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DIFFERENTIAL SCANNING CALORIMETRY OF MILK FAT GLOBULE MEMBRANES

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Differential scanning calorimetry was employed as an aid in examining the structure of the bovine milk fat globule membrane. At least six major endotherms are observed between 10 and 90°C, corresponding to order-disorder transitions of discrete structural domains of the membrane. These endothermic transitions occur at 16, 28, 43, 58, 68, and 75°C. The transitions occurring between 10 and 50°C were reversible, suggesting the involvement of lipid. However, the high temperature transitions were irreversible. The calorimetric C transition, centered at 43°C, was shown to involve neutral lipid, since the endotherm was reversible, insensitive to proteolysis, and similar to the endotherm of the isolated neutral lipid fraction of the milk fat globule membrane. The glycolipid and phospholipid fractions of the milk fat globule membrane yielded endotherms outside of the temperature range of the C transition. Another endotherm, the D transition (58°C), was found to involve the denaturation of the major membrane coat protein, butyrophilin (band 12). Evidence for this assignment included the following observations: (i) the nearly selective proteolysis of butyrophilin resulted in the complete removal of the D transition, (ii) the butyrophilin-enriched, Triton X-100-insoluble pellet of milk fat globule membrane yielded a relatively normal D transition, and (iii) the irreversible, disulfide-stabilized aggregation of butyrophilin occurred in the membrane solely at the temperature of the D transition. Furthermore, no other prominent milk fat globule membrane polypeptide formed these non-native disulfide crossbridges during the D transition. The sources of the other major endotherms of the milk fat globule membrane have not yet been assigned.

Introduction

As a model for studies of the nature of plasma membranes and selective membrane budding, the milk fat globule membrane has received increasing interest. Extensive biochemical and morphological evidence implies that the milk fat globule membrane is derived from the apical plasma membrane of mammary epithelial cells (for review, see Refs. 1, 2). Within the cytoplasm of these cells, milk fat droplets form in the endoplasmic reticulum and then migrate to the apical region of the mammary cell. Once in contact with the apical membrane, the lipid droplet is secreted by a process of encapsulation. This process involves the surrounding of the droplet by a portion of the plasma membrane which is then pinched off in the final stages of milk secretion. The milk fat globule is thus enclosed by a membrane with roughly the same composition and sidedness as the apical membrane of the mammary cell [1–6]. By isolating the cream of freshly drawn milk, high yields of this membrane can be obtained for extensive study.

Differential scanning calorimetry (DSC) provides a means for selectively examining the behav-

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Abbreviations: DSC, differential scanning calorimetry: PMSF, phenylmethylsulfonyl fluoride.

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ior of specific proteins and lipid domains within a heterogeneous biological membrane. Once each of the calorimetric endotherms of the membrane scan has been identified with a specific membrane component, considerable structural and functional information can be obtained on that component in situ, i.e. without need for purification [7,8]. Recent calorimetric investigations of the human erythrocyte membrane have provided substantial information on the properties of the cytoskeleton and the major transport protein of that membrane (Refs. 7–10; Davio, S.R. and Low, P.S., unpublished data). Other membranes have also been examined by DSC [11–13], but the studies are not yet as detailed. In the present work, we report the calorimetric profile of the milk fat globule membrane. At least six major endotherms can be detected in the temperature range of 10–85°C, and these presumably correspond to order → disorder transitions of discrete structural domains of the membrane. We present evidence that one of these, the C transition, involves lipid, while another, the D transition, arises from the unfolding of the major protein of the coat which lines the inner face of the membrane, butyrophilin (band 12).

