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The X-ray structure of a tetrapeptide bound to the active site of human cyclophilin A

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Human cyclophilin A (165 residues) has peptidyl-prolyl *cis-trans* isomerase activity. Here we report a high-resolution three-dimensional X-ray structure of a substrate, ac-Ala-Ala-Pro-Ala-amc (ac, acetyl; amc, amidomethylcoumarin) bound to the active-site of cyclophilin. The structure consisting of a dimer of complexes and 135 water molecules was refined to a crystallographic R-factor of 17.7% for all data in the range 8 Å–2.3 Å.

X-ray structure; *cis-trans* Isomerase; Cyclophilin

1. INTRODUCTION

cis-trans-Peptidyl-proline isomerases (PPIases) are a family of enzymes including cyclophilin and FK-binding protein (FKBP) which catalyse the *cis-trans* isomerisation of the Xaa-Pro amide bond. With model peptide substrates, the specificity of cyclophilin for the amino acid Xaa has been shown to be low [1]. Both FKBP and cyclophilin have been shown to accelerate the refolding of a number of proteins, presumably by catalysing the rate determining step of proline isomerisation [2,3]. Specific cellular targets of the PPIases are not yet known, but it has been suggested that they play a role in the folding of newly synthesised proteins or act as molecular switches [2–4]. The immunosuppressive drugs cyclosporin and FK506 bind to and inhibit the PPIase activity of their respective protein receptors cyclophilin and FKBP. Inhibition of the PPIase activity of the immunophilins is however not sufficient to cause immunosuppression [5–7]. The interaction between cyclophilin and cyclosporin is discussed in the following paper.

The PPIase activities of cylophilin and FKBP have been measured for model substrates like suc-Ala-Ala-Pro-Phe-pna (suc, succinyl; pna, *para*-nitroaniline) using a chymotrypsin-coupled assay [8,9] which relies on the fact that chymotrypsin cleaves off *p*-nitroaniline only when the Xaa-Pro amide is in the *trans* conformation. Cyclophilin is an efficient catalyst [9] and submicromolar amounts can cause a 6-fold speed-up of refolding of RNase T1 [2]. Cellular concentrations of cyclophilins could be as high as micromolar and various biological regulatory roles have been suggested for them [6]. A number of mechanisms for PPIase activity have been proposed based on various kinetic studies [7,9,10].

The details of the interactions between cyclophilin and the ac-Ala-Ala-Pro-Ala-amc substrate presented in this paper provide a clear definition of the active site of cyclophilin and should help explain the PPIase mechanism.

2. MATERIALS AND METHODS

X-ray diffraction data were collected on a FAST area detector using a rotating anode generator. Crystallization of the cyclophilin/tetrapeptide complex [11] and the architecture of cyclophilin [12] have already been reported. The structure was solved by MIR using 2 heavy atom derivatives. Model building was done using the program O [13] on an ESV10 graphics work station. Refinement (no non-crystallographic symmetry restraints) with XPLOR resulted in an R-factor of 17.7% for all data in the range 8.0 Å–2.3 Å (19689 reflections). The rms deviations for the C^{α} atoms of the two monomers after least squares superposition is 0.194 Å. A Luzatti plot indicates an accuracy of about 0.25 Å.

The model contains all atoms of the two enzymes (local dimer) per asymmetric unit. One full tetrapeptide (ac-Ala-Ala-Pro-Ala-amc) plus the Ala-amc part of a second disordered tetrapeptide were included for each cyclophilin molecule monomer, as well as 135 solvent molecules per dimer. The rms-deviations from ideal values for bond lengths and bond angles are 0.014 Å and 3.1°, respectively. The backbone torsion angles for all non-glycine residues are in allowed regions of a Ramachandran plot. Details of the structure solution by MIR and of the structure analysis will be published elsewhere.

