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Conformational Studies of the Human Vitamin A–Transporting Protein Complex*

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ABSTRACT: The human vitamin A–transporting protein complex and its two component proteins, prealbumin and the retinol-binding protein (RBP), have been studied by optical rotatory dispersion (ORD) and circular dichroism (CD). Some information about the absolute conformation of the two proteins was obtained from these measurements. Thus neither protein seems to contain any appreciable amount of α helix. Prealbumin may contain some regions of β structure, but more probable is that some nonperiodic, unordered, yet rigid, structure dominates both in this protein and in RBP. The major features of the ORD and CD spectra of RBP are caused by the protein moiety, vitamin A being responsible only for certain minor characteristics. Dissociation of the prealbumin–RBP complex is effected at low ionic strength, as has been described earlier; a major result of this study is that a conformational change associated with this phenomenon occurs in RBP and not in prealbumin. Another conformational change, distinct from the previous one, takes place when COOH-terminal arginine is cleaved from the polypeptide chain of RBP, a reaction that occurs physiologically. Finally, measurements of ORD and CD of the RBP–prealbumin complex reveal that at most minimal alterations of the conformations of the two proteins occur when they interact.

The human vitamin A–transporting protein complex consists of two proteins, the thyroxine-binding prealbumin and the retinol-binding protein (RBP),[1] which is the actual vitamin carrier (Kanai *et al.*, 1968; Peterson, 1969, 1971a). The two proteins are attached to each other by noncovalent bonds, but their interaction is very strong under physiological conditions, the apparent association constant being 2×10^7 M^{-1}. The complex is an example of a protein–protein interaction with a high degree of specificity (Peterson and Rask, 1971). The binding between prealbumin and RBP is abolished at low ionic strength, and concomitantly the fluorescent properties of the retinol bound to RBP are also altered (Peterson, 1971b, Peterson and Rask, 1971). This finding, together with the fact that there exists a modified form of RBP in normal serum which cannot bind to prealbumin (Peterson, 1971c)

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[1] Abbreviations used are: RBP, retinol-binding protein; ORD, optical rotatory dispersion; CD, circular dichroism.

suggest that the protein components of the prealbumin–RBP complex under certain conditions may undergo conformational changes incompatible with sustained interactions between the two proteins (Peterson, 1971c; Rask *et al.*, 1971b). To verify these suggestions and to obtain some information about the prealbumin–RBP complex and its individual components in aqueous solution it is necessary to collect detailed information about a number of important regions of the molecule. It is well known that optical rotatory dispersion and circular dichroism provide a powerful tool for elucidation of conformational changes. Accordingly, studies were initiated to investigate the optical properties of prealbumin and RBP when free and when interacting.

Materials and Methods

Proteins. The prealbumin–RBP complex was isolated from outdated plasma as described previously (Peterson, 1971a). Prealbumin was prepared according to the procedure recently outlined by Rask *et al.* (1971a). RBP was obtained from the prealbumin–RBP complex by gel chromatography on Sephadex G-100 at low ionic strength (Peterson, 1971a) or from urine by affinity chromatography on a column of prealbumin coupled to Sepharose (Vahlquist *et al.*, 1971). To free RBP from retinol, solutions of the protein were extracted with heptane (Peterson, 1971b). The resulting protein solution, which was slightly turbid, was centrifuged, and the supernatant was passed over the affinity chromatography column. Only protein which was retained on the column and which was subsequently eluted with deionized water was used for the analyses of retinol-poor RBP (Rask *et al.*, 1971b). The species of RBP devoid of retinol and unable to bind to prealbumin was isolated from urine (Rask *et al.*, 1971b).

