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

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ABSTRACT: The interactions of retinol with rat cellular retinol-binding protein (CRBP) and with rat serum retinol-binding protein (RBP) were studied. The equilibrium dissociation constants of the two retinol–protein complexes (K_d) were found to be 13×10^{-9} and 20×10^{-9} M for CRBP and for RBP, respectively. The kinetic parameters governing the interactions of retinol with the two binding proteins were also studied. It was found that although the equilibrium dissociation constants of the two retinol–protein complexes were similar, retinol interacted with CRBP 3–5-fold faster than with RBP; the rate constants for dissociation of retinol from CRBP and from RBP (k_{off}) were 0.57 and 0.18 min^{-1}, respectively. The rate constants for association of retinol with the two proteins (k_{on}) were calculated from the expression: $K_d = k_{off}/k_{on}$. The k_{on}'s for retinol associating with CRBP and with RBP were found to be 4.4×10^7 and 0.9×10^7 M^{-1} min^{-1}, respectively. The data suggest that the initial events of uptake of retinol by cells are not rate-limiting for this process and that the rate of uptake is probably determined by the rate of metabolism of this ligand. The data indicate further that the distribution of retinol between RBP in blood and CRBP in cytosol is at equilibrium and that intracellular levels of retinol are regulated by the levels of CRBP.

Retinol is a lipophilic compound that is found in aqueous phases in vivo largely bound to retinol-specific-binding proteins. In blood, retinol circulates bound to a specific plasma retinol-binding protein (RBP). RBP is a 21 000-Da protein which, in the circulation, is bound to another protein, transthyretin (TTR) (Goodman & Blaner, 1984; Blaner, 1989). Within cells, retinol is found bound to specific cellular retinol-binding proteins. Two cellular-binding proteins for retinol have been isolated and characterized, cellular retinol-binding protein (CRBP) and cellular retinol-binding protein II (CRBP II) (Chytil & Ong, 1987). CRBP is widely distributed in the body and has been reported to be present in the cytoplasm of all rat tissues examined (Kato et al., 1985). CRBP II is found only in the small intestine of adult rats (Crow & Ong, 1985; MacDonald & Ong, 1987).

The complete scope of the physiological function(s) of the retinol-binding proteins has not been elucidated as yet. It has been suggested, however, that one important function of these water-soluble binding proteins is to mediate the delivery of retinol, which is poorly soluble in water, to specific cells or to specific intracellular sites where retinoids are stored or utilized. RBP serves to transport retinol from its stores in the liver to target tissues (Blaner, 1989). CRBP and CRBP II both function to deliver retinol intracellularly to the enzyme lecithin:retinol acyltransferase (LRAT) for esterification and storage of the retinoid (Ong et al., 1987, 1988; Saari & Bredberg, 1988; Yost et al., 1988).

Regarding the mechanism by which retinol-binding proteins are involved in the process of delivery of retinol, 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). It was also proposed that CRBP delivers retinol to the nucleus via specific binding of this protein to sites in nuclei or chromatin (Crow et al., 1987). On the other hand, 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, 1990; Fex & Johannesson, 1987), 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, 1990a; Ho et al., 1989). These observations suggest that retinol bound to RBP, in the circulation, will spontaneously equilibrate with unbound retinol, that unbound retinol will freely cross the plasma membranes and enter the cytosol of target cells, and that the flux of retinol from blood into cells will be determined by the concentration of the unbound ligand. Since none of the steps of 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, it is not clear what function may be served by binding of RBP to specific receptors in the plasma membranes of target cells (Noy & Xu, 1990).

These considerations suggest that the initial events of uptake of retinol by cells occur spontaneously and are 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. Once inside the cell, retinol will distribute between high-affinity sites such as intracellular membranes and a cellular retinol-binding protein, which in most tissues will be CRBP. In the present study, the affinity for retinol of RBP and of CRBP and the rates of the interactions of retinol with the two proteins from the same species (rat) are compared. This study was undertaken in order to clarify the relationship between the pool of retinol in blood (bound to RBP) and the pool of retinol in cells (bound to CRBP), and to investigate whether the kinetics of the interactions of retinol with CRBP may play a role in the regu-

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lation of the intracellular disposition of retinol inside cells.

Materials and Methods

Lipids were obtained from Avanti Polar Lipids. *all-trans*-Retinol was obtained from Kodak. [^{3}H]Retinol and [^{14}C]dioleoylphosphatidylcholine were from New England Nuclear. All other chemicals were from Sigma Chemical Co. Scintillation liquid (Liquiscint) was from National Diagnostics. Male Wistar rats weighing about 300 g were obtained from Charles River Breeding Laboratories.

