View metadata, citation and similar papers at core.ac.uk
brought to you by CORE

CROATICA CHEMICA ACTA CCACAA **75** (3) 835–845 (2002)

ISSN-0011-1643
CCA-2835

Review

Retinoids in Mammals: A Crystallographic Perspective*

Giuseppe Zanotti[a,**] *and Rodolfo Berni*[b]

[a] *Department of Organic Chemistry, University of Padova and Biopolymer Research Center, C.N.R., Via Marzolo 1, 35131 Padova, Italy*

[b] *Department of Biochemistry and Molecular Biology, University of Parma, Parco Area delle Scienze 23/A, 43100 Parma, Italy*

Received April 8, 2002; revised July 5, 2002; accepted July 9, 2002

Retinoids are involved in several essential processes in mammals, including vision, morphogenesis, spermatogenesis and maintenance of epithelial tissue. Since they are labile compounds, nearly insoluble in water, they are present in body fluids and within the cell bound to specific retinoid-binding proteins. In plasma, a single protein, called retinol-binding protein, delivers the alcoholic form of vitamin A from its store sites to target cells. In the cytoplasm, four different cellular retinol-binding proteins and two retinoic acid-binding proteins have been discovered and structurally characterized to date. Finally, two classes of nuclear receptors for retinoic acid isomers have been characterized. The structure/function relationship for several retinoid-binding proteins is discussed here.

Key words: vitamin A, retinol, retinoids, retinoic acid, retinol-binding proteins, retinoid receptors, crystal structure.

INTRODUCTION

Retinoids constitute a large group of synthetic and naturally occurring compounds related to retinol, the vitamin A alcohol (Figure 1). By analogy with the prototypical retinol molecule, physiologically occurring retinoids

---

* Based upon the plenary lecture presented at the 10th Croatian-Slovenian Crystallographic Meeting, Lovran, Croatia, June 21–24, 2001.

** Author to whom correspondence should be addressed. (E-mail: giuseppe.zanotti@unipd.it)

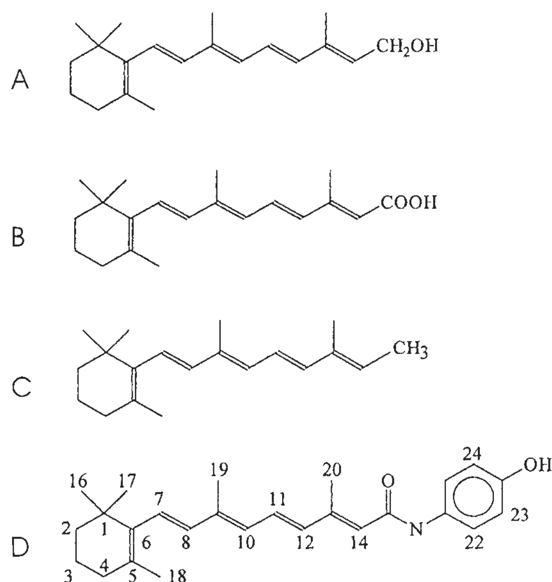

Figure 1. Some retinoids cited in the text. (A) all-*trans*-retinol, (B) all-*trans*-retinoic acid, (C) axerophtene, (D) fenretinide.

are characterized by a β-ionone ring, a polyene chain and a functional polar end group. In mammals, retinoids fulfil essential roles and their metabolism, biological effects and mechanism of action are topics of considerable research interest. Because of their chemical instability and quite low solubility in the uncomplexed form in an aqueous medium, natural retinoids need to be bound to specific retinoid-binding proteins to be protected, solubilized, and transported in body fluids and within the cell. Retinol circulates in blood bound to Retinol-binding Protein (RBP), which delivers the vitamin from its store sites to target cells.[1] The homologous Cellular Retinol-binding Proteins (CRBPs) and Cellular Retinoic Acid-binding Proteins (CRABPs) have been reported to be cytoplasmic carriers of retinoids.[1–3] In addition, the eye has distinct retinoid-binding proteins, unrelated to RBP and CRBPs/CRABPs: a Cellular Retinaldehyde-binding Protein and an Interstitial/Interphotoreceptor Retinoid-binding Protein. Besides being involved in vision, a process in which 11-*cis*-retinal plays a key role, retinoids are indispensable for morphogenesis, spermatogenesis and the maintenance of epithelial tissue. For the last mentioned processes, isomers of retinoic acid have been shown to be the active vitamin A derivatives. Both 9-*cis* and all-*trans* isomers of retinoic acid are transcriptional activators, which exert their effects through binding to specific nuclear retinoid receptors.[4] Finally, evidence has

been obtained that retinoids are involved in the regulation of the immune response by controlling lymphocyte survival.[5]

