Interactions of Retinol with Binding Proteins: Studies with Retinol-Binding Protein and with Transthyretin†

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ABSTRACT: The interactions within the molecular complex in which retinol circulates in blood were studied. To monitor binding between retinol-binding protein (RBP) and transthyretin (TTR), TTR was labeled with a long-lived fluorescence probe (pyrene). Changes in the rotational volume of TTR following its association with RBP were monitored by fluorescence anisotropy of the probe. Titration of TTR with holo-RBP revealed the presence of 1.5 binding sites characterized by a dissociation constant K_d = 0.07 μM. At 0.15 M NaCl, binding of RBP to TTR showed an absolute requirement for the native ligand, retinol. At higher ionic strength (0.5 M NaCl), RBP complexed with retinal also bound to TTR with high affinity (K_d = 0.134 μM). RBP containing retinoic acid did not bind to TTR even at the high salt concentration. The data suggest that the TTR binding site on RBP is in close proximity to the retinoid binding site and that the head group of retinoic acid, when bound to RBP, presents steric hindrance for the interactions with TTR. The implications of the data for selectivity in retinoid transport in the circulation are discussed. The kinetics of the steps leading to complete dissociation of the retinol–RBP–TTR complex was also studied. The first step of this process was dissociation of retinol, which had a rate constant of 0.06/min. Following loss of retinol, the two proteins dissociate. The rate of dissociation is slow (k = 0.055/h), however, indicating that the complex apo-RBP–TTR will be an important factor in regulating serum levels of retinol.

Vitamin A alcohol (retinol) is a lipophilic compound that is transported in blood bound to a specific plasma retinol-binding protein (RBP),[1] a single polypeptide chain with a molecular mass of 21 000 Da which contains one binding site for retinol. In blood, holo-RBP binds to transthyretin (TTR), a protein with a molecular mass of 54 980 Da composed of four identical subunits, which plays a role in the transport of both vitamin A and thyroid hormone. The three-dimensional structures of holo-RBP (Newcomer et al., 1984) and ot TTR (Blake et al., 1978) have been determined, but the protein surfaces involved in the interactions between the two proteins have not been defined, and little information is available regarding these interactions. The stoichiometry of binding of RBP to TTR has been investigated in several laboratories, which obtained evidence for one (Raz et al., 1970; Heller & Horowitz, 1974; Fex et al., 1979), two (Tragardh et al., 1980; Berni et al., 1990), or four (van Jaarsveld et al., 1973; Vahlqvist & Peterson, 1973) RBP binding sites on TTR. It was reported that the binding affinity of TTR for apo-RBP was significantly reduced as compared to the affinity for holo-RBP but that the interactions between the two proteins do not have a strict requirement for a specific ligand (Fex et al., 1979).

The sequence of events leading to the dissociation of the complex retinol–RBP–TTR may, theoretically, follow either of these paths:

reaction 1

retinol–RBP–TTR → retinol + RBP–TTR → RBP + TTR

reaction 2

retinol–RBP–TTR → retinol–RBP + TTR → retinol + RBP

No quantitative data are available regarding the kinetics of the various steps in the above reactions. It is, however, generally accepted that dissociation follows reaction 1, i.e., that the first step of the overall reaction is dissociation of retinol and that the affinity of RBP for TTR is largely decreased in the absence of the ligand, leading to rapid dissociation of the protein complex. The association of RBP with the larger protein serves to prevent its filtration through the kidney (Goodman, 1984), so that, following dissociation, RBP is filtered in the glomeruli (Raz et al., 1970; Heller, 1975; Vahlquist et al., 1973) or is taken up by the liver (Gjoen et al., 1987). An important question regarding the fate of RBP is, thus, how fast does the RBP–TTR complex dissociate following uptake of retinol into target cells? The kinetics of this dissociation process will play an important role in determining the lifetime of the apo-RBP–TTR complex and, in turn, the lifetime of RBP in the circulation.

The question of whether the tertiary complex dissociates via reaction 1 or 2 is also important for understanding the mechanism by which RBP is involved in the process of delivery of retinol to target cells. Studies of the kinetics of the interactions of retinol with RBP and with membranes have shown that this ligand dissociates from RBP spontaneously and rapidly (Noy & Xu, 1990a; Fex & Johannesson, 1987),

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[1] Abbreviations: *all-trans*-retinol, -retinal, and -retinoic acid, retinol, retinal, and retinoic acid; RBP, serum retinol-binding protein; TTR, transthyretin; DOPC, dioleoylphosphatidylcholine; NBD-DPPE, *N*-(7-nitro-2,1,3-benzodiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine.

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that it traverses membranes at a rate in the millisecond range, and that it dissociates from membranes with a $t_{1/2}$ of about 1 s (Fex & Johannesson, 1988; Noy & Xu, 1990b). These studies suggest that none of the steps in the process of transfer of retinol from the binding site on RBP to cellular cytoplasm requires facilitation to account for uptake of retinol by target cells, i.e., that the initial events of uptake of retinol by cells occur spontaneously. These events are thus regulated by the equilibrium distribution of retinol between the various phases involved: RBP, the plasma membranes of target cells, and the aqueous phase of cytoplasm. However, the affinity of RBP–TTR for retinol is higher vs the affinity of RBP alone (Noy & Xu, 1990a). The rate of dissociation of retinol from the RBP in serum hence may depend on the integrity of the protein complex and on the sequence of events leading to complete dissociation.

