A Small-Angle X-ray Scattering Study of the Binding of Cyclosporin A to Cyclophilin

BY ROBERT B. KNOTT

ANP Program, Australian Nuclear Science and Technology Organisation, Private Mail Bag, Menai, NSW 2234, Australia

MALCOLM CAPEL

Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

STEEN HANSEN

Department of Mathematics and Physics, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 FRB C, Denmark

AND ROBERT E. HANDSCHUMACHER*

Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, USA

(Received 6 January 1995; accepted 6 March 1995)

Abstract

The small-angle X-ray scattering (SAXS) technique was used to investigate structural characteristics of the protein cyclophilin in solution and to attempt to detect major changes induced by the binding of the immunosuppressant drug cyclosporin A. Maximum-entropy methods were used to analyse the experimental SAXS data. The measured radius of gyration, \( R_g \), for cyclophilin is 16.3 (5) Å. This is equivalent to a compact sphere of radius 21.0 Å. There is qualitative agreement between the experimental SAXS profiles and the derived distance-distribution function, \( p(r) \), for cyclophilin, and similar profiles calculated from the crystallographic structure. The notable discrepancy is the difference of approximately 1.5 Å in the estimated radius of the equivalent sphere. On binding cyclosporin A, the main structure-related change in cyclophilin observed under these experimental conditions is an increased propensity to form oligomers. Meaningful estimates of \( R_g \) for the monomeric complex are not possible because of the presence of a significant population of aggregates. In a second series of experiments, both native cyclophilin and the cyclophilin/cyclosporin A complex readily formed aggregates under the prevailing experimental conditions.

1. Introduction

Cyclosporin A (CsA) is a hydrophobic cyclic undeca-peptide (1.2 kDa) and a potent immunosuppressant drug with extensive clinical application primarily for the prevention of transplanted solid-organ rejection and graft-versus-host disease following bone marrow en-graftment (Borel, 1983; Wenger, 1986). In addition, there is mounting evidence for a wider range of CsA interactions with the immune system (Nussenblatt, Salinas-Carmona, Waksman & Gery, 1983; Stiller et al., 1984). Because of the impact CsA is having on clinical immunology, it is essential to investigate the behaviour of CsA in the cellular and subcellular regimes. The low-molecular weight cytosolic protein cyclophilin (CyP) (17.7 kDa) is a specific receptor for CsA (Handscharucher, Harding, Rice, Duggie & Speicher, 1984; Harding & Handscharucher, 1988). CyP is widely distributed in cells and tissues of eukaryotic organisms (Koletskey, Harding & Handscharucher, 1986), and has been shown to possess peptidyl-prolyl cis-trans isomerase (PPIase) activity (Takahashi, Hayuno & Suzuki, 1989; Fischer, Wittmann-Liebold, Land, Kieffhaber & Schmid, 1989), completely inhibited by the binding of CsA. The role of CyP in fundamental cellular processes, particularly in the immune system, is still being established.

It has long been recognised that the structural details of CsA and CyP are vital to the understanding of the molecular mechanisms that lead to observed immunological effects. Extensive structural studies of CsA, its derivatives and metabolites, have been carried out (Petcher, Weber & Reienger, 1976; Kessler, Loosli & Oschkinat, 1985; Loosli et al., 1985; Walkinshaw & Boelsterl, 1988; Knott, Schefer & Schoenborn, 1990). A number of studies on the CsA/CyP complex have also been reported (Dalgarno, Harding, Lazarides, Handscharucher,...)
The structure of recombinant human T-cell CyPA has also been determined by single-crystal X-ray diffraction methods and refined to a resolution of 1.63 Å (Ke, Zydowsky, Lui & Walsh, 1991). The crystal structure of the CsA/CyP complex has also been reported (Pfliegl et al., 1993). Further, the structure of human CyP complexed with a tetrapeptide was determined to 2.6 Å resolution (Kallen et al., 1991) and with a dipeptide substrate to 1.64 Å resolution (Ke, Mayrose & Cao, 1993). The solution structure of (i) the CsA/CyP complex (Thériault et al., 1993) and (ii) the *Escherichia coli* periplasmic CyP (eCyP) (Clubb, Ferguson, Walsh & Wagner, 1994) have been determined by nuclear-magnetic-resonance techniques. Important structural details are emerging from these studies, but more information is required.

