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Interactions Between Proteins and Polar Lipids

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I. INTRODUCTION

Proteins and polar lipids coexist in biological systems, sometimes unassociated with each other, but also as composite structures with specific actions (e.g., cell membranes and blood lipoproteins). They have a very important physical property in common—an inherent amphiphilic nature, which provides the driving force for formation of associated structures of lipids as well as for the folding of a polypeptide chain to form the unique conformation of a native protein. An important consequence of this dualistic character is that the molecules will orient at an interface. Composite biological structures are formed in this way, such as cell membranes, which separate one microenvironment from another. Therefore, it is not surprising that the major concern of the vast number of publications dealing with protein–lipid interactions have been focused on biological membranes. The main concern has been to gain a better understanding of biomembrane function, fusion, and other membrane processes, which has evoked a number of studies of these complex lipid–protein ''supramolecular'' systems. There are a number of reviews on this expanding area of research, such as Refs. 1–5 and the book edited by Watts (6). The classic technique for studying protein–lipid interactions in this context has been to study the properties of lipid or mixed lipid–protein monolayers at the air–aqueous interface by the surface film balance by itself or in combination with techniques such as surface rheology measurements and imaging techniques (4,7–14).

Many proteins have also the biological role of transporting molecules with hydrophobic properties, which bind to hydrophobic pockets in the protein. One such protein is the major whey protein in milk, β-lactoglobulin—a lipocalin type of protein. The biological role of β-lactoglobulin is thought to be to transport retinol (or possibly other hydrophobic ligands) (15–19). Several studies have clearly demonstrated that this protein has a high-affinity binding site for a range of ligands like phospholipids, fatty acids, cholesterol, and triglycerides (19–26). This has implications for the processing of milk and dairy products in terms of, for instance, thermal stability, as discussed by Sawyer et al. (27).

Water-soluble polar lipids and synthetic analogs (surfactants) are widely used in many technical and analytical applications. As we will discuss in the following, the interaction between proteins and polar lipids can lead both to stabilization of the protein structure and to unfolding. An example of the latter is the unfolding induced by sodium dodecylsulfate (SDS), used for analytical purpose in the SDS–polyacrylamide gel electrophoretic (SDS–PAGE) determination of molecular weights (28,29).

Proteins and lipids, separately as well as in mutual interaction, contribute significantly to the physical properties of many systems of technological interest (e.g., emulsions and foams). Further, protein structure, and thereby function and properties, are affected in various ways by polar lipids. The intention of the review is to discuss the diverse nature of (polar) lipid–protein interactions and how it can affect the physicochemical properties of lipid–protein systems. We will mainly focus on aspects relevant for the properties of emulsions and foams. However, it must be pointed out that lipid–protein interactions at interfaces are of fundamental importance also in many biological processes. One such example is the pulmonary surfactants system, consisting of a complex mixture of proteins and lipids, which is essential for the function of the lungs (30–32). Obviously, the function of the lungs involves the formation of lipid–protein film at the air–aqueous interface. Recently, the alveolar surface has been suggested to be lined by a liquid-crystalline phase (33–35), rather than a monolayer, which so far has been the traditional view as presented by Clements in the 1950s (36). Lipase-catalyzed lipolyses is another interfacial process that is of large physiological importance (37). In addition, lipase-catalyzed lipolyses has come to large industrial importance in many technical applications, including detergency and food processing (38). Because the lipases are water soluble and most of the natural lipids have low aqueous solubility, lipolyses takes place at the aqueous–lipid interface and therefore one often speaks of ''interfacial activation'' in connection with lipase activity (39,40).

The structure of lipids in aqueous systems is discussed in Chapters 3 and 4. General information of proteins and protein conformation can be found in most textbooks on biochemistry. There are also publications dedicated to protein structure, like the excellent book by Creighton (41) or quite a number of good reviews (42–45). Here, we will highlight only the structural and physicochemical properties of importance for the understanding of polar lipid–protein interactions.

II. LIPIDS

Lipids can be divided into two major groups: polar and nonpolar lipids. The nonpolar lipids, primarily the triglycerides, have small polar groups and, hence, show only limited interaction with aqueous systems. The polar lipids, however, with large charged or uncharged polar groups, giving these lipids an amphiphilic nature, associate in aqueous systems. The common feature for the self-assembly of the polar lipids in aqueous environment is the formation of a polar interface, which separates the hydrocarbon and water regions. The hydrocarbon chains can exist either in a fluid state, as in liquid-crystalline phases, or in a solid state, as in the lipid gel phases (46). Generally, the melting of the chains in an aqueous environment occurs at a much lower temperature compared to the melting of the pure lipid.

Polar lipids can be divided further into two classes on the basis of their interaction with water:

1. Lipids and synthetic analogs that are water soluble in monomeric and micellar form (i.e., surfactants)
2. Lipids with very low water solubility, but with the ability to swell into liquid-crystalline phases

The water-soluble polar lipids (e.g., ionized fatty acids, bile salts, and synthetic surfactants, charged or uncharged) have monomeric solubility in the millimolar range and form micelles at higher concentrations. The critical micelle concentration (cmc) is considered to be a narrow concentration range, within which aggregates start to form by a strong cooperative process (47). The driving force for micelle formation is the hydrophobic interaction (cf. Ref. 48). The cmc for single-chain amphiphiles decreases with increasing chain length; for ionic amphiphiles, cmc also depends on the ionic strength, because the addition of salt reduces the electrostatic repulsion between the charged head groups. Increased temperature has, however, only a moderate influence on cmc, once the temperature has exceeded the critical temperature, where the monomer

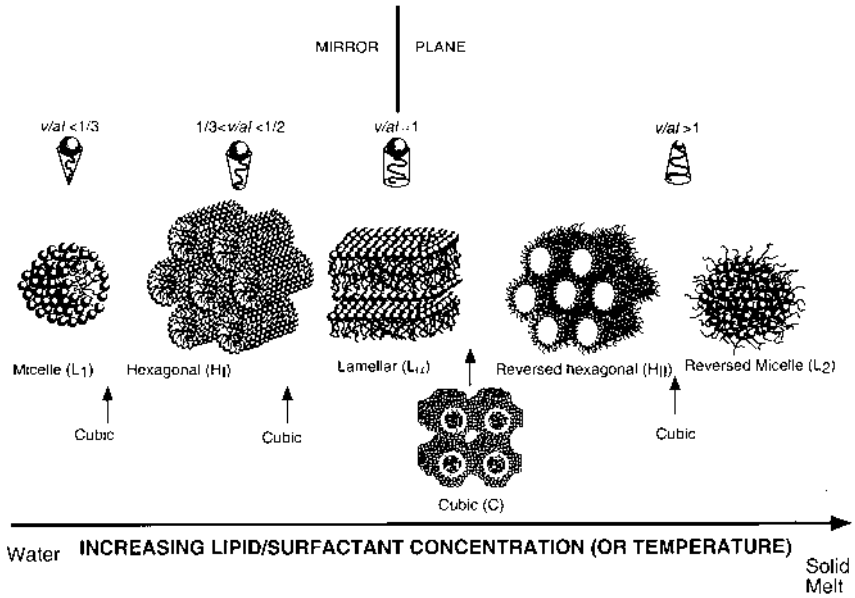

Figure 1 Commonly formed association structures by polar lipids. Phase transitions can be induced by changes in water content, temperature, or by interaction with other solution components, like proteins. The lamellar liquid-crystalline phase (L_α) can be regarded as the mirror plane, where the aggregates are of the "oil-in-water" type on the water-rich side and of "water-in-oil" type on the water-poor side (49). On both, the water-rich and water-poor sides of the L_α, there are two possible locations for cubic phases. Other "intermediate phases" may also occur. The formation of a particular phase can, in many cases, be understood by looking at the geometric packing properties of the amphiphilic molecule in the particular environment (50,51). This property can be expressed by the so-called packing parameter (v/al), which is defined as the ratio between the volume of the hydrophobic chain (v) and the product of the head group area (a) and the chain length (l).

solubility is equal to the cmc (Krafft temperature). At low water content, an inverse micellar structure, the L_2 phase, is formed, in which the hydrocarbon chains form the continuous medium and the aqueous medium is present within the micelles.

A common feature of the two classes of polar lipids is the tendency to form lyotropic liquid-crystalline phases. A summary of some of the different liquid-crystalline phases that can occur is given in Fig. 1. With decreasing water content, the phase behavior of polar lipids often follows the sequence hexagonal phase (H_I) $\rightarrow$ lamellar phase (L_α) for water soluble lipids and

lamellar phase (L_α) → reversed hexagonal phase (H_{II}) for lipids with low water solubility. Cubic liquid-crystalline phases (Q) often occur in between these. Phase transitions can also occur with changes in temperature; with increasing temperature, the sequence of thermal transitions is usually the same as with decreased water content. In many cases, the formation of a particular phase can be understood by looking at the geometric packing properties of the amphiphilic molecule in the particular environment (50,51), which is the cross-section area of the polar head group in relation to that of the acyl chain. This property can be expressed by the so-called packing parameter (v/al), which is defined as the ratio between the volume of the hydrophobic chain (v) and the product of the head group area (a) and the chain length (l). The packing parameter for a particular environment will determine the curvature of the interface and, thus, the particular phase. Generally speaking (see Fig. 1), a value of the packing parameter lower than unity (cone-shaped amphiphile) facilitates the formation of structures where the polar interface is curved toward the hydrocarbon phase (i.e., structures of "oil-in-water" type (L_1, H_I). On the other hand, a value larger than unity (reversed cone-shaped amphiphile) will give the reverse curvature and favor "water-in-oil" structures like H_{II} and L_2. When the packing parameter is changed, for instance, by the change of ionic strength or temperature or the addition of other molecules like proteins, phase transitions will ultimately arise. Increased temperature, for example, will increase chain mobility and thereby increase the volume of the lipophilic part of the molecules, explaining the often seen thermally induced transition $L_\alpha \rightarrow H_{II}$. Decreased hydration will decrease the head-group repulsion, resulting in a decreased interface area and, thus, in an increase of the packing parameter.

In nature and in many technical applications, the lipid aggregates consist of a mixture of different lipids, which either exist in a homogenous mixture or separate into domains. As discussed in the review by Raudino (52), the lateral distribution in these mixed aggregates is influenced by a number of factors like ionic strength, presence of polymers/proteins, as well as the composition of the lipids, and it is thus hard to give any general rules to predict when phase separation will occur.

Luzzati and co-workers determined the main features of the most commonly found mesophases in the early 1960s by x-ray diffraction [reviewed by Luzzati in 1968 (53); see also Chapter 3 of this book].

Results from spectroscopy studies have increased the understanding of the dynamic nature of these phases. The lamellar phase (L_α) consists of stacked infinite lipid bilayers separated by water layers, whereas the hexagonal phases consists of infinite cylinders, having either a hydrocarbon core (H_I) or a water core (H_{II}). As shown in Fig. 1, the cubic phases (Q) can exist in several locations in the phase diagram. The cubic phases have

been shown to exist in a number of lipid systems (54,55). They are isotropic and highly viscoelastic. Different structures of the cubic phases, depending on the particular lipid system, have been suggested (49,55–59). One type of cubic structure, consisting of rodlike aggregates connected three and three at each end, has been proposed by Luzzati et al. (56). The two polar rodlike networks formed in this way gives two continuous and unconnected systems of fluid hydrocarbon chains. We will, however, mainly focus on the other main type of cubic phase, which has been observed in aqueous dispersions of polar lipids with low aqueous solubility like monoglycerides, phospholipids, and glyceroglucolipids (46,54,57), as well as for water-soluble surfactants like ethoxylated fatty alcohols (60). This type of cubic phase is bicontinuous and based on curved nonintersecting lipid bilayers (cf. Fig. 10) that are organized to form two unconnected continuous systems of water channels (cf. Refs. 55,57, and 61 and Chapter 11 of this book). The two principal radii of curvature, R_1 and R_2, can be used to describe the curvature of any surface. The average curvature $1/2(1/R_1 + 1/R_2)$ is by definition zero at any point for a minimal surface. Thus, at all points, the surface is as concave as it is convex. If an interface is placed in the gap between the methyl end groups of the lipid in the bicontinuous bilayer type of cubic phase, it will form a plane that can be described as a minimal surface (57,62). A minimal surface exhibiting periodicity, like in the cubic lipid–aqueous phase, is termed an *infinite periodic minimal surface* (IPMS). It has been shown by Hyde that the packing parameter for a lipid in such a curved bilayer is larger than unity and can be related to the Gaussian curvature $[1/(R_1\ R_2)]$ of the IPMS. Three types of IPMS, described by different cubic space groups, have been shown to be important in lipid systems (57,59,62):

- The primitive lattice (*Pn3m*) which corresponds to the diamond (D) type of IPMS
- The body-centered lattice (*Ia3d*) which corresponds to the gyroid (G) type of IPMS
- The body-centered lattice (*Im3m*) which corresponds to the primitive (P) type of IPMS.

The occurance of micellar cubic phase, C_{mic}, space group *Fd3m*, where disjointed reversed micelles embedded in a three-dimensional hydrocarbon matrix are organized in a cubic symmetry has been reborted by Luzzati and co-workers (64). The formation of this type of C_{mic} phase has previously been reported for aqueous systems containing monoolein and oleic acid (64–67), for aqueous mixtures of sodium oleate and oleic acid (68), and, consequently, also during lipase-catalysed lipolysis of monoolein in aqueous dispersions under neutral/alkaline conditions (69,70).

Today cubic lipid–aqueous phases are recognized as important in biological systems (34,46,55,57,58,59,66,71–75). Some of these reports suggest that cubic lipid–aqueous phases can occur during the fusion of biological membranes. There are vast amount of studies of membrane fusion [cf. the comprehensive reviews by Kinnunen and Holopainen (2)], which is impossible to cover here. It is however worth mentioning that early studies on the fusion process for biological membranes reported that lipidic particles were discovered during the fusion event (76), indicating that additional liquid-crystalline phases occur during the fusion process. The nature of these intermediate phases during fusion is unclear, although the formation of interbilayer structures as inverted micelles have been proposed (77). In addition, the fusion rate is increased in systems which have a lamellar phase $\rightarrow$ inverted hexagonal phase transition (76,78,79). As will be discussed further, such a phase transition can be affected by proteins and polypeptides and there are also several reports on the promotion of fusion by proteins and polypeptides (52).

The liquid-crystalline lipid–aqueous phases can exist in excess of aqueous solution. One example of such lipid dispersions is unilamellar or multilamellar vesicles,* which are formed from lamellar (L_α), phases. The stability, size, and shape of vesicles can vary, depending on the composition of lipids and aqueous phase (for reviews, see Refs. 80–83). In analogy with liposomes, dispersions of a cubic lipid–aqueous phases, cubosomes, which were first discovered by Larsson et al. (57,75,84) are also formed with an excess of water. The stability of cubosomes, formed in MO–H_2O-based systems, and the corresponding dispersed H_{II} phase (hexosomes) in the MO–TO–H_2O system was found to increase in the presence of an amphiphilic block copolymer (polyoxamer) (84–86).

III. PROTEINS

An important consequence of protein–lipid interaction is the effect on the stability of the protein in solution as well as its behavior at interfaces. When discussing the stability of proteins, we may distinguish between the conformational stability of proteins and aggregation/precipitation phenomena due to reduced solubility at pH close to the isoelectric point, at high ionic strength (salting out), and/or caused by specific binding of ions (e.g., the formation of calcium bridges) or lipids. Although the two phenomena

*The term *liposomes* are, according to IUPAC recommendation, synonymous to lipid vesicles, but are sometimes used for multilamellar vesicles.

usually are connected, aggregation/precipitation can occur without major conformational changes of the protein (87). The conformational stability of a protein, which, of course, has no meaning for proteins lacking secondary structure, can be estimated by circular dichroism (cf. Ref. 41), compressibility measurement (cf. Ref. 88), and calorimetry (cf. Refs. 89–91). The stabilization of the protein structure have been extensively reviewed by a number of authors (cf. Refs. 43,44, and 89–92), and we will only focus on some aspects of significance in emulsion systems.

The native protein structure is a consequence of a delicate balance of forces, including electrostatic forces, hydrogen-bonding, van der Waals forces, conformational entropy, and so-called hydrophobic interactions (cf. Refs. 43 and 91–94). The amino acid sequence of the polypeptide chain (the primary structure) will determine the folding into structural units (the secondary structure), and the association of structural units into domains, tertiary and quaternary structures, gives each protein the unique conformation connected with its action and specificity. Naturally, cross-links, such as disulfide bridges, increase the stability of a protein.