3. RESULTS

There is an interesting stoichiometry of two cyclophilin molecules and four ac-Ala-Ala-Pro-Ala-amc molecules in the crystallographic asymmetric unit. The two independent cyclophilin molecules are related by a non-crystallographic dyad axis and each has one ac-Ala-

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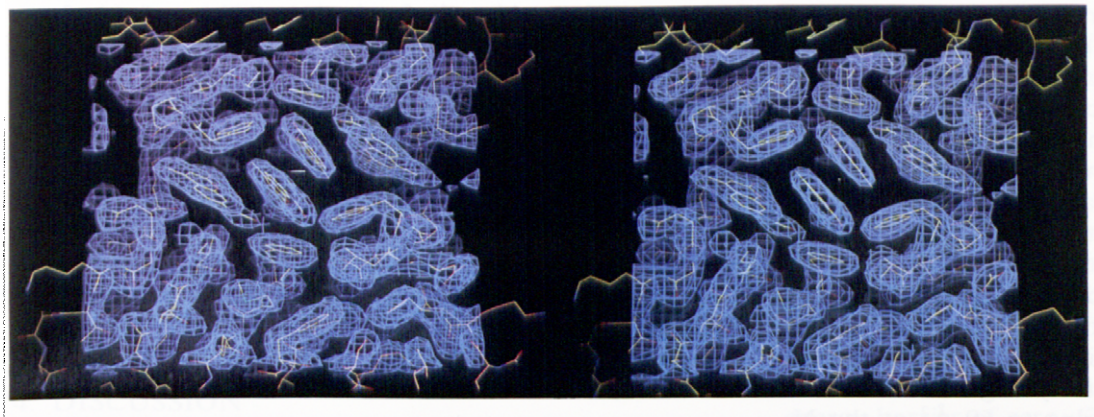

Fig. 1.

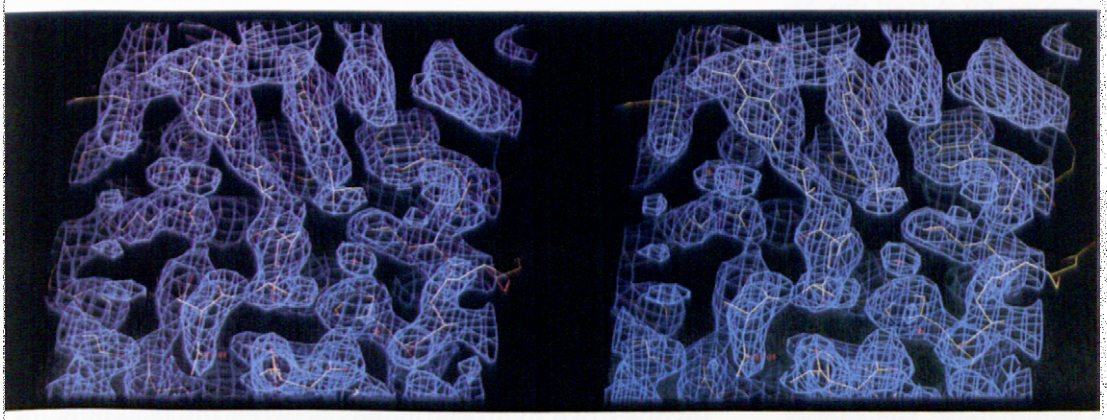

Fig. 2.

