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STRUCTURAL STUDIES OF THE HEN'S EGG LIPOVITELLINS. NMR AND FLUORESCENCE INVESTIGATIONS*

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The pH dependent reversible association-dissociation reaction of α- and β-lipovitellins from egg yolk has been studied by ^{1}H NMR and fluorescence probe methods. Increased mobility of the choline methyl groups has been demonstrated on dissociation. The lipid methylene resonance of β-lipovitellin shows clear doublet character suggesting that the fatty acid chains exist in distinct environments. The high field component increases with temperature but is suppressed on treatment with pronase, suggesting a significant role for proteins in maintaining the differences in lipid environments. 1-Anilino-8-naphthalene sulfonate has been shown to bind less effectively to the monomeric lipovitellins. This is in agreement with earlier results suggesting that dissociation may be accompanied by increased hydration and conformational changes.

I. Introduction

Serum lipoproteins play an important role in lipid transport and metabolism while egg yolk lipoproteins serve functions in embryogenesis [1,2]. A number of physicochemical techniques have been used to study the structure of serum lipoproteins [1]. However few investigations have been reported on egg yolk lipoproteins [3]. Hens egg yolk consists of two classes of lipoproteins viz., lipovitellins (α and β) and very low density lipoproteins (VLDL). Lipovitellins (Lv) consist of about 18% lipid while VLDL contains about 87% lipid [4,5]. Extensive studies on the structure of hen's egg yolk VLDL have led to a model consisting of a lipid core of neutral triglycerides surrounded by radially oriented phospholipids. The polar head groups of the lipids and the proteins are localised in the peripheral region of the particles [6]. The structure of the lipovitellins, on the contrary, have not been investigated in detail. In addition to their low lipid content another characteristic feature of egg yolk lipovitellins is their ability to undergo an association-dissociation reaction in aqueous solution, in a manner reminiscent of globular proteins [7]. Below pH 6.8 the lipovitellins exist in the dimeric form and dissociate into monomers when the pH is raised to ~ 10. This phenomenon is of particular interest as the lipovitellins appear to behave as individual molecules. Hens egg yolk VLDL and human serum lipoproteins do not exhibit this behaviour. This paper presents the results of ^{1}H NMR and fluorescence studies of the α- and β-

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Lv from egg yolk, with a view towards understanding the molecular organisation of these lipoproteins and the structural changes in the lipid region that accompany association.

II. Experimental

A. Materials

Ammonium salt of 1-anilino-8-naphthalene sulfonic acid (ANS), bovine serum albumin (BSA) and pronase Type VI (*ex. Streptomyces grieseus*) were obtained from Sigma Chemical Co., USA.

Lipovitellins from egg yolk were prepared by a modification of the procedure of Wallace [4], using a linear gradient on a TEAE-cellulose column. Protein depleted lipovitellins were prepared by treating 0.6 ml of a 50 mg protein/ml solution of α- or β-Lv in 0.3 M NaCl with 10 mg pronase in 0.1 ml of 50 mM Tris · HCl buffer at pH 7.5. The solution was dialysed against 250 ml 50 ml Tris · HCl (pH 7.5) containing 0.3 M NaCl for 24 h at room temperature to remove all the dialysable products of proteolysis. This was followed by 24 h dialysis against 0.3 M NaCl in D_2O. The lipovitellin samples then contained only 5% of the original protein content. The pronase is lost during the 24 h dialysis, as small fragments formed by self digestion. Lipovitellins with no protease added were processed in similar fashion and served as controls. Protein estimations were carried out by the Folin procedure [8].

B. NMR measurements

α- and β-Lv were studied at pH 6.5 and 10.3 where they exist predominantly in dimeric and monomeric forms respectively. Sodium carbonate–bicarbonate buffer 50 mM at pH 10.3 and 250 mM sodium phosphate buffer at pH 6.5 in D_2O containing 100 mM NaCl were used. The NMR samples contained 40–50 mg protein/ml, prepared by dialysing against the appropriate buffer. All spectra were recorded on a Varian HA-100 spectrometer at an ambient probe temperature of 31°C. Linewidths were determined on spectra obtained by signal averaging using a Varian C-1024 time averaging computer. Chemical shifts are expressed as δ ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

