The Enhancement of Fluorescence and the Decreased Susceptibility to Enzymatic Oxidation of Retinol Complexed with Bovine Serum Albumin, β-Lactoglobulin, and the Retinol-binding Protein of Human Plasma*

SIDNEY FUTTERMAN AND JORAM HELLER

From the Department of Ophthalmology, University of Washington School of Medicine, Seattle, Washington 98195, and The Jules Stein Eye Institute, U.C.L.A. Center for the Health Sciences, Los Angeles, California 90024

SUMMARY

The fluorescence yield of bound retinol in human retinol-binding protein (RBP) and its prealbumin complex (PA-RBP) were found to be nearly the same and to exceed that of free retinol in petroleum ether by about 9-fold.

Bovine serum albumin and β-lactoglobulin also formed strongly fluorescent water-soluble complexes with retinol. The fluorescence yield of retinol complexed with bovine serum albumin was about 4-fold and with β-lactoglobulin about 3-fold larger than retinol in petroleum ether. In the retinol-bovine serum albumin complex the molar ratio of retinol to protein was found to be approximately 1.

The greater degree of fluorescence enhancement, the greater stability of retinol in RBP and PA-RBP to spontaneous degradation at room temperature, the relative resistance to extraction of the bound retinol by ether, and the complete unavailability of the retinol in RBP and PA-RBP for oxidation by liver alcohol dehydrogenase, in contrast to the retinol in complexes formed with either bovine serum albumin or β-lactoglobulin, were interpreted as indicating that retinol is bound with greater affinity to the natural carrier protein than it is to bovine serum albumin or β-lactoglobulin.

Retinol was extracted from dry preparations of RBP and PA-RBP with ethanol in the cold, yielding apoprotein preparations that were able to rebind retinol, regenerating about 90% of their original fluorescence with the bound retinol becoming again completely unreactive toward liver alcohol dehydrogenase.

It has recently been established that unesterified retinol released from the liver stores of retinyl esters is transported in plasma in combination with a specific binding protein (mol wt 21,000) which is itself tightly bound in a 1:1 molar ratio to prealbumin (mol wt 64,000) (1-3). RBP* was found to contain 1 mole of retinol per mole of protein and the molar absorptivity of the bound retinol at 330 nm did not differ from that of free retinol in nonpolar solvents (1, 2).

The fluorescence properties of RBP and PA-RBP have recently been recorded and analyzed in some detail (4), but have not as yet been compared directly either with free retinol in a hydrocarbon solvent or with retinol in the presence of other proteins in aqueous solution. In the present study such comparisons have been made. Evidence is presented that proteins other than RBP can bind retinol and thereby enhance its fluorescence, but in contrast to RBP, they do not appear to render it unreactive with liver alcohol dehydrogenase.

EXPERIMENTAL PROCEDURE

Materials—All-trans-retinal was purchased from Eastman Kodak; nanograde petroleum ether from Mallinckrodt; bovine serum albumin, crystalline, from Pentex; β-lactoglobulin, three times crystallized, from Xana; liver alcohol dehydrogenase, lyophilized (2 units per mg), from Worthington; lactic dehydrogenase, Type III (570 units per mg), α-chymotrypsin, Type II, trypsin, Type III, sodium pyruvate, and DPN from Sigma.

Preparation of RBP and PA-RBP—The method of Petersen (2) was used to prepare RBP and PA-RBP from 5.8 liters of pooled normal human serum. The A₃₄₀:A₃₃₀ ratios obtained for the preparations of RBP and PA-RBP were 1.09 and 2.32, respectively. The RBP moved as a single, sharply defined band on electrophoresis in cellulose acetate membranes in barbital buffer, pH 8.9, and showed a single precipitin line when examined by immunodiffusion (2) using antiserum prepared by injecting rabbits with RBP. Concentrations of RBP and PA-RBP were determined from absorption measurements at 330 nm using ε₃₃₀ = 46,000 or by the method of Lowry et al. (5) using bovine serum albumin as a standard.

The abbreviations used are: RBP, retinol-binding protein; PA-RBP, prealbumin-retinol-binding protein complex.

