BBA 75892

LYSOLECITHIN–CASEIN INTERACTIONS

II. GEL FILTRATION, GEL ELECTROPHORESIS AND DENSITY-GRADIENT ULTRACENTRIFUGATION OF THE LYSOLECITHIN–α_{s1}-CASEIN SYSTEM

B. PARTRICK, M. D. BARRATT, M. A. F. DAVIS AND L. RAYNER

Biophysics Division, Unilever Research Laboratory, Colworth/Welwyn, The Frythe, Welwyn, Herts (Great Britain)

(Received December 6th, 1971)

SUMMARY

The complexes formed when lysolecithin is added to α_{s1}-casein have been studied by gel filtration, gel electrophoresis and density-gradient ultracentrifugation.

Gel filtration on Sephadex G-200 shows that the α_{s1}-casein tetramer and the lysolecithin–α_{s1}-casein complex have identical Stokes' radii (48 Å) over a wide range of lysolecithin:casein ratios.

Gel electrophoresis data show that the lysolecithin–α_{s1}-casein complex possesses approximately half the charge of the α_{s1}-casein tetramer and confirms the stoichiometry indicated previously by the ESR data.

In density gradients of sodium bromide, the α_{s1}-casein tetramer equilibrates at d 1.30–1.31 g/cm^3. As lysolecithin is added, the complex appears at d 1.22–1.24 g/cm^3. The lysolecithin–α_{s1}-casein ratio required to convert all of the α_{s1}-casein to complex agrees with that already established by ESR and gel electrophoresis.

From the combined data a model is proposed for the lysolecithin–α_{s1}-casein complex (in the presence of excess lysolecithin) consisting of two monomer units of α_{s1}-casein (mol.wt. = 28000) and approximately 56 molecules of lysolecithin.

INTRODUCTION

The interactions between various casein fractions and lysolecithin have already been examined by magnetic resonance techniques[1]. In this paper we describe the use of gel filtration, gel electrophoresis and density gradient ultracentrifugation to investigate the interactions between α_{s1}-casein and lysolecithin. α_{s1}-Casein was selected for this study because, as a tetramer (mol. wt. 4 × 28000)[2] at room temperature it is the simplest of the three main casein fractions.

The combination of the three techniques provides relatively unambiguous data relating to macromolecular size, charge and hydrated density.

MATERIALS AND METHODS

α_{s1}-Casein was prepared by the method of HIPP *et al.*[3] and gave a single band on polyacrylamide gel electrophoresis. Lysolecithin (cryst. *ex* egg) was purchased from Lipid Products Ltd., and was pure by thin-layer chromatography. Sephadex G-200 and Blue Dextran were obtained from Pharmacia, G.B., Ltd. Proteins of known Stokes' radii for calibration of the G-200 columns were obtained as follows: chymotrypsinogen (Armour Pharmaceuticals), conalbumin (Sigma) and γ-globulin (Mann Biochemicals).

Chemicals were reagent grade.

Gel filtration

Gel filtration was carried out using columns (55 cm × 2 cm^2) of Sephadex G-200 in 0.1 M NaCl, 0.02 % NaN_3, 0.01 M Tris (pH 7.0) equilibrated at less than 20 cm hydrostatic pressure. The eluate was monitored with an LKB Uvicord II at 280 nm and the volume was recorded by drop counting using an LKB Ultrorac. The number of drops/ml was calculated from the average drop weight.

The column void volume (V_0) was determined using Blue Dextran and the total volume (V_t) was calculated from the column dimensions. The internal gel volume (V_i) was calculated from the equation,

$$V_i = (V_t - V_0)0.95$$

The column was calibrated by measuring the elution volumes (V_e) of globular proteins of known Stokes' radii. The Stokes' radius for each protein was plotted against its penetration coefficient (K_D), a measure of the extent of penetration of the solute into the internal gel volume. K_D was calculated from the Gelotte equation[4,5].

$$V_e = V_0 + K_D \cdot V_i$$

A typical calibration curve is shown in Fig. 1a. Samples applied to the column (2 ml) contained α_{s1}-casein (20 mg) and lysolecithin (0–15 mg). Stokes' radii for α_{s1}-casein and the complex were estimated from the calibration curve.