Measurements of ORD and CD Spectra. ORD and CD spectra were measured with a Jasco Model J-20 spectropolarimeter. The proteins studied were dissolved in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. For studies at different ionic strengths, the buffer was 0.002 M Tris-HCl (pH 8.0) and the ionic strength was varied by addition of 2 M NaCl. The spectra were recorded at room temperature (25–27°), using protein concentrations of 0.1–1.0 mg/ml and cells with from 10- to 1-mm path lengths. The optical density never exceeded 1.5 except at wave lengths lower than about 205 nm. The results are given as plots of reduced mean residue rotation, $[m']$, or reduced mean residue ellipticity, $[\theta']$, *vs.* wavelength. Each curve represents the average of at least two measurements on different samples. The parameters $[m']$ and $[\theta']$ were computed in the usual manner (Björk and Tanford, 1971). The mean residue weights were calculated from the amino acid compositions of the proteins and were found to be 115 for RBP and 110 for prealbumin.

Protein concentrations were determined spectrophotometrically in a Hitachi Perkin-Elmer Model 139 spectrophotometer. Specific extinction coefficients of 17.5 for RBP and 13.5 for prealbumin were used (Peterson, 1971a).

Results

The ORD spectrum of native RBP, saturated with retinol and analyzed in a solvent of physiological ionic strength (0.16), is shown in Figure 1. The protein exhibits two superimposed Cotton effects, one minor with a flat minimum at about 250 nm and a sharper maximum at 235 nm, and a major effect with a trough at 216 nm ($[m'] \sim -3000°$) and a maximum at 202 nm ($[m'] \sim +4000°$). The corresponding CD spectrum (Figure 2A,C) has four well-resolved bands, a small positive one at 325 nm with a reduced mean ellipticity of about +200°, a small negative band at 240 nm with an ellipticity of about −300°, a positive band at 226 nm of somewhat larger magnitude ($[\theta'] = +700°$), and finally a main negative band at 212 nm ($[\theta'] \approx -1500°$). In order to investigate if any of these features may be due to bound retinol, RBP from which the vitamin had been removed by heptane extraction was studied. Some minor changes of the spectra were noted. In ORD (Figure 1) a progressive lowering of the mean residue rotation in the higher wavelength range (225–280 nm) with a preservation of the individual features of the original spectrum is seen with decreasing concentrations of vitamin A. No change was detected in the main trough and peak, however. In CD only one preparation of retinol-extracted RBP, in which 15% of the molecules contained the vitamin, was investigated (Figure 2A,C). The most prominent changes are also in this case in the higher wavelength range (Figure 2C). The broad positive band at 325 nm has diminished and the negative band at 240 nm has essentially disappeared. These two features are thus largely due to the presence of vitamin A. The small differences in the curves of the retinol-poor and retinol-saturated RBP seen below 210 nm (Figure 2A) are hard to evaluate since the experimental errors in this region are rather large.

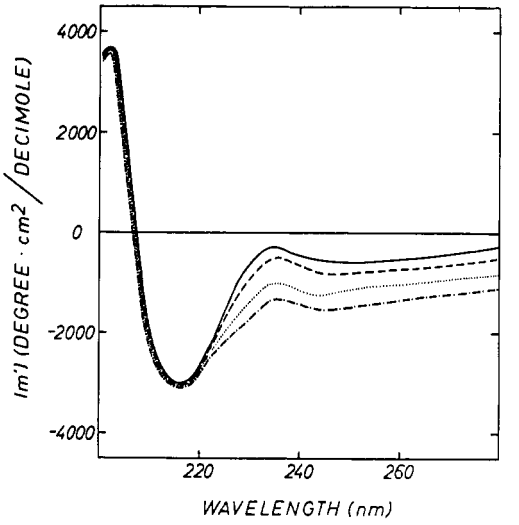

FIGURE 1: Optical rotatory dispersion of heptane-extracted RBP containing different amounts of retinol. The measurements were performed at physiological ionic strength. The molar ratios of retinol to RBP in the preparations used were: 1.0 (——); 0.76 (---); 0.43 (. . .); 0.15 (·-·-).