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Preparation of Retinol—A 5.0-mg sample of all-trans-retinal was dissolved in 2 ml of isopropyl alcohol and reduced quantitatively to retinol with excess sodium borohydride, approximately 100 mg, by warming to 40° and stirring the mixture for 2 to 3 min until it became essentially colorless. After dilution to 10 ml with isopropyl alcohol and brief centrifugation to remove the insoluble excess borohydride, suitable further dilutions were made in isopropyl alcohol. These solutions were immediately chilled and used within 1 hour after preparation. An εs0 of 46,000 was observed for retinol in isopropyl alcohol. Commercial reagent grades of isopropyl alcohol did not contain significant amounts of fluorescent contaminants. When solutions of retinol were added to aqueous reaction mixtures, the isopropyl alcohol concentration did not exceed 2.5% by volume and was insufficient to hinder the oxidation of retinol by alcohol dehydrogenase. Identical results were obtained when retinol prepared by borohydride reduction in 2 ml of isopropyl alcohol was extracted by mixing successively with 5 ml of petroleum ether and 5 ml of water, recovered from the upper phase by removing the solvent under a stream of nitrogen, and dissolved promptly in isopropyl alcohol for addition to reaction mixtures or in petroleum ether for fluorescence measurements.

Extraction of Retinol from PA-RBP and RBP—A 0.5-ml portion (75 nmoles) of PA-RBP was concentrated to dryness in a Corex centrifuge tube using a vacuum pump and warming the solution in a water bath at 37°. The dry residue was extracted for 2 min with 10 ml of cold ethanol at 40° using a stirring rod to suspend the sediment. After sedimenting for 1 min at 8000 × g in a Sorvall centrifuge, the supernatant fluid containing retinol was decanted. The sediment was promptly dried on the vacuum pump and dissolved in 18 ml of solution containing 70 mM KCl and 30 mM sodium phosphate buffer, pH 7.4.

The extraction of retinol from RBP was carried out similarly after first bringing the concentration of the solution to 0.5 M with respect to NaCl.

Fluorescence—Fluorescence measurements were routinely made using an Amino-Bowman spectrophotofluorometer with excitation at 330 nm, emission at 470 nm, and slit widths, respectively, of 2, 3, 3, 3, 3, and 2 nm. Fluorescence spectra were obtained with solutions in which λmax was not greater than 0.06 to minimize internal filter effects. Spectra were corrected as described by White et al. (8). Quantum yields of fluorescence were determined as described by Parker and Rees (7) using a value of 0.55 for the quantum yield of fluorescence of quinine sulfate (7).

RESULTS

Effect of Proteins on Retinol Fluorescence—The intensities of fluorescence for retinol in RBP and PA-RBP were found in preliminary experiments to be linearly related to concentration for concentrations up to 1.5 nmoles per ml, nearly equal in magnitude and approximately an order of magnitude greater than the fluorescence of free retinol in petroleum ether. To determine whether enhancement of retinol fluorescence was a property unique to the specific binding protein, the fluorescence of retinol was examined in aqueous solution in the presence of several other proteins (Fig. 1). When retinol was added to aqueous solutions of bovine serum albumin or β-lactoglobulin, there was a rapid increase in fluorescence with maximum fluorescence intensity being reached in less than 1 min. With retinol present in low concentration, the fluorescence intensity appeared to be nearly the same in the presence of bovine serum albumin and β-lactoglobulin and about 2½ fold higher than the fluorescence intensity obtained with an equivalent concentration of retinol in petroleum ether. Maximum fluorescence was obtained when the molar ratio of retinol to bovine serum albumin was approximately 1.

In contrast, the fluorescence of retinol dispersed in aqueous solutions of trypsin or α-chymotrypsin (Fig. 1) was reduced below that observed for retinol in petroleum ether and was comparable in intensity to the fluorescence of retinol in a predominantly aqueous solution without added protein, as represented by retinol in 20% isopropyl alcohol.

Evidence for Retinol Binding to Bovine Serum Albumin—Although the data obtained with bovine serum albumin and β-lactoglobulin could be interpreted as representing binding curves, it was apparent that the concentrations of retinol which saturated the proteins also showed self-quenching when retinol was dissolved in petroleum ether.

To obtain additional information about the interaction of retinol with protein, retinol was incubated with increasing concentrations of bovine serum albumin (Fig. 2). For each concentration of retinol, fluorescence increased with increasing concentration of bovine serum albumin until the molar ratio of protein to retinol was approximately 1. Furthermore, when a reaction mixture containing bovine serum albumin and excess retinol was subjected to gel filtration (Fig. 3), 19% of the retinol remained bound to the protein and was clearly separated from fluorescent material, presumably degradation products of retinol, that was retarded and appeared in the “small molecule” fraction.

Fluorescence Spectra of Retinol-Protein Complexes—The corrected emission spectra of RBP and PA-RBP were both blue-shifted with respect to free retinol in accord with the earlier observations of Peterson and Rask (3). No such shift was observed in the emission spectra of retinol-bovine serum albumin or retinol-β-lactoglobulin complexes (Fig. 4).

