ABSORPTION SPECTRA FOR THE COMPLEXES FORMED FROM VITAMIN-A AND β-LACTOGLOBULIN

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ABSTRACT The interactions between vitamin-A and β-lactoglobulin have been investigated. We have found that two different complexes can be formed: one involving vitamin-A, and one involving a derivative of vitamin-A that most probably has a retro-β-ionylidene structure. Room temperature absorption spectra for these complexes in phosphate buffer at pH 7.50 are reported and discussed.

INTRODUCTION

The ubiquitous role of 11-*cis* retinal as the chromophore in visual pigments argues strongly for the thesis that there is something quite special about the interactions between this molecule and its protein matrix in rhodopsin and the changes in these interactions that are produced by electronic excitation. In this case, a clear understanding of polyene-protein interactions and their spectroscopic consequences would contribute significantly to our ability to interpret the rapidly accumulating data on rhodopsin. It is to this end that we have initiated a program to produce well-characterized polyene-protein complexes and to study in detail their spectroscopic properties. The selection of the β-lactoglobulin-vitamin-A system for our initial studies was stimulated by the report of a structured absorption spectrum for the complex by Georghiou and Churchich(1) and further encouraged by the possibility of obtaining a high resolution x-ray structure.[1] We have thus set about to characterize further the binding of vitamin-A to β-lactoglobulin, to measure the spectroscopic properties of the complex (absorption, emission, and resonance Raman studies), and to produce crystals for x-ray-diffraction studies. We would like to report on the initial characterization of the complexes.

The Protein

β-lactoglobulin (BLG) is a globular protein found in the milk of all mammals. We have dealt exclusively with bovine BLG which exists under physiological conditions as a dimer of molecular weight 36,000. Depending on genetics, subunits of either type A or B may be formed. Thus there are three types of dimers, called A, B, and M (mixed). Differences in the isoelectric points of these phenotypes have been described by Tanford and Nozaki(2). Piez et al.(3) have separated types A and B by chromatography on diethylaminoethylcellulose and gel electrophoresis and reported amino acid analyses. The state of aggregation of this protein is strongly pH dependent. Townend et al.(4) found that mixed BLG undergoes a reversible dissociation below pH 3.5 due to nonspecific electrostatic repulsion between the 18,000 mol wt

[1]Sawyer, L. Private communication.

subunits. Townend et al.(5,6) further reported that between pH 3.7 and 5.2 BLG undergoes a reversible tetramerization (eight subunits) at temperatures close to 0°C.

Near the isoelectric point (pH 5.20) and up to neutral pH BLG exists as a dimer (two subunits) having a molecular weight of 36,000. Tanford et al.(7) reported that at pH 7.5 a novel reversible transformation was detected by changes in specific optical rotation with pH. Apparently, no general unfolding but rather a refolding of part of the peptide chains is involved. Groves et al.(8) reported that at pH $\geqq$ 8.0 BLG undergoes irreversible denaturation.

Because of the genetic variability of the subunits and the strong dependence of the degree of aggregation on pH and temperature, many different forms of BLG crystals can be obtained. This has been reviewed by Aschaffenburg(9). The x-ray-diffraction studies at Edinburgh (see footnote 1) are concerned with lattice types *x* and *z* in Aschaffenburg's notation(9), which crystallize at pH 6 from 2 M $(NH_4)_2SO_4$ and pH 8 from 2 M K_2HPO_4, respectively. Accordingly, our work on complex characterization has been at pH's in the 6 to 8 range, most often pH 7.5 as realized in 0.1 M KH_2PO_4-NaOH buffer.

EXPERIMENTAL PROCEDURES

Reagents

β-lactoglobulin was obtained either from fresh cow's milk, following the method of Aschaffenburg and Drewry(10), or purchased from the United States Biochemical Corp.(Cleveland, Ohio.) We could find no differences in behavior between the commercially available protein and that extracted from milk in our laboratory. In the extraction procedure the protein is salted out of the reaction mixture and collected by filtering over celite. After dilution to solubilize the protein, and removal of the celite by filtration, the protein was crystallized by dialysis in H_2O at pH 5.2. The crystals were collected by centrifugation, allowed to dry, and kept at ~4°C until used.

