Eur Biophys J (2007) 36:213–224
DOI 10.1007/s00249-006-0121-3

ORIGINAL PAPER

# A molecular dynamics study of Cyclophilin A free and in complex with the Ala-Pro dipeptide

**Pekka Mark · Lennart Nilsson**

Received: 29 August 2006 / Revised: 27 November 2006 / Accepted: 28 November 2006 / Published online: 16 January 2007
© EBSA 2007

**Abstract** Six different molecular dynamics simulations of Cyclophilin A, three with the protein free in water and three with the Ala-Pro dipeptide bound to the protein, have been performed, and analysed with respect to structure and hydration of the active site. The water structure in the binding pocket of the free Cyclophilin A was found to mimic the experimentally obtained binding *cis* conformation of the dipeptide. Cyclophilin A is a peptidyl–prolyl *cis–trans* isomerase (PPIase), but the mechanism of the *cis/trans* isomerization is not exactly clear. This study was performed to understand better the binding between dipeptide and Cyclophilin A, but also two previously proposed isomerization mechanisms are discussed.

## Introduction

Cyclophilin A (CyPA) is a small, globular protein with several biological activities: it has peptidyl-prolyl *cis–trans* isomerase (PPIase) activity (Fischer et al. 1989; Takahashi et al. 1989); in complex with the immunosuppressant drug cyclosporin A (CsA), which it binds with high affinity, it has been found to block the activation of quiescent T cells; it inhibits the in vitro activity of a Ser/Thr phosphatase, calcineurin (CN) (Braun et al. 1995); it has been reported to bind human immunodeficiency virus type 1 (HIV-1) gag protein (Gamble et al. 1996; Yoo et al. 1997; Zhao et al. 1997) to be required for the infectious activity of HIV-1 virions, where it is the first protein found to be incorporated into the virion during virus assembly and to perform an important function in the next cycle of viral infection, but the exact role of CyPA in viral infection is still unclear (Franke et al. 1994; Thali et al. 1994; Ackerson et al. 1998; Sherry et al. 1998). Similar functions have been identified in other proteins, with PPIase activity in the FK506 binding protein (FKBP) and the parvulin protein families (Hennig et al. 1998; Göthel and Marahiel 1999; Pliyev and Gurvits 1999; Ivery 2000). The FKBP-FK506 complex blocks calcineurin and T cell activation at the same stage as the CyPA-CsA complex (Braun et al. 1995). Several structures of CyPA (Ke 1992; Ottiger et al. 1997), and other cyclophilins (Ke et al. 1991; Clubb et al. 1994; Mikol et al. 1998), with various ligands (Kallen et al. 1991, 1998; Kallen and Walkinshaw 1992; Spitzfaden et al. 1992, 1994; Ke et al. 1993a, b, 1994; Mikol et al. 1993, 1994; Pflügl et al. 1993, 1994; Theriault et al. 1993; Konno et al. 1996; Zhao and Ke 1996a, b; Taylor et al. 1997) have been determined, and there are a number of biochemical investigations, using wild-type and mutant forms of CyPA, into the binding of cyclosporin A (Cardenas et al. 1995; Scholz et al. 1997, 1999), as well as of the peptidyl-prolyl *cis–trans* isomerase activity of human Cyclophilin A (Zydowsky et al.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00249-006-0121-3) contains supplementary material, which is available to authorized users.

P. Mark · L. Nilsson (✉)
Department of Biosciences and Nutrition,
Karolinska Institutet, 141 57 Huddinge, Sweden
e-mail: lennart.nilsson@biosci.ki.se

*Present Address:*
P. Mark
School of Biotechnology, The Department of Theoretical Chemistry, Royal Institute of Technology, AlbaNova University Center, 106 91 Stockholm, Sweden

1992). The mechanism of the *cis–trans* isomerization of the peptidyl-prolyl bond is not exactly clear, but several mechanisms have been proposed (Fischer et al. 1993; Ke et al. 1993a; Orozco et al. 1993; Konno et al. 1996; Zhao and Ke 1996a, b; Eisenmesser et al. 2002; Hur and Bruice 2002).

We have performed molecular dynamics simulations of Cyclophilin A when free and complexed with an Ala-Pro dipeptide in water, starting from the Ala-Pro dipeptide binding conformation of the human Cyclophilin A, which was obtained with X-ray crystallography (Ke et al. 1993a). The simulations are analysed in terms of structure, hydration and flexibility around the active site. To allow a more precise determination of the water structure in the binding pocket one of the three simulations of Cyclophilin A free in water was performed with the protein structure restrained to the start conformation, allowing only the solvent molecules around the protein to move freely. It should be noted that experimentally obtained rates of the catalysis are five orders of magnitude slower than the simulation time used in this study, so even though the motions found in different trajectories may be responsible for enabling isomerization, they do not show the whole mechanism.

This study was performed to better understand the binding of *cis* conformation of the Ala-Pro dipeptide to Cyclophilin A. Our interest was also to study the hydration around the empty active site and investigate if the water structure in the empty active site can provide structural information of relevance for the characterization of a putative ligand.

## Methods

All molecular dynamics simulations and analysis were performed using the CHARMM program (Brooks et al. 1983) with the all-atom CHARMM 22 force field for proteins (MacKerell et al. 1998) and the SPC/E water model (Berendsen et al. 1987). The starting coordinates for the Cyclophilin A protein and the bound Ala-Pro dipeptide were obtained from the PDB code 2cyh (Ke et al. 1993a). Human Cyclophilin A consists of 165 amino acid residues, but in the crystal structure the first methionine residue was missing. This residue was also included in the starting structure in all simulations and was modelled to have approximately the same conformation as obtained with NMR (Ottiger et al. 1997). Molecular dynamics simulations were carried out in the NVT ensemble, using a cubic simulation cell with 60 Å side length and periodic boundary conditions. To obtain a neutral total charge of the simulated system one chloride counterion was included. The particle-mesh Ewald method (van Gunsteren and Berendsen 1990; Darden et al. 1993) was used to calculate the long-range interactions using the following parameters: $r_c$ = 12.0 Å, $\kappa$ = 0.34 Å$^{-1}$and a grid spacing of 1 Å. The nonbonded list was generated with 14.0 Å list size and was updated when any atom had moved 1 Å or more since the last list update. The simulation temperature was controlled with the Hoover extended system constant temperature algorithm (Nosé 1984, 1990; Hoover 1985) using a thermal piston with a ''mass'' of 1,000 kcal mol$^{-1}$ ps$^2$. In all simulations, the SHAKE algorithm (Ryckaert et al. 1977) was used to keep the water molecules rigid and to constrain all hydrogen atom-heavy atom bond lengths of Cyclophilin A and dipeptide. Integration of Newton' s equations of motion was carried out with the Verlet leap-frog algorithm with a time step of 2 fs (van Gunsteren and Berendsen 1990). The dielectric constant was 1.0 and all coordinates were saved every 100 steps.

Six different MD simulations were performed at 298 K using a solvent density of 0.998 g/cm$^3$ in a cubic box with periodic boundary conditions (Table 1). Three simulations with the Cyclophilin A free in water and three simulations with the complex of Cyclophilin A and the Ala-Pro dipeptide in water were performed. The solute (CyPA or CyPA:Ala-Pro), together with the crystallographically determined water molecules, was centred in the box containing 7,208 water molecules, and then all water molecules that had the oxygen atom closer than 2.5 Å from any solute heavy atom or a crystal water were removed.