In the present study, the kinetics of the various steps leading to the dissociation of the tertiary complex retinol–RBP–TTR were investigated. In addition, binding of RBP to TTR in the presence of various retinoids was studied in order to clarify the ligand specificity of the interactions between the two proteins.

MATERIALS AND METHODS

Lipids were obtained from Avanti Polar Lipids. *all-trans*-Retinol, -retinal, and -retinoic acid were purchased from Kodak. NBD-DPPE and 1-pyrenesulfonyl chloride were from Molecular Probes Inc. All other chemicals were obtained from Sigma Chemical Co.

Proteins. RBP and TTR were purified from bovine serum (Pel-Freeze Co.) by affinity chromatography on TTR–Sepharose and RBP–Sepharose, respectively (Rask et al., 1971). RBP was originally prepared by the method outlined in Rask et al. (1971); TTR for the subsequent affinity columns was purified using the RBP column. The purified RBP migrated as a single homogeneous band on SDS–PAGE (Laemmli, 1970) with a mass of approximately 21 000 Da. Purified TTR migrated with a mass of 14 000 Da, corresponding to the size of the protein's subunits.

Concentrations of proteins and of retinoids were determined from their extinction coefficients (Horowitz & Heller, 1977; Tragardh et al., 1980). Apo-RBP was obtained by extracting retinol from RBP into ethyl ether. Equal volumes of a solution of holo-RBP in water and ether were stirred gently at room temperature for 4 h. Over 90% of bound retinol was removed by this procedure as judged by the ratio of absorbance at 280 and 330 nm. Approximately 20% of RBP was denatured by this procedure. Denatured protein was separated from the native protein by centrifugation. To reconstitute apo-RBP with retinol, retinal, or retinoic acid, the ligands were added to the protein from a concentrated solution in ethanol to form a complex with a protein/ligand mole ratio of 1:1. In control experiments it was verified that reconstitution of apo-RBP with retinol resulted in holo-RBP which was indistinguishable from the native protein in regards to its binding to TTR and to the rate of dissociation of retinol.

Labeling of TTR with Pyrene. TTR was covalently labeled with pyrenesulfonyl chloride as described (Liu et al., 1981). In order to ensure that labeling does not affect the parameters of binding of RBP to TTR, labeling of TTR was carried out in the presence of RBP. Pyrenesulfonyl chloride (120 nmol) in acetone (25 μL) was added to a solution containing 60 nmol of TTR and 60 nmol of holo-RBP in 3.5 mL. Buffer included 50 mM Tris (pH 8.0) and 0.15 M NaCl. The mixture was incubated at 4 °C overnight and dialyzed extensively against a buffer containing 50 mM Tris (pH 7.4) and 0.15 M NaCl to remove unreacted probe. To separate labeled TTR from RBP, the proteins were chromatographed on a RBP–Sepharose affinity column (see above). This step also served to further verify the ability of labeled TTR to bind RBP.

Membranes. Small unilamellar vesicles were prepared by sonication. Dioleoylphosphatidylcholine (DOPC) in chloroform solution was pipetted into a stainless steel cup and the organic solvent evaporated under a stream of argon or nitrogen. Buffer containing 150 mM NaCl and 10 mM HEPES (pH 7.4) was added and the suspension sonicated in a Heat-System sonicator to clarity. Vesicles were centrifuged at 100000*g* for 15 min to pellet multilamellar vesicles. For vesicles containing the probe NBD-DPPE, DOPC was mixed with the probe in chloroform solution and vesicles were prepared by cosonication of the lipids. The concentration of NBD-DPPE within the bilayers was 1.5 mol % of total lipids. Concentration of lipids was determined by phosphorus content (Dittmer & Wells, 1969).

Fluorescence measurements were performed using an ISS-GregPC (Champaign, IL) or a SEPX Fluorolog 2 (Edison, NJ) spectrofluorometer. Both instruments were equipped with Glan-Thompson polarizers.

Titration of RBP-Bound Retinoic Acid. These experiments were carried out by monitoring the shift in the absorption spectrum of retinoic acid that accompanies the protonation of the carboxyl group (Noy, 1992). Apo-RBP was reconstituted with retinoic acid at a mole ratio of 1:1 in a 0.5 M NaCl solution. The concentration of the complex was 7 μM. Titration was carried out by addition of dilute solutions of either HCl or NaOH, and the pH was measured by a Sigma Tris electrode using a Radiometer research-grade pH meter. The absorption of the mixture at various pHs was measured using a Cary 1 spectrophotometer (Varian). To ensure that equilibrium was reached, samples were incubated at room temperature in the dark, and pH values and the absorption at 340 and 380 nm were measured at 15-min intervals until constant values were observed. Solutions were purged with argon before use to minimize oxidation of retinoic acid, and mixtures were kept in the dark whenever possible. Titrations and absorption measurements were carried out in dim light.

Rate of Transfer of Retinol from RBP or from TTR–RBP to Vesicles Containing NBD-DPPE. Transfer of retinol from the proteins to vesicles was monitored by using the fluorescent lipid probe *N*-(7-nitro-2,1,3-benzodiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-DPPE). The absorption spectrum of NBD-DPPE overlaps extensively with the emission spectrum of retinol, and there exists an energy transfer between retinol and NBD when the two fluorophores are embedded in the same bilayer (Noy & Xu, 1990). NBD fluorescence at 600 nm (where the fluorescence of retinol is negligible) can thus be observed upon excitation of retinol (at 330 nm). Movement of retinol from protein to bilayers in which the probe is incorporated results, therefore, in an increase in NBD fluorescence. NBD-DPPE was incorporated into vesicles of DOPC by cosonication. Holo-protein(s) (0.6–2.2 nmol) was(were) mixed in a cuvette with vesicles of DOPC containing 1.5 mol % NBD-DPPE in a buffer containing 20 mM sodium phosphate, pH 7.4, and 150 mM NaCl. The final volume was 1 mL. Fluorescence (excitation, 330 nm; emission, 600 nm) was followed at intervals until equilibrium was reached. The mixture was kept in the dark between measurements to minimize photodegradation of retinol.

