Aust. J. Biol. Sci., 1976, **29**, 317–23

Studies on the Apoproteins of the Major Lipoprotein of the Yolk of Hen's Eggs II.* The Dimer–Tetramer Transition of Apovitellenin I

R. W. Burley and W. A. Davies

Division of Food Research, CSIRO, P.O. Box 52, North Ryde, N.S.W. 2113.

Abstract

Further studies have been made of the physical properties of hen's apovitellenin I, the principal low-molecular-weight protein from the high-lipid low density lipoprotein of the yolk of hen's eggs. The methods used included chromatography, sedimentation, viscosity, optical rotation, and spin labelling; the solvents used were aqueous urea, and, for some experiments, aqueous formamide. It is concluded that at neutral pH the protein is present in these solvents as an aggregate of molecular weight 36 000 corresponding to a tetramer. Below about pH 4·5 solutions of the tetramer increased greatly in viscosity; furthermore, a covalently bound spin label increased in mobility. These changes were reversible and were apparently the result of dissociation of the tetramer to a dimer. This dissociation did not involve a change in the proportion of α-helix.

In contrast to the results of previous experiments, it now seems probable that the apovitellenin I dimer is stabilized by an interchain disulphide bond.

Introduction

In part I (Burley 1975) it was shown that the apoproteins (termed 'apovitellenins') of the major lipoprotein of hen's egg yolk could be freed from lipid, dissolved in aqueous urea, and separated into a series of fractions by gel filtration chromatography on Sephadex. Four proteins of low molecular weight, referred to as apovitellenins I_{sub}, I, II and I*a*, in order of decreasing apparent molecular weight, were isolated from the mixture. Some of these are now being studied in more detail. Physical properties of the largest fraction (apovitellenin I), including its ability to undergo a dimer–tetramer interconversion, are described here.

Materials and Methods

Preparation of Egg-yolk Lipoprotein

The hen's eggs used were from flocks of Australorp or White Leghorn hens kept under commercial conditions at the Division of Animal Genetics, CSIRO, in Sydney. They were freshly laid and either warm or a few hours old when opened. Small differences were observed between eggs of the two breeds of hen; lipid was often more easily removed from the lipoprotein of Australorp hens for reasons that are not clear.

Egg-yolk high-lipid lipoprotein was isolated as described previously in Burley (1975) although 3 M NaCl was used for flotation instead of 4 M, and for some experiments 24% (0·7 M) sucrose was used instead of NaCl, without apparent difference in the behaviour of the lipoprotein.

Solvents

Concentrated aqueous urea was either acidic (6 M urea, 0·025 M HCl, pH 3·3) or neutral (6 M urea, 0·025 M sodium acetate, 0·001 M EDTA; or 6 M urea, 0·05 M TES, pH 6–7). Aqueous formamide (20% v/v, 5·0 M) was used for some experiments.

* Part I, *Aust. J. Biol. Sci.*, 1975, **28**, 121–32.

Spin Label

The spin label, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl) maleimide, was a gift of Dr J. C. Seidel, Boston Biomedical Research Institute, Mass., U.S.A.

Preparation of the Total Apoprotein Mixture and the Isolation of Apovitellenin I

Lipid was separated from the apoprotein by both of the methods described in Burley (1975), namely (1) the *neutral-pH method* in which the lipoprotein in a solution of EDTA at pH 6–7 was added to a mixture of chloroform and methanol at 20°C to give, after filtering and drying in air, the apoprotein as a powder with a high proportion of moisture, and (2) the *low-pH method* in which the lipoprotein was treated with a chloroform–methanol–water (0·025 M HCl, pH 2·5) mixture at 2°C from which the apoprotein was obtained as a solution in 6 M urea, pH 3·3. For both methods better yields of low-molecular-weight proteins were obtained if the initial concentration of lipoprotein was 6–7% instead of 10%. It was also found that in the first method apovitellenin I was isolated in slightly higher yield if lipid was removed in the presence of a lower concentration of EDTA (0·07 M initially instead of 0·2 M), although in this case there was apparently some loss of apovitellenin I*a* and II.

Apovitellenin I was isolated from the total apoprotein mixture by chromatography in acidic 6 M urea as described in Burley (1975). The protein was finally purified on a long column of Sephadex G75 to remove contaminating apovitellenin II and the higher aggregate, apovitellenin I_{sub} (see Fig. 1).

