Crystallographic Studies on Complexes between Retinoids and Plasma Retinol-binding Protein*

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The three-dimensional structures of complexes between bovine plasma retinol-binding protein (RBP) and three retinol analogs with different end groups (fenretinide, all-*trans* retinoic acid, and axerophthene) have been determined to 1.8–1.9-Å resolution. Their models are very similar to that of the bovine retinol·RBP complex: the root mean square deviations between equivalent α-carbons in the two proteins range from 0.17 to 0.24 Å. The retinoid molecules fit in the β-barrel cavity assuming the same conformation of the vitamin, and the substitutions have no consequences on the overall protein structure. While confirming that an intact hydroxyl end group is not an absolute requirement for a correct retinoid binding to RBP, this study has shown the occurrence of conformational changes, although limited, in the rather flexible loop region at the entrance of the β-barrel upon fenretinide and retinoic acid binding. These changes are suitable for accommodating the end groups of the above retinoids. Instead, no such changes have been revealed in RBP complexed with axerophthene, a retinol analog bearing a hydrogen atom in place of the hydroxyl end group. The protein conformational changes in the above loop region, the steric hindrance of bulky end groups of bound retinoids, and the lack of the retinol hydroxyl group appear to be responsible for the possible reduced affinity of retinoids for RBP relative to retinol and, at the same time, for the abolished or reduced affinity of retinoid·RBP complexes for transthyretin relative to retinol-RBP.

Retinoids constitute a large group of synthetic and naturally occurring compounds related to retinol, playing a key role in embryo development, cell growth, and differentiation. These fundamental processes appear to be mediated by the interaction of selected isomeric forms of the activated vitamin A retinoic acid (*e.g.* all-*trans*-retinoic acid and 9-*cis*-retinoic acid) with two families of nuclear receptors, the retinoic acid receptors, and the retinoid X receptors (Petkovich *et al.*, 1987;

Giguere *et al.*, 1987; Heyman *et al.*, 1992). Because of their chemical instability and quite low solubility in the uncomplexed form in the aqueous medium, natural retinoids need to be bound to specific retinoid-binding proteins to be protected, solubilized, and transported in body fluids and in the cell (Soprano and Blaner, 1994; Ong *et al.*, 1994). In blood, retinol circulates bound to retinol-binding protein (RBP),[1] which delivers the vitamin from its store sites to target cells. RBP is a single polypeptide chain of 21 kDa, containing one binding site for retinol. HoloRBP is found bound, in the circulation, to transthyretin (TTR), a protein of 55 kDa composed of four identical subunits. Besides participating in vitamin A transport, TTR is involved in the transport of thyroxine. The formation of the holoRBP·TTR complex is believed to prevent filtration, through kidney glomeruli, of the relatively small RBP.

The high resolution crystal structures of RBP (Newcomer *et al.*, 1984; Cowan *et al.*, 1990; Zanotti *et al.*, 1993a, 1993b), cellular retinol-binding proteins CRBP and CRBPII (Cowan *et al.*, 1993; Winter *et al.*, 1993), and epididymal retinoic acid-binding protein E-RABP (Newcomer, 1993) have been described. In all cases the natural ligand occupies the cavity of a β-barrel. In holoRBP the retinol cyclohexene ring is bound innermost in the barrel, the isoprene chain is fully extended, and the hydroxyl end group reaches the protein surface. A protein conformational change essentially confined to one of the loops near the entrance of the β-barrel (residues 32–37) has been shown to occur upon the release of retinol from holoRBP (Zanotti *et al.*, 1993a, 1993b). This change is likely to be responsible for the low affinity of apoRBP for TTR relative to the holoprotein. Whereas certain modifications in the area of the retinol cyclohexene ring appear to be incompatible with the binding to RBP, retinoids modified in the area of the hydroxyl group bind well to the carrier protein (Cogan *et al.*, 1976; Berni *et al.*, 1993a, 1993b). A crystallographic study has shown that one of such retinoids binding to RBP, *N*-ethyl retinamide, fits in the RBP β-barrel internal cavity occupying the same position of the retinol molecule (Zanotti *et al.*, 1993c). However, the replacement of the retinol hydroxyl group and quite small changes in the loop region near to the entrance of the β-barrel drastically affect the interaction between *N*-ethyl retinamide RBP and TTR. These findings and those of previous studies with apoRBP (Zanotti *et al.*, 1993a, 1993b) were interpreted as a strong evidence that the above loop region in RBP participates in the interaction with TTR.

The increasing interest in synthetic retinoids exhibiting pharmacological and antitumor activities raises some relevant questions as to their possible interactions with retinoid-binding proteins, enzymes, and nuclear retinoid receptors. First, syn-

[1] The abbreviations used are: RBP, retinol-binding protein; TTR, transthyretin.

