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Protein and ligand adaptation in a retinoic acid binding protein

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Abstract

A retinoic acid binding protein isolated from the lumen of the rat epididymis (ERABP) is a member of the lipocalin superfamily. ERABP binds both the all-trans and 9-cis isomers of retinoic acid, as well as the synthetic retinoid (E)-4-[2-(5,6,7,8)-tetrahydro-5,5,8,8-tetramethyl-2 napthalenyl-1 propenyl]-benzoic acid (TTNPB), a structural analog of all-trans retinoic acid. The structure of ERABP with a mixture of all-trans and 9-cis retinoic acid has previously been reported. To elucidate any structural differences in the protein when bound to the all-trans and 9-cis isomers, the structures of all-trans retinoic acid–ERABP and 9-cis retinoic acid ERABP were determined. Our results indicate that the all-trans isomer of retinoic acid adopts an 8-cis structure in the binding cavity with no concomitant conformational change in the protein. The structure of TTNPB-ERABP is also reported herein. To accommodate this all-trans analog, which cannot readily adopt a cis-like structure, alternative positioning of critical binding site side chains is required. Consequently, both protein and ligand adaption are observed in the formation of the various holo-proteins.

Keywords: binding protein; retinoic acid; X-ray structure

It has long been known that retinol plays an important role in male fertility. In the early 1960s, it was found that male rats raised on a retinol-free diet supplemented with retinoic acid were blind and sterile but "otherwise healthy." The simplest interpretation of this observation was that it is retinol and not retinoic acid (which cannot be reduced in vivo) that is required for male fertility. However, retinoic acid may play a role in spermatogenesis or sperm maturation as well, and its inability to substitute for retinol may be due to the fact that retinoic acid cannot cross the blood-testis barrier. Instead, retinol is converted to retinoic acid in situ. In the testis, spermatogonia undergo a series of transformations and subsequently emerge as spermatozoa. Upon exiting the testis, the spermatozoa must traverse the epididymis and in transit through the organ acquire the ability to fertilize an egg. Both cellular and extracellular retinoic acid binding proteins are abundant in testis and epididymis (see Ong et al., 1994). Furthermore, high levels of the transcripts for the retinoic acid receptors (RAR)-α and -β have been observed in the epididymis and testis (e.g., Kim & Griswold, 1990; Wan et al., 1992).

A retinoic acid binding protein abundant in the lumen of the epididymis (epididymal retinoic acid binding protein (ERABP)) that binds both 9-cis and all-trans retinoic acid is a member of the lipocalin superfamily. The crystal structure of the protein has been described in the absence and presence of the ligand (Newcomer, 1993; Newcomer et al., 1993). The original crystals of the holoprotein were prepared with all-trans retinoic acid, however, during the time required for the growth of crystals significant amounts of 9-cis retinoic acid can accumulate. The clear sickle shape of the ligand electron density led the authors to check for the presence of 9-cis retinoic acid in the crystals and the ability of the protein to bind 9-cis retinoic acid. The crystal was found to contain a mixture of the 9-cis and all-trans isomers and hence any conformational differences between the protein ligand complexes could not be discerned (Newcomer et al., 1993). To elucidate any such conformational differences, we have prepared crystals of ERABP with 9-cis and all-trans retinoic acid with highly purified isomers under conditions in which isomerization is minimized.

Synthetic retinoids have been developed as possible therapeutic agents, as retinoic acid based compounds have been shown to be effective in oncology and dermatology. One early such retinoic acid analog is (E)-4-[2-(5,6,7,8)-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propenyl]-benzoic acid (TTNPB), which has been shown to be 80% as effective as retinoic acid in RAR activation assays (Manglesdorf et al., 1990). TTNPB was developed as a conformationally restricted analog of the all-trans isomer, yet TTNPB competes well with all-trans retinoic acid for binding to ERABP, despite the fact that the X-ray structure clearly revealed a binding site that appears more complementary in shape to the 9-cis isomer of the ligand, and one in which all-trans retinoic acid must adopt a cis-like conformation in order to bind. The all-trans and 9-cis isomers of retinoic acid when bound to ERABP have their trimethylcyclohexenyl rings and carboxylates situated in the same

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volumes of the protein binding site. The carboxylates participate in a charged cluster, which is critical for ligand recognition, as retinol or retinal do not bind to ERABP. It is this positioning of the carboxylate that necessitates a rotation about the C8-C9 bond of the isoprene tail of the all-trans isomer. Since conformationally restricted TTNPB cannot adopt a cis-like conformation and position its carboxylate for the critical protein:ligand interaction observed with the natural ligands, we extended our studies to include the structure determination of ERABP with TTNPB.

