Supramolecular Structure of the Casein Micelle

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ABSTRACT

The supramolecular structure of colloidal casein micelles in milk was investigated by using a sample preparation protocol based on adsorption of proteins onto a poly-L-lysine and parlodion-coated copper grid, staining of proteins and calcium phosphate by uranyl oxalate, instantaneous freezing, and drying under a high vacuum. High-resolution transmission electron microscopy stereo-images were obtained showing the interior structure of casein micelles. On the basis of our interpretation of these images, an interlocked lattice model was developed in which both casein-calcium phosphate aggregates and casein polymer chains act together to maintain casein micelle integrity. The caseins form linear and branched chains (2 to 5 proteins long) interlocked by the casein-stabilized calcium phosphate nanoclusters. This model suggests that stabilization of calcium phosphate nanoclusters by phosphoserine domains of α_{s1} -, α_{s2} -, or β -casein, or their combination, would orient their hydrophobic domains outward, allowing interaction and binding to other casein molecules. Other interactions between the caseins, such as calcium bridging, could also occur and further stabilize the supramolecule. The combination of having an interlocked lattice structure and multiple interactions results in an open, sponge-like colloidal supramolecule that is resistant to spatial changes and disintegration. Hydrophobic interactions between caseins surrounding a calcium phosphate nanocluster would prevent complete dissociation of casein micelles when the calcium phosphate nanoclusters are solubilized. Likewise, calcium bridging and other electrostatic interactions between caseins would prevent dissociation of the casein micelles into casein-calcium phosphate nanocluster aggregates when milk is cooled or urea is added to milk, and hydrophobic interactions are reduced. The appearance of both polymer chains and small aggregate particles during milk synthesis would also be expected based

on this interlocked lattice model of casein micelles, and its supramolecule structure thus exhibits the principles of self-aggregation, interdependence, and diversity observed in nature.

Key words: casein micelle, structure, electron microscopy

INTRODUCTION

Casein micelles are particles of colloidal size that can be described as supramolecules, or a system consisting of multiple molecular entities held together and organized by means of noncovalent intermolecular binding interactions (International Union of Pure and Applied Chemistry, 1997). They serve as the prime nutritional source of calcium, phosphate, and amino acids to meet the growth and energy requirements of mammalian neonates. The term casein micelle was applied to these colloidal supramolecules before it was understood that they did not have a classical micellar structure. They have been under investigation for more than half a century, with various models of their structure proposed and reviewed many times (e.g., McMahon and Brown, 1984; Rollema 1992; de Kruif and Holt, 2003). They consist predominantly of the casein proteins and calcium phosphate, and although they may vary in size, their general structure is thought to be similar across species, yet the question still remains of whether they have a particulate or homogeneous internal structure.

In bovine milk, the caseins consist of 4 major proteins, α_{s1} -casein, α_{s2} -casein, β -casein, and κ -casein, that are secreted in their numerous genetic and posttranslational variations (Farrell et al., 2004). The secondary structure of the caseins has often been referred to as random coil, although this is misleading; a better description is to consider the caseins as being intrinsically unstructured proteins (Farrell et al., 2006) like other secretory calcium-binding proteins (Smith et al., 2004). Their physiological function in the mammary gland results from their different partially folded conformations, and from structural transitions between them. Other terms used to describe the considerable conformational flexibility of the caseins include molten globule structure (Malin et al., 2005) and rheomorphic structure (Holt and Sawyer, 1993). Recently, Farrell et al. (2006) determined that α_{s1} - and α_{s2} -caseins are

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predicted to be natively unfolded proteins with extended coil-like (or premolten globule-like) conformations, whereas β - and κ -caseins would possess molten globule-like properties.

Because of their lack of a rigid 3-dimensional tertiary conformation, caseins can react very rapidly to environmental changes and function in the mammary cells by sequestering small clusters of calcium phosphate, thus preventing precipitation and calcification of the mammary milk synthesis and transport system (Horne, 2002; de Kruif and Holt, 2003). This conformational flexibility further allows them to interact with multiple target molecules, and in that sense, caseins can also be classified as part of the scavenger class of unfolded proteins (Smith et al., 2004).

