Release of retinol and denaturation of its plasma carrier, retinol-binding protein

Valentina E Bychkova[1], Alexandra E Dujsekina[1], Andrea Fantuzzi[2], Oleg B Ptitsyn[1,3] and Gian-Luigi Rossi[2]

Background: Retinol is tightly packed inside the structure of its plasma carrier (retinol-binding protein, RBP). It was found that retinol release from RBP to aqueous solutions is facilitated by either very low pH or very high temperatures (i.e. by non-physiological conditions that cause protein denaturation). It was also found that alcohols induce protein conformational transitions to denatured states. On this basis, it may be suggested that retinol release *in vivo* is facilitated by the partial unfolding of the carrier resulting from the concerted action of the moderate local decrease of pH and the moderate local decrease of dielectric constant in proximity to the target membranes.

Results: *In vitro*, at 37°C, retinol is removed from its plasma carrier by the concerted action of the moderately low pH and the moderately low dielectric constant of solutions containing a low ionic strength buffer and methanol in variable proportions. Release of retinol is accompanied by a conformational transition of RBP from the native to the molten-globule state.

Conclusions: The physiological function of RBP – targeted delivery of retinol – is mimicked *in vitro* by the facilitated release of retinol (associated with a partial unfolding of the protein carrier) in solutions exhibiting pH and dielectric constant values that are within the range of values expected in the *in vivo* microenvironment.

Addresses: [1]Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia. [2]Institute of Biochemical Sciences, University of Parma, 43100 Parma, Italy. [3]Laboratory of Experimental and Computational Biology, National Cancer Institute, NIH, Bethesda, MD 20892-5677, USA.

Correspondence: Gian-Luigi Rossi
E-mail: rossi@ipruniv.cce.unipr.it

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Introduction

The molecular mechanism of retinol release from its plasma carrier, the retinol-binding protein (RBP), is still unknown. There is evidence that retinol is delivered to target cells via binding of RBP to a surface receptor [1–4]. A recent report on retinol transfer from its plasma carrier to its cellular carrier, mediated by permeabilised human placental membranes, emphasises the involvement of an active receptor in this process [5]. It is known, however, that RBP-bound retinol can be released either to liposomes or to plasma membranes even in the absence of receptor [6–8] and that the process is facilitated by moderately low pH [6]. It has also been shown that retinol can be completely removed from RBP, in the absence of membranes, by organic solvent extraction [9], very low pH [10] and heating [10–12].

Because retinol is tightly packed within non-polar side-chains inside a deep pocket of RBP [13], its dissociation constant is rather low. It has been reported to be 190 nM at pH 7.4 and 27°C [14]. By loosening the packing, release of retinol may be substantially facilitated. In fact, we have shown that, in acidic solutions, RBP molecules tend to release retinol and, at the same time, undergo a conformational transition to the molten-globule state [10]. This state is typical of globular proteins under mild denaturing conditions (see [15,16] for recent reviews). The pH-induced unfolding can favour retinol release near biological membranes where the negative electrostatic potential decreases the local pH value with respect to that of the bulk medium [17–19]. The mere decrease of pH by one or two units may, however, be too small to ensure efficient retinol delivery. It can be suggested that protein denaturation in proximity to a membrane surface occurs because of both a local decrease of the pH and a local decrease of the dielectric constant of the medium [15,20]. In fact, a recent report predicts the distance-dependent value of the dielectric constant near polyelectrolytes in solution [21]. This analysis, which is supported by a comparison of the theoretical and experimental values of the local dielectric constant within the major and minor grooves of DNA molecules, shows that the dielectric constant near a charged membrane decreases from a value of 70 at a distance of 5 Å from the surface to < 30 within a distance of 1 Å. These values are not very sensitive to the ionic strength of the surrounding medium.

Are the effects of a biological membrane on approaching proteins mimicked by water–alcohol mixtures at moderately low pH? It has been shown that the denaturing action of different alcohols on ubiquitin [22] and β-lactoglobulin [23,24] is associated with the decreased average dielectric constant of the water–alcohol mixture rather than with the specific properties of the alcohol used. Moreover, it has

been shown that the 'concerted' action of moderately low pH and moderate concentrations of methanol leads cytochrome *c* to a molten-globule-like state [25].