All-*trans* retinol was used as obtained from Hoffman-La Roche, Inc. (Nutley, N.J.) or was prepared from all-*trans* retinal (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) by $NaBH_4$ reduction following the method of Brown and Wald(11). The commercially available compound and that obtained from freshly reduced aldehyde behaved identically in all respects.

Complex Preparation

We have found that two distinct complexes can be prepared, depending on the relative concentrations of vitamin-A and protein. At vitamin-A-to-protein molar ratios of ~1:1, a complex in which the chromophore is all-*trans* retinol is the primary product. At vitamin-A-to-protein molar ratios of 2:1 or higher, a complex in which the chromophore is a retro derivative of vitamin-A is formed. This is discussed more completely below.

In a typical preparation of the vitamin-A complex, an equimolar amount of vitamin-A in ethanol (~0.23 mg/ml) is added to a ~0.08% by weight solution of β-lactoglobulin in K_2HPO-NaOH buffer (pH 7.50). In all cases, the final volume of ethanol in the reaction mixture was kept below 5% of the total volume to minimize protein degradation.

The preparation of the retro complex followed procedures suggested by Song.[2] In this preparation, a 20- to 30-fold molar excess of vitamin-A in ethanol is added to protein solution (~4 mg/ml) in phosphate buffer (pH 7.50). The final ethanol concentration in the reaction mixture was kept below 2% by volume. The complex was then purified by chromatography on Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.). Typical protocols are detailed together with the observed absorption spectra in the next section.

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[2]Song, P. S. Private communication.

PROPERTIES OF THE COMPLEXES

In this initial survey, the complexes have been characterized on the basis of their near ultraviolet absorption spectra. In the presence of the protein, the absorption spectrum of vitamin-A is significantly changed, as can be seen in Fig. 1. In addition, the protein absorption band at 278 nm shifts slightly and changes shape. In experiments where vitamin-A is added to buffer solutions that do not contain BLG, the chromophore absorption is completely lost in tens of minutes. In the presence of the protein, the chromophore absorption is stable for weeks.