Materials and Methods

Preparation of milk fat globule membrane. Cream was obtained from fresh bovine milk and washed three times with 20 mM sodium phosphate, 125 mM NaCl, pH 7.4. Milk fat globule membranes were then prepared from cold washed cream as described previously [14]. The Triton X-100-insoluble components were isolated from milk fat globule membrane by washing the milk fat globule membrane twice in 10 mM sodium phosphate, pH 7.4, resuspending the membrane in a 1% (v/v) Triton X-100 solution of the same buffer (final protein concentration, 1.85 mg/ml) and incubating the milk fat globule membrane for 30 min at 37°C [15]. The insoluble material was pelleted by centrifugation for 40 min at $45000 \times g$, washed once in 1.0% Triton X-100 and then twice in phosphate-buffered saline, pH 7.4.

Trypsin digestion. Membrane samples (3.7 mg/ml) suspended in phosphate-buffered saline, pH 7.4, were incubated for 1 h at 24°C with 25 μg trypsin/ml (Sigma: 10200 U/mg). The digestion was terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 200 μg/ml. The membranes were then pelleted and washed twice in phosphate-buffered saline, pH 7.4.

Lipid fractionation. Lipids were extracted from milk fat globule membrane with chloroform-methanol and washed to remove non-lipid contaminants [16]. Gangliosides were recovered from the aqueous washes [16]. Total lipid extracts were fractionated into neutral lipid, neutral glycosphingolipid (cerebrosides) and phospholipid fractions on a silicic acid columm [17]. Cerebrosides were eluted from the column with acetone-methanol and were freed of contaminating phospholipids by mild alkaline methanolysis [18]. The various lipid fractions, dissolved in chloroform-methanol, were dried down by evaporating the solvent, and the lipid film was suspended in phosphate-buffered saline, pH 7.4, by vortexing at room temperature.

Differential scanning calorimetry. Heat capacity measurements were obtained on a Microcal 1 differential scanning calorimeter (Amherst, MA) equipped with matched 1 ml platinum cells. Membrane samples equilibrated in the desired buffer were loaded into the sample cell, and an equal volume of the identical buffer system was added to the reference cell of the calorimeter. The heating rate in these experiments was 1 K per min. The membrane concentrations used in the calorimeter were determined by drying the samples to a constant weight at 96°C, and these concentrations were normally about 1% by weight.

Analytical procedures. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to Fairbanks et al. [19] using 5.6% disc gels in a buffer that contained 0.1% SDS. Membrane samples were dissolved in solubilization buffer (0.02 M Tris, 0.002 M EDTA, 2% SDS, 1% 2-mercaptoethanol, pH 8) and clarified when appropriate by centrifugation for 30 min at $45000 \times g$. The Coomassie blue-stained bands on the developed gels were numbered according to Mather and Keenan [20].

Thermal gel analysis was performed essentially as described by Lysko et al. [21], using membrane samples (5.7 mg/ml protein) in phosphate-buffered saline, pH 7.4. The samples were heated in a water bath at a rate of approx. 1 K/min and 200-μl

aliquots were removed at 2 K intervals. The membrane samples were allowed to cool to room temperature and stored at 4°C overnight. Each sample was solubilized (1 : 1, v/v) by addition of the above solubilization buffer without 2-mercaptoethanol. Electrophoresis was performed on the various samples as described above. The Coomassie blue-stained gels were scanned at 550 nm in a Gilford UV-visible spectrophotometer.

Protein concentrations were determined using the procedure of Lowry et al. [22].

Results

DSC scans of two preparations of milk fat globule membranes suspended in phosphate-buffered saline, pH 7.4, along with the corresponding scans of reheated samples, are shown in Fig. 1. In the top scan, six endothermic transitions can be seen between 10 and 90°C, and these are labelled A through F. The temperatures (°C) of these transitions are as follows: A, 16; B, 28; C, 43; D, 58; E, 68; F, 75. The second heating scan of these samples shows partial reversibility of the A transition, with an apparent shift of C into B giving rise to a broad transition centered at 38°C. The D, E and F endotherms are clearly absent from the second scan of these membranes, suggesting their irreversibility. The lower set of scans in Fig. 1 were obtained from a different preparation of milk fat globule membrane. The transitions seen in the initial scan are labeled A′ (13°C), A″ (16°C), B (28°C), C (45°C), D (60°C), E (69°C), and F (76°C), with the primes indicating these transitions may have a different origin than the A transition of the upper scan. The reheat of these membranes exhibited a clearer reversibility of the A′ and A″ transitions not seen in the previous scan (Fig. 1). However, the apparent lowering of the C transition into B, and the irreversibility of D, E, and F are similar to the behavior observed upon reheating the former preparation.