We report here the structures of ERABP with all-trans retinoic acid, 9-cis retinoic acid, and TTNPB, as well as the 1.65 Å native structure with dimethyl sulfoxide (DMSO) bound in the hydrophobic binding cavity. Our results indicate that although the protein bound conformations of the all-trans and 9-cis isomers of retinoic acid are superimposeable and the protein itself displays no conformational differences between the two holo-forms, the conformationally restricted all-trans retinoic acid analog TTNPB is shifted relative to the retinoic acid isomers in the binding cavity, and the placement of the TTNPB carboxylate requires an alternative positioning of amino acid side chains.

Results and discussion

ERABP is a member of the lipocalin superfamily and thus displays the structural framework common to the lipocalins: an eight-stranded up-and-down β-barrel with a carboxy-terminal α-helix. The two layers of β-sheet that form the barrel stack orthogonally and one end of the open barrel is sealed by amino acid side chains from the amino-terminus; the opposite end of the barrel provides access to an internal binding cavity, which is hydrophobic and complementary in shape to the ligand. Lipocalins, which bind retinol, retinal (Lepperdinger et al., 1996), and retinoic acid, have been identified. Structures for retinol (Cowan et al., 1990; Zanotti et al., 1993) and retinoic acid binding lipocalins (Newcomer, 1993) have been described.

Comparison of the holo- and apo-ERABP structures

It was previously observed that crystals of apo-ERABP are significantly better in quality than crystals of the holo-protein. Therefore, to obtain a high resolution structure of ERABP with ligand(s), the ligand solubilized in DMSO was added to the mother liquor in which the apo-crystals were harvested. Although the crystal packing is relatively tight (V_m = 2.1 Å^3/D), the binding site is open and the access relatively unobstructed. However, a difference Fourier calculated with data collected on crystals soaked at least one week in TTNPB revealed no electron density for the retinoid. Refinement of the structure to 1.65 Å resolution did, however, reveal a molecule of the DMSO carrier positioned in the binding cavity. The inability to diffuse ligand into the apo-ERABP crystals necessitated the preparation of holo-crystals by cocrystallization.

Difference Fouriers calculated with X-ray data from the cocrystallized crystals prepared with all-trans and 9-cis retinoic acid as well as TTNPB had clear ligand density. A representative simulated annealing omit map is given in Figure 1.

For the sake of simplicity, the comparison of the molecules is with monomer "B," as the ligand density for B is markedly better for all but the TTNPB cocrystal structure. (An explanation for this observation is given below.) An overlay of all four structures is given in Figure 2A. Note that the bulk of the conformational differences between the structures is limited to the loop regions. These regions in all the structures are mobile, as inferred from the fact that the temperature factors in these regions are significantly higher than the average overall temperature factor. The all-trans, 9-cis, and TTNPB cocrystal structures superimpose onto the apo(DMSO) protein well with root-mean-square deviations (RMSDs) of 0.47, 0.43, and 0.66 Å, respectively. The larger deviation for the TTNPB structure is a consequence of the fact that the placement of this ligand in the binding site requires that those side chains at the carboxylate end of the binding site must reposition. Furthermore, the bulkier ring region appears to require minor adjustments in the aromatic cluster at the ring end of the binding site. In contrast, no significant conformational differences in protein structure are observed when comparing the all-trans and 9-cis retinoic acid holo structures (RMSD 0.21 Å). It is the conformational flexibility of the ligand that allows both isomers to bind without concomitant structural differences in the protein.