THE PLASMA CARRIER OF RETINOL

Retinol is the prevailing retinoid in blood, and is mainly transported through the circulation from the storage site richest in vitamin A, the liver, to the surface receptors of a variety of target cells (Figure 2). This transport is specifically performed by RBP, a monomeric plasma protein of 21 kDa, which contains one binding site for retinol and belongs to the superfamily of extracellular Lipid-binding Proteins (eLBPs or lipocalins).[6] RBP circulates in plasma bound to another plasma protein, transthyretin (TTR). Besides being involved in the plasma transport of retinol-RBP, TTR is one of the plasma carriers of the thyroid hormone thyroxine. The formation of the RBP-TTR macromolecular complex is supposed to prevent filtration through renal glomeruli of the relatively small RBP molecule.

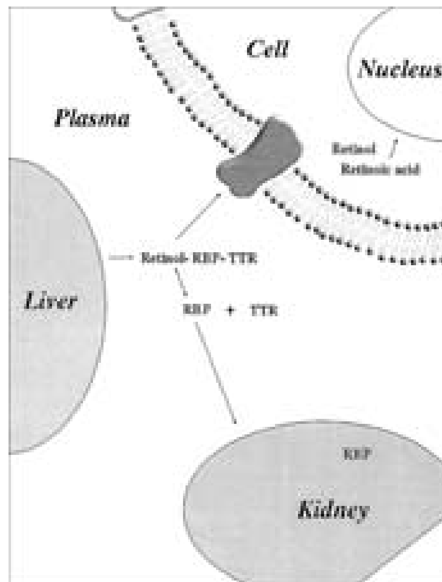

Figure 2. A schematic representation of vitamin A transport from the liver store site to target cells. The alcoholic form of vitamin A, retinol, is released in plasma bound to retinol-binding protein (RBP), which in turn forms a complex with another protein, transthyretin. When the ligand is released to the cell receptor, the complex dissociates and RBP is catabolized in renal glomeruli. Retinol and retinoic acid in the cell cytoplasm are bound to specific binding proteins, CRBPs and CRABPs (see text for details).

The crystal structures of human,[7,8] bovine,[9] pig[10] and chicken[11] RBPs have been determined (Figure 3). The RBP polypeptide chain folds into a single domain, made up of a N-terminal coil, eight antiparallel β-strands, a short α-helix and a C-terminal strand. The core of the protein is the internal cavity of the eight-stranded up-and-down β-barrel, where the ligand binds: the β-ionone ring and the polyene chain are completely buried inside the internal cavity, of a shape highly complementary to that of the ligand, whilst the alcoholic end group is at the protein surface, in contact with the solvent.