Two different minimization schedules were used:

(a) Harmonic restraints (20.0 kcal mol$^{-1}$ Å$^{-1}$) on all protein, dipeptide and crystal water atoms and minimization with the steepest descents method (Brooks et al. 1983) for 100 steps. Harmonic restraints (10.0 kcal mol$^{-1}$ Å$^{-1}$) on all protein, dipeptide and crystal water atoms and minimization with the adopted-basis set Newton–Raphson (ABNR) method (Brooks et al. 1983) 100 steps. Thereafter two rounds of 100 steps ABNR minimization with harmonic restraints (5.0 kcal mol$^{-1}$ Å$^{-1}$) applied in the first round to all protein and dipeptide atoms and to the backbone atoms only in the second round. Finally 100 steps of ABNR minimization without any restraints.
(b) Harmonic restraints (20.0 kcal mol$^{-1}$ Å$^{-1}$) on all protein, dipeptide and crystal water atoms and minimization with the steepest descents method

**Table 1** Systems simulated

| Simulation | Minimization[a] | Solute | Simulation period (ns) | Pressure[d] (atm) | Potential energy[d] (kcal/mol) |
|---|---|---|---|---|---|
| 1 | A | CyPA | 4.0[b]/3.0[c] | –149.5 (50.2) | –73958.6 (64.4) |
| 2 | B | CyPA | 4.0/3.0 | –151.7 (49.2) | –73971.4 (67.8) |
| 3 | A | CyPA | 0.68/0.6 | –194.2 (46.4) | –73750.5 (60.6) |
| 4 | A | CypA + Ala-Pro | 4.0/3.0 | –140.9 (48.3) | –74010.7 (68.3) |
| 5 | B | CyPA + Ala-Pro | 4.0/3.0 | –139.9 (50.8) | –73999.3 (67.0) |
| 6 | No (A) | CyPA + Ala-Pro | 3.6/3.6 | –136.5 (49.0) | –74020.9 (65.2) |

[a] See Methods

[b] Total time

[c] Time used for analysis

[d] The mean values with standard deviation (in parenthesis) calculated from averages over 2 ps over the analysed part of the simulation

for 100 steps followed by 200 steps of ABNR minimization with harmonic restraints (10.0 kcal $mol^{-1}$ $Å^{-1}$) on all protein, dipeptide and crystal water atoms.

After the minimization procedure, harmonic restraints (10.0 kcal $mol^{-1}$ $Å^{-1}$) on all solute atoms were applied and the system was heated to 298 K during 20 ps. During the first 90 ps of simulation the harmonic restraints were removed and the whole simulation system was free to move. Each of these simulations (Table 1; simulations #1, #2, #4 and #5) was 4.0 ns long and the first 1.0 ns was used for equilibration and was not included in the analysis. In one simulation (Table 1; simulation #3) of Cyclophilin A free in water, the protein was restrained to the starting structure with harmonic restraints (5.0 kcal $mol^{-1}$ $Å^{-1}$) during the whole simulation and only the solvent molecules were free to move. After the heating procedure a 600 ps long trajectory was produced. For one simulation (Table 1; simulation #6) of Cyclophilin A complexed with the Ala-Pro dipeptide in water, the torsion force constants were reduced on the peptide bond of the Ala-Pro dipeptide. This simulation was started after 800 ps of the trajectory from simulation #4 and the trajectory was extended to 3.6 ns.

Hydration of the Cyclophilin A was estimated using the average number of hydrogen bonds to water, and their average lifetime. The criteria used to define a hydrogen bond were: hydrogen to acceptor atom distance ≤2.4 Å and acceptor-hydrogen-donor angle ≥135°. All hydrogen bonds were calculated from the trajectory with a 5.0 ps time resolution.

Water distributions around Cyclophilin A were calculated using the oxygen atom on the water molecules. Each frame of the simulated trajectory was reoriented using the initial structure of the solute as a reference, and the population of water oxygen atoms around the solute molecule was calculated using a grid with volume elements of 0.5 Å side.

The self-diffusion coefficient for water was calculated from the mean square displacement of the oxygen atom of all water molecules using the Einstein relation (Allen and Tildesley 1987)

$$\left\langle (\mathbf{r}(t+t') - \mathbf{r}(t'))^2 \right\rangle \longrightarrow [t \to \infty]\, 6Dt$$

where $\mathbf{r}(t)$ is the position of the oxygen atom of a water molecule at time $t$, $D$ is the self-diffusion coefficient, and the brackets denote averaging over all water molecules and time origins $t'$. The self-diffusion coefficient was estimated from the slope of the linear part, at long times, of the mean square displacement versus time.

## Results

### Temperature, stability, and equilibration

In all six simulations the system temperature was controlled with the Hoover thermostat and stable trajectories with less than 1 Å root mean square deviation (RMSD) of the backbone atoms from the initial structure were produced with the exact target temperature of 298 K. (Table 1) (see Supplementary data for RMSD). Molecular dynamics simulations were carried out in the NVT ensemble without any pressure control. The slightly negative system pressure (~–150 atm) obtained in all simulations is smaller than typical instantaneous pressure fluctuations, and is not expected to significantly influence the result. The calculated self-diffusion coefficients for the water from all simulations were similar, $D \approx 2.5 \times 10^{-9}$ $m^2$ $s^{-1}$, which is slightly lower than in pure water simulations (Mark and Nilsson 2001a, 2002a). The two minimization procedures gave almost similar starting structures for the protein, 0.39 and 0.09 Å RMSD for all heavy atoms from the X-ray structure (Ke et al. 1993a) for minimization procedures A and B, respectively.

Cyclophilin A free in water

Two different unrestrained simulations of Cyclophilin A free in water were performed, and while it is clear from the low overall RMSD values that the conformation space of the protein was limited in the simulations, there was a certain amount of flexibility in the N- and C-termini and in loops as can be seen in snapshots of the protein structure at the end of the simulations (see Supplementary data).

When the binding pocket of the *cis* Ala-Pro dipeptide was examined more carefully side-chain conformational changes were observed when compared to the starting structure (Fig. 1). Residue Phe113, which is located in the bottom of the hydrophobic part of the binding pocket, was found to have a new conformation, which affected the shape of the binding pocket. Residues Phe60 and Trp121 are also part of the binding pocket and were found to make some smaller conformational changes. Lys125 is located close to the binding pocket and was observed to lose the hydrogen bond with the backbone O of Trp121, instead forming a new hydrogen bond to His126, which belongs to the hydrophobic part of the binding pocket as well. Gln63 at the bottom of the binding pocket lost the hydrogen bond with His54 and formed a new hydrogen bond with Gln111. Arg55, which makes specific hydrogen bonds to the Ala-Pro dipeptide was also found to have a new conformation. The Gly104 backbone NH in the loop region close to the binding pocket was observed to change the hydrogen bond from Asn102 to Thr107 due to loop movements in both simulations. All conformational changes mentioned above were found in both trajectories. All these conformational changes together make the binding pocket slightly larger when Cyclophilin A is free in water. As a simple measure of the size of the active site pocket we calculated average values over the simulations for three distances across the pocket (Arg55 $C_{\delta}$–His126 $C_{\delta 1}$ ca 9.6 Å, Met61 $S_{\delta}$–Asn102 O ca 10.6 Å, and Glu63 $O_{\varepsilon 1}$–Leu122 $C_{\gamma}$ ca 11.5 Å), and found the differences (0.1–0.3 Å) to be smaller than the observed error of the mean of ca 0.5 Å. A number of hydrogen bonds between different side-chains that were broken and formed again during the simulations were found on the protein surface.

