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A CUBIC PROTEIN-MONOOLEIN-WATER PHASE

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A cubic monoacylglycerol-protein-water phase has been identified by low-angle X-ray diffraction, and the main features of the ternary phase diagram monoolein/lysozyme/water are presented. The thermal stability of the protein in the lipid-protein cubic phase has been examined by differential scanning calorimetry. According to the physical properties of the phase it is proposed that the protein molecules are located in the water medium, i.e. in the water channel systems of the cubic structure earlier suggested. The ability of various proteins to form this cubic phase has been studied, and it was found that the formation of this phase is favoured by an isoelectric point (p*I*) far from pH 7 in a salt-free solution, thus by high electrostatic repulsive forces.

Introduction

The interaction of proteins with lipids, with special regard to the formation of aqueous liquid-crystalline phases, has been studied in various systems (cf. Refs. 1 and 2). One general conclusion from these results has been that mesomorphic lipid-protein-water phases are formed only when there is a possibility for ionic interaction between lipid and protein molecules [3]. The cubic phase described in the present paper is in this respect different from the phases earlier observed. As reported below proteins with a wide variation in size (about 14–150 kDa) are able to form a cubic phase with monoolein. The dimensions of the lattice depend on the proportion of protein and water present. The formation of this lipid-protein phase is discussed in relation to the cubic monoolein-water structure earlier proposed, with a continuous lipid bilayer which separates two water channel systems [4].

Little is known from earlier studies about the protein structure in such lipid-protein liquid-crystalline phases. In a Raman spectroscopy study of insulin-phospholipid-water phases [5], it was reported that the protein kept its native structure provided the lipid chains were below a certain length. The present work has been focused on the thermal stability of the protein structure in a cubic lipid-protein phase.

Materials

The 1-monoolein used (more than 99% pure, lot No. 110F-0481) was from Sigma Chemical Co. Lysozyme (L-6876, lot No. 57C-8025), α-lactalbumin (L-4379, lot No. 50F-8105), soybean trypsin inhibitor (T-9003, lot No. 68B-0560), myoglobin (M-0380, lot No. 85C-0138), pepsin (P-7012, lot No. 16C-7201), bovine serum albumin (A-7511, lot No. 16C-7201), conalbumin(C-0755, lot No. 39C-8000), and glucose oxidase (G-2133, lot No. 66C-0201) were all purchased from Sigma Chemical Co. Lysozyme, myoglobin and pepsin were dialyzed against distilled water for 24 h followed by freeze-drying to constant weight.

Methods

Preparation of samples. Samples were prepared at 40°C by slow addition of the aqueous protein solution (prepared in double distilled water) to the liquid monoacylglycerol. The samples were then allowed to equilibrate at room temperature for a maximum of 24 h (or until no change in the polarizing microscope could be detected).

A one-phase region of the cubic phase is simpler to identify than other mesophases. This phase is completely transparent, very viscous and optically isotropic. Still it was checked by centrifugation (8000 × *g* during 48 h) that no phase separation can be achieved especially that no excess protein solution was present.

X-ray diffraction. The samples were identified by a Guinier type of low-angle camera according to Luzzati and a Kiessig type of point-focus camera as described earlier [6].

Microscopy. For microscopic examination of the samples an Olympus Vanox polarizing microscope was used. A heating gradient, ranging from 25°C to 110°C was achieved by a Mettler FP52 heating table, using a heating rate of 10 K/min.

Differential scanning calorimetry. Calorimetric measurements were performed in a Perkin-Elmer DSC-2C calibrated according to Ref. 7. Samples (approx. 5 mg) were weighed into Perkin-Elmer standard volatile sample aluminium pans (part No. 219-0062), using an empty pan as reference.

Results

The phase diagram

Fig. 1 shows the main features of the ternary phase diagram monoolein/lysozyme/water at 40°C. The phases were identified in the polarizing microscope and by X-ray diffraction.

A limiting factor for the lysozyme concentration in this ternary system is the high viscosity of the protein solution above about 45%(w/w). Due to this experimental difficulty we have only information on the phase equilibria in the concentration region below 45% (w/w) lysozyme in water (see Fig. 1). Furthermore, the phase equlibria above a lipid concentration of 80%(w/w) were extremely slow, and we have therefore omitted also this region of the phase diagram.

