Retinol-Binding Protein Is in the Molten Globule State at Low pH†

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ABSTRACT: Using far- and near-UV circular dichroism, viscosity, tryptophan fluorescence, NMR spectra, binding of a hydrophobic probe, and microcalorimetry, we have shown that the apo form of human retinol-binding protein (RBP) at neutral pH is in a rigid state with properties similar to those of holo-RBP. On the contrary, at acidic pH apo-RBP is in the molten globule state which has been earlier revealed for a number of proteins under mild denaturing conditions. We have also shown that, at equilibrium, the pH-induced retinol release from holo-RBP parallels denaturation of the apoprotein. These findings are consistent with our hypothesis that the transformation of RBP into the molten globule state is involved in the mechanism whereby retinol is delivered to target cells. In particular, a local acidic pH near the membrane surface of target cells might cause the transition of RBP to the molten globule state as well as the release of retinol.

The transport of the liposoluble vitamin A alcohol from its storage sites in the liver to target tissues is accomplished by a specific transport protein, retinol-binding protein (RBP),[1] which is associated in plasma with thyroxine-binding transthyretin [Goodman (1984) and references therein]. RBP is a single polypeptide chain of approximately 21 kDa that contains a single site for retinol. The structure of human holo-RBP has been determined and refined to 2-Å resolution. It consists of a single globular domain and its most striking feature is the existence of an antiparallel β-barrel which completely encapsulates the retinol molecule (Newcomer et al., 1984; Cowan et al., 1990).

Evidence has accumulated indicating that retinol is delivered to target cells via binding of RBP to cell-surface receptors (Heller, 1975; Rask & Peterson, 1976; Ottonello et al., 1987; Sivaprasadarao & Findlay, 1988a,b). The identification and partial characterization of a retinal pigment epithelial membrane receptor for plasma RBP has indeed been recently reported (Båvik et al., 1991). Other lines of evidence have suggested that retinol can also be released from its carrier to liposomes or to plasma membranes without interaction between RBP and cell-surface receptors (Fex & Johannesson, 1987; Noy & Xu, 1990a; Noy & Blaner, 1991).

Retinol can be dissociated in vitro from the retinol–RBP complex by treatment with organic solvents (Goodman & Raz, 1972) or by heating (Goodman & Leslie, 1972). It has also been reported that the delivery of retinol to liposomes can be significantly facilitated by lowering the pH from 7 to 4.5 (Fex & Johannesson, 1987). No direct evidence has yet been presented to indicate that the release of retinol is associated with structural changes in the RBP molecule. The removal of retinol from RBP leaves a large empty volume in the hydrophobic interior of the protein molecule; this might be expected to trigger drastic conformational changes of RBP (Newcomer et al., 1984). However, the isomorphism of human holo- and apo-RBP crystals (Monaco et al., 1984) as well as far-UV CD spectra of holo- and apo-RBP in solution at neutral pH (Rask et al., 1972; Gotto et al., 1972) are consistent with the lack of considerable conformational differences between apo- and holo-RBP.

The aim of our work was to study the physical properties of the apo-RBP molecule at neutral and acidic pH in water solutions. We have found that at neutral pH apo-RBP, obtained by extracting retinol with an organic solvent, has a rigid 3D structure which is similar to that of the holoprotein. On the contrary, at acidic pH the apo-RBP molecule is in the denatured "molten globule" state, revealed earlier for other proteins (Dolgikh et al., 1981, 1985; Ptitsyn, 1987, 1992; Kuwajima et al., 1989). This state, intermediate between the native and the completely unfolded states, is rather compact and has well-pronounced secondary structure but has no rigid tertiary structure. Its intramolecular mobility is much higher than that of the native protein.

MATERIALS AND METHODS

Materials. Pure human holo-RBP (with a A_{280}:A_{330} ratio of 1.05–1.10) was obtained as previously described (Berni et al., 1985). Apo-RBP, devoid of the characteristic retinol absorbance at 330 nm, was obtained by extracting a holo-RBP solution with diethyl ether, as described by Cogan et al. (1976). Upon extraction and separation of the organic solvent phase, the diethyl ether remaining in the aqueous solution was evaporated under vacuum. Apo-RBP, obtained as described above, was used for studies at both neutral and acidic pH. The study at acidic pH was also performed with apo-RBP obtained by pH-induced release of retinol (see below). Protein concentrations were determined by their absorbance at 279 nm, using $\epsilon_{1cm}^{1mg/mL} = 1.74$ and 2.02 for the apo- and holo-RBP, respectively. The extinction coefficients were determined by the method described by Jaenicke (1974). ANS was a product from Serva (Germany). Optically pure GuHCl was purchased from Reachim (Riga, Latvia) and twice recrystallized from aqueous solutions. Retinol was obtained from Fluka (Buchs,