The SAXS technique is a valuable low-resolution method of determining structural features of proteins in solution and of investigating any changes induced by drug or substrate binding. SAXS is not sensitive to small local changes in structure, but will detect conformational changes in protein structure. SAXS profiles of CyP and the CsA/CyP complex in solution were collected at selected protein concentrations, \( c_p \), and the distance-distribution function, \( p(r) \), was calculated. From this function, the radius of gyration, \( R_g \), was calculated. The maximum-entropy method was used to evaluate the SAXS profiles. The results were compared with results obtained from the crystallographic data for human T-cell CyPA.

### 2. Experimental procedures

#### 2.1. Sample preparation

Two series of experiments were carried out. Cyclophilin was isolated from (i) bovine thymus glands and (ii) human spleen tissue, using methods previously described (Harding, Handschumacher & Speicher, 1986). During all phases of the experiments, it was essential to maintain buffer conditions that ensured the stability of the CyP under the experimental conditions. This was achieved by high salt concentration, the addition of antioxidants [β-mercaptoethanol (BME) or dithiothreitol (DTT)], low \( c_p \) (1 g l\(^{-1}\)) during storage, low temperature (277 K) and pH in the range 6.8–7.2. Solutions were purged under argon whenever possible. For the bovine thymus CyP, the buffer was 0.150 M NaCl, 0.020 M Tris, 0.025 M DTT, pH 7.2, 0.02% sodium azide, and for human spleen CyP, it was 0.100 M Na\(_2\)SO\(_4\), 0.100 M K\(_2\)HPO\(_4\), 0.005 M BME, pH 6.9, 0.05% sodium azide. During the experiment, the solutions were maintained at 279 K and cyclically exposed to the high intensity X-ray beam for 30 s per cycle. The total irradiation time was typically 5 min for each sample. The CsA/CyP complex was prepared at low \( c_p \) by incubating for 24 h a 2 M equivalent of CsA dissolved in ethanol (0.020 M). The solution was gently agitated at regular intervals. Previous experiments have demonstrated that, under these conditions, a stable CsA/CyP complex is formed (Handschumacher, Harding, Rice, Drugge & Speicher, 1984). Solutions of various \( c_p \) were prepared from the parent solution by volume division and solute concentration using Amicon Centricon-10 Microconcentrator # 4205. The \( c_p \) was determined by UV absorption on a Beckman DU-64 Spectrophotometer, by Albumin Base Biorad and/or by amino-acid analysis. After the experiments, the \( c_p \) were again measured and native protein solutions were assayed for CsA binding. The results verified that no significant denaturing of the CyP had resulted from its exposure to the X-ray beam.

#### 2.2. Small-angle X-ray scattering

In small-angle scattering, the intensity \( I \) is measured as a function of the length of the scattering vector \( q = 4\pi \sin(\theta)/\lambda \), where \( \lambda \) is the wavelength of the radiation and \( \theta \) is half the scattering angle. For scattering from a dilute solution of monodisperse molecules of maximum intraparticle distance \( D \), the intensity can be written in terms of the \( p(r) \) function:

\[
I(q) \propto \int_0^D [p(r)[\sin(qr)/qr] dr. \tag{1}
\]

For a scattering particle of uniform scattering density, the \( p(r) \) function is proportional to the probability distribution for the distance between two points within the particle.

The estimates of \( R_g \) were obtained from the second moment of the \( p(r) \) function, viz

\[
R_g^2 = \frac{1}{D} \int_0^D r^2 p(r) \, dr \int_0^D p(r) \, dr, \tag{2}
\]

where \( D \) was estimated from the \( p(r) \) function. It must be noted that attempts to separate the contribution of the aggregation from the value for \( R_g \) include significant systematic errors. The quoted values of \( R_g \) should be interpreted accordingly. Statistically accountable errors are usually not the most significant source in this type of data analysis.

The SAXS data were collected on the Small-Angle Scattering Instrument on the X12B beamline at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (Capel, 1993). The samples were loaded in Kapton-window cells with 1 mm path length and total volume of 25 µl. At least two samples for each \( c_p \) were loaded, together with corresponding buffer solutions and empty cells; plus two standards (collagen and cholesterol myristate) for \( q \) calibration.
The scattered intensity profiles for all samples were collected in the range $0.025 < q < 0.3 \text{ Å}^{-1}$, corrected for beam transmission, and the buffer contribution subtracted using standard procedures.