C. Fluorescence studies

ANS was recrystallised from water before use. Spectra were recorded using protein solutions containing 215–225 μg/ml protein of α- and β-Lv, prepared by dialysis against pH 6.5 and 10.3 buffers. The protein content was estimated after dialysis and minor changes in concentration, due to volume changes, were adjusted using appropriate buffers. BSA and sonicated egg yolk lecithin at concentrations of 100 μg/ml at

pH 6.5 and 10.3 were used as control samples. The excitation and emission wavelengths for ANS in the presence of lipovitellins were 380 nm and 470 nm respectively. Fluorescence measurements were made using a Perkin-Elmer Model 203 spectrofluorimeter. All spectra are uncorrected. Fluorescence titrations were carried out by addition of concentrated aliquots of ANS to lipovitellins. The apparent dissociation constants for the dye binding to lipovitellins were determined using a double reciprocal plot method [9,10].

III. Results

A. NMR studies

The α- and β-Lv exist predominantly in the dimeric form at pH ~ 6.5 and dissociate on raising the pH to ~ 10.3 The ^{1}H NMR spectra of α- and β-Lv from egg yolk at these two pH values are shown in figs. 1 and 2 respectively. The assignment of the methyl (0.9 δ), methylene (1.3 δ), $-CH_2-CO-$ (2.2 δ) and $-N^+(CH_3)_3$ (3.3 δ) resonances followed from earlier studies on lipid model systems [11]. The fact that the lipovitellins contain a high proportion of lecithin (75% of the phospholipids) is borne out by the high intensity of the choline signal at 3.3 δ relative to the methylene resonance at 1.3 δ, as compared to hen's egg yolk VLDL [12,13]. The dissociation of α-Lv at alkaline pH is accompanied by a sharpening of the choline resonance from 12 Hz at pH 6.5 to 8 Hz at pH 10.3. The narrowing of the choline resonance is less pronounced in β-Lv where it changes from 8 Hz at pH 6.5 to 6.7 Hz at pH 10.3. An interesting feature of the ^{1}H NMR spectrum of β-Lv is the doublet character of the fatty acid $-CH_2-$ resonance at 1.3 δ. At pH 6.5 a shoulder at high field is visible and at pH 10.3 a clear splitting of this resonance is observed (fig. 2). It is pertinent to note that the

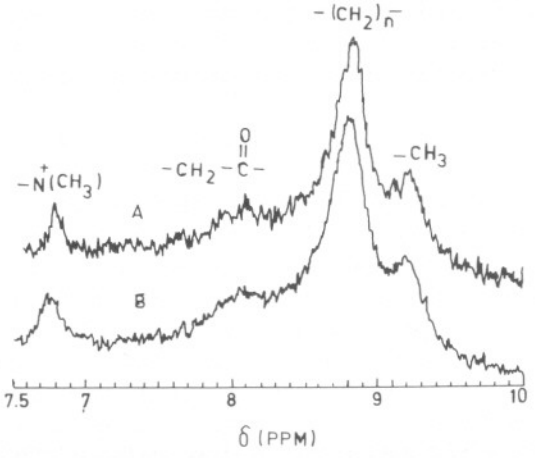

Fig. 1. 100 MHz proton NMR spectra of α-Lv. A: pH 10.3; B: pH 6.5.

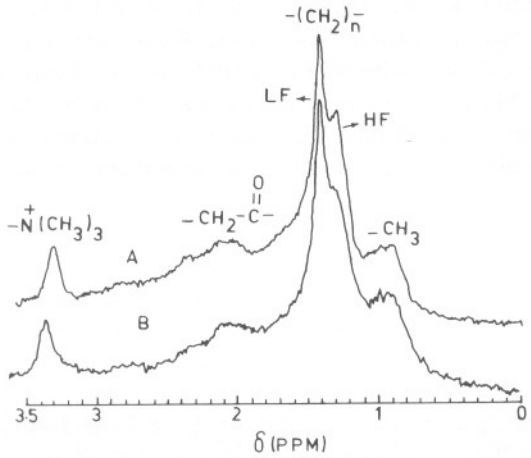

Fig. 2. 100 MHz proton NMR spectra of β-Lv. A: pH 10.3; B: pH 6.5. LF, low field; HF, high field.