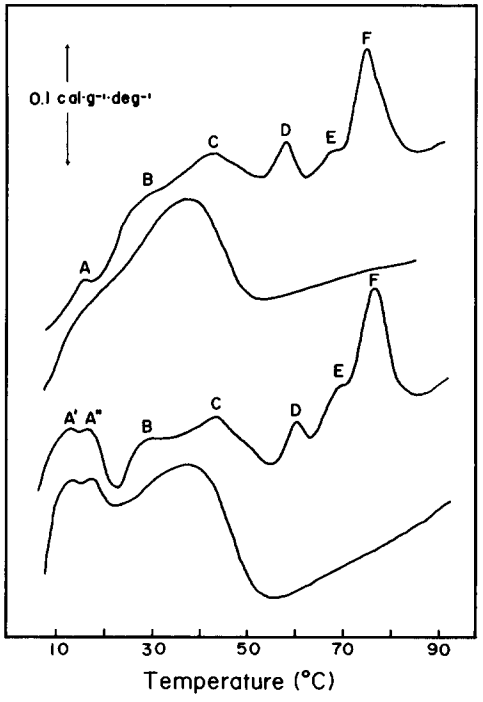

Fig. 1. The heat capacity as a function of temperature of two different preparations of milk fat globule membranes suspended in phosphate-buffered saline, pH 7.4. After each initial scan, the calorimeter was cooled to approx. 5°C and a second heating scan was carried out on the same sample (shown below each initial scan). The heating rate in all cases was approx. 1 K/min.

The variability between milk fat globule membrane preparations illustrated in Fig. 1 was typical of the many preparations we examined during this investigation. Significant differences in the calorimetric profiles at temperatures below 40°C were common among different membrane preparations. However, the temperatures of the D, E and F transitions were more constant from one sample to the next, even though the magnitudes (enthalpies) of these latter transitions were also somewhat variable. We do not know the source of this variability.

A closer look at the reversibility of the endothermic transitions is shown in Fig. 2, along with the corresponding control (scan f). In membranes that were heated to 20°C (scan a), cooled and then reheated, the A transition was totally reversible (scan b). However, after the same membranes had been heated through the A, B, and C transitions (scan b) and then cooled, the A and B transitions were only partially reversible in the subsequent scan, while C appeared uneffected (scan c). Reheating membranes that were previously elevated to 63°C shows the irreversible behavior of the D endotherm (scan d). However, the apparent shift of C into B and the loss of the E and F transitions did not occur until the membranes had been heated