The corrected excitation spectra of RBP and PA-RBP (Fig. 5) showed the effective energy transfer from protein to retinol de-
FIG. 2. Effect of concentration of bovine serum albumin on enhancement of fluorescence of retinol. All-trans-retinol, 8 nmoles (○—○) or 16 nmoles (△—△), was added to solutions containing 70 mm KCl, 30 mm phosphate buffer, pH 7.4, and bovine serum albumin as indicated in a final volume of 2.0 ml.

FIG. 3. Binding of retinol to serum albumin. One milliliter of solution containing 0.15 mM bovine serum albumin, 1.0 mM all-trans-retinol, 30 mM phosphate buffer, pH 7.4, and 70 mM KCl was applied to a column (1.5 × 90 cm) of Sephadex G-100 previously equilibrated in 0.02 M Tris-chloride buffer, pH 8.0, at 4°C. Elution was carried out using the same Tris-chloride buffer with a flow rate of 6.4 ml per hour. Fractions of 1.6 ml were collected and $A_{280}$ and fluorescence intensity were measured.

described by Peterson and Rask (3) whereas only relatively weak or absent energy transfer was observed in retinol-protein complexes formed from bovine serum albumin and β-lactoglobulin. In addition, the latter spectra showed a shift toward longer wavelengths. A similar shift of approximately 6 nm to the red with respect to free retinol was also observed in the absorption spectrum of the retinol-bovine serum albumin complex.

Fluorescence Quantum Yields—The fluorescence quantum yields of retinol in RBP and PA-RBP were practically identical and about 8.5 times larger than the fluorescence quantum yield for retinol in petroleum ether (Table I). The fluorescence quantum yield for retinol complexed with bovine serum albumin and with β-lactoglobulin were only about half that of retinol in RBP and PA-RBP, although still considerably higher than that for retinol in petroleum ether.

Reactivity of Retinal-Protein Complexes with Liver Alcohol De...

TABLE I

Fluorescence quantum yields of retinol

Fluorescence quantum yields were calculated from the corrected emission spectra (Fig. 4) as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Quantum yield</th>
<th>All-trans-retinol</th>
<th>RBP</th>
<th>PA-RBP</th>
<th>Retinol-serum albumin complex</th>
<th>Retinol-β-lactoglobulin complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute</td>
<td>0.099</td>
<td>0.079</td>
<td>0.077</td>
<td>0.067</td>
<td>0.091</td>
</tr>
<tr>
<td>Relative</td>
<td>1.0</td>
<td>8.8</td>
<td>8.5</td>
<td>4.1</td>
<td>3.5</td>
</tr>
</tbody>
</table>
of the bound retinol.

of the yellow color of retinol.

globulin, with loss of fluorescence accompanied by the appearance complex and more slowly when the retinol was bound to fl-lactoglobulin. Retinol-protein complexes were formed in solutions (2.0 ml) containing 1 mg of either fl-lactoglobulin (m---m) or bovine serum albumin (BSA) (d---d), 5.3 nmoles of all-trans-retinol, 140 μmoles of KCl, and 60 μmoles of phosphate buffer, pH 7.4. The complexes were then incubated at room temperature in a final volume of 2.3 ml after supplementation, where indicated "ADH," with DPN, pyruvate, lactic dehydrogenase, and alcohol dehydrogenase as above. BLG, fl-lactoglobulin.

hydrogenase—RBP and PA-RBP appeared to be stable in aqueous solution at room temperature. No decrease in fluorescence intensity was observed with time (Fig. 6). The retinol bound in RBP and PA-RBP was completely unavailable for oxidation by DPN in the presence of liver alcohol dehydrogenase. RBP and PA-RBP themselves were not inhibitory to the activity of the alcohol dehydrogenase because addition of free retinol to the incubation medium containing RBP or PA-RBP and the dehydrogenase led to prompt oxidation of the retinol.

Retinol complexes of bovine serum albumin and fl-lactoglobulin were not entirely stable at room temperature (Fig. 9). The fluorescence intensity of these complexes decreased slowly both in the dark and under the usual conditions of subdued room lighting. The bound retinol was readily oxidized in the presence of DPN and liver alcohol dehydrogenase. Enzymatic oxidation occurred rapidly in the case of the retinol-bovine serum albumin complex and more slowly when the retinol was bound to fl-lactoglobulin, with loss of fluorescence accompanied by the appearance of the yellow color of retinol.