A species of RBP, which is unable to bind to prealbumin and which contains negligible quantities of a retinol, is encountered both in serum and urine (Peterson, 1971c). This species was recently shown to lack the COOH-terminal arginine present in its retinol-containing counterpart; no other differences in primary structure between the two proteins could be detected (Rask *et al.*, 1971b). This modified form of RBP shows ORD and CD spectra rather different from those of retinol-containing RBP. In ORD the main minimum has shifted to 220 nm and decreased in magnitude, and the small Cotton effect centered around 240 nm is much less pronounced. In CD (Figure 2B,C) the two positive bands at 325 and 226 nm are much attenuated or completely absent

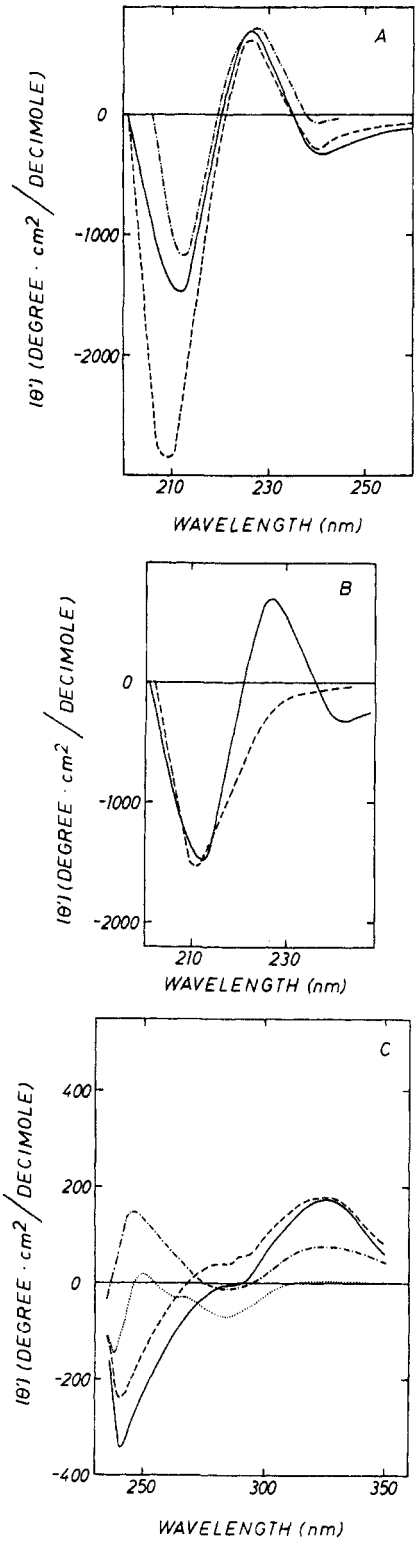

FIGURE 2: Circular dichroism measurements. (A) Between 200 and 260 nm of retinol-containing RBP (molar ratio retinol to protein 1.0) at physiological ionic strength (——) and at low ionic strength (0.007) (---) and of heptane-extracted RBP at physiological ionic strength (-·-·-). (B) Between 200 and 250 nm at physiological ionic strength of retinol-containing RBP (molar ratio retinol to protein 1.0) (——), and of RBP devoid of retinol and the COOH-terminal arginine (---). (C) Between 240 and 350 nm of retinol-containing RBP (molar ratio retinol to protein 1.0) at physiological ionic strength (——); of heptane-extracted RBP having a molar ratio of retinol to protein of 0.12, at physiological ionic strength, (-·-); of RBP devoid of retinol and the COOH-terminal arginine at physiological ionic strength (····); and of retinol-containing RBP (molar ratio retinol to protein 1.0) at an ionic strength of 0.007 (---).

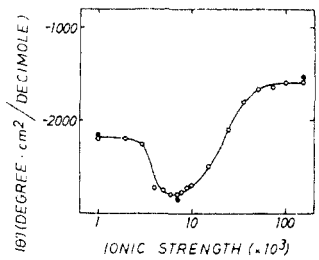

FIGURE 3: Measurements of the ellipticity at 212 nm of retinol-containing RBP in solutions of varying ionic strength. The measurements were carried out by adjusting the ionic strength from 0.001 to appropriate values by addition of NaCl (O) or by lowering the ionic strength by dialysis (●).

and instead a new small positive band at 250 nm is evident. The main 212-nm band is virtually unaffected.