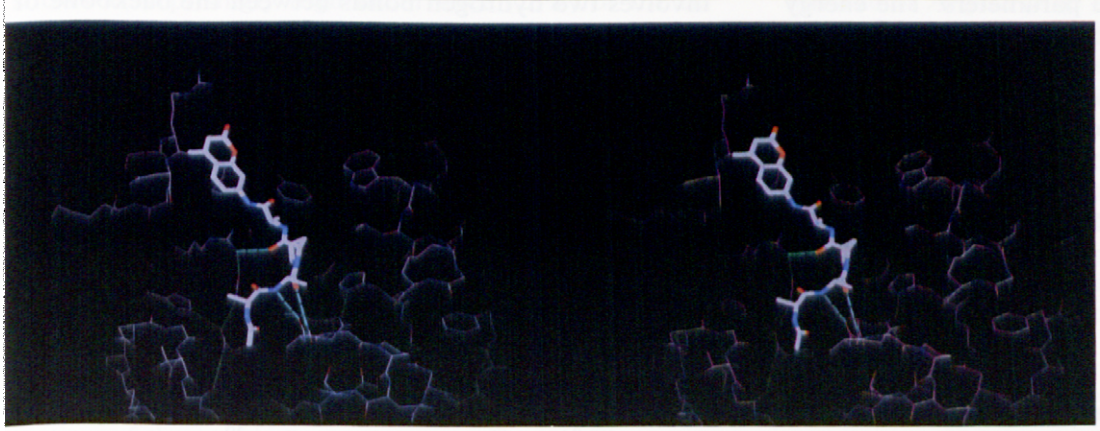

Fig. 3.

Fig. 1. View of the (2Fo-Fc)-electron density using all data from 8 Å–2.3 Å (contoured at 1σ) showing the dimer interface. The local dyad is indicated by the white line. Features roughly below the dyad, from left to right (belonging to one monomer) are: coumarin ring of ordered tetrapeptide, coumarin ring of disordered tetrapeptide and side chain of Trp-121.

Fig. 2. View of the (2Fo-Fc)-electron density (8 Å–2.3 Å, 1σ) for the ordered (coumarin ring on the upper left) and the disordered tetrapeptide (only atoms belonging to one monomer are shown in density). A simulated-annealing omit map (omitting the tetrapeptide) is very similar to the depicted electron density. Indicated in red are solvent molecules; they obey the local dyad symmetry (the two monomers were refined independently).

Fig. 3. A stereo-view of how the ordered tetrapeptide binds to the active site of cyclophilin. Important hydrogen bonds are indicated in green (dashed, intermolecular; undashed, intramolecular). We believe that the interactions with Arg-55 and Asn-102 (and maybe His-126) are important for the catalytic mechanism.

Ala-Pro-Ala-amc molecule bound in a *cis* conformation in its active site. Two additional partially disordered ac-Ala-Ala-Pro-Ala-amc molecules are accommodated in the crystal lattice with the four crystallographically independent planar coumarin groups stacking together (Fig 1), with interplanar distances between 3.6 and 3.8 Å. The Trp-121 residues from both cyclophilin molecules are also within van der Waals contact of two

coumarin groups and it is likely that this cluster of six aromatic groups (four coumarins and two indoles) induces the crystallographically observed dimer.

The X-ray structure of native cyclophilin has also been determined [14] and was crystallised with only one molecule in the asymmetric unit.

Both ac-Ala-Ala-Pro-Ala-amc molecules not bound in the enzyme active site are also related by the non-crystallographic twofold rotation axis, however only the Ala-amc part is visible in the electron density with the ac-Ala-Ala-Pro part disordered in the solvent. The enzyme-bound ac-Ala-Ala-Pro-Ala-amc molecules lie in well defined electron density (Fig. 2), though the temperature factors are relatively high and increase from 40 Å^2 in the coumarin ring to 60 Å^2 at the N-terminal end. These values compare with B-values of about 15 Å^2 for atoms in the protein active site.

3.1. *Substrate conformation*

The two independent enzyme-bound ac-Ala-Ala-Pro-Ala-amc molecules have very similar conformations and form very similar van der Waals and hydrogen bonded interactions with cyclophilin (the biggest differences are located in the ac-Ala part, correlating with the highest B values). Though the overall shape of the substrate is well defined in the electron density envelope, precise values of certain torsion angles are sensitive to the choice of force field parameters. The energy term for the *cis* Ala-Pro amide bond in XPLOR is $E=k(1+\cos(2\omega+180))$ kcal/mol. Refinement of the tetrapeptide was carried out with force constant values of $k=0$, 5, and 10 kcal/mol (corresponding to torsional barriers of 0, 10, and 20 kcal/mol, respectively) giving ω angles for substrate A of 45°, 30° and 22° respectively and ω angles for substrate B of 22°, 15° and 10°, respectively. The estimated standard deviation for these angles is between 4° and 10°. A list of substrate ϕ and ψ angles is given in Table 1. A number of protease X-ray structures have been examined as complexes with proline containing inhibitors [15]. In these cases the Xaa-Pro amide has always been found in the *trans* conformation. In the initial stages of model building of the cyclophilin-tetrapeptide complex, the *trans* peptide was fitted into the electron density [12], but subsequent refinement with additionally collected data to higher resolution clearly favoured the *cis* isomer.