The $p(r)$ function for the scattering particle was obtained by indirect Fourier transformation of the experimental $l(q)$ profile (Glatter, 1977). A number of protocols are available, and three selected methods of indirect Fourier transformation of SAXS data have been compared and a number of limitations highlighted (Hansen & Pedersen, 1991). In the experiments reported here, maximum-entropy methods of data analysis (Jaynes, 1983) were used for handling the added complexity of particle aggregation, a phenomenon that presented a significant experimental problem.

2.3. Maximum entropy

The estimation of the $p(r)$ function from noisy and truncated SAXS data is an underdetermined problem. When the maximum-entropy method is used for underdetermined problems, there are many possible solutions. The solution $p = (p_1, p_2, \ldots, p_N)$ is chosen that is closest (in the sense of having the maximum entropy) to a prior estimate $m = (m_1, m_2, \ldots, m_N)$ of the distribution of interest. This must be done while simultaneously fulfilling the constraints from measured data. The expression for the entropy that has been used in the present analysis is

$$S(p, m) = \sum_{j=1}^{N} -p_j \ln (p_j/m_j) + p_j - m_j, \quad (3)$$

where $p_j$ is taken as the value of the $p(r)$ function at the point $r_j$. For the prior, $m$, in SAXS from a dilute sample of monodisperse molecules, it will often be sufficient to assume the $p(r)$ function for a sphere if nothing is known about the shape of the scattering particle. Alternatively, the $p(r)$ function for an ellipsoid of revolution of free axial dimensions may be used (Müller & Hansen, 1994). The axial dimensions for the prior, $m$, should then be the dimensions for the ellipsoid that give the solution with the maximum entropy (Skilling, 1988). Finally, if some form of independent knowledge about the shape of the scattering particle is available, then the prior, $m$, can be constructed in accordance with this additional information.

For all the examples presented in this paper, an ellipsoid of revolution with free axis was selected for the prior. Using the $p(r)$ function for an ellipsoid of revolution allows for a smoother transition to zero for large distances; thus, the ellipsoid is better able to accommodate a small degree of aggregation in the sample. The maximization of the entropy of the distribution, $p$, was done subject to the constraints from the measurement of $M$ data points $l(q_1), l(q_2), \ldots, l(q_M)$ as given by the usual expression for $\chi^2$:

$$\chi^2 = \sum_{i=1}^{M} \left[ I(q_i) - \sum_{j=1}^{N} A_{ij}p_j \right]^2 / \sigma_i^2, \quad (4)$$

where $\sigma_i$ is the standard deviation for the Gaussian noise at data point $i$. The matrix $A$ describes the Fourier transition in (1):

$$A_{ij} = \sin (q_j r_i) / (q_j r_i) \Delta r, \quad (5)$$

where $\Delta r = r_{i+1} - r_i$. The maximization of the entropy was achieved through the solution of

$$V(S + \lambda \chi^2) = 0,$$

where $\lambda$ is a Lagrange multiplier allowing the $\chi^2$ to obtain a predetermined value (Steenstrup, 1985). In the absence of additional constraints from measured data, maximization of the entropy of $p$ will give the prior $m$ as the result.

A general problem for error estimation when using regularization techniques like the maximum-entropy method is the influence of the Lagrange multiplier on the hessian. This can lead to unrealistically small error estimates when a prior close to the final estimate is being used (see e.g. Hansen & Wilkins, 1994). Systematic errors like these and those mentioned previously from the unknown amount of aggregation will dominate the error on the estimates (see also Svergun & Pedersen, 1994).

The number of data points $M$ used for the calculations was 130–180 in the $q$ interval $[0.025, 0.24] \text{ Å}^{-1}$. This gives a $\Delta q < 0.002 \text{ Å}^{-1}$, well below the recommended sampling-point distance of $\pi/6D$ (Walter, Kranold & Behecher, 1974). The number of points $N$ used for estimation of the $p(r)$ function itself was 150–200. For the maximum-entropy method, the value for $N$ used is not important as long as it is sufficient to accommodate the features present in the measured data. For the measurements of the intensities shown in Figs. 1–5, the data have been rebinned for the purpose of presentation.