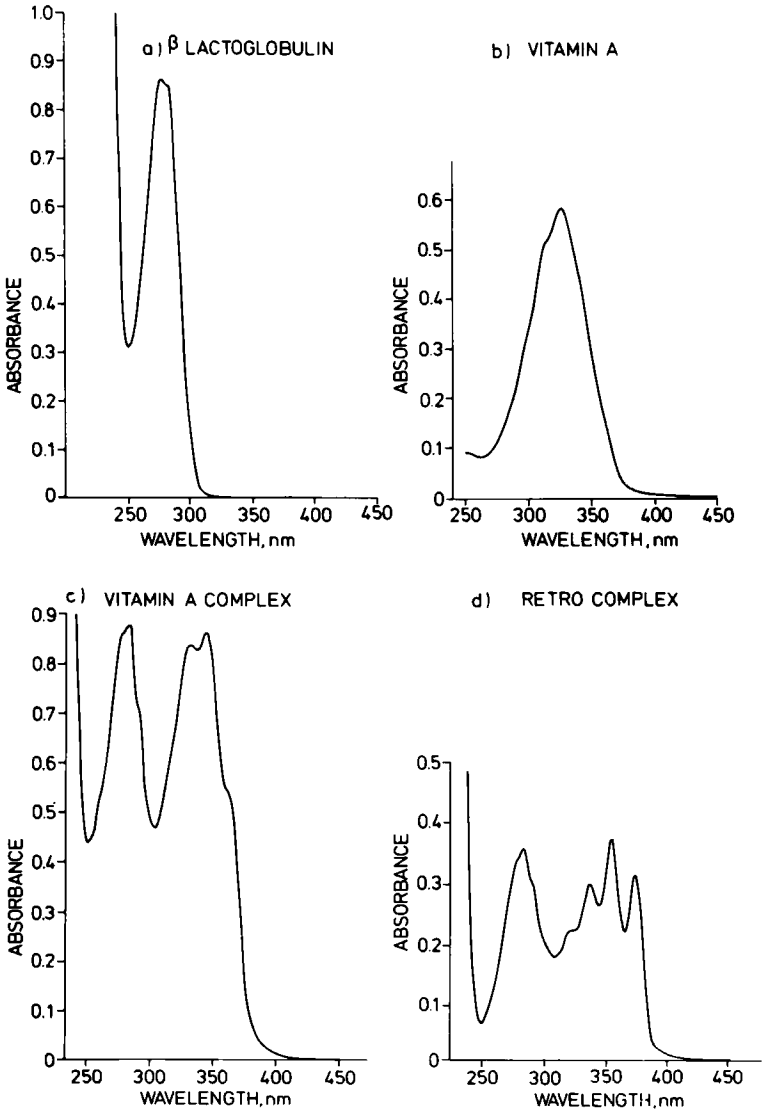


FIGURE 1 Room temperature absorption spectra. (*a*) BLG 2.5×10^{-5}M in phosphate buffer at pH 7.50. (*b*) Vitamin-A 1.1×10^{-5}M in ethanol. (*c*) Complex between BLG and vitamin-A in phosphate buffer at pH 7.50. (*d*) Complex between retro derivative of vitamin-A and BLG in phosphate buffer at pH 7.50.

These altered absorption spectra and the greatly enhanced stability of the polyene chromophore in polar solution argue convincingly for complex formation. Both the vitamin-A complex and the retrovitamin-A complex can be thermally denatured and the chromophore extracted into hexane. For the vitamin-A complex, the absorption spectrum of the extracted chromophore is the same as that observed before complexation, confirming the structure of the bound chromophore. In the case of the retro complex, the extract exhibits a structured absorption spectrum, as would be expected for a retro derivative. A more detailed description of the preparation and properties of each of the complexes is given below.

Complex with Vitamin-A

In a typical preparation, 0.25 ml of 2.4×10^{-3}M vitamin-A in ethanol was pipetted into 9.75 ml of BLG (0.9 mg/ml in phosphate buffer, pH 7.50). The absorption spectrum of the resulting complex is compared with those of the BLG and vitamin-A stock solutions in Fig. 1. The chromophore absorption maximum is shifted red by ~20 nm and shows shoulders at 366, 345, and 330 nm. The tyrosine-tryptophan peak for the protein at 278 nm is also altered slightly. Assuming that the molar extinction coefficient of the chromophore is unchanged in the complex (ϵ-52,800 l mol^{-1} cm^{-1}), we estimate that almost 40% of the vitamin-A is lost in the aqueous solution and 60% is bound into BLG.

Once formed, the complex is quite stable. Over a period of 3 d at room temperature, the absorption spectrum did not change except for a slight rise in the baseline due to the turbidity induced by bacterial degradation. The complex may be dissociated by treatment with hexane at 76°C. The spectrum of the hexane layer corresponds exactly to that of vitamin-A whereas the protein absorbance at 278 nm remains with the aqueous layer. Typical spectra are shown in Fig. 2. Attempts to extract the chromphore under gentler conditions were not successful.

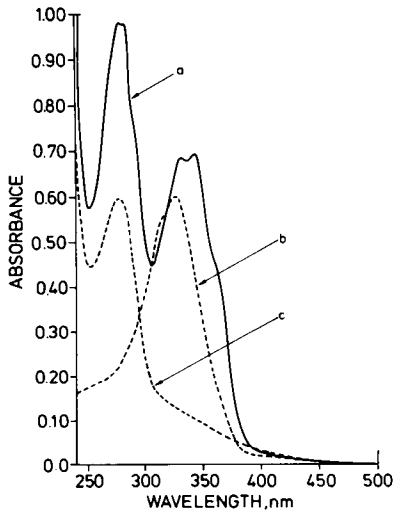


FIGURE 2 Chromophore extraction. Curve *a* is the absorption spectrum of the BLG-vitamin-A complex in phosphate buffer at pH 7.50. Curve *b* is the spectrum of the hydrocarbon phase, and curve *c* is the spectrum of the aqueous phase after extraction by hexanes at 76°C.

