for key interactions are shown in Figure 8. A hydrogen bond exists between the Asp³⁷ side chain and the amino groups of Ala2 and often Ala3, which are proximal (Figures 6 and 7). Two intrachain hydrogen bonds are also

common: Ame6-Ace1, and Phe5-Ala2, which stabilize the turn structure. Another interesting interaction is the close contact (ca. 2.0 Å) between the amide hydrogen of Phe5 and the N of Pro4. There are no hydrogen bonds between the oxygen of Ala3 and the protein. The analysis of Figure 8

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