Studies on the Fluorescence of the Human Vitamin A-transporting Plasma Protein Complex and Its Individual Components*

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SUMMARY

The fluorescence properties of the human prealbumin-retinol-binding protein (RBP) complex and of its individual components are described. At neutral pH, RBP and the protein complex have two fluorescence bands: one at 335 nm and the other, associated with the retinol, at 470 nm. Prealbumin has only a fluorescence at 335 nm (quantum yield 0.11). The quantum yield of retinol increased by 50% and the emission spectrum was blue shifted on RBP forming a complex with prealbumin. RBP, at low ionic strength where the binding of RBP and prealbumin is abolished, exhibited a decrease in the quantum yield of retinol.

With the protein fluorescence of RBP and the fluorescence of its cofactor, it was also possible to show the occurrence of transfer of excitation energy from the protein moiety to the retinol (efficiency of transfer: 60%). Studies on the accessibility of the aqueous medium (or protons) to the retinol site revealed that on complex formation between RBP and prealbumin most of the retinol-solvent interactions are abolished, suggesting that one of the functions of prealbumin is to stabilize the retinol-binding site of RBP.

The estimated apparent association constant is $2 \times 10^7$ M$^{-1}$ for the interaction of retinol-containing RBP and prealbumin. The binding constant of prealbumin and vitamin A-free RBP was also determined. A similar value was found and it is thus concluded that retinol has no major influence on the tertiary structure of RBP.

EXPERIMENTAL PROCEDURE

Materials

Proteins—Prealbumin was obtained as described elsewhere (13). The prealbumin-RBP complex was isolated according to a published procedure (10). As previously shown, the highly purified preparations of prealbumin and the prealbumin-RBP complex are virtually devoid of thyroxine (14). RBP was isolated from the prealbumin-RBP complex (10) or from urine of patients with tubular proteinuria (9) by means of affinity chromatography on a prealbumin-coupled Sepharose column (15). To obtain RBP devoid of vitamin A, protein samples were subjected to extraction with heptane as described elsewhere (16). After the heptane extraction RBP was passed over the prealbumin-coupled Sepharose column and only protein retained on the column was used in the studies. Protein concentrations were determined spectrophotometrically and related to the appropriate molar extinction coefficients (10).

Other Materials—D$_2$O (99.8%, w/w) was obtained from Norsk Hydro-Elektrisk. Urea (Mallinckrodt) was recrystallized prior to use. All other chemicals were the best available grade from commercial sources.

Methods

Fluorescence and Absorption Measurements—Most fluorescence measurements were carried out with use of an Aminco-Bowman Spectrophotofluorometer (1). The fluorescence of which gives information about the structure of its environment in the protein.

The human retinol-binding protein (7-10) is strongly fluorescent due to its content of retinol. The retinol molecule is probably tightly bound at a unique site in the protein (11, 12), which offers a variety of possible uses of fluorescence as a tool in the study of conformational changes in this protein. Such changes are of particular interest in the case of RBP in view of the fact that under physiological conditions it forms a stable protein-protein complex with the thyroxine-binding prealbumin (7, 8, 10).

In this study, the various types of fluorescence emission and excitation spectra in RBP, prealbumin, and the prealbumin-RBP complex have been measured. Additionally, the fluorescence quenching has been investigated for the retinol-protein and protein-protein interactions pertaining to this system.
spectrofluorometer, but measurements of some emission spectra were performed on a Zeiss FM 4 C spectrofluorometer. All spectra recorded were corrected for instrumental variations (17). The validity of the obtained correction factors was tested by comparing the corrected spectra of NADH (18).

Unless otherwise stated, all fluorescence measurements were performed at ambient temperature (23 ± 2°C) in a buffer composed of Tris-HCl (0.02 M) and NaCl (0.15 M) adjusted to pH 8.0. In experiments performed at low ionic strength 0.002 M Tris-HCl buffer, pH 8.0, was used. Quantum yields were determined with quinine sulfate in 0.1 N H2SO4 as a reference substance and taking 0.55 as its quantum yield (19, 20). Samples used for fluorescence measurements always had an optical density below 0.08 at the exciting wave length.

