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Structure of pig plasma retinol-binding protein at 1.65 Å resolution

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Abstract

The crystal structure of pig plasma retinol-binding protein (RBP) has been determined at 1.65 Å resolution. The space group is $P2_12_12_1$, with a = 45.81 (4), b = 53.14 (5), c = 72.97 (8) Å and one protein molecule in the asymmetric unit. The structure has been solved using the molecular replacement method and refined with restrained least squares to an R factor of 0.1844 and an R_{free} of 0.237 for 18 874 and 1001 independent reflections, respectively. The relatively high resolution structure of pig holoRBP has revealed some new structural details. Moreover, it has provided a description of the binding site for Cd^{2+}, a metal ion which is required for protein crystallization. The hepta-coordination of the RBP-bound cadmium ion involves different residues of two symmetry-related RBP molecules, consistent with the participation of the cation in intermolecular interactions that in turn promote protein crystallization.

1. Introduction

Retinol-binding protein (RBP) is the specific carrier of retinol in plasma. It consists of a single polypeptide chain of about 21 kDa and contains one binding site for retinol. The interaction with RBP is required to solubilize and protect the poorly soluble and highly unstable vitamin A alcohol to a large extent. RBP circulates in plasma bound to another protein, thyroxine-binding transthyretin (TTR). The three-dimensional structures of human and bovine holo and apo RBPs have been determined (Cowan *et al.*, 1990; Zanotti, Ottonello *et al.*, 1993; Zanotti, Berni *et al.*, 1993). The structure of the complex between chicken RBP and human TTR has also been determined (Monaco *et al.*, 1995). RBP is a single-domain protein, mainly consisting of an anti-parallel β-barrel, which forms the retinol-binding site. The retinol cyclohexene ring lies deep inside a hydrophobic cavity, whereas the isoprene chain extends to the protein surface, where the hydroxyl end group is in contact with the solvent. To define better details of the structure of RBP and of its interaction with the retinol molecule, the structure of pig holoRBP in an orthorhombic crystal form, which diffracts to 1.65 Å resolution with conventional sources, has been determined.

2. Experimental

HoloRBP was purified from pig plasma by using the chromatographic steps reported previously for the purification of human and chicken RBP (Malpeli *et al.*, 1996), except for the Blue-Sepharose CL-4B chromatography, which was replaced by gel filtration on Sephadex-G100 (Pharmacia) in the presence of 4 *M* urea, 20 m*M* Tris–HCl, pH 7.3. Pig holoRBP crystals were obtained at 277 K by the sitting-drop vapor-diffusion method, at a final protein concentration of approximately 8 mg ml^{-1} and in the presence of 8%(*v*/*v*) 2-methyl-2,4-pentanediol, 3 m*M* cadmium acetate, 0.1 *M* Tris–acetate, pH = 6.8.

X-ray diffraction data were measured with a Siemens HI-STAR multiwire proportional counter, using as a source a M18XHF-SRA Cu rotating anode. 413 and 180 frames with the detector placed at 2θ angles of -30 and $-5°$, respectively, were collected from a single crystal. The crystal-to-detector distance was fixed at 100 mm. Statistics on data reduction are summarized in Table 1.

The structure was solved with the molecular replacement method using the package *X-PLOR* (Brünger, 1990), starting from the coordinates of the orthorhombic crystal form of bovine RBP (Zanotti, Berni *et al.*, 1993). The refinement was carried out using the program *SHELXL*93 (Sheldrick, 1993). The restraints applied were those generated by the program itself. Only the Cd^{2+} ion was refined anisotropically. All H atoms, except those in disordered chains, were introduced in calculated positions and allowed to ride on the atom to which they were bound. After the final cycle of refinement, the highest peak present in the Fourier-difference map was 0.36 e Å^{-3}. Statistics on the final model and parameters are reported in Table 2.

The *PROCHECK* (Laskowski *et al.*, 1993) statistical tests for main and side chains do not indicate any outliers. The Ramachandran plot shows that 91% of the residues are in the most favored regions, whilst only two residues, Tyr111 and Asn66, are in the so-called 'generously allowed' regions. For serines in positions 8, 21 and 46 two orientations of the Oγ were visible. The average temperature factors for main-chain and side-chain atoms are 20 and 26 Å^2, respectively. The mean B factor for the ligand atoms is 22 Å^2.