Gel electrophoresis

Gel electrophoresis was carried out using 5 % polyacrylamide slabs (18 cm × 12 cm) (5 g, Cyanogum 41 (B.D.H. Ltd); 0.25 ml, *N,N,N,N*-tetramethyldiaminoethane; 0.5 ml freshly prepared 10 % ammonium persulphate solution per 100 ml distilled water) in 0.07 M Tris, 0.005 M citrate buffer (pH 8.4) for 18 h at currents below 25 mA (to minimise heating). The gels were stained using amido black (in 50 % aqueous methanol) and destained using 1 % acetic acid solution.

Density gradient ultracentrifugation

Gradients were prepared by layering solutions of sodium bromide. Six steps consisting of 60, 50, 40, 30, 20 and 10 % sodium bromide in 1 % sucrose, 10^{-3} M EDTA (pH 7.4) were used. The sample was incorporated into the 10 % sodium bromide solution and layered as part of the gradient. Centrifugation was carried out at 60000 rev./min (258000 × *g*) for 24 h in a Beckman L2-65 ultracentrifuge using

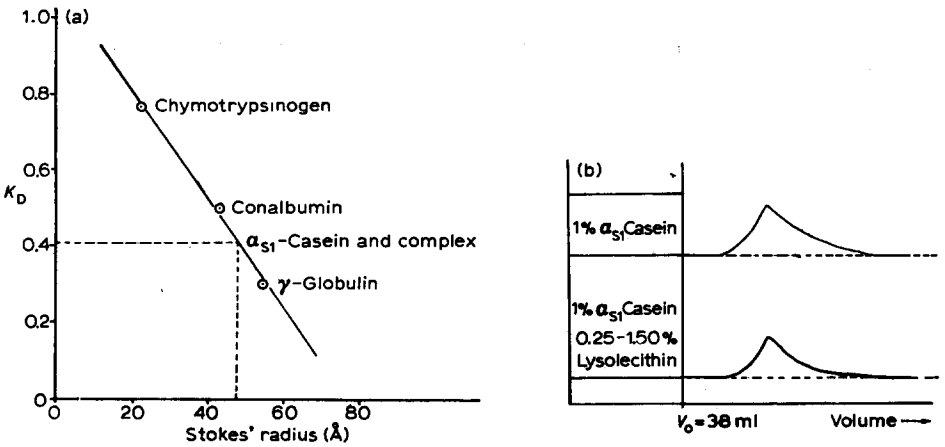

Fig. 1. (a) Stokes' radius calibration curve. (b) Gel filtration elution profiles for α_{s1}-casein and α_{s1}-casein – lysolecithin complex.

an SW-65 rotor. After centrifugation the gradients were fractionated by piercing the bottom of the tubes with a hypodermic needle and collecting 25–30 samples.

Refractive indices, measured on an Abbé refractometer were used to determine densities for each sample. Protein concentration was plotted as absorbance at 280 nm, measured after diluting an aliquot (0.1 ml) of each fraction to 1.1 ml with distilled water.

RESULTS

Gel filtration

The elution profiles for (a) α_{s1}-casein and (b) α_{s1}-casein (1 %) and lysolecithin (0.25–1.5 %) are shown in Fig. 1b. Data obtained from a typical Sephadex G-200 column (V_t = 110 ml, V_0 = 38 ml, V_i = 68.5 ml) are shown in Table I.

Gel electrophoresis

Absolute migrations of α_{s1}-casein and a range of lysolecithin–α_{s1}-casein mixtures in a typical gel slab are shown in Table II. The gel slab is illustrated in Fig. 2.

TABLE I

DATA OBTAINED FROM A TYPICAL SEPHADEX G-200 COLUMN

Sample	V_e(*ml*)	K_D	*Stokes radius* (Å)
Chymotrypsinogen	90.95	0.77	22.5 (known)
Conalbumin	72.0	0.50	43.0 (known)
γ-Globulin	58.75	0.30	55.0 (known)
Blue Dextran	38.0	—	—
α_{s1}-Casein	66.0	0.41	48 (estimated)
1 %α_{s1}-Casein/0.25 % lysolecithin	66.0	0.41	48 (estimated)
α_{s1}-Casein/0.5 % lysolecithin	66.0	0.41	48 (estimated)
α_{s1}-Casein/1.0 % lysolecithin	66.0	0.41	48 (estimated)
α_{s1}-Casein/1.5 % lysolecithin	66.0	0.41	48 (estimated)