The efficiency of energy transfer (T) was calculated by either one of the following methods (2): (a) from the quantum yield of the donor (the protein moiety) in the presence or in the absence of the acceptor (retinol), Qd, and Q0, respectively:

\[ T = 1 - \frac{Q_d}{Q_0} \tag{1} \]

(b) from the amount of fluorescence of the acceptor at 470 nm by excitation at the donor and the acceptor absorption maxima.

\[ T = \frac{F_d}{F_a} \cdot \frac{A_A}{A_D} \tag{2} \]

where \( F_d \) is the fluorescence intensity at 470 nm by excitation at 280 nm (\( \lambda_d \)); \( F_a \) is the fluorescence intensity at 470 nm by excitation at 330 nm (\( \lambda_a \)); \( A_A \) and \( A_D \) are the optical densities of the solutions at \( \lambda_A \) and \( \lambda_D \), respectively. This relation is only valid if the extinctions are very low. However, Equation 2 can be modified and its validity extended to solutions with high absorption (21).

Absorption measurements were recorded with a Beckman DBG spectrophotometer. In both fluorescence and absorption measurements blanks were run to correct for base-line variations with wave length.

**RESULTS**

**Fluorescence Excitation and Emission of Prealbumin, RBP, and Prealbumin-RBP Complex**—RBP has in addition to its absorption band at 280 nm a band with maximum at 330 nm due to the presence of retinol. Upon excitation of the protein at these two wave lengths, two types of fluorescence can be obtained: one with maximum at 335 nm caused by the protein, and the other at 470 nm caused by the vitamin. By excitation at 280 nm prealbumin only has a protein fluorescence at 335 nm, whereas the prealbumin-RBP complex exhibits a fluorescence emission spectrum qualitatively very similar to that of RBP. Fig. 1A shows the emission spectra of prealbumin at physiological and low ionic strength, respectively. The differences in environment obviously caused no alterations of the spectra. Fig. 1B shows the fluorescence emission spectra of RBP subjected to buffers of physiological and low ionic strengths, respectively. It can be seen that RBP under these conditions has two fluorescence maxima: at 335 nm and 470 nm. The protein fluorescence at 335 nm for RBP at physiological ionic strength is somewhat smaller in magnitude than that caused by retinol at 470 nm. The relative magnitude of the fluorescence maxima is, however, reversed for the sample of RBP subjected to an environment of low ionic strength. RBP devoid of vitamin A has a protein fluorescence which is about twice as intense as the corresponding fluorescence for the retinol-containing species. As will be shown below, there is transfer of excitation energy from the protein moiety to retinol which can account for the lowered emission at 335 nm for the RBP with retinol attached to it.

The prealbumin-RBP complex exhibits the same two types of

![Fig. 1](image-url)
Fluorescence of Vitamin A-transporting Protein Complex

FIG. 2. Emission spectra of RBP (---) and the prealbumin-RBP complex (—). The spectra were recorded with excitation at 330 nm, and the optical densities at this wave length were 0.05 and 0.03, respectively.

FIG. 3. Excitation spectra of the 335-nm fluorescence of retinol-containing RBP at physiological (----) and low (— — —) ionic strength, and of RBP devoid of retinol (——) at physiological ionic strength. The optical densities were in each case 0.08 at 280 nm. The spectrum of the retinol-free RBP was recorded with an instrument sensitivity half of that used for the two other spectra.

FIG. 4. Excitation spectra of the 470-nm fluorescence of RBP (-----) and prealbumin-RBP complex (—) at physiological ionic strength. The optical densities were in each case 0.08 at 280 nm and 0.08 and 0.03 at 330 nm, respectively.

fluorescence as RBP (Fig. 1C) but the magnitude of the protein fluorescence relative to that of retinol has increased considerably. Upon excitation of RBP and of the prealbumin-RBP complex at 330 nm the only fluorescence obtained is that of retinol. It is, however, evident from Fig. 2 that this fluorescence exhibits a blue shift of approximately 5 nm on formation of the prealbumin-RBP complex.

The fluorescence excitation spectra of retinol-containing RBP at two different ionic strengths and of RBP devoid of the vitamin are shown in Fig. 3. The maximum at 280 nm coincides with the absorption maximum for the protein, and the relative intensities of the excitation at this wave length are compatible with retinol quenching the tryptophan emission.

The excitation spectra of RBP and of the prealbumin RBP complex measured at an emission wave length of 470 nm show two maxima (Fig. 4). These maxima at 280 nm and at 330 nm coincide with the absorption maxima of the proteins. The difference recorded at 280 nm between RBP and the prealbumin-RBP complex is consistent with energy transfer from prealbumin to the retinol moiety (see below).

