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Crystallization and preliminary X-ray data for the human transthyretin–retinol-binding protein (RBP) complex bound to an anti-RBP Fab

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A macromolecular complex of human transthyretin, human retinol-binding protein and an anti-retinol-binding-protein Fab was crystallized by vapour diffusion in sitting drops. Diffraction from these crystals at cryogenic temperatures was consistent with the space group *C*222, with cell parameters a = 159.34, b = 222.40 and c = 121.27 Å. Crystals diffracted to a resolution limit of 3.36 Å using synchrotron radiation. Based on a 2:2:1 stoichiometry for the Fab–retinol-binding-protein–transthyretin complex and the presence of one such complex per asymmetric unit, a reasonable V_m coefficient of 2.74 Å^3 Da^{-1} could be estimated.

1. Introduction

Retinol-binding protein (RBP) and transthyretin (TTR) are two plasma proteins involved in at least three important interactions. They bind small ligands, *i.e.* the vitamin retinol and the hormone thyroxine, respectively, and form a moderately tight RBP–TTR complex in the blood. Additionally, evidence has been presented for their interaction with surface receptors of target cells. As a result of constraints imposed by these various interactions, as well as by structural requirements, the three-dimensional structures of RBP and TTR were found to be well preserved in vertebrate species distant in evolution (Zapponi *et al.*, 1992; Sunde *et al.*, 1996). The crystal structures of apo and holo forms of both RBP (Cowan *et al.*, 1990; Zanotti, Berni *et al.*, 1993; Zanotti, Malpeli *et al.*, 1993) and TTR (Blake *et al.*, 1978; Wojtczak *et al.*, 1996) have been described. The structure of the heterologous complex between chicken RBP and human TTR has also been determined, and showed that one tetramer of TTR was bound to two molecules of RBP and that the amino-acid residues of RBP which were involved in protein–protein contacts were close to the retinol-binding site (Monaco *et al.*, 1995).

A structural investigation on a homologous TTR–RBP complex might prove helpful for gaining further insight into the physiologically occurring mode of binding of the two interacting proteins. The presence in the crystallization experiments of a Fab specific for human RBP was found to promote crystallization of the complex of human TTR and RBP. We report here crystallization and preliminary X-ray data for the complex between human TTR, human RBP and an anti-RBP Fab.

2. Experimental

Six murine monoclonal antibodies of the IgG1 subclass, specific for human RBP, were produced and purified as described by Pereira *et al.* (1993). The Fab obtained from the A8P3 MAb was found to be suitable for co-crystallization with the RBP–TTR complex. The Fab fragment of the A8P3 MAb was obtained by papain digestion, using a substrate:enzyme (*w*:*w*) ratio of 100:1, at 310 K for 16 h in the presence of 0.1 *M* sodium phosphate and 2 m*M* EDTA pH 7.2. The occurrence of proteolysis leading to the Fab and Fc fragments was monitored by SDS–PAGE on the MAb digest. The MAb digest was concentrated in an Amicon cell and the buffer was simultaneously exchanged with 20 m*M* Tris–HCl pH 7.5 within a couple of hours at 277 K. Fab was then purified by means of anion-exchange fast protein liquid chromatography on a Protein Glass DEAE-5PW column (Waters Associate Inc., Milford, MA), equilibrated with 20 m*M* Tris–HCl pH 7.5 and developed with a linear NaCl gradient (0–0.2 *M*). Human holoRBP and TTR were purified from fresh plasma as described (Malpeli *et al.*, 1996).

Single crystals of the complex between anti-RBP Fab, human holoRBP and human TTR grew at 295 K in sitting-drop vapour-diffusion experiments. 5 µl sitting drops were obtained by mixing the protein solution, containing Fab (2.5 mg ml^{-1}), holoRBP (2.1 mg ml^{-1}) and TTR (2.75 mg ml^{-1}) (Fab:RBP:TTR molar ratio of 1:1:0.5) in 20 m*M* Tris–HCl pH 7.5, with an equal volume of the precipitant reservoir solution (2.35 *M* ammonium phosphate, 10 m*M* sodium citrate and 10 m*M* β-mercaptoethanol, pH 5.0). Absorption spectra of the complex in the crystal were obtained by means of single-crystal microspectrophotometric

Table 1
Data-collection and processing statistics.

Resolution interval (Å)	Number of independent reflections	Completeness (%)	Multiplicity	*R* factor	Percentages of reflections with $I > 3\sigma(I)$	$\langle I > /\sigma(I)\rangle$
∞–6.94	2665	81.6	3.1	0.10	92	4.5
6.94–5.15	4392	87.1	3.2	0.12	87	5.5
5.15–4.28	5586	88.5	3.1	0.14	85	4.3
4.28–3.74	6692	91.0	3.0	0.26	69	2.0
3.74–3.36	6413	77.7	2.6	0.34	57	1.9
∞–3.36	25748	84.4	3.0	0.16	75	3.3

analysis, using a Zeiss MPM 800 microspectrophotometer.