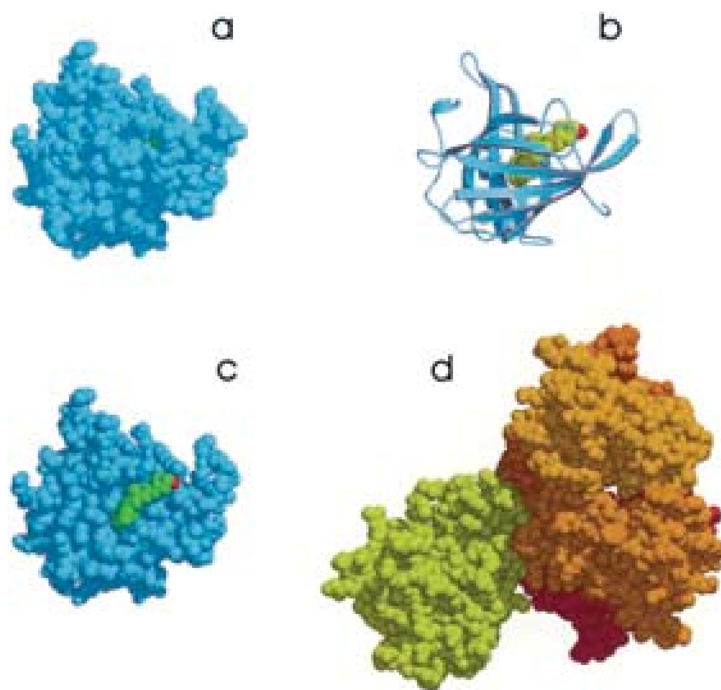

Figure 3. Plasma retinol-binding protein (RBP). (a) van der Waals representation of bovine plasma RBP (blue spheres) with retinol bound within the cavity (green spheres, the oxygen atom of OH group in red; coordinates from PDB 1HBP). (b) Cartoon representation of the same model. (c) Same as (a), but the model has been cut by a plane parallel to the paper to make the ligand inside the β-barrel cavity more visible. (d) van der Waals representation of the complex between RBP (yellow) and transthyretin, a tetramer made up of four identical subunits (from red to orange). Coordinates from PDB 1RLB. Only one RBP molecule is shown to represent the physiological complex in plasma.

The protein-retinol interactions are essentially hydrophobic, with the exception of the hydroxyl group, which participates in the hydrogen bond in-

teraction, mediated by a water molecule, with a carbonyl oxygen of the main chain. The high degree of complementarity between retinol and the ligand-binding cavity makes the binding very specific, so that chemical modifications of the retinol skeleton abolish the binding ability. Instead, the positioning of the terminal portion of the tail of the ligand allows the protein to easily accommodate the acid or aldehyde form of the vitamin, as well as retinoids bearing bulky end groups, as we have previously shown.[12,13] Specifically, the crystal structures of bovine RBP complexed with all-*trans*-retinoic acid, *N*-(4-hydroxyphenyl)retinamide, *N*-ethylretinamide and axerophthene illustrate how analogs of the retinol molecule, modified in correspondence to its alcoholic end group, still interact effectively with the protein moiety.

The extraction of retinol from the cavity of the holoprotein by using organic solvents induces minor but significant conformational changes: the superposition of the models of the apo and holo forms of human[7] and bovine[8] RBPs shows differences in correspondence of residues 33–36, which are present in one of the ligand entrance loops. In particular, the side chains of Leu-35 and Phe-36 change their orientation in the apoprotein and occupy some of the space left by the vitamin. The rest of the cavity is probably filled with non-ordered water molecules or with molecules of the organic solvent used to extract the ligand from the holoprotein.

The molecular mechanism of ligand binding and release for plasma RBP remains to be clarified. Since the β-barrel internal cavity of RBP has the shape of a calyx, the open end of the calyx is very likely the portal involved in retinol entering/exiting. Indeed, in agreement with this hypothesis, in the holoprotein, the retinol hydroxyl group is protruding from the protein surface towards the solvent from this end. However, the crystal structure of the apoprotein is similar to that of the holoprotein and, as such, does not allow the retinol molecule to enter. Therefore, it must be assumed that the apo and holo structures represent similar and static structural situations whereas, during the apo/holo transition, the protein undergoes remarkable ligand-dependent conformational changes, accompanying the process of the binding/release of the vitamin. It has in fact been proposed[14] that the protein molecule is more flexible than what appears from its crystal structure, and that the release of retinol might require a reversible transition of the protein to a transient, poorly structured state, perhaps a »molten globule« state. It has been shown that low pH conditions favor the release of retinol, along with mild denaturing conditions.[14] A molecular hypothesis for this behavior has been put forward in the past by this laboratory:[15] a number of conservative, positively charged side chains located on the retinol-binding face of the RBP molecule are involved in salt bridges with conservative, negatively charged groups. At acidic pH values, close to the pK_a value of the aci-

dic groups, these salt bridges are broken and, consequently, the retinol-binding face of RBP would hold from 8 to 12 positively charged groups. Such groups might ensure a proper orientation of the RBP molecule relative to the negatively charged cell membrane surface. The disruption of salt bridges and the electrostatic repulsion of positive charges might soften the structure near the entrance of the retinol-binding pocket and trigger the release of the ligand.