Casein micelles are highly hydrated and sponge-like colloidal particles. Of the approximately 4 g of water/g of protein contained within the colloidal particle, only approximately 15% is bound to the protein, with the remainder being simply occluded within the particle (de Kruif and Holt, 2003; Farrell et al., 2003). Supramolecule size distribution varies from 20 to 600 nm in diameter, with a median size between 100 and 200 nm (Schmidt et al., 1974; Bloomfield and Mead, 1975; de Kruif, 1998), depending on whether the method used to measure particle size generates a number average diameter or a weight average diameter (Udabage et al., 2003). Using a number distribution yields a median diameter of 1.1×10^2 nm, whereas a weight distribution yields a median diameter of 2.2×10^2 nm.

For many years, the most popularly accepted model for casein micelles was a submicelle model based on clustering of casein molecules into small subunits, which were further clustered to form the secreted colloidal particle (see reviews by McMahon and Brown, 1984, and Rollema, 1992). Other research (including our earlier work) had not been able to detect a particulate internal structure of casein micelles (McMahon and McManus, 1998; Holt et al., 2003; Dalgleish et al., 2004), and a homogeneous network of casein polymers containing nanoclusters of calcium phosphate has become the preferred model (de Kruif and Holt, 2003).

Such a network model, however, does not fully explain the combination of particulate or thread-like structures observed in the transmission electron microscopy (TEM) cross-sectional freeze-fracture replica images reported by Heertje et al. (1985) or, more recently, by Karlsson et al. (2007). Cross-sectional TEM images of rat mammary glands have also shown both fibrillar material (Helminen and Ericsson, 1968) and particulate aggregates (Farrell, 1973) being present during bioassembly of casein micelles in Golgi-associated vacuoles. Evidence for the particulate aggregates was also provided by Hojou et al. (1977), who used

ion beam sputtering and etching to disintegrate casein micelles into star-like particles approximately 7 to 13 nm in diameter, observed by using TEM. In contrast, Marchin et al. (2007) concluded from small-angle x-ray scattering analysis that casein micelles were most likely to consist of a complex network of protein chains, with the only particulate substructure being attributed to calcium phosphate nanoclusters.

On the basis of the combination of possible association characteristics of the caseins, calcium phosphate solubility, and the ability of the phosphoserine residues of α_{s1} -casein, α_{s2} -casein, and β -casein to stabilize calcium phosphate as amorphous nanoclusters, it is probable that the internal structure of casein micelles contains both globular and linear aggregates of proteins.

In the absence of calcium, Thurn et al. (1987) reported that at high ionic strength, α_{s1} -casein can form into polymer-like chains with its hydrophobic regions joined end to end. Malin et al. (2005) found predominantly dimers for all 3 genetic variants of this protein at 37°C and at physiological ionic strengths. β -Casein, on the other hand, forms more spherical particles in the absence of calcium (Swaisgood, 2003). All of the caseins can form some type of self-association structure when in solution, but how they associate in a mixed system containing calcium phosphate is unclear. The noncrystallizing nature of the individual caseins and their aggregates limits the use of techniques such as x-ray crystallography and multidimensional proton nuclear magnetic resonance to study their structure. Electron microscopy has thus been an important tool in deciphering the supramolecular arrangement of the caseins within the casein micelle. The challenge has been how to prepare and view casein micelles so that the resultant electron micrographs exhibit minimal variation of the casein micelle supramolecule from its native form (McMahon and McManus, 1998).

Surface images can be obtained by using scanning electron microscopy without metal coating (Dalgleish et al., 2004), and cross-sections of the internal structure can be seen by using TEM of freeze-fractured cryo-protected casein micelle suspensions (Heertje et al., 1985; Karlsson et al., 2007). Total (surface and internal) images can be obtained by TEM of freeze-dried surface-immobilized casein micelles without resin embedding and sectioning (McMahon and McManus, 1998) and by cryo-TEM of thin vitrified films of casein micelle suspensions (Marchin et al., 2007). Our objective was to use the freeze-dried TEM method to generate stereopair images of casein micelles to further elucidate their supramolecular organization. From this work, casein micelles appeared to exist as a completely interlocked supramolecular structure. We propose a new model structure for casein micelles that includes both protein

chains and protein-calcium phosphate aggregates. Both the polymerization tendencies of the caseins and their calcium phosphate binding ability would be expected to play key roles during casein micelle synthesis and in their observed properties.