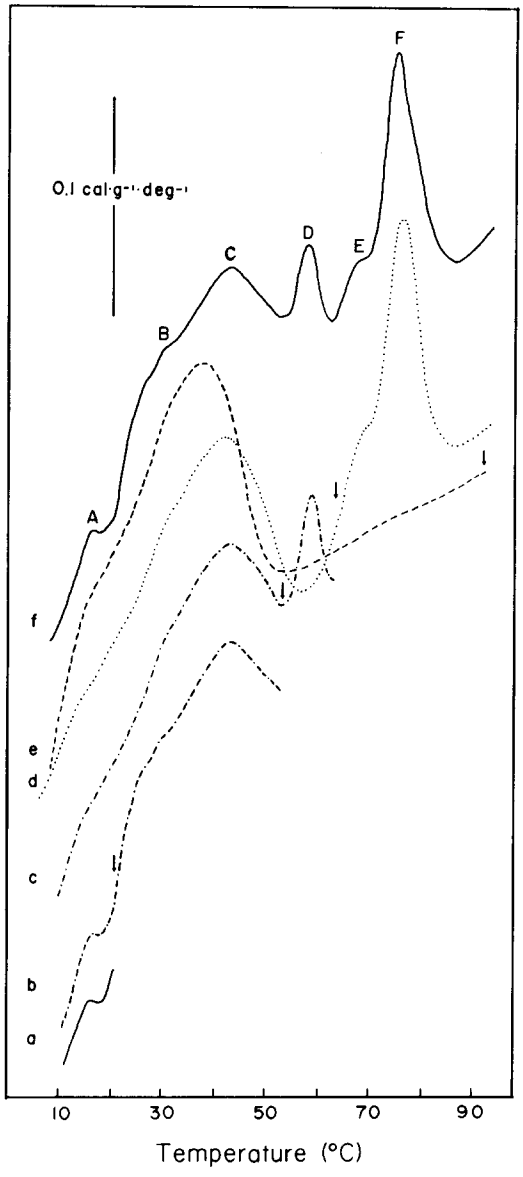

Fig. 2. Analysis of the reversibility of the major calorimetric transitions of the milk fat globule membrane. Membranes were heated to the indicated temperature (↓), cooled to approx. 5°C, and then reheated in the calorimeter to a higher temperature. The arrows (↓) which designate the pretreatment temperature in °C are located as follows: scan b, 20; scan c, 52; scan d, 63; and scan e, 90. Scan a and scan f (control) were conducted on unheated membranes.

to 90°C (scan e). Thus, the D, E and F endotherms were clearly irreversible, while A, B and C were either totally or partially reversible, depending upon the temperature to which the membranes had been previously heated.

To explore the possibility that the reversible transitions might involve lipid, total lipid extracts from milk fat globule membrane were fractionated into neutral lipid (predominantly triacylglycerol [1,23]), phospholipid, cerebroside, and ganglioside fractions, and these fractions were separately subjected to DSC (Fig. 3). The scan of the total lipid extract (scan A) yielded major endotherms at 10, 32 and 36°C. Neutral lipid, which constitutes approx. 60% of the total lipid of milk fat globule membrane [1,23], also gave rise to three transitions centered at 11, 35 and 43°C (scan B). The reheat of this sample had no effect on the 11°C transition, but shifted the latter endotherms down by approx. 10 K along the temperature axis (not shown). The calorimetric scan of the phospholipid fraction (~25% of total milk fat globule mem-

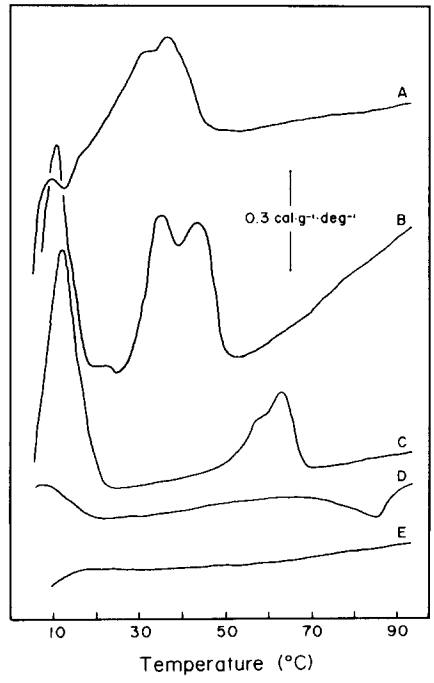

Fig. 3. Calorimetric scans (A–E) of various lipid fractions and the total lipid extract from bovine milk fat globule membrane. Total lipid extract (A), neutral lipid (B), cerebroside (C), phospholipid (D), and ganglioside (E) fractions were prepared as described in Methods. The various lipids, dissolved in chloroform-methanol, were dried to a thin film in a small flask under a stream of N_2. After suspending in phosphate-buffered saline, pH 7.4 by vortexing, the suspensions were scanned in the calorimeter.

brane lipid) yielded a reversible transition at approx. 8°C and an irreversible endotherm that began at approx. 85°C. Neutral glycosphingolipids (0.2% of the total lipid) generated three reversible transitions at 12, 56 and 62°C, while the ganglioside fraction (0.5% of the total lipid) showed no endothermic transition in either the initial or reheated sample. Gangliosides typically display no endotherm between 0 and 100°C [24].