Holo-RBP circulates in plasma bound to another protein, transthyretin (TTR), forming the ternary retinol-RBP-TTR complex. At present, the crystal structures of two such complexes are available: the heterologous complex between chicken RBP and human TTR[16] and the homologous complex between human RBP and human TTR.[17] In both cases, the highest resolution of the data is quite limited, about 3.2 Å. The stoichiometry of the complex involves one TTR and two RBPs (Figure 3). TTR is a symmetric tetramer and, in both cases, two RBP molecules are bound to it in a symmetric way. The orientation of the TTR-bound RBP molecules is different in the two complexes, but this is probably due to a different influence of the crystal state in the two cases. Recently, we have crystallized the human RBP-TTR complex associated with an *anti*-RBP Fab. Despite the very small size of the crystals, using the synchrotron radiation beamline of Elettra (Trieste, Italy), a data set to a resolution of 3.2 Å has been obtained.[18] The crystals belong to the $C222$ space group, with a = 159.3 Å, b = 222.4 Å and c = 121.27 Å. The structure determination of this macromolecular complex, consisting of three proteins, is under way in our laboratory.

THE CYTOPLASMIC CARRIERS OF RETINOIDS

A number of *in vitro* studies have suggested that CRBPs could play important roles in vitamin A homeostasis, being involved in retinol internalization, retinol esterification and oxidation, and retinyl ester hydrolysis.[2,3] Additionally, retinol-CRBP complexes, rather than uncomplexed retinol, might serve as substrates of enzymes involved in the retinol metabolism.[2,3] Despite the wealth of *in vitro* data obtained with purified proteins and/or subcellular fractions, the precise functional roles of CRBPs remain to be defined. Similarly to CRBPs, it has been proposed that CRABPs serve to solubilize and protect their ligand in the aqueous cytosol, and that they transport retinoic acid between different cellular compartments. Apart from the aforementioned intracellular retinoid-binding protein present in the eye, six cytoplasmic retinoid-binding proteins have been identified in mammals: four CRBPs, and two CRABPs.[2,3,19,20] They are monomeric proteins of approximately 15 kDa and belong, along with the Fatty Acid Binding Proteins

(FABPs), to the superfamily of iLBPs (intracellular Lipid Binding Proteins). The crystal structures of all these proteins are known and, despite a considerable variance in their primary structures, they possess a highly similar overall three-dimensional structure:[1] ten antiparallel β-strands form a β-barrel with a shell-like shape, closed at one end by a helix-turn-helix motif and containing within its internal cavity the hydrophobic ligand-binding site (Figure 4). In the case of CRBPs/CRABPs, the retinoid is completely buried inside the cavity, so that it is totally isolated from the outside solvent. The binding of retinol in CRBPs is strongly stabilized by an H-bond interaction of the hydroxyl group of the vitamin with Gln-108 in CRBP I and II,[1,2] and with His-108 in CRBP III.[19] In the case of CRABP, the negatively charged carboxylate group of the vitamin makes a salt bridge with Arg-131 and Arg-132 in CRABP I and II, respectively.[1] In the cytoplasmic retinoid-binding proteins, the protein cavity is definitely larger than the ligand and in all cases several ordered solvent molecules, from seven to nine, are present wi-