TABLE I

Cell parameters for the crystals of the retinoid·RBP complexes, compared with those of the bovine holo and apoRBP (Zanotti et al., 1993b). Space group is $P2_12_12_1$, $Z = 4$

	a	b	c
		Å	
HoloRBP	46.08	49.12	76.10
ApoRBP	46.55	48.97	76.87
Fenretinide-RBP	46.63	49.26	76.63
Retinoic acid-RBP	46.21	48.81	75.81
Axerophthene-RBP	46.38	49.20	76.65

TABLE II

Conditions used in the data collection. 2θ is the detector angle, in degree. The rotation angles are also in degrees

	Fenretinide-RBP (1)	Fenretinide-RBP (2)	Retinoic acid-RBp	Axerophthene-RBP
Crystal to detector distance (cm)	10.0	7.5	10.0	10.0
2θ (°)	−23	0.0	−20	−20
Rotation degree/frame	0.25	1.0	0.25	0.25
No. of frames	400	70	400	400
Exposure time/frame (s)	60	420	80	60

TABLE III

A, total number of reflections collected, number of independent reflections, and internal R factor for the three complexes. B, independent reflections as a function of resolution and percentages of reflections measured.

A				
	Fenretinide-RBP		Retinoic acid-RBP	Axerophthene-RBP
Total no. of reflections	$19,496^a$ $23,466^b$	$49,962^c$	16,527	16,650
Independent reflections	$7,360^a$ $8,822^b$	$12,128^c$	7,294	10,137
R	4.7^a 7.5^b	6.2^c	10.5	7.0

B						
Resolution interval	Axerophthene-RBP		Retinoic acid-RBP		Fenretinide-RBP	
	Independent reflections	%	Independent reflections	%	Independent reflections	%
Å						
∞−3.1	3,038	85	2,481	69	3,251	91
3.1−2.4	3,090	90	2,039	60	2,841	83
2.4−2.1	2,847	85	1,895	56	2,574	77
2.1−1.9	1,162	35	879	26	2,244	67
1.9−1.8	0	0	0	0	1,218	36
∞−1.8	10,137	60	7,294	43	12,128	71

[a] Xentronix area detector.
[b] Imaging plate area detector.
[c] Merged data.

thetic retinoids might interact *in vivo* with retinoid-binding proteins and enzymes involved in the metabolism of vitamin A, thus interfering with the transport and metabolism of physiologically occurring retinoids. Notably, it has been shown that some retinoids may affect the plasma transport of retinol, possibly as a result of their interaction with RBP (Berni *et al.*, 1993b). Second, at least part of the aforementioned activities of synthetic retinoids is believed to be mediated by the interaction with nuclear retinoid receptors. Consistent with this proposed mechanism of action, a correlation was found to exist for conformationally restricted aromatic analogs of retinoic acid between their binding affinity to retinoic acid receptor-β and their competence to activate retinoic acid receptor transcriptional activity (Lombardo *et al.*, 1994). Studies on the molecular details of the interactions of retinoids with binding proteins and receptors may provide the basis for identifying and designing binding specificities of retinoids for target proteins. X-ray crystallographic studies can unravel details of retinoid-protein recognition at the molecular level. As mentioned above, retinol analogs modified in the area of the hydroxyl group exhibit binding affinity to apoRBP. This has offered the opportunity to prepare and crystallize retinoid·RBP complexes suitable for x-ray diffraction investigations. The present structural study was devised to gain insight into the molecular interactions between retinoids with different end groups (fenretinide, retinoic acid, and axerophthene) and RBP.

EXPERIMENTAL PROCEDURES

Materials—Bovine holoRBP was purified from fresh plasma as described (Berni and Lamberti, 1989) and was quantified by its absorbance at 280 nm, using an absorption coefficient $A_{1\%, 1cm}$ of 18.6. Bovine apoRBP was prepared by extracting retinol from holoRBP with ethyl ether according to Zanotti *et al.* (1993b). All-*trans*-retinol and all-*trans*-retinoic acid were purchased from Fluka (Buchs, Switzerland). Fenretinide (*N*-(4-hydroxyphenyl)-all-*trans*-retinamide) was a generous gift of R. W. Johnson Pharmaceutical Research Institute (Spring House, PA). Axerophthene was obtained by synthesis as described (Newton *et al.*, 1978) and purified by reversed-phase high pressure liquid chromatography. Retinoids were quantified using the following absorption coefficients in ethanol: all-*trans*-retinol, $\epsilon = 46,000$ M^{-1} cm^{-1} at 325 nm; all-*trans*-retinoic acid, $\epsilon = 45,000$ M^{-1} cm^{-1} at 350 nm; fenretinide, $\epsilon = 55,630$ M^{-1} cm^{-1} at 361.5 nm; axerophthene, $\epsilon = 49,950$ M^{-1} cm^{-1} at 323 nm.

Crystallization of Retinoid·RBP Complexes—To prepare retinoid·RBP complexes, a 2-fold molar excess of the retinoid was incubated with freshly prepared bovine apoRBP (6 mg/ml) at 20 °C for 1 h, in 0.005 M sodium phosphate, 0.15 M NaCl, at pH 7.0. Crystals of each retinoid·RBP complex were then obtained, using methods and conditions that were suitable for the crystallization of bovine apoRBP (Zanotti *et al.*, 1993b). In addition, microcrystals of native retinol-RBP were used to seed the solutions of the retinoid·RBP complexes. The presence in the crystals of the RBP-bound retinoids was established on the basis of the absorption spectra characteristic of the retinoid·RBP complexes by means of single crystal microspectrophotometric analyses. The crystals are isomorphous to those of bovine holo and apoRBP. A summary of the crystal data is given in Table I.