Binding of RBP to TTR. Pyrene-labeled TTR was titrated with RBP and the titration was followed by measuring the anisotropy of pyrene fluorescence (excitation, 330 nm; emission, 378 nm). The maximum error of the reported anisotropy was ±0.002 with typical errors of that value. Corrections were made for background scattering. Control samples showed no instrumental drift in signal within the time range of the experiments. Protein association could be observed by the increase in anisotropy. Fluorescence anisotropy is related to the rotational volume of the complex through the Perrin equation, $A_0/A - 1 = RT\tau/\eta V_r$, where A_0 is the anisotropy in the absence of rotational motion, R and T are the gas constant and the absolute temperature, respectively, τ is the fluorescence lifetime, η is the solvent viscosity, and V_r is the rotational volume. In the absence of changes in lifetime and shape, increases in anisotropy directly correspond to changes in rotational motion. In these studies, addition of RBP to pyrene-labeled TTR did not affect the fluorescence intensity, indicating a constant fluorescence lifetime. Hence, the observed increase in anisotropy was due to an increase in rotational volume. The Perrin equation assumes spherical rotations. Since the proteins under study may not be spherical, the exact relation between anisotropy and V_r may only be estimated by this equation.

Analysis of the data was adapted from the method described by Cogan et al. (1976). The mass law equation

$$K_d = (nTR)/\mathrm{TR} \quad (1)$$

was used to derive a working equation for the evaluation of the apparent dissociation constant, K_d. In eq 1 T, R, and TR are the concentrations of free TTR, free RBP, and the complex TTR–RBP, respectively. n is the number of binding sites for RBP per mole of TTR. If α is defined as the fraction of free binding sites on TTR and T_0 and R_0 are the total TTR and RBP concentrations, respectively, eq 1 can be written in the form

$$T_0\alpha = (1/n)[R_0\alpha/(1-\alpha)] - K_d/n \quad (2)$$

A plot of $T_0\alpha$ vs $R_0\alpha/(1-\alpha)$ will have a slope of $1/n$ and an intercept of K_d/n. The value of α was calculated for every point on the titration curve of probe anisotropy vs total RBP concentration using the relation

$$\alpha = (A_{max} - A)/(A_{max} - A_0) \quad (3)$$

where A_0, A, and A_{max} are the anisotropy values at R_0, R, and saturation, respectively.

Rate of Dissociation of RBP from TTR. Pyrene-labeled TTR and holo-RBP were mixed in a cuvette at a mole ratio of 1:1 (2 μM) in a buffer containing 0.15 M NaCl and 20 mM HEPES, pH 7.4. In order to induce dissociation of the TTR–RBP complex, DOPC vesicles were added (1 mM). The dissociation of the TTR–RBP complex was followed by the decrease in anisotropy of pyrene fluorescence (excitation, 340 nm; emission, 378 nm). The data for the rate of change in pyrene anisotropy was transformed to rate of formation of free TTR. This was done by using the linear relationship between anisotropy and the fraction of free TTR obtained from the titration of TTR with RBP. This calibration curve is shown in Figure 1.

RESULTS

Binding of Holo-RBP to TTR. To study the binding affinity of TTR for RBP, TTR was covalently labeled with a pyrene probe, and the increase in anisotropy of the probe's fluorescence resulting from the association of the two proteins was used to monitor binding. As described under Materials and Methods, the ability of labeled TTR to bind RBP was protected by labeling the complete TTR–RBP complex rather than TTR alone. In addition, the two proteins were then separated by RBP affinity chromatography. Only TTR that bound to the column was used in further experiments. Pyrene-labeled TTR was titrated with holo-RBP and the anisotropy of the fluorescent probe was measured. There was a marked increase in anisotropy of the pyrene fluorescence upon addition of holo-RBP, indicating the formation of TTR–RBP complex. Anisotropy reached a maximum value which was the same regardless of the initial concentration of TTR used, suggesting that saturation of TTR with RBP was achieved. A typical titration curve of TTR with holo-RBP is shown in Figure 2 (top) (open circles). Also shown in Figure 2 (top) (filled circles) is the titration of TTR with apo-RBP. Although some association between TTR and apo-RBP could be observed, the affinity of binding was very low and saturation was not reached even at a large excess of apo-RBP. Analysis of data for binding of holo-RBP to TTR according to eq 2 (see Materials and Methods) is shown in Figure 2 (bottom). From the intercept and the slope of the straight line in Figure 2 (bottom), the number of TTR binding sites for RBP and the equilibrium dissociation constant can be calculated. These were 1.50 ± 0.47 mol of RBP/mol of TTR and 0.07 ± 0.008 μM ($n = 4$). As can be seen from the straight line in Figure 2 (bottom), binding did not resolve into multiple binding sites. The data thus indicate the existence of a mixed population of TTR possessing either one or two independent binding sites for RBP with similar dissociation constants.