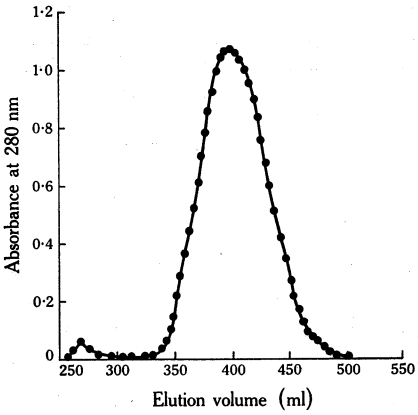

Fig. 1. Chromatography of apovitellenin I on a column (3·7 by 120 cm) of Sephadex G75 fine in 6 M urea–0·025 M HCl, pH 3·3. The very small leading peak represented some high-molecular-weight yolk apoproteins added as a marker. Void and bed volumes were 265 and 520 ml respectively. The flow rate was 20 ml/h.

Spin-labelling of Apovitellenin I

To the freeze-dried protein (20 mg) in 6 M guanidine hydrochloride–0·02 M tris buffer, pH 8·0, the above spin-labelled maleimide (3 mg) was added in 100 μl of ethanol. The solution was left for 16 h at 20°C and the spin-labelled protein was separated from unreacted reagent and its byproducts on a column of Sephadex G25 (2·5 by 45 cm) in neutral 6 M urea, pH 6–7.

Physical Measurements

All measurements were made at 20·0°C unless stated otherwise. Protein concentrations were determined from the optical absorption at 280 nm using $E_{1cm}^{1\%} = 13{\cdot}3$ for hen's apovitellenin I (Burley 1975). Dilutions were made with dialysate where appropriate and for most measurements protein solutions were passed through micropore filters.

For sedimentation equilibrium measurements either a Beckman model E ultracentrifuge with Rayleigh optics or a Spinco model E with Schlieren optics was used. Molecular weights were derived by two methods, details of which are given by Chervenka (1969). For most experiments Schlieren optics were used with 1-mm columns, the results being calculated by Lamm's method. For one series of experiments (at pH 7·0) Rayleigh optics were used and the initial cell concentrations were determined by means of synthetic boundary experiments. By this method it was established that equilibrium was reached in 48 h. Results were calculated as for the 'conventional method' in which r^2 was plotted against $\log c$, where r is the distance along the cell and c is the number of fringes. For both methods protein solutions were dialyzed against solvent and the dialysate used for the reference cell.

Viscosities were measured on an Ostwald microviscometer with a flow time of 226 s for water.

For measurement of optical rotation a Perkin–Elmer spectropolarimeter model 141, which uses filters in the range 365–589 nm, was used. Apparent percentages of α-helix were calculated from the Moffit–Yang equation assuming values of -630 for b_0 and 212 for λ_0. Refractive indices were measured on a Zeiss differential refractometer.

Measurements of electron spin resonance (e.s.r.) were made on a Varian E4 e.s.r. spectrometer using the small quartz cells. The procedures were in general those described previously (Sleigh and Burley 1973).

The molecular weight of hen's apovitellenin I monomer was taken as 9402 derived from its amino acid sequence (T.A.A. Dopheide and A. S. Inglis, personal communication) and the calculated value for the partial specific volume was 0·751 ml/g.

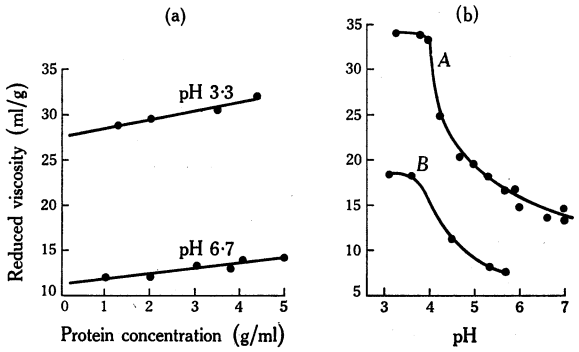

Fig. 2. Viscosity of solutions of apovitellenin I. (*a*) Effect of protein concentration in 6 M urea at pH 3·3 and pH 6·7. (*b*) Effect of pH at one protein concentration. *A*, 6 M urea, protein concentration 0·45%; *B*, 5 M aqueous formamide, protein concentration 0·40%. The pH was increased by adding 1·0 M NaOH. The effect of dilution has not been corrected for but was very small.

Results

Fig. 2*a* shows the viscosity of solutions of apovitellenin I in 6 M urea, pH 3·3 and 6·7, as a function of the concentration of protein. Fig. 2*b* curve *A* shows the effect of pH at one protein concentration. Evidently a sharp transition occurred between pH 4 and 4·5 followed by a less abrupt change to about pH 6. This transition was reversible on adding acid or alkali.