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[1] Abbreviations: RBP, retinol-binding protein; UV, ultraviolet; NMR, nuclear magnetic resonance; CD, circular dichroism; 3D structure, three-dimensional structure; $[C]_p$, partial specific heat capacity; GuHCl, guanidine hydrochloride; ANS, 8-anilino-1-naphthalenesulfonate; N, A, and U, neutral, acidic, and unfolded forms of RBP, respectively. pH* denotes the pH value for D_2O solutions used for NMR measurements.

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Switzerland). Sodium chloride, sodium phosphate, diethyl ether, HCl, and NaOH were reagent-grade products from Merck (Darmstadt, Germany).

The buffers used for all measurements were a low ionic strength solution (0.005 M sodium phosphate buffer, pH 7 or 2) or a high ionic strength solution (0.05 M sodium phosphate buffer and 0.1 M NaCl, pH 7.4). The desired pH of the protein solutions was obtained by mixing appropriate volumes of pH 7.5 and 2.0 buffer solutions, to avoid aggregation and insolubilization of RBP at intermediate pH values. Protein solutions were equilibrated at low ionic strength by dialysis against 0.005 M sodium phosphate buffer.

Equilibrium Sedimentation Measurements. Equilibrium sedimentation measurements were carried out with a MOM 3170 ultracentrifuge (Hungary) at 28 500 rpm. The apparent molecular mass of RBP was determined in solutions at different acidic pH values (in the range 1.5–3.0) and ionic strengths (from 0.05 M sodium phosphate buffer containing 0.1 M NaCl down to 0.005 M sodium phosphate buffer without NaCl), for protein concentrations 0.2–0.3 mg/mL.

Circular Dichroism. CD measurements were carried out with a JASCO 41A or a JASCO 500 spectropolarimeter equipped with a cell holder thermostatically controlled by circulating water from a bath. The path lengths of cells varied from 0.15 to 1.0 mm for different regions of far-UV CD spectra and from 5.0 to 10.0 mm for near-UV CD spectra. Protein concentrations varied from 0.15 to 1 mg/mL. Molar ellipticity was calculated according to

$$[\theta]_\lambda^{MRW} = \theta_\lambda MRW/lc$$

where θ_λ is the measured ellipticity (in degrees) at a wavelength λ, MRW is the mean residue weight calculated from the protein sequence (MRW = 115 for human apo-RBP), l is the cuvette path length (in millimeters), and c is the protein concentration (in grams per milliliter). Molar ellipticity $[\theta]_\lambda^{MRW}$ was plotted vs wavelength in the 185–350-nm region.

The denaturation of RBP, which is accompanied by the decrease of ellipticity in the near-UV region, was monitored by the decrease of the difference $[\theta]_{246} - [\theta]_{285}$. The release of retinol was detected by the decrease of $[\theta]_{325}$. pH dependences for apo-RBP denaturation and for the release of retinol from holo-RBP were obtained at 37 °C in 0.05 M sodium phosphate buffer containing 0.1 M NaCl and were expressed as relative content of the denatured apo-RBP (f_d) and of the RBP that has lost retinol (f_a), respectively, as a function of pH. f_d and f_a were calculated according to

$$f = (X_{pH7} - X_{pH})/(X_{pH7} - X_{pH2})$$

where $X = ([\theta]_{246} - [\theta]_{285})$ for f_d and $X = [\theta]_{325}$ for f_a.

Fluorescence. Fluorescence measurements were carried out with a Perkin-Elmer MPF-3L or Aminco SPF-1000CS spectrofluorometer. Polarization of tryptophan fluorescence was measured at 340 nm (excitation at 290 nm). ANS binding was monitored by the intensity of ANS fluorescence in the region between 420 and 620 nm (excitation at 340 nm). The protein concentrations used for fluorescence measurements were 0.02–0.1 mg/mL.