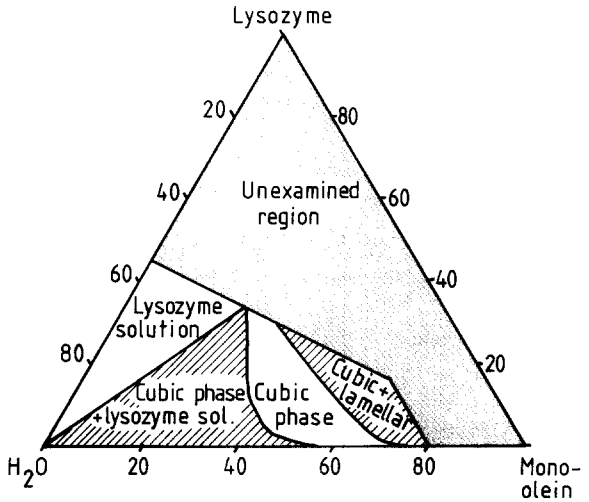

Fig. 1. Ternary phase diagram of the system monoolein/lysozyme/water at 40°C.

At 40°C the binary system monoolein/water forms a 'viscous isotropic' phase [8] at water concentrations from 20 to 40% (w/w). This structure has been characterized by low-angle X-ray diffraction [4,6] to be a body-centered cubic structure. This cubic phase has been proposed to be composed of bilayer units forming a continuous three-dimensional network, which separates two identical water-channel systems. The same type of cubic phase is formed in the ternary system lysozyme/monoolein/water according to X-ray diffraction data. As can be seen from the phase diagram (Fig. 1), the phase boundaries for the cubic phase are shifted towards higher water content as compared to the binary monoolein/water system, already at low lysozyme concentrations.

X-ray diffraction

X-ray diffraction data on the cubic phase reveal, as a result of the raised water content, an increase of the lattice dimensions when lysozyme is present. Another interesting effect due to the protein is the temperature fall-off of the reflection intensities, which is reduced so that many more reflections can be observed. An illustrative example of such a phase exhibiting 28 reflections is given in Table I.

Microscopy

Thermal phase transitions were followed in the

TABLE I

X-RAY DIFFRACTION DATA OF THE CUBIC PHASE OF MONOOLEIN/LYSOZYME/WATER (31.6:34.2:34.2, w/w)

All diffraction lines fit into a body-centered cubic lattice (a_0 = 235 Å) but only the low-order reflections can be indexed unambiguously. For lower lysozyme concentrations the lattice is smaller, e.g. the composition monoolein/lysozyme/water (56.0:6.6:37.4, w/w) gives a_0 = 215 Å. The values of a_0 for monoolein-water cubic phases at different water contents are given by Lindblom et al. [6].

d (Å)	hkl	a_0	I [a]	d (Å) (contin.)	I [a]
96.6	211	236.6	vs	17.33	w
82.97	220	234.7	s	16.14	w
68.49	222	237.3	w	15.03	w
54.62	411, 330	231.7	w	14.24	vw
41.09	440	232.4	m	13.36	w
36.26	541	235.0	m	12.32	w
32.77	640	234.2	w	11.39	w
29.02		Mean			
		value	w	10.74	w
28.02		234.6	w	10.22	w
27.08			w	9.79	vw
23.45			vw	9.40	vw
21.50			w	9.13	vw
20.81			w	9.05	vw
19.44			w	8.55	vw

[a] I = intensity, using an intensity scale of vs = very strong, s = strong, m = medium, w = weak, vw = very weak.

polarizing microscope. The temperature induced transitions of the binary system monoolein/water have been reported by Lutton [8]. In the excess of water region (above 40% (w/w) water) there is a transition at heating of the cubic phase at 90°C to a hexagonal phase. Further heating to above 100°C results in a transition from the hexagonal phase to an isotropic fluid (L2-phase according to Ekwall's nomenclature [9]). The same phase transitions were found to take place also when the cubic monoolein/water phase contained protein. Thus, the hexagonal phase was found to appear at a temperature of 91–93°C, evident in the polarizing microscope from the characteristic texture of the sample. The transition into the L2-phase, evident from the disappearance of birefringence and a 'melting' of the sample, was observed at a temperature of about 96–102°C. The reversed behaviour was seen on cooling.