The interior of a globular protein is very densely packed, having a quite constant mean packing density (0.74), a value also found for crystals of small organic molecules (94). Thus, van der Waals forces and hydrogen-bonding, which are short-range interactions, play an important role for the stability of folded proteins (91). As first pointed out by Kauzmann (95), it is clear that the so-called hydrophobic interactions play an important role in stabilizing the protein structure. The nonpolar amino acid residues will provide a strong driving force for folding, leading to an accumulation of hydrophobic residues in the core of the protein molecule. The polar amino acid residues (uncharged and charged) will have a high affinity for aqueous solvent and will, consequently, be located on the outside of the protein. The nature of hydrophobic interactions in this context is not yet fully understood (cf. Refs. 43,91, and 92), because it still is difficult to analyze them separately from other forces contributing to the stabilization of the protein structure (91).

It is important to bear in mind that proteins are only marginally stable at room temperature. This means that the exchange of only one amino acid residue, by, for instance, genetic engineering, might destabilize or stabilize the protein considerably. In the following, it will be demonstrated that the binding of lipids to the protein also can have the same effects. In many systems involving proteins and lipids, the protein–lipid interaction takes place at an interface. As discussed extensively by Norde et al. (96–98), the delicate balance between forces that stabilize and destabilize the protein might be shifted in the proximity of an interface, leading to unfolding upon adsorption. The loss of entropy upon protein folding is the main

force counteracting the stabilization of the protein structure (43). Thus, unfolding upon adsorption is an entropically favored process (96–98). Furthermore, at an interface, the unfolded hydrophobic domains might be oriented in such a way that their exposure to the aqueous environment is minimized. In fact, Norde argues that the entropy gained by the unfolding of the protein upon adsorption can be a significant driving force for adsorption (96–98). However, they also observed that adsorption of protein on apolar surfaces might lead to an increase order of the protein secondary structure as observed for enzymes like α-chymotrypsin and serine proteinase savinase on Teflon (98–100).

The folding and unfolding of proteins have been shown, under certain conditions, to occur via an intermediate state, the molten globule state (101–106). This state, which is somewhere between the native and completely unfolded state, is characterized by a retained secondary structure, but with a fluctuating tertiary structure. The protein molecule is also more expanded and exposes more hydrophobic domains. The molten globule state is hard to detect by calorimetric measurements, because the unfolding of the molten globule is accompanied with little or no heat absorption (104). As discussed by Dickinson and Matsumura (106), the molten globule state can be achieved in a number of ways, as pH changes, increase of temperature, the use of denaturation agents, breaking of disulfide bridges, and removal of ligands or cofactors bound to the protein. It is also suggested that the protein can adopt a molten globule state when interacting with an interface. In fact, it was found that α-lactalbumin was more surface active under conditions where it exists in the molten globule state. It has been proposed that the molten globule state of the protein may be required for the translocation of proteins across biological membranes (107,108).

It should be noted that although it is possible to classify proteins as globular, unordered (lacking secondary structure), and fibrous, there are marked differences between proteins belonging to the same class, due to their complex and intriguing design. Thus, it is much harder for proteins than for polar lipids to follow rules of thumb for their behavior. Protein properties such as conformation, charge distribution, association, and activity are also strongly influenced by environmental condition (e.g., pH, ionic strength, type of ion, and temperature). In this context, it is important to point out the effect of type, valence, and ionic strength of an added electrolyte. This can have a profound effect on interactions involving proteins and other polyelectrolytes, in particular under physiologically relevant conditions as discussed by Ninham et al. (109–111). They argue that the present theory is not sufficient to distinguish between van der Waals interactions and electrostatic interactions. In addition, as part of their biological role,

some proteins have specific binding sites for lipids. These binding sites can even be specific for a certain class of lipids. Thus, it is important to consider protein–lipid interactions in relation to the features of each individual protein.

IV. INTERACTION BETWEEN GLOBULAR PROTEINS AND WATER-SOLUBLE POLAR LIPIDS

A. In Solution

Ionic surfactants interact with most proteins. A high surfactant concentration will generally lead to unfolding of the protein structure. The interaction between nonionic surfactants are weaker and seldom affect the structure of proteins. Several reviews concerning the interaction between water-soluble polar lipids and protein are focused on the interaction between ionic surfactants [e.g., sodium dodecyl sulfate (SDS) and globular proteins at low and intermediate temperatures] (11,112–118). Because vast amounts of the surfactant–protein work is devoted SDS, we will use this system as an example, and at the end of this subsection, we will discuss some exceptions.

We can distinguish between two types of binding of water-soluble lipids to proteins:

1. A high-affinity type of binding that occurs at low lipid concentration
2. Nonspecific cooperative interaction taking place at higher concentrations (115,116).

An example of a binding isotherm, where the two types of binding occur, is given in Fig. 2. In this isotherm, for the binding of SDS to lysozyme, the regions of high-affinity noncooperative binding, at low surfactant concentration, is well separated from the cooperative binding observed at a higher concentration. For comparison, an example of a binding isotherm for the binding of a nonionic surfactant, *n*-octyl-β-glucoside, to the same protein is also inserted. In this case, only nonspecific cooperative binding occurs.

The specific binding is mediated by ionic and hydrophobic interactions and usually occurs below the cmc of the surfactant (115,119–122).

The nonspecific interaction occurs close to or above the cmc and usually leads to a destabilization of the native conformation. The cmc of the surfactant is thus an important parameter and conditions that affect cmc will generally affect the binding (cf. Refs. 116 and 123). The saturation of all of the binding sites generally corresponds to 1–2 g of surfactant per gram of protein (115,116,124). The extent of interaction and unfolding depend

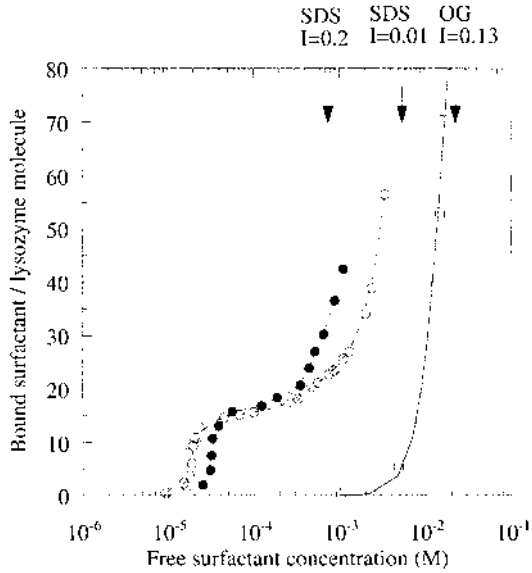

Figure 2 Binding isotherms for binding of surfactants to lysozyme in aqueous solution at 25°C. The isotherms (○, ●) for SDS have regions of both high-affinity noncooperative binding, at low surfactant concentration, and cooperative binding at high concentration. The influence of ionic strength on the binding isotherm is shown: ○, ionic strength (I) = 0.0119 M and ●, ionic strength = 0.2119 M at pH 3.2. For comparison, an example of a binding isotherm where only nonspecific cooperative binding occurs is also inserted. This isotherm, describing the binding of the nonionic *n*-octyl-β-glucoside (OG) to lysozyme (□) was measured at pH 6.4 and ionic strength of 0.132 M. The protein concentration was 0.13% (w/v). The arrows indicate the cmc for the different surfactants and ionic strengths. The data are adopted from Ref. 115 and the experimental details are given in Refs. 160 and 115 for SDS and OG, respectively.

mainly on the nature of the surfactant hydrophilic group, surfactant chain length, ionic strength, pH, temperature, and organic additives as well as on the protein itself (116). Organic additives include the presence of impurities in proteins as well as in the lipids. For instance, it has been demonstrated by Lunkenheimer and co-workers that commercial SDS samples usually contains a substantial amount of decanol, which actually is more surface active than SDS in itself (125–127). Similarly, it has been shown by Clark et al. that β-lactoglobulin contain bound fatty acids, which may alter the binding of other surface-active compounds (128). Clearly, the presence of amphiphilic impurities may give anomalous effects on the binding of other surfactants.

1. Anionic

The effect of surfactant protein interaction on the structural stability of proteins depends strongly on the mode of interaction. In fact, as shown in Fig. 3, the same surfactant can act to both stabilize and destabilize, depending on surfactant concentration as well as other solution conditions. At low surfactant-to-protein ratios, a high-affinity interaction between certain proteins and surfactants occurs. This interaction stabilizes the protein structure against thermally induced unfolding; thus, the thermally induced transition is shifted toward a higher temperature, as illustrated in Fig. 3 and previously reported by Hegg (129) for SDS and β-lactoglobulin. Similar findings have also been reported for other protein–surfactant complexes such as between fatty acids or SDS and bovine serum albumin (130) as well as between palmitic acid and β-lactoglobulin (131). As discussed earlier,

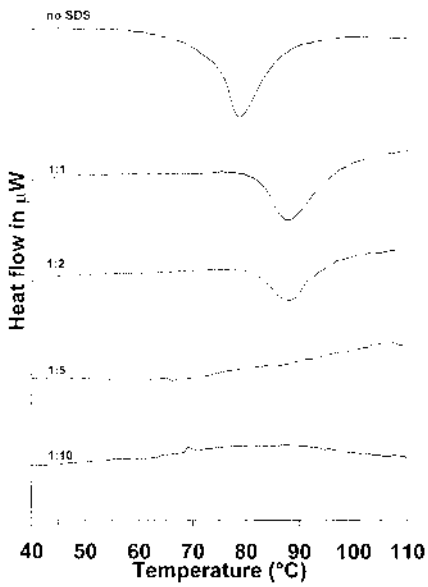

Figure 3 The thermograms from top to bottom shows the thermally induced unfolding of β-lactoglobulin (1.4 m*M* in 60 m*M* NaCl, pH 6) when increasing the protein/SDS molar ratio. The cmc of SDS is 0.47 m*M* at 25°C and ~1 m*M* at 90°C, when taking into account the ionic strength of the protein solution. Assuming that one SDS molecule is bound per β-lactoglobulin monomer, 3 m*M* SDS has to be added to reach the cmc of the surfactant at 90°C. The data are adopted from Ref. 123, where the experimental details are given also.

increasing the free-surfactant concentration to the cmc gives rise to nonspecific cooperative binding, which, in turn, can lead to unfolding of the protein, as illustrated in Fig. 3 (123). This is in agreement with earlier reports, in which a surfactant ratio above 10 mol of SDS per mole of serum albumin or 1 mol of SDS per mole of β-lactoglobulin monomer was observed to cause unfolding of the protein (129,130).

Surfactant like alkylsulfates or alkylethersulfates interacting with proteins with opposite net charge (e.g., lysozyme or gelatine) might cause precipitation of the protein–surfactant complex due to neutralization of the net charge (119,132–137). Although the protein is precipitated, only small changes in the secondary structure occur. At an increased surfactant concentration, the complex is dissolved and the protein starts to be unfolded. Generally, denaturation of proteins by long-chain alkyl sulfates such as SDS results in a structure with large fractions of the polypeptide chain in an α-helical conformation (48,138,139). As a simple rule, proteins with a low content of α-helix in their native form, such as concanavalin A, β-lactoglobulin, and ovalbumin, will increase in α-helix content upon interacting with SDS. The reverse is observed for proteins with a high α-helix content in their native form (e.g., myoglobin and serum albumin) (138). The structure resulting from the interaction is thought to consist of helical segments with flexible joints and with most of the hydrophobic side chains exposed to the surfactant. The successive binding of SDS opens up the molecules, due to the increased electrostatic repulsion, and unveils new hydrophobic domains, which can bind additional surfactants. This association stabilizes α-helical folding at the expense of nonrepetetive structure. The free energy gained by this process in most cases by far exceeds the unfavorable free-energy change of disrupting the native conformation (48). Light-scattering studies confirm the expansion of the hydrodynamic radius of the protein upon interaction with SDS (140). Several models of the structure of complexes between SDS and proteins at high surfactant concentration, like the correlated necklace, rodlike structure, and flexible helix, have been considered (cf. Refs. 116 and 141). However, small-angle neutron-scattering data strongly indicates a structure resembling a necklace (141,142), where the polypeptide chain with high flexibility is decorated with SDS micelles (138,141), as shown in Fig. 4. This interaction is reported to take place via the monomeric form of the surfactant (116,138).

It should also be kept in mind that not all proteins are fully unfolded by SDS. For instance, it has been shown that the activities of glucose oxidase, papain, pepsin, and bacterial catalase were not affected by high concentration of SDS, correlated to the low binding of SDS (143,144).

Thus far, we have mainly addressed the interaction at low protein concentrations. Morén and Khan (136) investigated the phase behavior of

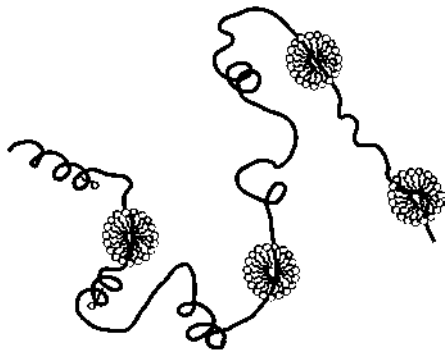

Figure 4 Schematic representation of the so-called necklace model for the interaction between SDS and proteins. The solid line represents the unfolded polypeptide chain, which still contains secondary structure. Micellelike clusters are cooperatively formed on the polypeptide chain.

the anionic SDS, positively charged lysozyme, and water over a wide concentration range and one of the phase diagrams they determined is given in Fig. 5a. Stenstam et al. later investigated in detail the stoichemistry of the formed complex and their findings are summarized in Fig. 5b (137). Small amounts of SDS, at a ratio to lysozyme corresponding to charge neutralization of the protein, were found to give precipitation. A net attractive force exists between the surfactant–protein complexes and hydrophobic interactions dominate (Fig. 5b). Further addition of SDS lead to a redissolution of the precipitate, which was complete when the number of SDS molecules was equal to the number of positive charges on the protein (19). At this ratio between SDS and lysozyme, a narrow strip of a bluish gel phase occurred when the protein concentration was between 7% and 20% (w/w). At a higher ratio between SDS and lysozyme, the interaction between the surfactant protein complexes is net repulsive and electrostatic interactions dominate (Fig. 5b). Consequently, an isotropic solution is formed. Morén and Khan also investigated the effect of varying alkyl chain length, $C_{12}SO_4$, $C_{10}SO_4$, C_8SO_4, and C_6SO_4 on the lysozyme–sodium alkyl sulfate–water ternary systems (145). The surfactant with shortest hydrophobic tail (C_6SO_4) forms the largest solution region with lysozyme prior to precipitation, and the extension of the solution region decreases with increasing surfactant chain length. The extension of the precipitation region toward higher surfactant concentrations increases with decreasing surfactant chain length. The surfactant concentration required to redissolve the precipitate at dilute protein concentrations therefore seems to follow the cmc for the surfactant in water, which also increases with decreasing surfactant chain

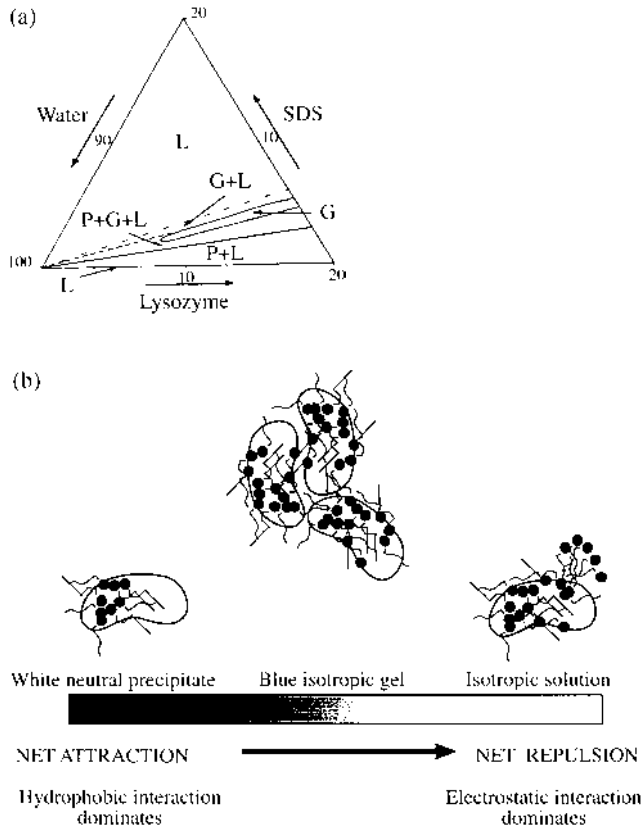

Figure 5 (a) Phase diagram of the lysozyme–SDS–water ternary system, where L indicates solution, G gel, and P precipitate. (Adapted from Ref. 136, where experimental details are given.) (b) Schematic representation of the interaction between protein surfactant complexes in the lysozyme–SDS–water system. (Adapted from Ref. 137, where the experimental details are given.)

length. A single gel phase was only observed for the $C_{12}SO_4$ and $C_{10}SO_4$ systems and not in the presence of C_8SO_4 and C_6SO_4. A similar type of gel phase is expected to occur in more food-relevant surfactant–lipid and protein–aqueous ternary system and therefore offer potentially very interesting possibilities to vary the functional properties of foods and food ingredients.