Measurements of Quantum Yields—Table I summarizes the values obtained for the quantum yields. It is obvious that the protein fluorescence of RBP containing retinol has a considerably lower quantum yield than the species devoid of the vitamin. It is also interesting to note that the quantum yield of the RBP protein fluorescence increases somewhat on lowering the ionic strength, whereas the quantum yield of the retinol fluorescence decreases under the same conditions (cf. Table I). The prealbumin-RBP complex exhibits a decreased quantum yield of the protein fluorescence (0.06) compared to the value theoretically computed from the quantum yields of the individual protein (0.09). It is evident from the table that the quantum yield of
TABLE I
Quantum yields of various types of fluorescence found in prealbumin, RBP, and prealbumin-RBP complex

Values for quantum yields given are the average of five determinations with different protein preparations.

| Protein                    | Maximum Excitation | Maximum Emission | Quantum yield |
|----------------------------|---------------------|------------------|---------------|
| RBP-retinolbc              | 280                 | 335              | 0.04          |
| RBP-retinolbc              | 280                 | 335              | 0.05          |
| Prealbumin                 | 280                 | 335              | 0.11          |
| Prealbumin-RBPc           | 280                 | 335              | 0.06          |
| RBP-retinolbc             | 330                 | 470              | 0.04          |
| RBP-retinolbc             | 330                 | 470              | 0.03          |
| Prealbumin-RBPc           | 330                 | 470              | 0.06          |

a RBP-retinol denotes the vitamin-protein complex in a 1:1 molar ratio.
b Determined at physiological ionic strength.
c Determined at low ionic strength.
d RBP, devoid of retinol.
e The complex contained prealbumin, RBP, and retinol in a 1:1:1 molar ratio.

retinol for the protein complex is approximately 50% higher than for RBP.

Energy Transfer—When RBP is excited at 280 nm it fluoresces not only at 335 nm but also at 470 nm, whereas in RBP devoid of retinol only the fluorescence with maximum at 335 nm is encountered (Fig. 1B). Similarly, the excitation spectrum of the 470-nm fluorescence gives rise to a distinct maximum at 280 nm (Fig. 4). These findings, together with the fact that the vitamin A-free form of the protein has a higher quantum yield in its protein fluorescence (Table I) although both proteins have very similar absorbance indices at 280 nm, suggest the occurrence of energy transfer from the protein moiety to retinol.

Assuming that the difference in quantum yield between RBP containing and devoid of retinol is the result only of the occurrence of resonance energy transfer, a transfer efficiency of approximately 60% would be expected (Equation 1). Indeed, this is the value found by calculating the transfer efficiency from a comparison of the retinol fluorescence obtained by excitation at the donor and the acceptor maxima (Equation 2 and Reference 21). The transfer thus calculated was found to be independent of the protein concentration (Fig. 5), suggesting an intramolecular mechanism of energy transfer from the protein moiety to retinol. Furthermore, the donor emission spectrum exhibits the same shape with and without retinol (Fig. 1B) which is a requirement for resonance energy transfer (2).

It is evident from Fig. 1C that the prealbumin-RBP complex exhibits sensitized fluorescence at 470 nm and from Fig. 4 that the excitation spectrum gives rise to a distinct maximum at 280 nm. However, a transfer efficiency of 33% would be expected on account of the quantum yield measurements whereas the transfer efficiency calculated from Equation 2 gives a value of only 20%. It is thus not conceivable to ascribe the protein fluorescence quenching of the prealbumin-RBP complex to resonance energy transfer only, but other types of processes seem to be involved.

Fig. 5. Effect of protein concentration on the efficiency of energy transfer in RBP. The transfer efficiency was calculated as described in Reference 21.

Fig. 6. Temperature and isotope effects on the retinol fluorescence of the prealbumin-RBP complex (A) and RBP (B). The proteins were dissolved in the standard buffer which was prepared with H2O (■ and ○) or D2O (□ and ○). The quantum yields (4) were measured with an excitation at 330 nm. T denotes absolute temperature.