X-ray Diffraction Data Collection and Processing—The x-ray source for data collection was a Siemens M18X-HF rotating anode generator operated at 50 kV and 90 mA, with an apparent focus size of 0.3 × 0.3 mm. Copper radiation was selected by a graphite crystal monochromator. A 0.5-mm collimator was used. Data were measured on a XENTRONIX X1000 area detector system, mounted on a three-axis goniometer. For crystals of the fenretinide·RBP complex, a R-AXIS II imaging plate area detector, installed on a Rigaku RU200 rotating anode generator, was also used. Details of the conditions used in the data collection of each derivative are reported in Table II. Scaling and merging was performed with the program SAINT (Siemens Industrial Automation). No sigma cut-off was applied and all the reflections were considered observed.

Table III gives the statistics of data collection and the percentage of reflections measured as a function of resolution for the different data sets. The relatively limited number of independent reflections collected must be attributed to the small size of the crystals, especially for the retinoic acid·RBP complex, for which the crystal was 0.2 mm in the longest dimension and 0.1 mm in the shortest.

Model Building and Refinement—For all the complexes, the coordinates of bovine holoRBP, deprived of the retinol molecule, were used as the starting model in the refinement. Some cycles of restrained least-squares on atomic coordinates and B factors were performed and a $|2F_{obs} - F_{calc}|$ electron density map calculated, using only the coordinates

TABLE IV
Resolution interval of the data used, crystallographic R factor (defined as $R = \Sigma \; \| \, |F_{obs}(\boldsymbol{h})| - |F_{calc}(\boldsymbol{h})| \, \| \; / \; \Sigma \; |F_{obs}(\boldsymbol{h})|)$ and r.m.s. standard deviations for the final model

	Resolution	R	Root mean square deviations on			
			Covalent bonds	Bond angles	Planarity	Torsion angles
	Å		Å	°	Å	°
Fenretinide-RBP	9–1.8	0.204	0.016	3.7	0.011	21.0
Retinoic acid RBP	7–1.9	0.184	0.008	2.8	0.006	19.8
Axerophthene	7–1.9	0.193	0.006	2.6	0.005	19.3

TABLE V
Number of atoms in the final models, crystallographic R factor, and number of independent reflections used in the refinement

	No. of protein atoms	No. of ligand atoms	No. of solvent atoms	R factor	No. of independent reflections
Fenretinide-RBP	1,411	29	164	0.204	12,128
Retinoic acid-RBP	1,422	22	165	0.184	7,294
Axerophthene-RBP	1,424	20	163	0.193	10,137

of protein atoms. At this point an electron density was visible inside the cavity formed by the β-barrel. The retinoid model was fitted using the coordinates of retinol, appropriately modified for each retinoid. After a few cycles of refinement, the new map was carefully inspected and minor adjustments applied to some side chains and to the solvent molecules. Some more cycles of restrained least-squares reduced the *R* factors to the final values reported in Table IV. Statistics on the final models are summarized in Table V. Refinement was performed with the restrained least-squares program TNT (Tronrud *et al.*, 1987) and maps displayed on a PS300 Evans & Sutherland graphic system using the program FRODO (Jones, 1978).

RESULTS

The Structure of the Fenretinide·RBP Complex—Fenretinide is a retinol analog in which a hydroxyphenyl amide group replaces the hydroxyl group of the vitamin (Fig. 1). An increasing interest has arisen for this synthetic retinoid which is at present being assessed as an antitumor chemopreventive agent in humans (Costa *et al.*, 1994). *In vitro* studies have previously shown that fenretinide binds well to apoRBP; however, the retinoid·RBP complex does not display affinity for TTR (Berni and Formelli, 1992). The crystal structure of the fenretinide·(bovine)RBP complex compares quite well with that of bovine retinol-RBP: the root mean square deviation calculated for the corresponding α-carbons is 0.24 Å (Fig. 2*a*). The retinoid fits in the central cavity of the β-barrel, in the site previously occupied by the vitamin: the cyclohexene ring and the isoprene tail of the ligand take the place of the corresponding portions of retinol, while the hydroxyphenyl amide group protrudes from the cavity toward the solvent, replacing the retinol hydroxyl group and a water molecule hydrogen bonded to it (Fig. 3). The electron density for the ligand is reasonably clear and continuous, except for a small break around C7 (Fig. 4; see also Fig. 1 for the numbering system used for the retinoid molecules). The resolution is not high enough and allows only a limited discussion of the bound ligand conformation. Nevertheless, in the tail of the retinoid we can clearly see three different planes. The first one from C7 to C14, including methyl groups C19 and C20: this part of the molecule is planar in all the structures of retinoids till now determined. A second plane is formed by the amide group that, due to a rotation around the C14-C15 bond, makes an angle of 85° with the previous one. It must be considered that the distance O ... C20 is 3.2 Å and should become 2.4 Å in case of perfect coplanarity. The third plane is that of the phenol ring, which is tilted with respect to the isoprene tail of the retinoid by about −45°. Finally, the

attachment of the isoprene chain to the ring for fenretinide is not different from that established for retinol in the native holoprotein. A comparison of two relevant torsion angles for all the examined complexes is reported in Table VI. The conformation of the cyclohexene ring is half-chair-like, but C3 is apparently pointing in the opposite direction with respect to retinol-RBP; however, the resolution is not high enough to decide if this is a real difference in conformation or simply an artifact of the refinement.