Within the type of surfactant, the binding is dependent on the nature of the polar head group; for example, for an anionic surfactant, the

interaction decreases in the order alkyl sulfates > alkyl sulfonates > alkyl benzene sulfonates > carboxylates ≈ alcohols (146,147).

2. Nonionic

The interaction between nonionic surfactants and proteins is generally weak (118,146,148–151). In the case of ionic surfactants, the specific ionic interaction, absent for a nonionic surfactant (Fig. 2), occurs in addition to the hydrophobic interaction, leading to more severe effects on the protein structure. For instance, each β-lactoglobulin monomer binds only one Tween-20 (152), or one sucrose ester (153), or one Triton X-100 (149). Generally, minor changes of the structure upon interaction are observed (148,150). An unordered, flexible protein, β-casein, was found to bind less than one sucrose ester per protein molecule, possibly due to incorporation of the surfactant in β-casein micelles (153). The reason for the weaker interaction between proteins and nonionic surfactants has been assigned to the lower cmc, which gives a too low monomer concentration to attain cooperative binding to the protein (148). The cmc is increased when the chain length is decreased, which may change this situation; The binding of octyl glucoside to various proteins was found to occur in a cooperative manner at surfactant/protein molar ratio of 100 and more, without any evidence of protein denaturation (150).

Also, the nature of the nonionic polar head groups will affect the interaction. For a series of Triton X surfactants, increasing the hydrophilic oxyethylene chain length was found to decrease the strength of interaction with bovine serum albumin (BSA), due to steric hindrance (151). The calorimetric data indicated that some conformational changes of BSA occurred during the saturation of the low-affinity, noncooperative binding sites (151).

Some studies have also been carried out with the zwitterionic surfactant lysophosphatidylcholine (LPC), which was found to bind cooperatively to puroindoline, a lipid-binding protein isolated from wheat flour, at a molar ratio of 5–1 (154). One LPC molecule was also found to bind with less affinity to β-lactoglobulin than Tween-20 (21). The binding of Tween-20, as opposed to LPC, had a much more disruptive effect on the interfacial film of the protein, attributed to the bulkier head group of Tween-20. This implies that also a nonionic surfactant can disrupt the structure of a protein, provided that the binding is strong enough and the hydrophilic head group large enough to sterically induce conformational changes.

3. Cationic

Cationic surfactants generally seem to exhibit an intermediate action on water-soluble proteins. Reports in the literature indicate a cooperative

interaction with proteins, but with less affinity and, thus, with less perturbation of the folded state, compared to the effect of the anionic ones (123, 155–159). If the binding is governed both by electrostatic and hydrophobic interactions, anionic and cationic surfactants will obviously occupy different sites. Nozaki et al. have suggested that the lower affinity of many proteins for cationic compared to anionic surfactants can be explained by the fact that the cationic arginine and lysine side chains contribute more CH_2 groups than anionic aspartate and glutamate side chains (157). This implies that the combined electrostatic and hydrophobic interactions will be more favorable for anionic surfactants. As a consequence, the cooperative binding step will start at a higher concentration for cationic relative to anionic surfactants (116).

4. Effect of Solution Conditions

Increased ionic strength affects the interaction between protein and ionic surfactants by reducing the electrostatic attraction between surfactants and amino acid residues with opposite net charges. Generally, the high-affinity noncooperative binding is strongly influenced by the electrostatic interaction between surfactant and protein. Thus, this part of the binding isotherm will be shifted toward higher surfactant concentration upon the addition of salt, as observed for lysozyme and SDS (Fig. 2) (115,160). Increasing the ionic strength will, on the other hand, favor the cooperative binding by screening the repulsion between the charged surfactant head groups. This part of the surfactant-binding isotherm will therefore be shifted toward lower surfactant concentrations, parallel to the decrease of surfactant cmc. Here, it is important to point out that the presence of highly charged proteins as a polyelectrolyte as well as temperature will affect the formation of micelles. This has been amply demonstrated by Waninge et al. who studied thermally induced unfolding of β-lactoglobulin at a concentration of 1.4 m*M* in 60 m*M* NaCl, pH 6, in the presence of various molar ratio SDS, and their main findings are illustrated by the thermograms in Fig. 3 (123). From Fig. 3, we note that the peak corresponding to the thermal unfolding disappears when the protein/SDS molar ratio increases above 1:2. This corresponds to a SDS concentration of about 3 m*M*. The cmc for SDS is about 8.1–8.2 m*M* in water (161,162). However, the cmc for ionic surfactants decreases with ionic strength and increases with temperature (161–164). Taking account to these effects for the presence of β-lactoglobulin, which has a net charge of –5, at a concentration of 1.4 m*M* in 60 m*M* NaCl, the cmc of SDS is expected to be 0.47 m*M* at 25°C and ~1 m*M* at 90°C. When taking into the specific binding of one SDS molecule per β-lactoglobulin monomer, 3 m*M* SDS

has to be added to reach the cmc of the SDS at 90°C. Thus, any effect of nonspecific cooperative interaction between the surfactant and the protein is expected to take place at this SDS concentration. In Fig. 3, we observe an apparent loss of protein structure. The unfolding of the protein structure at low temperature, which is observed in the presence of most anionic surfactants such as SDS at high concentration, is expected to be maintained at increased temperature. However, because the cmc generally increases with temperature, we might arrive at the situation where the cooperative binding ceases to exist at the high temperature, maybe even below the temperature at which thermally induced unfolding take place. Interestingly, Waninge et al. observed that the conformational changes invoked by the nonspecific cooperative binding of SDS at 25°C could be reversed by extensive dialysis (123). Referring to the discussion of the molten globule state earlier, it is tempting to compare it with the structure obtained by surfactant unfolding.

Although cationic surfactants seem to cause less unfolding of globular proteins at low temperature than anionic, some reports indicate that they can destabilize globular proteins at increased temperature (158,165). However, these reports also indicate that the unfolding process at the same time becomes considerably more reversible. The heat denaturation of ovalbumin, which in practice is completely irreversible, was found to be completely reversible in the presence of high concentrations of cationic surfactants (165). This was explained by decreased intermolecular and intramolecular interactions at high temperature, due to the interaction between the unfolded protein and surfactant, which facilitates the reformation of the native complex on cooling.

As a rule of thumb, an increase in pH will shift the binding of anionic surfactants to higher concentrations (166). A decrease of pH will have the same effect on the binding of cationic surfactants (167). At low surfactant concentrations (i.e., well below the cmc), cationic amphiphiles increase the solubility of proteins on the acidic side of the isoelectric point (pI), whereas precipitation can occur on the alkaline side of the pI. Anionic amphiphiles will affect solubility in the opposite direction. The solubilising effect is also observed at high temperatures.

We conclude that because the binding generally is thought to occur via monomers, any change affecting the cmc will also affect the cooperative binding at concentrations close to and above the cmc. Under some conditions, the formation of surfactant micelles will be energetically favored before binding to the protein. If the cmc is of the same order of magnitude as the concentration necessary for binding to occur, the lowering of the cmc caused by increasing ionic strength might even prevent binding.

B. At Interfaces

The stabilization of food emulsions and foams can be achieved by proteins, polar lipids, or mixtures of them. However, the mechanisms by which they stabilize emulsions and foams can be quite different. Here, we refer to the work of Clark and his colleagues (168), which have, based on results such as those from fluorescence recovery after photobleaching experiments, suggested a mechanism for stabilization of foams and emulsions. Generally, polar lipids are capable of reducing the interfacial tension more than proteins, whereas the protein molecules can be anchored at multiple sites at the interface. Therefore, many proteins stabilize foams and emulsions by forming intermolecular interactions between the adsorbed protein molecules, which encapsulates the dispersed phase. Such a mechanically rigid layer immobilizes the proteins and prevents perturbations, droplet coalescence, or flocculation. In contrast, lipids usually stabilize the dispersed droplet or bubble by forming a densely packed and much less rigid monolayer with high mobility. Any disturbance of the interface, such as expansion of the interface, will induce a (temporary) surface-tension gradient. This is, of course, unfavorable and the system tries to restore an even distribution of the surface free energy. During the lateral diffusion, or adsorption from the bulk, of the surface-active components, a flow counteracts the disturbance. This dynamic process is usually called the Gibbs–Marangoni effect (169). Thus, the rate of exchange of polar lipids at the interface is much higher than for proteins. The addition of polar lipids/surfactants to a protein film can, if the concentration becomes too high, destroy the integrity of the film by disrupting protein–protein interactions and, consequently, destabilize the foam or the emulsion. This points at another important reason to study the interaction between proteins and polar lipids in the adsorbed layer as well as during the competitive adsorption of the individual components. The surfactant-to-protein ratios as well as the properties of the components and the interface will determine the composition of the film.

In several investigations, surface-tension measurements have been used to study protein–lipid interactions (cf. Refs. 10,12,13,170, and 171). However, it must be kept in mind that any impurity with a higher surface activity than the studied components will accumulate at the interface, giving a lowering of the surface tension (125–127) and thus affecting the interpretation of the data. The presence of impurities (e.g., fatty acids) bound to β-lactoglobulin did have a profound effect on the interfacial behavior of mixtures with Tween-20, as judged from surface elasticity measurements at the air–aqueous interface (128). It was observed that the film containing purified β-lactoglobulin could maintain a more rigid film, at a much higher concentration of Tween-20 as compared to the sample containing impurities.

We will discuss some experimental data on the basis of possible factors that influence the way mixtures of proteins and polar lipids behave at an interface. These can be summarized as follows:

1. The surface activity of the individual components
   (a) Competitive adsorption. The lipids and proteins compete for the interface, where the most surface-active and/or abundant molecule wins, depending on the ratio between surfactants and proteins in solution.
   (b) Displacement. The polar lipids may, due to their higher surface activity, displace the proteins from the interface. This displacement can be hampered by a strong interaction between the protein and the interface and/or protein–protein interactions.
2. Protein–lipid interactions
   (a) Increased surface activity of the lipid–protein complex
      (i) The binding will cause unfolding and/or increase hydrophobicity of the protein that will lead to an increased affinity to the surface.
      (ii) The binding (of ionic amphiphiles) will cause precipitation at the interface due to charge neutralization.
   (b) Decreased surface activity of the lipid–protein complex
      (i) The binding will make the protein more soluble and hence lower the affinity for the interface.
      (ii) The binding will lead to precipitation of protein–lipid complex in the bulk, which will cause loss of surface-active material.
   (c) Protein–lipid interactions at the interface
      (i) The interaction will give more efficient packing at the interface and thus give a higher total surface concentration.
      (ii) The interaction will disrupt the protein–protein interaction in the interfacial film.

We will use some of our surface-tension data on protein–surfactant mixtures to demonstrate the effect of different modes of interaction. Different modes of interaction are observed for the same system depending on the lipid/protein ratio. The data will also serve as the basis for the discussion according to the above factors. The interaction between a protein

(ovalbumin) and three types of surfactant [an anionic (SDS), a nonionic (1-monocaproin) and a cationic (hexadecylpyridinium chloride)] as observed from surface-tension isotherms is illustrated in Fig. 6 [the experimental procedure is given elsewhere (171)]. The protein concentration used was 21 μM. To avoid precipitation, the measurements were carried out at pH 5.6 and 4.0 for SDS and hexadecylpyridinium chloride, respectively [i.e., below and above the isoelectric point (4.5–4.9) of ovalbumin]. The interaction between ovalbumin and ionic surfactants is considered to be nonspecific. Serum albumin has, however, specific binding sites for the surfactants (114) and, for comparison, the data for the surface tension of BSA (13 μM, 0.05 M phosphate buffer, pH 5.6) and SDS are also inserted in Fig. 6b. The surface-tension isotherms of the pure surfactants are shifted according to the differences in cmc although they have a similar shape. Hence, it is useful to discuss data in terms of the ratio between added number of surfactant monomers and protein molecules.

1. Competitive Adsorption

No synergistic effect is observed for the mixture of the nonionic surfactant, monocaproin, and ovalbumin (Fig. 6a). The component giving the lowest surface tension displaces the other at the interface. No molecular interactions seem to take place in either the bulk solution or at the interface. Also, for the cationic and anionic surfactants, the surface tension at high enough surfactant concentration is dominated by the contribution from surfactants. Thus, it is generally observed for a range of surfactant–protein systems that the protein dominates at low surfactant concentration, whereas the surfactant dominates at high surfactant concentration, as they generally give a lower surface tension [13].

The effect of interfacial properties of a solid surface on the competitive adsorption among a protein, fibrinogen, and a nonionic surfactant, pentaethyleneglycol mono *n*-dodecyl ether ($C_{12}E_5$), was investigated by using a wettability gradient silica surface (172). Fibrinogen was preferentially adsorbed on the hydrophilic surface at all concentrations, whereas the surfactant dominated at the hydrophobic and intermediate part of the gradient at surfactant concentrations close to the cmc and above. The preferential adsorption of the surfactant extended to a wider range of the gradient if the temperature was increased to a value close to the cloud point of the surfactant.

2. Displacement

Mackie et al. have, based on extensive studies using atomic force microscopy (AFM), Brewster-angle microscopy (BAM), fluorescence microscopy,

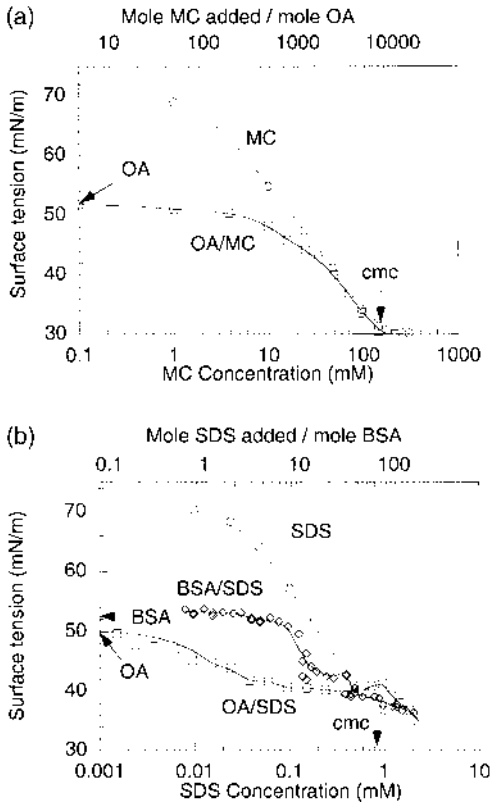

Figure 6 (a) Surface-tension isotherms of 21 μ*M* ovalbumin (OA) (□) in the presence of the nonionic monocaproin (MC) in water adjusted to pH 5.6, where the surface tension of the pure protein is marked with an arrow on the ordinate. Surface tension of pure MC is also shown (○) and the cmc is marked with an arrow on the abscissa. The surface-tension measurements were performed according to the drop-volume method as a function of time. The surface-tension value after 2000 s has been used for the isotherms. Further details are given elsewhere (171). (b) Surface-tension isotherms of 21 μ*M* ovalbumin (OA) (□) and 13 μ*M* BSA (◇) in the presence of the anionic SDS in 0.05 *M* phosphate buffer, pH 5.6. The surface tension of the pure proteins are marked with arrows on the ordinate. The surface tension of pure SDS is also shown (○) and the cmc is marked with an arrow on the abscissa. Other conditions are the same as given for part (a). (c) Surface-tension isotherms of 21 μ*M* ovalbumin (OA) (□) in the presence of the cationic hexadecylpyridinium chloride (HPC) in water adjusted to pH 4.0. The surface tension of the pure protein is marked with an arrow on the ordinate. The surface tension of pure HPC is also shown (○) and the cmc is marked with an arrow on the abscissa. Other conditions are the same as given in part (a).