2.4. Model calculations

The SAXS profiles for human T-cell CyP A unligated (Ke, Zydowskyy, Lui & Walsh, 1991), and ligated with a dipeptide (Ke, Mayrose & Cao, 1993) were calculated from the crystallographic coordinates (entries 1CPL and 1CYH, respectively) obtained from the Protein Data Bank (Bernstein et al., 1977) at the Brookhaven National Laboratory. The effective atomic scattering-length densities were used in the calculations (i.e. the atomic scattering-length densities were reduced by the scattering-length density of the solvent). As the resolution for the present experiments is lower than 10 Å, the moderation of the calculated scattering intensity due to spatial effects of the order of just a few Å will be of only minor importance. Consequently, smaller-scale effects from (i) the solvent-excluded volume and (ii) the scattering form factors for individual atoms were neglected.
From the calculated SAXS profiles of CyP A, a low-resolution \( p(r) \) function was obtained by truncating the scattered intensity at \( q = 0.5 \, \text{Å}^{-1} \), extrapolating by a \( q^{-4} \) power law and Fourier transforming the resulting SAXS profile.

3. Results and discussion

Figs. 1 and 2 show data for bovine thymus CyP experiments with four \( c_p \) values. In Fig. 1(a) are shown the measured \( I(q) \) data and the maximum-entropy fits. The estimates of the \( p(r) \) function corresponding to the fits are shown in Fig. 1(b). In the calculation of \( R_g \), \( D \) was selected to be 50 Å for all four data sets. With neglect of the lowest \( c_p \) sample, the average \( R_g \) was calculated to be 16.3 (5) Å. The dependence of \( R_g \) on \( c_p \) was not significant. For these buffer conditions, there is evidence of minor particle aggregation. Fig. 2(a) shows the data and maximum-entropy fits for the CsA/CyP complex. The corresponding \( p(r) \) functions are shown in Fig. 2(b). An increased propensity for aggregation, possibly induced by the binding of CsA to CyP, is evident from the calculated \( p(r) \) functions now extending to 120 Å. The estimated average \( R_g \) is \( \sim 32 \, \text{Å} \).

Human spleen CyP data are shown in Figs. 3 and 4. A total of four \( c_p \) values were investigated. The \( I(q) \) data for CyP are shown in Fig. 3. The aggregation is so severe that a reliable estimate of \( R_g \) is impossible. \( I(q) \) data for the CsA/CyP complex are presented in Fig. 4. Despite a determined effort at working in conditions that eliminated protein aggregation previously seen in the bovine thymus CyP experiments, the human spleen CyP data
clearly indicate a greater population of aggregates. There is 95% homology in amino-acid sequence between the CyPs from the two sources used. There are, therefore, unlikely to be major structural differences between the two species. The sample-preparation procedures were identical. The only difference was the buffer used in each case. Based on extensive experimentation, the buffer selected for the human-spleen CyP was assessed to be the most appropriate.

In the studies of the single-crystal structure of CyP and the CsA/CyP complex, the issue of aggregation has been discussed (Zurini et al., 1990; Mikol, Kallen, Pfügl & Walkinshaw, 1993). The physiological relevance of this phenomenon has been questioned since it has further been reported (Mikol et al., 1993) that light-scattering experiments in solution under physiological conditions suggest that CyP is a monomer with a hydrodynamic radius of 21.5 Å. The SAXS results are in general agreement with the size of the monomer if the thickness of the hydration shell (0.5 Å) is subtracted. However, the SAXS results strongly suggest that there may be very little difference between the conditions for formation of a monomer and an oligomer. Note that, because of the truncation of the experimental data at low q, it is impossible to determine the maximum size of the oligomer. Given the variation of the c_p, there is material in solution scattering at low q. The preparation for each sample was identical, but independent after the initial subdivision of the low-c_p stock solution. There is clearly a major variation in the apparent concentration of monomer in each sample.

Fig. 5(a) shows the I(q) data for the highest-c_p bovine thymus CyP compared with (i) the low-resolution crystallographic data and (ii) a sphere of radius 20 Å. It is clear that a spherical shape is a good overall approximation to the calculated p(r) function for CyP.

Fig. 3. (a) SAXS profiles for human spleen cyclophilin. The protein concentrations of the samples are (i) 24.3 (+), (ii) 17.1 (⊙), (iii) 11.7 (□) and (iv) 6.1 (×) g l⁻¹. The fitted maximum-entropy data for each sample are also presented. (b) The distance-distribution functions, p(r), for the four samples in (a) [(i) long-dashed line, (ii) solid line, (iii) short-dashed line, (iv) dotted line].