In order to be accommodated in the loop region surrounding the opening of the β-barrel, the bulky terminal group of fenretinide causes the displacement of the side chains of Leu-35, which rotates around the Cα-Cβ bond, and of Leu-63, which rotates around the Cβ-Cγ bond (Fig. 5). In addition, a long-shaped electron density close to the hydroxyl group of fenretinide has been interpreted as two water molecules, making hydrogen bond interactions between them and with the hydroxyl group of the ligand, at distances of 2.7 and 2.8 Å. The fenretinide end group also affects the position of the main chain from residue 60 to 63, which is slightly displaced from its previous position in retinol-RBP. It is worth noting that this part of the molecule is rather disordered in all the crystal structures of RBP till now determined, and the exact entity of this variation is difficult to state. The side chains of residues Arg-60, Leu-64, and Asn-65 are also slightly influenced by the presence of the ligand. With regard to the rest of the protein, only some polar or charged side chains, exposed to the solvent, exhibit a different conformation relative to retinol-RBP: Arg-19, Glu-44, Glu-49, Asn-50, Glu-81, Lys-87, Lys-89, Lys-99, Glu-112, Gln-149, Arg-166, and His-170. Most of these variations have been previously noticed when the structures of holoRBP, apoRBP, and of the N-ethyl retinamide·RBP complex have been compared. They possibly represent side chains that are present in more than one conformation in the crystal or whose orientations are influenced by crystallization conditions. Anyhow, all these residues are far from the substituent inside the β-barrel cavity and differences in side chain orientations cannot be attributed to it.

The Structure of the All-trans-retinoic Acid·RBP Complex— The model of the retinoic acid·RBP complex is also very similar to that of retinol-RBP (Fig. 2*b*). As in the previous case, the root mean square deviation for the corresponding α-carbons of the two complexes is 0.24 Å. The bound retinoic acid affects the positions of the Leu-35 and Leu-97 side chains, both close to the carboxyl end group of the ligand. To avoid short distance con-

tacts, the former simply rotates around the Cα-Cβ bond, the latter around the Cβ-Cγ bond. Additionally, some side chains on the protein surface, far from the bound retinoid, exhibit a slightly different conformation in the two complexes: Arg-10, -19, -153, -163, and -166, Lys-58, -87, -89, and -150, Glu-81, Gln-149, and Met-27. The electron density for the bound retinoic acid (Fig. 6) is not very satisfactory: some breaks in the density are visible in the zone of the ring, while the orientation of the isoprene tail is clearly identifiable, despite the relatively low resolution. This situation might be due to a relatively low occupancy of the ligand and/or to a partial decomposition of the labile retinoic acid. Furthermore, as previously stated, the crystals of the retinoic acid·RBP complex were particularly small and did not allow us to measure a full data set at 1.9 Å resolution (see Table III). In the complex, the acid occupies the same position of the alcoholic form of the vitamin in retinol-RBP, with the carboxyl group pointing toward the solvent. The plane of the latter group does not coincide with the plane of the isoprene tail of retinoic acid but makes with it an angle of about −35°; this angle is −8° in the triclinic modification of vitamin A acid (Stam, 1972). In the electron density map, a maximum is visible close to the bifurcation of the carboxyl group, and we interpret it either as a water molecule or as a positively charged ion in close contact with the carboxyl group. Clearly, this latter situation would imply a deprotonated state for retinoic acid. However, the region surrounding the carboxyl group

of the acid bound to RBP is strongly hydrophobic. In addition, no positively charged side chain is present that could stabilize the retinoic acid carboxylate group. A hydrophobic environment might explain the unusually high pK_a value that has previously been determined for the carboxyl group of the bound retinoic acid (pK_a = 6.6) (Noy *et al.*, 1992). Therefore, due to the acidic pH of the crystallization medium (pH 5.3–5.4), the RBP-bound retinoic acid in the crystal should essentially be in the protonated form.

The Structure of the Axerophthene·RBP Complex—The structure of the axerophthene·RBP complex is, among the structures discussed in this paper, the most similar to the native one (Fig. 2c). The root mean square difference between the corresponding Cα carbons of the two models is in fact 0.17 Å. This could be