Proteins. Bovine RBP was purified from bovine serum (Pel-Freeze Co.) by the procedure outlined in Berni et al. (1985). Rat RBP was purified from rat plasma by using standard chromatographic procedures described elsewhere (Blaner & Goodman, 1990). The purified RBP migrated as a single homogeneous band on SDS–PAGE (Laemmli, 1970) with a mass of approximately 21 000 daltons. Rat CRBP was purified from rat testes homogenates by using published procedures (Kato et al., 1984). Purified CRBP was judged to be homogeneous as evidenced by SDS–PAGE, and migrated with an approximate mass of 15 000 daltons.

Concentrations of proteins and of retinol were determined from their extinction coefficients (Horowitz & Heller, 1977). Apo-RBP was obtained by extracting retinol from RBP into heptane. Equal volumes of a solution of holo-RBP and heptane 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 absorbances at 280 and 330 nm.

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 100 mM NaCl, 10 mM HEPES (pH 7.0), and 1 mM ascorbate 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. The concentration of lipids was determined by the phosphorus content (Dittmer & Wells, 1969). Plasma membranes were isolated from rat liver on sucrose density gradients (Song et al., 1969).

Fluorescence measurements were performed with a SPEX DM1B fluorometer.

Equilibrium Dissociation Constant of Retinol–RBP. This was measured by fluorometric titration as described by Cogan et al. (1976). Apo-RBP was titrated with retinol dissolved in ethanol, and the titration was followed by measuring the fluorescence of the RBP–retinol complex (excitation, 290 nm; emission, 490 nm). The mass law equation

$$K_d = nPR/PR \quad (1)$$

was used to derive a working equation for the evaluation of the apparent dissociation constant (K_d). In eq 1, P, R, and PR are the concentrations of free RBP, free retinol, and retinol bound to RBP, respectively. n is the number of binding sites for retinol per mole of RBP. If α is defined as the fraction of free binding sites on the protein molecule and P_0 and R_0 are the total protein and retinol concentrations, respectively, eq 1 can be written in the form of eq 2. A plot of $P_0\alpha$ vs

$$P_0\alpha = (1/n)[R_0/(1 - \alpha)] - K_d/n \quad (2)$$

$R_0\alpha/(1-\alpha)$ will have a slope in $1/n$ and an intercept of K_d/n. The value of α was calculated for every point on the titration curve of fluorescence intensity vs total retinol concentration by using the relation

$$\alpha = (F_{max} - F)/(F_{max} - F_0) \quad (3)$$

where F_0, F, and F_{max} are the fluorescence intensities at R_0, at R, and at saturation, respectively. Calculations and corrections were performed as in Cogan et al. (1976).

Rate of Transfer of Retinol from CRBP or from RBP to Vesicles. Holoprotein (0.6–1.5 nmol) was mixed in a cuvette with vesicles of DOPC in a buffer containing 20 mM sodium phosphate, pH 7.0, and 200 mM NaCl. The final volume was 1 mL. Fluorescence (excitation, 290 nm; emission, 490 nm) was followed at 30-s intervals until equilibrium was reached. The mixture was kept in the dark between measurements to minimize photodegradation of retinol. Transfer of retinol from CRBP to vesicles was also monitored by using the fluorescent lipid probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-DPPE). The absorption spectrum of NBD-DPPE overlaps extensively with the emission spectrum of retinol. Movement of retinol from protein to bilayers in which the probe is incorporated results in quenching of retinol fluorescence. NBD-DPPE was incorporated into vesicles of DOPC by cosonication, vesicles were mixed with holo-CRBP, and the movement of retinol to the vesicles was followed by the decrease in retinol fluorescence (excitation, 350 nm; emission, 490 nm).

Distribution of Retinol between CRBP or Bovine RBP and Plasma Membranes of Rat Liver Cells. Holoprotein was mixed with a trace amount of [^{3}H]retinol to give a specific radioactivity of retinol of about 60 000 cpm/nmol. The mixture was incubated at 4 °C in the dark for 1 h to achieve equilibration between protein-bound and free ligand. The labeled protein (1–2 nmol) was then mixed with plasma membranes (100–200 nmol of phospholipids) in a final volume of 0.5 mL. The mixture was incubated at 20 °C and then centrifuged in an Eppendorf centrifuge for 30 min; 0.3 mL of the supernatant containing the protein was counted for [^{3}H]retinol. The rest of the supernatant was decanted, and the bottom of the Eppendorf tube (i.e., the plasma membranes pellet) was cut, dropped into a scintillation vial, and counted for [^{3}H]retinol. To correct for the amount of retinol in the supernatant that is dissolved in the aqueous phase, the distribution constant of retinol between the plasma membranes and water was measured. This was done by the same procedure but without the protein. The results were corrected according to that constant which was found to be 0.5×10^6 (molal fraction).