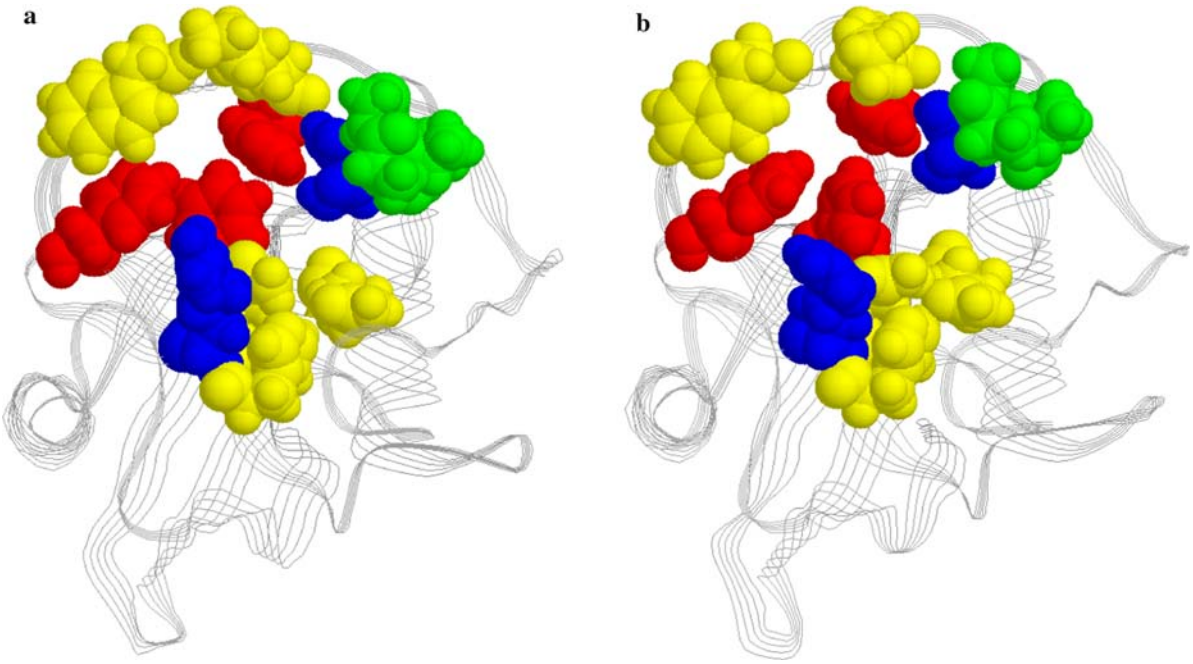


**Fig. 1** **a** Starting structure of Cyclophilin A free in water. **b** The last conformation from the simulations of Cyclophilin A free in water. Essential residues in the binding pocket of the cis isomer of the Ala-Pro dipeptide are shown in different *colours*. The hydrophobic part of the binding pocket including Phe60, Phe113 and His126 is coloured *red*. The residues making specific hydrogen bonds to the Ala-Pro dipeptide, Arg55 and Asn102 are coloured *blue*. The residues Ala103, Gly104 and Thr107 in the loop region close to the binding pocket are coloured *green*. The other residues close to the binding pocket, His54, Gln63, Gln111, Trp121 and Lys125, are coloured *yellow*. This figure, and Figs. 4 and 6, produced with the program RasMol (Sayle and Milner-White 1995)

### Cyclophilin A complexed with the Ala-Pro dipeptide in water

Residues Phe60, Met61, Phe113, Trp121, Leu122, and His126, form the hydrophobic part of the binding pocket where the proline residue of the *cis* Ala-Pro dipeptide is interacting with Cyclophilin A. Residues Arg55 and Asn102 make specific hydrogen bonds to the C- and N-termini of the Ala-Pro dipeptide, respectively. The side-chain of Arg55 makes hydrogen bonds to the C-terminal of the *cis* Ala-Pro dipeptide, whereas the backbone carbonyl of Asn102 hydrogen bonds to the N-terminal of the dipeptide. The complex is also stabilised by a hydrogen bond between the Arg55 and the residue Gln63, and by one water molecule that makes hydrogen bonds to the C- and N-termini of the dipeptide and the residue Gln63. All these interactions keep the Ala-Pro dipeptide bound to Cyclophilin A tightly as seen in the crystal structure 2cyh (Ke et al. 1993a) (Fig. 2).

Three different water simulations of Cyclophilin A complexed with the *cis* isomer of the Ala-Pro dipeptide were performed. Two of them were started with similar minimization procedures as Cyclophilin A free in water, and the third one, where the dihedral force constants were reduced for the peptide bond of the Ala-Pro dipeptide, was started after 800 ps trajectory from the simulation #4. In all these simulations the protein remained close to the starting structure (see Supplementary data for RMSD).

Similar side-chain conformational changes of Cyclophilin A were found in the binding pocket of the *cis* Ala-Pro dipeptide when complexed with the dipeptide and compared to the simulations of Cyclophilin A free in water (Fig. 1). All these residues show similar movements in the different trajectories. The proline residue of the *cis* Ala-Pro dipeptide was interacting with the hydrophobic part of the binding pocket, and residues Arg55 and Asn102 were making specific hydrogen bonds to C-terminal and N-terminal of the *cis* Ala-Pro dipeptide, respectively. The complex with Cyclophilin A and the *cis* isomer of the Ala-Pro dipeptide was also stabilised by a hydrogen bond between Arg55 and Gln63 in all three complex simulations. This hydrogen bond was not found in the simulations of Cyclophilin A free in water. The residue Gln63 also lost the hydrogen bond with His54 and formed a new hydrogen bond with Gln111 as seen in simulations of Cyclophilin A free in water. Residue Phe113 remained in the initial conformation as long as the *cis* Ala-Pro dipeptide was tightly bound to Cyclophilin A. When the N-terminal of the *cis* Ala-Pro dipeptide was found to break the hydrogen bond with Cyclophilin A, Phe113 assumed a new conformation similar to that in the simulation of free Cyclophilin A. In simulation #5 the dipeptide was tightly bound to

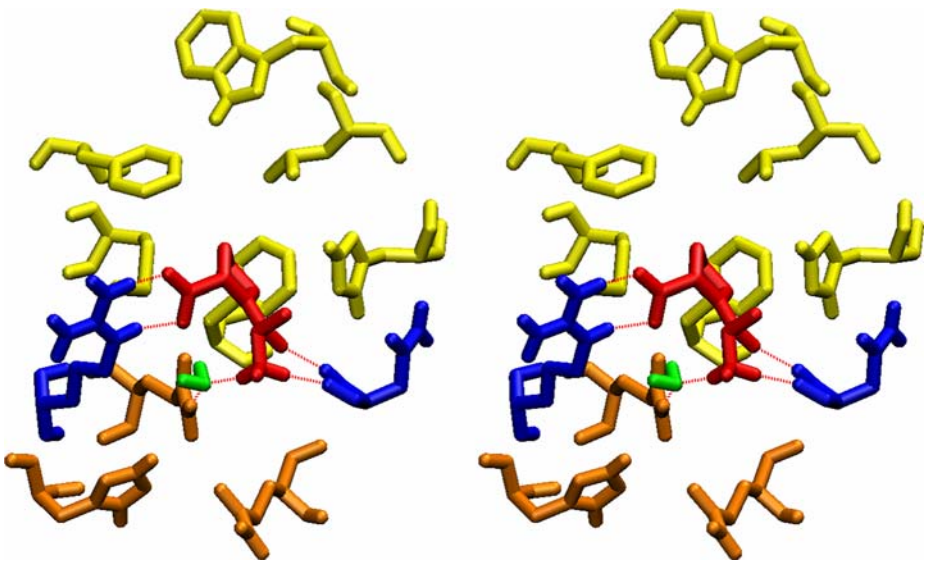

**Fig. 2** Stereo view showing essential residues in the binding pocket of cyclophilin A complexed with the cis isomer of the Ala-Pro dipeptide (*red*) (2cyh, Ke et al. 1993a). The hydrophobic part of the binding pocket including Phe60, Met61, Phe113, Trp121, Leu122 and His126 is coloured *yellow*. The residues Arg55 and Asn102 that make specific hydrogen bonds to the cis isomer of the Ala-Pro dipeptide are coloured *blue*, the water molecule making hydrogen bonds to Cyclophilin A and the dipeptide is coloured *green*, and the other residues in the binding pocket, His54, Gln63 and Gln111, are *orange*. Hydrogen bonds to the dipeptide are shown as *red dashed lines*. Figure produced with the program VMD (Humphrey et al. 1996)

CyPA during the whole 4.0 ns trajectory. In simulations #4 and #6 the N-terminal of the dipeptide was found to break the hydrogen bond with Cyclophilin A after about 3.0 and 2.0 ns, respectively.