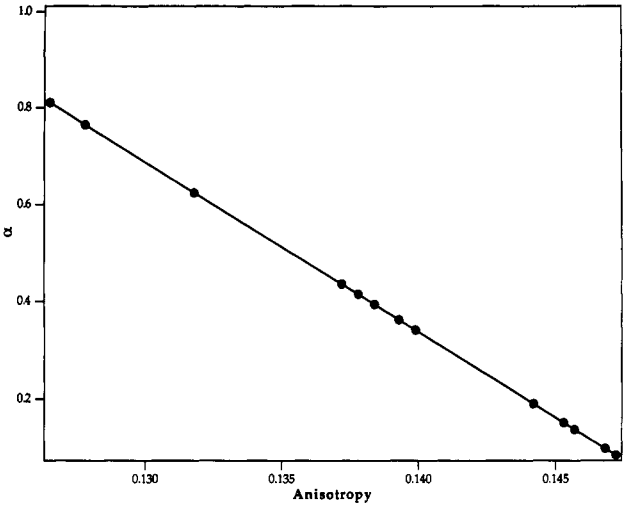

FIGURE 1: Fraction of bound TTR vs anisotropy. The calibration curve for transforming anisotropy values to fraction of bound TTR was constructed from the titration of TTR with holo-RBP (Figure 2). α corresponds to the fraction of bound TTR at each point along the titration curve. $\alpha = (A - A_0)/(A_{max} - A_0)$, where A, A_0, and A_{max} are the anisotropy values at individual points, at [RBP] = 0, and at saturation, respectively.

Binding of TTR to RBP in the Presence of Retinal or Retinoic Acid. The data in Figure 2 (top) indicated that the association of apo-RBP with TTR was weak vs the association of the holoprotein. To investigate the ligand specificity of the interactions between the two proteins, TTR was titrated with RBP reconstituted with retinal or with retinoic acid. The fluorescence anisotropy measurements indicated that neither RBP–retinal nor RBP–retinoic acid had a significant affinity for TTR. This was evident from a small change in anisotropy which did not reach saturation levels upon titration of TTR with retinal–RBP and a complete lack of change in anisotropy upon titration with RBP complexed with retinoic acid. To

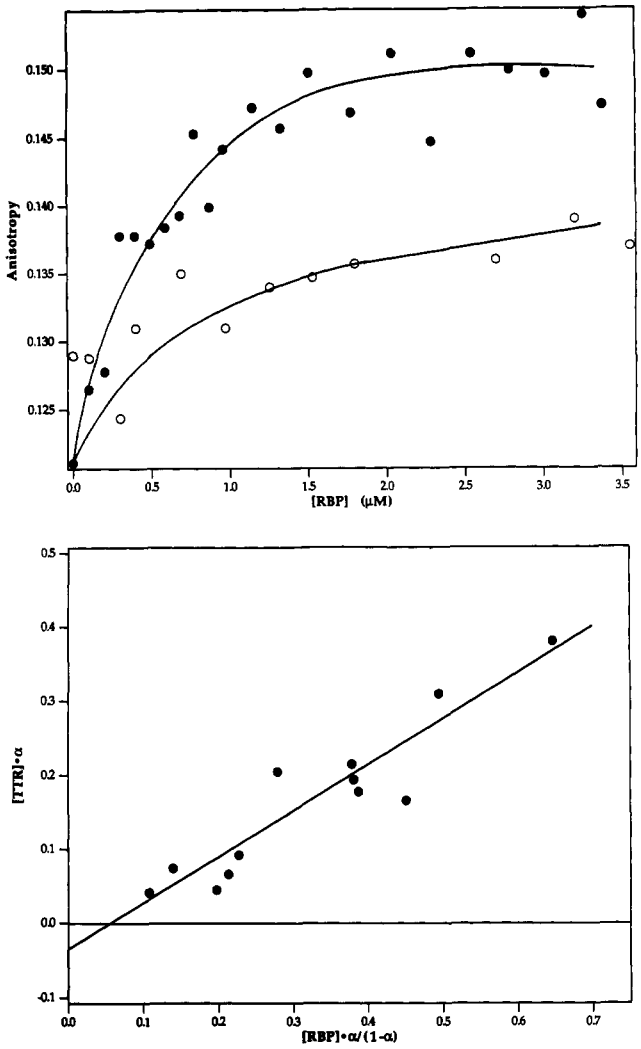

FIGURE 2: Fluorescence anisotropy titration of TTR with RBP. The titration system consisted of 1 mL of 0.5 μM of pyrene-labeled TTR in 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 0.1 mM DTT. Holo- or apo-RBP was added from solutions in the same buffer. Apo-RBP was obtained by extraction into ether (see Materials and Methods). Excitation wavelength, 330 nm; emission wavelength, 378 nm. Anisotropy was measured as described under Materials and Methods. (Top) Titration of TTR with holo-RBP (●) and with apo-RBP (○). (Bottom) Analysis of data for binding of holo-RBP. Corrected data are plotted according to eq 2, $P_0\alpha$ vs $R_0\alpha/(1-\alpha)$ (see Materials and Methods).