Although the endotherms of membrane-spanning proteins may be resistant to proteolysis (Ref. 25 and Davio, S.R. and Low, P.S., unpublished data), one procedure for identifying the transitions of peripheral membrane proteins is to determine which endotherms are sensitive to proteolytic digestion. Thus, milk fat globule membranes were treated with 25 μg trypsin/ml for 1 h at 24°C in order to remove the readily accessible peripheral membrane proteins. SDS-polyacrylamide gels of the digested membranes (Fig. 4A, gel B) illustrate the nearly selective loss of butyrophilin (band 12), the partial cleavage of band 3 (xanthine oxidase) and the appearance of a new band below band 16. Otherwise, the Coomassie blue banding pattern of the gel appeared relatively unmodified. The DSC scan of the digested membranes displayed a striking loss of the D transition, suggesting some relationship between the endotherm and the cleaved proteins in band 12 or possibly band 3 (Fig. 4B, middle scan). The total insensitivity of the irreversible E and F transitions to the tryptic digestion further suggests that these endotherms may derive from the denaturation of membrane polypeptides which are either resistant to or protected from the proteolysis. Significantly, the major endotherm of the human erythrocyte membrane has been shown to derive from the unfolding of the proteolytically resistant, membrane-spanning domain of the anion transport protein, band 3 (Ref. 25 and Davio, S.R. and Low, P.S., unpublished data).

The effect of trypsin digestion on the A and B transitions need not imply the involvement of protein denaturation in these endotherms. The low temperatures and the reversibility of the transition largely dismiss this interpretation. More likely, the endotherms may involve the melting of lipid phases which are either dependent on proteolytically sensitive proteins for their stability or are perturbed by the peptides released during the tryptic digestion.

The loss of the D transition upon removal of butyrophilin (band 12) implicates the 68 kDa protein in the transition. Because of our interest in this protein, we elected to examine this preliminary assignment in greater detail. Triton X-100 extraction of milk fat globule membrane has been shown to yield a relatively pure coat preparation devoid of most, if not all lipid, and enriched in bands 12 and 3 [15]. To determine if the D transition might be present in these preparations, we conducted the Triton X-100 extraction, washed the pellet to remove the residual detergent, and examined the pellet with calorimetry after resuspension in phosphate-buffered saline, pH 7.4. Fig. 4B (lower scan) confirms that the D transition is indeed retained in the Triton X-100 insoluble material. Surprisingly, the E and F transitions are also present in this scan, demonstrating that these endotherms must also originate in the coat extract. Since this Triton X residue lacks band 10 and essentially all membrane lipid, these components can be dismissed as major participants in the D, E and F transitions. The enrichment of bands 3 and 12 in this suspension further selects these polypeptides as likely sources of the irreversible endotherms (see Fig. 4A, gel C).

A more unambiguous identification of the calorimetric unfolding transition of band 12 can be obtained using a newly developed procedure of Brandts and coworkers [21] termed thermal gel analysis. In thermal gel analysis a suspension of whole membranes is heated slowly from room temperature to approx. 100°C, and at each 2 K interval an aliquot of the suspension is removed and examined by SDS-polyacrylamide gel electrophoresis, as described in Methods. Membrane proteins which have not yet thermally denatured migrate in their usual manner and appear at the expected positions on the developed gel. However, proteins which have thermally denatured form intermolecular disulfide bridges between sulfhydryl groups which are normally inaccessible in the native protein. These disulfide crosslinked aggregates fail to enter the gel in the absence of an exogenous reducing agent. Thus, the temperature of thermal denaturation of each membrane protein can be determined from the pretreatment temperature which renders the protein impermeable to the SDS-polyacrylamide gel.