Results

Transfer of Retinol from CRBP to Plasma Membranes from Rat Liver. 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, plasma membranes from rat liver were used. The distribution constant (ratio of mole fractions) of retinol between this membrane and an aqueous phase is on the order of 10^6 (Noy & Xu, 1990a). Thus, the membrane has a high affinity for retinol. It was also found that the affinity of plasma membrane from rat liver for retinol is consistent within the same membrane preparation (SEM was 8% of the value, $n = 8$), which indicates that it can serve as a good reference phase. In addition, the membrane can be easily separated from the soluble binding protein by centrifugation. To monitor transfer of retinol initially bound to CRBP to the membranes, the protein was labeled with [^{3}H]retinol (see Materials and Methods), incubated with plasma membranes, and separated from the membranes by centrifugation, and supernatant (containing CRBP) and pellet (containing the membranes) were counted for the presence of

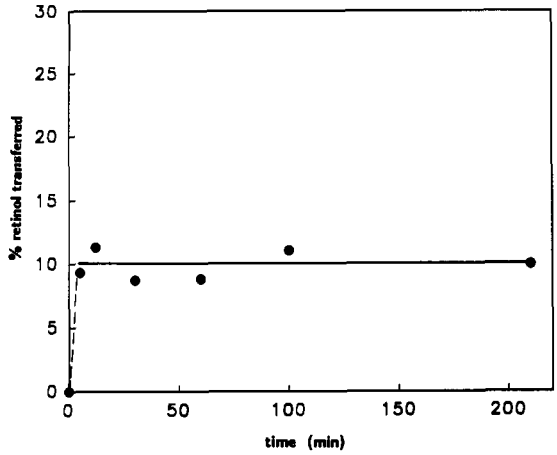

FIGURE 1: Transfer of retinol from CRBP to plasma membranes from rat liver. One nanomole of CRBP labeled with [^{3}H]retinol was mixed with plasma membranes (150 nmol of lipids) and incubated for the designated time periods. Protein was separated from the membranes by centrifugation, and supernatants and pellets were counted for the presence of [^{3}H]retinol.

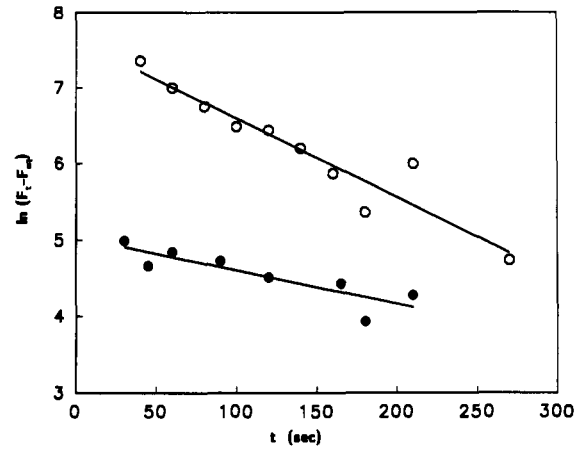

FIGURE 2: Transfer of retinol from CRBP to unilamellar vesicles of DOPC. (○) Holo-CRBP (2.5 nmol) (100% saturation) was mixed with DOPC vesicles (150 nmol of lipids) containing 1 mol % NBD-DPPE. Transfer of retinol from the protein to the lipids was followed by monitoring the decrease in the fluorescence of retinol (excitation, 350 nm; emission, 490 nm). (●) Holo-CRBP (2.5 nmol) was mixed with DOPC vesicles (600 nmol of lipids). Transfer of retinol from protein to vesicles was followed by the decrease of retinol fluorescence (emission, 490 nm) upon excitation of the protein (excitation, 290 nm). The final volume was 1 mL; the buffer contained 20 mM potasisum phosphate and 200 mM NaCl, pH 7.0. The data are plotted as ln ($F_t - F_e$) vs time. F_t and F_e denote the fluorescence at time t and at equilibrium, respectively.

[^{3}H]retinol. Figure 1 shows the time course of the transfer of retinol from CRBP to the plasma membranes. It is clear from these data that equilibrium distribution of retinol between CRBP and the membranes is established within 5 min and that the rate of transfer of retinol from CRBP to membranes is too fast to be measured by this method.