Identical chromophore spectra were obtained upon extraction irrespective of the time after complex formation, which ranged from a few minutes to several days.

When using the 60% yield for the complex formation derived from the free chromophore extinction coefficient, the yield for the extraction process described above is in excess of 90%. If, in the original reaction mixture, the molar ratio of vitamin-A to protein is significantly greater than 1:1, there is evidence that a second chromophore with a more structured absorption spectrum is formed that also binds to BLG. The left side of Fig. 3 shows the result of the hexane extraction procedure on a solution of complex prepared from a solution that initially contained a 2:1 molar ratio of vitamin-A to BLG. The right side of Fig. 3 show the chromophore obtained by extracting the aqueous phase from the first extraction a second time with hexane. This chromophore, which we believe has a retro-β-ionylidene configuration, also forms a complex with BLG, as described in the next section.

Retro-Vitamin-A Complex

Here we have followed procedures suggested by Song (see footnote 2). In a typical preparation, 0.1 ml of 0.18 M vitamin-A in ethanol was pipetted into 5 ml of BLG (4.4 mg/ml in phosphate buffer, pH 7.50). Thus, the original reaction mixture contains ~31 mol of vitamin-A per mole of BLG. Development of the complex may be followed by diluting a portion of the original reaction mixture and monitoring the absorption spectrum as a function of time. In this specific example, 0.1 ml of the original reaction mixture was diluted to 1/50 the original concentration. As can be seen in Fig. 4, the chromophore absorption develops structure and decreases in intensity. We believe that the relatively sharply defined vibrational

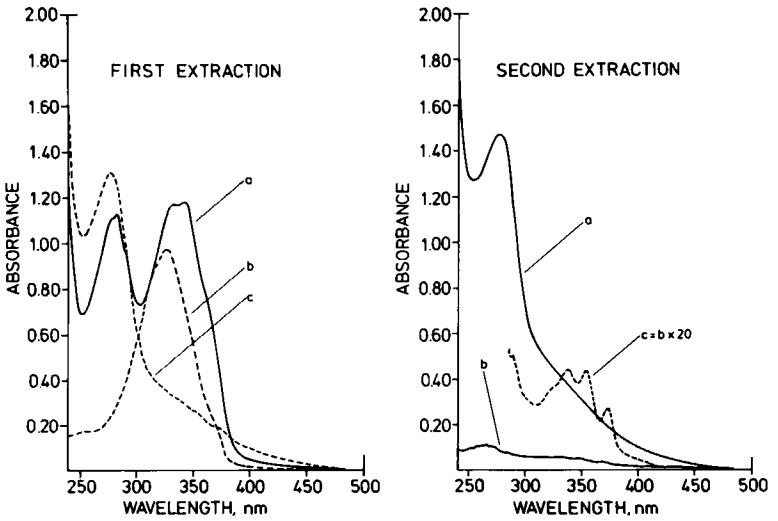


FIGURE 3 Evidence for a second complex. These absorption spectra apply to a BLG-vitamin-A complex prepared with a twofold molar excess of vitamin-A. In the left-hand box, curve *a* is the absorption spectrum of the reaction mixture before extraction, curve *b* is the absorption spectrum of the hydrocarbon layer, and curce *c* is the spectrum of the aqueous layer after the first extraction by hexanes at 76°C. In the right-hand box, curve *a* is the absorption spectrum of the aqueous layer from the first extraction, and curves *b* and *c* are spectra for the hydrocarbon layer resulting from a second extraction by hexanes at 76°C.