Diffraction data were collected under cryogenic conditions at the diffraction beamline of the ELETTRA synchrotron (Trieste, Italy), using two crystals of approximate size 0.2 × 0.01 × 0.03 mm. Cryofreezing was accomplished by exchanging the crystal mother liquor with a cryoprotectant solution consisting of 29% saturated ammonium sulfate (pH 5.0) and 35%(*v*/*v*) glycerol. The crystals were left for about 5 min in the cryosolvent at room temperature. A fibre loop was used to scoop the crystals out of solution and place them rapidly in the nitrogen-gas cold stream, the temperature being maintained at 100 K by means of an Oxford Cryosystems Cryostream. During the measurements, the synchrotron ring was operated at 1.99 GeV, with a current ranging from 250 to 190 mA. The 1.3 Å wavelength was monochromated by the (111) face of a silicon double-crystal monochromator. Diffraction data were measured using an imaging-plate recorder (MAR Research; diameter 180 mm), positioned at a distance of 220 mm. Oscillations of 0.7 and 1.0° were performed for a total of 133 frames. Data were processed with the software *MOSFLM* (Leslie *et al.*, 1986). Data collection and processing statistics are given in Table 1.

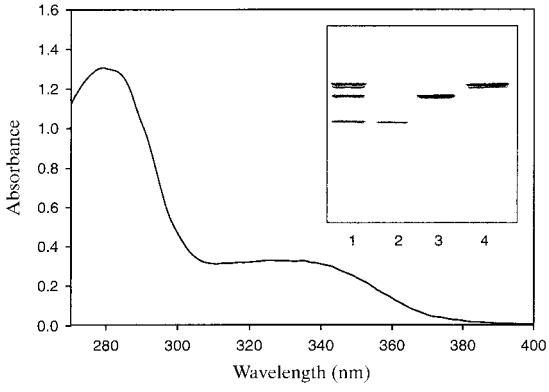

Figure 1
Absorption spectrum of a single crystal of the protein complex formed in the presence of human RBP, anti-RBP Fab and human TTR. The spectrum was determined by means of microspectrophotometric analysis after extensive washing of the crystal with the precipitant reservoir solution used for protein crystallization. Inset: SDS–PAGE of the components of single crystals obtained in the presence of human RBP, anti-RBP Fab and human TTR. Lane 1: single crystals of the complex were extensively washed with the crystallization precipitant reservoir solution and then dissolved in water to perform the electrophoretic analysis. Lanes 2, 3 and 4 correspond to purified human TTR (subunit, 13.7 kDa), human RBP (21 kDa) and anti-RBP Fab (light chain and heavy-chain fragment, approximately 25 kDa), respectively. The proteins were electrophoresed on a 15%(*w*/*v*) SDS–PAGE gel and stained with Coomassie blue.

3. Results and discussion

To establish that the crystals obtained in the presence of human RBP, anti-RBP Fab and human TTR really consist of the complex of the three proteins, we used two independent techniques: single-crystal microspectrophotometry and gel electrophoresis. An absorption band characteristic of RBP-bound retinol, with a maximum around 330 nm, along with the protein absorption band centred at 280 nm, were monitored by microspectrophotometric analysis of single crystals (Fig. 1). Since an A_{280}:A_{330} ratio of approximately four can be estimated from the spectrum shown in Fig. 1, compared with the A_{280}:A_{330} ratio of one which is characteristic of pure holoRBP, it appears that holoRBP is complexed to other molecules in the crystals obtained from the three proteins. The presence of a protein complex in the crystal could be unambiguously demonstrated by assaying the protein composition of single crystals by means of SDS–PAGE (Fig. 1, inset). It is worth noting that a doublet can be revealed in the electrophoretic pattern for both Fab alone (lane 4) and Fab present in the complex (lane 1). The doublet is attributable to the two components of Fab, *i.e.* the immunoglobulin light chain and the heavy-chain fragment.

In regard to the stoichiometry of Fab, holoRBP and TTR in the protein complex, the extinction coefficients for Fab ($\sim 70 \times 10^3\ M^{-1}\ cm^{-1}$), RBP ($\sim 42 \times 10^3 M^{-1}\ cm^{-1}$) and TTR ($\sim 79 \times 10^3 M^{-1}\ cm^{-1}$) at 280 nm and for RBP-bound retinol ($\sim 42 \times 10^3 M^{-1}\ cm^{-1}$) at 330 nm, respectively, account for a A_{280}:A_{330} ratio of about 3.6 in the case of a 2Fab:2RBP:1TTR stoichiometry. This value compares well enough with the A_{280}:A_{330} ratio of four estimated from the spectrum shown in Fig. 1. It should be noted that 4:4:1 and 1:1:1 stoichiometries would lead to A_{280}:A_{330} ratios of 3.1 and 8, respectively. Additionally, a 2:2:1 stoichiometry would be in agreement with the known 2:1 stoichiometry for the RBP–TTR complex in solution (Berni *et al.*, 1994) and in the crystal (Monaco *et al.*, 1995).

The suggested 2:2:1 stoichiometry in the crystalline protein complex appears to be corroborated by preliminary X-ray diffraction data. Crystals belong to the orthorhombic space group *C*222 with unit-cell dimensions a = 159.34, b = 222.40, c = 121.27 Å. A reasonable V_m coefficient of 2.74 $Å^3\ Da^{-1}$, with a 55% solvent content in the unit cell, is consistent with a total molecular mass of approximately 200 kDa per asymmetric unit, which corresponds to a complex formed by two Fab (50 kDa), two RBP (21 kDa) and one TTR (homotetramer, 55 kDa). Diffraction data could be measured to 3.36 Å resolution, and statistics are reported in Table 1.

The three-dimensional structure of the macromolecular complex is currently being solved in our laboratory using the molecular-replacement method. The structural model of the Fab–RBP–TTR complex will lead to the description of the mode of binding of homologous RBP and TTR. Moreover, this structure will unravel the molecular architecture of a macromolecular complex involving three protein molecules and characterized by the binding interfaces between RBP and both TTR and Fab.

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