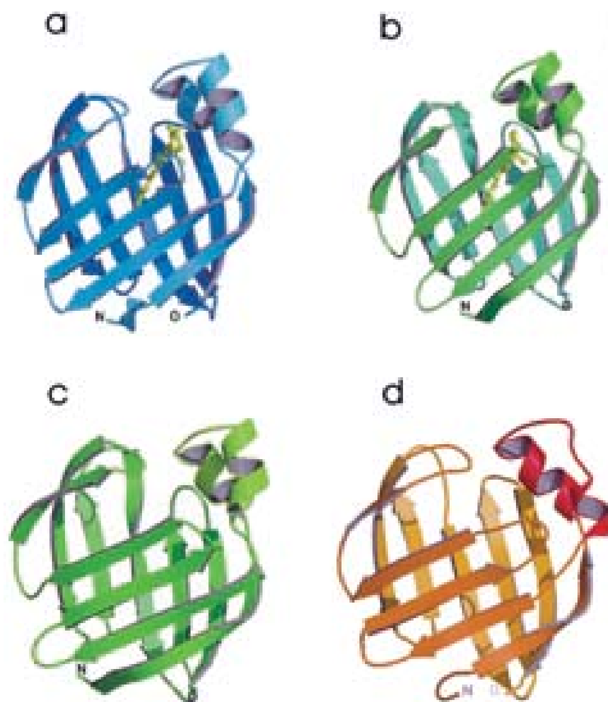

Figure 4. Cartoon representation of some cellular retinol-binding proteins (CRBPs). (a) holo-CRBP I (coordinates from PDB 1CRB), (b) holo-CRBP II (coordinates from PDB 1OPB), (c) apo-CRBP II (coordinates from PDB 1OPA), (d) apo-CRBP III (coordinates from PDB 1GGL). These proteins have a globular compact shape, made up of ten antiparallel β-strands and a helix-turn-helix motif.

thin the cavity along with the retinoid. All these water molecules are well defined in the crystal structures, and form intermolecular hydrogen bonds as well as hydrogen bonds with the side chains of residues lining the β-barrel internal cavity. They may play a structural role and limit, presumably along with non-ordered water molecules, the volume of the cavity accessible to the ligand.

Despite the abundance of experimental data available, there are several open questions about the functional and structural properties of the members of the CRBP and CRABP protein families. In particular:

a) To what extent are they specific with regard to ligand binding, the function displayed and tissue distribution?
b) What is the mechanism of release of the ligand from the interior of the cavity and of its binding? Because limited conformational differences between the crystalline holo and apoproteins have been reported, one must hypothesize that significant, but transient, protein conformational changes take place to allow ligand binding/release.
c) What is the exact function of different CRBPs and CRABPs, apart from that obvious of binding the retinoid? Several hypotheses have been put forward, but none of them seems to be conclusive. Since several proteins that bind the same retinoid may be present in a particular tissue, it is likely that they can not simply have the function of binding and solubilizing the ligand. At least some of these proteins must perform more specialized functions. In particular, it has been proposed that specific recognition properties of CRBPs/CRABPs may allow a specific delivery of retinoids to enzyme systems or other acceptors. Recently, the role of CRABP in shuttling retinoic acid from the cytoplasm to the cell nucleus and the delivery of this retinoid to retinoic acid receptors has been reported.[21]

THE NUCLEAR RETINOID RECEPTORS

It is in the nucleus that retinoic acid performs the important function of regulating gene transcription.[4] This action is displayed through the binding of the retinoid to nuclear receptors, proteins containing several domains, such as a DNA-binding domain and a ligand-binding domain. Two families of retinoic acid receptors have been characterized to date: the RARs (retinoic acid receptors) and the RXRs (retinoid X receptors). The former bind all-*trans* and 9-*cis*-retinoic acid and the latter bind 9-*cis*-retinoic acid.

X-ray structures have been determined for the ligand binding domains of RARsγ, RXRsα and RXRsβ[22–24] and found to be quite similar: twelve α-helices form a sort of helical sandwich, in which a layer of α-helices and two

β-strands are surrounded by two layers of α-helices. The retinoic acid molecule binds in the center of this structure (Figure 5). Significant conformational changes, presumably associated with the activation of the receptors to transcription factors, are induced by ligand-binding.