Because of the possible involvement of solvent in the transition shown in Fig. 2*b*, the effect of pH on viscosity was also examined in aqueous formamide (5·0 M). In this solvent the intrinsic viscosities were lower than in 6 M urea, but there was a marked transition in the same region, as indicated in Fig. 2*b* curve *B*. In aqueous formamide the protein precipitated above about pH 6 at the concentration used for Fig. 2*b*.

Increasing the ionic strength with NaCl did not immediately alter the viscosities of the solutions of apovitellenin I in 6 M urea, but it was observed that at pH 3·3 solutions of 0·5% had set to a firm gel in 4 days at 20°C if the ionic strength was greater than about 0·1. This effect was not studied in detail.

Fig. 3*a* shows the effect of concentration of urea on the viscositiy of apovitellenin I at pH 3·3 and 6·0. Measurements at the higher pH were made on very dilute solutions (0·1 mg/ml) at urea concentrations less than 3 M because of the decreased solubility above about pH 6.

Fig. 3*b* shows the effect of the concentration of urea and formamide on the amount of α-helix in apovitellenin I at two pH values, where possible, measured by optical rotatory dispersion in the visible region. In aqueous formamide the protein had a slightly lower proportion of α-helix, but for each solvent pH evidently had little effect. The change in viscosity (Fig. 2*b*) was therefore not accompanied by change in the helical structure.

In another attempt at elucidating the nature of the pH-induced transition, the spin-labelling of apovitellenin I was examined. The piperidinyl-maleimide spin label reacted slowly with the protein (see Methods) to give a spin-labelled derivative with

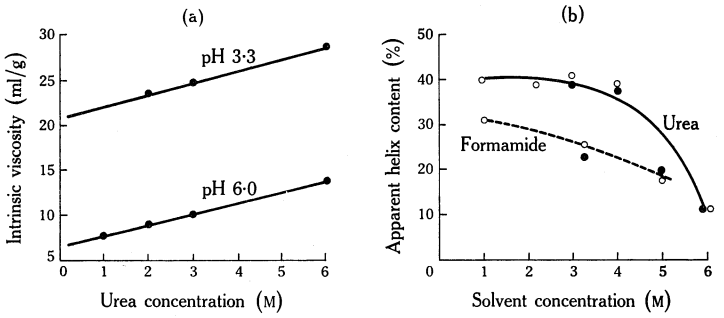

Fig. 3. (*a*) Intrinsic viscosity of apovitellenin I as a function of concentration of urea at pH 3·3 and 6·0. (*b*) α-Helix in apovitellenin I as a function of solvent concentration. Measurements were made by optical rotatory dispersion assuming $b_0 = -630$. The upper curve (——) refers to aqueous urea at pH 3·3 (○) and 6·5 (●). The lower curve (– – –) refers to aqueous formamide at pH 3·3 (○) and 6·0 (●).

a reasonably mobile spectrum in 6 M urea. Fig. 4, for example, shows the spectrum at pH 3·3 and 7. There was clearly a decrease in mobility on increasing the pH, indicating more restriction to the movement of the spin label. This change was reversible and approximately followed the changes in viscosity shown in Fig. 2*b*. Tests on small spin-labelled molecules showed that the effect of the viscosity of the urea solution on the e.s.r. spectrum was just detectable and that there was no change on altering the pH.

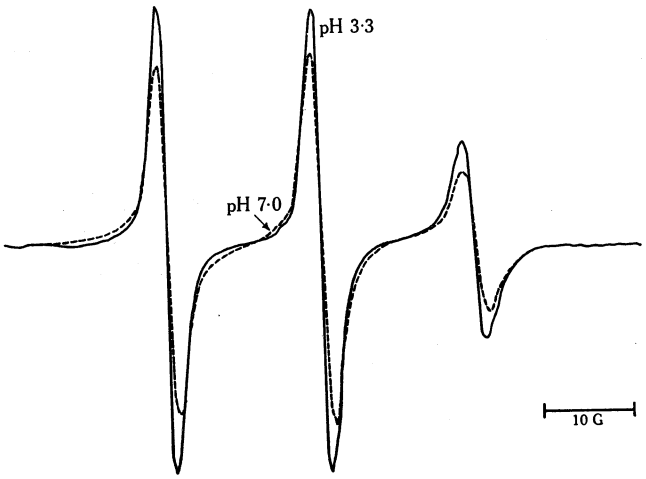

Fig. 4. E.s.r. spectra (first-derivative curves) of hen's apovitellenin I spin-labelled with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl) maleimide. Protein concentration 0·1%. Measurements were at 20°C and the pH was increased by adding 1·0 M NaOH. The small effect of dilution was not corrected for; it would have decreased the difference between the two curves slightly.