3. Results and discussion

3.1. *The overall protein structure*

The overall topology of pig RBP is practically identical to that of the previously described human and bovine proteins (Cowan *et al.*, 1990; Zanotti, Berni *et al.*, 1993). The amino-acid sequence of pig RBP is different, as compared with human and bovine RBPs, for 12 and seven amino acids out of a total of 183 residues, respectively. Such differences are essentially confined to regions that are not involved in the interaction with retinol and in the interaction with TTR (see Fig. 1). The overall models of the three mammalian proteins of known structure are quite similar, as demonstrated by the r.m.s deviation

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Table 1. *Processing statistics*

20 127 unique reflections were obtained from a total of 84 968 measurements from one crystal, with a merging R factor of 0.064. % refers to the possible total reflections in the shell. No sigma-cutoff was applied.

Resolution range (Å)	No. of reflections	Completeness (%)	Multiplicity	R_{merge} (%)	$\langle I/\sigma(I)\rangle$
55–3.30	2898	98.6	5.5	4.8	65.2
3.30–2.62	2816	99.9	5.3	5.8	22.7
2.62–2.29	2741	98.8	4.7	8.5	11.7
2.29–2.08	2657	96.7	4.3	11.5	9.0
2.08–1.93	2597	95.0	4.0	16.2	6.1
1.93–1.82	2545	92.6	2.9	24.0	3.7
1.82–1.73	2372	87.3	1.9	32.3	2.5
1.73–1.65	1501	55.6	1.2	37.1	1.8
55–1.65	20127	90.7	4.2	6.4	20.8

between equivalent Cα atoms, from residues 3 to 174: 0.43 and 0.30 Å for the comparison between human and pig RBPs and between bovine and pig RBPs, respectively. It must be pointed out that residue 116, an Ala according to the cDNA sequence (Trout *et al.*, 1991), was modelled as a Val: the $|2F_{obs} - F_{calc}|$ map, calculated without any side chains for this residue, clearly indicates the presence of a valine. This residue is also a valine in human and bovine RBPs.

3.2. *The RBP-bound retinol molecule*

The conformational flexibility of the all-*trans* retinol molecule is intrinsically limited by its conjugated double-bonds system, so that in practice a certain degree of freedom is allowed only for the rotation around the two torsion angles formed by C4—C5—C6—C7 and C5—C6—C7—C8 atoms (for the numbering system used for retinol see Zanotti *et al.*, 1994). Such angles define the orientation of the isoprene tail relative to the β-ionone ring and the conformation of the ring itself. The values of these angles are −172 and +60° for the present structure, in agreement, at least for the present resolution, with the values found for retinol in human (+172 and +62°: Cowan *et al.*, 1990) and bovine RBP (+165 and +33°: Zanotti, Berni *et al.*, 1993) and with the values found for retinol analogs bound to RBP (Zanotti *et al.*, 1994). Definitely larger deviations from these torsion angles have

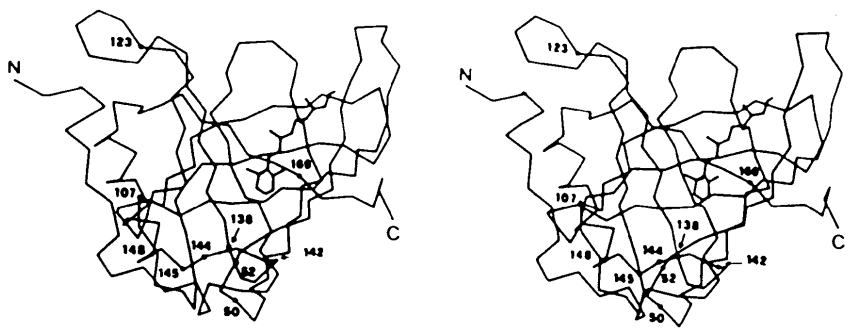

Fig. 1. Stereoview of the Cα chain trace of pig RBP, where labels indicate those residues that are different as compared with human protein. The retinol molecule is also shown. The sequence for pig RBP is taken from the cDNA sequence (Trout *et al.*, 1991). Residue 116, an Ala in the original sequence, is clearly a Val on the basis of its electron density; residue 167 is possibly different from Leu, but the assignment is not unequivocal.