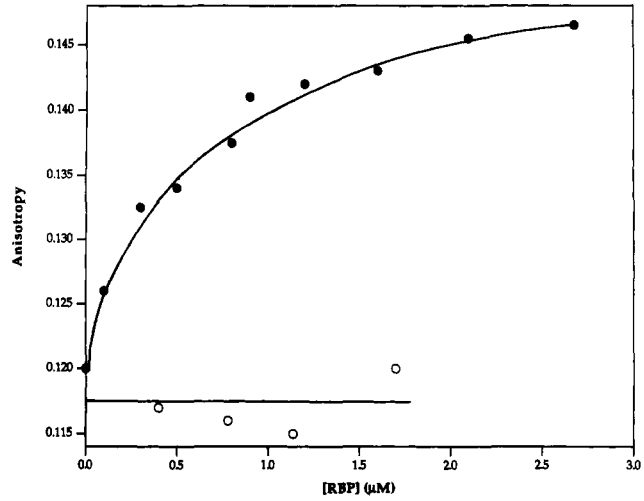

FIGURE 3: Fluorescence anisotropy titration of TTR with RBP complexed with retinal or retinoic acid. Apo-RBP was complexed with retinal (●) or retinoic acid (○) as described under Materials and Methods. Titrations were carried out as described in the legend to Figure 2 except that the NaCl concentration was 0.5 M.

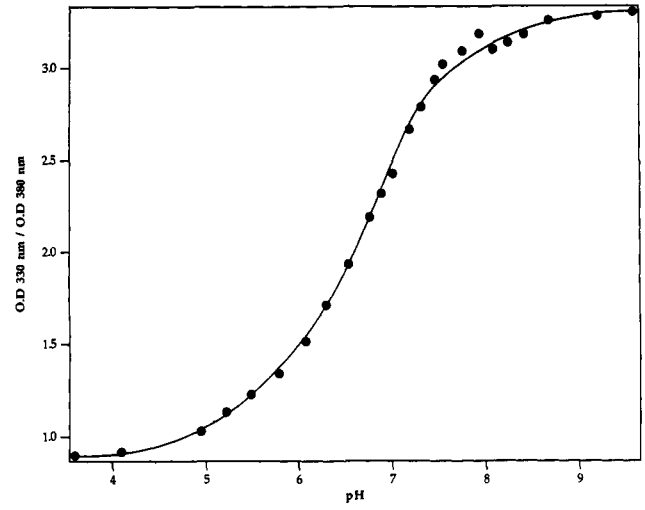

FIGURE 4: pH titration of retinoic acid bound to RBP. Retinoic acid was complexed to apo-RBP and the titrations were carried out as described under Materials and Methods. The absorption ratio A_{330nm}/A_{380nm} was monitored as a function of pH. The concentration of RBP was 7 μM.

ensure that RBP was not modified by the ether extraction used to obtain the apoprotein (see Materials and Methods), TTR was also titrated with apo-RBP reconstituted with retinol. The dissociation constant of this complex was 0.09 μM, essentially identical with the dissociation constant obtained with native holo-RBP. It was reported that the formation of the TTR–RBP complex strongly depends on the ionic strength of the medium (van Jaarsveld et al., 1973). Measurements of the binding of holo-RBP to TTR in the presence of 0.5 M NaCl (data not shown) showed 1.22 ± 0.04 binding sites/protein and K_d = 0.093 ± 0.03 μM (n = 6); i.e., the affinity of binding of RBP to TTR in the presence of retinol was not significantly affected by increasing the salt concentration from 0.15 to 0.5 M. Titrations of TTR with RBP reconstituted with retinal or retinoic acid in the presence of 0.5 M NaCl are shown in Figure 3. RBP reconstituted with retinal, but not with retinoic acid, was found to bind to TTR under these conditions. Analysis of the data revealed the presence of 1.56 binding sites for retinal–RBP/molecule of TTR (mean of two experiments; individual values were 1.48 and 1.64) and K_d = 0.134 μM; i.e., the affinity of TTR for retinal-containing RBP in 0.5 M NaCl was about 2-fold lower than that for retinol–RBP.

RBP reconstituted with retinoic acid, unlike RBP with retinol and retinal, did not associate with TTR even at high ionic strength conditions. Since retinoic acid is the only ligand of these three retinoids that may be ionized, one possible explanation for these observations is that retinoic acid bound to RBP is negatively charged and that the negative charge hinders the association between the two proteins. To investigate this possibility, the pK of retinoic acid bound to RBP was measured. It was previously shown that ionization of retinoic acid can be followed by monitoring the red shift in the absorption maximum of retinoic acid that occurs upon deprotonation (Noy, 1992). Figure 4 shows the absorbance ratio 330/380 nm of a solution containing retinoic acid bound to RBP (mole ratio of 1:1) as a function of pH. The value of the pK of RBP-bound retinoic acid found from this titration curve was 6.6. Hence, at pH 7.4, a predominant fraction of retinoic acid bound to RBP will be negatively charged. If this negative charge inhibits binding of retinoic acid-containing

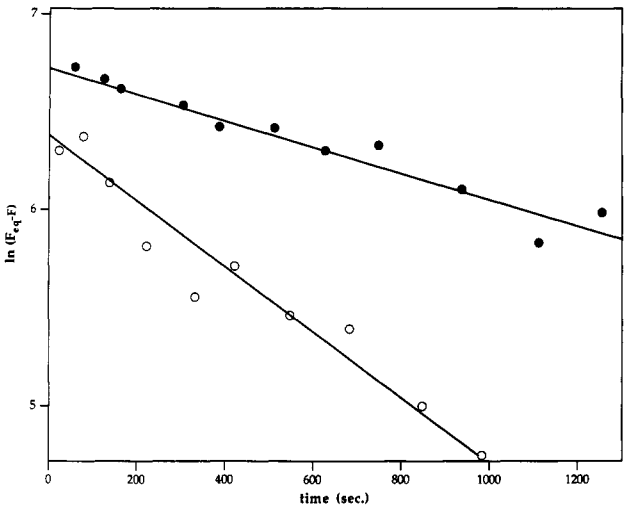

FIGURE 5: Transfer of retinol from RBP or from TTR-RBP to unilamellar vesicles of DOPC. RBP (2.2 nmol, containing 2 nmol of retinol) were mixed in a cuvette with vesicles of DOPC (2200 nmol of lipid) containing 1.5 mol % NBD-DPPE in the presence (●) or the absence (○) of 2.5 nmol of TTR. The final volume was 1 mL. Transfer of retinol from the protein to the vesicles was followed by monitoring NBD fluorescence upon excitation of retinol (excitation, 350 nm; emission, 600 nm) which was followed at intervals until equilibrium was reached. Data are plotted as $F - F_c$ vs time. F and F_c denote the fluorescence at time t and at equilibrium, respectively.