Fig. 4. (a) SAXS profiles for the complex formed by human spleen cyclophilin and cyclosporin A. The protein concentrations of the samples are (i) 22.5 (+), (ii) 15.8 (⊙), (iii) 11.2 (□) and (iv) 4.5 (×) g l⁻¹. The fitted maximum-entropy data for each sample are also presented. (b) The distance-distribution functions, p(r), for the four samples in (a) [(i) long-dashed line, (ii) solid line, (iii) short-dashed line, (iv) dotted line].
Fig. 5b), except for the larger distances, where aggregation may influence the result. Accommodation of this effect required an equivalent sphere of 21 Å when calculated from the $R_g$ of 16.3 Å. However, as indicated in Fig. 5(b), the sphere of 20 Å is subjectively a more appropriate equivalent-sphere radius. A sphere fitted to the overall shape of the CyP $p(r)$ function from the crystallographic coordinates has a radius of approximately 18.5 Å. The radius of an equivalent sphere calculated from the volumes of the constituent amino acid residues (Perkins, 1986) is 17.5 Å. With the assumption that aggregation is responsible for the deviation at large distances between the experimental $p(r)$ function and the $p(r)$ function calculated from the crystallographic coordinates, there still remains a difference of 1.5 Å between the equivalent spheres for the two $p(r)$ functions. Models based on the swelling of the structure in solution are difficult to construct given the hydrophobicity of the CyP core.

The $p(r)$ functions for the twelve models constructed from nuclear-magnetic-resonance data for eCyP in solution (Clubb et al., 1994) were calculated using the procedure outlined in §2.4. The coordinates from the Protein Data Bank (entry 1CLH) were used. The results are presented in Fig. 6 together with the results from Fig. 5. There is small but significant structural difference between the twelve models when viewed at this resolution. As shown in Table 1, there is not a strong correlation between $R_g$ and $D$. The molecule changes shape slightly between the models. There is only 36% sequence homology between eCyP and human CyP.

<table>
<thead>
<tr>
<th>Model no.</th>
<th>$R_g$ (Å)</th>
<th>$D$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.1</td>
<td>50.2</td>
</tr>
<tr>
<td>2</td>
<td>14.9</td>
<td>49.8</td>
</tr>
<tr>
<td>3</td>
<td>15.0</td>
<td>50.6</td>
</tr>
<tr>
<td>4</td>
<td>15.2</td>
<td>50.8</td>
</tr>
<tr>
<td>5</td>
<td>15.1</td>
<td>49.8</td>
</tr>
<tr>
<td>6</td>
<td>15.3</td>
<td>52.2</td>
</tr>
<tr>
<td>7</td>
<td>14.8</td>
<td>49.1</td>
</tr>
<tr>
<td>8</td>
<td>14.9</td>
<td>47.6</td>
</tr>
<tr>
<td>9</td>
<td>15.0</td>
<td>50.5</td>
</tr>
<tr>
<td>10</td>
<td>15.1</td>
<td>51.7</td>
</tr>
<tr>
<td>11</td>
<td>15.2</td>
<td>51.6</td>
</tr>
<tr>
<td>12</td>
<td>15.3</td>
<td>50.0</td>
</tr>
<tr>
<td>CyP A</td>
<td>14.4</td>
<td>44.6</td>
</tr>
<tr>
<td>CyP A (SAXS)</td>
<td>16.3</td>
<td>*</td>
</tr>
</tbody>
</table>

* Estimated at 50 Å but with substantial error due to aggregation.
Comparison of the high-resolution structures (Clubb et al., 1994) indicates similar conformation with major flexibility restricted to regions of irregular secondary structure. Calculated p(r) functions indicate that eCyP in solution has a calculated R, that is ~0.5 Å larger than the human CyP crystal structure. The maximum intraparticle distance is 3–6 Å larger.

This study has sought to elucidate structural features of CyP in solution. These preliminary results highlighted some experimental difficulties in producing a monodisperse population of CyP and CsA/CyP complex in solution. For data analysis, the utility of the maximum-entropy method has been demonstrated. More information is required to explain the small but significant difference between the CyP structure data calculated for the crystal phase with the structure in solution.

This work was supported by grants from the Australian Department of Industry, Trade and Commerce (RBK) and from the Danish Natural Science Research Council (SH). Work carried out at the Brookhaven National Laboratory is under the auspices of the US Department of Energy.

References