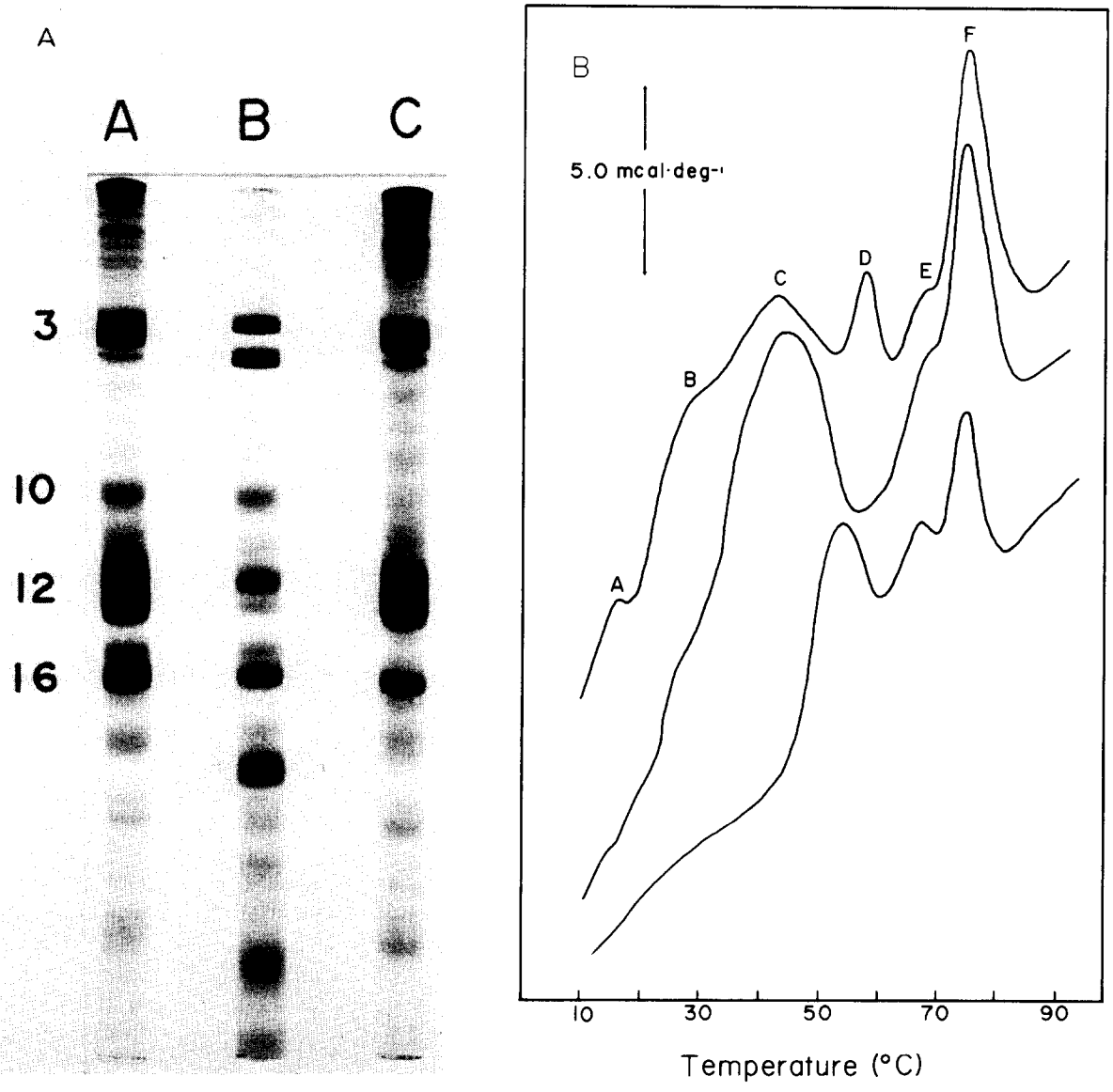

Fig. 4. (A) SDS-polyacrylamide disc gels of the milk fat globule membrane samples used in the calorimetric scans described in Fig. 4B. Freshly prepared milk fat globule membranes (gel A) suspended in phosphate-buffered saline, pH 7.4, were incubated for 1 h at 24°C with 25 μg/ml trypsin. The digestion was terminated by addition of PMSF and the trypsin-treated membranes (gel B) were washed twice in phosphate-buffered saline, pH 7.4. Triton X-100-insoluble material (gel C) was obtained by incubating the milk fat globule membrane in the presence of 1.0% Triton X-100 for 30 min at 37°C. The insoluble material was pelleted by centrifugation and washed twice in isotonic phosphate buffer. The molecular weights of the major milk fat globule membrane protein bands are as follows [20]: 3 (155000); 10 (89000); 12 (68000); 16 (43000). (B) The heat capacity as a function of temperature of the membrane samples described in Fig. 4A. The protein contents of the samples used in the calorimeter were as follows: intact milk fat globule membrane (14.3 mg/ml total protein) (upper scan), trypsin digested (13.6 mg/ml total protein) (middle scan), and Triton X-100 extract (17.5 mg/ml total protein) (lower scan). The buffer in each case was phosphate-buffered saline, pH 7.4.