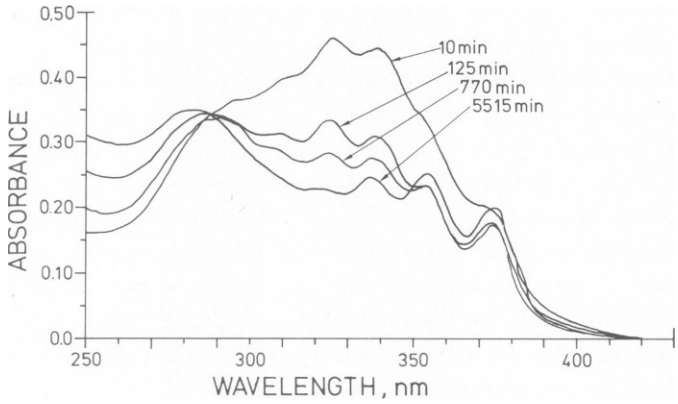


FIGURE 4 Absorption spectrum as a function of time for a solution of BLG in phosphate buffer at pH 7.50 to which a 30-fold molar excess of vitamin-A in EtOH was added at t = 0 min.

fine-structure comes from the formation of a retro-vitamin-A derivative capable of displacing vitamin-A from the protein. The decrease in overall intensity comes from the competing destruction of the unbound chromophore in water solution. After 15h the spectrum remains stable with maxima at 375, 354, 337, and 322 nm. In this example the remaining mixture was pipetted onto a Sephadex column. The column contained 70 gm Sephadex G-100 that had been swelled in 1.2 l of 0.1 M phosphate buffer (pH 7.5) to give a final bed 3.6 cm diam. by 79 cm high. Elution was at a rate of ~1 ml/min and the progress of the vitamin-A-containing band was followed by fluorescence. After 5h fraction collection was begun. Those fractions that showed the strongest chromophore absorbance also showed the strongest protein absorbance. Typical spectra are shown in Fig. 5. The absorbance spectra of these fractions showed no change over the course of 10 d. As was the case before chromatography, the maxima were at 375, 355, 337, and 322 nm. As before, the chromophore could be extracted by heating to 76°C and then shaking with hexanes. The results of such an extraction are also shown in Fig. 5. As can be seen in the figure, the chromophore absorption is red-shifted ~6 nm when it binds to the protein. The structured maxima for the free chromophore in hexanes occur at 369, 349, 333, and 316 nm.

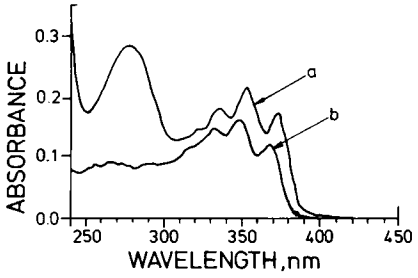


FIGURE 5 Extraction of the retro-chromophore. Curve *a* is the absorption spectrum of the BLG retroretinylidene complex in phosphate buffer at pH 7.50 after Sephadex chromatography. Curve *b* is the absorption spectrum of the hydrocarbon layer after extraction by hexanes at 76°C.

DISCUSSION

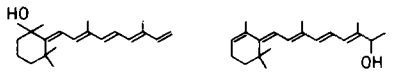
General Comments

The rapid degradation of vitamin-A in an aqueous environment has long been appreciated and has been the focal point of several studies, including those of Lucy and co-workers(12). The enhanced stability of this chromophore when complexed to bovine serum albumin, β-lactoglobulin, and the retinol-binding protein of human plasma has been studied by Futterman and Heller(13), who also reported on the fluorescence properties of these complexes. The substantial increase in fluorescence yield (to four times the yield observed for retinol in petroleum ether) and the resistance to oxidation that results when vitamin-A is incubated with BLG are diagnostic of complex formation. The results reported here reinforce this conclusion and offer further insight into the nature of the complexes.

BLG-Vitamin-A Complex

The question of the stoichiometry of this complex remains open. Because of the existence of competing reactions that lead both to the degradation of vitamin-A and the formation of a new chromophore that can bind to BLG, it is not possible to prepare solutions with known concentrations of both reaction components. Further, because there is considerable overlap between the absorption spectra of the reaction partners and the complex, it is impossible to resolve the contributions of the individual components to the spectrum observed for the reaction mixture. However, by assuming that the molar extinction coefficients of vitamin-A and BLG are not changed upon complexation, some information on the possible stoichiometry can be obtained. At low vitamin-A to BLG molar ratios (1:1 to 3:1), we find that of order 50% of the vitamin-A is almost immediately lost. The resulting protein-to-vitamin-A extinction ratio is then consistent with a stoichiometry of one molecule of vitamin-A to one molecule of BLG (i.e., see the curve labeled 10 min in Fig. 4). However, the absorbance in the chromophore region undergoes further decay, well-described by a first-order rate of 1/700 min^{-1} to a final steady value. This long-time absorbance ratio is then consistent with a stoichiometry of one molecule of vitamin-A per two molecules of BLG. Whether this decay represents the destruction of roughly half of the vitamin-A that had been weakly bound to nonspecific sites, or a degradation of the protein, is uncertain.