Extraction of RBP and PA-RBP with Ethanol and Rebinding of Retinol to Apo-RBP and Apo-PA-RBP—Solutions of RBP and PA-RBP did not undergo any increase in fluorescence in the presence of added retinol (Fig. 7) indicating that functional binding sites on the protein were already saturated with retinol.

Extraction of lyophilized RBP or PA-RBP with petroleum ether or diethyl ether resulted in a loss of less than 5% of the fluorescence of the proteins and recovery of trace amounts of retinol in the organic phase. In contrast, extraction of lyophilized preparations of the retinol-bovine serum albumin or retinol-fl-lactoglobulin complexes with ether removed 50 to 75% of the bound retinol.

Extraction with ethanol in the cold removed over 90% of the bound retinol from RBP and PA-RBP as judged by the loss of fluorescence of the proteins and recovery of retinol in the ethanol. When the apoproteins were redissolved in aqueous solution and freshly prepared retinol was added, a rapid reconstitution of the complexes, RBP, and PA-RBP, occurred as indicated by the recovery of fluorescence (Fig. 7). The rebinding of retinol to the apoproteins was essentially complete in the 15 to 20 s that elapsed before a measurement could be taken after the addition of retinol. Saturation of the fluorescence enhancement was reached with a ratio of retinol to apoprotein of approximately 1. The fluorescence intensity attained on rebinding was within 90% of that observed for the unextracted control. The regenerated complexes were found to be just as unreactive toward DPN and liver alcohol dehydrogenase as were the native RBP and PA-RBP.

In most of the reconstitution studies all-trans-retinol was employed. Since the other geometrical isomers were found to display a considerably lower fluorescence yield, in accord with published data (8), and in the reconstitution experiments the recovery of fluorescence using the all-trans isomer was about 90%, it is possible to draw the tentative conclusion that the isomer present in the native carrier is all-trans-retinol.

DISCUSSION

The fluorescence intensity of retinol in solution is known to be greater in hydrocarbons than in highly polar solvents (8, 9). In water containing only enough polar organic solvent to hold the retinol in solution, retinol fluorescence was extremely weak. It was, therefore, surprising that the fluorescence of retinol in RBP was approximately an order of magnitude more intense than the fluorescence of free retinol in petroleum ether, since the fluorescence intensity of retinol in petroleum ether ranks among the higher values recorded for retinol in various solvents (8, 9). Presumably, RBP possesses a nonpolar binding region in which the retinol is buried. In addition to providing a nonpolar environment, the binding of retinol to a large molecule might also be expected to restrict the mobility of the retinol, thereby reducing
somewhat the nonradiative decay of energy following excitation and consequently enhancing fluorescence. This interpretation is supported by the occurrence of the large blue shift in the emission spectra of RBP and PA-RBP (4), evidence (10) that a chromophore is present in a relatively nonpolar environment.

This study provides evidence that the capacity to form a fluorescent complex with retinol in aqueous solution is a property that is not unique to the protein of the carrier for retinol in blood, RBP, but is also displayed by bovine serum albumin and β-lactoglobulin among the four other proteins examined. The binding of retinol to bovine serum albumin adds 1 more small molecule to the multitude of diverse substances of low molecular weight that bind to this protein (11, 12). Although no detectable retinol is bound to serum albumin in vivo, the possibility exists that this protein could serve as an auxiliary carrier if excess free retinol were introduced into the circulation.

Free retinol in aqueous solution or in organic solvents is markedly unstable, decaying within minutes to a few hours, depending on the solvent, temperature, light, and other factors, to a complex mixture of products. On the other hand, retinol in purified preparations of RBP and PA-RBP seems to be stable by comparison with free retinol as judged by spectral and fluorescence criteria, and probably decays only after protein denaturation.

The greater resistance to extraction with ether and the lack of reactivity toward alcohol dehydrogenase of retinol in PA-RBP and RBP, taken together, support the view that retinol is bound with greater affinity in the natural carrier protein than it is in the artificial complexes formed using bovine serum albumin and β-lactoglobulin. The physiological mechanism by which retinol is removed from its tightly bound state in PA-RBP remains to be elucidated.

The extraction of dry RBP and PA-RBP with ethanol in the cold was found to be a convenient way to obtain apo-RBP and apo-PA-RBP suitable for studies on the rebinding of retinol. The almost complete recovery of fluorescence and the characteristic lack of reactivity of retinol in the reconstituted holo-RBP and holo-PA-RBP toward alcohol dehydrogenase suggest that the protein survives the extraction procedure in a state sufficiently native to enable binding studies to be carried out.

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REFERENCES

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Sidney Futterman and Joram Heller


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