However, the distribution of retinol between CRBP and plasma membranes can be used to measure the equilibrium dissociation constant of the retinol–CRBP complex. The method most often used to measure this quantity for retinol-binding proteins is spectrofluorometric titration, a method in which an apo-binding protein is titrated with free retinol (Cogan et al., 1976). The titration usually is followed either by the increase in retinol fluorescence or by the decrease of the inherent fluorescence of the binding protein that occurs upon binding of the ligand [e.g., see Ong and Chytil (1978), Noy and Xu (1990), and Ho et al. (1989); and see below]. The main difficulty encountered with this method is the requirement for an apoprotein. Retinol-binding proteins are usually purified at least partially saturated with ligand, and procedures for extracting bound retinol sometimes result in denaturation of part, or all, of the protein. In the case of CRBP, retinol can be extracted to obtain the apoprotein, and the dissociation constant for this retinol–protein complex has been measured (Ong & Chytil, 1978), which provides an opportunity to test an alternative way for measuring dissociation constants for retinoid-binding proteins from which the ligand cannot be extracted without denaturation. We determined the equilibrium dissociation constant of the retinol–CRBP complex by comparing the affinity for retinol of CRBP relative to plasma membranes to the affinity for this ligand of bovine RBP relative to plasma membranes. Bovine RBP was used as a "reference" protein because the dissociation constant for this protein is known from a previous study (Noy & Xu, 1990). The distributions of retinol between CRBP or bovine RBP and plasma membranes of rat liver at equilibrium (K_{eq}) were measured as described above. K_{eq}'s for CRBP and bovine RBP (expressed as the ratio of mole fractions of retinol in the protein and the membranes) were 843 ± 57 (n = 4) and 160 (n = 2), respectively. The affinity for retinol of CRBP relative to bovine RBP was obtained by using the expression K_{eq}(CRBP/bovine RBP) = [K_{eq}(CRBP/PM)]/[K_{eq}(bovine RBP/PM)] = 5.3. The equilibrium dissociation constant of the retinol–bovine RBP complex is 70 × 10^{-9} M (Noy & Xu, 1990); the K_d for the retinol–CRBP complex is thus 13 × 10^{-9} M. This value is in good agreement with the K_d measured by fluorometric titration which was reported to be 16 × 10^{-9} M (Ong & Chytil, 1978).

Rate of Dissociation of Retinol from CRBP. The data in Figure 1 indicate that the rate of dissociation of retinol from CRBP is too rapid to be measured by a method that relies on centrifugation to separate "donor" from "acceptor" phases. The kinetics of the dissociation of retinol from CRBP were therefore studied by optical methods. As discussed above, a component, other than CRBP, with high affinity for retinol, was added in order to induce dissociation of an amount of retinol that is large enough to monitor the reaction accurately. The component chosen for this purpose was unilamellar vesicles of phospholipids. Transfer of retinol from CRBP to vesicles of DOPC was followed after mixing holoprotein with vesicles by two optical methods: (1) by monitoring the rate of arrival of the ligand at the vesicles; (2) by monitoring the rate of its dissociation from CRBP.

(1) The fluorescent lipid probe NBD-DPPE was incorporated into vesicles of DOPC by cosonication. Transfer of retinol from CRBP to vesicles was followed by the time-dependent decrease in the fluorescence of retinol following the mixing of holo-CRBP with vesicles that contained NBD-DPPE, as described under Materials and Methods. Figure 2 (open circles) shows the results of a typical time course plotted as a first-order kinetic plot. The data show that transfer follows a single first-order reaction with a rate constant of 0.68 ± 0.069 min^{-1} (n = 6).

Transfer of retinol from two other retinol-binding proteins—bovine RBP and the bovine interphotoreceptor RBP—to lipid vesicles has been shown to proceed via the aqueous phase (Noy & Xu, 1990; Ho et al., 1989), i.e., by dissociation of retinol from the protein (eq 4) followed by diffusion of free retinol through the aqueous phase and association with the vesicles (eq 5). Alternatively, retinol could

$$\text{retinol–CRBP} \leftrightarrow \text{retinol} + \text{CRBP} \quad (4)$$

$$\text{retinol} + \text{bilayer} \leftrightarrow \text{bilayer–retinol} \quad (5)$$

Table I: Rate Constants for Transfer of Retinol from CRBP to Vesicles of DOPC at Various Lipid/Protein Ratios[a]

lipid/protein ratio (mol/mol)	k (min^{-1})
47.5	0.71
118.0	0.66
165.0	0.96
236.0	0.53
330.0	0.69

[a] Rates of transfer were measured as described under Material and Methods. Holo-CRBP was mixed with varying amounts of unilamellar vesicles of DOPC containing 1 mol % NBD-DPPE to give the designated lipid/protein mole ratios. Data were collected and analyzed as described in Figure 2.

be directly transferred from a protein to vesicles during collisions, as in eq 6.

$$\text{CRBP–retinol} + \text{bilayer} \leftrightarrow \text{bilayer–retinol} + \text{CRBP} \quad (6)$$

To clarify the mechanism of the transfer of retinol from CRBP to lipid bilayers, the rate of transfer was measured at various protein/lipid mole ratios. 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 proceeds 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. The data (Table I) show that transfer from CRBP proceeds with the same rate constant even though the donor/acceptor ratio was varied by 7-fold, indicating that transfer occurred via eqs 4 and 5 rather than by direct collisions.

