Some Observations on the Ultraviolet-Absorption Spectra of β_A - and β_B -Lactoglobulin

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Bovine β -lactoglobulin was shown by Aschaffenburg & Drewry (1955) to exist in two variants, A and B, the incidence of which is determined by heredity. Since then Ogston & Tombs (1957), Tombs (1957) and Timasheff & Townend (1961) have shown that the variants differ in certain respects, although in others, as is common with genetically determined pairs of proteins, they are very similar. The main difference is that below the isoelectric point in acetate buffers β_A -lactoglobulin forms a tetramer but β_B -lactoglobulin undergoes isomerization and does not polymerize. Titration studies indicate a difference of only two carboxyl groups (Tanford & Nozaki, 1959), and the amino acid composition differs slightly (Gordon, Basch & Kalan, 1961; Piez, Davie, Folk & Gladner, 1961). Kalan, Gordon, Basch & Townend (1961) have shown that part of the difference in constitution consists of the substitution of an aspartic acid residue in β_A -lactoglobulin for a glycine in β_B -lactoglobulin. Ogston & Tombs (1957) found very little difference in ultraviolet-absorption curves at high pH and Tanford & Nozaki (1959) found no difference at all in the absorption near 280 m μ in neutral solvents.

Spectral shifts on denaturation and related structural changes in proteins have been much studied (Yanari & Bovey, 1960; Glazer & Smith, 1961) but no results have been described for the β -lactoglobulins, and no comparison of a pair of genetically linked proteins has so far been made by these methods. Glazer & Smith (1961) described a new peak in protein difference spectra near 230 m μ and which was stated to be present in β -lactoglobulin. They apparently used a mixture of the A and B varieties. It seemed possible that difference spectra of the two forms might throw some light on the structural differences between them.

METHODS AND MATERIALS

β_A - and β_B -Lactoglobulin were a gift from Dr R. Aschaffenburg. All reagents were of A.R. grade. pH was determined with a wide-range glass electrode and a Pye Dynacap pH-meter. Ultraviolet-absorption measurements were made with a Hilger Uvispek spectrophotometer fitted with a synthetic silica prism. In the measurement of difference spectra four 0.5 cm. cuvettes were used as follows: in the

reference position, protein in solvent x , and another cuvette contained solvent y . In the sample position, protein in solvent y , and a separate cuvette contained solvent x . In this way any contribution from the solvent was cancelled out; it should be noted that two cuvettes were in the light-path at all times. The spectra were measured in two sections, between 210 and 250 m μ at about 0.005% of protein and between 250 and 310 m μ at about 0.1% of protein. In each case the spectrum of β_A - or β_B -lactoglobulin in phosphate buffer, pH 7.1 (15.6 g. of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 35.8 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}/\text{l.}$), was measured by using β_A - or β_B -lactoglobulin in other solvents as the reference solution.

Wavelength calibrations were checked against known substances, the error between 220 m μ and 300 m μ being less than 0.5 m μ . Stock solutions were made by dissolving the crystalline proteins in 0.1 M-NaCl solution: dilutions were made by weighing the required amount of stock solution into a volumetric flask.

RESULTS AND DISCUSSION

Measurements of difference spectra below 240 m μ were attended by difficulties not encountered at longer wavelengths. They are mainly because absolute extinctions are high and change rapidly with wavelength. It was found easy to produce a completely false 'peak' by a small difference in concentration between the two samples to be compared, combined with stray-light effects. This is particularly alarming because in appearance and position false peaks may be almost identical with the genuine peak. Some care was necessary to avoid concentration errors and errors due to instrumental failure at high absorption at short wavelengths. The instrument used was known to give a satisfactory performance at least up to extinctions of 1.5 and 210 m μ , and this was not exceeded in these experiments. In addition, the magnitude of the difference-spectrum peak at 230 m μ showed a linear relationship to the protein concentration in the range used. This shows that stray-light effects were not important (see also Glazer & Smith, 1961) at this wavelength. Errors of concentration were more troublesome but were overcome by weighing solutions instead of pipetting them.

The difference spectra of β_A - or β_B -lactoglobulin produced by alkali between 260 and 310 m μ are shown in Fig. 1. They are very similar to each other, and a difference spectrum of β_A -lactoglobulin, with

β_B -lactoglobulin as the reference, showed neither maxima nor minima from 210 $m\mu$ to 310 $m\mu$ when both proteins were in the same solvent. The spectra near 280 $m\mu$ may be interpreted in the usual manner in terms of perturbations of the aromatic chromophores (Yanari & Bovey, 1960), in this case by ionization of the tyrosine.