RBP to TTR, than lowering the pH below the pK of RBP-bound retinoic acid should result in protonation of the carboxyl head group and, in turn, in enhanced binding. The anisotropy of pyrene fluorescence upon titration of pyrene-labeled TTR with retinoic acid-containing RBP at pH 5.8 was monitored. At this pH about 80% of the retinoic acid is expected to be fully protonated; however, no association of the two proteins was observed under these conditions. This did not reflect denaturation of RBP at this pH, as was evidenced by the only slightly reduced (K_d = 0.11 μM) affinity of TTR for retinol–RBP at pH 5.8 vs at pH 7.0.

Rates of Dissociation of Retinol from RBP and from TTR–RBP. The kinetics of dissociation of retinol from the complex RBP–retinol or from the complete complex TTR–RBP–retinol were studied. Studies of the dissociation of ligands that are poorly soluble in water, like retinol, from binding proteins require the introduction of a phase, other than the binding protein, with an affinity for the ligand. Such a phase is needed to induce dissociation of the ligand to an extent that can be accurately measured. For this purpose, unilamellar vesicles of phospholipids were used. Transfer of retinol from RBP or from TTR–RBP to vesicles of DOPC following the mixing of holoprotein(s) with vesicles was monitored by the rate of arrival of the ligand at the vesicles. The lipid probe NBD-DPPE was incorporated into vesicles of DOPC by cosonication. Since the absorption spectrum of NBD overlaps extensively with the emission spectrum of retinol (Noy & Xu, 1990a), the presence of retinol and NBD within the same bilayer results in quenching of retinol fluorescence. Transfer of retinol from the binding proteins to vesicles was hence followed by the time-dependent decrease in the fluorescence of retinol following the mixing of holoprotein(s) with vesicles that contained NBD-DPPE, as described under Materials and Methods. Figure 5 shows typical time courses for dissociation of retinol from RBP or from TTR–RBP plotted as first-order kinetic plots. The data show that transfer in both cases followed a single first-order reaction with rate constants of 0.116 ± 0.014 and 0.042 ± 0.0023 min^{-1} (n = 4) for the dissociation of retinol from RBP and from TTR–RBP, respectively.

Table I: Parameters of the Interactions of Retinol with RBP and with TTR–RBP[a]

protein	K_d (M)	k_{off} (min^{-1})	k_{on} (M^{-1} min^{-1})
RBP	0.7×10^{-7}	0.116	1.6×10^{6}
TTR–RBP	0.35×10^{-7}	0.042	1.2×10^{6}

[a] Rate constants for dissociation (k_{off}) of retinol from the proteins were taken from the data in Figure 1. Equilibrium dissociation constants (K_d) were obtained from Noy and Xu (1990a). Rate constants of the association of retinol with the proteins (k_{on}) were calculated by the expression $K_d = k_{off}/k_{on}$.

Transfer of retinol from a binding protein (BP) to lipid vesicles may proceed via the aqueous phase, i.e., by dissociation of retinol from the protein (reaction 3) followed by diffusion of free retinol through the aqueous phase and association with the vesicles (reaction 4):

reaction 3

$$\text{retinol–BP} \leftrightarrow \text{retinol} + \text{BP}$$

reaction 4

$$\text{retinol} + \text{bilayer} \leftrightarrow \text{bilayer–retinol}$$

Alternatively, retinol could be directly transferred from a protein to vesicles during collisions. If transfer occurred during collisions, than increasing the concentration of the acceptor (lipid bilayer) phase should result in an increased rate of transfer. If, on the other hand, transfer proceeded via the water, the rate of transfer would be determined by the rate of dissociation from the protein and will be independent of the donor/acceptor concentration ratio. As with a previous report regarding the dissociation of retinol from RBP (Noy & Xu, 1990a), the rate of dissociation of retinol from the TTR–RBP complex was found to be independent of vesicle concentrations, indicating that transfer occurred via reactions 3 and 4 rather than by direct collisions (data not shown). The rate of transfer of retinol from the proteins to vesicles hence directly represents the rate of dissociation from the proteins (Noy & Xu, 1990a; Noy & Blaner, 1992). The data thus indicate that the dissociation of retinol from the TTR–RBP complex is about 2.5-fold slower vs the dissociation of this ligand from RBP alone.

Rates of Association of Retinol with RBP and with TTR–RBP. These can be calculated from the rate constants for dissociation (k_{off}) and the equilibrium dissociation constants of retinol with RBP and with TTR-RBP (K_d) by using the expression $K_d = k_{off}/k_{on}$. The values of k_{off} were obtained as in Figure 5. K_d's for binding of retinol to bovine RBP and TTR–RBP were measured previously (Noy & Xu, 1990a). The rate constants characterizing the interactions of retinol with the binding protein(s) are shown in Table I. It is clear from the data that the rates of association of retinol with free and with TTR-bound RBP are similar and that the higher affinity for retinol displayed by the complete protein complex vs by RBP alone stems predominantly from the slower rate of dissociation of the ligand from the former complex.