For Lys125 a similar conformation as in the simulations of Cyclophilin A free in water was found, but no hydrogen bond with His126 was formed in simulations #4 and #5. In simulation #6 an interaction between the Lys125 side-chain NH3-group and Trp121 backbone carbonyl was found. The Gly104 backbone NH in the loop region close to the binding pocket was also found to switch the hydrogen bond from Asn102 to Thr107 due to loop movements in all complex simulations. The structural changes seen in the active site of Cyclophilin A in the presence of the Ala-Pro dipeptide were small; only Arg55 and Gln63 changed their conformations significantly and the rest of the active site was found to have quite similar conformation, thus the binding pocket of the *cis* Ala-Pro dipeptide seems to be pre-formed to fit the *cis* isomer of the Ala-Pro dipeptide and the active site is quite rigid.

The Ala-Pro dipeptide flexibility was reduced in comparison to the simulations of the *cis* Ala-Pro dipeptide free in water (Mark and Nilsson 2001b). The carboxylate, methyl and amino groups of the Ala-Pro dipeptide show limited rotations when bound to Cyclophilin A (Fig. 3). The binding conformation was

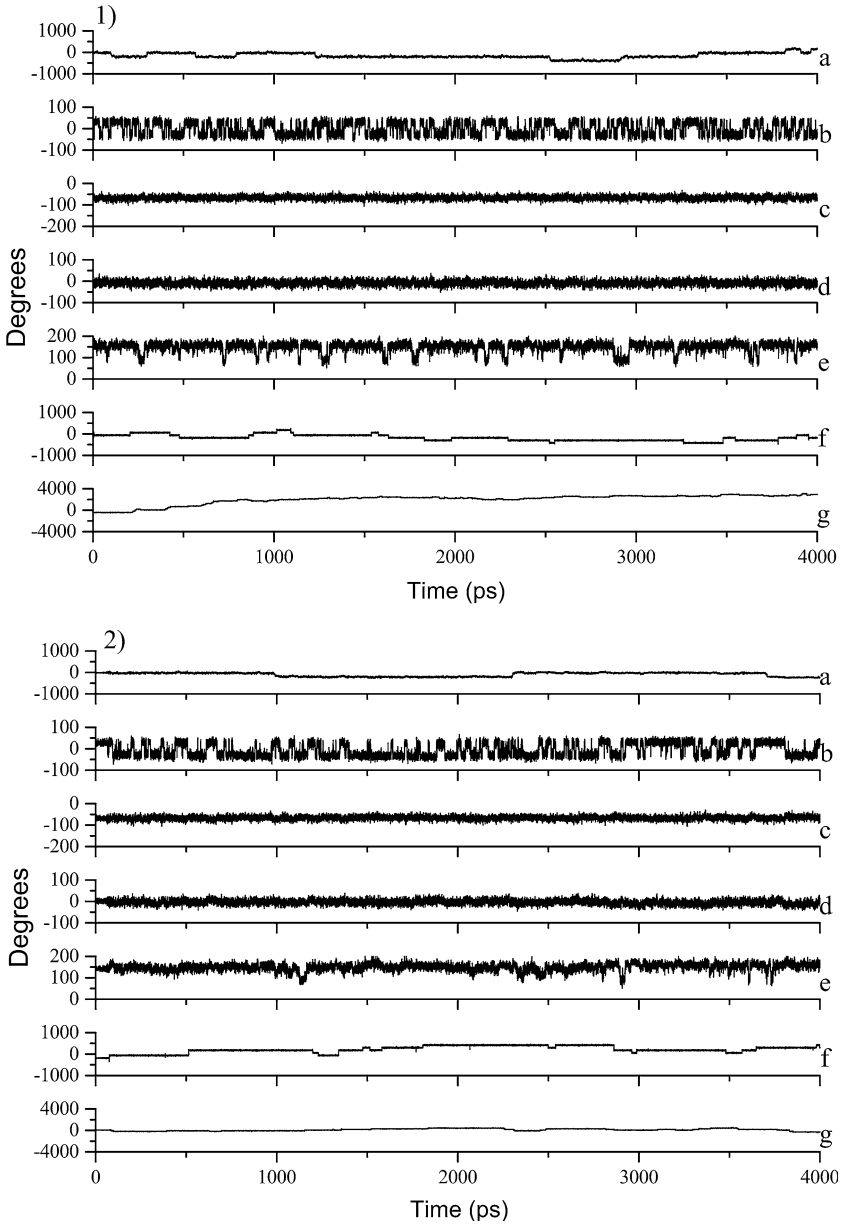


**Fig. 3** Torsion angles as a function of time for the Ala-Pro dipeptide. **1** Molecular dynamics simulation of the Ala-Pro dipeptide in water (Mark and Nilsson 2001b). **2** Molecular dynamics simulation of the Ala-Pro dipeptide complexed with Cyclophilin A in water, simulation #4. Key: *a* carboxylate group rotation, *b* proline puckering, *c* Phi ($\phi$), *d* peptide bond ($\omega$), *e* Psi ($\psi$), *f* methyl group rotation, *g* amino group rotation

found to be the major conformation of the *cis* isomer of the Ala-Pro dipeptide free in water. The minor conformation of the *cis* isomer observed for the Ala-Pro dipeptide free in water, where the N- and C-termini of the dipeptide form a salt bridge, was also obtained a few times when the *cis* Ala-Pro dipeptide was complexed with Cyclophilin A; the population of this conformation was, however, much smaller than in the *cis* isomer of the Ala-Pro dipeptide simulated free in water (see Fig. 3 dihedral e). The only difference between the two forms of the *cis* isomer is in the alanine backbone torsion angle $\varphi$ (90° and 155° for the minor and major conformations, respectively).

No spontaneous *cis*/*trans* isomerizations were observed for the *cis* Ala-Pro dipeptide, either free (Mark and Nilsson 2001b) or bound to CyPA.

## Cyclophilin A hydration

Hydration of the Cyclophilin A was calculated from all trajectories, using the last 600 ps of each one for investigation. All residues in the binding site of the *cis* isomer of the Ala-Pro dipeptide were found to be solvated to some extent. Trp121 was less solvated in the binding pocket compared to previous simulations of a free Trp in water (Mark and Nilsson 2002b), in part due to interactions between Trp121 and the side-chain phenol ring of Phe60. All calculated hydrogen bonds to water for the essential residues in the binding pocket of the *cis* isomer of the Ala-Pro dipeptide from simulations are given in Table 2. Residues Arg55, Gln63 and Asn102 were less hydrated when in the presence of the Ala-Pro dipeptide. In simulation #5 the dipeptide was tightly bound to Cyclophilin A and hydration of different residues in the binding pocket that were affected by the dipeptide binding are given in bold letters in Table 2. Several crystal waters were found close to the binding pocket after 3.4 ns simulations and these crystal waters are given in italic letters in Table 2. All these crystal waters are inside the protein and stabilizing side chain conformations in the binding pocket of the *cis* isomer of the Ala-Pro