Microcalorimetry. Calorimetric measurements were carried out using a DASM-4 capillary scanning microcalorimeter equipped with gold cells of 0.5-mL volume (Bureau of Biological Instrumentation of the Russian Academy of Sciences, Russia). To extend the heating range above 100 °C, all measurements were performed under an excess pressure of 2.4×10^5 Pa. The heating rate was 1 K min^{-1}. The protein concentration was in the range 0.4–1.1 mg/mL. A partial specific volume for RBP of 0.725 was used. The partial specific heat capacity of the protein in solution was determined as described by Privalov et al. (1986).

NMR Measurements. ^{1}H-NMR spectra were recorded with a 400-MHz Bruker WH 400 spectrometer. Accumulation of signal was about 150. pH* 7.4 buffer was used for the neutral (N) form and pH* 2 buffer for the acidic (A) and unfolded (U) forms of RBP (pH* is the direct reading of the pH meter). Proteins were lyophilized from solutions at the desired pH and then dissolved in D_2O or GuDCl solutions at the same pH*. The NMR spectra of the different forms of RBP were found to be temperature-independent in the range 25–40 °C.

Viscosity. The reduced viscosity (η_{red}) of a protein solution is defined by

$$\eta_{red} = (\eta/\eta_0 - 1)/c$$

where η is the viscosity of the protein solution, η_0 is the viscosity of the solvent, and c is the protein concentration (while the limit of η_{red} as $c \rightarrow 0$ is the intrinsic viscosity $[\eta]$). The reduced viscosities of protein solutions were obtained by the use of an Ostwald capillary viscometer (with a water flow time of 140 s) and have been calculated according to

$$\eta_{red} = (t/t_0 - 1)/c$$

where t is the flow time for the protein solution, t_0 is the flow time for the solvent, and c is the protein concentration (in grams per milliliter). The temperature was kept at 20 ± 0.05 °C by the use of two thermostats. The measurements were done at three protein concentrations varying from 2 to 4 mg/mL and an average value for η_{red} was obtained.

RESULTS

pH-Induced Release of Retinol from RBP. As mentioned above, there is some evidence that the release of retinol from holo-RBP to liposomes is facilitated at low pH (Fex & Johannesson, 1987). We have studied the pH dependence of retinol release from holo-RBP in water solutions. The near-UV CD spectrum of holo-RBP has a maximum at 325 nm (see below, Figure 3), which is absent in apo-RBP and has been attributed to the bound retinol molecule (Goodman & Leslie, 1972). Therefore, we have used the ellipticity at this maximum as a measure of the relative content of bound retinol (see Materials and Methods). We have observed that the pH-induced retinol release can be very slow at 37 °C: for example, approximately 1 h at pH 3.0 and 5 h at pH 3.5 are required to reach equilibrium. Hence, we measured UV CD spectra at different pH values several times until they became time-independent.

The pH dependence for the release of retinol from holo-RBP is presented in Figure 1. The figure shows that retinol can be completely released from holo-RBP in aqueous solution (in the absence of lipids or organic solvents) at pH ≤ 3 (at the physiological temperature 37 °C).

Figure 1 also presents for comparison the curve of the pH-induced denaturation of apo-RBP monitored by the difference $[\theta]_{246} - [\theta]_{285}$ (see Materials and Methods). It shows that the denaturation of apo-RBP is finished at pH ≤ 3. At these pH values apo-RBP has almost completely lost its rigid 3D structure as evidenced by its near-UV CD spectrum (see below, Figure 3).

Both the rigid 3D structure of apo-RBP and its ability to rebind retinol are at least partly restored when the pH-denatured apo-RBP is transferred back to neutral pH. It was

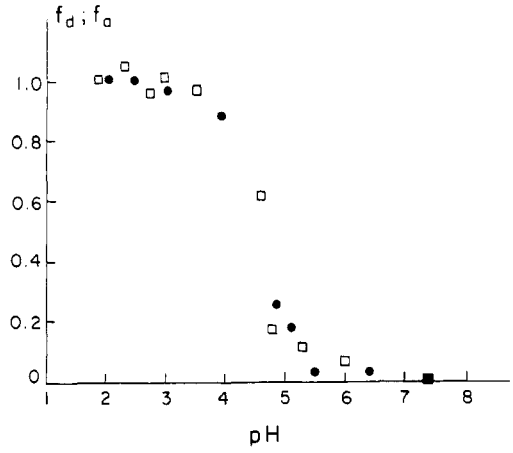

FIGURE 1: pH dependence profiles for the release of retinol from holo-RBP and for apo-RBP denaturation. The relative content of RBP which has released retinol (f_a; □) and of denatured apo-RBP (f_d; ●) are plotted vs pH. $f = (X_{pH7} - X_{pH})/(X_{pH7} - X_{pH2})$, where $X = [\theta]_{325}$ for f_a and $X = ([\theta]_{246} - [\theta]_{285})$ for f_d (see Materials and Methods). Buffer, 0.050 M sodium phosphate and 0.1 M NaCl; temperature, 37 °C; protein concentration, 0.2 mg/mL; cuvette path length, 5 mm.