(2) The rate of transfer of retinol from CRBP to vesicles was also measured by monitoring the dissociation of retinol from the protein. Energy transfer between tryptophanyl residues which are located in the vicinity of the binding site on CRBP and bound retinol (Sacchettini et al., 1987) was utilized for this purpose. Thus, excitation of the protein moiety of the retinol–CRBP complex at 290 nm results in energy transfer from the protein to bound retinol and in fluorescence at the emission band of retinol (around 490 nm). When retinol dissociates from the protein, energy transfer ceases with concomitant decrease in fluorescence. The rate of decrease in fluorescence upon mixing of holo-RBP with phospholipid vesicles was used directly to determine the rate of transfer of retinol from the protein to the vesicles. Holo-CRBP was mixed with vesicles, and the fluorescence was monitored at intervals until equilibration was reached. Figure 2 (closed circles) shows the results of a typical time course plotted as a first-order plot. The transfer process monitored by this method also follows single first-order kinetics with a rate constant $k_{off} = 0.45 \pm 0.05$ min^{-1} ($n = 6$).

The slowest step of the transfer process is, most likely, the dissociation of retinol from CRBP. Subsequent steps are diffusion to the bilayer and association with it which are rapid [e.g., see Noy et al. (1985), Storch and Kleinfeld (1986), and Doody et al. (1980)]. The observed rate constant for transfer represents, therefore, the rate constant for dissociation, k_{off}. This conclusion is supported by the close agreement of the rate constants measured by following the rate by which retinol leaves the CRBP-binding site and the rate of arrival at the bilayer. Thus, the measurements described above (Figure 2) show that the half-life for dissociation of retinol from CRBP is 1–1.5 min, indicating that retinol spontaneously and rapidly dissociates from CRBP into the aqueous phase (see Discussion).

Equilibrium Dissociation Constant of the Retinol–RBP Complex. The dissociation constant of the retinol–RBP complex was measured by fluorometric titration utilizing the energy transfer between tryptophanyl residues which are located in the vicinity of the binding site on RBP and bound retinol (Goodman & Leslie, 1972). Analysis of the data obtained from the titration according to eq 2 (see Materials and Methods) is shown in Figure 3. The apparent dissociation constant and the number of binding sites were 0.6 mol of retinol/mol of protein and 20 ($\pm$2) $\times$ 10^{-9} M ($n = 3$). The reported value of K_d for human RBP is 150 $\times$ 10^{-9} M (Cogan et al., 1976; Muhilal & Glover, 1975); the reported value for bovine RBP is 70 $\times$ 10^{-9} M (Noy & Xu, 1990). Thus, the binding affinities of RBP from the different species for retinol show large variations despite the close similarity of their primary structure and the observation that the binding site for retinol appears to be highly conserved in the three species (Sundelin et al., 1985; Berni et al., 1990).

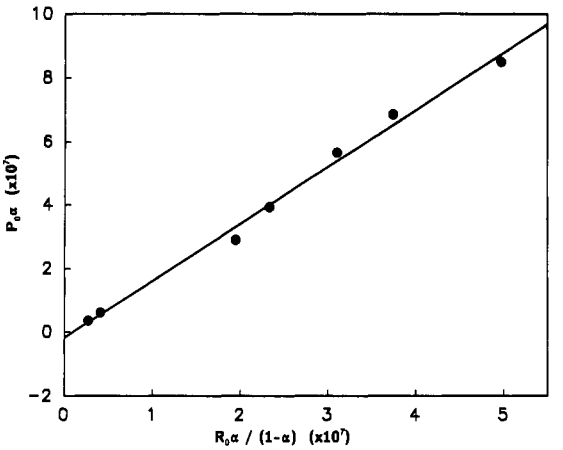

FIGURE 3: Fluorometric titration of apo-RBP with retinol. The titration system consisted of 1 mL of 1.96 μM bovine apo-RBP in 20 mM potassium phosphate, pH 7.0, and 150 mM NaCl. Retinol was added from a solution in ethanol. Most of the data was obtained by using 1–6 L of ethanol. The maximal volume of ethanol added was 17 L. Excitation wavelength, 290 nm; emission wavelength, 480 nm. Corrected data are plotted according to eq 2, $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$ (see Materials and Methods).

Rate of Dissociation of Retinol from RBP. The rate of transfer of retinol from RBP to vesicles was followed by the decrease in energy transfer between RBP and bound retinol (excitation, 290 nm; emission, 490 nm). The data, shown in Figure 4, indicate that transfer of retinol from RBP to vesicles follows single, first-order kinetics with a rate constant of 0.18 $\pm$ 0.03 min^{-1} ($n = 4$). The rate constant did not change over a 7-fold variation in the concentration of vesicles (data not shown). It can be concluded from these data that transfer of retinol from RBP to vesicles, similar to the transfer of retinol from CRBP described above, proceeds via the aqueous phase and that the rate constant for transfer represents the rate constant for dissociation of retinol from this protein.