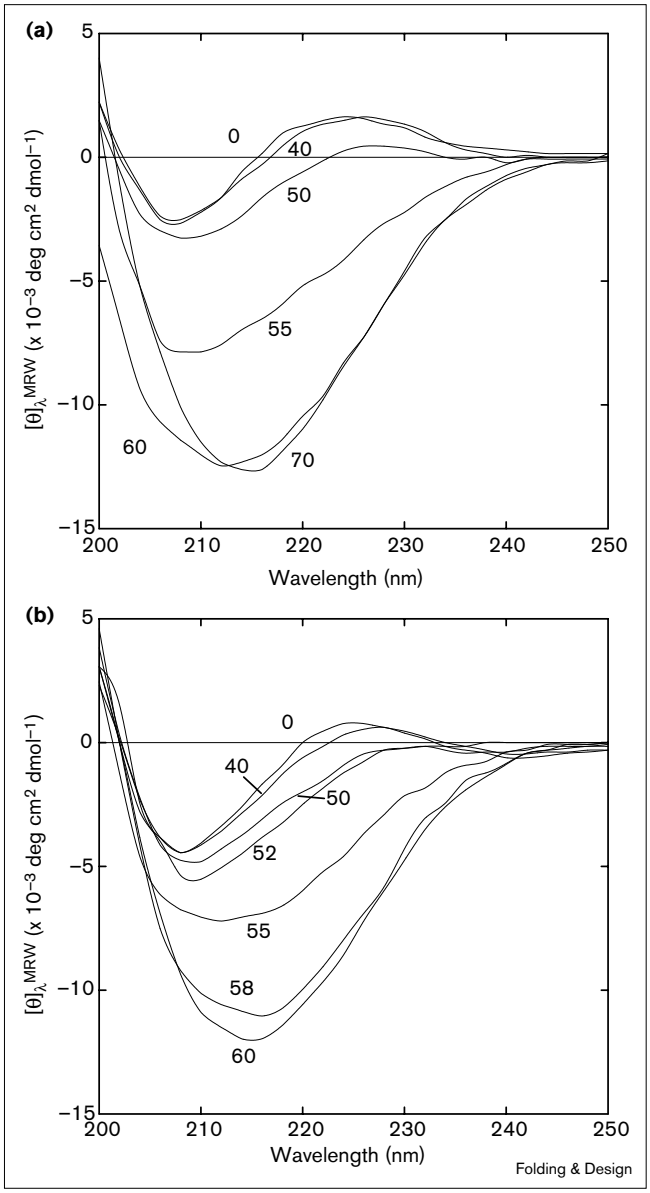
The aim of the present work is to simulate the microenvironment in which retinol release occurs *in vivo*, with water–methanol mixtures of moderately low pH and moderately low dielectric constant; to determine the range of conditions under which retinol dissociates; and to analyse the conformational state of the protein after ligand release.

Results and discussion

Methanol-induced RBP denaturation and retinol release at pH 8.5

Figure 1 shows the far-UV CD spectra of native apo-RBP and holo-RBP in phosphate buffer, pH 8.5, as well as the spectra obtained by exposing apo-RBP and holo-RBP to variable concentrations of methanol. It should be noted that the spectra of both apo-RBP and holo-RBP in the native state bear greater resemblance to the spectrum of a completely unfolded protein than to that of a typical β-protein in the native state [10]. These 'anomalous' spectra are almost certainly a result of the influence of aromatic sidechains and S–S bonds, which are known to contribute to the far-UV CD spectra of rigid native proteins [26–29]. Proteins in the molten-globule state have a secondary structure content similar to that of native proteins, but do not have a rigid tertiary structure. In fact, the far-UV CD spectrum of apo-RBP in the molten-globule state, which is obtained by acidic denaturation, in which the contributions of aromatic sidechains and S–S bonds are either absent or strongly reduced, bears more resemblance to that of a typical β-protein [10]. The differences in the far-UV CD spectra between the native state of apo-RBP and holo-RBP and the denatured states of apo-RBP can be used to monitor protein unfolding under different conditions. In conditions of up to 50% v/v methanol, the spectra remain nearly unchanged, but become very pronounced when the methanol concentration is increased from 50% to 60% v/v. At 55% v/v methanol, the spectra are typical of a β-protein. Apo-RBP denaturation can also be measured by changes in the near-UV region (Figure 2). The changes occur in the same interval of alcohol concentration as the changes in the far-UV CD spectra. In Figure 2, the near-UV CD spectrum of native holo-RBP, containing the very strong positive band around 325 nm that results from tightly bound retinol, is presented for comparison. The decrease of this band, under denaturing conditions, can be used to monitor retinol release. In the case of holo-RBP, the ellipticity at 325 nm, as well as retinol fluorescence at 460 nm (data not shown), are essentially abolished by 55% v/v methanol, indicating complete retinol release.