MATERIALS AND METHODS

Milk Samples

Raw bovine milk was obtained from the Caine Dairy Research and Teaching Center (Utah State University, Logan, UT) and the fat was skimmed centrifugally. Pasteurized skim bovine milk was obtained from the Gary H. Richardson Dairy Products Laboratory (Utah State University). Samples of human, mare, and pygmy goat milk were also obtained locally for comparison with bovine milk and are so designated; otherwise, milk refers to bovine milk. Calcium-depleted casein micelles were prepared by adding 0.5 g of disodium EDTA and 0.8 g of tetrasodium EDTA per 100 mL of milk. Glutaraldehyde-fixed casein micelles were prepared by adding a 10% glutaraldehyde solution (Electron Microscopy Sciences, Fort Washington, PA) to milk in the ratio of 1:4.

Electron Microscopy

TEM Grid Preparation. Strips of parlodion (nitrocellulose) film (SPI Supplies, West Chester, PA) were dissolved in amyl acetate (Electron Microscopy Sciences) to form a 2% solution. Copper grids (600 mesh, Electron Microscopy Sciences) were coated with parlodion solution and then allowed to air-dry, producing a 40-nm-thick layer of parlodion. The grids were dipped into a 0.01 mg/L solution of poly-L-lysine (Electron Microscopy Sciences) to produce an activated surface with a positive electrostatic charge, then air-dried in a dust-free environment and stored until needed.

Sample Preparation. Milk samples were diluted 1:100 with either distilled water or skim milk ultrafiltrate to reduce casein concentration to about 240 mg/L. Immediately after dilution (<10 s), casein micelles were immobilized onto poly-L-lysine-coated TEM grids based on the cryopreparation method of Nermut (1973) as described by McManus and McMahon (1997).

A poly-L-lysine-coated grid was placed on the surface of the diluted milk for 60 s to allow adsorption of proteins onto the grid. Nonelectrostatic attached material was washed off the grid by placing the grid (sample side down) on top of a drop of double-distilled water for 10 s, then repeating for another 10 s in fresh double-distilled water. The grid was placed on top of a drop of 12 mM solution of uranyl oxalate (50:50 uranyl acetate and oxalic acid) for 60 s and dipped in water to remove

excess stain. The thin layer of water that remained attached to the grid via surface tension and the immobilized casein micelles were then instantaneously frozen by immersing the grid into Freon 22 (Mallinckrodt Inc., Paris, KY) that had been cooled to -159°C with liquid nitrogen. The grid was placed on a 1-kg brass block similarly cooled and placed inside a vacuum chamber that was then evacuated to 10^{-4} mbar, under which conditions the frozen water was sublimated as the sample slowly warmed to room temperature overnight.

TEM Imaging. The freeze-dried samples were viewed with a Zeiss 902 energy-filtered transmission electron microscope (Zeiss Electron Optics, Thornwood, NY) at magnifications of 50,000 $\times$, 85,000 $\times$, and 140,000 $\times$ at 80 kV. Stereoscopic images were obtained by rotating the microscope stage in 8° increments with a goniometer and rephotographing the same casein micelle at the new angle. Images were captured on Kodak Electron Image SO 163 negative film (Ted Pella Inc., Redding, CA) and digitized by scanning the negative images at 500 pixels per inch.

Image Analysis

Pairs of images of individual casein micelles were examined by using stereoscopic glasses (Abrams Instrument Corp., Lansing, MI) to visually observe their 3-dimensional structure. A peripheral section of a stereopair of images of a typical casein micelle was digitally magnified and printed so observations could be made on a section of the supramolecule without too many overlapping planes of network structure, and a single 2-dimensional plane of connected electron-dense locations were identified by using stereoscopic glasses. The digital image was then modified by using Photoshop software (Adobe Systems Inc., San Jose, CA) and pixels not in the plane, or an immediate neighbor to the plane, were converted to white. The remaining electron-dense locations were then color-coded according to how many adjacent neighbors they had so that they could be described in terms of their polymerization functionality.