**Table 2** Hydrogen bonds to water from Cyclophilin A

| Simulation | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Acceptor/donor | | | | | | |
| HIS 54 | | | | | | |
| HD1 | *0.93*[a]*/61.7*[b] | *0.78/27.6* | *1.0/600* | *0.92/61.1* | *0.63/22.1* | *0.90/49.1* |
| NE2 | 0.57/17.0 | 0.79/17.6 | 0.30/10.0 | 0.97/36.3 | 0.59/13.1 | 0.98/39.3 |
| O | 1.10/12.0 | 0.32/9.5 | 1.41/20.1 | 1.33/14.8 | 1.09/13.9 | 1.10/17.4 |
| ARG 55 | | | | | | |
| HE | 0.88/23.0 | 0.84/18.0 | 0.88/21.2 | 0.02/10.0 | **0.06/5.8** | 0.01/5.0 |
| HH11 | 0.88/18.9 | 0.93/25.5 | 0.94/70.6 | 0.93/28.0 | 0.93/22.2 | 0.93/24.1 |
| HH12 | 0.82/9.8 | 0.84/12.0 | 0.87/19.3 | 0.07/40.0 | 0.37/11.6 | 0.04/5.0 |
| HH21 | 0.89/11.1 | 0.86/12.0 | 0.99/35.0 | 0.01/5.0 | **0.13/8.0** | 0.01/5.0 |
| HH22 | 0.83/9.3 | 0.84/8.3 | 0.83/10.8 | 0.03/10.0 | **0.16/11.9** | 0.01/5.0 |
| GLN 63 | | | | | | |
| OE1 | 1.21/24.2 | 1.18/13.9 | 1.35/17.2 | 0.02/5.0 | **0.53/26.7** | 0/0 |
| HE2 | 0.44/6.5 | 0.44/7.2 | 0.47/8.8 | 0.62/10.3 | *0.27/26.7* | 0.61/8.7 |
| ASN 102 | | | | | | |
| HN | 0.23/7.9 | 0.39/13.1 | 0.68/17.1 | 0.54/12.0 | **0/0** | 0.29/21.9 |
| OD1 | 0.95/14.6 | 0.58/9.3 | 1.43/16.1 | 1.56/17.0 | 0.81/21.1 | 1.26/19.9 |
| HD21 | 0.17/6.3 | *0.57/12.1* | 0/0 | 0.02/5.0 | 0.02/5.0 | 0.04/6.3 |
| HD22 | *0.13/6.2* | 0.02/5.0 | *0.87/32.5* | *0.68/15.8* | *0.52/11.1* | *0.58/9.2* |
| O | 1.27/9.9 | 1.19/9.4 | 1.24/7.8 | 1.59/9.9 | **0.16/5.9** | 0.85/10.0 |
| GLN 111 | | | | | | |
| HN | 0.68/13.2 | 0.88/37.5 | 0.47/15.6 | 0.65/19.5 | 0.80/19.2 | *0.91/49.5* |
| OE1 | 0.77/12.1 | 0.51/11.7 | 1.0/33.3 | 0.49/7.0 | 0.95/14.3 | 0.16/6.8 |
| O | *0.80/24.0* | *0.85/26.8* | *0.80/40.0* | *0.78/23.5* | *0.66/18.8* | *0.90/41.5* |
| TRP 121 | | | | | | |
| HE1 | 0/0 | 0.12/5.8 | 0.03/5.0 | 0.02/5.0 | 0.42/8.6 | 0.02/5.0 |
| O | 1.38/12.2 | 1.23/10.2 | 0.02/5.0 | 1.32/10.5 | 1.37/9.5 | 0.76/11.7 |
| HIS 126 | | | | | | |
| NE2 | 0.81/15.6 | 1.05/16.6 | 1.73/17.0 | 1.33/22.1 | 0.97/38.7 | 1.24/33.9 |
| O | *0.91/38.9* | *0.98/117.0* | *0.87/12.1* | *0.99/297.5* | *0.96/95.8* | *1.0/600* |

Crystal waters in italic letters. Hydration affected by dipeptide binding in bold letters

[a] Average number

[b] Average lifetime (ps)

**Table 3** Hydrogen bonds to water from the Ala-Pro dipeptide

| Simulation | 4 | 5 | 6 |
|---|---|---|---|
| Acceptor/donor | | | |
| Ala 1 | | | |
| HT1 | 0.93[a]/15.1[b] | 0.35/14.0 | 0.83/16.0 |
| HT2 | 0.88/12.6 | 0.78/17.9 | 0.88/21.0 |
| HT3 | 0.94/14.1 | 0.63/34.1 | 0.72/13.0 |
| O | 0.98/10.4 | 0.01/5.0 | 0.65/12.6 |
| Pro 2 | | | |
| OT1 | 1.98/17.8 | 1.06/22.7 | 1.80/18.0 |
| OT2 | 2.14/21.8 | 2.29/21.5 | 2.38/15.5 |

[a] Average number

[b] Average lifetime (ps)

dipeptide. Hydration of the dipeptide (Table 3) was similar as when it was simulated free in water (Mark and Nilsson 2001b).

In simulation #3 the protein was kept rigid. This simulation was performed to give a more well-defined water structure in the binding pocket in the absence of bound ligand. Hydration of Cyclophilin A was found to not depend on the protein flexibility. The Cyclophilin A was similarly hydrated when the simulation of the rigid protein was compared to the two other simulations. The number of water molecules making hydrogen bonds with the rigid protein was very similar when the calculations of the flexible protein model were compared.

The flexible protein model may give a more dynamic hydration shell, but interactions between protein and surrounding water molecules were not much affected. The lifetimes of the hydrogen bonds were found to be longer in this study when compared with our previous study of the free Ala-Pro dipeptide (Mark and Nilsson 2001b). When the hydration of the binding pocket of the Ala-Pro dipeptide was determined from the simulation with the rigid protein, the water structure in the binding pocket was found to mimic the conformation of the *cis* isomer of the Ala-Pro dipeptide (Fig. 4). The water structure in the binding pocket for the Ala-Pro dipeptide was similar in the simulations with the protein restrained and unrestrained, but the water structure was not as well defined from simulations with the flexible protein, due to the motions of the protein (Fig. 4).

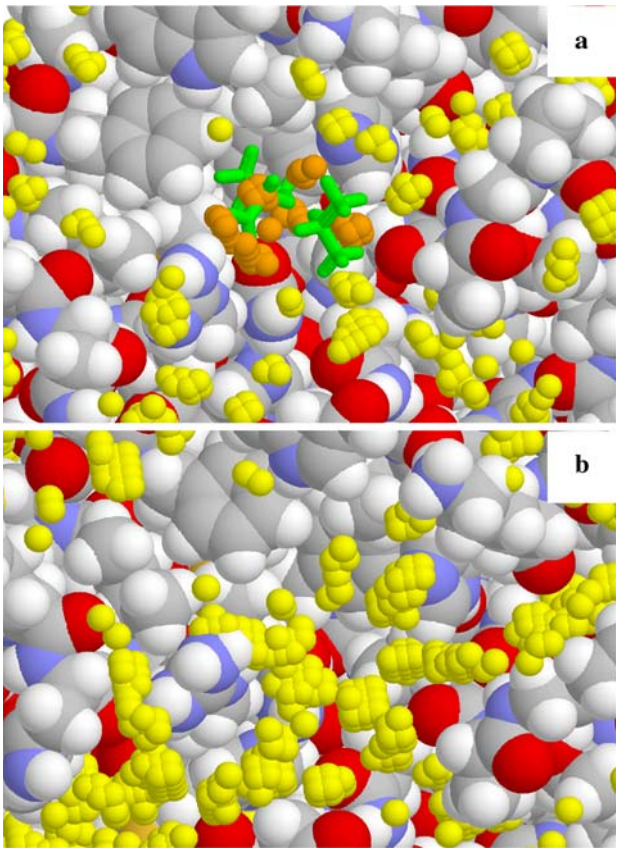


**Fig. 4** **a** The most populated average water structure in the binding pocket mimic the experimentally obtained binding conformation of the Ala-Pro dipeptide, namely the cis isomer. The *cis* isomer of the Ala-Pro dipeptide shown in *green*. The most populated water sites that mimic the cis isomer of the Ala-Pro dipeptide in the binding site are shown with *orange spheres*. All these water molecules are replaced when the cis isomer of the Ala-Pro dipeptide is binding to Cyclophilin A. The most populated other water sites are shown as *yellow spheres*. **b** The most populated average water structure in the binding pocket calculated from simulation #1 with the flexible protein. The most populated water sites are shown as *yellow spheres*

*Cis/trans* isomerization mechanism of Cyclophilin A

Many different isomerization mechanisms have been proposed in the literature (Fischer et al. 1993; Ke et al. 1993a; Orozco et al. 1993; Konno et al. 1996; Zhao and Ke 1996a; 1996b; Eisenmesser et al. 2002; Hur and Bruice 2002). In one of these mechanisms (Hur and Bruice 2002), based on a structure where the C-terminal of the peptide together with the proline residue are bound to Cyclophilin A, it is the N-terminal peptide segment that switches conformation in the transition from *cis* to *trans* isomer, but also a totally opposite reaction trajectory that involves a rotation of the C-terminal peptide segment around the prolyl peptide bond while the N-terminal part remains bound to Cyclophilin A has been suggested (Eisenmesser et al. 2002).