Details of the binding site

As mentioned above, the structure of ERABP with a mixture of all-trans and 9-cis isomers of retinoic acid has previously been

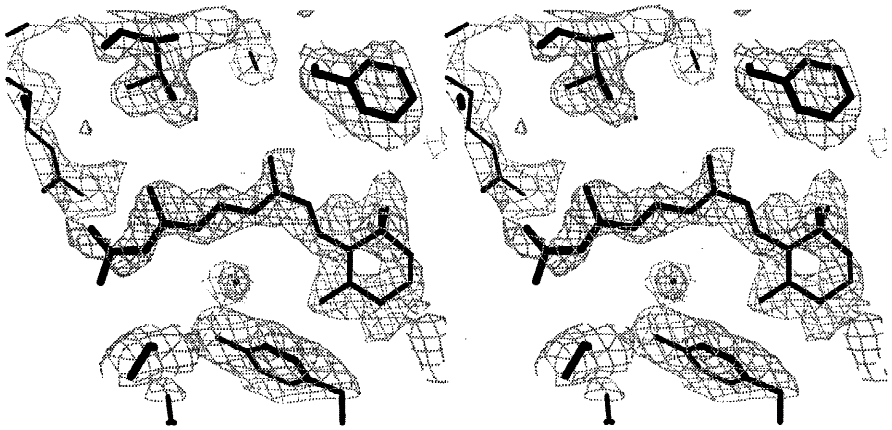

Fig. 1. Simulated annealing omit map for all-trans retinoic acid ERABP at 2.1 Å resolutions contoured at 1σ.