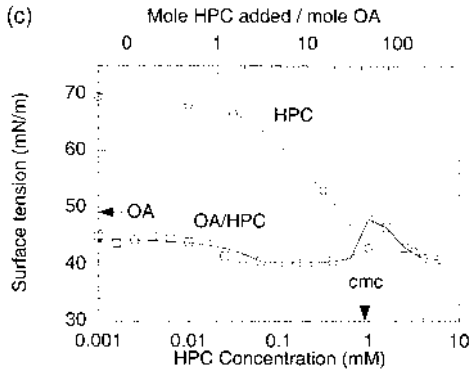

Figure 6 Continued.

and surface rheological techniques, developed an "orogenic" displacement model (173–175). This model is based on the adsorption of the displacing surfactant/lipid into localized defects in the heterogeneous protein network. These nucleation sites then grows, which leads to a compression of the protein network. During this initial stage, the protein film seems to be denser but not thicker. However, above a certain critical density, the film thickness starts to increase with the expanding surfactant domains while maintaining the protein film volume. Eventually, a protein network cannot withstand the high surface pressure, but it will collapse and the protein will be released and desorb from the interface. This model points at the importance of protein–protein and protein–interface interactions in relation to the interaction of the surfactant with defects in the protein network as well as with the interface.

Indirectly, the neutron reflectivity study on the binding of SDS onto preadsorbed layers of BSA at the hydrophilic silicon oxide–water interface by Lu et al. confirm the "orogenic" displacement model (176). Their results show a uniform layer distribution of SDS at low surfactant concentrations, whereas the distributions become unsymmetrical as the SDS concentration increases. The binding of SDS results in an expansion of the preadsorbed BSA layer from 35 Å in the absence of SDS to some 80 Å at $3 \times 10^{-4}\,M$ SDS, which Lu et al. interpreted as a considerable structural deformation of the protein. They based this interpretation on the close agreement between the volume ratio of SDS to BSA in the mixed layer of 0.45 and the literature value for the binding of SDS onto denatured protein in the bulk reported by Tanner et al. (140). The specular neutron reflection is sensitive to the density profile normal to the interface, but it does not give any lateral resolution. Therefore, the observations by Lu et al. (176) can also be explained by the "orogenic" displacement model (173–175).

The effect of the interfacial protein film age on the displacement of the protein from the surface of emulsion drops by nonionic water-soluble surfactants [Tween-20 and octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$)] showed that β-lactoglobulin is harder to replace the longer the residence time was (177,178). Apart from the possible conformational changes that occur during the adsorption process, which can hamper displacement, it has been reported that β-lactoglobulin might polymerize through disulfide exchange at the oil–water interface (179). Consequently, the displacement of β-casein, which is a flexible and unordered protein without sulfhydryl groups, did not depend on the age of the film. Furthermore, it was observed that it was harder to replace β-lactoglobulin from a emulsion prepared close to the p*I* of the protein than at neutral pH, whereas the replacement from emulsions prepared at pH 3 was easier and no time effects was observed. Mackie et al. also studied displacement of β-lactoglobulin and β-casein by Tween-20, but from the air–water interface (173). They also found that β-casein was more easily displaced (i.e., β-lactoglobulin films breaks at higher surface pressures). Furthermore, stress invoked by penetration of the surfactant was found to propagate homogenously through the β-casein film, which, in turn, resulted in the growth of circular surfactant domains at the interface. β-Lactoglobulin, on the other hand, was found to form elastic (gellike) networks at the air–water interface and the penetration of the surfactant therefore resulted in the growth of irregular (fractal) surfactant domains. Interestingly, Tween-20 preferentially displaced β-casein before β-lactoglobulin from a mixed β-casein/β-lactoglobulin film at the air–water interface (174).

The effect of altering the protein conformational stability on the displacement of adsorbed protein layers by surfactants was also visualized at the solid–liquid interface by McGuire et al. (180). They found that the removal of wild-type and structural stability mutants of bacteriophage T4 lysozyme from hydrophobic and hydrophilic silica surfaces by a cationic detergent, decyltrimethylammonium bromide (DTAB), generally increased with the stability of the mutants.

Wahlgren and Arnebrant (181) investigated the effect of the surface properties on the displacement of adsorbed β-lactoglobulin (negative net charge) and lysozyme (positive net charge) by the cationic surfactant cetyltrimethyl ammonium bromide (CTAB) and the anionic SDS. They used hydrophobic (hydrophobised silica), negative (hydrophilic silica), neutral (chromium oxide), as well as positively charged (nickel oxide) surfaces and found four types of behavior for surfactant concentrations well above the cmc:

1. Surfactant binds to the protein and the complex desorbs on dilution. This was observed for SDS and β-lactoglobulin as well as

lysozyme on a negative silica surface and can be explained by simple electrostatic considerations. No adsorption from SDS–protein mixtures occurred.

2. The surfactant replaces the protein at the interfaces. This requires that the surfactant interacts more strongly with the surface than the protein, as was observed for CTAB with negative silica and SDS and CTAB with the hydrophobic surface when the adsorbed layer consisted of β-lactoglobulin.
3. The surfactant adsorbs reversible on top of the protein layer. The protein–surface interaction is the stronger one and the surfactant is thus unable to solubilize the protein from the interface. This was observed for CTAB interacting with both proteins at the chromium oxide surface and SDS interacting with β-lactoglobulin at the nickel oxide surface.
4. Partial removal of the protein. This can be explained as due to the presence of multiple binding sites for the protein, which will give a combination of mechanism 1 and 2.

Others have made similar observations. For instance, Green et al. also studied the interaction between SDS and preadsorbed lysozyme at the hydrophilic silicon oxide–water interface by neutron reflectivity measurements (182). SDS binds cooperatively to the preadsorbed protein layer at intermediate surfactant concentrations, with no desorption of lysozyme from the interface. The protein was partly removed when the SDS concentration was increased to above 0.5 mM, whereas a surfactant concentration of 2 mM was required to completely remove both protein and surfactant from the interface in accordance with the type 1 behavior discussed.

3. Increased Surface Activity of the Lipid–Protein Complex

When comparing the data for the interaction between SDS and ovalbumin and the corresponding data for BSA, we clearly observe the different mode of interaction (Fig. 6b). The gradual decrease in surface tension with increasing surfactant concentration observed for ovalbumin and SDS mixtures can be explained by more efficient packing at the interface as discussed here. In addition, it has been argued that the electrostatic interaction between surfactant and protein might increase the hydrophobicity of the protein and, hence, its surface activity. The specific binding of SDS to BSA does not affect the surface tension until the concentration corresponding to saturation of the high-affinity binding sites is reached (i.e., 9–10 mol SDS per mole protein) (114), where a sharp decrease in surface tension is observed. This arises probably from an increase in the free-monomer concentration of SDS. The second plateau, indicating constant surfactant

monomer concentration, which is observed at increased surfactant concentration, is likely to be connected with saturation of the cooperative binding sites. As the surfactant concentration further increases, the surface-tension isotherms for the two protein–surfactant mixtures coincide. The second plateau observed in surface tension isotherms for ovalbumin and hexadecylpyridinium chloride (HPC) mixtures just below cmc of HPC (Fig. 6c) can be related to the electrostatic interaction between HPC and globular proteins that has been observed below the cmc in bulk solution (158). It is noteworthy that the surface tension is slightly lower than for pure HPC, suggesting that the complex is more surface active. Green et al. used specular neutron reflection and surface-tension measurements to study the adsorption of lysozyme and SDS at the air–water interface (183). Their results show that the lysozyme–SDS complexes are much more surface active than the unbound species as the surface excesses for both lysozyme and SDS increases and surface tension decreases upon addition of SDS (region A). Interestingly, the molar ratio of SDS to lysozyme was found to remain constant at about 7, although the total surface excesses increase with SDS concentration up to a surfactant concentration of $2.5 \times 10^{-4}\,M$. This indicates that the complex that adsorbed on the interface had a rather well-defined stoichiometric composition.

A further increase in SDS concentration beyond $2.5 \times 10^{-4}\,M$ led to a sharp decrease in the total surface excess, whereas the molar ratio of SDS to lysozyme increased. Eventually, as more SDS was added, the mixed protein–surfactant layer was replaced by a pure SDS monolayer.

The surface activity of the complex depends also on the properties of the interface, as shown by Wilde and Clark (152) for liquid interfaces. They found that the complex between Tween-20 and β-lactoglobulin was more surface active at the oil–water interface than at the air–water interface, where the same surface activity as for the nonbound protein was observed. The complexes adsorbed at both type of interfaces was, however, displaced by Tween-20 at the same surfactant-to-protein ratio. Here, we need to emphasize the difference in nature between the two types of liquid interface, the liquid–air and the one between two condensed media, which explains the experimental observations. The oil–water interface allows hydrophobic residues to become dissolved in and interact favorably with the oil phase, which is not possible at the air–water interface. We have also previously discussed that the unfolding of protein induced by the action of surfactants or by the presence of an interface generally leads to exposure of hydrophobic residues; that is, the unfolded protein can be substantially more "oil soluble" than the native one. This relates to the following section, dealing with molecular interactions, where it will be demonstrated that changes in oil-phase composition and hence solvent

properties also can lead to changes in the structure of the adsorbed protein film. At the hydrophobized silica–aqueous interface, it was found that the complex between β-lactoglobulin and SDS, formed at a high surfactant–protein ratio, but still below cmc of SDS, was more surface active than pure β-lactoglobulin (184).

4. Decreased Surface Activity of the Lipid–Protein Complex

The maxima in the surface-tension isotherm at HPC concentrations between 0.8 and 2.5 m*M* probably reflects an increased HPC–ovalbumin interaction in bulk solution (Fig. 6c). The formed highly charged complex is less surface active and an increase in surface tension is thus observed. The surface-tension maximum has been found to depend on ovalbumin concentration and is shifted toward higher HPC concentration at increased ovalbumin concentration (corresponds to 30 mol HPC per mole ovalbumin, independent of protein concentration) (185). The adsorption from mixtures of human serum albumin (HSA) and nonionic surfactant, decyl-dimethyl-phosphine-oxide (C_{10}DMPO) at the air-water interface was reported by Miller et al. (186). They reported an anomalous surface-tension increase for the mixtures at low surfactant concentrations to values higher than for the protein at the same concentration without the surfactant. Thus, it seemed that the surfactant–protein complex was less surface active. The likely explanation is that the nonionic surfactant is associated with HSA via hydrophobic interaction and thus makes the protein more hydrophilic and hence less surface active. Miller et al. also observed that the concentration range, where the coverage of protein and surfactant are comparable in the mixed surface layer was quite narrow (186).

The precipitation of protein in the bulk solution due to neutralization by added surfactant can also cause a decrease in surface concentration due to loss of surface-active material. Garcia Dominguez et al. (187) have shown that the surface-tension reduction of lysozyme and insulin at pH 3.5 (i.e., below pI) decreased when an anionic surfactant (SDS) was added, due to precipitation of the protein.

5. Protein–Lipid Interactions at the Interface

A synergistic effect on surface tension is seen for mixtures of proteins with both the anionic and cationic surfactants (Fig. 6b and 6c). For ovalbumin and SDS mixtures (Fig. 6b), a gradual decrease of the surface tension with increasing surfactant concentration is observed. This might be assigned to the more efficient packing in the formed mixed surfactant–protein layer compared to the one formed by the individual components at this concentration (171). Even at the lowest concentration of cationic surfactant

(0.05 mol HPC per mole ovalbumin), where the pure surfactant has the same surface tension as water, a decrease in surface tension for the protein–surfactant mixture, compared to pure ovalbumin, is observed (Fig. 6c). It is unlikely that any bulk interaction will affect the interfacial behavior at this low HPC-to-ovalbumin ratio. Therefore, the lowering in the surface tension probably arises from molecular interactions in the adsorbed surface film, giving a more condensed surface layer. Buckingham et al. (188) found a strong synergistic lowering of the surface tension of a mixed solution of SDS and poly-L-lysine at conditions at which no precipitation, micelle, or complex formation take place in the bulk solution. A similar behavior was observed for mixtures of low-molecular-weight surfactants of opposite charges (189). This effect has been assigned to the formation of electro-neutral complexes in the interfacial film.

Not only the composition of the interfacial layer but also the mechanical properties (e.g., the dilational viscosity) of the layer are important for the stability of emulsions and foams (8,11,14,190). In particular, both surface and bulk rheology as well as the disjoining pressure of the thin lamellae determine the stability of foams (14,191). Hence, in technical applications, thickeners are often added. The mechanical properties of interfacial films can, to a large extent, be controlled by the intermolecular interactions. This means that the protein stabilization of a foam is mainly due to protein–protein interaction and the destabilization is thought of as a disruption of these interactions. Sarker et al. (21) discussed the effect of the surfactant properties on the stability of interfacial films in foams. The addition of small amount of lysophosphatidylcholine (LPC) was found to increase the foam stability of β-lactoglobulin foams (21). A further increase of the surfactant concentration led to a decrease of the foam stability. The surface tension versus molar ratio of LPC and β-lactoglobulin shows an inflection point close to a molar ratio of unity, corresponding to the binding of the surfactant to the protein. No increase of foam stability was, however, observed for mixtures of Tween-20 and β-lactoglobulin, instead, the stability decreased with increasing surfactant concentration (192). The same observations were made for the stability of an oil-in-water emulsion, where it was found that small amount of Tween-20 increased the rate of shear-induced coalescence of β-lactoglobulin-stabilized emulsion droplets (178). The marked reduction in surface shear viscosity even at low surfactant-to-protein ratios confirmed that loosening of the protein layer occurred. The protein–surfactant complex is thought of being less surface active and a further increase of the surfactant concentration will lead to replacement of protein and protein–surfactant complexes with the surfactant at the interface (192,193). The mobility of the protein in a protein-stabilized thin liquid film, as measured with the fluorescence recovery after photobleaching technique (FRAP),

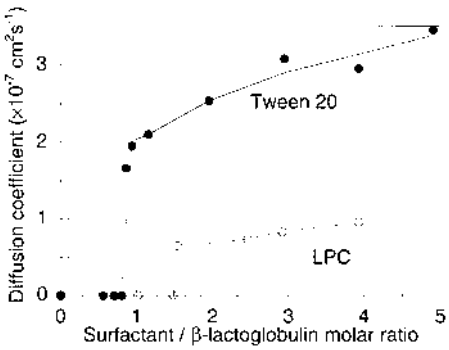

Figure 7 The effect of surfactant addition on the lateral diffusion in the adsorbed mixed layer of surfactant and β-lactoglobulin, measured with the FRAP technique. The diffusion coefficients of the fluorescent probe 5-*N*-(octadecanoyl)aminofluourescein and fluourescein isothiocyanate isomer 1 labeled β-lactoglobulin measured in the presence of L-α-lysophosphatidylcholine (○) and Tween-20 (●), respectively, are shown as a function of the molar ratio between surfactant and β-lactoglobulin. The data are adopted from the work of Sarker et al. (21) and Coke et al. (192), respectively, in which the experimental details also are given.

increases at lower surfactant-to-protein ratio for Tween-20 than for LPC (Fig. 7). This was attributed to the stronger binding of Tween-20, compared with LPC, to β-lactoglobulin (21) and will also explain why the foam becomes unstable at a lower surfactant concentration when Tween-20 is used. The foaming properties of puroindoline from wheat was found to be improved by the addition of LPC to protein molar ratio of 1–10 (154). This was assigned to the forming of a complex, which prevents the interfacial aggregation of the protein. Once the surfactant concentration becomes large enough, the protein–protein interactions within the surface film will be prevented, the mobility increased, and, thus, the foam stability decreased.

An ionic surfactant can also induce flocculation of protein-stabilized emulsions and this is dependent on the nature of the protein–lipid interaction, as discussed by Chen and Dickinson (134,135,194). An anionic surfactant, sodium lauryl ether sulfate (SLES), at sufficient concentration has been found to flocculate gelatine-stabilized oil-in-water emulsion (134). A further increase in surfactant concentration was found to lead to a restabilization of the flocculated emulsion. In bulk solution, the anionic surfactant will, at high enough concentrations, cause precipitation of the positively charged gelatine. At a further increased surfactant concentration, the precipitate was redispersed. Gelatine was initially displaced by SLES from the interface (194), but an increase of the surfactant concentration led to an increase of gelatine concentration at the interface and the

surface charge became partly neutralized (135), causing flocculation. A further increase of the surfactant concentration led to a decrease of the gelatine surface concentration (194) and a restabilization of the emulsion (134). It was also observed that the addition of SLES to a β-lactoglobulin-stabilized emulsion not did cause any flocculation, although some kind of complex was formed in bulk solution. It should be kept in mind that β-lactoglobulin was negatively charged under the experimental conditions used. This confirms the electrostatic nature of the observed SLES-induced flocculation of the emulsions stabilized by the positively charged gelatine. Flocculation of β-lactoglobulin-stabilized emulsions was, however, observed in the presence of gelatine and SLES. Because it only occurred above the cmc of the surfactant, it was suggested to depend on cross-linking of the emulsion droplets by surfactant micelles (134).