Simulations #4 and #5 with Cyclophilin A complexed with the *cis* isomer of the Ala-Pro dipeptide did not give any indication of the *cis*/*trans* isomerization mechanism of the Ala-Pro dipeptide supported by Cyclophilin A. In both simulations #4 and #5 the

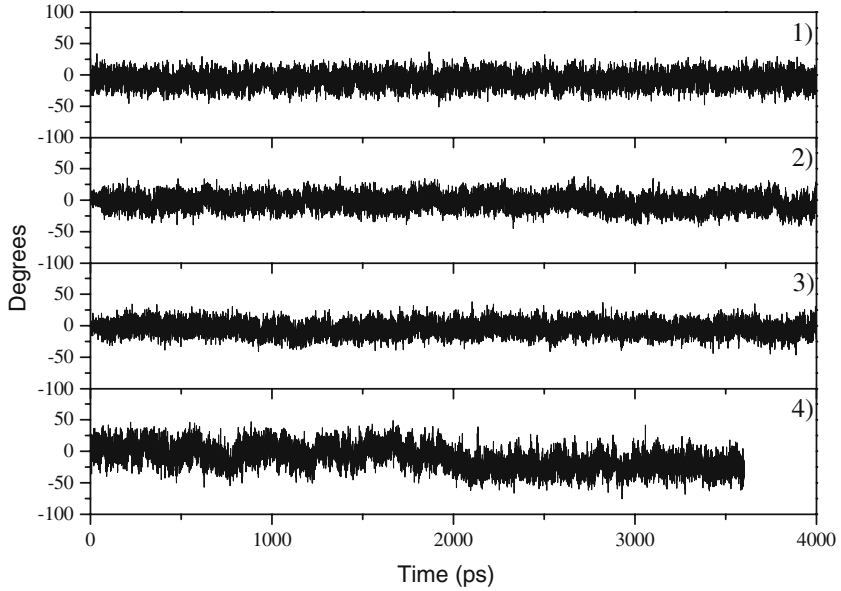


**Fig. 5** The peptide bond torsion angle as a function of time for the Ala-Pro dipeptide. *1* Molecular dynamics simulation of the Ala-Pro dipeptide in water (Mark and Nilsson 2001b). *2* Molecular dynamics simulation of the Ala-Pro dipeptide complexed with Cyclophilin A in water, simulation #4. *3* Molecular dynamics simulation of the Ala-Pro dipeptide complexed with Cyclophilin A in water, simulation #5. *4* Molecular dynamics simulation of the Ala-Pro dipeptide complexed with Cyclophilin A in water, simulation #6. The force constants were reduced from the peptide bond of the Ala-Pro dipeptide in simulation #6

dipeptide stayed in its starting conformation during the whole simulation time (4 ns) (Fig. 5), but in simulation #4 the N-terminal of the dipeptide was breaking the hydrogen bond with Cyclophilin A after about 3.0 ns and the N-terminal of the dipeptide became completely solveted with water. In simulation #6 the dihedral force constants were reduced for the peptide bond of the *cis* Ala-Pro dipeptide, and here we observed increased fluctuations of the peptide bond torsion angle in the dipeptide (Fig. 5). In this simulation too, the N-terminal of the dipeptide was breaking the hydrogen bond with Cyclophilin A after about 2.0 ns, and the N-terminal of the dipeptide became completely solvated. After the N-terminal of the dipeptide lost the hydrogen bond with Cyclophilin A, residue Arg55 was found to support the Ala-Pro dipeptide *cis/trans* isomerization. The side chain of Arg55 was making a hydrogen bond with the carbonyl oxygen atom in the Ala-Pro peptide bond (Fig. 6), an interaction that is favourable for the transition of the dipeptide to the *trans* isomer. The possibility of the N-terminal of the Ala-Pro dipeptide in the *cis* conformation to break the hydrogen bonds with Cyclophilin A in favour of solvation by surrounding water molecules together with interactions between Arg55 and the carbonyl oxygen atom in the Ala-Pro peptide bond support a *cis/trans* isomerization mechanism where the proline residue and the C-terminal are locked to Cyclophilin A, and the N-terminal part of the dipeptide is rotated around the prolyl peptide bond (Hur and Bruice 2002). A part of this mechanism was found in our simulation, although the complete *cis/trans* isomerization was not observed.

The single point mutations Arg55Ala, Phe60Ala and His126Gln result in loss of more than 99% of the *cis/trans* isomerization activity for Cyclophilin A and the mutations Phe113Ala and Trp121Ala cause the loss of 97 and 91% of the *cis/trans* isomerization activity, respectively, when compared to the wild type protein (Zydowsky et al. 1992). All of these mutations probably affect the active site shape. Residues Arg55, Phe60, Phe113, Trp121 and His126 are essential for the *cis/trans* PPIase activity. The residues Phe60, Phe113, Trp121 and His126 together form an important part of the hydrophobic cavity where the proline residue of polypeptides is binding. The peptide bond has to be in the cis isomer for the residue preceding the proline to fit the active site and to make the specific hydrogen bonds with the residues Arg55 and Asn102. All these interactions stabilise the binding of the *cis* isomer of polypeptides with Cyclophilin A.

## Discussion

Simulations of Cyclophilin A free in water show that the binding site for the *cis* isomer of the Ala-Pro dipeptide is opening when Cyclophilin A is not com-

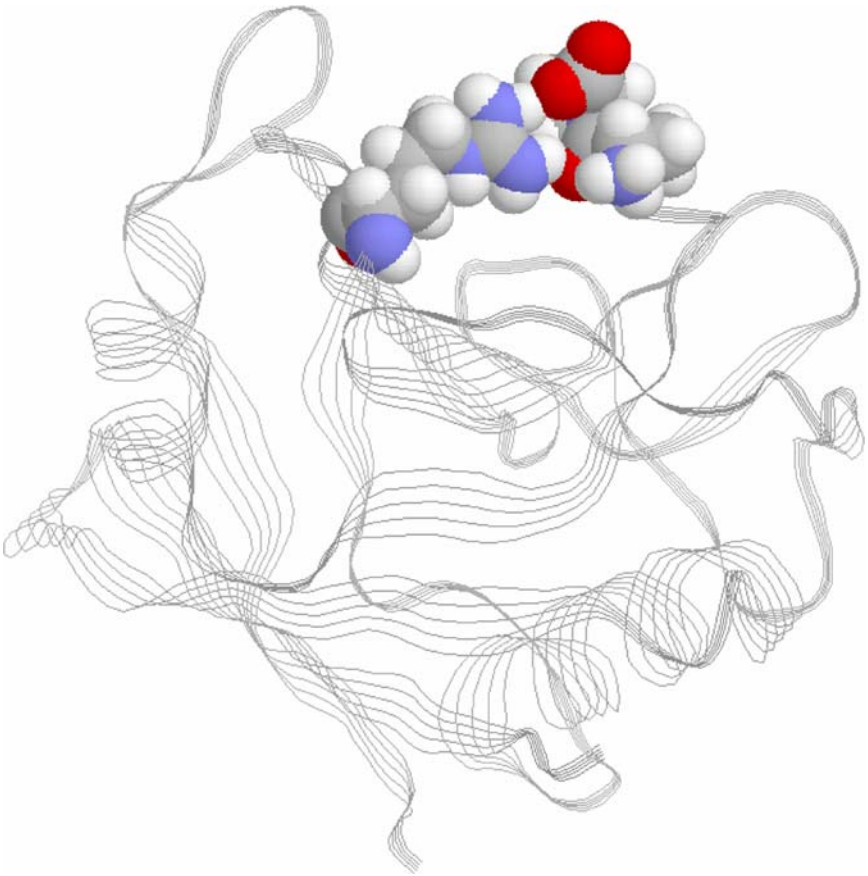

**Fig. 6** Snapshot of the Ala-Pro dipeptide conformation when residue Arg55 is hydrogen bonded with the carbonyl oxygen atom in the peptide bond of the Ala-Pro dipeptide

plexed with the dipeptide. The side-chain dynamics were collected during the nanosecond time scale and some new side-chain conformations were found in the binding site for the *cis* isomer of the Ala-Pro dipeptide. The water structure in the binding site for the *cis* isomer of the Ala-Pro dipeptide was calculated with the best precision when the rigid protein structure was used. The water structure in the binding pocket was found to mimic the experimentally obtained binding conformation of the Ala-Pro dipeptide, namely the *cis* conformation, an observation that may be important for drug design in the future.