Rate Constants of Association of Retinol with CRBP and with RBP (k_{on}). These parameters can be calculated from the equilibrium dissociation constants (K_d) and from the rate constants for the dissociation of retinol from the two retinol–protein complexes (k_{off}) by using the relation $K_d = k_{off}/k_{on}$. Direct measurement of k_{on} for bovine RBP has previously shown the validity of calculating this value by the above expression (Noy & Xu, 1990). Thus, the rate constants for the association of retinol with CRBP and with RBP are 4.4 $\times$ 10^7 and 0.9 $\times$ 10^7 min^{-1} M^{-1}, respectively.

Discussion

The data presented above show that the equilibrium dissociation constants of the rat CRBP–retinol and of the rat

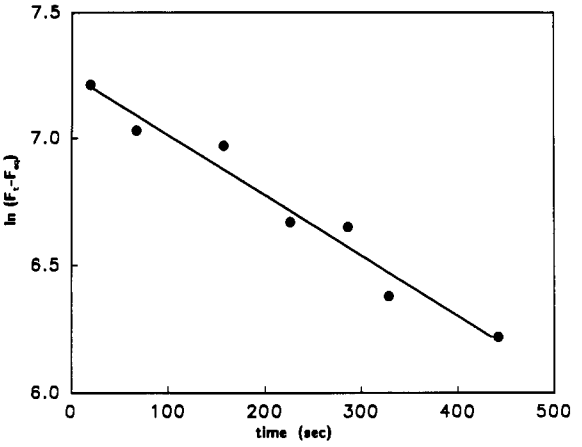

FIGURE 4: Transfer of retinol from RBP to unilamellar vesicles of DOPC. One nanomole of RBP (containing 0.55 nmol of retinol) was mixed with vesicles of DOPC (950 nmol of lipid). Transfer of retinol from protein to vesicles was followed by the decrease in retinol fluorescence (emission, 490 nm) upon excitation of the protein (excitation, 290 nm). Data are plotted as $(F_t - F_e)$ vs time. F_t and F_e denote the fluorescence at time t and at equilibrium, respectively.

RBP–retinol complexes are 13×10^{-9} and 20×10^{-9} M, respectively. It was previously shown that the binding affinity for retinol of the complete serum-binding protein TTR–RBP is 2-fold higher than that of RBP alone (Noy & Xu, 1990). In the circulation, where RBP is complexed to TTR, the dissociation constant for retinol from the TTR–RBP–retinol complex will be approximately 10×10^{-9} M. Thus, the binding affinity of RBP for retinol in the plasma is similar to that of the intracellular CRBP. Measurements of the kinetic parameters of the interactions of retinol with CRBP and with RBP showed that the dissociation of retinol from CRBP was over 3-fold faster than the dissociation from RBP (k_{off}'s were 0.57 and 0.18 min^{-1} for dissociation from CRBP and from RBP, respectively) and that the rate of association of retinol with CRBP was about 5-fold faster than the association of this ligand with the serum protein (k_{on}'s were 4.4×10^7 and 0.9×10^7 M^{-1} min^{-1}, respectively). It seems, then, that CRBP is capable of binding and releasing retinol faster than its serum counterpart. This observation may be rationalized if the function of CRBP is to serve as a storage compartment for retinol in cytosol. Thus, there may be a physiological need for CRBP to respond faster to intracellular "shortage" or "excess" of vitamin A, while the serum RBP serves as an overall buffer for retinol with a corresponding slower response.

The observed rapid rate of release of retinol from CRBP conflicts with recently reported results obtained by using NMR techniques in which the half-life for release of retinol from CRBP expressed in *Escherichia coli* was found to be on the order of hours (Li et al., 1991). The origin of this discrepancy is not clear to us. However, in the present study, retinol was shown to rapidly dissociate from CRBP by two independent methods: by the distribution of radioactively labeled ligand (Figure 1) and by fluorometric measurements (Figure 2). It is possible, therefore, that CRBP, when expressed in *Escherichia coli*, is subtly different from the native protein and possesses different binding characteristics.