TABLE II

ABSOLUTE MIGRATIONS OF α_{s1}-CASEIN AND RANGE OF LYSOLECITHIN–α_{s1}-CASEIN MIXTURES ON GEL ELECTROPHORESIS

Sample	*Migration (cm)*
(a) α_{s1}-Casein (1 %)	4.0
(b) α_{s1}-Casein (1 %)	4.0
Lysolecithin (0.25 %)	2.3
(c) α_{s1}-Casein (1 %)	4.2 (low intensity)
Lysolecithin (0.5 %)	2.4
(d) α_{s1}-Casein (1 %)	
Lysolecithin (1 %)	2.4

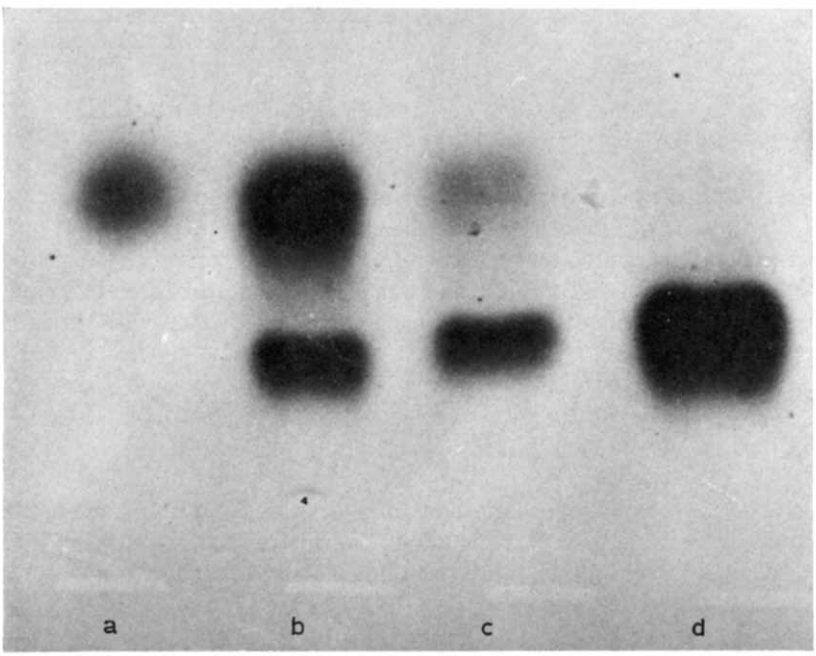

Fig. 2. Gel electrophoresis of α_{s1}-casein (1 %) with lysolecithin (a) 0 %, (b) 0.25 %, (c) 0.5 % and (d) 1 %.

Density-gradient ultracentrifugation

Fig. 3 shows the effect of adding increasing amounts of lysolecithin to α_{s1}-casein. As the amount of lysolecithin is increased, the peak at d 1.30–1.31 g/cm^3 (α_{s1}-casein) decreases and a peak at d 1.22–1.24 g/cm^3 begins to appear. Between 4 and 6 mg of lysolecithin is required to convert 10 mg of α_{s1}-casein entirely into complex. Addition of further lysolecithin (up to 10 mg) caused no further change in the density gradient profile of the protein.

DISCUSSION

The behaviour of the α_{s1}-casein tetramer and the α_{s1}-casein–lysolecithin complex on Sephadex G-200 indicates that both species have Stokes' radii close to 48 Å. In