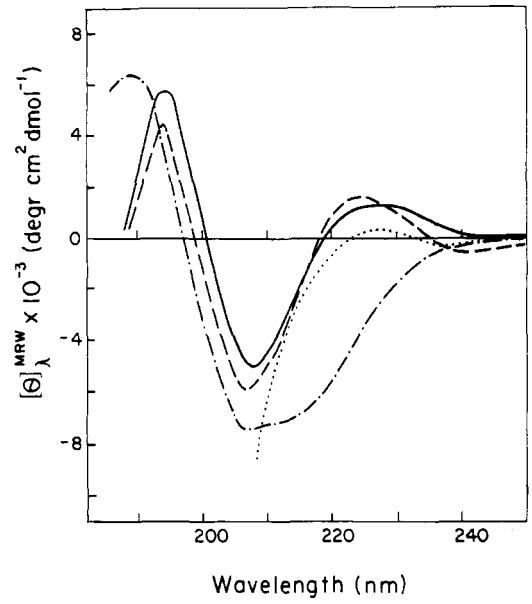

FIGURE 2: Far-UV CD spectra for various forms of RBP. Neutral forms at pH 7.4: apo-RBP (—) and holo-RBP (– – –). Denatured forms of apo-RBP (at pH 2.0): A form (– · –) and U form (···). Buffer, 0.005 M sodium phosphate; protein concentration, 0.5 mg/mL (1 mg/mL for the U form); temperature, 10 °C; cuvette path length, 0.15–1.0 mm.

difficult to obtain more quantitative data due to the propensity of the protein to aggregate and precipitate near its isoelectric point.

We have also studied the temperature-induced release of retinol from holo-RBP in 0.05 M sodium phosphate buffer, pH 7.4, and 0.1 M NaCl. The results show that retinol is completely released from holo-RBP at $T > 75$ °C and that apo-RBP is denatured at these temperatures (data not shown). Both temperature-induced release of retinol and its rebinding at 37 °C are much faster than the same processes occurring under the effect of pH (see above).

Physical States of Apo-RBP at Neutral and Low pH. A detailed investigation of the physical states of apo-RBP at neutral and low pH has been carried out. The forms of RBP present at low and neutral pH values will be called A (acidic) and N (neutral), respectively. The completely unfolded RBP in the presence of 6 M GuHCl will be called U (unfolded) form. The N form of apo-RBP has been obtained by extraction of retinol from holo-RBP with diethyl ether. We have observed by equilibrium sedimentation measurements that at low pH (≈2) and in 0.05 M sodium phosphate buffer containing 0.1 M NaCl, apo-RBP molecules undergo association: the apparent molecular mass was at least 4 times as large as that of the monomeric protein (data not shown). Searching for conditions in which apo-RBP is a monomer at low pH, we have checked different pH values from 1.5 to 3.0 and different ionic strengths, down to 0.005 M sodium phosphate buffer in the absence of NaCl. We have found that the A form of apo-RBP has the lowest apparent molecular mass of approximately 28 kDa (still higher than that of the native RBP, which is 21 kDa) at pH 2.0 and in 0.005 M sodium phosphate buffer without NaCl. The data are consistent with a mixture of approximately 70% monomers of apo-RBP and no more than 30% dimers. Most of the data presented below were obtained at this low ionic strength, though we have verified that the main results are valid also for high ionic strength.