FIG. 2. **Root mean square (*rms*) differences (Å) of equivalent atoms of main chain (*solid line*) and side chains (*dashed line*) as a function of the residue number.** *A*, fenretinide·RBP complex *versus* holoRBP; *B*, retinoic acid·RBP complex *versus* holoRBP; *C*, axerophthene·RBP complex *versus* holoRBP.
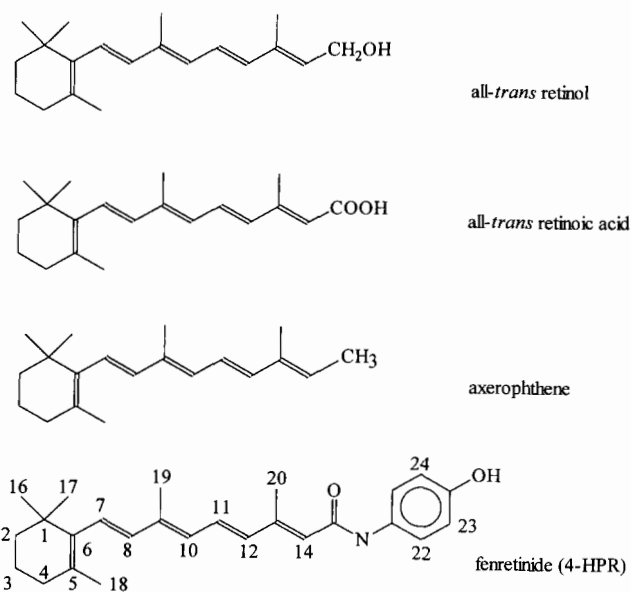

FIG. 1. **Structural formulas of all-*trans*-retinol and all-*trans*-retinoids considered in this study.** The numbering system used is shown for fenretinide.
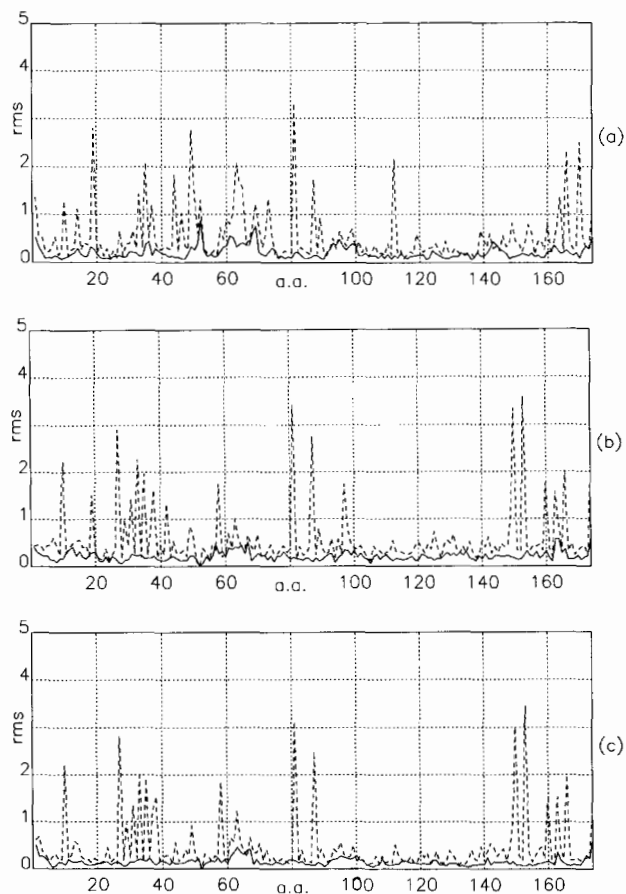

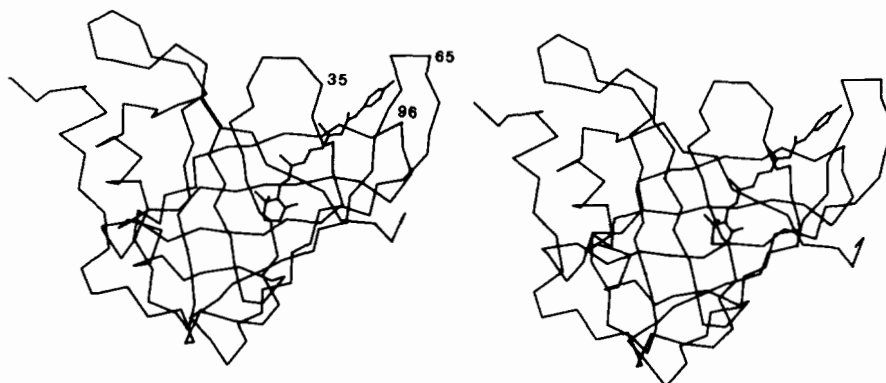FIG. 3. **Stereo drawing of the α-carbon chain trace for the renretinide·RBP complex, with the retinoid in the β-barrel central cavity.**

