Volume 276, number 1,2, 63-66 FEBS 09168 December 1990

Crystallisation and preliminary X-ray diffraction studies of cyclophilin–tetrapeptide and cyclophilin–cyclosporin complexes

Mauro Zurini[1], Joerg Kallen[1], Vincent Mikol[1], Gaston Pfluegl[2], Johan N. Jansonius[2] and Malcolm D. Walkinshaw[1]

[1]*Preclinical Research, Sandoz Pharma AG, CH-4002 Basel* and [2]*Biocentre, University Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland*

Received 8 August 1990; revised version received 9 Oktober 1990

Recombinant human cyclophilin has been co-crystallised with a number of peptides to give crystals suitable for X-ray analysis. The crystal complexes for which heavy-atom derivatives have been prepared and X-ray data collected are: cyclophilin with *N*-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin (I) which crystallises in space group $P2_12_12_1$ with a = 108.2, b = 123.0, c = 35.8 Å, and cyclophilin with cyclosporin (II) which crystallises as tetragonal plates in space group $P4_12_12$ or $P4_32_12$ with a = b = 94.98, c = 278.55 Å.

Cyclophilin; Cyclosporin; Protein crystallisation; *Cis–trans* isomerase; Linear dichroism

1. INTRODUCTION

Cyclophilin is a ubiquitous cytosolic protein ($M_r = 18\,000$) with a high affinity for the immunosuppressant drug cyclosporin A (Sandimmun) [1]. Cyclophilin has also been shown to have peptidyl-prolyl *cis–trans* isomerase activity and can accelerate the refolding of several proteins in vitro [2,3].

Cyclosporin A (II) is a cyclic undecapeptide fungal metabolite which is used to prevent graft rejection after transplant surgery. The exact biochemical mechanism is unknown, though it inhibits the initial steps in T-cell activation and suppresses the expression of interleukin-2 [4]. It is still not proven that specific cyclosporin–cyclophilin binding is the principal mode of action for cyclosporin activity, though it has been shown that in lower eukaryotes *Neurospora crassa* and *Saccharomyces cerevisiae*, cyclophilin mediates a cytotoxic cyclosporin A effect [5]. It seems, therefore, that cyclophilin is indeed a specific and biologically relevant receptor for cyclosporin, but it is still difficult to explain how such a ubiquitous protein provides a specific target for an immunosuppressive drug in T-lymphocytes. It is also not yet clear how relevant the *cis–trans* isomerase activity is in the mechanism. This activity is inhibited by cyclosporin A, and it may be significant that the chemically unrelated immunosuppressant FK506 also inhibits the *cis–trans* prolyl isomerase activity of another cytosolic protein, macrophilin [6,7].

Correspondence address: M.D. Walkinshaw, Preclinical Research, Sandoz Pharma AG, CH-4002 Basel, Switzerland

The 3-D structure of cyclophilin and its complexes with peptides, including cyclosporin A, should provide an insight into the mechanism of enzymatic action and also provide a template for the design of novel drugs which may mimic or improve the action of cyclosporin A.

2. MATERIALS AND METHODS

2.1. *Crystallisation*

Large quantities of pure recombinant human cyclophilin were obtained using a novel affinity chromatography procedure [8]. Most tetrapeptides were obtained from Bachem AG, Bubendorf, Switzerland. All crystallisation experiments were carried out using the hanging drop method in Corning 24-well tissue culture plates at room temperature. 10 μl-drops were equilibrated against 1 ml precipitant solution. Most trials were carried out using an ICN robot pipetting station with software developed in-house.

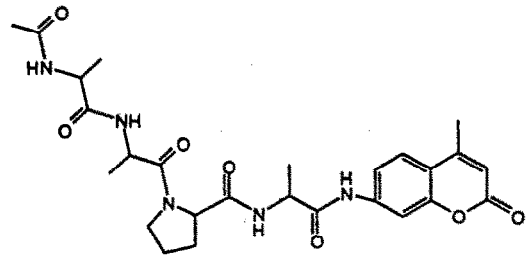
2.2. *Absorption spectroscopy*

Absorption spectra with linearly polarised light were recorded using a Zeiss microspectrometer 03. Spectra were taken in the wavelength range from 270 to 550 nm, with a step size of 2 nm (bandwidth 5 nm). A small needle-like crystal was inserted between two quartz slides which were separated by glass cover slips. Baselines were recorded in a region of mother liquor.