(*a*) *Secondary Structure.* Figure 2 shows far-UV CD spectra of apo-RBP in N, A, and U states and, for comparison, the spectrum of native holo-RBP at 10 °C. The spectrum of apo-RBP at neutral pH is similar to that of holo-RBP and has a rather unusual shape: molar ellipticities in the region between 220 and 235 nm are positive unlike typical spectra for β-proteins, particularly β-lactoglobulin, whose 3D structure is similar to that of RBP (Papiz et al., 1986). The A form of apo-RBP has a CD spectrum which is much more typical for a β-protein and differs remarkably from the spectrum of the U form (in 6 M GuHCl), thus suggesting the existence of the high content of secondary structure in the A form of this protein. The unusual shape of the CD spectrum of the N forms of apo- and holo-RBP probably reflects the influence of aromatic side chains. In fact, in rigid protein structures these side chains are in an asymmetrical environment which can be responsible for the positive molar ellipticity in the region 220–235 nm, while in flexible protein states aromatic side chains are in a more symmetrical environment and, therefore, the positive contribution to the molar ellipticity is abolished. This interpretation, proposed by Sears and Beychok (1973), Dolgikh et al. (1985), and Ptitsyn (1987), has been confirmed by the deconvolution of far-UV CD spectra of proteins to establish the contributions of backbone and aromatic side chains (Bolotina & Lugauska, 1985) [see also direct calculations of these contributions for bovine pancreatic trypsin inhibitor (Manning & Woody, 1989)]. The existence of a rigid asymmetrical environment of aromatic side chains in apo-RBP at neutral pH and its substantial weakening at acidic pH is confirmed by near-UV CD spectra as well as by ^{1}H-NMR of apo-RBP (see below, Figures 3 and 4).

(*b*) *Compactness.* The volume of protein molecules is proportional to intrinsic (or reduced) viscosities. The reduced viscosities (η_{red}) for the N, A, and U states of apo-RBP are presented in Table I. The value of η_{red} for RBP at neutral pH is typical for globular proteins. The value of η_{red} measured for the A form of apo-RBP is more than twice as large as that measured for the N state but is considerably lower than that obtained for the U state. One must take into account that the η_{red} measured for the A form of RBP gives the highest estimate of the molecular volume at acidic pH. In fact, remarkable dependences of η_{red} on protein concentration are usually found at acidic pH values. Therefore, molecular volumes obtained at these pH values by extrapolation to zero protein concentration may be twice as small as those obtained at the protein

Table I: Physical Properties of Different States of Apo-RBP at 20 °C

parameter	N^a	A^b	U^c
$\eta_{red}{}^d$	4.4 ± 0.2	9.7 ± 1.0	24.5 ± 1.0
$\lambda_{max}{}^e$	335	335–336	350
$[C]_p{}^f$	0.34	0.45	

[a] In 0.005 M sodium phosphate buffer, pH 7.4. [b] In 0.005 M sodium phosphate buffer, pH 2.0. [c] In 0.005 M sodium phosphate buffer and 6 M GuHCl, pH 2.0. [d] In mL g^{-1}, measured at protein concentrations of 2–4 mg/mL. [e] Maximum of emission wavelength (nm) for the intrinsic protein fluorescence (excitation at 290 nm). [f] In cal g^{-1} K^{-1}.

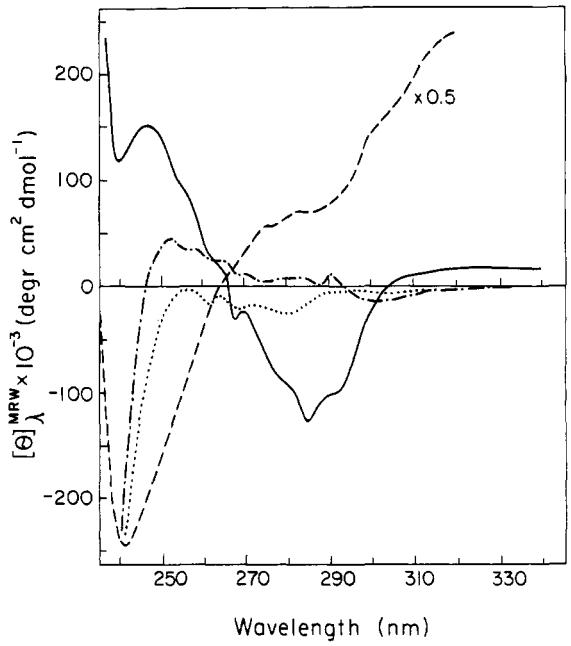

FIGURE 3: Near-UV CD spectra for various forms of RBP. Symbols and experimental conditions are the same as for Figure 2. For holo-RBP, all $[\theta]_\lambda$ values were multiplied by 0.5. Cuvette path length: 10 mm (5 mm for the unfolded state).

concentrations used in our experiments (2–4 mg/mL). These data indicate that the compactness of the apo-RBP molecule at acidic pH is closer to that of the N form of RBP than to that of the U form.