Figure 1



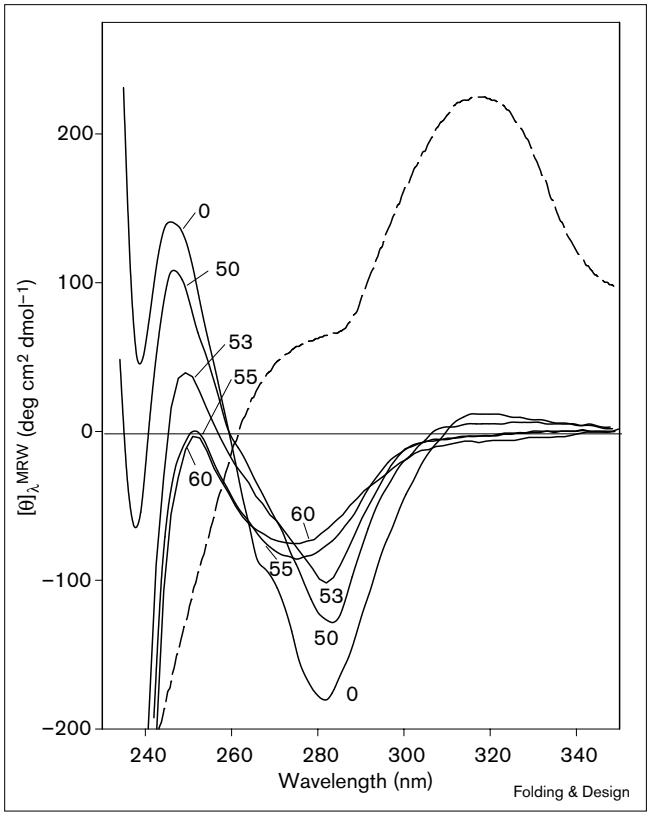
Far-UV CD spectra of **(a)** apo-RBP and **(b)** holo-RBP in 0.005 M sodium phosphate buffer and different methanol concentrations (v/v), shown by numbers near the curves; pH 8.5 and T = 37°C. Protein concentration, 0.03 mg/ml; and cuvette pathlength, 5 mm.

RBP denaturation and retinol release under the concerted action of pH and methanol

As was shown previously [10], both the release of retinol and protein denaturation occur in a purely aqueous medium at very low pH. We therefore investigated whether the two processes might be facilitated *in vitro* by the combined action of moderately low pH and moderately low methanol concentrations.

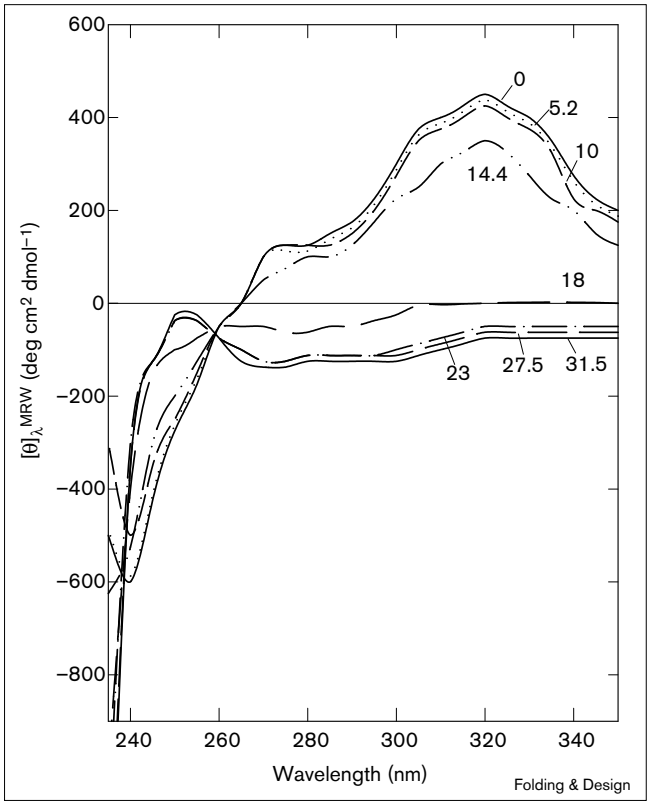
As an example, Figure 3 reports the near-UV CD spectrum of holo-RBP at pH 3.5 in the absence of methanol; the spectrum is the same as that at pH 8.5. In the presence of 14–18% v/v methanol, cooperative retinol release occurs, as indicated by the decrease and disappearance of