The difference spectra of β_A -lactoglobulin between 210 $m\mu$ and 250 $m\mu$ at various alkaline pH values are also shown in Fig. 1. The spectra for β_B -lactoglobulin were very similar and Fig. 2 (a) shows the development of the peak as the pH became more alkaline. The process involved has pK 11.2 for both proteins, although the curves diverge slightly at higher pH. A pK of this magnitude could correspond to a rather weak tyrosyl hydroxyl group dissociation. The variation of the wavelength of the maximum with the height of the peak is shown in Fig. 2 (b). Differences in the positions of the maxima of β_A - and β_B -lactoglobulin of the order of 1 $m\mu$ were observed but these are of doubtful significance. There was a clear tendency for the maximum to shift to shorter wavelengths as the peak grew larger and the pH higher.

The β -lactoglobulins are known to be exceptionally stable in acid and this is supported by the limited development of the difference spectrum at low pH. Fig. 3 shows the effect of acid, and alkali followed by acid, on β_A - and β_B -lactoglobulin. The

maxima in the range 250–310 $m\mu$ were due to tyrosine, tryptophan and phenylalanine as shown. Between 210 and 250 $m\mu$ a single peak, small compared with those produced by alkali, appeared at 230–233 $m\mu$. The difference spectra of β_A - and β_B -lactoglobulin at pH 4.6 in acetate buffers and also at pH 3.4 in acetic acid (not illustrated) showed no very definite maxima or minima and were identical. It is possible, however, to produce a difference spectrum with urea in this pH range, which is of particular interest because differences between β_A - and β_B -lactoglobulin in polymerization are most marked at this pH.

Fig. 1. Difference spectra of β_A - and β_B -lactoglobulin. In all cases measurements were made on protein in 0.1 M-phosphate, pH 7.0, with protein in NaOH soln. (at the stated pH) as the reference standard. Curve (A), β_A -lactoglobulin, pH 12.2; curve (B), β_B -lactoglobulin, pH 12.2. Curves (1)–(5) are those for β_A -lactoglobulin; pH values were 10.0, 10.8, 11.2, 12.8 and 12.9 respectively. The five curves were matched at 245 $m\mu$. All values of ΔE have been calculated to 0.1% of protein and 0.5 cm. optical depth. Experimental points have been omitted for clarity; measurements were made at intervals of 1 $m\mu$ or less. The right-hand scale applies to curves at wavelengths above 250 $m\mu$; the left-hand scale applies to curves at lower wavelengths.

Fig. 2. (a) Development of the 230 $m\mu$ peak with increasing pH. $\circ$, β_A -Lactoglobulin; $\bullet$, β_B -lactoglobulin. (b) Variation of wavelength of the absorption maximum with the height of the peak. $\circ$, β_A -Lactoglobulin; $\bullet$, β_B -lactoglobulin. The ordinate scales represent the difference between ΔE values at the absorption maximum and at 245 $m\mu$, calculated for 0.1% of protein in a 0.5 cm. cuvette.

When treated with 8M-urea at pH 7.2–8.2 β_A - and β_B -lactoglobulin both showed a clear difference spectrum with maxima in the 230 m μ region and also at 285 and 293 m μ . On acidification of the urea to pH 4.6 with hydrochloric acid, maxima were obtained in similar positions (Fig. 4) and the two proteins were again identical in behaviour.

We have been unable to detect the difference-

Fig. 3. Absorption spectra of β_A - and β_B -lactoglobulin at low pH. The general conditions were as described for Fig. 1. Curve (1) and curve (A) β_A -lactoglobulin in HCl, pH 1.4; curve (2) and curve (B), β_B -lactoglobulin in HCl, pH 1.4; curve (3) and curve (C), β_A -lactoglobulin was adjusted to pH 12.2 with NaOH, then to pH 0.8 with HCl; curve (4) and curve D, β_B -lactoglobulin similarly adjusted to pH 12.2 and pH 0.8. The chief amino acid maxima are indicated. All results were calculated to 0.1% of protein and 0.5 cm. optical depth. Phe, Phenylalanine; Try, tryptophan; Tyr, tyrosine.

Fig. 4. Difference spectra of the β -lactoglobulins in the presence of urea. Curves (1) and (2), β_A - and β_B -lactoglobulin in 8M-urea, pH 8.2; (3) and (4), β_A - and β_B -lactoglobulin in 8M-urea + HCl, pH 4.6. Curves (A) and (B), β_A - and β_B -lactoglobulin in 8M-urea, pH 7.2; (C) and (D), β_A - and β_B -lactoglobulin in 8M-urea + HCl, pH 4.6. Other conditions were as described for Fig. 1.

spectrum peak with a maximum at 236 m μ described by Glazer & Smith (1961), though under similar conditions we have found maxima at 232–234 m μ in acid and in alkali at 230 m μ for both proteins. In case the 236 m μ peak was a consequence of the use of a mixture of β_A - and β_B -lactoglobulin instead of the individual protein, we also used a mixture (50% of β_A - and 50% of β_B -lactoglobulin), but the spectrum did not differ from that of the individual proteins. This relatively small difference between our results and those of Glazer & Smith could easily be due to instrumental variation.