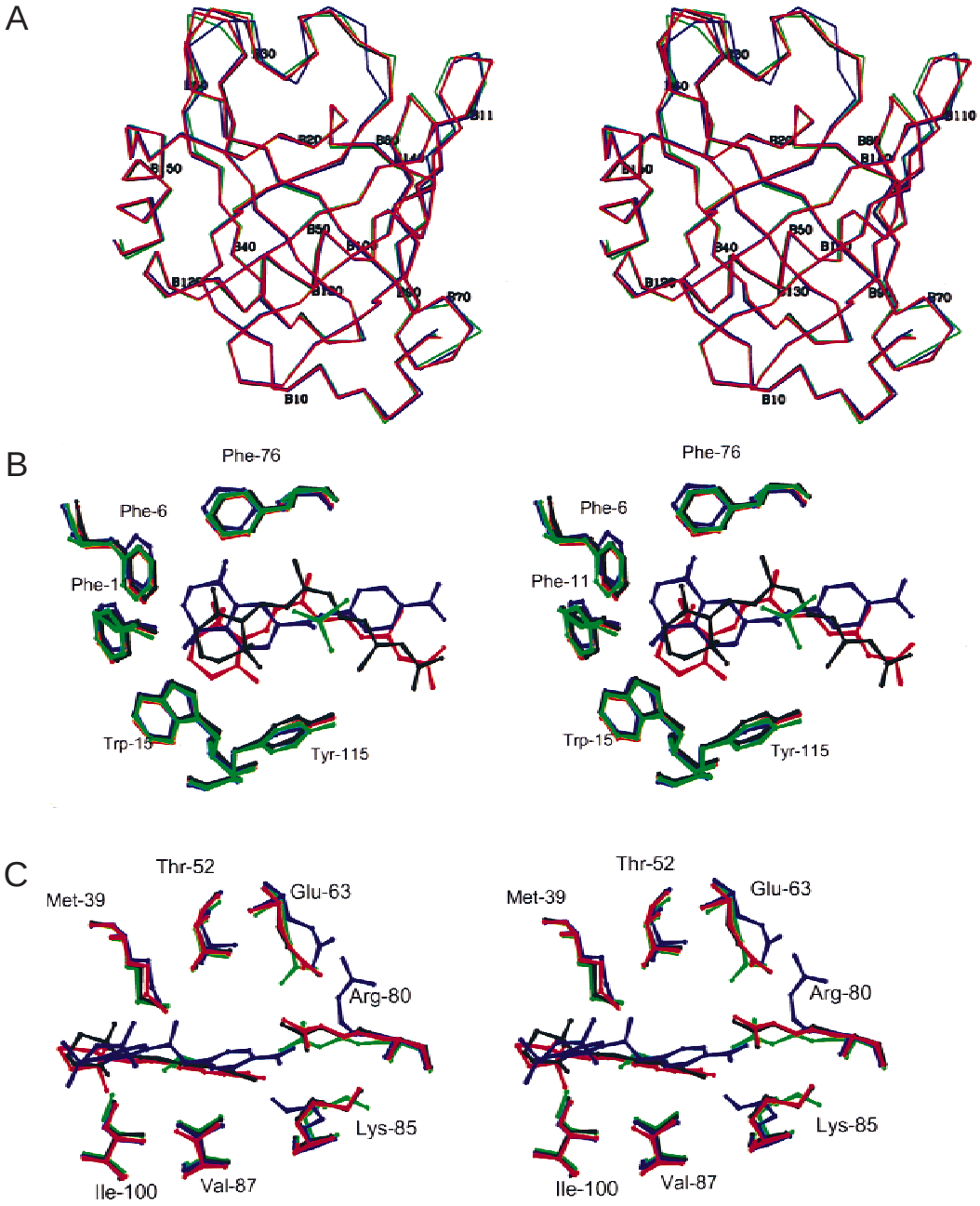

Fig. 2. Superpositions of apo (green), all trans retinoic acid (red), 9-cis retinoic acid (black), and TTNPB (blue) ERABP structures. **A:** Backbone trace. **B:** Aromatic cluster of the binding site. **C:** Neutralization of the ligand carboxylate by protein side chains.

described. In that structure the ligand is clearly sickle shaped, and all-trans retinoic acid adopts an 8-cis structure (where the C7-C8-C9-C10 torsion angle is equal to 0) in the binding cavity. The binding site of ERABP is complementary to the amphipathic ligand in both shape and chemical nature, and largely excludes water, in contrast to what is described for the retinoic acid binding site of the cellular retinoic acid binding protein (Kleywegt et al., 1994). An aromatic cluster at the trimethylcyclohexenyl ring end of the ligand encircles the ligand (Fig. 2B), the isoprene tail is surrounded by hydrophobic amino acids, and the carboxylate tail is neutralized in a web of charged amino acids surrounding the entrance to the binding cavity (Fig. 2C). Interestingly, when crystals are prepared with 9-cis retinoic acid and all-trans retinoic, it is crystals of the former that are of lesser quality. However, despite the relatively poor quality of the 9-cis data, it is clear that the ligands interact with the protein in an equivalent fashion, despite the fact that the structures of the free ligands are not superimposeable and the fact that all-trans retinoic must adopt a cis-like conformation.

In contrast, the all-trans retinoic acid analog TTNPB is shifted in its placement in the binding site, presumably a result of the bulkier ring moiety and the additional conformational restraints of

the "tail" region. The carboxylate of the TTNPB is thus positioned ~3.5 Å from the carboxylate of the all-trans and 9-cis structures, and the amino acids of the protein that form the charged pair web at the carboxylate end of the amphipathic binding site are alternatively positioned to neutralize the TTNPB carboxylate. In Figure 3, overlays of the protein bound 9-cis and all-trans isomers as well as the all-trans isomer and TTNPB are provided. It is clear from this figure that neither the "ring" or "tail" of TTNPB superimpose with the corresponding moieties of the natural ligand that TTNPB mimics.

Conformational differences observed between the two molecules of the asymmetric unit

ERABP crystallizes with two molecules in the asymmetric unit. In Figure 4 the molecules "A" and "B" from the four structures (apo, all-trans and 9-cis retinoic acid, and TTNPB ERABP) are superimposed. Note that loops at the open end of the barrel in molecule "A" are displaced from those in molecules "B." An important protein ligand interaction, which involves Arg80 and the carboxylate of the 9-cis and all-trans isomers of retinoic acid, is absent in molecule A, as crystal packing contacts do not allow placement of this loop as it is positioned in molecule B. This observation is consistent with the fact that ligand density in molecule A for the 9-cis and all-trans isomers of retinoic acid is not as well defined as that in molecule B. This disparity in occupancy is not observed for the TTNPB-ERABP structure because the ligand itself, due to its conformational restraints, requires alternative positioning of Arg80. Despite the protein conformational difference, TTNPB binds as well as the biological ligands. When incubated with equimolar amounts of TTNPB and either all-trans or 9-cis retinoic acid, ~60% of the ligand extracted from the protein is TTNPB (Newcomer et al., 1993).