The gel scans of milk fat globule membrane samples heated to various temperatures in the range of the D transition are shown in Fig. 5A. Significantly, butyrophilin (band 12) is the only

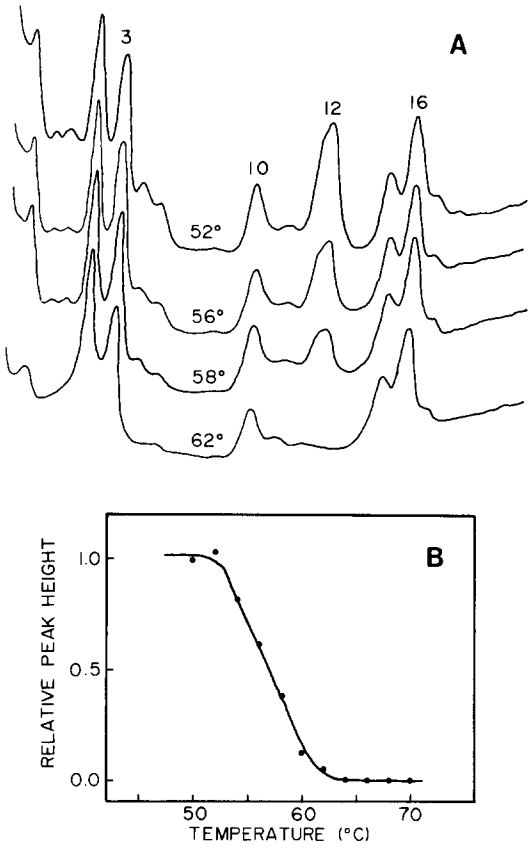

Fig. 5. (A) Thermal gel analysis of the major milk fat globule membrane proteins over the temperature range of the D transition. Membrane samples suspended in phosphate-buffered saline, pH 7.4, were heated in a water bath at a heating rate of approx. 1 K/min and samples were removed for electrophoretic analysis at the temperatures indicated. Polyacrylamide gel electrophoresis in 0.1% SDS was performed on the various samples in the absence of a reducing agent, and the Coomassie blue-stained gels were scanned at 550 nm in a Gilford UV-visible spectrophotometer. The assignment of the major coomassie blue bands is taken from Ref. 20. (B) The normalized peak height of band 12 from the thermal gel scans is plotted as a function of temperature. The temperature of half-maximal disappearance of band 12 is 57°C.