The molecular weight of apovitellenin I in 6 M urea at pH 7·0 was measured by sedimentation equilibrium using Rayleigh optics (see Methods). Fig. 5*a* shows measurements at one concentration, and Fig. 5*b* shows the effect of concentration of protein in the range 0·5–5·0 mg/ml. There were no signs of protein heterogeneity. The

extrapolated value for the molecular weight is given in Table 1, which also gives values in 6 M guanidine hydrochloride with and without a sulphydryl reducing agent, and the minimum value calculated from the amino acid sequence. Evidently at pH 7·0 apovitellenin I was present in 6 M urea as a tetramer.

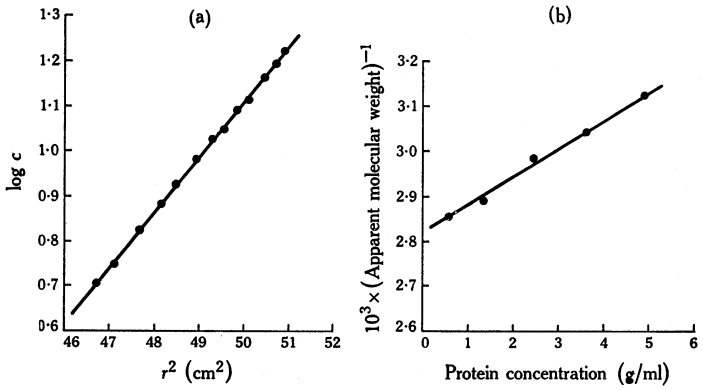

Fig. 5. Determination of the molecular weight of apovitellenin I in 6 M urea, 0·05 M TES buffer, 0·03 M NaCl, pH 7·00, by sedimentation equilibrium with Rayleigh optics at 14290 rev/min for 72 h at 19·3°C. (*a*) Plot of log c *v.* r^2, where r (cm) is radial distance along the solution and c is the concentration (in fringes) at r for one protein concentration (2·42 mg/ml). (*b*) Effect of protein concentration on apparent molecular weight.

Table 1. Molecular weights of hen's apovitellenin I at acid and neutral pH

Method	Solvent	pH	Molecular weight
Sedimentation equilibrium	6 M urea	7·0	35 600[A,B]
	6 M urea	7·0	37 000[B]
	6 M urea	3·3	18 000[B]
	6 M guanidine hydrochloride	5·7	18 000
	6 M guanidine hydrochloride + 0·1 M mercaptoethanol	6·7	10 000[C]
Minimum value from amino acid sequence			9 402

[A] Obtained using Rayleigh optics, standard error for five determinations 1200; for the other values Schlieren optics were used with approximately twice the standard error.
[B] Ionic strength 0·08.
[C] Value from Burley (1975).

The effect of pH on the molecular weight of apovitellenin I in 6 M urea was also determined by sedimentation equilibrium. For these measurements Schlieren optics were used (see Methods) which are less precise than the Rayleigh optics used for Fig. 5. Nevertheless, they revealed a large change on decreasing the pH. Thus Fig. 6*a* shows the extrapolation to infinite dilution at pH 7·0 and 3·3 for samples of apovitellenin I from one preparation. Values below about 2 mg/ml were not taken into account. Measurements at pH 3·3 were sensitive to ionic strength, as expected for a pH far from neutrality, and were complicated at ionic strengths greater than about 0·1 by extensive aggregation, as mentioned already. In addition, different preparations

of protein gave slightly different results. This was not noticed at neutral pH. Fig. 6*b* shows the relationship between molecular weight and pH. Evidently the change in molecular weight occurred in approximately the same region as the sharp change in viscosity (Fig. 2*b*). Extrapolated molecular weights from the Schlieren method are given in Table 1. The result at pH 7·0 is clearly in agreement with that for the other method. At pH 3·3 the molecular weight corresponded to that of a dimer. A similar value was found in guanidine hydrochloride solution except in the presence of a reducing agent, when, as reported in Burley (1975), the protein was monomeric.

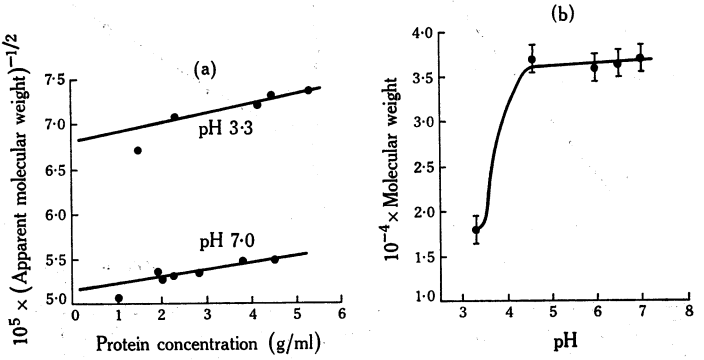

Fig. 6. Molecular weight of apovitellenin I in 6 M urea determined by sedimentation equilibrium with Schlieren optics. (*a*) Effect of protein concentration on apparent molecular weight at pH 3·3 and 7·0 plotted by the procedure of Marler *et al.* (1964), the ionic strength being 0·08 for each. (*b*) Effect of pH on molecular weight. The bars represent standard errors.