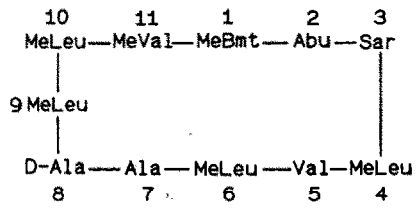
2.3. *Density measurements*

The Ficoll density gradient method [9] was used. Ficoll 400 (Pharmacia) solutions were layered in fractions of decreasing density in a glass tube (inner diameter 2 mm). The gradient was smoothed by centrifugation and crystals were placed at the top of the gradient with a drop of mother liquor. The tube was spun at about $1000 \times g$ and the position of the crystal in the tube was measured as a function of time (up to one hour). The gradient was calibrated over 5 points, with drops of a mixture of chloroform and toluene (both of which had been saturated with water). For each of the five different chloroform/toluene mixtures, the density was determined pycnometrically.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50



(I)



(II)

2.4. *X-ray diffraction measurements*

Crystals were mounted and sealed in Debye-Scherrer glass capillary tubes (0.01 mm thickness) with a plug of well solution. The X-ray source for photographic and area detector studies was an FR571 rotating anode generator (Delft Instruments, Delft, Holland) run at 40 kV, 70 mA. Data were collected on an Enraf-Nonius FAST area detector system and also on an image plate using the DESY synchrotron source (in collaboration with Drs C. Betzel and K. Wilson).

3. RESULTS AND DISCUSSION

3.1. *Cyclophilin-tetrapeptide crystal complexes*

Native cyclophilin could be crystallised as very thin long needles ($0.005 \times 0.005 \times 2.0$ mm^3) with the following initial conditions: *Well:* 100 mM imidazole-HCl, pH 6.5, 46% saturated ammonium sulphate (AS), (saturated at 20°C), 0.04% (w/v) sodium azide; *Drop:* 25 mM imidazole-HCl, pH 6.5, 12.5% saturated AS, 0.01% sodium azide, 0.8 mM cyclophilin.

Much better crystals were obtained by co-crystallising cyclophilin with a variety of tetrapeptides which are likely substrates for the *cis–trans* isomerase activity. *Well:* 100 mM Tris-HCl, pH 8.2, 46% saturated AS, 0.04% sodium azide; *Drop:* 50 mM Tris-HCl, pH 8.2, 42% saturated AS, 0.02% sodium azide, 6 mM tetrapeptide, 0.6 mM cyclophilin, 5% methanol.

The small concentration difference in AS between drop and well was found to be important for good crystal growth [10]. The standard 50% dilution of precipitant in the drop only gave precipitate. Fig. 1 shows a typical crystal of cyclophilin complexed with the peptide Ac-AAPA-amidomethylcoumarin (I).

Crystal dimensions can reach $0.2 \times 0.3 \times 1.5$ mm^3. They have space group $P2_12_12_1$ with cell dimensions $a = 108.2$, $b = 123.0$, $c = 35.8$ Å and diffract to better than 2.4 Å resolution. A full native and two useful heavy-atom derivative data sets have been collected for this crystal form. Absorption spectroscopy was used to examine the crystals and to test for the presence of the

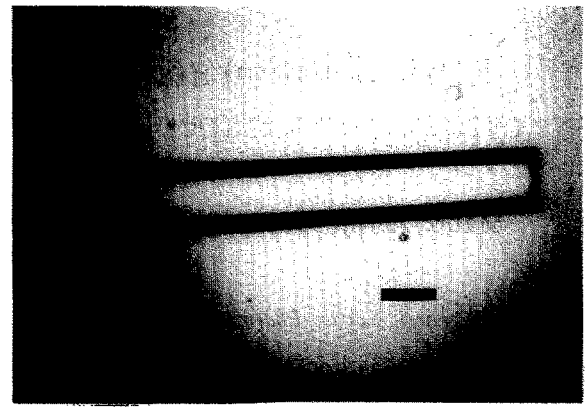


Fig. 1. A crystal of the complex of cyclophilin with Ac-AAPA-amidomethylcoumarin (I) (approximate dimensions $0.3 \times 0.3 \times 3.0$ mm^3). The crystal is lying on a face of the (110)-form. The needle axis is parallel to the c-axis of the unit cell. The bar represents 0.2 mm.

coumarin group. The prominent faces (Fig. 1) were identified as belonging to the (110)-form by measuring the interfacial angles.

Absorption spectra were measured with light which was linearly polarised parallel and normal to the needle axis c in the (110)-face. The spectra (Fig. 2) clearly demonstrate that the coumarin chromophore (giving rise to the 330 nm band) is oriented in the crystal indicating that the tetrapeptide (I) is bound in a specific way to cyclophilin.