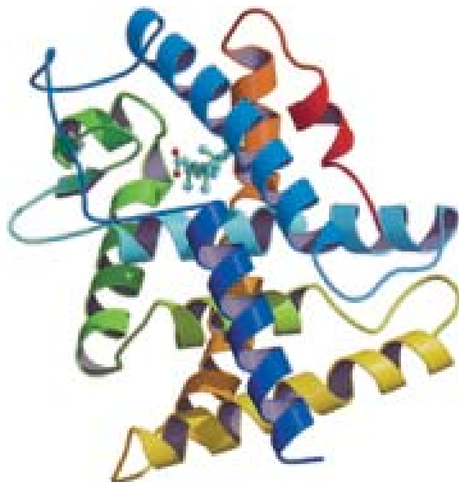

Figure 5. Cartoon representation of the retinoid binding domain of human RAR-γ complexed with all-*trans*-retinoic acid (coordinates from PDB 2LBD).

CRYSTAL STRUCTURES OF OTHER RETINOID-BINDING PROTEINS

A protein that shares a similarity of folding motifs with plasma RBP[25] is epididymal retinoic-acid binding protein (E-RABP), a major secreted protein found in the lumen of epididymis. Its physiological role is probably that of supplying the retinoic acid necessary in either sperm maturation or epididymal function. Since E-RABP, differently from RBP, binds retinoic acid, there are considerable structural differences in the binding sites of the two proteins.

Proteins of the lipocalin family other than RBP are known to bind retinol *in vitro*, for example β-lactoglobulin, a protein found in milk of several mammals. Nevertheless, vitamin A in the milk is associated with fat globules, and retinol bound to β-lactoglobulin extracted from milk has not been detected yet.

All-*trans*-retinol and 11-*cis*-retinal are known to bind to interphotoreceptor retinoid binding protein (IRBP), the major soluble component of inter-

photoreceptor matrix. This large protein, made up of four contiguous repeats, is critical to the function, integrity, and development of the vertebrate retina. The crystal structure at 1.8 Å resolution of one of the functional units of the protein from *Xenopus laevis* has been recently determined.[26] The module consists of two domains, separated by a hydrophobic ligand binding site, that have not structural similarity with lipocalins.

CONCLUSIONS

Different classes of proteins originate from mammals that bind retinoids either extracellularly or intracellularly. In plasma, a single protein (RBP) binds and transports retinol. Its main function is that of solubilizing and protecting the retinol molecule in plasma and of transporting/delivering it to the receptors of target cells. In the cytoplasm, at least six different retinoid-binding proteins are present: they share a relatively limited sequence identity, but represent a very similar structural fold, and they also bind retinol (CRBPs) or retinoic acid (CRABPs) in a similar fashion. Their functional roles have not been well established yet. Finally, in the nucleus, two different families of retinoic acid receptors (RARs and RXRs) have been identified and their function in the control of gene transcription has been established. The crystal structure of the retinoid binding domain of nuclear receptors is totally different from those of the extracellular and intracellular carriers of retinoids, in agreement with the distinct role of nuclear receptors compared to those of the carrier proteins.

REFERENCES

1. M. E. Newcomer, R. S. Jamison, and D. E. Ong, *Subcell Biochem.* **30** (1998) 53–80.
2. D. E. Ong, M. E. Newcomer, and F. Chytil, *Cellular retinoid binding proteins*, in: M. B. Sporn, A. Roberts, and D. S. Goodman (Eds.), *The Retinoids, Biology, Chemistry and Medicine*, Raven Press, New York, 1994, pp. 283–318.
3. E. Li and A. W. Norris, *Annu. Rev. Nutr.* **16** (1996) 205–234.
4. P. Chambon, *FASEB J.* **10** (1996) 940–954.
5. C. A. Ross and U. G. Hammerling, *Retinoids and the immune system*, in: M.B. Sporn, A. Roberts, and D. S. Goodman (Eds.), *The Retinoids: Biology, Chemistry and Medicine*, Raven Press, New York, 1994, pp. 521–544.
6. D. R. Flower, A. C. T. North, and C. E. Sansom, *Biochim. Biophys. Acta* **1482** (2000) 9–24.
7. S. W. Cowan, M. E. Newcomer, and T. A. Jones, *Proteins* **8** (1990) 44–61.
8. G. Zanotti, S. Ottonello, R. Berni, and H. L. Monaco, *J. Mol. Biol.* **230** (1993) 613–624.
9. G. Zanotti, R. Berni, and H. L. Monaco, *J. Biol. Chem.* **268** (1993b) 10728–10738.