Discussion

The above results help to establish the physical properties of apovitellenin I, the principal low-molecular-weight apoprotein of the high-lipid lipoprotein of hen's egg yolk, after it had been isolated in concentrated urea solutions at low pH. The most notable property of this protein is the large reversible change in the viscosity of its solutions when the pH is changed from 3 to 6 (Figs 2 and 3*a*). Although most measurements were made in aqueous urea because of the insolubility of the protein in salt solutions, sufficient tests were carried out in aqueous formamide to be certain that reactions of the protein with cyanate from the urea, which are possible above about pH 4 (e.g. Hagel *et al.* 1971), were not responsible for this change (Fig. 2*b*).

The change in viscosity evidently did not involve the helical structure of the protein, which is rather low in concentrated urea (Fig. 3*b*). The e.s.r. spectrum of a spin-labelled derivative was, however, altered (Fig. 4). There was a decrease in the size of the e.s.r. signal, and a change in the relative heights of the three peaks on raising the pH. Although the label was probably bound covalently, the site of spin labelling has not yet been determined; nevertheless the present results indicate an average decrease in the mobility of the label on increasing the pH. This was caused either by a decrease in the rotational mobility of the protein, as would be expected for the formation of a larger molecule, or by an increase in the adhesion of the spin label to the protein surface (for example if the label were more completely enclosed by protein); or possibly a combination of these was responsible.

Measurements by sedimentation equilibrium (Figs 5 and 6, Table 1) suggest that the molecular weight of apovitellenin I doubles between pH 3 and 7, from a dimer (molecular weight about 18 000) to a tetramer (molecular weight about 36 000). It is suggested that the large increase in viscosity between pH 3 and 6 (Fig. 2) is a result of a change in the shape of the molecules. Such a change would be expected if a compact globular tetramer became a highly asymmetrical or rod-shaped dimer. It should be mentioned as further evidence that the dimer has an abnormal shape that it behaved abnormally during gel-filtration chromatography in 6 M urea. Thus on Sephadex G75 or G100 it was easily separated from apovitellenin II although this has about the same molecular weight (see Fig. 4, Burley 1975). We were not, however, able to separate these proteins on columns of Biogel P150.

Because the dimer–tetramer transition depends on pH it is reasonable to assume that the tetramer is stabilized by ionic forces (Fig. 2). The reasons for the stability of the dimer in 6 M urea are less clear. In Burley (1975) it was assumed from the low molecular weight of apovitellenin I found by polyacrylamide-gel electrophoresis in dodecyl sulphate in the presence and absence of dithiothreitol that it occurred as a monomer with one sulphydryl group. Quantitative reaction with a maleimide derivative was used as further evidence for a single relatively unreactive sulphydryl. More recently, however, contradictory evidence has been found from which it appears possible that the usual form of hen's apovitellenin I at lower pH is a dimer stabilized by an interchain disulphide group. Thus the behaviour of the hen's low-molecular-weight apoprotein on polyacrylamide-gel electrophoresis has been found to be very sensitive to the source of the acrylamide and other factors not properly understood so that the monomer and dimer are not always separated (F. S. Shenstone and A. G. Scott, personal communication). Furthermore, experiments on the spin labelling of emu's apovitellenin I, which has no cysteine or cystine residues, gave evidence for slow reaction with maleimides (Burley, unpublished data); and in an experiment in which lipid was removed from the hen's lipoprotein in the presence of iodoacetamide at pH 7, no carboxymethyl cysteine could be detected in the isolated apovitellenin I after acid hydrolysis, although cystine was present.

In conclusion, our results strongly suggest that hen's apovitellenin I is a protein able to undergo specific interactions to give two small and well-defined aggregates.

Acknowledgments

We are much indebted to Dr B. Chandler and Dr A. H. Reisner, Division of Animal Genetics, CSIRO, for measurements on the Beckman model E ultracentrifuge, and to Mr R. L. Goates of the same Division for maintaining a supply of hen's eggs. We also thank Mr W. C. Osborne for expert technical assistance.

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Manuscript received 2 February 1976