Investigation of the conformation of RBP at low ionic strength is of interest, since the prealbumin–RBP complex dissociates under these conditions. In ORD no differences outside experimental error were found between the spectra of RBP at high and low ionic strength in the accessible wavelength range. These spectra therefore are not shown. In CD, however, a large difference is seen in the main negative band, which has approximately twice the magnitude and also has shifted from 212 to 209 nm (Figure 2A). The differences at higher wavelengths are only minor (Figure 2C). The large difference between the CD spectra of RBP at high and low ionic strengths should be associated with a difference also in the ORD spectra, since the two methods are interrelated. It is probable that such a difference, although not observed, exists in the lower wavelength range of ORD (below 205 nm), where experimental errors are so large as to preclude meaningful measurements of the small rotations of RBP.

The conformational change that occurs with decreasing ionic strength was followed more closely by measuring the ellipticity at 212 nm (Figure 3). Two transitions are evident. The first one, with a midpoint at an ionic strength of about 0.02, results in a pronounced decrease in ellipticity to about $-2800°$, whereas during the second transition (midpoint $\Gamma/2 = 0.004$) the ellipticity again increases to $-2200°$. These conformational changes are completely reversible as shown in Figure 3.

The conformation of prealbumin, a protein which is able to form a complex with RBP, was also investigated (Figure 4A,B). Both the ORD and CD curves show less detail than those of RBP. There is a minimum in the ORD curve at 227 nm ($[m'] \approx -3000°$), but the maximum of the Cotton effect is below 200 nm, where measurements were not carried out due to large experimental errors. In CD there is a major negative band at 214 nm with a reduced mean residue ellipticity of $-5500°$; no fine structure in the aromatic region was found. The conformation of prealbumin is entirely unaffected by changes of the ionic strength, as also is shown in Figure 4.

Finally, the ORD and CD curves of the complex between RBP and prealbumin were measured at physiological ionic strength. These curves are given in Figure 5A,B and are compared with the theoretical curves for an equimolar mixture of RBP and prealbumin, computed from the individual curves presented earlier. As can be seen there is complete agreement between the two curves both in ORD and CD, signifying that at most minimal conformational changes occur in either of the two proteins on complex formation.

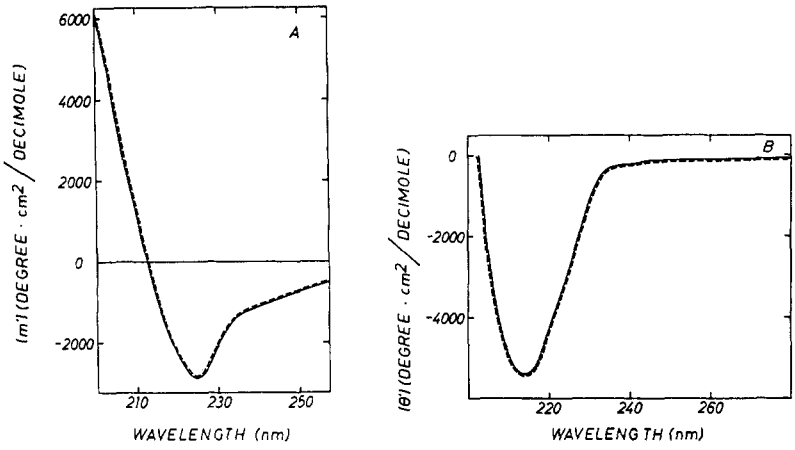

FIGURE 4: Measurements of the ORD (A) and CD (B) of prealbumin at ionic strengths of 0.16 (——) and 0.007 (---).