3.2. *Description of the active site*

Cyclophilin residues which have one non-hydrogen atom within 3.8 Å of any non-hydrogen atom of the active site-bound ac-Ala-Ala-Pro-Ala-amc for both crystallographically independent complexes are :

Arg-55, Ile-57, Phe-60, Gln-63, Ala-101, Asn-102, Gln-111, Phe-113, Leu-122, His-126 and Arg-145. The enzyme active site as defined by this environment is a channel sitting on top of two antiparallel β strands (with contacts from residues Phe-60, Gln-63, Gln-111 and Phe-113). Loops protrude out from the surface of the barrel and provide a distinctive grooved protein surface. One loop from residues 101 to 110 contains the contact residues Ala-101 and Asn-102. A narrow pass separates this loop from a second loop (residues 69 to 74). The narrow pass between these two loops provides a possible location of the extended substrate binding site. The final important topological feature is the wall on one side of the binding site which is composed of residues 118–126 and has a close to helical conformation with Leu-122 and His-126 in contact with the peptide substrate. The *cis*-proline of ac-Ala-Ala-Pro-Ala-amc sits in a rather deep pocket made principally by Phe-60, Met-61, Phe-113 and Leu-122.

3.3. *Hydrogen bonding*

The active site has an interesting network of hydrogen bonds between amino acid side chains and a few solvent molecules. There are also three hydrogen bonds formed between the peptide and cyclophilin and they are shown in Fig. 3. The guanidinium group of Arg-55 forms a hydrogen bond to the carbonyl oxygen of Pro-3 in the peptide (distances Arg-55NH1–OPro-3 are 2.8 Å and 3.0 Å, respectively for the two momoners). It also makes a hydrogen bond to Gln-63 which in turn is hydrogen bonded to the side chain of Gln-111.

A possible recognition motif for the natural substrate involves two hydrogen bonds between the backbone of the substrate (Ala-2), and the backbone of Asn-102 (distances Asn-102O–NAla-2 are 3.4 Å and 3.1 Å, respectively, and distances Asn-102N–OAla-2 are 3.4 Å and 3.3 Å, respectively for the two monomers). The formation of a short stretch of antiparallel sheet is also found in some complexes of serine proteases [15,16].

Table 1

ϕ and ψ torsion angles for the two ordered tetrapeptides bound to the two crystallographically independent monomers (A,B)

Residues		ϕ, ψ (degrees)
Ala-1	A	. 36
	B	. 108
Ala-2	A	108, 134
	B	145, 130
Pro-3	A	72, 179
	B	73, 176
Ala-4	A	124, 66
	B	123, 78

The side chain of His-126 is close to the alanyl-prolyl *cis* amide bond. The X-ray refinement suggests a conformation in which the protonated His-126 acts as a hydrogen bond donor to carbonyl oxygen of Met-100 and to a solvent water molecule. This places $C\varepsilon^1$ of His-126 3.0 Å and 3.4 Å respectively away from the carbonyl oxygen of the *cis* amide. A rotation of 180° about the χ^2 angle of His-126 would permit a weak hydrogen-bond to be formed to the *cis*-amide carbonyl oxygen. (In our original modelling of the peptide in the *trans* configuration we postulated a water molecule bridge between His-126 and the peptide [12]).