Figure 2



Near-UV CD spectra of apo-RBP in 0.005 M sodium phosphate buffer and different methanol concentrations (v/v), shown by numbers near the curves; pH 8.5 and T = 37°C. Protein concentration, 0.10–0.18 mg/ml; and cuvette pathlength, 5 mm. The near-UV CD spectrum of native holo-RBP (in the absence of methanol) is shown by the dashed line (all values of $[\theta]_\lambda^{MRW}$ for holo-RBP are multiplied by 0.5).

Figure 3



Near-UV CD spectrum of holo-RBP in 0.005 M sodium phosphate buffer and the spectra resulting from the addition of different methanol concentrations (v/v), shown by numbers near the curves; pH 3.5 and T = 37°C. The titration was carried out directly in the cuvette by addition of 95% v/v methanol. The pH was adjusted to remain constant in the course of the titration. Protein concentration, from 0.215 mg/ml at 0% v/v methanol to 0.145 mg/ml at 31.5% v/v methanol; cuvette pathlength, 5 mm. The spectrum at pH 3.5 in the absence of methanol is practically identical to that at neutral pH.

the band centred at 325 nm. Far-UV CD spectra also change (becoming much more pronounced) in approximately the same range of methanol concentrations (data not shown).

Figure 4 shows the dependencies of the relative amounts of native protein and bound retinol (f_N) on methanol concentration at pH 8.5 and pH 3.5:

$$f_N = \frac{(X - X_M)}{(X_N - X_M)} \quad (1)$$

where X is the value of the measured parameter and X_N and X_M are the values determined for the 'pure' native and methanol-denatured states. The X_M value was taken as the value observed at 55% v/v methanol, a concentration at which all retinol is released from the protein. The figure clearly shows that retinol release and a protein conformational transition are associated processes. It also shows the concerted action of protons and methanol in promoting both the release of retinol and the denaturation of RBP.

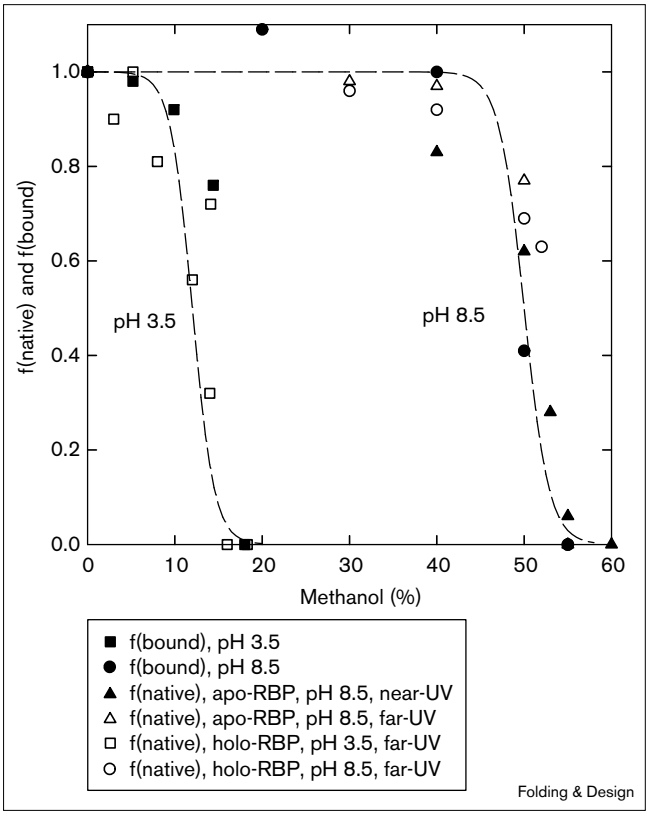
A phase diagram

We have measured the methanol dependence of retinol release and RBP denaturation at different pH values (2.0, 3.5, 4.0 and 8.5) and the pH dependence of retinol release and RBP denaturation at different methanol concentrations (0, 30% and 40% v/v). The results are illustrated in Figure 5 as a phase diagram, in which the pH and methanol concentration values are combined at stages at which the release of retinol and RBP denaturation are observed. The average dielectric constant of the water–methanol mixtures is also given [30,31].