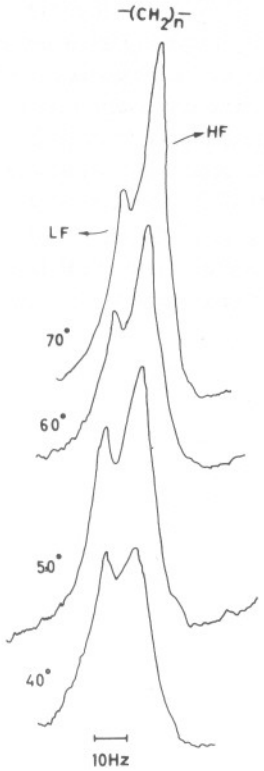

Fig. 3. Lipid methylene resonances of β-Lv in 0.3 M NaCl–D_2O (pH 6.1) as a function of temperature. LF, low field; HF, high field.

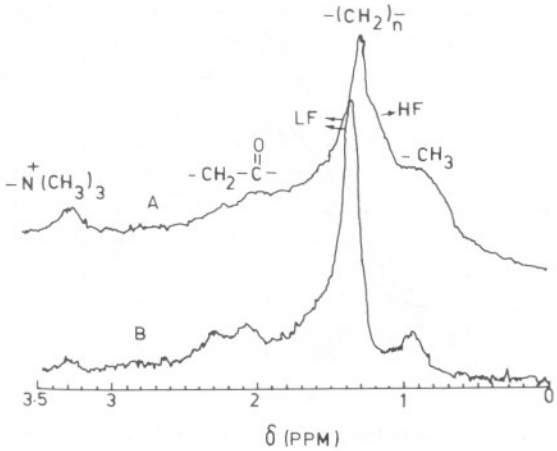

Fig. 4. 100 MHz proton NMR spectra of β-Lv. A: β-Lv in 0.3 M NaCl–D_2O, pH 6.1. B: β-Lv after pronase treatment in 0.3 M NaCl–D_2O, pH 6.1.

corresponding resonances in α-Lv (fig. 1) and VLDL do not show any evidence of doublet character.

The effect of raising the temperature on the methylene resonances of β-Lv is shown in fig. 3. The low field signal diminishes in intensity with increasing temperature, while the high field peak increases in intensity. α-Lv and VLDL do not show any evidence for a doublet nature of the methylene peak over this temperature range. In order to ascertain the effect of proteins on the appearance of the lipid NMR spectra, lipoproteins that had been treated with proteases were studied. Treatment of α- and β-Lv with pronase results in the removal of over 95% of the protein. Figure 4 compares the NMR spectra of β-Lv before and after treatment with pronase. It is seen that the removal of proteins results in the disappearance of high field component of the methylene resonance at 1.3 δ, and the enhancement of the low field component. In the case of α-Lv pronase treatment did not affect the appearance of the methylene resonance.

B. Fluorescence studies

The anionic fluorescent probe ANS has found extensive use in studies of membranes and lipid model systems [14]. The binding of ANS to α- and β-Lv is accompanied by a large enhancement of emission intensity and a blue shift of the fluorescence maximum to 470 nm. This is indicative of the probe binding at hydrophobic sites on the lipoprotein [15]. Figure 5A shows the effect of changing the pH on the fluorescence spectra of ANS bound to α- and β-Lv. It can be seen that on increasing the pH from 6.5 to 10.3 there is a clear reduction in the intensity of emission. Since ANS is an anionic probe, binding is likely to fall off at high pH values due to the development of charge repulsions arising from negatively charged groups on the lipoproteins. Figure