Differential scanning calorimetry

Fig. 2 shows a typical thermogram of a sample of the cubic phase containing lysozyme. Protein denaturation occurs at 64°C (T_D in Fig. 2), involving an enthalpy of denaturation of about 300 kJ/mol protein. Both the temperature and the enthalpy values are typical for the samples of the cubic phase containing lysozyme in various concentrations (ranging from 6% (w/w) lysozyme to 34% (w/w) lysozyme). That is, no difference related to protein concentration either in temperature or in denaturation enthalpy can be seen. The values obtained are in good agreement with results of Fujita and Noda [10] from studies on lysozyme at low and intermediate water content. Thus in the lysozyme concentration range examined here, lysozyme in the cubic phase shows native qualities as regard temperature and enthalpy.

Lysozyme is known to be reversible on heat denaturation [11]. It was found that the reversibility of lysozyme in the cubic phase, however, depends on kinetic parameters, i.e. heating and cooling rate, and also on the maximum temperature the sample has been exposed to. According to DSC-measurements at reheating of the samples, reversibility ranges from around 90% (high and intermediate heating/cooling rates, the sample not exposed to temperatures above 100°C) to 0% (slow

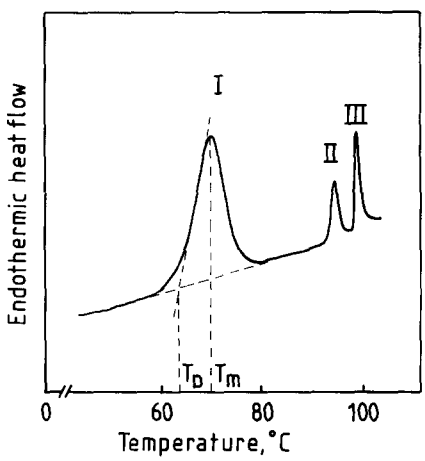

Fig. 2. Thermogram of a cubic phase sample with the composition monoolein/lysozyme/water (35.6:25.1:39.3, w/w). Peak I corresponds to denaturation of the protein, peak II corresponds to the phase transition cubic → hexagonal and peak III corresponds to the phase transition hexagonal → L2 (inversed micellar phase).

heating/cooling rates, maximum temperature above 100°C). Thus, the degree of reversibility seems to be dependent on the time-temperature profile above the hexagonal to L2-phase transition, which involves a 'melted' phase. This transition occurs, dependent on the composition of the samples, between 96–102°C, i.e. near the boiling point of the water.

The phase transition identified by the polarizing microscope can also be seen in the thermograms (cf. Fig. 2). The transition from cubic phase to hexagonal phase occurs as an endothermic peak at 96°C, and on further heating there is a second endothermic peak revealing the transition into the L2-phase at 99°C. Both these transitions involve enthalpies in the magnitude of 0.75 kJ/mol monoolein.

Other proteins forming the cubic phase with monoolein and water

It has earlier been reported that a very hydrophobic wheat protein fraction, gliadin, could be solubilized into the cubic monoolein-water phase [12]. Thus 10% (w/w) of A gliadin dispersed in 90% (w/w) monoolein, formed the cubic phase when water was added. Gulik-Krzywicki et al. [13] in a recent freeze-etching electron microscopy study of the cubic structure, has found that an aqueous cytochrome *c* solution can form this cubic phase with sunflower oil monoacylglycerol.

To examine whether the effect of lysozyme on the cubic monoolein-water phase is a phenomenon general to hydrophilic proteins, a few other globular proteins were examined. The composition of these new protein samples was chosen to be approx. 40% (w/w) monoolein/18% (w/w) protein/42% (w/w) water. The proteins studied were α-lactalbumin (M_r 14200, p*I* 4.2–4.5) [14], soybean trypsin inhibitor (STI) (M_r 20100, p*I* 4.0) [15], myoglobin (M_r 17000, p*I* 7.0) [16], pepsin (M_r 35000, p*I* 1.0) [16], bovine serum albumin (BSA) (M_r 67000, p*I* 4.5–5) [17,18], conalbumin (M_r 77000, p*I* 6.5–6.8) [19,20], and glucose oxidase (M_r 150000, p*I* 4.2) [21].