V. INTERACTIONS BETWEEN PROTEINS AND WATER-INSOLUBLE LIPIDS

In this section, we will discuss interactions involving lipids with low solubility, where the lipids exist as dispersed particles, liposomes or vesicles, liquid crystalline phases, as well as monolayers at interfaces. Many of the principles discussed in the earlier sections also apply for protein–lipid interactions in condensed systems. Polar lipids, which normally are water insoluble, associate into a variety of structures in aqueous solution. This process will have an impact on interactions with proteins. For water-soluble surfactants, the association with proteins mainly involves a surfactant in the monomeric state, whereas for insoluble lipids, the association structures also have to be considered. We also note that even polar lipids that are considered water insoluble have a certain monomer solubility, which, although small (about 10^{-7} for mono-olein and about 10^{-10}–10^{-12} M for phospholipids), makes it possible for them to interact with proteins in the monomeric form—in particular, if the protein has a high-affinity binding site for the lipids. This is demonstrated in Fig. 8, which shows the themograms from differential scanning calorimetry (DSC) measurements of β-lactoglobulin, distearoylphosphatidic acid (DSPA), and β-lactoglobulin + an aqueous dispersion of DSPA. We note that the peak corresponding to the thermally induced unfolding transition of β-lactoglobulin in the presence of DSPA is shifted toward a higher temperature compared to the one recorded for the pure protein. This confirms the presence of a specific interaction between phosphatidic acid and β-lactoglobulin that thermally stabilize the protein. This was also observed in the presence of dipalmitoylphosphatidic acid (DPPA), but no such interaction was observed

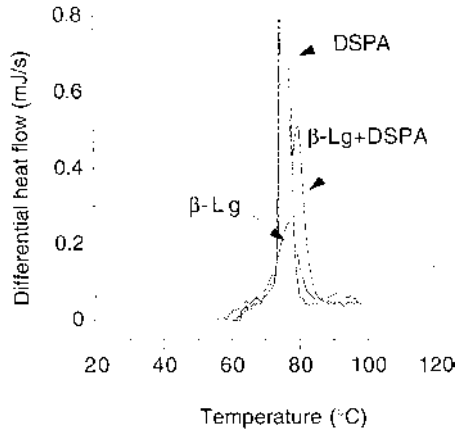

Figure 8 The interaction between DSPA and β-lactoglobulin (β-Lg) is demonstrated by the results from differential scanning calorimetry, where the thermogram of the protein–lipid mixture is compared with those of the pure components. Thermograms of DSPA, 5% (w/v) (—), β-Lg 5% (w/v) (– · –), and a mixture of β-Lg 5% and DSPA 5% (w/v) (— - - —) in 1% sodium chloride at pH 7. A scanning rate of 10°C/min was used. (Data from Ref. 22, where the experimental details are given.)

when the protein was mixed with phosphatidylcholine, phosphatidylethanolamine, or phosphatidylglycerol (22). Neither could any interaction be observed if the lipid contained unsaturated fatty acid residues. Thus, the results show that the interactions between β-lactoglobulin and phospholipids are strongly dependent on the acyl chain as well as the head group. A small negatively charged head group is needed for the interaction to take place. Such an interaction can have important implication for the functional properties of the protein. We discussed earlier that fatty acids bound to β-lactoglobulin could affect the interfacial behavior of the protein (128). Kurihara and Katsuragi (277) reported that a lipid–protein complex formed between β-lactoglobulin and phosphatidic acid could mask a bitter taste. This property was suggested to be specific for phosphatidic acid, as no effect was observed for mixtures of β-lactoglobulin and phosphatidylcholine, triacylglycerol, or diacylglycerol.

The large number of studies using lipid monolayers at the air–aqueous interface and spread or adsorbed proteins have given us the basic knowledge of the interaction between proteins and polar lipids with low aqueous solubility. Therefore, we will start to discuss some of the main conclusions that can be drawn from such studies. The following subsections will address the interaction with oil–aqueous interface, vesicles, and liquid-crystalline phases.

A. Lipid Monolayers at the Air–Aqueous Interface

1. Driving Force for Lipid–Protein Interactions

Electrostatics. Phospholipid–β-lactoglobulin interactions at the air–aqueous interface has been investigated by Bos and Nylander (195) using the surface film balance. Some of their findings are summarized in Fig. 9, where

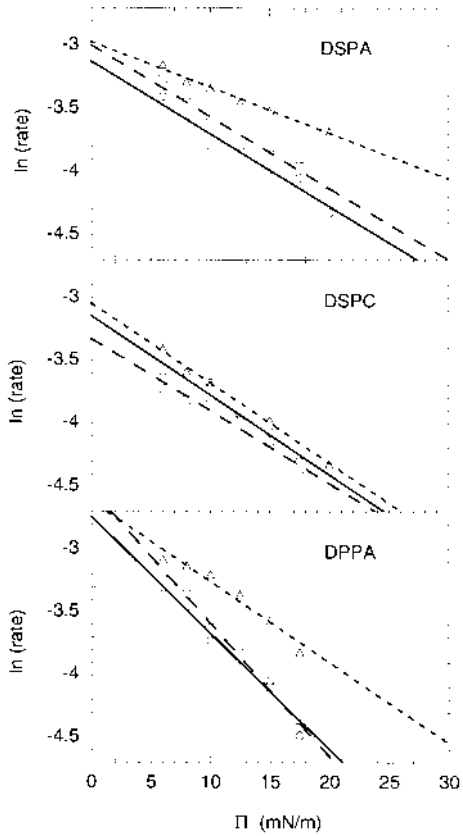

Figure 9 The rate of incorporation of β-lactoglobulin into monolayers of DSPA, DSPC, and DPPA versus surface pressure (Π). The data were recorded at constant surface pressure by measuring the area increase of the lipid monolayer spread on a protein solution contain 1.15 mg/L in 10 m*M* phosphate buffer of pH 7, with 0 m*M* (—○—), 50 m*M* (– –□– –), or 150 m*M* (- - -△- - -) sodium chloride. The rate (in mg/m^2) was calculated from the area increase by using the Π–area isotherm of spread monolayers of β-lactoglobulin. Data from Ref. 195, where the experimental details are given.

the rate of incorporation of β-lactoglobulin into monolayers of DSPA, distearoylphosphatidylcholine (DSPC) and DPPA is shown versus surface pressure (Π) at pH 7. The rate was calculated using a simple first-order kinetics model (8), where only the surface pressure barrier is taken into account. The highest rate of adsorption of β-lactoglobulin into a phospholipid monolayer was observed for anionic DSPA. The incorporation of the protein take also place at a higher surface pressure into a DSPA monolayer than into a monolayers of the other lipids. Because the β-lactoglobulin, with a zero net charge at pH≈5.2 (196), has a positive net charge at pH 4, a larger rate of adsorption into the negatively charged phosphatidic acid monolayers would be expected under acidic conditions. However, almost the same rates were found (195). As discussed earlier, anionic lipids seems to interact more strongly with proteins (i.e., to its cationic amino acid residues) compared to lipids with no or positive net charge. The incorporation into the zwitterionic DSPC monolayers is as expected less salt dependent than what was observed for the phosphatidic acid monolayers, where the rate increases with increasing ionic strength of the subphase. Probably, this is a consequence of a decreased repulsion within the phosphatidic acid protein monolayer at a higher ionic strength. The findings by Bos and Nylander (195) is somewhat contradictory to the findings of Cornell and Patterson, who studied the adsorption β-lactoglobulin to a negatively charged lipid monolayer, composed of a mixture of palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylglycerol (POPG) (65/35 mol%). They only observed a substantial binding of β-lactoglobulin at pH 4.4, which is when the protein carries a net positive charge, but not at higher pH (pH 7) (197). The differences probably arises from the different lipids and methodology used by Cornell et al. (197–199). Cornell et al. measured the amounts of protein adsorbed to the lipid layer by transferring the layer to a solid support. During the transfer, the surface pressure was kept at 30–35 mN/m, thus preventing insertion of portions of the protein in the lipid monolayer (199). Only protein molecules that interact strongly with the lipid head groups are transferred to the solid supported. Another difference is that their surface pressure data of the protein penetration is recorded under constant area, not at constant pressure, as in our study. In addition, Cornell et al. used lipids with their chains in the liquid state, which, as will be discussed, can influence the interaction. Cornell (198) also observed a specific interaction between β-lactoglobulin and egg yolk phosphatidic acid (e-PA) in spread mixed films at low pH (1.3 and 4) where β-lactoglobulin carries a positive net charge. No interaction was observed for e-PA in the neutral pH range or for egg yolk phosphatidylcholine (e-PC). Similar observations was made for the interaction between α-lactalbumin or BSA with mixed monolayers of POPC and POPG, where adsorption was

observed below the isoelectric point of the protein, where the lipid layer and the protein carry opposite net charge, but less was adsorbed around and almost nothing above the isoelectric point (199). The interaction was reduced in the presence of calcium as well as at increased ionic strength. Cornell et al. thus concluded that the interaction is of an electrostatic origin.

The work of Quinn and Dawson concerning the interaction between cytochrome-*c* (positive net charge below pH 10) and phospholipids from egg yolk also stresses the importance of the electrostatic interaction, although conformational changes of the protein are of importance (200,201). They measured the pressure increase caused by the penetration/adsorption of the protein to the lipid monolayers as well as the amount adsorbed by using ^{14}C-labeled protein. Their results show that the limiting pressure for penetration is 20 and 24 mN/m for phosphatidylcholine and phosphatidylethanolamine, respectively, whereas penetration into the phosphatidic acid and diphosphatidylglycerol (cardiolipin) monolayers occurred up to pressures close to the collapse pressure of the film (<40 mN/m). Furthermore, the penetration into the e-PC monolayers was not affected by increasing the sodium chloride concentration to 1 *M*. Cytochrome-*c* bound to the e-PC monolayers could not be removed by increasing the ionic strength. This is in contrast to the cardiolipin and e-PA monolayers, where the penetration was reduced when the sodium chloride content was increased to 1 *M*. It was also possible to partly desorb some cytochrome-*c* from e-PA monolayers. However, the pH dependence of the interaction was found to be quite complex, which suggests that subtle changes in the protein conformation also affect the interaction.

The importance of the electrostatic interaction with the phospholipid head group has also been shown by the work of Malmsten et al. (202,203), who studied the interaction of human serum albumin, IgG, and fibronectin from human plasma with phospholipid layers spin-coated onto methylated silica surfaces. Generally, he found no interaction between the proteins and lipids with no net charge or with shielded charges (e.g., phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and phosphatidylinositol), whereas interaction was observed with the surfaces containing unprotected charges (e.g., phosphatidic acid, diphosphatidylglycerol, and phosphatidylserine).

Hydrophobic Interactions. As observed in Fig. 8, the rate of adsorption of β-lactoglobulin into DPPA monolayers was significantly lower than into the monolayers, where the corresponding lipid had a longer chain length. This points to the importance of hydrophobic interactions for the incorporation. It was also observed that the incorporation was much faster

into the lipid monolayer than into its own proteinous layer, being less "oil-like" than the lipid layer (195). In addition, repulsive steric and electrostatic forces might contribute the lower rate of incorporation. Quinn and Dawson (201) found that the threshold surface pressure, above which no penetration of cytochrome-*c* took place in phosphatidylcholine monolayers, was considerably lower when DPPA was used instead of hydrogenated e-PC. The latter lipid contained fatty acid with a longer chain length, about 60% C_{18} and 30% C_{16}. Du et al. (204) studied the influence of the alkyl chain length of glucolipids (dialkyl glycerylether-β-D-glucosides and dialkyl glyceryl-ether-β-D-maltosides) on the interaction between lipid monolayers and glucose oxidase. The interaction, as shown by an increase in surface pressure, was found to increase with increasing lipid chain lengths for both types of lipid. These results suggest that the hydrophobic interaction is the predominant force. Furthermore, it is interesting to note that the interactions were not so strong with the lipids having the more bulky head group (i.e., the dialkyl glycerylether-β-D-maltosides), although the Π–A isotherms for the corresponding dialkyl glycerylether-β-D-glucosides were similar. This illustrates that a bulky head group can sterically hamper the protein–lipid (hydrophobic) interaction.

Effect of Lipid Fluidity. The complete hydrogenation of e-PC was found not to affect the surface pressure threshold for penetration of cytochrome-*c* compared to the native e-PC (201). However, the change in surface pressure due to the penetration of the protein versus initial surface pressure was less steep for the saturated one. A similar trend was observed for the e-PE samples (200). The conclusion was that the limiting pressure for penetration to take place is likely to be determined by the work necessary for the penetration, that is $\int \Pi \, dA$, where an area, A, of the interface has to be created for the protein to penetrate. Once the penetration is feasible, the magnitude will depend on the space between the molecules, and, thus, the degree of penetration is expected to be lower for the hydrogenated sample (201). The surface pressure threshold below which penetration of cytochrome-*c* into the anionic diphosphatidylglycerol (cardiolipin) monolayer took place was also found to decrease when the lipid was fully hydrogenated (201). Ibdah and Phillips found the same trend in their study of the effect of lipid composition and packing on the penetration of apolipoprotein A-I into lipid monolayers (205). In the biological system, this protein interacts with the phospholipid membrane of the serum high-density lipoprotein (HDL) particles (see Section V.B). Their results show that this protein adsorption occurs, to a larger extent, on expanded monolayers than on condensed monolayers (i.e., protein adsorption decrease in the order e-PC > egg sphingomyelin > DSPC). Furthermore, it was found that

protein adsorption generally decreased with an increasing amount of cholesterol in the lipid monolayer. It was suggested this was due to the condensing effect of cholesterol.

Effect of Protein Structure. Evidence of the importance of the protein structure was provided by Hanssens and Van Cauwelaert (206), who studied the penetration of α-lactalbumin in monolayers of DPPC and cardiolipin at physiological pH (pH 7.4) and at pH 4.6 with and without calcium. Reports have indicated that the protein is transferred to an intermediate, partially unfolded (molten globule) state at low pH and by depletion of calcium (101,103,104), which would expect to facilitate the penetration of α-lactalbumin into the monolayer. Furthermore, it has been reported that the calcium-free form of α-lactalbumin is more hydrophobic (207). Indeed, penetration occurred at low pH and was prevented if the protein was adsorbed from a calcium solution (206).

It is not easy to in situ monitor the structural changes of the protein on binding to a lipid monolayer. However, they can be monitored indirectly by recording the circular dichroism (CD) spectra of the mixed protein–lipid film transferred to a quartz plate. This technique was used to recorded CD spectra for β-lactoglobulin, α-lactalbumin, or BSA bound to mixed monolayers of POPC and POPG (197,199). The spectra show that the protein bound to the lipid monolayer was similar to the one recorded in solution, indicating that the conformation of the protein did not change significantly when interacting with the lipid monolayer.

2. Structure of the Interfacial Film

Even from the study of the penetration of protein versus surface pressure, it is also possible get some hints about the structure of the mixed layer. Cornell et al. (197,199) observed penetration of β-lactoglobulin, α-lactalbumin, or BSA into mixed monolayers of POPC and POPG at such high surface pressure that it is unlikely that the proteins could penetrate into a protein layer. Thus, they concluded that the formation of pure protein patches is unlikely and that portions of the protein is suggested to be intercalated into the lipid monolayer. Bos and Nylander made similar observation for the interaction between β-lactoglobulin and DSPC and DSPA monolayers (195).

Fluorescence microscopy and Brewster-angle microscopy (BAM) can be used to in situ image the structure of the film at the air–aqueous interface, although the lateral resolution is limited by the resolution of the optical microscope. Fluorescence microscopy together with the surface film balance technique was used by Heckl et al. to study the structure of

mixed phospholipid–cytochrome-*c* and cytochrome-*b* films (208). They found that proteins primarily were located in the fluid membrane phase, which coexisted with solid lipid domains without protein. The penetration into the lipid monolayer was reduced with increasing pressure. Cytochrome-*c* (positively charged) was found to interact with dimyristoylphosphatidic acid (DMPA) monolayers but not with dipalmitoylphosphatidylcholine (DPPC) layers, showing the electrostatic nature of the interaction. Schönhoff et al. concluded from their study of the incorporation of membrane proteins into DPPA/DOPA monolayers that incorporation mainly takes place in the fluid phases of the matrix (209). Zhao et al. used BAM to image the kinetics of β-lactoglobulin penetration into DPPC monolayers at the air–aqueous interface from a 500-n*M* solution in 10 m*M* phosphate buffer, pH 7 (210). For instance, at an initial surface pressure of 7.8 mN/m, it took 0.17 min until domains, with similar morphology as those appearing during the compression of a pure DPPC monolayer, appeared. These domains were found to consist only of the lipid, as confirmed by grazing incidence x-ray diffraction, and β-lactoglobulin penetration was found to occur without any specific interaction with DPPC. β-Lactoglobulin was not able to penetrate into a condensed DPPC monolayer (i.e., above a surface pressure of about 20 mN/m).