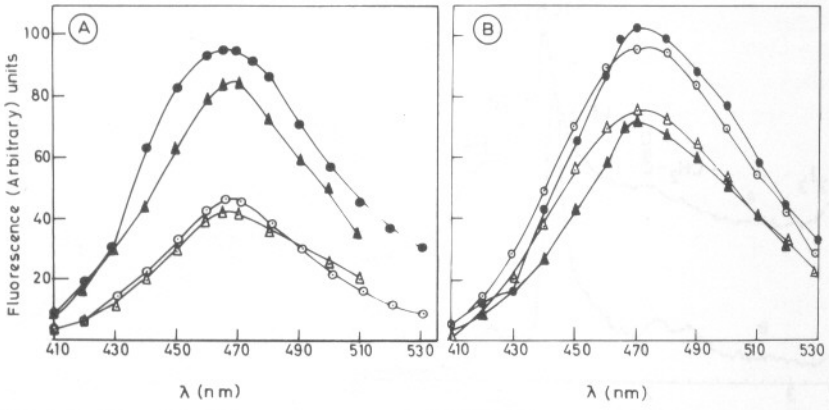

Fig. 5. A: Uncorrected fluorescence spectra of ANS in the presence of lipovitellins: (○——○) α-Lv pH 6.5; (●——●) α-Lv pH 10.3; (▲——▲) β-Lv pH 6.5; (△——△) β-Lv pH 10.3. B: uncorrected fluorescence spectra of ANS in the presence of egg lecithin and BSA. (●——●) lecithin pH 6.5; (▲——▲) lecithin pH 10.3; (○——○) BSA pH 6.5; (△——△) BSA pH 10.3.

5B shows the effect of altering the pH from 6.5 to 10.3 for ANS bound to egg yolk lecithin dispersions and BSA. It is seen that in both cases there is a decrease in the fluorescence intensity at high pH. However it is clearly seen that the reduction in emission for BSA (20%) and lecithin (30%) is less than that observed for the lipo-

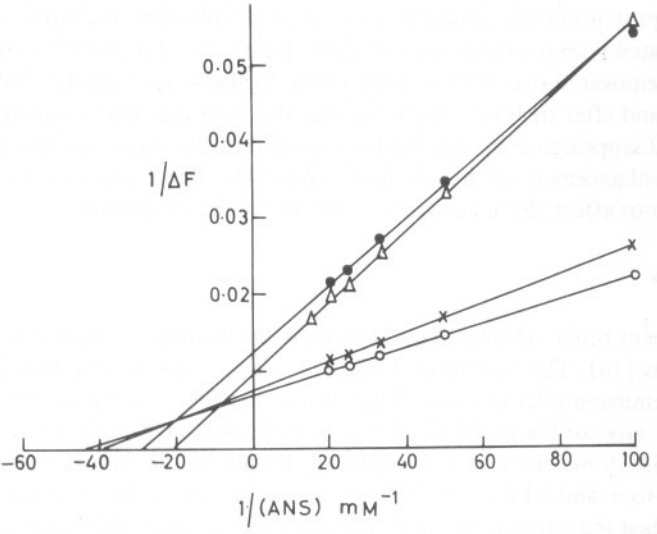

Fig. 6. Double reciprocal plots of the fluorescence of ANS bound to lipovitellins as a function of probe concentration. (○——○) α-Lv pH 6.5; (●——●) α-Lv pH 10.3; (x—x) βg-Lv pH 6.5; (△——△) β-Lv pH 10.3.

vitellins (α-Lv 47%; β-Lv 53%). It is likely that charge repulsions alone may not account for the lowered ANS fluorescence at high pH in the presence of lipovitellins. The reduced emission intensity may reflect structural changes that accompany dissociation.

Fluorescence titrations were carried out to determine the binding affinities of ANS for lipovitellins in the monomeric and dimeric states. Figure 6 shows the double reciprocal plots obtained for ANS binding to α- and β-Lv at pH 6.5 and 10.3. The apparent average dissociation constants ($K_{app} \times 10^2$, mM) obtained were as follows:

α-Lv at pH 6.5 – 3.0; α-Lv at pH 10.3 – 4.0
β-Lv at pH 6.5 – 2.5; β-Lv at pH 10.3 – 4.1.

IV. Discussion

The α- and β-Lv yield relatively broad ^{1}H NMR spectra in aqueous solution (figs. 1 and 2), as compared to VLDL from egg yolk recorded under the same instrumental conditions [12,13]. In VLDL the high lipid content results in a structure containing a highly fluid core with lipid–protein interactions confined to a small region of the lipid surface. This results in proton NMR spectra that closely resemble lipid solutions in organic solvents [11]. On the contrary the broader resonances in the lipovitellin spectra may result from a more restricted environment for the lipids due to dominant interactions with the protein components. It has been shown that removal of the lipid from the lipovitellins causes the proteins to aggregate strongly and high concentrations (10%) of SDS in the presence of 2-mercaptoethanol are required for solubilisation [16]. On the other hand the proteins of VLDL are soluble even in 0.5% SDS in the absence of sulfhydryl reagents [17]. The exposure of the egg yolk lipoproteins to aqueous solutions saturated with chloroform results in absorption of the organic solvent by the lipoproteins [18]. While α- and β-Lv show enhanced susceptibility to phospholipase-C after chloroform absorption, there is no compelling evidence for lipid exposure in VLDL. These observations together with the NMR data suggest that lipid–protein interactions are crucial in maintaining the structural integrity of the lipovitellins.