When simulations of Cyclophilin A complexed with the cis isomer of the Ala-Pro dipeptide were compared with the simulations when Cyclophilin A free in water quite similar results were found; only residues Gln63, Arg55, Phe113 and Lys125 were found to change conformation when the Ala-Pro dipeptide was bound to Cyclophilin A.

In two of the complex simulations the N-terminal of the Ala-Pro dipeptide was found to loose its hydrogen bonds with Cyclophilin A and be completely exposed to the solvent. This flexibility of Cyclophilin A and the Ala-Pro dipeptide complex may explain a part of the possible *cis/trans* isomerization mechanism (Hur and Bruice 2002), in which the C-terminal part of the dipeptide together with the proline residue is locked to the Cyclophilin A binding site and the N-terminal part of the dipeptide is rotated around the prolyl peptide bond. Arg55 was found to support this *cis/trans* isomerization mechanism in our simulation and experimental mutation studies have also shown that Arg55 is essential for *cis–trans* peptidyl–prolyl isomerase activity (Zydowsky et al. 1992). Reducing the dihedral force constants from the peptide bond of the Ala-Pro dipeptide did not help to produce the whole isomerization procedure in our simulation. Even though the torsional fluctuations increased when the force constants were reduced (see Fig. 5), the isomerization barrier was still too high to be crossed over at room temperature. The experimental *cis* → *trans* rate constants for the isomerization of the peptide bond, Xaa-Pro in water is $2.5 \times 10^{-3}$ $s^{-1}$ at 25°C (Grathwohl and Wüthrich 1981) and the isomerization of the substrate, Suc-Ala-Phe-Pro-Phe–4-NA catalysed by Cyclophilin A (Eisenmesser et al. 2002) was calculated to have a rate constant of about 9,000 $s^{-1}$. The trajectories produced in this study were all 4.0 ns long and in comparison with the experimental rate constants the lack of isomerization step of the Ala-Pro dipeptide from the

trajectories was not a big surprise. These three complex simulations showed that the *cis/trans* isomerization of the Ala-Pro dipeptide was not happening spontaneously on a time scale of ns in a standard molecular dynamics simulation, but some motions that may be responsible for enabling isomerization could be found in different trajectories.

**Acknowledgments** We thank Dr. Jan Norberg for critical reading of the manuscript. This work was supported by the Magnus Bergvall Foundation, the Swedish Natural Science Research Council and the Swedish Research Council for Engineering Science.

## References

Ackerson B, Rey O, Canon J, Krogstad P (1998) Cells with high Cyclophilin A content support replication of human immunodeficiency virus type 1 gag mutants with decreased ability to incorporate Cyclophilin A. J Virol 72(1):303–308

Allen MP, Tildesley DJ (1987) Computer simulations of liquids. Oxford Science Publications, Oxford

Berendsen HJC, Grigera JR, Straatsma TP (1987) The missing term in effective pair potentials. J Phys Chem 91:6269–6271

Braun W, Kallen J, Mikol V, Walkinshaw MD, Wüthrich K (1995) Three-dimensional structure and actions of immunosuppressants and their immunophilins. FASEB J 9(1):63–72

Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M (1983) CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. J Comput Chem 4:187–217

Cardenas ME, Lim E, Heitman J (1995) Mutations that perturb Cyclophilin A ligand binding pocket confer cyclosporin a resistance in *Saccharomyces cerevisiae*. J Biol Chem 270(36):20997–21002

Clubb RT, Ferguson SB, Walsh CT, Wagner G (1994) Three-dimensional solution structure of *Escherichia coli* periplasmic cyclophilin. Biochemistry 33(10):2761–2772

Darden T, York D, Pedersen L (1993) Particle mesh Ewald: an Nlog(N) method for Ewald sums in large systems. J Chem Phys 98:10089–10092

Eisenmesser EZ, Bosco DA, Akke M, Kern D (2002) Enzyme dynamics during catalysis. Science 295(5559):1520–1523

Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX (1989) Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. Nature 337(6206):476–478

Fischer S, Michnick S, Karplus M (1993) A mechanism for rotamase catalysis by the FK506 binding protein (fkbr). Biochemistry 32(50):13830–13837

Franke EK, Yuan HE, Luban J (1994) Specific incorporation of Cyclophilin A into hiv-1 virions. Nature 372(6504):359–362

Gamble TR, Vajdos FF, Yoo S, Worthylake DK, Houseweart M, Sundquist WI, Hill CP (1996) Crystal structure of human Cyclophilin A bound to the amino-terminal domain of hiv-1 capsid. Cell 87(7):1285–1294

Grathwohl C, Wüthrich K (1981) NMR studies of the rates of proline *cis-trans* isomerization in oligopeptides. Biopolymers 20(12):2623–2633

Göthel SF, Marahiel MA (1999) Peptidyl-prolyl *cis-trans* isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci 55(3):423–436

Hennig L, Christner C, Kipping M, Schelbert B, Rücknagel KP, Grabley S, Küllertz G, Fischer G (1998) Selective inactivation of parvulin-like peptidyl-prolyl *cis/trans* isomerases by juglone. Biochemistry 37(17):5953–5960

Hoover WG (1985) Canonical dynamics: equilibrium phase-scape distribution. Phys Rev A 31(3):1695–1697

Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. J Mol Graph 14(1):33–38

Hur S, Bruice TC (2002) The mechanism of *cis–trans* isomerization of prolyl peptides by cyclophilin. J Am Chem Soc 124(25):7303–7313

Ivery MT (2000) Immunophilins: switched on protein binding domains? Med Res Rev 20(6):452–484

Kallen J, Walkinshaw MD (1992) The x-ray structure of a tetrapeptide bound to the active site of human Cyclophilin A. FEBS Lett 300(3):286–290

Kallen J, Spitzfaden C, Zurini MG, Wider G, Widmer H, Wüthrich K, Walkinshaw MD (1991) Structure of human Cyclophilin and its binding site for Cyclosporin A determined by x-ray crystallography and NMR spectroscopy. Nature 353(6341):276–279

Kallen J, Mikol V, Taylor P, Walkinshaw MD (1998) X-ray structures and analysis of 11 cyclosporin derivatives complexed with Cyclophilin A. J Mol Biol 283(2):435–449

Ke H (1992) Similarities and differences between human Cyclophilin A and other beta-barrel structures. Structural refinement at 1.63 A resolution. J Mol Biol 228(2):539–550

Ke H, Zydowsky LD, Liu J, Walsh CT (1991) Crystal structure of recombinant human t-cell cyclophilin a at 2.5 A resolution. Proc Natl Acad Sci USA 88(21):9483–9487

Ke H, Mayrose D, Cao W (1993a) Crystal structure of cyclophilin a complexed with substrate ala-pro suggests a solvent-assisted mechanism of *cis-trans* isomerization. Proc Natl Acad Sci USA 90(8):3324–3328