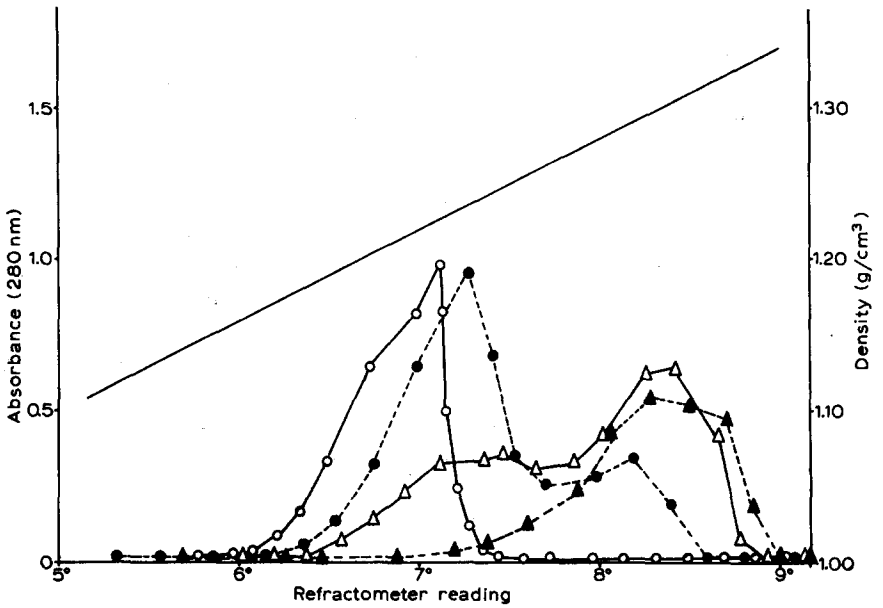

Fig. 3. Density gradient profile of α_{s1}-casein (10 mg) ▲---▲, and with lysolecithin (2 mg) △—△, (4 mg) ●---● and (6 mg) ○—○.

contrast gel electrophoresis demonstrates a clear difference in mobility between the α_{s1}-casein tetramer and the complex, the complex having about one half the mobility of the free protein.

The mobility of solute molecules in gel columns is governed by their size, shape and surface characteristics. With the addition of charge density, these factors also apply to gel electrophoresis. The reduced mobility of the complex (about half that of the protein) on gel electrophoresis together with the information that both the protein and complex show identical behaviour on gel filtration suggests that the complex has about half the charge of the free protein. As lysolecithin contributes no net charge to the complex, we may deduce that the complex contains half as much protein as the α_{s1}-casein tetramer, *i.e.* two monomer units.

In the light of the electrophoresis data, the identical behaviour of the protein and complex on gel filtration would appear to be coincidental. The complex, containing two units of protein, might be expected to have a smaller volume than the α_{s1}-casein tetramer. The surface characteristics of the complex are probably quite similar to those of the protein. The lysolecithin molecules, being bound hydrophobically[1] with their polar groups outwards, would not be expected to alter the charged character of the surface. The similarity in gel filtration behaviour is probably due therefore, to a difference in shape between the complex and the protein, compensating for a difference in volume.

In addition to defining the charge:size ratio of the complex, the gel electrophoresis data also confirm the stoichiometry indicated by the spin label studies described in Part I[1]. As increasing amounts of lysolecithin are added to the α_{s1}-casein, the high-mobility spot decreases in intensity until at 0.5 % lysolecithin, 1.0 % α_{s1}-casein, only a small quantity of free protein is observed.

From the density-gradient ultracentrifugation data, we are able to obtain the hydrated densities of α_{s1}-casein and the α_{s1}-casein lysolecithin complex and also confirm the stoichiometry of the complex. All the protein floats at a density of 1.23 g/cm^3 on the addition of between 4 and 6 mg of lysolecithin to α_{s1}-casein (10 mg). From these data, the maximum number of lysolecithin molecules (mol. wt. = 500) which may bind per α_{s1}-casein monomer (mol. wt. = 28000) is 28. It is also worthy of mention that the stability of the complex in high salt concentrations reinforces our suggestion that the lysolecithin is bound to the protein hydrophobically.

Under conditions of excess lysolecithin, we may calculate a molecular weight for the α_{s1}-casein–lysolecithin complex. Combining the gel electrophoresis result with the stoichiometry data, the complex consists of two α_{s1}-casein monomers (mol. wt. = 28000) and about 56 molecules of lysolecithin (mol. wt. = 500) to give a molecular weight of about 84000.

We are now in a position to suggest a model for the α_{s1}-casein–lysolecithin complex. At the same time it is also pertinent to discuss the gross structure of the α_{s1}-casein tetramer.

From the following equation[6] we may calculate the hydration of α_{s1}-casein:

$$\bar{v}_{\text{protein}} = \frac{1+\Gamma}{\rho_{\text{hyd}}} - \Gamma/\bar{v}_{\text{water}}$$

where ρ_{hyd} is the hydrated density, $\bar{v}_{\text{water}}$ and $\bar{v}_{\text{protein}}$ are the partial specific volumes of water and the protein and Γ is the net hydration. Using $\bar{v}_{\text{protein}} = 0.728$ (ref. 7) for α_{s1}-casein a hydration of 0.15 g water per g protein is obtained. The same hydration is used for both α_{s1}-casein and the complex. The volumes (V) for the α_{s1}-casein tetramer and the complex may thus be calculated from their molecular weights and their corrected densities.