The proton NMR spectra of α- and β-Lv differ only in the appearance of the fatty acid methylene resonance at 1.3 δ. The high field shoulder evident in the β-Lv spectrum at pH 6.5 is pronounced at pH 10.3 but is absent in α-Lv at both pH values. This suggests that the fatty acid side chains may reside in two distinct environments and that interconversion of lipid molecules between these states is slow on the NMR time scale. A small change is also obtained for the linewidth of the choline resonances in the two lipoproteins. These spectral differences reflect small but distinct differences in the structures of α- and β-Lv. The observations are of interest as the two lipoproteins have similar lipid contents and differ only slightly in the extent of protein phosphorylation [4]. They also have similar molecular weights and sedimentation coefficients [3] and yield identical CD spectra, suggesting broadly similar secondary structures for the pro-

tein components [19]. α- and β-Lv differ however in their dissociation patterns as a function of pH in that β-Lv dissociates above pH 6.8 whereas α-Lv remains largely associated till pH 9.5. Further the two lipoproteins differ in the extents to which they absorb chloroform. More recently the protein components of α- and β-Lv have been shown to yield different sodium dodecyl sulfate–acrylamide gel patterns [16]. The NMR results presented here also point to differences in lipid organisation of the two lipovitellins.

The ability of the lipovitellins to form specific dimers is a property that must be dependent on a well-defined structure of the monomers. It has been shown that hybrid dimers of α- and β-Lv do not form [20]. Burley and Cook using -SH blocking reagents and phosphoprotein phosphatase have shown that sulfhydryl groups and protein phosphoryl moieties are not involved in the association-dissociation reaction [21]. The reduction of lipid content from 17% to 13% by controlled exposure to organic solvents stabilises the monomers at neutral pH [22]. The NMR results presented here show a distinct narrowing of the choline resonance of α-Lv on dissociation suggesting a restriction of motional freedom of the choline head group in the dimer. In β-Lv the line narrowing is less pronounced on dissociation. However it should be noted that the choline groups appear to have narrower lines in β-Lv dimer as compared to α-Lv dimer. The doublet character of the lipid methylene resonance at 1.3 δ of β-Lv becomes pronounced on dissociation. Removal of 95% of the protein by pronase treatment results in the abolition of the high field resonance. This suggests that the two environments of the lipid methylene protons are detectable in both forms of β-Lv. It has also been observed that increasing the temperature of β-Lv in 0.3 NaCl where it is predominantly in the dimeric form results in an increase in the high field resonance. Presumably enhanced fluidity of the lipid phase results in an increase in the high field component. It is noteworthy that removal of protein as well as lowering a temperature reduces the high field resonance. The insertion of protein residues into a tightly packed lipid aggregate may result in enhanced lipid fluidity. Consequently, removal of protein may then cause a greater ordering of the lipid phase. It has been reported that dissociation decreases with increasing temperatures, an observation that stresses the importance of hydrophobic interactions in the association process [3]. The NMR results lead us to conclude that changes in lipid organisation occur within the β-Lv dimer on increasing the temperature.

Preliminary studies have shown that the fluorescent probe ANS binds with almost equal affinities to both α- and β-Lv. The decrease in ANS emission at high pH is partially due to charge repulsions between negatively charged groups on the lipoprotein and the anionic probe. However, using lecithin and BSA as controls, it appears that a reduction in the ANS fluorescence results from a decrease in the number of binding sites available on dissociation. The apparent dissociation constants also show a small but consistent increase with pH for both α- and β-Lv. This is understandable since charge repulsion at higher pH lowers the affinities of binding. Thermodynamic measurements have shown that there is a large negative entropy change (– 42 eu) on dissociation suggesting increased hydration of the monomer or a conformational

change in lipoprotein [7]. The reduced ANS binding on dissociation that has been observed here supports this interpretation. The CD spectra of α- and β-Lv remain unchanged on dissociation suggesting that changes in protein structure are minimal [19]. While the spectroscopic data presented here do not yield specific information on the protein components, clear evidence exists for altered lipid environments on dissociation.

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