α-Lactalbumin, bovine serum albumin and pepsin resemble lysozyme when they form the cubic phase. They give after equilibration for 24 h a viscous transparent phase, which is optically isotropic, and shows the same characteristics as the cubic monoolein-water phase. Also myoglobin forms the cubic phase together with monoolein. The behaviour is different, however, as a prolonged equilibration time was needed (for this weight ratio 7 days) in order to obtain a homogeneous cubic phase.

α-Lactalbumin and lysozyme are small proteins similar in size and closely related regarding their amino acid composition and structure [22], which can explain the resemblance in their phase behaviour. Bovine serum albumin, on the other hand, is a much larger protein, which indicates that the geometry of the cubic lattice is flexible, allowing hydrophilic proteins of different sizes to be introduced.

As lysozyme and pepsin carry a high net charge (p*I* near 11 and 1, respectively), whereas myoglobin has an p*I* of 7, electrostatic repulsion forces might be of importance in the formation of the ternary cubic phase. These three proteins had been desalted before the addition to the monoacylglycerol, i.e. the shielding effect of salt present in the aqueous protein solutions has been diminished. If these proteins are added prior to desalting the phase behaviour is quite different. The myoglobin/monoolein sample gives after equilibration a cubic monoolein-water phase in contact with excess protein, whereas pepsin and lysozyme enter the cubic phase, but at an extremely slow rate.

From this it might be concluded that high apparent net charge repulsion between the protein molecules favours the formation of the cubic monoacylglycerol-protein-water phase. The other proteins examined behave in a similar way, thus even the largest protein used in this investigation, glucose oxidase (M_r 150000), can form the cubic phase.

Discussion

The cubic protein-monoacylglycerol-water phase described above exhibits similar intensity distribution of the X-ray diffraction pattern as the monoolein-water phase [6]. Thus, there are no reasons to suggest any structural differences in relation to the bilayer model proposed [4]. It can be assumed that the protein molecules are not attached to the lipid bilayer matrix but is located in the water channel systems according to the following evidence:

1. Lysozyme activity can be observed outside the cubic phase (a suspension of dried cells of *Micrococcus lysodeikticus* was added to the cubic phase containing lysozyme and enzymatic activity was observed in the suspension outside the cubic phase).

2. Diffusion of coloured proteins can be followed visually. It can thus be seen how proteins such as myoglobin move into the cubic monoacylglycerol-water phase from an outside protein solution or out from the cubic monoacylglycerol-protein-water phase to an outside water solution.

3. A cubic monoacylglycerol-lysozyme-water phase can be dried over vacuum until all the water is removed, and the same aqueous phase can then be reconstituted by water exposition with the protein still in its native state as measured by DSC.

4. The enthalpy and temperature of thermal denaturation are the same for the protein in the cubic phase and in aqueous solution.

The difference in behaviour of the hydrophilic proteins examined can possibly be explained by the difference in net charge. Electrostatic repulsion forces between the protein molecules might influence the formation of the cubic lattice. Proteins with isoelectric point (p*I*) far from pH 7, thus with high net charge in a water solution, seem to have a higher ability to increase the dimension of the cubic lattice than proteins with p*I* near 7. The effect of the salt concentration, i.e. the shielding of the electrostatic repulsion, is in accordance with this model. Thus, when the protein carries an apparent net charge, the formation of the cubic protein-monoacylglycerol-water phase is facilitated, and equilibration time required is shortened.

The cubic monoacylglycerol-water phase can be used to induce membrane fusion. Monoolein, for example, is a well-known fusogenic substance, and evidence for the formation of a cubic structure in connection with fusion has been reported [23]. A possible mechanism might be that a fragment of the cubic phase adheres to the actual membrane by a local joint between the bilayer of the cubic phase and the membrane bilayer. When the bilayers are joined, the lipid composition will tend to be equally distributed by the rapid lateral diffusion. If the cubic 'particle' constitutes a minor part of the total bilayer system, it should thus be expected to be solubilized in the membrane. If there also is a joint between the cubic fragment and another membrane, such a diffusion should lead to fusion, and any protein in the cubic phase will also be involved in such a fusion process.

Acknowledgements

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