The molten-globule-like state

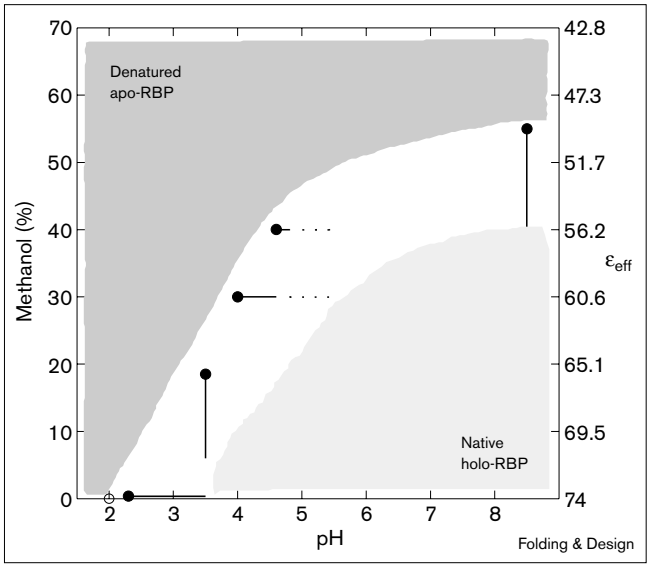
The conformational states of RBP under conditions that cause the release of retinol can be compared with the molten-globule state induced by acidic denaturation in aqueous solutions [10]. Figure 6 shows the far-UV CD spectrum of methanol-denatured RBP at pH 8.5 (55% v/v

Figure 4



Methanol-induced and pH-induced release of retinol from holo-RBP and denaturation of RBP in relative units (see Equation 3). f(bound), the fraction of retinol molecules bound to RBP measured by the molar ellipticity $[\theta]_{325}$ for bound retinol at pH 3.5 and pH 8.5; f(native), the fraction of native RBP molecules measured by the near-UV molar ellipticity $[\theta]_{280}$ for apo-RBP at pH 8.5 as well as by far-UV molar ellipticity $[\theta]_{224}$ for apo-RBP at pH 8.5 and holo-RBP at pH 3.5 and pH 8.5; conditions are the same as in Figures 1–3.