FIG. 4. **A Fourier difference map of the β-barrel cavity, calculated with coefficients $F_{obs}-F_{calc}$.** Contour levels were drawn at 1.5σ. Only protein atoms were included in the calculation of phases; consequently, maxima representing solvent molecules are visible. The fenretinide molecule is fitted into the density: a small break, around C7, is clearly visible.
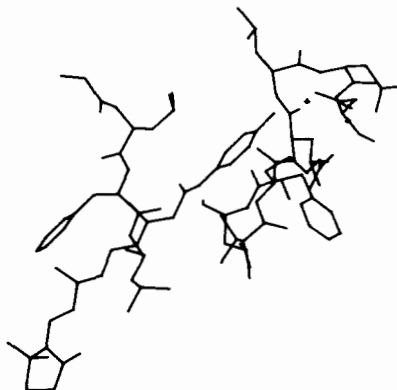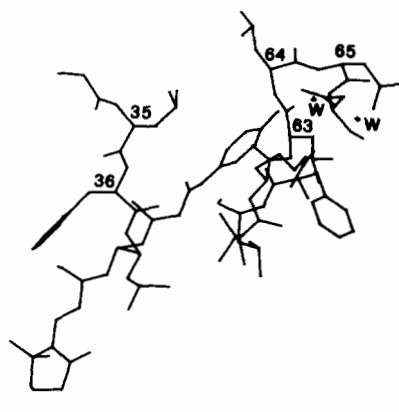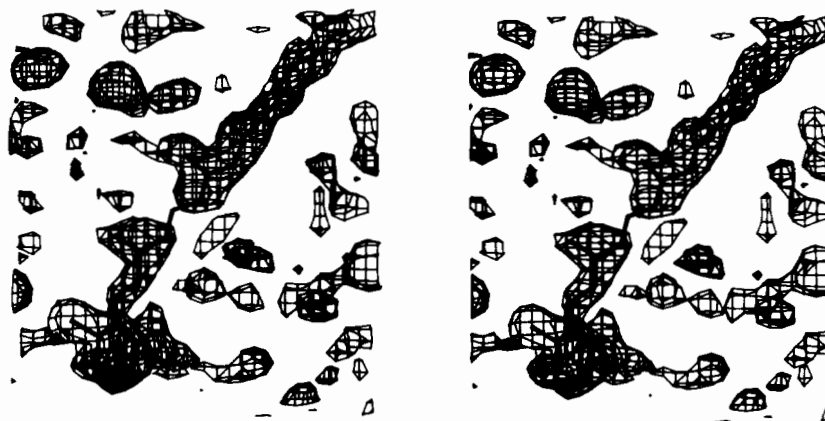

FIG. 5. **Details of the cavity accommodating the fenretinide·RBP complex, with the fenretinide molecule surrounded by some of the protein side chains whose positions are affected by the binding of the retinoid.** Two water molecules interacting with the hydroxyl group of the retinoid are also shown.

FIG. 6. **Details of the electron density map inside the cavity accommodating retinoic acid in the retinoic acid·RBP complex.** Maps were calculated with coefficients $2F_{obs}-F_{calc}$ using only protein atoms and drawin with contour levels at 1σ.
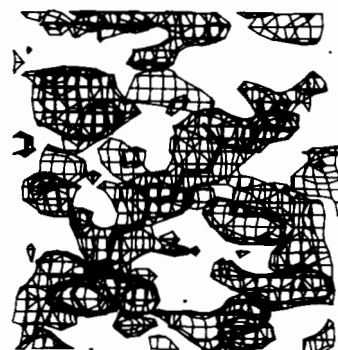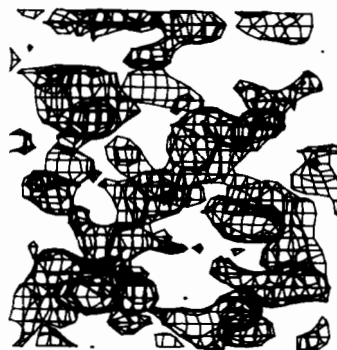
easily predicted, since the only difference between the two ligands is the replacement of the retinol hydroxyl group with a hydrogen atom (Fig. 1). The electron density of the ligand is shown in Fig. 7: it occupies the position of the retinol, without inducing any remarkable change in the protein structure. The only significant difference relative to retinol-RBP is the lack of the water molecule that forms a bridge, via hydrogen bond interactions, between the retinol hydroxyl group and a nearby main chain carbonyl oxygen. Minor differences are the changes in side chain orientation observed for some residues at the protein surface: Arg-10, -153, -163, and -166, Lys-29, -58, -87, and -150, Glu-33, -49, and -81, and Met-27.