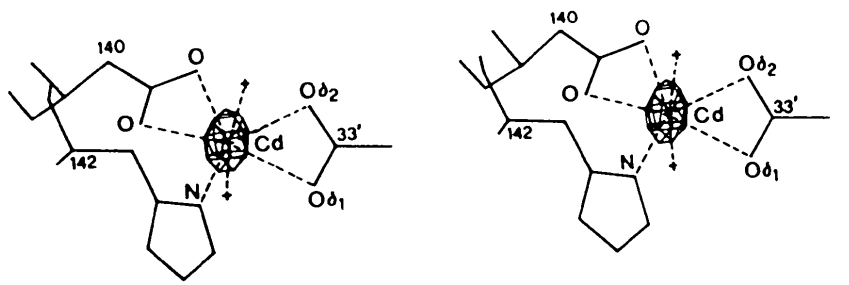

Fig. 2. Stereoview of the cadmium site formed by two RBP molecules and two water molecules, with electron density calculated with coefficients $|2F_{obs} - F_{calc}|$ and phases from protein atoms only. Contours are drawn at 13σ level. Interatomic distances between the metal ion and the following protein and water atoms are: 140O^{γ_1}, 2.52 Å; 140O^{γ_2}, 2.40 Å; 142N^{δ_1}, 2.35 Å; 33′O^{γ_1}, 2.35 Å; 33′O^{γ_2}, 2.50 Å; 350, 2.38 Å; 351, 2.31 Å. The ′ indicates a residue of a symmetry-related RBP molecule.

Table 2. *Summary of parameters for the refined model of pig RBP*

Crystal data (Å)	$P2_12_12_1$, $Z = 4$ $a = 45.81$ (4), $b = 53.14$ (5), $c = 72.97$ (8)	
Number of reflections		R factor (%)
Used in refinement	18874	18.44
With $F > 4\sigma(F)$	15686	15.70
For R_{free}	1001	23.72
Goodness of fit	1.096	
Weighting scheme	$1/[\sigma^2(F_{obs}^2) + (0.4584P)^2 + 516.53P]$ where $P = [\max(F_{obs}^2,0) + 2F_{calc}^2]$	
No. of protein atoms	1428	
No. of atoms in the β-barrel cavity	21	
Solvent region	124 water molecules, 1 Cd^{2+} ion	
R.m.s. deviations from ideal values		
1–2 distances (Å)	0.03	
1–3 distances (Å)	0.03	
Planarity (Å)	0.2	

been reported for retinoids in the solid state (see, *e.g.*, references in Cowan *et al.*, 1990).

The isoprene tail is, as expected, roughly planar, with the significant exception of the two C atoms at the end of the tail, C14 and C15, and of the hydroxyl group (Fig. 3). The latter group definitely points towards residue 98, forming with its peptide N atom a hydrogen-bond interaction (O···N98, 2.82 Å). Evidence for the latter interaction was not obtained in the case of the structure of human and bovine holoRBPs, presumably because of the lower resolution as compared with structure described here. Additionally, the retinol hydroxyl group forms a second hydrogen bond with a water molecule, which in turn interacts with the peptide O atom of residue 96 (OH···W387, 2.54 Å; W387···O96, 3.03 Å). Thus, the retinol hydroxyl group may significantly contribute to the strength of binding of the vitamin to the protein. This is in agreement with the results of competition binding experiments showing a lower binding affinity of retinoids for RBP as compared with retinol (Berni R., unpublished results) and with structural data for the binding of retinol analogs to RBP (Zanotti, Malpeli *et al.*, 1993; Zanotti *et al.*, 1994). The non-perfect planarity of the rest of the tail, and in particular of the last two C atoms, may be attributed to the constraints imposed by the protein side chains and by the very limited space available in the β-barrel cavity.

3.3. *The cadmium binding site*

Cadmium ions represent an absolute requirement for the growth of orthorhombic crystals of pig RBP, as well as human (Newcomer *et al.*, 1984) and bovine (Berni *et al.*, 1990) RBPs. However, RBP-bound cadmium ions could not be identified in the electron-density maps of the human or bovine RBPs. On the contrary, a quite high peak in the electron-density map of pig RBP has been attributed to a Cd^{2+}. Notably, the Cd^{2+} ion forms a bridge between two RBP molecules, thus providing the molecular basis for the role of this heavy metal in promoting the intermolecular interactions present in the crystals of pig RBP. The hepta-coordination of the Cd^{2+} ion is shown in Fig. 2: the two carboxylic O atoms of Asp140 and the N atoms of His142 of a RBP molecule, and the two carboxylic O atoms of Glu33 of a symmetry-related protein molecule form the base of a pentagonal bipyramid, whose vertices are occupied by two water molecules. Distortions in the coordination geometry could be explained by steric needs of the protein side chains. However, a distorted bipyramidal geometry has also been observed previously in Cd^{2+} complexes (Shiba & Bau, 1978).