A role for Arg80 in protein ligand recognition has been verified by the results of site-directed mutagenesis experiments. Sundaram et al. (1998) prepared the E63I, R80I double mutant of ERABP. Unlike the wild-type protein, the double mutant ERABP binds retinol, retinal, and retinoic acid. The K_d for retinoic acid is roughly twice that for wild-type ERABP. The authors suggest that the modest reduction in affinity for retinoic acid of the double mutant indicates that the primary interactions responsible for ligand affinity are the hydrophobic contacts.

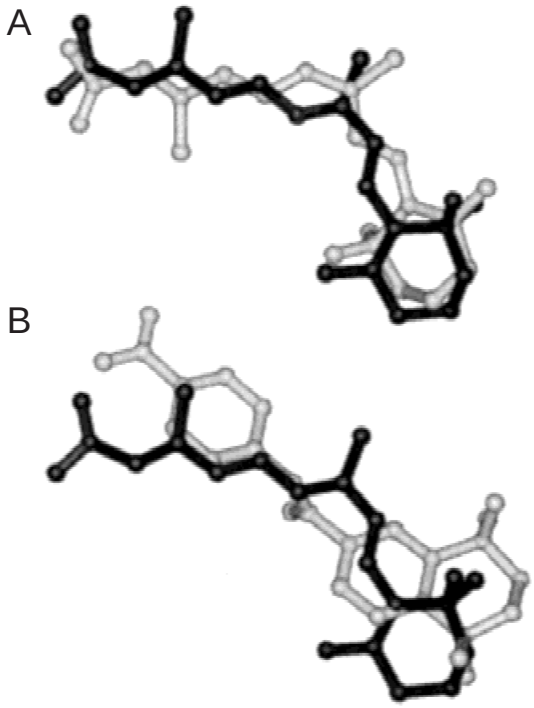

Fig. 3. Superpositions of protein bound ligands. **A:** All-trans retinoic acid (black), 9-cis retinoic acid (gray). **B:** All-trans retinoic acid (black), TTNPB (gray).

Comparison to other binding sites for all-trans and 9-cis retinoic acid

The retinoic acid nuclear receptor RAR binds both all-trans and 9-cis retinoic acid, while the retinoid X receptor (RXR) binds only 9-cis retinoic acid. In the structures of RAR-γ with 9-cis and all-trans isomers of retinoic acid, the ligands also are superimposeable (Klaholz et al., 1998). In this case, a difference in the ring-tail dihedral angle of the isomers (the angles are 45 and 26° for the all-trans and 9-cis isomers, respectively, for the C5-C6-C7-C8 dihedral) allows for the two ligands to assume the same overall shape and one in which the isoprene tail of the all-trans isomer is fully elongated. The structure of RAR with a synthetic retinoic acid analog was also reported. Like TTNPB, this analog has a tetrahydro-tetramethyl-napthalenyl (TTN) moiety on one end, and benzoic acid on the carboxylate end. In the holo structure, the tetramethyl portion of the TTN moiety is positioned as one might predict in the volume occupied by the trimethylcyclohexenyl ring of retinoic acid. The carboxylate of the analog superimposes with the carboxylate of the natural ligands, the 9-cis and all-trans isomers of retinoic acid. The binding site accommodates all-trans retinoic acid without the substantial ligand conformational change observed for ERABP, and consequently the conformationally restricted synthetic analog can position in the binding site such that both ring and tail ends superimpose with all-trans retinoic acid. This positioning of both ends of the ligand is crucial for the nuclear receptors, in which the ligand must be fully encapsulated to invoke the conformational change that is required for the activation function of the receptors (Bourguet et al., 1995; Renaud et al., 1995). Similarly, in the cellular retinoic acid binding proteins, all-trans retinoic acid binds in an extended conformation and ligand analogs utilize the same protein–ligand interactions observed for the natural ligand (Kleywegt et al., 1994). In ERABP, however, the carboxylate end of the ligand binding cavity is relatively exposed, and only minor side-chain movement is necessary to accommodate a repositioning of the carboxylate. However, this option is not used for the binding of the all-trans isomer retinoic acid: the protein invokes a conformational change in the ligand.