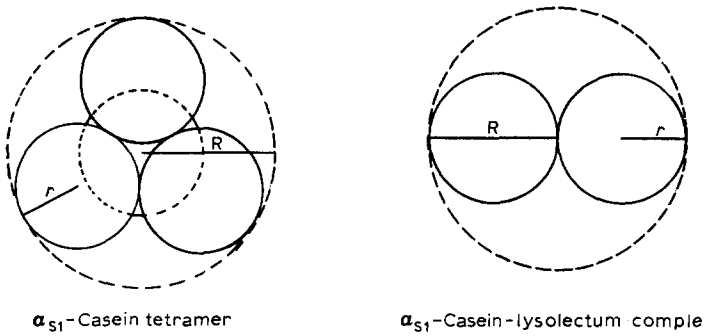

Fig. 4. Proposed models for the α_{s1}-casein tetramer and the α_{s1}-casein–lysolecithin complex.

We must now assume a tetrahedral arrangement of 4 spherical subunits for α_{s1}-casein and a rod-shaped structure of 2 spherical subunits based on the two protein units for the complex. The volumes (v) and radii (r) of these subunits may thus be calculated.

The proposed structures are illustrated in Fig. 4.

The radius of the sphere required to envelop the tetrahedron is given by $R = r + (r/\sin\alpha)$ where 2α is the tetrahedral angle. For the complex the radius of the swept sphere is simply $R = 2r$. The combined data are shown in Table III.

TABLE III

COMBINED DATA FOR THE α_{s1}-CASEIN TETRAMER AND THE α_{s1}-CASEIN–LYSOLECITHIN COMPLEX

Parameter	*α_{s1}-Casein tetramer*	*α_{s1}-Casein–lysolecithin complex*
Mol. wt.	112000 (4 × 28000)*	84000
ρ_{hyd}	1.31 g/cm^3 (observed)	1.23 g/cm^3 (observed)
V	$1.63 \cdot 10^5$ Å^3	$1.30 \cdot 10^5$ Å^3
v	$4.08 \cdot 10^4$ Å^3	$6.50 \cdot 10^4$ Å^3
r	21.3(5) Å	25.0 Å
R	47.5 ± 1.0 Å	50.0 ± 1.0 Å

* Ref. 2.

A square planar arrangement for the α_{s1}-casein tetramer would give $R = 51.6 \pm 1.0$ Å. (The errors for these calculations are based on varying the net hydration of the protein and complex from 0.1 to 0.2 g water per g protein).

Comparing the calculated radii with the observed Stokes' radii of 48 Å measured by gel filtration for α_{s1}-casein and the complex, it becomes clear how the two species can have the same Stokes' radius in spite of the fact that their molecular weights are significantly different.

Conclusion

Combining the data from Part I with those presented above, the following conclusions may be drawn regarding the interaction of lysolecithin with α_{s1}-casein:

When lysolecithin is added to α_{s1}-casein, a complex is formed in which the fatty acid chains of the lysolecithin molecules are bound hydrophobically to the protein whilst the polar-head groups of the lysolecithin molecules remain free. As the amount of added lysolecithin is increased, the α_{s1}-casein tetramer dissociates to form complexes containing two monomer units of α_{s1}-casein.

There is a maximum ratio of 28 lysolecithin molecules which can be bound per monomer of protein. Under conditions of excess lysolecithin, the complex has a molecular weight of about 84000, and a hydrated density of 1.23 g/cm^3.

A rod-shaped structure for the complex is proposed, based on the combined data from all the techniques.

REFERENCES

1 M. D. BARRATT AND L. RAYNER, *Biochim. Biophys. Acta*,
2 D. G. SCHMIDT AND B. W. VAN MARKWIJK, *Biochim. Biophys. Acta*, 154 (1968) 613.
3 N. J. HIPP, M. L. GROVES, J. H. CUSTER AND T. L. MCMEEKIN, *J. Dairy Sci.*, 35 (1952) 272.
4 L.SIEGEL AND K. MONTY, *J. Dairy Sci.*, 112 (1966) 346.
5 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
6 J. VINOGRAD AND J. E. HEARST, *Fat. Chem. Org. Naturst.*, 20 (1960) 372.
7 H. A. MCKENZIE AND R. G. WAKE, *Aust. J. Chem.*, 12 (1959) 734.