The lateral organization in mixed protein–lipid films at the air–aqueous interface can be studied by spectroscopic techniques and high-resolution imaging techniques such as electron microscopy and atomic force microscopy (AFM), after transferring the films to a solid support. Using electron microscopy, Cornell and Carroll found that only lipids with the chains in liquid state (e-PA, dioleoylphosphatidylcholine, and dioleoylphosphatidylethanolamine) formed homogenous films with β-lactoglobulin, whereas DPPA and DSPC formed heterogeneous layers (211). The use of AFM as powerful technique for studying the lateral organization in mixed films of proteins and soluble surfactants has already been demonstrated with the development of the ''orogenic'' displacement model (173–175). Diederich et al. studied the interaction between bacterial surface layer proteins (S-layer proteins) and phosphatidylethanolamine (DMPE and DPPE) monolayers using dual-label fluorescence microscopy, Fourier transform infrared (FTIR) spectroscopy, and electronmicroscopy (212). When the monolayer is in the two-phase region, with one isotropic and one anisotropic fluid phase, the S-layer protein adsorbed preferentially to the isotropic phase. However, two-dimensional crystallization could be nucleated in the boundaries between the two phases, but proceeded mainly underneath the anisotropic phase. The FTIR measurements clearly indicate that the protein crystallization leads to an increased order of the lipid acyl chains.

3. Monolayer Stability

One might expect that monolayer made up of lipids with a very low aqueous solubility would be stable. However, this is far from general. The metastablility of monolayers can be caused by processes such as rearrangement within the layer, dissolution into the subphase, and transformation to a three-dimensional phase, which can occur at pressures above the equilibrium spreading pressure (213,214). Furthermore, the stability of the monolayers can be affected by the spreading solvent and the techniques used for spreading the lipid (215,216). The stability of the monolayer can also be considerably changed by the ion composition of the aqueous subphase. For instance, the stability of an arachidic (*n*-eicosanoic, $C_{20:0}$) acid monolayer was found to increase in the order $H^+ < Li^+ < Na^+ < Ca^{2+} < Mg^{2+}$ (213). There are several examples of proteins that are thought to have the role to stabilize a lipid monolayer or bilayer. One such example is the milk fat globule membrane that has been suggested to consist of the monolayer of polar lipids, which covers the fat globule surface, and an outer lipid-based bilayer (217,218). The milk fat globule membrane is expected to be inhomogeneous, with a significant amount of proteins in the membrane. An aqueous layer containing different proteins, like xanthine oxidase, is present between the monolayer and bilayer. One of the roles that have been assigned to xantinoxidase is to stabilize the milk fat globule membrane (218). Interestingly, Kristensen et al. found that the presence of a xanthine oxidase can increase the stability of a monolayer composed of sphingomyelin from the milk fat globule membrane (219). They investigated the interaction between one of the major proteins, xanthine oxidase, and the major lipids, sphingomyelin and phosphatidylcholine, in the milk fat globule membrane at the air–aqueous interface by using the monolayer technique. Both lipids have a similar phopshorylcholine head group, which is zwitterionic in the neutral pH range, although the belt regions linking the phopshorylcholine group with the acyl chains are different. The Π–A isotherms of sphingomyelin and phosphatidylcholine are shown in Figs. 10a and 10b, respectively. The isotherms for sphingomyelin monolayers spread on pure buffer and a xanthine oxidase solution are shown. The slope of isotherm and the area of the compressed monolayer for pure sphingomyelin (Fig. 10a) are smaller than expected for these types of lipid. In addition, the large hysteresis and the dependence on the compression speed, not observed for distearoylphosphatidylcholine, confirms that the sphingomyelin monolayer is metastable. The difference in stability of monolayers formed by two different lipids can probably be related to the different conformations of choline groups in the two types of lipid, where intramolecular hydrogen-bonding is possible between the phosphate group and the amide and hydroxyl groups

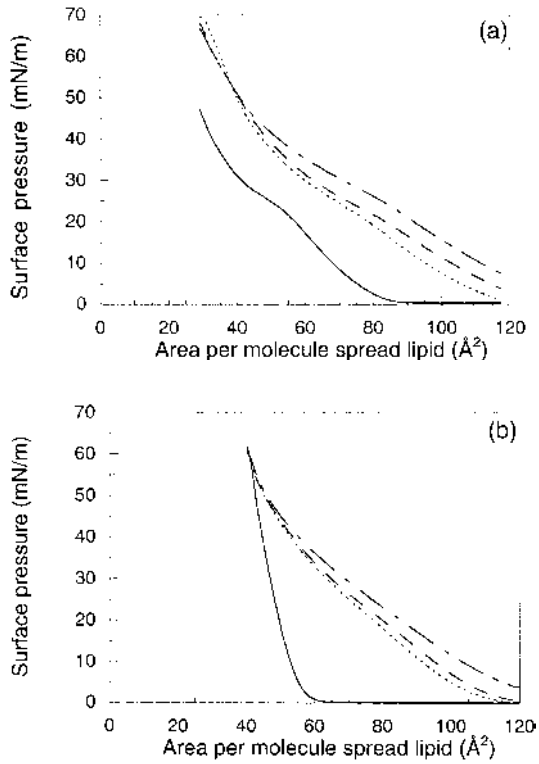

Figure 10 Dynamic surface pressure (Π) as a function of the molecular area of the spread amount lipid for compression of (a) sphingomyelin and (b) DSPC monolayers on a phosphate-buffered subphase (40 m*M* phosphate containing 0.1 *M* sodium chloride, pH = 7.4) with or without xanthine oxidase (5 mg/mL). The isotherms recorded for the lipid spread on pure buffer (—) and at 5 (- - -), 10 (– – –), and 20 (– - –) min elapsed between spreading and compression. The lipid (25 μg) was spread from a chloroform/methanol (2:1, v/v) solution on a maximum area of $50 \times 450\,mm^2$ and a compression speed of 12.5 mm/min was used. (Data from Ref. 219, where the experimental details are given.)

in the belt region of sphingomyelin (220). An increase in Π at maximum compression of the sphingomyelin monolayer, which reflects an increase in the monolayer stability, was observed in the presence of sphingomyelin. Furthermore, the area per sphingomyelin molecule increases in the presence of xanthine oxidase even at high Π values. This is in contrast to the results from the parallel study of the phosphatidylcholine monolayers with and without xanthin oxidase, where the interacting protein could be completely

squeezed out from the lipid monolayer at high enough surface pressures without affecting the collapse pressure. This indicates that the interaction between xanthine oxidase and sphingomyelin is much stronger than that between the protein and phosphatidylcholine.

B. Protein-Lipid Interactions at the Oil–Aqueous Interface

Most studies of protein–lipid interactions at the oil–aqueous interface has been carried out using model emulsions. The purity of polar lipid and the way it is added (e.g., to the oil or the water phase) are bound to affect the interactions with proteins, which, in turn, affect the emulsion stability. Yamamoto and Araki (221) studied this by comparing the interfacial behavior of β-lactoglobulin, in the presence of lecithin (PC) in the water or in the oil phase, with the stability of corresponding emulsions. In the presence of protein, crude lecithin was found to increase the stability of emulsion and lower the interfacial tension more effectively than a pure lecithin preparation. When crude lecithin was added to the oil phase, the interfacial tension was found to decrease and the emulsion stability increased compared to when the lecithin was dispersed in the aqueous phase. One might speculate whether these findings can be related to the presence of fatty acid and/or charged phospholipids in the crude lecithin. Aynié et al. studied the interaction between nitroxide homologs of fatty acids and milk proteins by following the mobility of the nitroxide radicals using electron spin resonance (222). At pH 7, the importance of the lipid–protein interaction was not determined by the structure of the protein, but positively correlated with the number of positive charges on the protein. Thus, it was observed that the importance of the interaction in the emulsions decreased in the order α_{s1}-casein > β-lactoglobulin > β-casein, suggesting that the interaction was of an electrostatic nature. The different proteins also affect the organization of the lipid monolayer, where α_{s1}-casein, in contrast to β-lactoglobulin and β-casein, induce, an ordering of a monolayer of nitroxide fatty acids on the surface of an emulsion droplet (222). This can probably be assigned to the stronger interaction of α_{s1}-casein with lipids compared to the other proteins.

Bylaite et al. studied the stability and droplet size of β-lactoglobulin- and lecithin [phosphatidylcholine from soybean (sb-PC)]-stabilized emulsions of caraway essential oil as well as the amount of protein on the emulsion droplets (223). It should be noted that sb-PC was dispersed in the oil phase. Some of their data are given in Fig. 11, where the amount of β-lactoglobulin adsorbed on the oil–aqueous interface is shown versus amount added of s-PC. These data show that sb-PC is likely to replace some of the protein at the oil–aqueous interface, although it is unable to completely replace the protein. The maximum reduction in the amount of

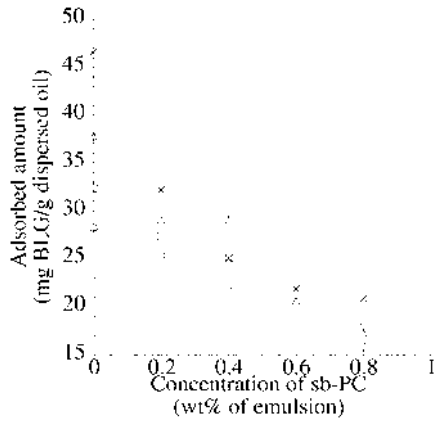

Figure 11 Adsorbed amount of protein at the caraway essential oil–water (△, ×) and olive oil–water (○, □) interfaces in emulsions stabilized by 1 (△, ○) and 2 (× □) wt% β-lactoglobulin and variable amount of soybean-PC. Emulsions were prepared from 15 wt% oil in a 60 m*M* phosphate buffer of pH 6.7. (Data Ref. 223 where the experimental details are given.)

β-lactoglobulin adsorbed is by a factor of 3 for the caraway oil. These findings are in agreement with other studies, where lecithin was found to be less efficient in displacing milk proteins from the oil–water interface compared to other surfactants (224,225). Bylaite et al. found that emulsions with triglyceride oil generally proved to be more stable compared to those made with caraway essential oil as the dispersed phase (223). However, the stability of the emulsions could be improved considerably by adding sb-PC. An increase in the protein concentration also promoted emulsion stability. Fang and Dalgeish arrived at a somewhat different conclusion for casein-stabilized emulsions (226). They found that the presence of DOPC destabilized casein-stabilized emulsions of soybean oil in 20 m*M* imidazole/HCl at pH 7.0. This seemed to be independent on whether DOPC was present during emulsification or if it was added to the emulsion as dispersed aggregates. At a high concentration of casein, the emulsions were stable, and the decrease in surface load was a direct indication of the removal of casein from the interface by the presence of DOPC. The higher the DOPC concentration, the greater was the effect on emulsion stability and surface load. DPPC and egg PC either enhanced or did not affect the stability of the emulsion.

Bylaite et al. applied ellipsometry to study the adsorption of the lipid from the oil and the protein from the aqueous phase at the oil–water interface (223). Independently of the used concentration, close to monolayer coverage

of sb-PC was observed at the caraway oil–aqueous interface. On the other hand, at the olive oil–aqueous interface, the presence of only a small amount of sb-PC led to an exponential increase of the layer thickness with time beyond monolayer coverage. This interesting observation was assigned to the formation of a multilamellar layer of sb-PC at the olive oil–aqueous interface, when sb-PC reached the solubility limit in the olive oil.

The displacement of caseinate from the interface of emulsion droplets by monoglycerides, mono-oleoylglycerol, and monostearoylglycerol dissolved in the oil phase was found to correlate with the adsorption of the monoglycerides at the oil–water interface (227). The amount of monooleoylglycerol increased gradually with concentration and reached a plateau when approaching an oil-phase concentration of 1 wt%. Under these conditions, all of the caseinate was displaced from the interface. The saturated lipid, monostearoylglycerol, was much more efficient in displacing the protein. Already, at a concentration in the oil phase of between 0.2 and 0.3 wt%, the adsorbed amount of monostearoylglycerol increased sharply and reached much higher surface concentrations than monooleoylglycerol. At 0.3 wt%, all of the caseinate was removed from the interface.

C. Protein Interactions with Lipid Vesicles

The mechanisms that determine the stability, size, and shape of vesicles are complex and widely discussed (for reviews, see, e.g., Refs. 80–83). The spherical shape is generally the most stable shape for equal distribution of molecules between the two monolayers constituting the bilayer (81). These spherical vesicles can be large multilamellar vesicles (MLV) and large (LUV) and small (SUV) unilamellar vesicles (81). The bending of the lipid bilayer to form a vesicle imposes a strain on a symmetric bilayer, as the inner monolayer has a negative curvature, whereas the outer has a positive curvature. The magnitude of this curvature energy can be difficult to estimate, but it is thought to be significant enough in many cases to make the vesicles inherently unstable and energy has to be added to form them (81–83). The result of the tension can be a nonspherical vesicle (228). A mixture of phospholipids, which corresponds to the composition in the milk fat globule membrane, gives both spherical vesicles and tubular structures (229). In particular compositions (e.g., 80% DOPE, 12% DOPC, and 8% sphingomyelin) that at high lipid content give liquid-crystalline phases at the boundary of lamellar to reversed hexagonal phase tend to give microtubular structures at high water content rather than vesicles. A larger proportion of multilamellar vesicles were observed in buffer and divalent salts than in pure water. A small increase in the interlayer spacing

of the mulitlamellar vesicle was observed in the presence of β-lactoglobulin and β-casein.

1. Driving Force for the Protein–Vesicle Interaction

The driving mechanism for the interaction of proteins with the lipid bilayer of the vesicles are basically as for the interaction a lipid monolayer at the air–aqueous interface. In parallel to the Quinn and Dawson study discussed earlier (200,201), Rytömaa et al. (230) found a strong electrostatic contribution when cytochrome-*c* binds to cardiolipin–phosphatidylcholine liposomes. This interaction did not take place if the negatively charge lipid cardiolipin was absent in the membrane. Furthermore, the protein was dissociated from the vesicle in the presence of 2 m*M* $MgCl_2$ and 80 m*M* NaCl at pH 7. The apparent affinity of cytochrome-*c* to the vesicles also increased when the pH was dropped to 4. The interaction was found to be completely reversible for pH changes; that is, if the pH was increased to 7, the protein could be dissociated from the vesicle by adding salt.

Price et al. studied the adsorption of fibrinogen to neutral liposomes, composed mainly of phosphatidylcholine (PC) and cholesterol and negative liposomes, composed mainly of phosphatidic acid (PA) and cholesterol, as well as to the corresponding liposomes in which a polyethylene glycol (PEG)-modified phosphatidylethanolamine had been introduced (231). They found that negatively charge liposomes adsorbed more fibrinogen than the corresponding neutral liposomes. PEG modification was found to have no effect on neutral liposomes in terms of fibrinogen adsorption. However, PEG modification, which sterically stabilizes the liposome, markedly reduced the adsorption to the negative liposomes.

Brooksbank et al. conducted an extensive study on the interaction of β-casein, κ-casein, α_{s1}-casein, and β-lactoglobulin with negatively charged e-PG and zwitterionic e-PC vesicles using photon correlation spectroscopy (232). Their data on the adsorption of β-casein are shown in Fig. 12. All of the studied proteins were found to give a thicker layer on the negatively charged vesicles, although they all carried a negative net charge under the conditions used (160 m*M* sodium chloride at pH 6.2). Brooksbank et al. (232) suggested that binding to the vesicle surface takes place mainly through hydrophobic interactions and the differences in thickness of the adsorbed layers on the two types of vesicles were explained in terms of the protein charge distribution. For instance, the hydrophilic, N-terminal, part of β-casein has a net charge of −12, whereas the remainder of the molecule carries almost no net charge. Thus, on the negatively charge vesicle surface, the molecules adopt a more extended configuration, as the N-terminal part is likely to be pushed away from the surface by means of

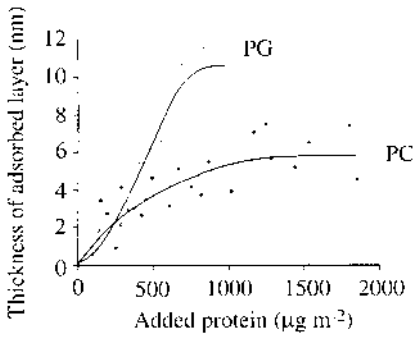

Figure 12 Thickness of adsorbed layer on β-casein on negatively charged e-PG and zwitterionic e-PC vesicles as a function added protein expressed as microgram of protein per square meter of available liposome surface. The liposomes were dispersed in 160 m*M* and the pH was about 6.2. The data are taken from a photon correlation spectroscopy study by Brooksbank et al, where further experimental details are given (232).

electrostatic repulsion. This explains the thicker layers on this surface as shown in Fig. 12. A similar reasoning can be applied for κ-casein. The apparently very thick adsorbed layer of α_{s1}-casein was explained by bridging flocculation of the vesicles mediated by the protein. The middle section of α_{s1}-casein carries a negative net charge, whereas the two ends have no net charge. One of the uncharged ends pertrudes into the vesicle bilayer and the middle section is repelled from the vesicle surface, leaving the other uncharged end of the peptide chain free to interact with another vesicle. The charge distribution on β-lactoglobulin is more even and the interpretation of the results was not as straightforward.