It is reasonable to conclude, however, that in the final complex the vitamin-A is bound in a rather specific site. This conclusion follows from the observation that complexation introduces vibrational fine-structure into the normally diffuse vitamin-A absorption spectrum. Studies on model compounds by Christensen and Kohler(14) have shown that the diffuseness of the absorption spectra of retinyl polyenes results because in solution a number of conformations differing in the angle between the polyene chain and the β-ionylidene ring are present. This model has been put into quantitative form by Hemley and Kohler(15) and fit to the absorption bandwidths of several carotenoids. Given this model for the broadening, the association of a structured absorption spectrum with the fixing of the β-ionylidene ring-polyene chain torsional angle, either through specific external perturbation provided by the environment or by the formation of a retro compound, follows. Since the vitamin-A chromophore can be extracted from the complex under rather mild conditions, it is clear that in this case the formation of vibrational structure results not from the formation of a retro



Compound 1 Compound 2

FIGURE 6 Two possible structures for a polar retro compound formed from vitamin-A in water solution.

compound but from perturbation by the protein. If these perturbations were different for different sites, the structure would again be washed out. Thus, the presence of a specific and selective binding site is implied.

Retro-Compound Complex

The primary issues here are the identity of the chromophore, its mode of formation, and the nature of the complex. The absolute identity of the chromophore will have to await the development of techniques for producing it in sufficient quantity so as to permit structural analysis via nuclear magnetic resonance, etc. Nonetheless, some inferences are now possible. First, we can suggest that a retro β-ionylidene linkage is present in a compound containing the same number of conjugated double bonds as retinol. The former follows from the structured spectrum of the free chromophore and the considerations presented in the last section; the latter follows because the chromophore absorption remains near 330 nm. Using thin-layer chromotography techniques, we have determined that this compound is most likely more polar than vitamin-A itself. These observations suggest two possibilities for the identity of the chromophore.

In anhydrous ethanolic solutions, vitamin-A can be protonated, losing water to form the retro hydrocarbon, anhydrovitamin-A(16,17). Anhydrovitamin-A can then undergo further reaction to give what has been called isoanhydrovitamin-A. Isoanhydrovitamin-A has a structured absorption spectrum with reported maxima at 366, 348, and 332 nm(18). This is very similar to our observation of maxima at 369, 349, 333, and 316 nm. At first, it was suggested that isoanhydrovitamin-A resulted from anhydrovitamin-A by the loss of a double bond via solvation of the terminal vinyl group(19). Later elemental analyses showed the presence of oxygen(20), suggesting that the compound was probably a retro vitamin-A ethyl ether formed by the addition of an ethoxy group to a carbonium ion. That is, the compound could have structures similar to those shown in Fig. 6. By analogy, we suggest that the retro chromophore in our case results from the hydroxylation of an anhydrovitamin-A carbonium ion formed when vitamin-A is added to polar solution. Either of the compounds shown in Fig. 6 would be consistent with all of the observations to date.

While the characterization of these compound is somewhat tentative now, the direction of future work is clear. Certainly the prospect that a well-defined protein-vitamin-A complex can be produced justifies considerable further effort.

We have benefited from the efforts of Leslie Greengard, who studied the decay of retinol in buffer solution, and Charla Reinganaum, who developed techniques for concentrating the complexes. Dr. Kohler gratefully acknowledges support from the Alexander von Humboldt Stiftung and the hospitality of Professor Albert Weller at the Max Planck Institute for Biophysical Chemistry in Göttingen, where this manuscript was prepared.

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