DISCUSSION

The results presented in this study clearly indicate the competence of retinol analogs modified in the area of the hydroxyl group to bind correctly to RBP. While the positions in the RBP β-barrel internal cavity of retinol, fenretinide, and axerophthene superimpose almost perfectly, a small difference in the general orientation of the retinoid inside the cavity can be observed for retinoic acid (Fig. 8, *a* and *b*). This might be attributed to the strongly hydrophilic carboxyl group of retinoic acid, which induces some variations in the binding mode; however, we cannot exclude an artifact due to the relatively low resolution of the retinoic acid-RBP structure. Nevertheless, the binding is essentially the same for all RBP-bound retinoids till now considered, including *N*-ethyl retinamide (Zanotti *et al.*, 1993c). The conformation of the protein-bound retinoids is also practically the same. In Table VI we report the two torsion angles, C4-C5-C6-C7 and C5-C6-C7-C8, relevant for the attachment of the isoprene tail to the ring: all values agree quite well, considering the limited resolution. Since the values reported in the literature for vitamin A and its analogs in the solid state present large variations (see Stam, 1972; Cowan *et al.*, 1990 and references therein), we can conclude that the binding of retinoids to RBP leads to a preferential conformation of the bound ligands.
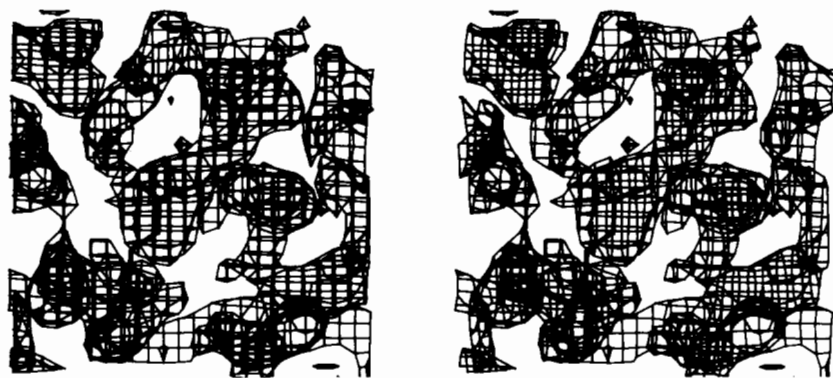
FIG. 7. **Details of the electron density map inside the cavity accommodating axerophthene in the axerophthene·RBP complex.** Maps were calculated and drawn as for Fig. 6.
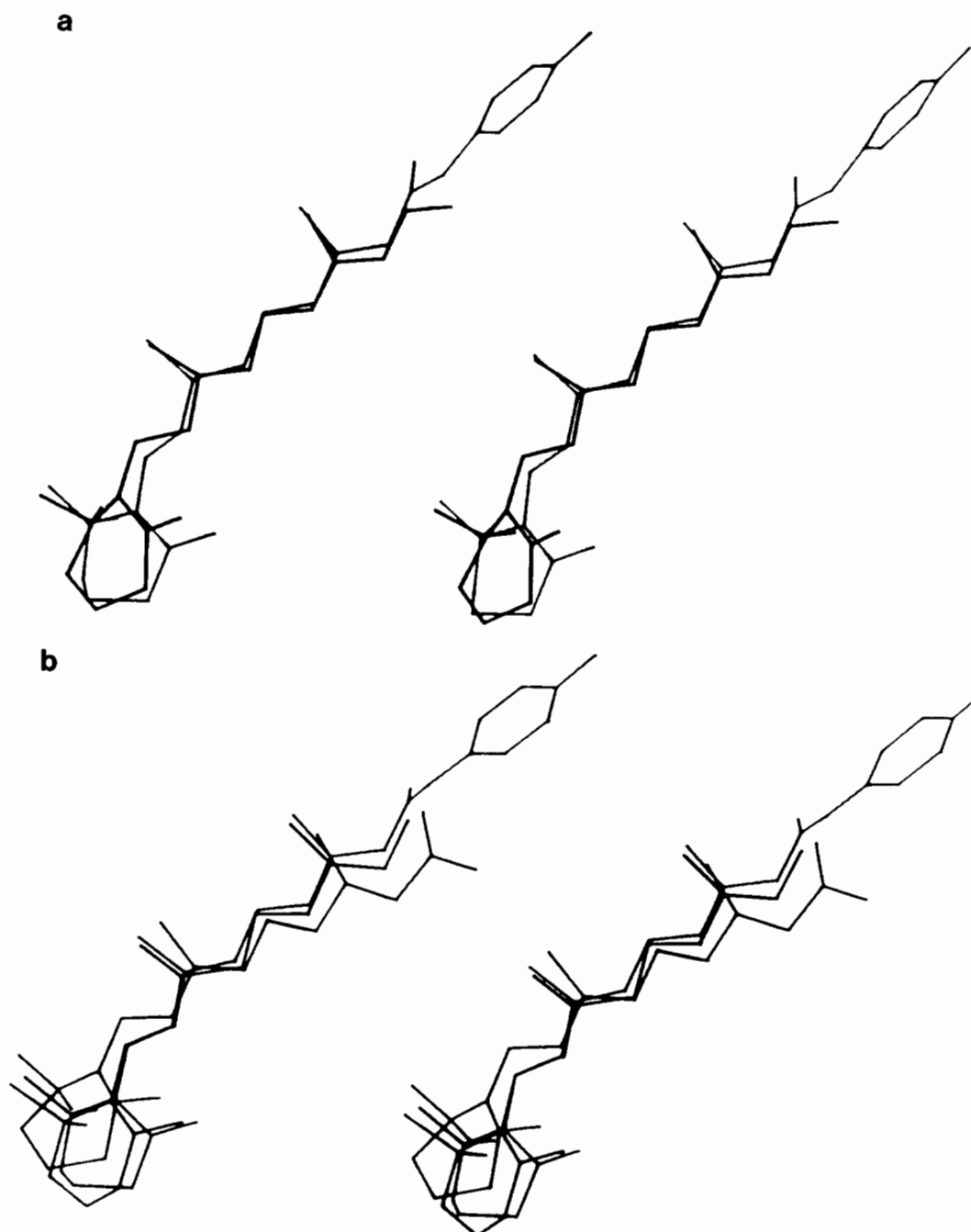
a

b

FIG. 8. *a*, stereo drawing of the fenretinide molecule superimposed on that of bound retinol in the bovine holoRBP; *b*, the RBP-bound retinoids fenretinide, retinoic acid and axerophthene are superimposed.