As discussed by Kinnunen, the introduction of the H_{II}-forming double-chain lipid (a lipid with packing parameter >1; see Fig. 1) in a lamellar membrane imposes a considerable stress on the membrane (1). This frustrated membrane is said to be in the L_{ε} state according to the Kinnunen terminology (1). Free energy can be gained by allowing some of the lipids in the frustrated membrane to adopt the so-called extended or splayed chain conformation, where one of the acyl chains extends out from the bilayer, whereas the other chain remains in the membrane. Such an extend chain can also be accommodated within a proper (hydrophobic) cavity of protein interacting with the membrane (1). This is an interesting alternative explanation for the hydrophobic interaction between peripheral proteins and membranes that has been discussed in this review. The splayed chain conformation has also been suggest to be one mechanism for membrane fusion (2). This and other implications of

the splayed chain confirmation has recently been discussed by Corkery (233).

2. Influence of the Protein Structure on the Vesicle Interaction

Kim and Kim studied the interaction between α-lactalbumin and phosphatidylserine–phosphatidylethanolamine vesicles (1:1 molar ratio) versus pH (234). They found that the interaction, which almost did not exist at neutral pH, increased with decreasing pH (Fig. 13). What is interesting to note (Fig. 13) is that vesicle fusion, as estimated from the increase of the initial rate of Tb fluorescence increase, correlates with the binding of the protein to the vesicles. The binding was suggested to be due to hydrophobic interaction via protein segments penetrating into the lipid bilayer, as it was impossible to dissociate it by increasing the pH. This was further confirmed by using proteolytic enzymes, which were found to cut off both ends of the polypeptide chain, leaving only the segment that penetrates into the bilayer. This penetration protein loop was also believed to induce fusion of the vesicles.

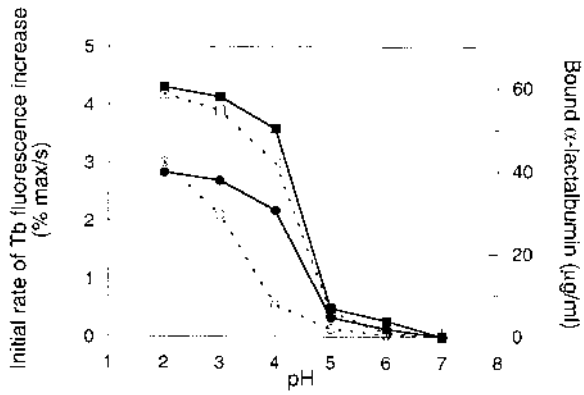

Figure 13 The initial rate of Tb fluorescence increase (- - -○ - - -, - - -□ - - -) upon α-lactalbumin induced fusion of phophatidylserine–phosphatidylcholine (1:1 molar ratio) vesicles is shown as a function of pH. The pH-dependent binding of α-lactalbumin is shown as the amount of protein bound per milliliter of vesicle suspension (—●—, —■—), which contained 1 m*M* lipid molecules (determined from the phosphor content) per milliliter of suspension. The results for initial protein concentrations of 50 (○, ●) and 100 (□, ■) μg/mL are presented. As the curves for the fusion process represents kinetic data and the binding studies represent equilibrium data when the fusion process is over, only a qualitative comparison is possible. (Data Ref. 234, where the experimental details are given.)

The importance of the protein conformation on the interaction with vesicles was also shown in the study of Brown et al. (235). They found no interaction between native β-lactoglobulin and DPPC vesicles, but β-lactoglobulin, modified by exposing it to a 2:1 mixture of chloroform and methanol, did interact with the vesicles. Moreover, the lipid–protein complex formed had an α-helix content of at least 25–30% larger than for the native protein. The interaction was found to lead to aggregation of the vesicles at pH 7.2, whereas no aggregates were observed at pH 3.7. This was explained by the larger net charge at pH 3.7 (+20) compared to pH 7.2 (−10). These results imply that protein modification, either during processing or by special treatment, can increase the helix content, which, in, turn can be boosted by lipid interaction. The lipid–protein complexes formed have been suggested as a way to improve the emulsification processes (236,237).

3. Lateral Phase Separation in Vesicle Bilayers

Raudino and Castelli reported that the presence of lysozyme could induced lateral phase separation in vesicle bilayers composed of a mixture of phosphatidic acid and phosphatidylcholine (238). Their differential scanning calorimetry study of the lipid chain melting transition showed good mixing in the absence of the protein and the single peak was shifted toward higher temperatures as the phosphatidic acid content increased. In the presence of lysozyme however, lateral phase separation did occur as the chain melting transition peak was split into two peaks. In addition, they found that the temperature of protein unfolding increased with the fraction of phosphatidic acid, suggesting a stabilization of the protein due to the interaction with phosphatidic acid.

It is important to bear in mind that microheterogeneity of the bilayer not only occurs for mixtures of different lipids but also close to the gel-to-fluid phase transition of the lipid. Hønger et al. studied the relation between phospholipase A_2-catalyzed hydrolysis of one-component phosphatidylcholine vesicles and the microheterogeneity of the lipid bilayer (239). They varied the microheterogeneity by changing the temperature in the vicinity of the gel-to-fluid phase transition as well as using lipid chain lengths between C_{14} and C_{18} and found a strong correlation between the maximal lipase–lipid interaction and the maxima in the interfacial area between gel and fluid domains.

D. Protein Interaction with Liquid-Crystalline Phases/Gel Phases

As discussed previously, the interaction between water-soluble lipids and proteins usually takes place via monomers, and the associated surfactant

structures is generally formed in the same range, or higher, of surfactant monomer concentration as needed for the interaction to take place. For lipids with low aqueous solubility, on the other hand, the association structures, different lyotropic mesophases, are generally already present when mixed with the protein and have thus a profound impact on the protein–lipid interaction. It is therefore important to be familiar with the phase behavior of the particular lipid used because often seemingly conflicting results can be derived from differences in the phase structure of the lipids. For instance, we have observed that the interaction between β-lactoglobulin and phosphatidic acid only occurred when the lipids were present as a dispersion, but not when they were mixed in the gel state with the protein (22).

Even if no specific interaction occurs, proteins can have an impact on the liquid-crystalline phase or gel phase due to the limited space of the aqueous cavity. This was demonstrated by Minami et al., who investigated the incorporation of lysozyme, β-lactoglobulin, and α-lactalbumin in a sphingomyelin gel phase containing 0.6 wt% sodium palmitate and 80 wt% aqueous solution (240). The dimension of the aqueous layer in the gel phase was suggested to limit the amount of protein that could be incorporated. Above this limit, phase separation will occur with a gel phase and an ''outside'' protein-rich solution. The protein will, at high enough concentration, probably also compete for the water in the interlamellar spacing, which will eventually lead to a reduction of the aqueous layer thickness. This effect was demonstrated for high-molecular-weight polymers in equilibrium with the phosphatidylcholine lamellar phase (241). The polymer was unable to enter the aqueous layer, but still exerted an osmotic stress that was large enough to compress the lamellar lattice, as shown by x-ray diffraction data. This method has been used to measure the interaction between the lipid bilayers (241,242).

Proteins are, of course, also able to enter into the aqueous layer of a lamellar phase and thereby affect the swelling. This was shown by Rand (243), who studied the penetration of bovine serum between negatively charged lecithin/cardiolipin bilayers at pH 3.3, where the protein has a positive netcharge. BSA is also likely to adopt a more expanded structure at this pH, thus exposing more hydrophobic segments. He found that the interlamellar spacing of the lamellar phase, decreased with an increasing cardiolipin/bovine serum albumin ratio. This was related to a reduction of the negative charge of the lipid layer as the amount of bound protein increases.

We will start our discussion by giving some example of the interplay between the lipid structures and protein in terms of the effect on the curvature of the lipid–aqueous interface, because curvature has an important role

in condensed matter, as discussed in the book by Hyde et al. (59). McCallum and Epand found that changing the curvature of biological membranes could modify membrane-bound insulin receptor autophosphorylation and signaling (244). This was demonstrated by adding compounds that raised the bilayer to the reverse hexagonal (H_{II}) transition temperature of model membranes. This promoted a decrease of curvature and inhibited the insulin stimulation of the receptor phosphorylation.

The next part will focus on a specific structure, the bicontinuous cubic liquid-crystalline phase, where the interaction with the lipid bilayer as well as the physical entrapment within the cubic phase takes place. Finally, we will briefly discuss the effect of enzyme interactions, namely lipase action, on liquid crystalline phases.

1. Protein Interactions That Increase the Curvature of the Lipid–Aqueous Interfaces

Proteins or peptides that penetrate into the hydrophobic domain of the lipid bilayer generally provoke an increase of curvature of the lipid–aqueous interface (i.e., becomes more concave toward the aqueous space). Quite a few of the membrane-bound peptides have these properties, such as Gramicidin A, a hydrophobic polypeptide, which forms channels for monovalent cations in phospholipid membranes (245). This peptide was found to favor the transition lamellar phase $\rightarrow$reversed hexagonal (H_{II}) phase in dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) systems in an excess of water, as observed by nuclear magnetic resorance (NMR) studies (246).

Not only can proteins or peptides that penetrate into the lipid bilayer induce phase transitions, but also proteins that are certain to interact with the head groups of the phospholipid bilayer can give rise to similar effects. This has been demonstrated for cytochrome-*c*, which has a positive netcharge and has been shown to interact with negatively charged phospholipids (247). The binding of cytochrome-*c* to anionic cardiolipin liposomes induced the formation to an inverted hexagonal, H_{II}, structure (247). No interaction and, hence, no phase transition was observed in the presence of liposomes composed of neutral zwitterionic lipids like PC and phosphatidyl ethanolamine (PE). A phase transition to the H_{II}-phase was observed if a sufficient fraction of these lipids was replaced for cardiolipin, Interestingly, the protein was found to interact with liposomes with the anionic lipid phosphatidylserine (PS), but did not induce any phase transition. The interaction between cardiolipin and cytochrome-*c* was also studied by Spooner and Watts, using deuterium and phosphorus-31 NMR measurements (248). Likewise, they found that the interaction can, depending

on the lipid stoichiometry, cause a transition from a lamellar to a non-bilayer structure. The binding of the protein with the liquid-crystalline bilayers of cardiolipin was also found to cause extensive derangement of the cytochrome-*c* secondary structure (248,249).

Studies of the interaction between cytochrome-*c* and suspensions of DMPG or admixtures of dioleoylglycerol (DOG) or DOPC with DOPG also showed that binding of cytochrome-*c* could promote an increase in surface curvature of the lipid aggregates from a bilayer structure (250). This is deduced from NMR data where an isotropic peak occurs in the presence of cytochrome-*c*, indicating cubic lipid phases, small spherical vesicles, or extended bilayers with high local curvature. The structure of cytochrome-*c* was found to change on binding to the lipid, and two forms, depending on the lipid composition, were identified with resonance Raman measurements:

I. Close to the native conformation in solution
II. Unfolded with the heme crevice opened

The changes in protein structure could be correlated with the curvature of the lipid bilayer, as illustrated in Fig. 14, as the ratio between the unfolded (II) and native (I) cytochrome-*c* (cyt c) in DOPC/DOG dispersions versus DOG mol%. The presence of DOG was found to induce spontaneous curvature in the DOPG lipid bilayer in the pure lipid system, which at a DOG content of $\sim 50\%$, leads to the transition to a reversed hexagonal (H_{II})

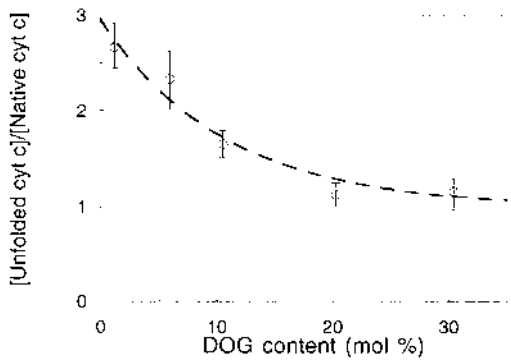

Figure 14 Concentration of unfolded (II) and native (I) cytochrome-*c* (cyt c) in DOPC / DOG dispersions versus DOG mol% determined from Raman resonance spectra. The concentrations of lipid and cytochrome-*c* were 300 and 20 μM, respectively, in an aqueous buffer (1 mM HEPES, 1 mM EDTA) of pH 7.5. (Data Ref. 250, where the experimental details are given.)

phase. In the absence of DOG (i.e., a strict bilayer structure), the binding of the more unfolded form (II) of cytochrome is favored, whereas the fraction of the more native globular protein structure (I) increases with the amount of DOG (Fig. 14) and thus with curvature of the surface. The physical state of the lipid was also found to affect the proportions of the two structural forms of cytochrome-*c*. In the fluid state of pure DMPG, the fraction of the more unfolded form (II) was larger (85%) than when the lipid was in the gel state (80%). It is noteworthy that they found that the bound fraction of the more unfolded form (II) to the fluid DOPG bilayer structure was substantially lower (75%), indicating that not only the fluidity of the bilayer matters but also the type of lipid.

The interaction between cytochrome-*c* and mono-olein in the cubic phase was studied by Razumas et al. by differential scanning calorimetry (DSC) and optical microscopy (251). In line with the above-reported studies, they also found that the presence of cytochrome-*c* at high enough concentrations favored lipid aggregates with a larger curvature. Thus, they observed that the phase transition cubic $\rightarrow H_{II} \rightarrow L_2$ in the mono-olein–cytochrome-*c*–water system took place at a lower temperature than in the binary mono-olein–water system (251). Similar effects were observed when glucose oxidase was included into mono-olein–aqueous cubic phase (252). The temperature of the phase transition cubic $\rightarrow H_{II}$ in the mono-olein–glucose oxidase aqueous system decreases with the increase in glucose oxidase concentration.

2. Protein Interactions That Decrease the Curvature of the Lipid–Aqueous Interfaces

Fraser et al. investigated the ability of a range of basic proteins and polylysine to convert a reversed hexagonal (H_{II}) phase, consisting of DOPE and mixtures of DOPE and PS, to stable lamellar (L_α) phases at pH 9, where DOPE is anionic, and at pH 7, when it is zwitterionic (253). The proteins investigated were all capable of binding to the H_{II} phase at pH 9, but only myelin basic protein and polylysine did induce transition to the L_α phase. Lysozyme formed a new H_{II} phase in which the protein was included. A lowering of the pH seemed to release the proteins, except for mellittin, which also seemed to penetrate into the hydrophobic core of the lipid aggregates. The presence of PS in the H_{II} phase at pH 7 increased the protein binding, but only interaction with myelin basic protein gave a lamellar phase. Based on earlier studies, Fraser et al. suggested that the myelin basic protein stabilized the lamellar phase by interacting with the DOPE headgroup and thereby increasing its effective size (253). They concluded that the properties of myelin basic protein in terms of stabilizing the lamellar

structure could be related to the role of the protein to stabilize the myelin sheath multilayers.

3. Cubic Lipid–Protein Aqueous Phases

Lipid bilayers can be folded into such intriguing structures as cubic liquid-crystalline lipid–aqueous phases. In these structures not only the surface properties and the curvature but also the dimensions of the aqueous space affect protein–lipid interactions. The main features of the bicontinuous cubic phase is illustrated in Fig. 15. The mono-olein–aqueous system is a thoroughly studied example of such a system, where two types of cubic phase have been observed on the water-rich side of the lamellar phase (84,254–257). We have already discussed the biological implications of lipid-cubic phases. Here, we will highlight some of the main features that are of importance for the functionality of lipid cubic phases. First, it is the bicontinuity of the cubic phase. This is illustrated Figs. 16a and 16b, where the mobility of glucose solubilized in the aqueous channels and vitamin K solubilized in the lipid bilayer, respectively is illustrated. Figure 16a shows the concentration profiles of glucose in the cubic mono-olein–aqueous phase equilibrated against water as determined by holographic laser interferometry (258). These profiles could be fitted to Ficks second law, which gave a diffusion coefficient four times lower than the value in the aqueous solution. The mobility of the molecules in the aqueous channels of the cubic phase is certain to be affected by the dimensions of the channels and the size of the solute. Thus, electrochemical studies of the transport of cytochrome-*c* in the mono-olein–aqueous cubic phase gave values of diffusion coefficients that were about 70 times lower than the bulk values (251). Figure 16b shows the mobility of mono-olein and vitamin K_1 dispersed in the lipid bilayer as the NMR self-diffusion coefficients plotted versus lipid volume fraction in the cubic phases. It is noteworthy that the mobility of the introduced vitamin K_1 follows that of mono-olein, indicating complete dispersion of vitamin K_1.