Concluding remarks

In solution retinoic acid is a relatively well-constrained amphipathic ligand: the trimethylcyclohexenyl ring at one end and carboxylate at the opposite end are fixed in their spatial relationship, a consequence of the conjugated double bond system of the isoprene tail. Yet, protein bound retinoic acid can assume alternative conformations. Rotation about the ring-tail dihedral is necessary to relieve unfavorable intramolecular interactions, and protein bound

Fig. 4. Superposition of molecules "A" (grey) and "B" (black) for all four structures. The side chains of Arg80 and Lys85 are depicted, as well as ligand for the all-trans retinoic acid holo structure.

retinol, and retinoic acid ring-tail dihedrals vary, presumably a result of the intramolecular interactions and the geometric constraints of the different binding sites. Similarly, rotation within the isoprene tail is possible if binding site geometry dictates. And although the synthesis of synthetic analogs, which are conformationally restricted, may provide information about the protein binding site in the absence of protein structure information, it is apparent from the results of these studies that the interpretation of structure function relationships can be complicated by the fact that both protein and ligands may adapt upon the formation of a protein ligand complex.

Materials and methods

Purification and crystallization

ERABP was purified as previously described (Newcomer & Ong, 1990). Crystals of the apo-protein were grown by hanging drop-vapor diffusion. Equal volumes of protein (at 6–8 mg/mL) and well solution were suspended above a well solution of 40% saturated ammonium sulfate, 10 mM Tris-HCl (pH 8.0), 10–12% glycerol, 2.5 mM EDTA at room temperature. Initial attempts to soak large apo-crystals in mother liquor to which retinoid dissolved in DMSO were added resulted in crystals in which DMSO, but not retinoid, was clearly revealed in the binding cavity. Holo-crystals were then grown from preparations of protein (4 mg/mL) to which ligand was added (dissolved in DMSO). These crystals appear at a significantly lower concentration of ammonium sulfate (31%). Although distinct in their morphologies, apo- and holo-crystals are isomorphous. The apparent difference in unit cell dimensions given in Table 1 is simply a result of alternative indexing, specifically the arbitrary assignment of the direction of the *b*-axis. Ligand isomerization was minimized by limiting exposure of the crystallization experiments to light. In addition, microseeding reduced the time necessary for crystal growth.

Data collection

Data were collected at room temperature on San Diego Multiwire Area Detectors mounted on a Rigaku RU-200 rotating anode. A single crystal mounted in a glass capillary was required for each of the four data sets. Statistics are provided in Table 1.

Table 1. *Data collection statistics*

	Apo	TTNPB	All-trans retinoic acid	9-cis-retinoic acid
Unit cell	a = 37.37 Å	39.89	39.86	40.08
	b = 58.71	59.06	59.14	59.64
	c = 66.72	65.31	65.18	65.93
	β = 109.21°	105.6°	105.6°	105.9°
Resolution	1.64 Å	2.0	2.15	2.8
R_{sym}	8.0 (35.1)	8.1 (34.7)	8.7 (30.9)	11.3 (40.4)
I/σ	9.9 (1.5)	7.9 (2.0)	7.9 (2.1)	8.6 (2.1)
Total number observations	104,061	49,644	34,523	22,672
Unique reflections	30,574	18,098	15,045	7,410
Completeness of data	87 (83)	91 (86)	92 (90)	98 (95)

Table 2. *Refinement statistics*

	Apo	TTNPB	All-trans retinoic acid	9-cis-retinoic acid
Number of protein atoms	2,537	2,537	2,537	2,537
Number of ligand atoms	4	52	44	44
Number of water molecules	319	186	206	268
Sigma cutoff	None	2	2	2
Resolution (Å)	1.65	2.0	2.15	2.8
$R_{crystal}$	21.7 (38.5)	21.7 (26.1)	20.7 (26.6)	22.8 (29.2)
R_{free}	29.3 (44.1)	28.2 (32.5)	25.3 (29.2)	28.1 (30.3)
Average *B*-factors (Å^2)				
Protein	27.5	22.7	24.1	24.1
Solvent	51.8	43.8	34.7	40.1
Ligand (A, B)	21.8	43.5, 42.0	73.5, 47.9	78.6, 43.3
Overall	30.2	24.5	23.7	26.2
RMSD bond lengths (Å)	0.013	0.012	0.013	0.015
RMSD bond angle (°)	1.8	1.6	1.8	2.0