Temperature and Isotope Effects on Retinol Fluorescence of RBP and Prealbumin-RBP Complex—Increasing the temperature from 6 to 40° brings about a decrease in the quantum yield of the retinol fluorescence both for RBP and for the prealbumin-RBP complex (Fig. 6). From the temperature dependence of the quantum yield it was found that the energy of activation of the quenching process is about 2.8 kcal per mole for RBP and about 1.9 kcal per mole for the prealbumin-RBP complex (24). By measurements of the quantum yield in D2O it was found that the energy of activation is similar both for RBP and for the prealbumin-RBP complex (2.8 and 1.9 kcal per mole, respectively). The isotope effect, although small, was significantly different for
Effect on Protein and Retinol Fluorescence of Prealbumin and RBP Complex Formation—The prealbumin fluorescence at 335 nm undergoes dramatic changes with addition of RBP containing retinol. Fig. 7 shows the progressive quenching of the prealbumin fluorescence on increments of the molar ratios of RBP to prealbumin. It is evident that interpolation of the linear regions of the quenching curve intersects at a 1:1 molar ratio of the two proteins. All values were corrected for dilution.

The fluorescence of retinol at 470 nm on excitation at 330 nm increases when prealbumin is added to RBP (Fig. 7). It is evident from the figure that the enhancement is completed after formation of the prealbumin-RBP complex. This result is consistent with the observed differences in quantum yield of RBP and the prealbumin-RBP complex (Table I).

Characteristics of Affinity between RBP and Prealbumin—The finding that the prealbumin fluorescence in quenched to a considerable extent on complex formation with retinol-containing RBP made investigations on the affinity between the two proteins possible. Furthermore, thyroxine bound to prealbumin quenches the protein fluorescence of RBP devoid of retinol on complex formation of the two proteins.2 This quenching process was used for the estimation of the association constant of prealbumin and the heptane-extracted, vitamin A-free species of RBP (22). Fig. 8 shows two Scatchard plots resulting from the binding of the retinol-containing and the retinol-free RBP, respectively to prealbumin. The apparent association constants were the same within experimental error (1.6 × 10^7 and 1.2 × 10^7 M^-1, respectively). This result shows that the retinol content of RBP is not a prerequisite for binding to prealbumin. Furthermore, it may tentatively be inferred that removal of retinol does not seriously change the conformation of RBP (16, 25).

The thermodynamic quantities involved in the RBP binding of prealbumin were investigated by performing fluorescence-quenching experiments at various temperatures within the range 4 to 45°. Fig. 9 shows the resulting plot of log K versus 1/T_absolute. It is evident from the figure that the binding of RBP

Fig. 7. Effects on the prealbumin fluorescence emission at 335 nm (excitation at 280 nm) (○) and of the retinol emission at 470 nm (excitation at 330 nm) (●) at various molar ratios of RBP to prealbumin. The optical densities were never allowed to exceed 0.08 at the excitation wave length in the two experiments. In the experiment where the prealbumin fluorescence emission was measured, a constant concentration of a prealbumin solution was used to which various amounts of a concentrated RBP solution were added to obtain the desired molar ratios of the two proteins. The contribution of RBP to the emission at 335 nm was subtracted from the measured values by using blanks of RBP with appropriate concentrations. In the experiment where the retinol fluorescence emission was measured, a constant concentration of an RBP solution was used to which various amounts of a concentrated prealbumin solution were added to obtain the desired molar ratios of the two proteins. All values were corrected for dilution.

Fig. 8. Binding of retinol-containing (●) and retinol-free (○) RBP to prealbumin determined by fluorescence quenching in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The data were plotted according to Scatchard (23). r is the molar ratio of bound RBP to prealbumin and c the concentration of free RBP.

RBP and the prealbumin-RBP complex. For example, at 20° the quantum yield of RBP was 0.063 in D_2O and 0.049 in H_2O giving an isotope effect of 1.30. The corresponding values for the prealbumin-RBP complex are 0.060 and 0.055, respectively, giving an isotope effect of only 1.09.

Fig. 9. Variation of the apparent association constant K with respect to temperature for the prealbumin-RBP interaction determined with the fluorescence-quenching technique (see Fig. 8) in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl.

Effect on Protein and Retinol Fluorescence of Prealbumin and RBP Complex Formation—The prealbumin fluorescence at 335 nm undergoes dramatic changes with addition of RBP containing retinol. Fig. 7 shows the progressive quenching of the prealbumin fluorescence on increments of the molar ratios of RBP to prealbumin. It is evident that interpolation of the linear regions of the quenching curve intersects at a 1:1 molar ratio of the two proteins. In this region the tryptophan fluorescence of prealbumin is considerably quenched.

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to prealbumin is only slightly affected by heat. Table II shows the computed values for $\Delta H^\circ$, $\Delta S^\circ$, and $\Delta G^\circ$ for the interaction. The large negative Gibbs free energy is composed of a negative enthalpy change and a positive entropy change.