10. G. Zanotti, M. Panzalorto, G. Marcato, G. Malpeli, C. Folli, and R. Berni, *Acta Crystallogr., Sect. D* **54** (1998) 1049–1052.
11. G. Zanotti, V. Calderone, M. Beda, G. Malpeli, C. Folli, and R. Berni, *Biochim. Biophys. Acta* **1550** (2001) 64–69.
12. G. Zanotti, G. Malpeli, and R. Berni, *J. Biol. Chem.* **268** (1993) 24873–24879.
13. G. Zanotti, M. Marcello, G. Malpeli, C. Folli, G. Sartori, and R. Berni, *J. Biol. Chem.* **269** (1994) 29613–29620.
14. V. E. Bychkova, R. Berni, G. L. Rossi, V. P. Kutyshenko, and O. B. Ptitsyn, *Biochemistry* **31** (1992) 7566–7571.
15. O. B. Ptytsin, G. Zanotti, A. I. Denesyuk, and V. E. Bychkova, *FEBS Lett.* **317** (1993) 181–184.
16. H. L. Monaco, M. Rizzi, and A. Coda, *Science* **268** (1995) 1039–1041.
17. H. M. Naylor and M. E. Newcomer, *Biochemistry* **38** (1999) 2647–2653.
18. G. Malpeli, G. Zanotti, F. Gliubich, A. Rizzotto, S. K. Nishida, C. Folli, and R. Berni, *Acta Crystallogr., Sect. D* **55** (1999) 276–278
19. C. Folli, V. Calderone, S. Ottonello, A. Bolchi, G. Zanotti, M. Stoppini, and R. Berni, *Proc. Natl. Acad. Sci. USA* **98** (2001) 3710–3715.
20. S. Vogel, C. L. Mendelsohn, J. R. Mertz, R. Piantedosi, C. Waldburger, M. E. Gottesman, and W. S. Blaner, *J. Biol. Chem.* **276** (2001) 1353–1360.
21. A. Budhu, R. Gillilan, and N. Noy, *J. Mol. Biol.* **305** (2001) 939–949.
22. P. F. Egea, A. Mitschler, N. Rochel, M. Ruff, P. Chambon, and D. Moras, *EMBO J.* **19** (2000) 2592–2601.
23. J. P. Renaud, N. Rochel, M. Ruff, V. Vivat, P. Chambon, H. Gronemeyer, and D. Moras, *Nature* **378** (1995) 681–689.
24. J. D. Love, J. T. Gooch, S. Benko, C. Li, L. Nagy, V. Krishna, K. Chatterjee, R. M. Evans, and J. W. R. Schwabe, *J. Biol. Chem.* **277** (2002) 11385–11391.
25. D. E. Ong. M. E. Newcomer, J.-J. Lareyre, and M.-C. Orgebin-Crist, *Biochim. Biophys. Acta* **1482** (2000) 209–217.
26. A. Loew and F. Gonzales-Fernadez, *Structure* **10** (2002) 43–49.

SAŽETAK

Retinoidi u sisavaca: kristalografsko gledanje

Giuseppe Zanotti i Rodolfo Berni

U sisavaca retinoidi sudjeluju u nekoliko bitnih procesa koji uključuju vid, morfogenezu, spermatogenezu i održavanje epitelnih tkiva. Oni su prisutni u tjelesnim tekućinama, a u stanici su vezani na specifične retinoid-vežuće proteine, jer su labilni spojevi skoro netopivi u vodi. U plazmi jedan jedini protein, nazvan retinol-vežući protein, isporučuje alkoholni oblik vitamina A iz njegovih mjesta skladištenja u ciljne stanice. U citoplazmi su do sada otkrivena i strukturno okarakterizirana četiri različita retinol-vežuća proteina i dva proteina koji vežu retinsku kiselinu. Konačno, okarakterizirane su dvije klase nuklearnih receptora za izomere retinske kiseline. Ovdje se raspravlja odnos strukture i funkcije za nekoliko retinoid-vežućih proteina.