major polypeptide which disappears from the gel (denatures) during this transition. A plot of the measured peak height from the gel scans versus the heating temperature (Fig. 5B) reveals that the midpoint temperature of band 12 denaturation (57°C) is nearly identical to the midpoint temperature of the D transition (58°C). Since bands 3, 10 and 16 do not begin to disappear from the gel scans below pretreatment temperatures of 65°C, these polypeptides can be virtually eliminated as candidates for the D transition. Thus, the irreversibility of D, its sensitivity to mild trypsin digestion, the presence of D in scans of the Triton X-100 pellet, and the thermal gel analysis all suggest that the D transition involves protein unfolding, and specifically the denaturation of butyrophilin (band 12).

Discussion

Differential scanning calorimetry of isolated milk fat globule membranes yields at least three reversible endothermic transitions between 10 and 50°C, and three irreversible transitions in the temperature range of 50–90°C (Fig. 1). The calorimetric C transition (43°C) appears to involve lipid since the transition is reversible and essentially insensitive to proteolysis (Fig. 2, 4B). Lipid fractions separated from the total lipid extracts of milk fat globule membrane were scanned in the calorimeter in an attempt to identify the specific lipid component involved in this transition. The phospholipid, ganglioside and cerebroside fractions yielded no transitions between 25 and 45°C, i.e. the temperature range of C (Fig. 3). Furthermore, cerebrosides and gangliosides represent only 0.2% and 0.5% of the total lipid, respectively [1,23], and thus, would not be expected to contribute significantly to any of the major membrane endotherms. Neutral lipid, which constitutes between 56 and 80% of the total lipid, yielded a broad endotherm within the range of the C transition. Thus, neutral lipid would appear to be the sole candidate for the major lipid species of the C transition. Whether neutral lipid and/or phospholipid (approx. 25% of the total lipid) are responsible for the reversible A (A′-A″) and B transitions cannot be determined from these experiments. However, the fact that multiple reversible transitions are observed is quite peculiar, since to our knowledge no biological membrane has been reported to exhibit more than a single reversible endotherm. If the reversible endotherms of the milk fat globule membrane all derive from lipid phase transitions, then it would seem that certain lipids must segregate into discrete domains in the milk fat globule membrane.

The irreversible D transition (58°C) was shown to involve the protein-unfolding of butyrophilin.

This protein appears to be located exclusively on the apical surface membrane of milk-secreting epithelial cells in a dense coat covering the cytoplasmic aspect of the plasma membrane [26]. It has been speculated that butyrophilin functions as a cytoskeletal protein involved in the recognition, budding and vectorial discharge of milk lipid globules at the cell apex. Together with other coat material butyrophilin forms a dense 10–50 nm thick layer sandwiched between the milk fat globule membrane and the outer shell of the lipid droplet [15,26]. Butyrophilin enriched insoluble coat material can be obtained from milk fat globule membrane by removal of the lipid and loosely associated protein with non-ionic detergents such as Triton X-100 [15]. In agreement with our identification of the D transition, calorimetric analysis of this insoluble fraction showed a relatively normal D transition. Since the non-ionic detergent had little effect on the extracted D transition, it is likely that butyrophilin retained its 'native' configuration even though more than 90% of the initial lipid was removed [15].

With the D transition identified, DSC can be employed to specifically study the effects of various solution conditions on this protein in situ. Preliminary studies demonstrate that ionic strength, pH and Ca^{2+} all exert a significant effect on the D transition. Changing the buffer from isotonic phosphate-buffered saline to 10 mM sodium phosphate greatly reduced the enthalpy of the transition, while readjusting the buffer back to isotonic phosphate-buffered saline reversibly restored D to its normal magnitude. Lowering the pH of the buffer to pH 6 or adding 10 mM $CaCl_2$ substantially elevated the temperature of the D transition. Thus, it may be that band 12 is structurally sensitive to solution conditions which could exist in the cell or in the milk.

Acknowledgements

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