3.2. *Cyclophilin-cyclosporin crystal complexes*

Three distinct crystal forms of a cyclophilin-cyclosporin A complex were obtained. The conditions used for crystal growth were: *Well:* 100 mM Tris-HCl, pH 8.2, 48% saturated AS, 0.04% sodium azide, 10% DMSO, isopropanol (2.0%, 2.2%, 2.4%); *Drop:* 50 mM Tris-HCl, pH 8.2, 42% saturated AS, 0.02%

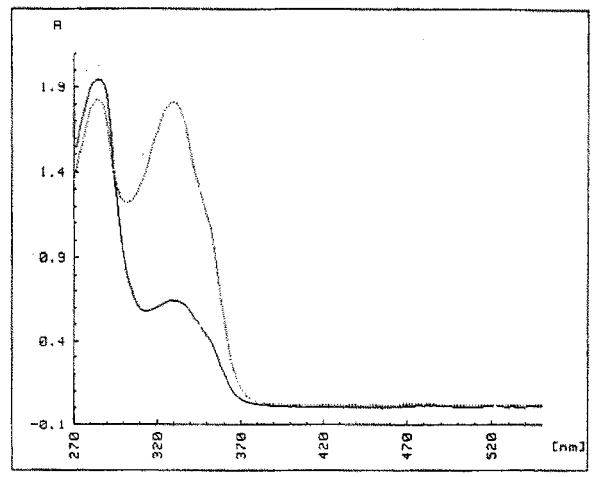


Fig. 2. A linear-dichroism absorption spectrum taken from a single crystal of the Ac-AAPA-amidomethylcoumarin complex lying on a (110)-face. Absorbance is plotted against wavelength (nm). The full and dotted lines are spectra obtained for linearly polarised light parallel to and perpendicular to the needle axis, respectively.

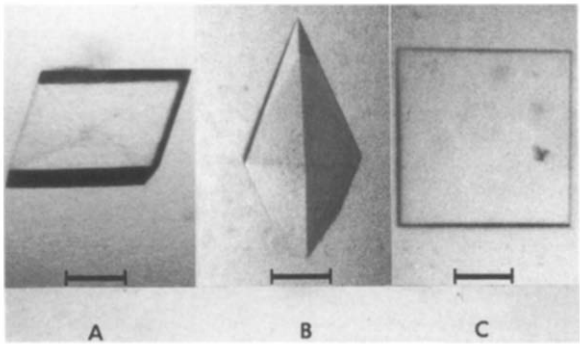


Fig. 3. (a) Bipyramidal crystal of the cyclophilin-cyclosporin complex. The bar represents 0.25 mm. (b) Rhombohedral crystal of the cyclophilin-cyclosporin complex. The bar represents 0.3 mm. (c) Tetragonal crystal of the cyclophilin-cyclosporin complex. The bar represents 0.17 mm.

sodium azide, 0.5 mM cyclophilin, 0.5 mM cyclosporin, 5.3% DMSO.

Crystals formed within 5 days. The exact quantities of both DMSO and isopropanol, and also the small difference of AS concentration between drop and well, were all found to be critical for the formation of the crystals. The hexagonal bipyramidal form (Fig. 3a), the rhombohedral form (Fig. 3b) and the tetragonal form (Fig. 3c), grew in the presence of 2%, 2.2% and 2.4% isopropanol, respectively. In some drops all 3 crystal forms were found to grow together.

The tetragonal form is the most suitable for detailed crystallographic study. A multiple seeding technique was used to obtain suitably large crystals. After 3–4 weeks thick, square plates or truncated tetragonal pyramids up to $1.5 \times 1.5 \times 0.2$ mm^3 had developed. The crystals have the space group $P4_12_12$ or $P4_32_12$ with $a = b = 94.98$, $c = 278.55$ Å and diffract to better than 3 Å.

A native data set to 2.8 Å as been collected using the DESY synchrotron source, and low resolution (5.5 Å) heavy-atom derivative data have been collected on a FAST area detector.

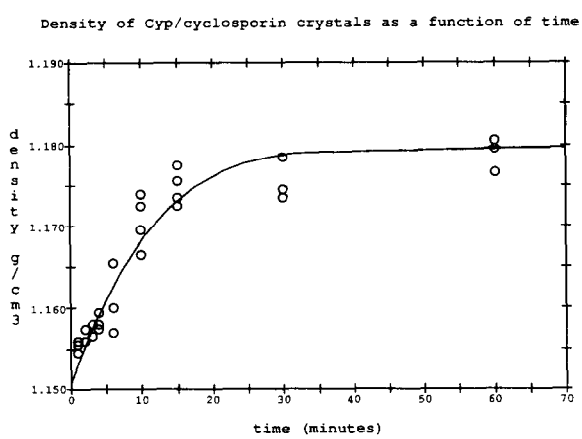


Fig. 4. Apparent density of the bipyramidal form of cyclophilin complexed with cyclosporin as a function of time.

3.3. *Crystal density measurements*

Crystal densities of cyclophilin complexed with both cyclosporin A and the tetrapeptide have been experimentally measured using the Ficoll method.