RESULTS AND DISCUSSION

Electron Microscopy

Casein Micelle Immobilization. On the TEM grids, proteins attach to the poly-L-lysine coating by electrostatic attraction, so proteins that contain negatively charged groups, such as carboxylate or phosphoserine groups, will bind to the poly-L-lysine, and the complete surface of the grid becomes covered with immobilized protein. When samples were prepared with undiluted milk, the grids were crowded with immobilized proteins, making it difficult to obtain quality im-

ages of individual casein micelles. Diluting with water reduced the amount of immobilized protein, and this was not expected to change the supramolecular structure of the casein micelle, given the short time (<10 s) between dilution and immobilization. A comparison was made in which milk was diluted with skim milk ultrafiltrate rather than water, but no noticeable differences in casein micelle structure were observed between samples prepared by the 2 dilution methods (data not shown).

Once the proteins were adsorbed onto the film by electrostatic attraction, further washing of the grid with water removed any secondary layers of proteins that were held onto the film solely by surface tension (data not shown). At low magnification, we observed casein micelles of various sizes as well as noncolloidal proteins attached to the grids, and the fine structure of the casein micelles was observable at high magnification (Figure 1). Such variation in casein micelle size was expected given their known size range of approximately 40 to 600 nm in diameter (de Kruif, 1998).

We consistently observed a halo around the casein micelles, indicating a reduced concentration of noncolloidal protein material bound to the surface of the grid in the area close to each casein micelle. Apparently, immobilization of the relatively large casein micelles physically excludes other proteins from binding to the grid in the immediate vicinity of the casein micelles.

When preparing casein micelles for examination by electron microscopy, it is important to realize that the integrity of these supramolecules depends on a combination of factors, including strong electrostatic linkages of caseins to calcium phosphate as well as protein-protein interactions such as H-bonding, salt bridging (via calcium ions), ion pairing, and hydrophobic interactions (McMahon and Brown, 1984). Furthermore, the casein molecule conformational flexibility that facilitates their rapid and accurate response to environmental change can easily lead to structural rearrangements during the chemical treatments used during sample preparation for electron microscopy. Such structural changes are known to occur during the fixation, alcohol dehydration, and critical point drying steps of scanning electron microscopy sample preparation. This makes interpretations of extracellular structures of biological specimens viewed at very high magnification rather limited because of uncertainties regarding artifact formation resulting from sample preparation (e.g., structural

Figure 1. Transmission electron micrographs of (A) low-magnification field of view of freeze-dried poly-L-lysine immobilized casein micelles on a parlodion-coated copper grid, and (B, C, D) high-magnification view of individual casein micelles of different sizes.

changes), surrounding materials (e.g., metal coatings, surface tension, ice), or image capture (e.g., contrast settings).

Uranyl Oxalate Staining. At high pH, uranyl ions aggregate into colloidal particles, resulting in the dark staining around solid objects that is used for negative staining. Using an equimolar solution of uranyl acetate and oxalic acid provides an acid medium so that the uranyl oxalate acts as a positive stain. On the basis of the behavior of uranyl ions in other biological systems, we assumed that the uranyl oxalate would bind to the calcium phosphate nanoclusters as well as the caseins. Uranyl ions form reversible but stable complexes with phosphoryl and carboxyl ligands on the outer surface of membranes (Rothstein, 1970), can react with phosphate groups in lecithin monolayers (Shah, 1969), can form ionic bonds with phosphate groups in DNA (Zobel and Beer, 1961), and can bind to free amino groups in proteins (Lombardi et al., 1971).

We had expected to observe some changes in protein structure because of the low pH of the uranyl oxalate, but none was observed, and the stained casein micelles had structures similar to those of the unstained casein micelles (Figure 2). The only difference between stained and unstained micelles was a difference in contrast, with the stained sample having higher electron density than the unstained sample. Thus, staining with uranyl ions appeared to help obtain images with better contrast, and therefore better clarity, without changing the casein micelle structure. In such a positively stained TEM image, the components of the casein micelle appear as dark images against a lighter background (see Figure 2B). This difference in contrast and brightness is a result of the difference in electron scattering or electron impermeability of the stained particle compared with the background material. Some of the background surface also appears dark because of noncolloidal proteins being immobilized on the poly-L-lysine linkers.