Structure determinations

The 2.1 Å resolution structure of ERABP served as the starting model for the four structures (with an appropriate rotation applied to the model for those crystal data sets indexed with $\beta = 105°$). An apo-protein crystal soaked in TTNPB dissolved in DMSO revealed no electron density for the retinoic acid analog, but had clear density for the DMSO in molecule B. The structure was refined to 1.65 Å resolution after an initial simulated annealing at 1,000 K by successive cycles of positional refinement in X-PLOR (Brünger, 1993) and interpretation of $2F_o - F_c$ and difference maps with the program O (Jones et al., 1991). Approximately 10% of the reflection data was omitted from the refinements and monitored throughout by calculation of the R_{free} (Brünger, 1992). The structures of the protein with all-trans retinoic acid, 9-cis retinoic acid, and TTNPB were refined similarly. Individual *B*-factors were refined for the native, all-trans retinoic acid, and TTNPB structures. For the 9-cis retinoic acid structure, one main chain and one side chain B per residue was refined. Ligand fits were confirmed by inspection of simulated annealing omit maps. The final refinement statistics for the four structures are given in Table 2.

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References

Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D. 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature 375*:377–382.

Brünger AT. 1992. Free *R*-value: A novel statistical quantity for assessing the accuracy of crystal structures. *Nature 355*:472–475.

Brünger AT. 1993. *X-PLOR: A system for X-ray crystallography and NMR*. New Haven, Connecticut: Yale University Press.

Cowan SW, Newcomer ME, Jones TA. 1990. Crystallographic refinement of human serum retinol binding protein at 2.0 Å resolution. *Proteins Struct Funct Genet 8*:44–61.

Jones TA, Zov ZY, Cowan SW, Kjelgard M. 1991. Improved methods for building models in electron density maps and the location of errors in these models. *Acta Crystallogr A47*:110–119.

Kim KH, Griswold MD. 1990. The regulation of retinoic acid receptor mRNA levels during spermatogenesis. *Mol Endocrinol 4*:1679–1688.

Klaholz BP, Renaud J-P, Mitschler A, Zusi C, Chambon P, Gronemeyer H, Moras D. 1998. Conformational adaptation of agonists to the human nuclear receptor RAR-gamma. *Nature Struct Biol 5*:199–202.

Kleywegt GJ, Bergfors T, Senn H, Le Motte P, Gsell B, Shudo K, Jones TA. 1994. Crystal structures of cellular retinoic acid binding proteins I and II in complex with all-trans-retinoic acid and a synthetic retinoid. *Structure 2*:1241–1258.

Lepperdinger G, Strobl B, Jilek A, Weber A, Thalhamer J, Flockner H, Mollay C. 1996. The lipocalin Xlcpl1 expressed in the neural plate of Xenopus laevis embryos is a secreted retinaldehyde binding protein. *Protein Sci 5*:1250–1260.

Manglesdorf DJ, Ong ES, Dyck J, Evans RM. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature 348*:224–229.

Newcomer ME. 1993. Structure of the epididymal retinoic acid binding protein at 2.1 Å resolution. *Structure 1*:7–18.

Newcomer ME, Ong DE. 1990. Purification and crystallization of a retinoic acid-binding protein from rat epididymis. *J Biol Chem 265*:12876–12879.

Newcomer ME, Pappas RS, Ong DE. 1993. X-ray crystallographic identification of a protein-binding site for both all-trans- and 9-cis-retinoic acid. *Proc Natl Acad Sci USA 90*:9223–9227.

Ong DE, Newcomer ME, Chytil F. 1994. Cellular retinoid binding proteins. In: Sporn M, Roberts AB, Goodman DS, eds. *The retinoids: Biology, chemistry, and medicine*. New York: Raven Press. pp 283–317.

Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D. 1995. Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. *Nature 378*:681–689.

Sundaram M, Sivaprasadarao A, van Aalten DMF, Findlay JBC. 1998. Expression, characterization and engineered specificity of rat epididymal retinoic acid-binding protein. *Biochem J 334*:155–160.

Wan YJ, Wang L, Wu TC. 1992. Detection of retinoic acid receptor mRNA in rat tissues by reverse transcriptase-polymerase chain reaction. *J Mol Endocrinol 9*:291–294.

Zanotti G, Berni R, Monaco HL. 1993. Crystal structure of liganded and unliganded forms of bovine plasma retinol binding protein. *J Biol Chem 268*:10728–10738.