Discussion

The interaction between RBP and prealbumin is highly specific, but the physiological importance of this binding is not well understood at present. However, two effects of the complex formation are evident. The binding of prealbumin to RBP thus restricts the elimination of the small RBP molecule through the glomerular barrier (Kanai *et al.*, 1968; Peterson and Berggård, 1971; Peterson, 1971a,c) and also to a large extent excludes water from contact with the retinol moiety of RBP (Peterson, 1971b; Peterson and Rask, 1971). From investigations of the prealbumin–RBP complex at low ionic strength and from experiments using specific antibodies against the two proteins the suggestion has been made that RBP, but not prealbumin, may undergo conformational changes at low ionic strength and when its COOH-terminal amino acid is cleaved off, a reaction that occurs physiologically (Peterson, 1971c; Peterson and Rask, 1971). Optical rotatory dispersion and circular dichroism have been employed to further characterize these conformational changes. In addition, some limited information on the absolute conformation of the two proteins and their complex can be extracted from such measurements.

Although the polyene retinol has a high absorbance in the ultraviolet region and is known to be strongly fluorescent it is evident from both the ORD and CD studies presented here that the optical activity of RBP to a major extent is due to the protein moiety. Retinol is responsible only for certain minor characteristics of the spectra in the high-wavelength region examined, which will be discussed below. The major features of the ORD and CD spectra of RBP are not compatible with any appreciable degree of α helix; indeed, calculations by equations given by Jirgensons (1969) and Greenfield and Fasman (1969) give essentially zero helix content. Furthermore, it is impossible to generate either the ORD or the CD spectrum by any combination of the spectra measured for poly-L-lysine in the α helix, β structure, or random coil form, which has been successfully done for some proteins (Greenfield *et al.*, 1967; Greenfield and Fasman, 1969). It is obvious, then, that a major part of RBP is folded into some periodic structure other than α helix or β structure, or more likely into some nonperiodic, unordered but rigid structure. This does not exclude, however, the existence of short segments of α helix or β structure.

The fine structure of the RBP spectra in the higher wavelength range is very intriguing, especially that of the CD spectrum, since much less detail is observable in the ORD spectrum. The small positive peak at 325 nm and the larger negative band at 240 nm probably arise from the presence of retinol in RBP. These features may be produced by the vitamin itself or, more likely, may be associated with its interaction with the protein moiety. The 240-nm band is interesting since similar bands exist in a few other proteins and have been attributed to an amino acid side-chain chromophore, *e.g.*,

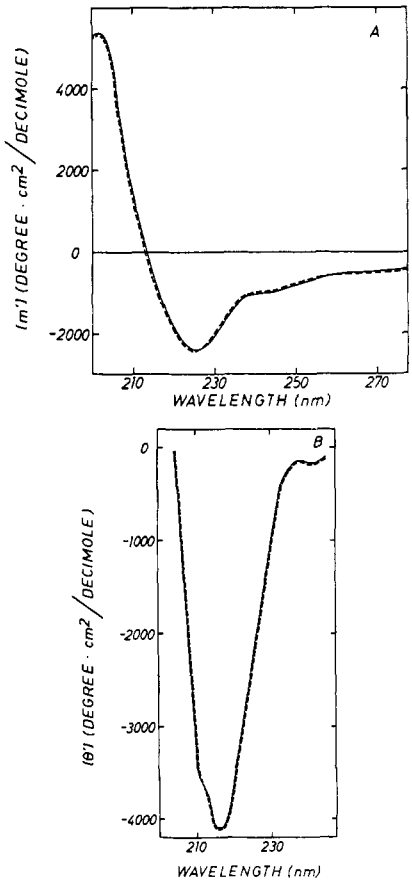

FIGURE 5: Measurements at physiological ionic strength of the ORD (A) and CD (B) spectra for the prealbumin–RBP complex (——). The same spectra computed for an equimolar mixture of prealbumin (Figure 4) and RBP (Figure 1 and 2) are included for comparison (---).

that of cystine (Beychok, 1966). The unequivocal demonstration of the nature of this band is, however, uncertain, especially in view of the fact that a similar negative band is present also in the random coil form of poly-L-glutamate and poly-L-lysine (Velluz and Legrand, 1965).