The interaction between RBP and prealbumin is very sensitive with respect to the ionic strength (12). Measurements of the apparent association constant at various ionic strengths (Fig. 10) show that the value of the constant rapidly falls in the region 0.008 to 0.004 and the interaction of the two proteins seems to be abolished at still lower values of the ionic strength.

### DISCUSSION

This study describes the fluorescence properties of the prealbumin-RBP protein complex and its individual components, and thus provides sensitive physicochemical parameters for the study of structure-function relationships in this system.

In addition to its protein fluorescence at 355 nm, RBP has a unique fluorescence at 470 nm associated with its content of retinol. The fluorescence of RBP, devoid of this ligand, and of prealbumin caused by the protein moieties are similar to the native fluorescence of many other proteins (26). The prealbumin-RBP complex exhibits the same types of fluorescence as RBP. The retinol fluorescence of RBP has a low quantum yield (0.04). However, it is interesting to note that free retinol in hydrocarbon solvents has a similar quantum yield (27, 28) whereas the value obtained in methanol only is 0.002 (28). The quantum yield of retinol increases about 50% when prealbumin is bound to RBP indicating that the environment of vitamin A is altered. Furthermore, the Stokes' shift is reduced by approximately 5 nm which is compatible with the new environment of the vitamin being more hydrophobic.

The retinol fluorescence is of great interest since it arises from a chromophore at a unique site (one per RBP molecule) the integrity of which should be a prerequisite for the maintenance of the biologically active conformation of RBP. The retinol molecule would therefore act as a natural reporter group in the protein. Hence, the lowered quantum yield of RBP at low ionic strength probably is related to the increased magnitude of the 212 nm band on circular dichroism (25). Furthermore, previous results from this laboratory have shown that the rate of release of retinol from the protein to a heptane phase was much enhanced on lowering the ionic strength whereas the release was considerably slowed down on RBP forming a complex with prealbumin (12).

The effect of D$_2$O on the emission at 470 nm of RBP and of the prealbumin-RBP complex was examined to obtain further information about the effect of environment on the retinol fluorescence. It is known that in the presence of D$_2$O the fluorescence of many molecules is enhanced. This has been explained by either of two mechanisms. If the excited state is quenched by a competing protonation or deprotonation reaction substitution of D$_2$O for H$_2$O should slow this process and thus enhance the fluorescence (29). Alternatively the quenching may be the result of solvent reorientation about the excited state (30). Regardless of the mechanism, the presence of a D$_2$O effect would thus be interpreted as strong circumstantial evidence for the ability of excited retinol to interact with solvent. It is therefore interesting to note that there is a pronounced difference in isotope effect between RBP and the prealbumin-RBP complex (about 30 and 10%, respectively), indicating that water, or at least protons, may reach part of the retinol molecule when bound to RBP whereas the conformation of the protein complex seems to exclude most of this solvent interaction. This in itself does not imply, however, that retinol in the complex is fully protected from the aqueous medium. Nevertheless, the results of the isotope experiments are fully compatible with the quantum yield measurements.

An interesting property of RBP reported here is the occurrence of energy transfer in the protein. The transfer of electronic excitation energy from the protein moiety to retinol occurs most probably via the resonance transfer mechanism described by Förster (31) since its efficiency is not a function of protein concentration and there is a good overlap between the protein fluorescence spectrum and the absorption spectrum of retinol when bound to the protein. The critical distance for 50% transfer ($R_0$) has been calculated$^3$ to be about 35 Å. This value of $R_0$ al-

### Table II

| Ligand | $K$ | $\Delta H^\circ$ | $\Delta S^\circ$ | $\Delta G^\circ$ |
|--------|-----|-----------------|-----------------|----------------|
| RBP... | $2.9 \times 10^7$ | $-9.3$ | $-9.9$ | $-3.9$ | +19.5 |

**Fig. 10.** Variation of the association constant $K_{app}$ with respect to ionic strength determined with the fluorescence-quenching technique (see Fig. 8). The buffer used contained 0.002 M Tris-HCl, pH 8.0, and was adjusted to the desired ionic strength with NaCl.
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fluorescence of RBP through the kidney glomeruli (7, 9, 33), stabilizes the RBP binding of retinol. Further studies may reveal other important biological effects explaining why this vitamin has to be transported by two proteins.

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