This feature is confirmed by fluorescence spectra of apo-RBP which show that the environment of tryptophan residues in the A state of RBP is much more similar to that in the N state of the protein than to that in the U state (see Table I, which shows the λ_{max} of the intrinsic fluorescence emission bands obtained for N, A, and U states of apo-RBP).

(*c*) *Environment of Side Groups.* Human RBP contains four tryptophans, eight tyrosines and 10 phenylalanines out of a total of 182 residues (Rask et al., 1979). Due to the absence of retinol in apo-RBP, its near-UV CD spectrum is quite different from that of holo-RBP (Figure 3). However, it is still well-pronounced, as is usually the case for rigid native proteins. The near-UV CD spectrum of the A form of apo-RBP is drastically reduced near 285 nm and to a smaller extent also near 250 nm as compared to the N form and is much closer to the spectrum of the U form (in 6 M GuHCl). This suggests that the environment of aromatic groups in the A form of RBP is time-averaged in such a way that these groups have lost their rigid environment.

The absence of the specific native environment of side groups in the A state of apo-RBP is confirmed by the comparison of ^{1}H-NMR spectra of the N, A, and U states of apo-RBP (Figure 4). The ^{1}H-NMR spectrum of the N form of apo-RBP (as well as of holo-RBP, not shown) is typical for native globular proteins and shows well-pronounced high-field resonances at <1 ppm (Wüthrich, 1976). This is consistent with the presence of rigid mutual positions of aromatic and aliphatic side chains in the protein core. The ^{1}H-NMR spectrum of the A form in this region is very similar to that of the unfolded protein, thus indicating that specific mutual positions of aliphatic and aromatic side chains are lost at low pH. Resonances at >1 ppm confirm that many side chains have lost their specific native environment in the A form of RBP. All these features have been observed earlier for the acidic forms of α-lactalbumin (Dolgikh et al., 1985; Baum et al., 1989) and carbonic anhydrase (Rodionova et al., 1989), which are in the molten globule state at acidic pH.

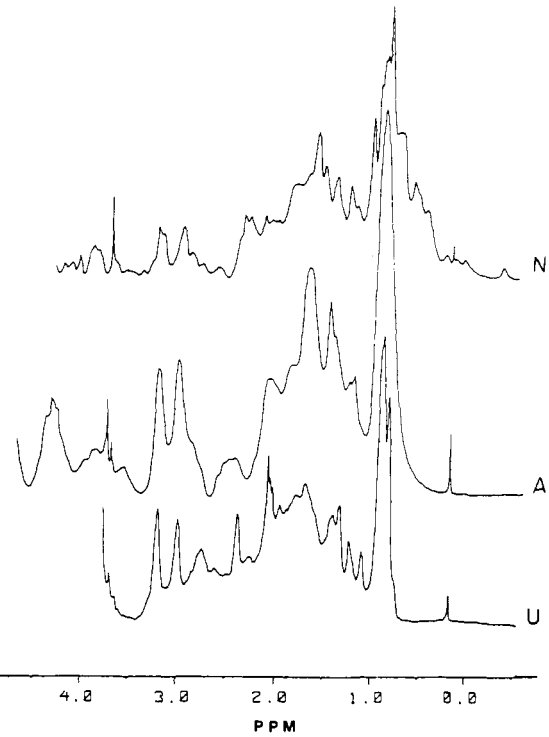

FIGURE 4: High-field ^{1}H-NMR spectra of the N, A, and U forms of apo-RBP. Conditions: A form at 2.5 mg/mL in 0.005 M sodium phosphate, pH* 2.0; N form at 4 mg/mL in 0.05 M sodium phosphate and 0.1 M NaCl, pH* 7.4; U form at 1 mg/mL in 0.005 M sodium phosphate and 7.2 M GuDCl, pH* 2.0. Temperature: 30 °C.