The fairly large positive band at 226 nm observed in the CD spectrum of RBP does not seem to have any correspondence in the ORD spectrum but may be masked by the high-background rotation. This 226-nm band is rarely seen in the CD spectra of proteins but has been observed in the case of soybean trypsin inhibitor (Jirgensons, 1969b). In this protein it disappeared after reduction and alkylation. Oxidized glutathione also has a positive CD band at 226 nm, which, however, is abolished on reduction (Coleman and Blout, 1968). Possibly the corresponding band in the RBP spectrum is in some way related to a disulfide bond.

Recent studies from this laboratory have shown that there exists a physiological form of RBP which is unable to bind to prealbumin and is devoid of retinol and the COOH-terminal arginine residue (Peterson, 1971c; Rask *et al.*, 1971b). This species of RBP has a considerably altered conformation compared to its retinol containing counterpart as revealed by the ORD and CD spectra. It is, however, at present impossible to ascertain the exact nature of this conformational difference. It is probably mainly a result of a rearrangement of the molecule induced by the loss of the COOH-terminal arginine, and not by the concomitant loss of retinol, since this ligand is responsible only for certain minor characteristics in the optical activity of unmodified RBP.

Apart from this conformational change of RBP that is associated with the cleavage of COOH-terminal arginine from the polypeptide chain, hydrodynamic and fluoresence studies have indicated that molecular architecture of RBP depends on the ionic strength of the environment (Peterson, 1971b; Peterson and Rask, 1971). This suggestion is validated by the present investigations. A conformational change is demonstrated by the CD spectrum of RBP at low ionic strength. The perturbation of the conformation of RBP induced by this environment largely affects that region of the molecule which interacts with prealbumin, since the affinity of RBP for this protein is abolished (Peterson and Rask, 1971), and the conformation of prealbumin is unaffected at low ionic strength (see below). The curve obtained by measuring the ellipticity at 212 nm at different ionic strengths exhibits two discrete transitions, suggesting two sequential conformational changes of the protein. Only the one occurring at the lowest ionic strength seems to involve a rearrangement of the site to which prealbumin is attached, since prealbumin and RBP show a diminished affinity for each other only at this and lower ionic strengths (Peterson and Rask, 1971).

Prealbumin exhibits much simpler spectra than RBP. Neither the minimum at 227 nm in ORD nor the minimum at 214 nm in CD are compatible with any appreciable amount of α helix (Greenfield *et al.*, 1967; Greenfield and Fasman, 1969) and, in agreement with this, calculations by the methods mentioned earlier gave zero helix content. The minima described are instead more suggestive of a structure consisting of 20–40% random coil and 60–80% β structure, judging from theoretical curves based on measurements of poly-L-lysine in the two different conformations (Greenfield *et al.*, 1967; Greenfield and Fasman, 1969). The validity of this reduction is, however, open to serious doubt and it is possible that also in the case of prealbumin some kind of rigid, nonperiodic structure is instead dominating.

Both ORD and CD spectra obtained for prealbumin at physiological ionic strength are identical with the corresponding spectra measured at low ionic strength; thus no major conformational change has occurred in the latter case. This finding corroborates other indications of the absence of such an effect (Peterson, 1971c; Peterson and Rask, 1971). One of the main conclusions of this paper, therefore, is that the conformational change responsible for the dissociation of the RBP–prealbumin complex at low ionic strength occurs entirely within the RBP molecule; the conformation of prealbumin is not affected at all.

The prealbumin–RBP complex shows spectra at physiological ionic strength both in ORD and CD which are identical with those computed from the curves of the individual proteins. Hence, another major conclusion is that on formation of the complex between prealbumin and RBP, the conformations of the two proteins are altered very slightly, if at all. This indicates that the interacting sites on prealbumin and RBP have such conformations that at most minimal structural rearrangements of the sites are necessary for the highly specific and strong interactions between the two proteins to occur.

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