It is clear from the above data that retinol spontaneously and rapidly dissociates from the rat CRBP, as well as from the rat RBP. It seems, therefore, in agreement with previous observations concerning the human and the bovine RBP (Fex & Johannesson, 1987; Noy & Xu, 1990) as well as the bovine interphotoreceptor retinol-binding protein (Ho et al., 1989), that the interactions of retinol with these binding proteins are dynamic in the sense that the ligand can freely distribute between the various cellular components with affinity for it. It was previously shown for the bovine RBP, and can now be concluded also for the rat RBP, that the rate of spontaneous dissociation of retinol from this protein is faster than reported rates of uptake of retinol by various cells, indicating that this step is not rate-limiting for uptake [for discussion, see Noy and Xu (1990)]. It can be further concluded from the data in the present paper that the association of retinol with CRBP is also faster than observed uptake rates and does not limit this process. It can be predicted, therefore, that the distribution of retinol between RBP in serum and CRBP in cellular cytoplasm will be at equilibrium.

The ability of CRBP to rapidly bind and release retinol combined with the thermodynamic need to maintain equilibrium provides a basis through which cellular levels of retinol can be modulated. Since serum retinol and RBP levels remain constant except in extremes of vitamin A nutriture or in disease, the above data would predict that the amount of retinol which can be drawn into a cell from the circulating pool of RBP–retinol will depend on the level of apo-CRBP present within the cell. The level of apo-CRBP within the cell can be modulated either by changes in the rates of retinol utilization that will directly influence the level of saturation of CRBP or by modulation of the overall concentration of this protein in cells. Regarding the latter point, tissue and cellular levels of CRBP are, indeed, known to vary in response to alterations in the physiological status of an organism, i.e., as a result of retinol nutritional deficiency (Crow & Ong, 1985; Blaner et al., 1987a), in retinol repletion of retinol nutritional deficiency (Rajan et al., 1990), in response to changes in cellular cAMP levels (Eskild et al., 1988), and during differentiation and development [e.g., see Wei et al. (1989)]. Thus, as cellular needs for retinoids change, cellular retinol uptake may be regulated through changes in the level of CRBP.

The concept that retinol distribution between the cytosolic CRBP-bound pool and the circulating RBP-bound pool is at equilibrium is supported by other data in the literature. The concentration of CRBP in the liver is similar to the concentration of RBP in serum (Kato et al., 1985; Harrison et al., 1987), and since the dissociation constants of the two retinol–protein complexes are also similar, an equilibrium distribution will be achieved when the concentration of retinol in serum is equal to the concentration of CRBP-bound retinol in cytosol. The concentration of CRBP-bound retinol in rat liver has been reported to be about 0.6 g/g of liver (Harrison et al., 1987); taking the volume of 1 g of liver to be 1 mL, the concentration of CRBP-bound retinol can be calculated to be about 2 M, which is similar to the concentration of RBP-bound retinol in serum. These values imply that the distribution of retinol between blood and intracellular cytoplasm, in the liver, is, indeed, at equilibrium. The concentration of retinol that is bound to CRBP inside cells in tissues, other than the liver, has not, to our knowledge, been measured. It remains uncertain, therefore, whether CRBP in the cytoplasm of cells in extrahepatic tissues is also equilibrated with RBP in serum in regard to retinol concentration. The data presented above, however, suggest that this will be so.

Since 1975, it has been repeatedly suggested that cell-surface receptors are important for cellular uptake of retinol (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). These reports have explored a variety of cell types and characterized

binding of ^{125}I-RBP to cell surfaces or isolated plasma membrane preparations [see Blaner (1989) for a review]. However, no study reporting the purification of the putative RBP receptor has appeared since 1975, and none of the published studies regarding the putative RBP receptor have provided insight into the distribution of the receptor in tissues and cells throughout the body. Moreover, the specific role that the putative receptor is postulated to play in the process of uptake of retinol into cells has not been specified. Thus, much biochemical and physiological data regarding such a receptor are still lacking. In contrast, one recent report has indicated that ^{125}I-RBP binding could not be detected in primary cultures of mouse keratinocytes (Creek et al., 1989). Studies of the spontaneous transfer of retinol between RBP and membrane fractions have provided direct evidence that cell-surface receptors for RBP would not be required for cells to take up retinol from the retinol–RBP complex (Fex & Johannesson, 1987; Noy & Xu, 1990). The data in the present report support this conclusion. The data suggest that no step in the process of uptake of retinol by cells requires facilitation and that, in fact, the regulation of uptake is based on thermodynamics, rather than on kinetic parameters; i.e., a sensitive and physiologically responsive regulatory mechanism for uptake of retinol can be understood based on the equilibrium distribution of retinol between retinol-binding proteins outside and inside the cell.