4. DISCUSSION

The rotational energy barrier between *cis* and *trans* amides is estimated to be between 18–21 kcal/mol [17,18]. The energy difference between *cis* and *trans* isomers of *N*-methylacetamide has been calculated at 2.6 kcal/mol [17,18], while the corresponding difference between the *cis* and *trans* states for Xaa-Pro imide bonds is estimated to be only 0.5 kcal/mol [19]. This small energy difference converts to an expected 30–41% occurrence of the *cis* form. Experimentally, between 10 and 30% of imide bonds in oligopeptides are *cis*. A review of protein X-ray structures has shown that only 6.5% of all Xaa-Pro imide bonds are *cis*. This survey shows a surprisingly high occurrence (25%) of the *cis* form in the sequence Tyr-Pro which compares with only 3% (4 out of 120) for the sequence Ala-Pro [20].

Using an improved spectrophotometric assay with the substrate suc-Ala-Ala-Pro-Phe-pna, the steady state kinetic parameters for recombinant human cyclophilin are K_m=0.87 mM and k_{cat}=12,700/s [9]. The catalytic efficiency of cyclophilin is defined as the ratio of the rate constants for the *cis-trans* isomerisation with cyclophilin present (k_{cat}) to the rate constant for the uncatalysed process. The estimated catalytic efficiency for cyclophilin at 37°C is 2×10^5. This value implies that PPIases lower the *cis-trans* activation free energy by about 7 kcal/ mol at 0°C. As expected, the equilibrium concentration of the two isomers was not affected. There are still major questions to be answered about the catalytic mechanism, but some recent studies can exclude certain possibilities.

The involvement of cysteine was one of the first proposals [21] but kinetic [10] and mutagenesis [22] data show that the mechanism does not involve nucleophilic catalysis by an active-site thiol; indeed the cysteine nearest to the substrate in our structure is Cys-115 which is about 8 Å from the tetrapeptide Pro.

PPIase catalysis of suc-Ala-Ala-Pro-Phe-pna, has also been shown to be independent of pH between 5.5 and 9 [10]. Furthermore, the steady state kinetic parameters k_{cat} and K_m are the same in H_2O and D_2O [9] which also suggests that the catalytic mechanism does not involve the formation of covalent bonds to hydrogen as the rate limiting step [9]. Both these results would be inconsistent with general-acid/general-base catalysis. The significance of our finding that His-126 is in close proximity to the Ala-Pro amide group remains unclear.

The novel mechanism of catalysis by distortion has been proposed for both cyclophilin [22] and FKBP [23]. The hypothesis is that the PPIase would bind and stabilise the substrate with a twisted high energy conformation imide bond. It was also possible to describe the X-ray structure of FK506 complexed with FKBP as providing a mimic for the twisted amide transition state. The ω angles for the Ala-Pro amide bond in X-ray structures of cyclophilin-bound substrates have values between 20° and 45° when refined with an ω torsional energy barrier of zero. (This work and unpublished results from a related complex (J.K., S. Pfeffer, M.W.)). These values correspond to torsional energies of between 6 and 10 kcal/mol (assuming a *cis-trans* free energy barrier of 20 kcal/mol). An alternative mechanism has been proposed [9] in which the transition state is stabilised by protonation of or hydrogen bonding to the tetrahedral nitrogen lone pair formed as the amide is rotated. The suggested hydrogen bond donors being serine, threonine or tyrosine.

There are no serine, threonine or tyrosine residues near the proline nitrogen and the only possibility for hydrogen bond donors would be a water molecule or His-126 or Arg-55 (Fig. 3). (The water molecule hydrogen bonded to His-126 is some 4.5 Å away from the prolyl nitrogen atom.)

Substrate specificity for cyclophilin was tested using a series of peptides with the general structure suc-Ala-Xaa-Pro-Phe-pna and found to show little dependence on Xaa [10]. This may be rationalised by assuming that the binding conformations of these substrates are similar to that found in this peptide complex. This would point the Xaa towards the solvent (for certain side-chain torsion angles) without necessarily affecting binding to cyclophilin.

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