Figure 5

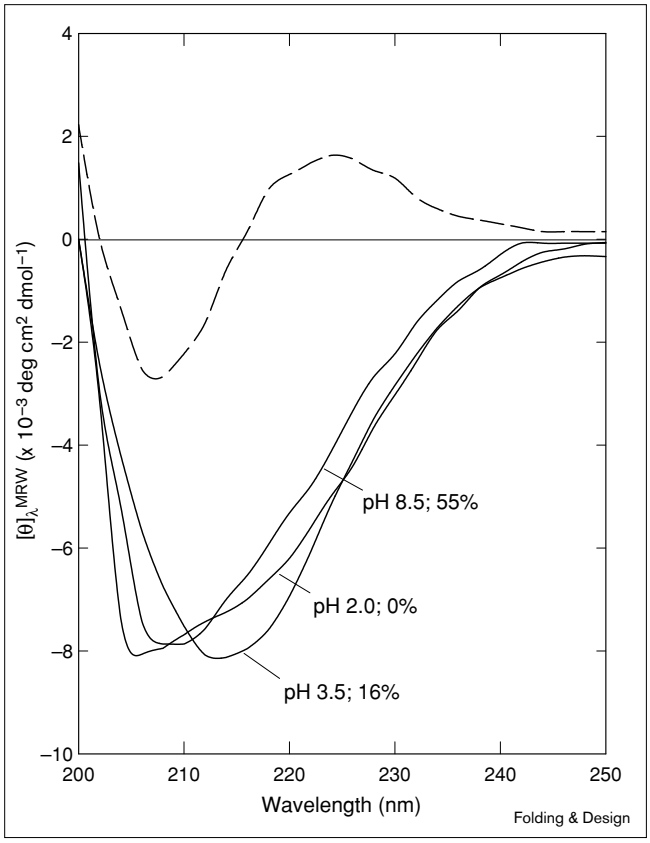


A phase diagram illustrating retinol release and protein denaturation as a function of pH and methanol concentration. ε_{eff}, the average dielectric constant of the water–methanol mixtures [30,31]. The horizontal and vertical bars represent the intervals of pH at a fixed methanol concentration and the intervals of methanol concentration at a fixed final pH, respectively, within which release of retinol (measured by $[\theta]_{325}$) and protein denaturation (measured by $[\theta]_{224}$) occur. The bars define a region that separates two states of RBP molecules; retinol release is facilitated within this region. Dots along a bar indicate regions where protein aggregation occurs. Full circles represent the molten-globule-like state of the protein. The open circle marks the molten-globule state of RBP that was described previously [10].

methanol), the spectrum of RBP denatured by a concerted action of moderately acidic pH (3.5), which would not cause protein denaturation by itself, and moderate methanol concentration (16% v/v), and the spectrum of RBP in the molten-globule state induced by pH 2.0 in the absence of methanol, as previously reported [10]. The far-UV CD spectrum of native apo-RBP is also shown. It is evident that the spectra of the denatured forms are similar to each other and significantly different from the spectrum of the protein in the native state. The near-UV CD spectrum of RBP practically vanishes for all denatured forms (see, for example, the spectrum of the protein in 18% v/v methanol shown in Figure 3).

The compactness of RBP molecules in these denatured states is evaluated by the diffusion coefficient and polarisation (P) of tryptophan fluorescence. Diffusion coefficients are measured for native holo-RBP at pH 7.5 and for the molten-globule state of apo-RBP at pH 2.0, in the absence of methanol. The $D_{20,w}$ value for the native state (11.3×10^{-7} cm^2 s^{-1}) corresponds to a Stokes' radius R_s = 19 Å, whereas the $D_{20,w}$ value for the molten-globule state (10×10^{-7} cm^2 s^{-1}) corresponds to R_s = 21.5 Å. Thus, the linear dimensions of the molten globule are ~15% higher than those for the native protein, a result in agreement with the data for α-lactalbumin [32–34], β-lactamase and carbonic anhydrase [35]. It was impossible to measure diffusion coefficients in water–methanol mixtures because the measurements involve an overnight dialysis, which leads to association of RBP molecules. On the other hand, it was possible to measure the depolarisation of tryptophan fluorescence in water–methanol mixtures. Table 1 shows that all values of 1/P measured for denatured RBP are close to the value for the native protein (~13) and much smaller than the value for a guanidine hydrochloride unfolded protein (~37). This suggests that RBP denatured by pH and/or methanol is nearly as compact as the native protein. Thus, we come to the conclusion that the states of RBP denatured by low pH, methanol, and the combined action of moderately low pH and moderately low concentrations of methanol have a substantial secondary structure, do not reveal a rigid tertiary structure and are

Figure 6



The far-UV CD spectra of RBP, after release of retinol, at pH 3.5 in 16% v/v methanol and at pH 8.5 in 55% v/v methanol are compared with the spectrum of RBP in the molten-globule state at pH 2.0 (in the absence of methanol). The distinct far-UV CD spectrum of native apo-RBP is shown by the dashed line.

compact (i.e. they meet the usual requirements for the molten-globule state).

Retinol release and protein aggregation

RBP molecules tend to aggregate in conditions favouring retinol release. This behaviour is additional evidence for the occurrence of the molten-globule-like state. In fact, it is known that the molten-globule state has more hydrophobic surface exposed to water than the native state and, consequently, an increased affinity for hydrophobic dyes — ANS, 8-anilino-1-naphthalenesulfonate; TID, 3(tri-fluoromethyl)-3-(m-[^{125}I] iodo-phenyl) diazirine — as well as for phospholipid vesicles and membranes (see [15] for a review).

Conclusions

The data presented in this work show that the specific function of plasma RBP, retinol delivery to target membranes, is simulated by retinol release in a simple artificial system: a water–methanol mixture characterised by a moderately low pH and moderately low dielectric constant.

Table 1

Depolarisation of tryptophan fluorescence in RBP under different conditions.

State	pH	[MeOH] (v/v; %)	1/P
Native	7.5	0	12.8
Molten globule	2.0	0	15.6
Unfolded in 6 M guanidine hydrochloride	7.4	0	36.5
Denatured by methanol	8.5	60	11.7
Denatured by concerted action of pH and methanol	3.5	18.8	12.3

P, polarisation of tryptophan fluorescence.