The dimensions of the water channels in the bicontinuous cubic phases, which depend on the degree of swelling and type of cubic phase are in the same range as the size of proteins (cf. Ref. 252). Furthermore, as liquid-crystalline phases they are quite flexible structures. These features have triggered a number of studies, which have shown that a large range of hydrophilic proteins with molecular weights up to 590 kDa can be entrapped in the aqueous cavity of the mono-olein–aqueous cubic phases (251,252,259–262). The entrapped proteins have been found to be protected in the cubic phase, with retained native confirmation (260,262–265). Enzymes can be kept for a very long time (months, in

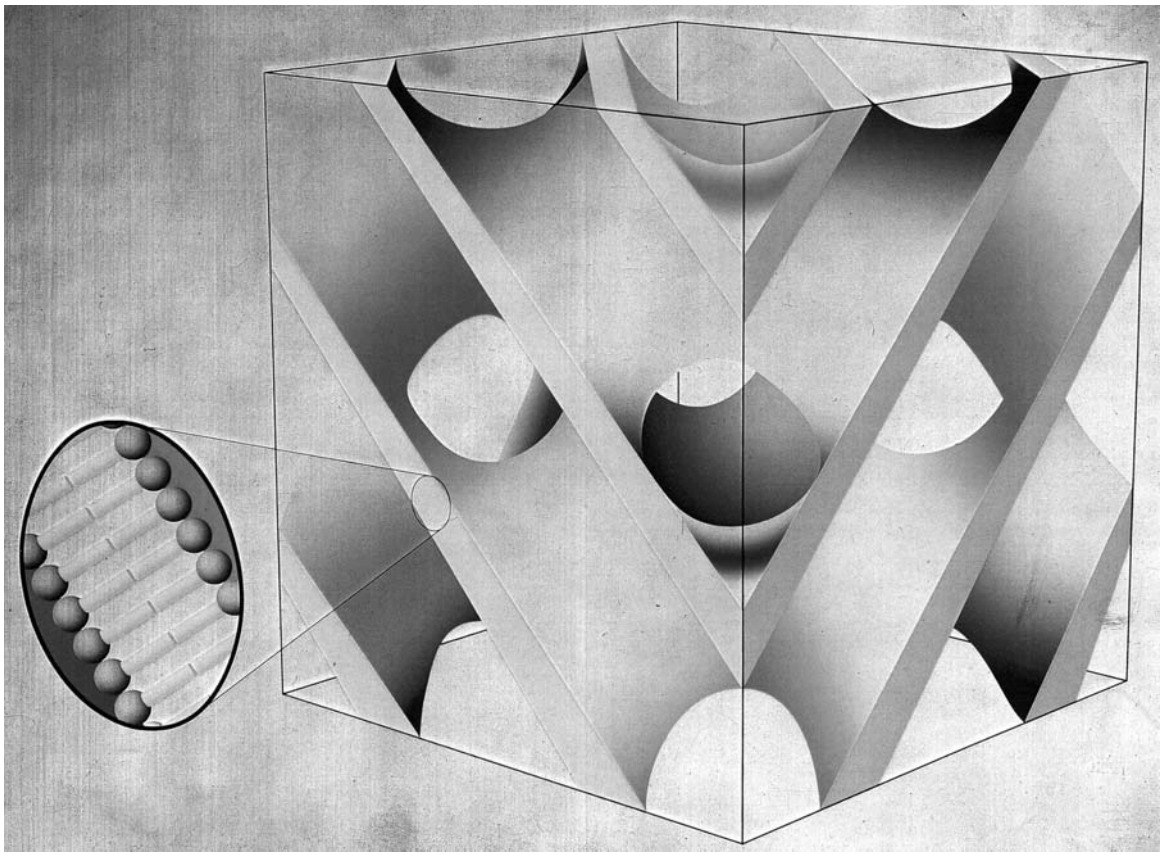

Figure 15 Schematic of the cubic phase, showing the two unconnected water channel systems. The location of the lipid bilayer is also indicated. The type of cubic phase shown can be described as a diamond of an infinite periodic minimal surface (IPMS) corresponding to a primitive cubic lattice (*Pn3m*).

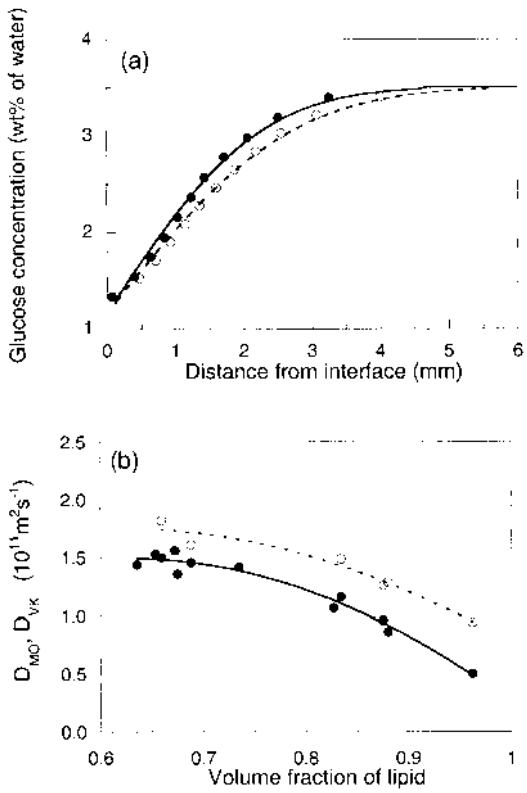

Figure 16 (a) Glucose concentration profiles in a mono-olein–aqueous cubic phase (62:38 wt%), where the aqueous solution initially contained 3.5 wt% glucose, after 3 h (●) and 4 h (○) equilibration against pure water. The concentration is given as the wt% glucose in the aqueous solution of the cubic phase. The solid and broken lines represent the best theoretical fit of Fick's law, giving diffusion coefficients of 1.39×10^{-10} m^2/s and 1.47×10^{-10} m^2/s after 3 and 4 h, respectively. The corresponding bulk value is 6.7×10^{-10} m^2/s. The data, obtained by holographic laser interferometry, are from Refs. 258 and 261, where the experimental details are given. (b) NMR self-diffusion coefficients at 25°C in mono-olein–aqueous cubic phases containing 0–5 wt% vitamin K_1 are shown as a function of the lipid volume fraction (including vitamin K_1). The self-diffusion coefficients were measured in the cubic (both gyroid and diamond type) and in the reversed micelle, L_2, phases. Self-diffusion coefficients of mono-olein (D_{MO}) (●) and vitamin K_1 (D_{VK}) (○) are shown. The lines are arbitrary fits to demonstrate the similar trends. The data are from Ref. 271, where the experimental details are given.

some cases), with retained activity, which is not possible in the aqueous solution (259,261).

Spectroscopic data have revealed changes in the molecular organization of the lipids evoked by the presence of the protein. FTIR measurements on the mono-olein–cytochrome-*c* aqueous system showed that the presence of cytochrome-*c* increased the conformational order of the mono-olein acyl chain and caused structural rearrangements in the polar-head-group region (251). These observations are in agreement with the decrease of the mono-olein packing parameter upon incorporation of cytochrome-*c*, which was deduced from the increase in unit-cell dimension of the cubic phase as determined by small-angle x-ray diffraction. The Raman scattering studies on the mono-olein–lysozyme–aqueous system demonstrated an increase in the number of hydrogen -bond C=O groups of mono-olein, but no increase in the acyl chain order relative to the binary lipid–aqueous system (260). A similar increase in the hydrogen-bonding, caused by the presence of a protein, was observed in the mono-olein–hemoglobin–aqueous system using FTIR spectroscopy (262). However, in this case, the protein incorporation also caused a decrease in the acylchain order.

The cubic monoglyceride phases have also the ability to solubilize lipophilic proteins like A-gliadin from wheat (266) and bacteriorhodopsin (267), as well as relatively large amounts of membrane lipids (260,261,268–270) and other hydrophobic compounds of biological relevance (268,271,272). These compounds are most probably dispersed in the lipid bilayer region of the cubic phase.

Apart from the biological significance of cubic lipid–aqueous phases, Razumas et al. demonstrated that cubic mono-olein–aqueous phases, containing enzymes, could be used as the biocatalytic layer in amperometric and potentiometric biosensors (259). Their results for biosensors, based on a variety of enzymes, show that the long-term stability decreases in the order lactate oxidase > creatinine deiminase > glucose oxidase > urease, (i.e.; is basically in the order of increasing molecular weight). Also, the cubic phases of other amphiphiles like ethoxylated fatty alcohols can be used to entrap glucose oxidase, to construct a simple glucose monitor (60). The bicontinuous cubic structures have, by virtue of their well-defined porosity, also a large potential in drug delivery systems (46). Stable particles of lipid–aqueous cubic phases, cubosomes, can also be produced for this purpose (46,56,75,84–86). Landau and Rosenbusch demonstrated that the bicontinuous phases based on mono-olein and monopalmitolein could provide matrices for the crystallization of membrane proteins like bacteriorhodopsin (267). They pointed out that the use of these types of cubic phase is advantageous as they provide nucleation sites, and the membrane proteins can be dissolved in the lipid bilayer. In addition, they support

growth by allowing lateral diffusion of the protein molecules in the membrane.

4. Lipase Action on Liquid-Crystalline Phases

Another food application, in which cubic lipid phases are believed to play an important role, is in the degradation of lipids. This is also an example of protein–lipid interaction. However, lipases act at such a low concentration that their presence as protein does not significantly affect the global lipid self-assembly structure. It is rather their catalytic activity that has an impact on the lipid self-assembly structure. It is also important to remember that the action of lipases only decreases the time taken to reach the equilibrium and does not affect the equilibrium composition as such. Thus, the changes in structure and composition would have occurred even without the lipase if given enough time. In a simple experiment, Wallin and Arnebrant demonstrated that a cubic phase was much faster decomposed by the action of lipase from *Thermomyces* (former *Humicola*) *lanuginosa* than the reference sample consisting of triolein and the aqueous phase (273). This was attributed to the much larger interfacial area in the cubic phase. In an in vitro study of lipolysis of triglycerides in a intestinallike environment, Patton and Carey observed (37), apart from the initially occurring crystalline phase, a viscous isotropic phase composed of monoglycerides and fatty acids, which is identical to the one formed in monoglyceride systems. In excess of bile salts, the lipolysis products are rapidly solubilized in mixed micelles. However, the bile acid amounts in vivo are not sufficient to solubilize all lipids after a meal rich in fats, which implies that the liquid-crystalline phases exist in vivo (274). Lipase and water must be free to diffuse through the phases formed by the lipolysis products, surrounding the diminishing fat droplet. Thus, the bicontinuity as well as the incorporation properties of the cubic monoglyceride phases are thought to be important features for the lipolysis process (275). Recently, Borné et al., in a series of studies, have investigated the affect of lipase action on the liquid-crystalline phase as well as other self-assemble structures such as vesicles and cubosomes (69,70,276). Some of their findings are summarized in Fig. 17, which shows a schematic representation of the change in structure of the different liquid crystalline phases as a function of time after adding *Thermomyces lanuginose* lipase. The observed changes in self-assembled structures could be predicted from either the mono-olein–oleic acid–aqueous ternary phase diagram, where the lipolysis give rise to a transition of cubic $\rightarrow$ reversed hexagonal $\rightarrow$ micellar cubic $\rightarrow$ reversed micellar phase + dispersion or mono-olein–sodium oleate–aqueous ternary phase diagram, where the corresponding sequence is lamellar $\rightarrow$ normal hexagonal. These difference in

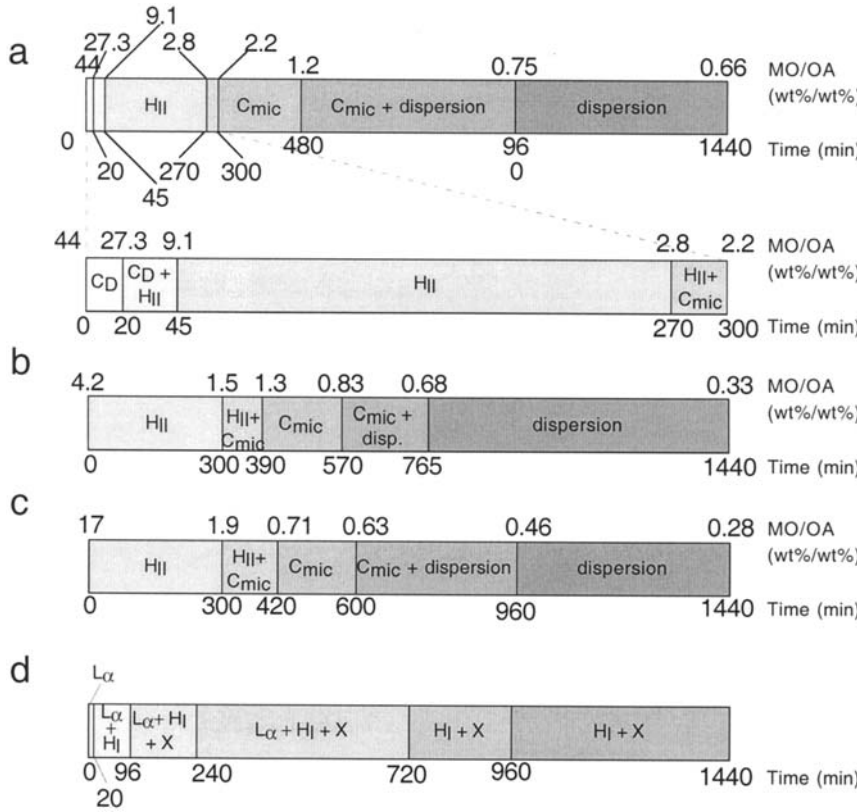

Figure 17 Schematic representation of the change in structure during lipolysis of mono-olein (MO) (or diolein DO) in different liquid-crystalline phases: (a) C_D phase (63 wt% MO, 37 wt% ^2H_2O), (b) oleic acid (OA)–H_{II} phase (65.4 wt% MO, 15.6 wt% OA, 19 wt% ^2H_2O), (c) DO–H_{II} phase (68 wt% MO, 18 wt% DO, 14 wt% ^2H_2O), and (d) L_α phase [10 wt% MO, 5 wt% sodium oleate (NaO), 85 wt% ^2H_2O]. The main liquid-crystalline phases as determined by small-angle X-ray diffraction (SAXD), are indicated in the figure as a diamond type of bicontinuous cubic phase, space group *Pn3m* (C_D), reversed hexagonal phase (H_{II}), normal hexagonal phase (H_I), lamellar phase (L_α), and micellar cubic phase, space group *Fd3m* (C_{mic}). These may exist in excess of water or in the presence of minor amounts of other phases. Some of the observed reflections in the diffractograms, obtained by SAXD, could not be unambiguously assigned to a structure. This unidentified structure is denoted by X. (Adapted from Ref. 70, where details are given.)

reaction sequences could be rationalized in terms of differences in degree of protonation of the fatty acid (70). The initially lamellar phase had a high pH (about 10), (i.e., a low degree of protonation) and thus the degradation as expected follows the mono-olein–sodium oleate–aqueous ternary phase diagram. The initially cubic and hexagonal phase had a low pH (4–7), (i.e., a high degree of protonation) and thus the degradation as expected follows mono-olein –oleic acid–aqueous ternary phase diagram. Adding *Thermomyces lanuginose* lipase to aqueous dispersions of cubic phases (cubosomes) and lamellar dispersions (vesicles) at high water content and gave the corresponding morphological changes as for the liquid-crystalline phases (276). The phase diagrams of the relevant systems can thus be used as maps to navigate through the changes in the self-assembly structure of the substrate and the product. Borné et al found similar specific activity of *Thermomyces lanuginose* lipase on the cubic as on the reversed hexagonal mono-olein-based liquid-crystalline phases, which was somewhat unexpected (70).

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