Assuming a density of 1 g/cm^3 for solvent regions and a partial specific volume for the protein of 0.737 g^{-1} · cm^3, the number of molecules per cell (n) can be calculated from

$$n = 2.324\ (V/M)\ (\varrho_c - 1)$$

where V is the cell volume and M is the molecular weight.

Crystal density (ϱ_c) is estimated by flotation in a Ficoll density gradient and the initial density is obtained by extrapolation to time zero. It has been observed that for less densely packed crystals, the Ficoll method leads to an increase of the apparent crystal density as a function of time [11], presumably due to an inclusion of Ficoll into the lattice. This was clearly the case for cyclophilin complexed with cyclosporin A in which the crystal density (ϱ_c) rose from 1.15 to 1.18 g/cm^3 after 40 min (Fig. 4).

The tetragonal form of cyclophilin complexed with cyclosporin A has a unit cell volume of 2 513 000 Å^3. The molecular mass of cyclophilin is 18 012 Da and the molecular mass of cyclosporin A is 1202 Da. The measured density of 1.151 g/cm^3 gives a calculated solvent content of 58% with 45.9 cyclophilin-cyclosporin A heterodimers in the unit cell. Spacegroups $P4_12_12$ and $P4_32_12$ have 8 asymmetric units per cell which would be consistent with either 6 (for $n = 48$) or 5 (for $n = 40$) heterodimers per asymmetric unit. Six cyclophilin-cyclosporin A heterodimers would give 55% solvent content and a V_m of 2.72 Å^3/Da; five heterodimers would give 63% solvent content and a V_m of 3.27 Å^3/Da.

For the cyclophilin-Ac-AAPA-aminomethylcoumarin complex ($V = 476\ 400$ Å^3, $M_r = 18\ 012 + 527$ Da), the measured density of 1.155 g/cm^3 is consistent with $n = 9.5$ and a solvent content of 57%. This indicates 8 molecules per cell and a V_m of 3.29 Å^3/Da and a calculated solvent content of 62%. For space group $P2_12_12_1$ this requires two crystallographically independent molecules per asymmetric unit.

4. CONCLUSION AND FUTURE WORK

X-ray diffraction data from native and heavy-atom derivative crystals of cyclophilin complexed with Ac-AAPA-amidomethylcoumarin have been used to calculate an electron density map to 3Å and chain tracing is underway. The more difficult crystallographic problem of the complex of cyclophilin with cyclosporin A which has 5 or 6 molecules per asymmetric unit, will be tackled using molecular replacement techniques once a model of cyclophilin becomes available. These struc-

tures will be able to answer a number of important questions concerning the action of cyclosporin A. Firstly, it will be possible to determine the conformation of bound cyclosporin. A number of crystal structures of different chemical derivatives of cyclosporin A tend to show the same conformation [12], however, recent NMR results [13] indicate that a very different ring conformation is present when bound to cyclophilin. An accurate description of the bound conformation will be of considerable use in designing cyclosporin analogues which may have immunosuppressant properties. The second major question is whether the *cis–trans* isomerase activity of cyclophilin is biologically relevant. The structure of the bound tetrapeptide substrate should provide some clues as to the enzymatic mechanism. Comparison of the two crystal structures will unambiguously show whether the tetrapeptide and cyclosporin A bind to the same site or whether there is an allosteric site on cyclophilin. If the enzymatic action is found to be relevant, this would open up a new direction in the design of immunosuppressant drugs based on enzyme substrate analogues.

REFERENCES

[1] Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J. and Speicher, D.W. (1984) Science 226, 544–547.
[2] Takahashi, N., Hayano, T. and Suzuki, M. (1989) Nature 337, 473–475.
[3] Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. and Schmid, F.X. (1989) Nature 337, 476–478.
[4] Emmel, E.A., Verweij, C.L., Durand, D.B., Higgins, K.M., Lacy, E. and Crabtree, G.R. (1989) Science 246, 1617–1620.
[5] Tropschug, M., Barthelmess, I.B. and Neupert, W. (1989) Nature 342, 953.
[6] Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L. (1989) Nature 341, 758–760.
[7] Harrison, R.K. and Stein, R.L. (1990) Biochemistry 29, 3813–3816.
[8] Stedman, K., et al. (1990) manuscript in preparation.
[9] Westbrook, E.M. (1985) in: Methods in Enzymology (Wyckoff, H.W., Hirs, C.H.W. and Timasheff, S.N., eds) Academic Press, pp. 187–196.
[10] Mikol, V., Rodeau, J.L. and Giegé, R. (1990) Anal. Biochem. 186, 332–339.
[11] Bode, W. and Schirmer, T. (1985) Biol. Chem. Hoppe-Seyler 366, 287–295.
[12] Walkinshaw, M.D. and Boelsterli, J.J. (1988) Z. Kristallogr. 185, 41–42.
[13] Wüthrich, K. et al. (1990) to be submitted.