TABLE VI

Dihedral angles (degrees) defining the relative position of the cyclohexene ring and of the isoprene tail

	C4-C5-C6-C7	C5-C6-C7-C8
Retinol[a]	−165	33
Retinol[b]	172	62
Fenretinide[c]	168	28
Retinoic acid[c]	−164	73
Axerophthene[c]	178	54
N-Ethyl retinamide[d]	−170	64

[a] Retinol in bovine holoRBP (Zanotti *et al.*, 1993b).
[b] Retinol in human holoRBP (Cowan *et al.*, 1990).
[c] RBP-bound retinoids studied in this work.
[d] RBP-bound N-ethyl retinamide (Zanotti *et al.*, 1993c).

Distinct modes of retinoid binding have previously been revealed for various retinoid-binding proteins. In fact, the orientation of the ligand inside the β-barrel cavity is completely different in RBP and the epididymal retinoic acid-binding protein relative to the structurally distinct cellular retinol-binding proteins (Newcomer, 1993; Cowan *et al.*, 1993; Winter *et al.*, 1993). In addition, in the epididymal retinoic acid-binding protein the isoprene chain of bound all-*trans*-retinoic acid is folded, adopting a conformation which resembles that of 9-*cis*-retinoic acid (Newcomer, 1993). The characteristic mode of binding of retinol to RBP described above is also found for retinoids bearing end groups different from the hydroxyl group. Clearly, the rigidity of the β-barrel internal cavity may not allow different modes of retinoid binding. On the other hand, the end groups of RBP-bound retinoids like N-ethyl retinamide (Zanotti *et al.*, 1993c), fenretinide, and retinoic acid are located in the rather flexible loop region surrounding the opening of the β-barrel. The flexibility of this region may allow the rearrangements, although limited, of some side chains that are suitable for accommodating the end groups of the above retinoids upon their binding to RBP.

While confirming that an intact hydroxyl end group is not an absolute requirement for a correct retinoid binding to RBP, the results of this study indicate on a structural basis the relevant consequences that the replacement of retinol with the retinoids considered in this study may have. In fact, such replacement abolishes the favorable situation of a water molecule that forms a bridge between the retinol hydroxyl group and a nearby main chain carbonyl oxygen (PHE-g2). In addition, the binding to RBP of both fenretinide and retinoic acid destabilizes to some extent the native protein structure by inducing some local conformational changes. These observations tend to indicate that the retinol·RBP complex is more stable than the retinoid·RBP complexes and, therefore, that the affinity of retinoids for RBP may be reduced with respect to retinol. In contrast, the results of direct fluorescence binding assays have previously indicated that retinol and retinol analogs possess similar affinities for RBP (Cogan *et al.*, 1976; Berni *et al.*, 1993a; Zanotti *et al.*, 1993c). However, a result contradicting this experimental evidence has recently been obtained. In a competition binding assay, it has been found that a 2-fold molar excess of retinoic acid and fenretinide was almost completely ineffective in displacing retinol from retinol-RBP; instead, in the reverse of the above experiment an equimolar amount of retinol exhibited the ability to displace RBP-bound fenretinide and retinoic acid to a significant extent (Berni *et al.*, 1993b). Therefore, in spite of the fact that modifications of the retinol hydroxyl group do not prevent a high affinity binding of retinoids to RBP, the retinol molecule may possess an affinity significantly higher than that of the retinoids for RBP, in accordance with the indications emerging from our structural investigation. The above situation appears to be somehow similar to that previously found for retinoid binding to CRBP. In fact, despite its high affinity for CRBP as determined in direct fluorescence binding experiments, retinaldehyde could not significantly compete with retinol for the binding to CRBP (Levin *et al.*, 1988; Li *et al.*, 1991). A model providing the molecular bases for the reduced affinity of retinaldehyde for CRBP relative to retinol has also been recently proposed (Cowan *et al.*, 1993; Banaszak *et al.*, 1994).

It has been found previously that the binding to RBP of fenretinide (Berni and Formelli, 1992) and of retinoic acid (Noy *et al.*, 1992) strongly interferes with the RBP-TTR recognition. An interference, although weaker, with the RBP-TTR interaction is also induced by the binding of axerophthene to RBP.[2] The present investigation may provide the structural bases for the above interferences. In fact, conformational changes in the loop region at the entrance of the β-barrel cavity and the steric hindrance of groups replacing the retinol hydroxyl group may drastically affect the RBP-TTR interaction, as in the case of fenretinide-RBP and retinoic acid-RBP. Instead, a weaker interference with the protein-protein recognition may be exerted by the bound axerophthene just because of the lack of the retinol hydroxyl group. The observation that despite the lack of the hydroxyl group the interaction between axerophthene-RBP and TTR is still present confirms the conclusion of our previous study with apoRBP (Zanotti *et al.*, 1993b) that the presence of the retinol hydroxyl group is not an absolute requirement for the protein-protein recognition. Finally, the finding of a weak interaction between axerophthene-RBP and TTR is consistent with the interpretation that the retinol hydroxyl group is involved, directly or through the water molecule hydrogen bonded to it, in the binding of retinol-RBP with TTR.

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