(*d*) *ANS Binding.* A very sensitive and specific test for the molten globule state of protein molecules is the binding of the hydrophobic fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS). It has been recently shown that ANS affinity to the molten globule state is much stronger than to native and unfolded proteins (Semisotnov et al., 1987, 1991; Ptitsyn et al., 1990). The binding of ANS to proteins can be easily monitored by its fluorescence, which is much more intense for protein-bound than for free ANS. Figure 5 shows the intense fluoroescence spectrum of ANS in the presence of the A state of apo-RBP and, for comparison, the much lower intensities of ANS fluorescence in the presence of the N and U (in 6 M GuHCl) states of RBP. This result indicates that the A form of RBP strongly binds ANS, a property typical for proteins in the molten globule state (Semisotnov et al., 1991).

(*e*) *Temperature Melting.* Temperature dependences for the partial specific heat capacity ($[C]_p$) show that the heat absorption peaks for apo- and holo-RBP are similar (Figure 6). The temperatures at the middle of transition are 77 and 79 °C, respectively. The calorimetric melting enthalpies

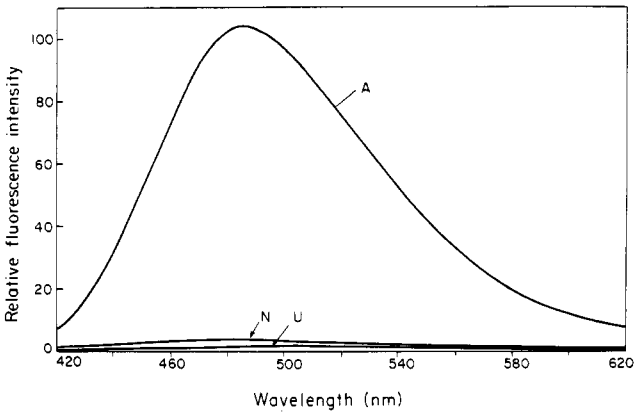

FIGURE 5: Fluorescence emission spectra of ANS in the presence of the A, N, and U forms of apo-RBP. The spectrum of free ANS was neglibible under the same conditions. The ANS:RBP molar ratio was 20:1. Excitation was at 340 nm. Buffer, 0.005 M sodium phosphate at pH 7.4 for the N form and at pH 2 for the A and U forms (the U form was in 6 M GuHCl); protein concentration, 0.1 mg/mL; temperature, 20 °C.

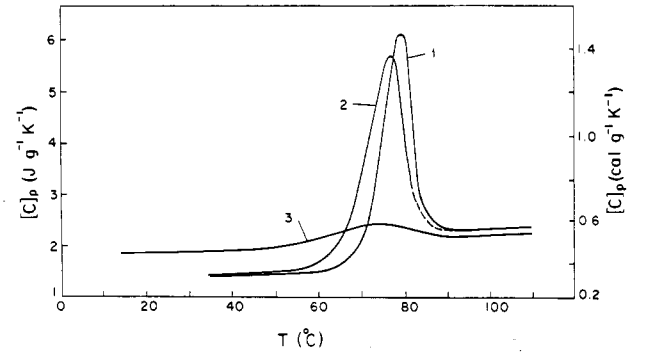

FIGURE 6: Temperature dependences of partial specific heat capacity ($[C]_p$) for various forms of RBP: (1) N form of holo-RBP at 0.5 mg/mL, pH 7.4; (2) N form of apo-RBP at 0.5 mg/mL, pH 7.4; (3) A form of apo-RBP at 1.1 mg/mL, pH 2.0. Buffer, 0.005 M sodium phosphate; cell volume, 0.5 mL; heating rates, 1 K min^{-1}. As apo-RBP at pH 7.4 tends to precipitate at high temperatures, the high-temperature part of its melting curve (2) was drawn as a dashed line extrapolating to the $[C]_p$ value obtained for holo-RBP at $T \geq 90$ °C.

(ΔH_{cal}) for neutral holo- and apo-RBP (i.e., the melting heat per mole of protein) are about −200 kcal/mol, while the van't Hoff melting heats (ΔH_{VH}) (i.e., the melting heat per mole of protein cooperative units) are about −100 kcal/mol. A value for the $\Delta H_{cal}/\Delta H_{VH}$ ratio of approximately 2 suggests the existence of at least two cooperative units (Tiktopulo & Privalov, 1978) in a molecule for both forms of RBP. More experimental evidence is required to clarify the significance of these units from a structural point of view. The similarity of heat absorption peaks for neutral apo- and holo-RBP suggests that the binding of retinol to RBP is mainly an entropic process, in agreement with the conclusion of Noy and Xu (1990b).