4. Conclusions

The high degree of conservation of the three-dimensional structures of three mammalian (human, bovine and porcine)

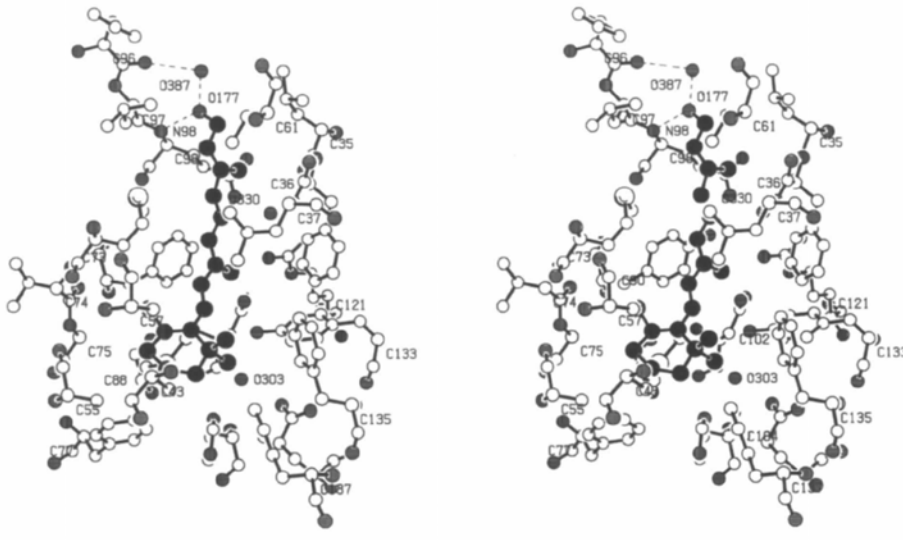

Fig. 3. Stereoview of the retinol molecule in the RBP central cavity. Protein residues and solvent molecules (O387, O303 and O330) that possess at least one atom at a distance less than 4.6 Å from an atom of the ligand are shown. Protein C atoms are represented as hollow spheres and C atoms of retinol as black spheres. O and N atoms as spheres with horizontal and vertical dashings, respectively. Only the Ca is labelled for every amino-acid residue. Dashed lines indicate the hydrogen-bond interactions involving the retinol hydroxyl end group.

RBPs may be correlated with the multiple interactions established by RBP, *i.e.* with retinol, transthyretin and, possibly, target cell-surface receptors. The relatively high resolution structure presented here has revealed an additional hydrogen-bonding interaction between the retinol hydroxyl group and the protein moiety. This finding further supports the notion of a higher binding affinity of retinol for RBP, as compared with retinoids lacking the retinol functional end group. Pig RBP crystallization is dependent on metal ions, such as Cd^{2+}, that promote intermolecular interactions involved in crystal packing.

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† Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1AQB).

References

Berni, R., Stoppini, M., Zapponi, C. M., Meloni, M. L., Monaco, H. L. & Zanotti, G. (1990). *Eur. J. Biochem.* **192**, 507–513.

Brünger, A. T. (1990). *Acta Cryst.* A**46**, 46–57.

Cowan, S. W., Newcomer, M. E. & Jones, T. A. (1990). *Proteins Struct. Funct. Genet.* **8**, 44–61.

Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) *J. Appl. Cryst.* **26**, 283–291.

Malpeli, G., Folli, C. & Berni, R. (1996). *Biochim. Biophys. Acta*, **1294**, 48–54.

Monaco, H. L., Rizzi, M. & Coda, A. (1995). *Science*, **268**, 1039–1041.

Newcomer, M. E., Liljas, A., Sundelin, J., Rask, L. & Peterson, P. A. (1984). *J. Biol. Chem.* **259**, 5230–5231.

Sheldrick, G. M. (1993). *SHELXL*93, *Program for Crystal Structure Refinement*. University of Göttingen, Germany.

Shiba, J. K. & Bau, R. (1978). *Inorg. Chem.* **17**, 3484–3488.

Trout, W. E., McDonnell, J. J., Kramer, K. K., Baumbach, G. A. & Roberts, R. M. (1991) *Mol. Endocrinol.* **5**, 1533–1540.

Zanotti, G., Berni, R. & Monaco, H. L. (1993). *J. Biol. Chem.* **268**, 10728–10738.

Zanotti, G., Malpeli, G. & Berni, R. (1993). *J. Biol. Chem.* **268**, 24873–24879

Zanotti, G., Marcello, M., Malpeli, G., Folli, C., Sartori, G. & Berni, R. (1994). *J. Biol. Chem.* **269**, 29613–29620.

Zanotti, G., Ottonello, S., Berni, R. & Monaco, H. L. (1993). *J. Mol. Biol.* **230**, 613–624.