The protein that has released retinol exhibits molten-globule properties. The calculated local dielectric constant [21] and pH [19] in proximity to a membrane indicate that similar denaturing conditions are likely to occur in the physiological microenvironment. We have verified that liposomes containing a mixture of neutral and negatively charged phospholipids, in a low ionic strength buffer, pH 6, not only favour retinol dissociation but also induce the conformational transition to the molten-globule state of the resulting apo-protein, as previously found for cytochrome *c* [36]. In such experiments, however, in contrast with those reported here, we could not determine directly the local pH and dielectric constant that are synergistic in the process.

Because retinol release and protein denaturation are intrinsically related, the protein denaturation might favour excretion of the resulting apo-RBP *in vivo*.

Materials and methods

Materials

Pure human holo-RBP (with an A_{280}:A_{330} ratio of 1.1–1.2:1) was prepared and used as previously outlined [37]. Apo-RBP was obtained by extracting retinol from holo-RBP by diethyl ether [14]. Diethyl ether was removed from aqueous solutions under vacuum. Protein concentrations were determined from absorbance at 279 nm using $\varepsilon_{1\ mg/ml}^{1\ cm} = 1.74$ and 2.02 for the apo-form and the holo-form, respectively [10]. Methanol was an analytical reagent for HPLC from LabScan Analytical Sciences (LabScan Limited, Ireland).

All measurements were carried out at 37°C, using protein dissolved in low ionic strength solutions (5 mM sodium phosphate buffer) and mixed with variable volumes (5–70% v/v) of methanol. Different pH values were obtained by addition of either HCl or NaOH. To prevent protein association at pH 4.0–4.7, solutions in this pH range were obtained by mixing a protein solution at pH 8.5 with the required amount (≤ 20% of the starting volume) of a solution of similar composition adjusted to pH 2.0. The pH values indicated are those of the final solutions, after addition of methanol, as measured by the pH meter.

Circular dichroism

CD measurements were carried out with JASCO 500 or 600 spectropolarimeters equipped with thermostatted cell holders. The temperature was controlled by circulating water from a thermostat. The path lengths of cells varied between 1 mm and 5 mm, to obtain spectra of

suitable intensity in different regions of far-UV, and 5 mm and 10 mm, to obtain near-UV spectra. Protein concentrations varied between 0.03 mg/ml and 0.25 mg/ml. Molar ellipticity was calculated according to:

$$[\theta]_{\lambda}^{MRW} = \frac{\theta_{\lambda} MRW}{lc} \quad (2)$$

where θ is the measured ellipticity (in mdeg) at a given wavelength λ, MRW is the mean residue molecular weight calculated from the protein sequence (MRW is 115 for human RBP), l is the cuvette path length in mm and c is the protein concentration in mg/ml. Measurements were carried out in the 200–350 nm region.

Fluorescence

Fluorescence experiments were performed using a Perkin-Elmer LS50B spectrofluorimeter. Spectra were registered in the 300–500 nm region (excitation at 285 nm or 330 nm), using protein concentrations in the range 0.03–0.15 mg/ml. Polarisation of tryptophan fluorescence was measured at 340 nm (excitation at 285 nm) at protein concentrations 0.01–0.03 mg/ml as:

$$P = \frac{I_{||} - GI_{\perp}}{I_{||} - GI_{\perp}} \quad (3)$$

The intensities of the vertical ($I_{||}$) and horizontal ($I_{\perp}$) fluorescence components were recorded at an angle of 90° to the vertical polarised excitation beam. The factor G (equal to $I_{\perp}'/I_{||}'$, where the primes indicate excitation polarised in a perpendicular direction) was used to correct for the unequal transmission of differently polarised light.

Diffusion

Diffusion coefficients were measured by the method of macroscopic diffusion [38] previously applied to the study of proteins in different conformational states [33]. The diffusion coefficient D is related to the hydrodynamic (Stokes) radius R_s by the equation:

$$D = \frac{kT}{6\pi\eta_0 R_s} \quad (4)$$

where T is the temperature, k is the Boltzmann constant and η_0 is the viscosity of a solvent at the given temperature. $D_{20,w}$ is the diffusion coefficient reduced to the viscosity of water at 20°C. Measurements were carried out at protein concentrations in the range 0.10–0.15 mg/ml.

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