Ke H, Zhao Y, Luo F, Weissman I, Friedman J (1993b) Crystal structure of murine cyclophilin c complexed with immunosuppressive drug cyclosporin a. Proc Natl Acad Sci USA 90(20):11850–11854

Ke H, Mayrose D, Belshaw PJ, Alberg DG, Schreiber SL, Chang ZY, Etzkorn FA, Ho S, Walsh CT (1994) Crystal structures of cyclophilin a complexed with cyclosporin a and *n*-methyl-4-[(E)-2-butenyl]-4,4-dimethylthreonine cyclosporin a. Structure 2(1):33–44

Konno M, Ito M, Hayano T, Takahashi N (1996) The substrate-binding site in escherichia coli cyclophilin a preferably recognizes a cis-proline isomer or a highly distorted form of the *trans* isomer. J Mol Biol 256(5):897–908

MacKerell AD Jr, Bashford D, Bellott M, Dunbrack RL Jr, Evanseck JD, Field MJ, Fischer S, Gao J, Guo H, Ha S, Joseph-McCarthy D, Kuchnir L, Kuczera K, Lau FTK, Mattos C, Michnick S, Ngo T, Nguyen DT, Prodhom B, Reiher III WE, Roux B, Schlenkrich M, Smith JC, Stote R, Straub J, Watanabe M, Wiórkiewicz-Kuczera J, Yin D, Karplus M (1998) All-atom empirical potential for molecular modeling and dynamics studies of proteins. J Phys Chem B 102:3586–3616

Mark P, Nilsson L (2001a) Structure and dynamics of the TIP3P, SPC, and SPC/È water models at 298 K. J Phys Chem A 105(43):9954–9960

Mark P, Nilsson L (2001b) Molecular dynamics simulations of the ala-pro dipeptide in water: conformational dynamics of *trans* and *cis* isomers using different water models. J Phys Chem B 105(33):8028–8035

Mark P, Nilsson L (2002a) Structure and dynamics of liquid water with different long-range interaction truncation and

temperature control methods in molecular dynamics simulations. J Comput Chem 23(13):1211–1219
Mark P, Nilsson L (2002b) A molecular dynamics study of tryptophan in water. J Phys Chem B 106(36):9440–9445
Mikol V, Kallen J, Pflügl G, Walkinshaw MD (1993) X-ray structure of a monomeric Cyclophilin A–Cyclosporin A crystal complex at 2.1 A resolution. J Mol Biol 234(4):1119–1130
Mikol V, Kallen J, Walkinshaw MD (1994) X-ray structure of a Cyclophilin B/cyclosporin complex: comparison with Cyclophilin A and delineation of its calcineurin-binding domain. Proc Natl Acad Sci USA 91(11):5183–5186
Mikol V, Ma D, Carlow CK (1998) Crystal structure of the cyclophilin-like domain from the parasitic nematode brugia malayi. Protein Sci 7(6):1310–1316
Nosé S (1984) A molecular dynamics method for simulations in the canonical ensemble. Mol Phys 52:255–268
Nosé S (1990) Constant-temperature molecular dynamics. J Phys Condens Matter 2:115–119
Orozco M, Tirado-Rives J, Jorgensen WL (1993) Mechanism for the rotamase activity of FK506 binding protein from molecular dynamics simulations. Biochemistry 32(47):12864–12874
Ottiger M, Zerbe O, Güntert P, Wüthrich K (1997) The nmr solution conformation of unligated human Cyclophilin A. J Mol Biol 272(1):64–81
Pflügl G, Kallen J, Schirmer T, Jansonius JN, Zurini MG, Walkinshaw MD (1993) X-ray structure of a decameric cyclophilin-cyclosporin crystal complex. Nature 361(6407):91–94
Pflügl GM, Kallen J, Jansonius JN, Walkinshaw MD (1994) The molecular replacement solution and x-ray refinement to 2.8 A of a decameric complex of human cyclophilin a with the immunosuppressive drug cyclosporin a. J Mol Biol 244(4):385–409
Pliyev BK, Gurvits BY (1999) Peptidyl-prolyl *cis-trans* isomerases: structure and functions. Biochemistry (Mosc) 64(7):738–751
Ryckaert J-P, Ciccotti G, Berendsen HJC (1977) Numerical integration of the Cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. J Comput Phys 23:327–341
Sayle R, Milner-White EJ (1995) RasMol: biomolecular graphics for all. Trends Biochem Sci 20:374–376
Scholz C, Schindler T. Dolinski K, Heitman J, Schmid FX (1997) Cyclophilin active site mutants have native prolyl isomerase activity with a protein substrate. FEBS Lett 414(1):69–73
Scholz C, Maier P, Dolinski K, Heitman J, Schmid FX (1999) R73A and H144Q mutants of the yeast mitochondrial cyclophilin cpr3 exhibit a low prolyl isomerase activity in both peptide and protein-folding assays. FEBS Lett 443(3):367–369
Sherry B, Zybarth G, Alfano M, Dubrovsky L, Mitchell R, Rich D, Ulrich P, Bucala R, Cerami A, Bukrinsky M (1998) Role of Cyclophilin A in the uptake of hiv-1 by macrophages and T lymphocytes. Proc Natl Acad Sci USA 95(4):1758–1763
Spitzfaden C, Weber HP, Braun W, Kallen J, Wider G, Widmer H, Walkinshaw MD, Wüthrich K (1992) Cyclosporin A-cyclophilin complex formation. A model based on x-ray and NMR data. FEBS Lett 300(3):291–300
Spitzfaden C, Braun W, Wider G, Widmer H, Wüthrich K (1994) Determination of the NMR solution structure of the Cyclophilin A-Cyclosporin A complex. J Biomol NMR 4(4):463–482
Takahashi N, Hayano T, Suzuki M (1989) Peptidyl-prolyl *cis-trans* isomerase is the Cyclosporin A-binding protein Cyclophilin. Nature 337(6206):473–475
Taylor P, Husi H, Kontopidis G, Walkinshaw MD (1997) Structures of cyclophilin-ligand complexes. Prog Biophys Mol Biol 67(2–3):155–181
Thali M, Bukovsky A, Kondo E, Rosenwirth B, Walsh CT, Sodroski J, Göttlinger HG (1994) Functional association of cyclophilin a with hiv-1 virions. Nature 372(6504):363–365
Theriault Y, Logan TM, Meadows R, Yu L, Olejniczak ET, Holzman TF, Simmer RL, Fesik SW (1993) Solution structure of the Cyclosporin A/Cyclophilin complex by NMR. Nature 361(6407):88–91
Yoo S, Myszka DG, Yeh C, McMurray M, Hill CP, Sundquist WI (1997) Molecular recognition in the hiv-1 capsid/cyclophilin a complex. J Mol Biol 269(5):780–795
van Gunsteren WF, Berendsen HJC (1990) Computer simulation of molecular dynamics: methodology, applications, and perspectives in chemistry. Angew Chem Int Ed Engl 29:992–1023
Zhao Y, Ke H (1996a) Crystal structure implies that cyclophilin predominantly catalyzes the *trans* to *cis* isomerization. Biochemistry 35(23):7356–7361
Zhao Y, Ke H (1996b) Mechanistic implication of crystal structures of the cyclophilin-dipeptide complexes. Biochemistry 35(23):7362–7368
Zhao Y, Chen Y, Schutkowski M, Fisher G, Ke H (1997) Cyclophilin A complexed with a fragment of hiv-1 gag protein: insight into hiv-1 infectious activity. Structure 5(1):139–146
Zydowsky LD, Etzkorn FA, Chang HY, Ferguson SB, Stolz LA, Ho SI, Walsh CT (1992) Active site mutants of human cyclophilin a separate peptidyl-prolyl isomerase activity from cyclosporin a binding and calcineurin inhibition. Protein Sci 1(9):1092–1099