Kinetics of Dissociation of RBP from TTR. The changes in fluorescence polarization of pyrene-labeled TTR associated with binding of RBP to this protein were utilized to monitor the rate of dissociation of the TTR–RBP complex. TTR–RBP–retinol at a mole ratio of 1:1:1 were mixed with vesicles of DOPC. Upon mixing, retinol partitioned from the protein complex into the vesicles (Figure 5), and since the affinity of TTR for apo-RBP is low (Figure 2, top), apo-RBP then

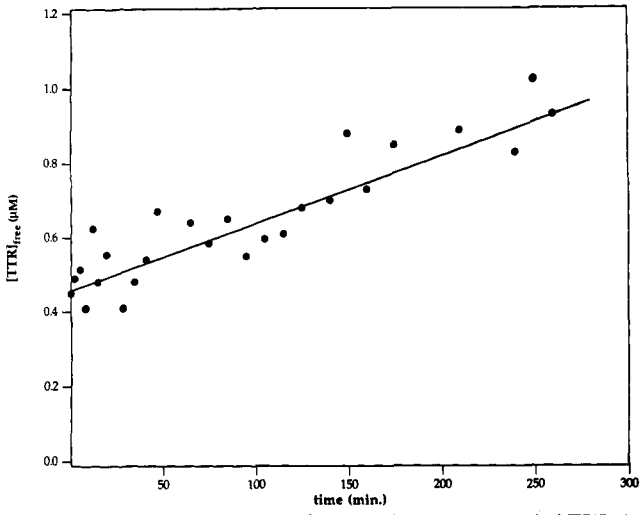

FIGURE 6: Dissociation of RBP from TTR. Pyrene-labeled TTR (2 μM) was mixed in a cuvette with 2 μM holo-RBP. Dissociation of the proteins was initiated by addition of unilamellar vesicles of DOPC (1100 μM) and was followed by monitoring fluorescence anisotropy (excitation, 330 nm; emission, 378 nm). Anisotropy values were transformed to concentration of free TTR by using the calibration curve depicted in Figure 1.

dissociated from TTR. The dissociation reaction was followed by monitoring the time-dependent decrease of polarization. In order to translate the rate of change in fluorescence anisotropy to rate of formation of free TTR, the linear correlation between the value of anisotropy with the fraction of free TTR (α in eqs 2 and 3) was used. Thus, a calibration curve was constructed from the data of the titration curve (Figure 2) as is shown under Materials and Methods. The time course of dissociation of the complex TTR–RBP is shown in Figure 6. The rate of dissociation of the two proteins was slow enough to allow for evaluation of the rate constant from the initial, linear phase of the reaction. The value of the rate constant for dissociation of RBP from TTR was 0.047 ± 0.005 h^{-1} ($n = 3$); i.e., $t_{1/2}$ of the reaction was about 15 h.

DISCUSSION

Experiments presented in this paper focused on studying the interactions within the protein complex which binds retinol in blood. The interactions of RBP with TTR were investigated by covalently labeling TTR with a long-lived fluorescence probe (pyrene) and monitoring changes in rotational volume as determined by fluorescence anisotropy of the probe that occurred upon association of the two proteins. The data show that in 0.15 M NaCl bovine TTR exists as a mixed population displaying either one or two binding sites for bovine holo-RBP and that the dissociation constants of the two sites were identical and had a value of 0.07 μM. The binding affinity of the two proteins was thus found to be 5-fold higher than previously reported (Berni et al., 1990). One possible explanation for this discrepancy is that proteins employed in the present study were isolated by affinity chromatography, while proteins used in the study of Berni et al. (1990) were purified using gel filtration and ion-exchange chromatography. The former procedure is likely to select for TTR and RBP with higher mutual affinity.

At physiological ionic strength (0.15 M NaCl), binding of RBP to TTR showed an absolute requirement for retinol, the native ligand. Apo-RBP or RBP complexed with retinal or retinoic acid displayed very weak or no association with TTR (Figures 2 and 3). These observations are of interest regarding the mechanism by which various retinoids are transported in the circulation. Though the binding affinity of RBP for retinol and for retinoic acid (as well as for other retinoids) is similar (e.g., Cogan et al., 1976), in blood, only retinol is found bound to RBP. On the other hand, retinoic acid which was injected into the circulation was found in blood bound to serum albumin (Smith et al., 1973). The results of the present study suggest that the selectivity for transport of retinol bound to RBP may originate not from the selectivity of binding of retinol to this protein but from the interactions of RBP with TTR, which are efficient in the presence of retinol but are hindered in the presence of retinoic acid. The data indicate that RBP complexed with retinoic acid will not bind to TTR. This is likely to result, in vivo, in the filtration of retinoic acid–RBP complex in the glomeruli and a short lifetime in the circulation.