Another implication of the results of the present study relates to the mechanism of intracellular distribution of retinol. The observed rapid dissociation of the CRBP–retinol complex indicates that CRBP-bound retinol will be equilibrated with unbound retinol inside cells. The molecular weight of the retinol–CRBP complex is about 50-fold higher than that of unbound retinol, so that the diffusion constant of the former will be about 10-fold smaller than the diffusion constant of the later (Tanford, 1961). The concentration of unbound retinol in cytosol [calculated from the K_d of the CRBP–retinol complex obtained in the present work and the concentration of this complex in the liver (Harrison et al., 1987)] is about 0.2 M, or about 10-fold lower than the concentration of the bound form. It follows from these values that, in cytosol, unbound retinol will diffuse to its target sites at a rate that is at least comparable to the rate of diffusion of the bound form and that the pool of retinol that is unbound can play an important role in the process of transfer of this vitamin to intracellular sites. It would be difficult, therefore, to rationalize how CRBP serves as a specific delivery vehicle for retinol except in systems (enzymes, gene-regulating sites, etc.) that require the presence of the retinol–protein complex, and not of unbound retinol, for activity, i.e., when the retinol–protein complex rather than unbound retinol is the substrate. The enzyme LRAT which catalyzes the formation of retinyl esters is an example of a system of this type. Thus, it has been shown that this enzyme requires the complex CRBP–retinol, and not unbound retinol, as its substrate (Ong et al., 1987, 1988; Saari & Bredberg, 1988; Yost et al., 1988; Shingleton et al., 1989 a,b). Another such system has been reported to exist in retinal pigment epithelium where it was shown that an enzyme involved in retinoid metabolism, 11-*cis*-retinol dehydrogenase, utilizes 11-*cis*-retinal bound to cellular retinal-binding protein as its substrate (Saari & Bredberg, 1982). The product of the reduction reaction catalyzed by this enzyme, 11-*cis*-retinol, remains complexed with the binding protein following the reaction (Saari & Bredberg, 1982), indicating that unbound retinoids do not participate in the enzyme-catalyzed reaction. Thus, CRBP, in addition to being essential for regulating the uptake of retinol by cells, plays an important role in the metabolism of this ligand.

In summary, the data presented here provide the physical–chemical basis from which the uptake of retinol into cells can be understood. Uptake, under normal conditions, where serum retinol and RBP levels are constant, will depend on cellular levels of apo-CRBP. As retinol is removed from CRBP by metabolism, cellular levels of apo-CRBP will increase, thus drawing retinol into cells to reestablish equilibrium. As cellular needs for retinoids change (e.g., during differentiation and development), the amount of retinol taken up by cells will be modulated through changes in CRBP levels. The data would suggest that cellular CRBP levels essentially determine cellular ability to take up retinol from the circulation. This conclusion is consistent with the observation that CRBP levels vary greatly between different tissues (Crow & Ong, 1987; Blaner et al., 1987a) and different cells within tissues (Blaner et al., 1985, 1987b). This variability has been interpreted to represent differing tissue and cellular needs for retinoids (Crow & Ong, 1985; Blaner et al., 1987a; Rajan et al., 1990). It seems now that cellular CRBP levels not only indicate retinoid need but also actively satisfy this need by participating in and by regulating retinol uptake into cells.

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Studies of the Rate-Limiting Step in the Tyrosine Hydroxylase Reaction: Alternate Substrates, Solvent Isotope Effects, and Transition-State Analogues†

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ABSTRACT: Tyrosine hydroxylase catalyzes the formation of dihydroxyphenylalanine from tyrosine, utilizing a tetrahydropterin and molecular oxygen as cosubstrates. Several approaches were taken to examining the identity of the rate-limiting step in catalysis. Steady-state kinetic parameters were determined with a series of ring-substituted phenylalanines. The V_{max} value was unchanged with substrates ranging in reactivity from tyrosine to 4-fluorophenylalanine. Neither 4-pyridylalanine *N*-oxide, a model of tyrosine phenoxide, nor 4-hydroxy-3-pyridylalanine *N*-oxide or α-amino-3-hydroxy-4-pyridone-1-propionic acid, models of a hydroxycyclohexadienone intermediate, was an effective inhibitor. There was no solvent isotope effect on either the V_{max} or the V/K_{Tyr} value. These results establish that no chemistry occurs at the amino acid in the rate-limiting step and no exchangeable proton is in flight in the rate-limiting step. The results are consistent with a model in which the slow step in catalysis is formation of the hydroxylating intermediate.

The enzyme tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of the catecholamine neurotransmitters, the hydroxylation of tyrosine to form dihydroxyphenylalanine (Kaufman & Kaufman, 1985) (Scheme I). This enzyme is one of several poorly understood aromatic amino acid hydroxylases which utilize tetrahydropterins as substrates and contain iron in the active site; phenylalanine hydroxylase and tryptophan hydroxylase are other members of this group (Benkovic, 1980; Shiman, 1985). The actual mechanism of hydroxylation by these enzymes and even the identity of the hydroxylating intermediate are unknown. The pterin product released from the enzyme during catalysis has been shown to be a 4a-hydroxytetrahydropterin for both tyrosine hydroxylase

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