The tryptophan peak is relatively prominent in the difference spectrum of the less-denatured form produced by simple acid treatment: this might be interpreted as showing that the tryptophyl residues are relatively more accessible than the tyrosyl residues, which are so little affected that they must be buried in the molecule. The tyrosine spectrum becomes much more marked when a preliminary alkali treatment is used.

The major conclusion to be drawn from this study is the remarkable identity of all aspects of the absorption spectra of β_A - and β_B -lactoglobulin. Although certain small differences of doubtful significance in the exact maxima were noted, both the aromatic-amino acid-residue spectra and the spectral shifts in changing environments were indistinguishable. The parts of the spectra and difference spectra between 220 m μ and 240 m μ that depend more on the peptide-chain configuration were also identical. It may be concluded that the aromatic chromophores do not interact with the extra carboxyl groups present in β_A -lactoglobulin, since this would be expected to produce measurable differences in the spectra.

SUMMARY

1. The ultraviolet-absorption spectra of β_A - and β_B -lactoglobulin have been measured between 220 m μ and 310 m μ . They are identical.

2. Shifts in the spectra caused by acid, alkali and urea denaturation of β_A - and β_B -lactoglobulin produced almost identical difference spectra between 225 m μ and 310 m μ .

3. It is concluded that in the lactoglobulin molecule the extra carboxyl groups present in β_A -lactoglobulin are not close to an aromatic chromophore, since this would be expected to produce a measurable difference in their spectroscopic behaviour.

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Steroid Metabolism in the Cat

2. BILIARY AND URINARY EXCRETION OF METABOLITES OF [4-¹⁴C]CORTICOSTERONE*

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When ¹⁴C-labelled progesterone is administered to human subjects and animals radioactive metabolites appear in the urine and bile or faeces. In rodents metabolites in bile or faeces account for the greater part (about 70%) of the administered steroid and metabolites in urine for about 30% (Grady, Elliott, Doisy, Bocklage & Doisy, 1952; Shen, Elliott, Doisy & Doisy, 1954). In man about 30% of the administered dose is excreted in the bile of subjects with bile fistulae, but only about 13% in the faeces of intact subjects; most of the remainder of the dose can be accounted for as metabolites in urine (Sandberg & Slaunwhite, 1958). However, when [4-¹⁴C]progesterone was administered intravenously to anaesthetized male cats virtually all of the excreted radioactivity appeared in the bile; in the six animals studied urine radioactivity did not exceed 1% of the dose (Taylor & Scratcherd, 1961). Since it is probable that progesterone is not secreted to any appreciable extent by male cats it seemed desirable to investigate the metabolism of a steroid hormone known to be secreted by this animal. Therefore [4-¹⁴C]-corticosterone has been administered intravenously to anaesthetized male cats and the excretion of labelled metabolites in bile and urine has been determined. A preliminary account of this work has been published (Scratcherd & Taylor, 1961).

MATERIALS AND METHODS

4-¹⁴C-labelled steroids. [4-¹⁴C]Corticosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione; 44.4 μ C/mg.) was obtained from the Endocrinology Study Section, U.S. Public

Health Services, Bethesda, Md., U.S.A. Paper chromatography in benzene-methanol-water (2:1:1, by vol.) showed that 96–98% of the radioactivity had the same R_F as an authentic sample of corticosterone. The labelled steroid was dissolved in ethanol and stored at –15°; no detectable decrease in purity had occurred after 7 weeks. For the single-dose experiments the steroid in 0.4 ml. of ethanol was diluted with 0.6 ml. of 0.154 M-NaCl; for infusion experiments about 1 mg. of unlabelled corticosterone (kindly supplied from the M.R.C. Reference Steroid Collection by Professor W. Klyne) was added to the [4-¹⁴C]corticosterone and dissolved in 2 ml. of ethanol and 18 ml. of 0.154 M-NaCl. [4-¹⁴C]Progesterone (pregn-4-ene-3,20-dione) was obtained from The Radiochemical Centre, Amersham, Bucks.; solutions for injection were prepared as described by Taylor & Scratcherd (1961).

Operative procedure. Male cats were prepared as described by Taylor & Scratcherd (1961), except for a change in the method of collecting bile. A cannula was made by attaching polythene tubing to a stainless-steel tube (0.5 mm. internal diam. $\times$ 4.5 cm. long), which was passed up the common bile duct to about 3 cm. above its entrance to the duodenum and tied in place. The cystic duct was tied off as near as possible to its junction with the common duct. The volume of the cannula, estimated by weighing it empty and then full of water, was 0.12 ml.

All other materials and methods were as described by Taylor & Scratcherd (1961), except that water-saturated ether-ethyl acetate (3:1, v/v) was used for extraction.

RESULTS

Table 1 shows the excretion of radioactivity in the bile of cats after a single rapid injection (cats 1 and 2) and after a 45 min. infusion (cats 3 and 4) of [4-¹⁴C]corticosterone. The volumes of bile collected and the concentration of total solids are also included to show that biliary function was main-

* Part 1: Taylor & Scratcherd (1961).