To investigate whether molecular contacts between RBP and TTR are based on charge interactions, the effects of increased ionic strength on protein association in the presence of retinol, retinal, and retinoic acid were studied. It was found that the dissociation constants of the complex comprised of TTR and retinol-containing RBP were 0.09 and 0.07 μM in the presence of 0.5 and 0.15 M NaCl, respectively; hence, in contrast to previous findings (van Jaarsveld et al., 1973), binding affinity between the two proteins was not found to be affected by the ionic strength when the ligand was retinol. However, the increased salt concentration dramatically affected binding to TTR of RBP complexed with retinal. At high salt concentration, titration of TTR with retinal-containing RBP revealed the presence of 1.56 binding sites/molecule of TTR, while no association was detected at 0.15 M NaCl. Binding of retinal–RBP was characterized by a dissociation constant of 0.134 μM. Thus, under high ionic strength conditions, the affinity of TTR for retinal–RBP was only 2-fold lower vs the affinity for retinol–RBP. The isoelectric points of TTR and RBP are 4.6 and 4, respectively (Peterson & Berggard, 1971; van Jaarsveld et al., 1973). Both proteins are thus negatively charged at physiological pH and it was proposed that the effect of salt on the interactions between them originates from masking of negative charges (van Jaarsveld et al., 1973). The above data indicate that the interactions between RBP and TTR are also sensitive to the composition of the head group of the retinoid bound by RBP. This conclusion is emphasized further by the observations that no association between TTR and RBP could be detected in the presence of retinoic acid even at the high salt concentration. Since retinoic acid contains an ionizable head group, one possible explanation for these observations is that retinoic acid bound to RBP is negatively charged and that the negative charge hinders the association between the two proteins.

To investigate this possibility, the pK of retinoic acid bound to RBP was measured. The pK of RBP-bound retinoic acid was found to have a value of 6.6 (Figure 4), so that at pH 7.4, a predominant fraction of retinoic acid bound to RBP will, indeed, be negatively charged. However, no association of the two proteins was observed at pH 5.8 where about 80% of the retinoic acid is protonated, pointing at the conclusions that structural, rather than charge, parameters prevent the association of RBP with TTR in the presence of retinoic acid. Within the RBP binding pocket, retinoids lie along the axis of the β barrel with the β ionone ring innermost and the polar head group almost at the protein surface; bound retinol is essentially buried within the protein with minimal access to bulk water (Newcomer et al., 1984). The data presented above suggest that the site of interactions of RBP with TTR may be in close proximity to the retinoid binding pocket and

that complete closure of this binding pocket may be important for protein–protein interactions. The carboxyl head group of retinoic acid may thus present steric obstacles to binding, while the carbonyl and hydroxyl head groups of retinal and retinol are accommodated within the binding site.

To clarify the sequence of events leading to dissociation of the complex retinol–RBP–TTR, the kinetics of the various steps of this process were studied. It was found that dissociation of retinol from the tertiary complex is about 2.5-fold slower than dissociation from RBP alone, which results in a 2.5-fold increase in affinity of binding of retinol in the tertiary complex vs by RBP. Loss of retinol from the complex leads to dissociation of RBP from TTR. The later step is slower by far than the former, and the complete dissociation process can be described as in reaction 1:

$$\text{retinol–RBP–TTR} \xrightarrow{0.042/\text{min}} \text{retinol} + \text{RBP–TTR} \xrightarrow{0.047/\text{h}} \text{RBP} + \text{TTR}$$

Since $t_{1/2}$ for dissociation of apo-RBP from TTR following the loss of retinol is about 15 h, the data indicate that, despite the low binding affinity between TTR and apo-RBP, the complex of these two proteins will remain in the circulation for an extended period of time. This complex is likely to rebind free retinol from serum or from plasma membranes of cells. This conclusion suggests that RBP is not cleared from blood immediately upon loss of retinol as is usually believed (Goodman, 1984), that it remains in blood for an extended period, and that TTR–apo-RBP, in addition to TTR–holo-RBP freshly secreted from the liver, is likely to play an important role in determining the level of retinol in serum.

It has been postulated from binding studies that uptake of retinol from blood into target cells involves binding of the serum RBP to receptors in the plasma membranes of these cells (Chen & Heller, 1977; Rask & Peterson, 1976; McGuire et al., 1981; Heller, 1975; Ottonello & Maraini, 1981; Pfeffer et al., 1986; Eriksson et al., 1986; Sivaprasadaro & Findlay, 1988; Bavik et al., 1991). However, the exact function that may be served by binding of RBP to specific receptors in the plasma membranes of target cells is unclear. Thus, the results of previous studies of the kinetics of interactions of retinol with RBP, with membranes, and with cellular RBP (CRBP) have indicated that none of the steps of the process of transfer of retinol from the binding site on RBP through cellular membranes to cytoplasm requires facilitation to account for rates of uptake of retinol by target cells; i.e., the distribution of retinol between blood, plasma membranes of target cell, and cytosol is at equilibrium (Noy & Xu, 1990a,b; Noy & Blaner, 1991). The conclusions of these studies were derived from the observations that the rate of dissociation of retinol from RBP is the slowest step of the initial events leading to uptake of retinol by target cells and that this step is orders of magnitude faster than observed rates of uptake of retinol by such cells. The results of the present study show that solvation of retinol precedes separation of the proteins. Hence, the protein species from which retinol dissociates into serum is not RBP alone but the complete complex TTR–RBP. The rate of dissociation of retinol from TTR-RBP was found to be about 2.5-fold slower than dissociation from RBP (Table I). This rate is faster by far than observed rates of entry of retinol into cells. The results of the present study thus confirm that the dissociation of retinol from binding proteins in serum does not limit uptake of this compound and that the distribution of retinol between proteins in serum and the plasma membranes of cells will be at equilibrium. Retinol will be taken up continuously only by cells that metabolize it. Specificity of uptake will thus be determined by retinol metabolism in various cells and not by events at the plasma membranes.

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Registry No. DOPC, 4235-95-4; retinol, 68-26-8; retinal, 116-31-4; retinoic acid, 302-79-4.