On the contrary, the heat capacity–temperature dependence for the A form of apo-RBP has only a very smooth and broad maximum (Figure 6), which suggests that at pH 2.0 apo-RBP molecules are almost melted already at room temperature. This maximum increases with protein concentration (data not shown) and, therefore, may reflect the formation of small protein aggregates at acidic pH and high temperatures. However, we cannot exclude that this smooth maximum is related, at least partly, to the melting of some traces of rigid tertiary structure present in the A form of apo-RBP. Figure 6 shows that the value of $[C]_p$ for the A state of apo-RBP is as large as 1.9 J g^{-1} K^{-1} (0.45 cal g^{-1} K^{-1}) already at 20 °C, which corresponds to the $[C]_p$ value typical for temperature-denatured proteins. On the other hand, $[C]_p$ for apo- and holo-RBP at neutral pH is equal to 1.40 J g^{-1} K^{-1} (0.34 cal g^{-1} K^{-1}) at 20 °C.

(f) Effect of Ionic Strength. The A form of apo-RBP at high ionic strength (0.05 M sodium phosphate buffer and 0.1 M NaCl, pH 2.0) has properties similar to those of the A form at low ionic strength. Similar is the far-UV CD spectrum and the near-UV CD spectrum is dramatically reduced as compared to that of the protein at neutral pH (data not shown). The A form of apo-RBP at high ionic strength binds ANS very strongly and practically has no heat absorption peak upon heating. A substantial association of the A form at high ionic strength makes the evaluation of the molecular compactness by hydrodynamic measurements difficult. However, fluorescence data obtained at low protein concentrations are consistent with the compact state of apo-RBP. In fact, λ_{max} at pH 2.0 and high ionic strength (336 nm) is very near to that for neutral pH (335 nm), while λ_{max} for the U form is 350 nm. Polarization of the tryptophan fluorescence of the A form is equal to 0.13, which is even larger than that of the N form (0.10), while fluorescence polarization for the U form is 0.06. All these data leave little doubt that the A form of apo-RBP is also in the molten globule state at high ionic strength.

DISCUSSION

We have shown that apo-RBP can exist in at least two quite different states in water solution. The N state, attained after extraction of retinol from holo-RBP with organic solvents, has the properties of a rigid native protein. The A state maintains pronounced secondary structure and is much more compact than the U state. On the other hand, rigid intramolecular contacts are dramatically reduced. This state does not exhibit a highly cooperative temperature melting and strongly binds ANS. All these features are typical for the molten globule state of proteins (Ptitsyn, 1992).

The molten globule state is also attained when holo-RBP is transferred to low pH solutions where it spontaneously releases retinol (data not shown). The question arises as to which state RBP is in when it releases retinol under physiological conditions near the cell surface. To answer this question we must bear in mind that a high density of negatively charged groups at the membrane surface can create a strong electrostatic potential which leads to a local acidic pH (Prats et al., 1986; Stegmann et al., 1989; McLaughlin, 1989). The most clear evidence for this effect has been obtained very recently by van der Goot et al. (1991), who have shown that the surface pH for negatively charged dioleoylphosphatidylglycerol liposomes is 1.6 units lower than that in the bulk. An even larger shift of pH (2.7 units) has been predicted by the same authors on the basis of the Gouy–Chapman theory. A pH value as low as 4.5 is sufficient to induce a significant release of retinol from holo-RBP and transition of apo-RBP into the molten globule state (Figure 1). A similar surface pH value might be found on the membranes of target cells if the shift of pH with respect to the bulk is not too different from that estimated for dioleoylphosphatidylglycerol liposome surfaces. This leads us to propose that the release of retinol from RBP near the cell membrane is facilitated by a local acidic pH and that the RBP molecule which has released retinol under these conditions is in the flexible molten globule state rather than in a rigid state.

We have suggested earlier that the molten globule state may be important for a number of processes occurring in vivo, including the translocation of proteins through membranes as

well as their folding and degradation (Bychkova et al., 1988). At least two of these predictions have been afterward confirmed by experiments: it has been shown that the chaperone GroEL recognizes dihydrofolate reductase and rhodanese in the molten globule state (Martin et al., 1991) and that the pore-forming domain of colicin A is inserted into liposome membranes in the molten globule state (van der Goot et al., 1991). Now we would like to extend this hypothesis to suggest that a transition from the native to the molten globule state of proteins might also be important for the delivery of ligands by carrier proteins (like RBP) to target cells.

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