If the particle being viewed is thicker, has heavy metal atoms attached, or is more dense than the background material, we are able to distinguish the image from the background. This implies that to detect the presence of a particle by using TEM, the combined thickness and electron density of the particle must be greater than that of the support film (e.g., nitrocellulose) to which it is attached. To test whether uranyl ions were binding to both proteins and calcium phosphate, we examined casein micelles that had been calcium depleted as well as calcium phosphate isolated from whey.

In calcium-depleted casein micelles, there was still high contrast (Figure 3A), indicating sufficient uranyl ions were bound to the proteins, giving adequate con-

Figure 2. Transmission electron micrographs of freeze-dried poly-L-lysine immobilized casein micelles (A) without uranyl oxalate impregnation, and (B) showing increased contrast obtained with uranyl oxalate impregnation.

trast with the background. Likewise, a calcium phosphate material from whey appeared as very electron-dense particles when stained with the uranyl oxalate solution (data not shown). In casein micelles it has been suggested that uranium can exchange with calcium (Knoop et al., 1973). There was partial disintegration of the casein micelles upon EDTA treatment, but some

Figure 3. Transmission electron micrograph of freeze-dried poly-L-lysine immobilized casein micelles that have been (A) calcium depleted with EDTA, and (B) fixed with glutaraldehyde.

particles still had the characteristic structure of intact casein micelles. Thus, with the uranyl oxalate staining protocol described above, the proteins and the calcium phosphate nanoclusters both appeared to bind the uranyl oxalate and be positively stained and imaged as electron-dense locations in the TEM micrographs.

Effect of Glutaraldehyde. We also examined calcium-depleted casein micelles that had been fixed in

glutaraldehyde before EDTA was added (Figure 3B). We had presumed that glutaraldehyde would simply crosslink the proteins so that when calcium was removed, the casein micelles would not disintegrate. In one sense this assumption was correct, but we also observed that the structure of the casein micelles changed as the proteins reacted with glutaraldehyde. The glutaraldehyde-fixed casein micelles were much more electron-dense, and we could no longer observe the fine structural arrangement of the proteins. We had made similar observations when examining casein micelle structure by using TEM of thin sections of glutaraldehyde-fixed milk samples entrapped in agar (McMahon and McManus, 1998) in which the samples became more electron-dense than unfixed samples. In addition, the surface of the casein micelle appeared smoother, with the exception of some large protuberances that would correspond with observations made by Dalgleish et al. (2004). It appears that crosslinking of the proteins with glutaraldehyde may change the structure of the casein micelles, or that the glutaraldehyde molecules themselves cause more heavy metal binding in the vicinity of the proteins. In either of these situations, the use of glutaraldehyde when analyzing casein micelles may not be appropriate, at least at the typical 2% level.

For the retention of fine structural detail during sample preparation, a cryo-method of sample preparation that avoids glutaraldehyde fixation is preferred (Wang et al., 2000). Cryo-fracturing and cryo-etching have been used to examine the structure of casein micelles (Heertje et al., 1985; Karlsson et al., 2007), but this still limits the observation to a cross-sectional plane, and some changes in structure can result from the addition of glycerol as a cryoprotectant. The method we used—immersing a grid with an attached monolayer of immobilized casein micelles in liquid N₂-cooled Freon to yield vitrification (freezing with a lack of ice crystal formation) of water surrounding and inside the casein micelles, followed by sublimation to remove the water molecules—allows for observation of the entire casein micelle and appears to retain fine structural detail. Surface images of casein micelles obtained by earlier rotary shadowing techniques (Kalab et al., 1982) or more recent scanning electron microscopy techniques (Dalgleish et al., 2004) are unable to image internal structure and show a dense, space-filling structure rather than the open, porous structure we observed.

Electron-Dense Locations. It is typical, when using TEM to observe a 3-dimensional spherical object such as a casein micelle, that the central region of the image is darker than the periphery. This is not indicative of a change in electron density but represents more scattering opportunities being present when the thick-

Figure 4. Stereo-pair of transmission electron micrograph of a freeze-dried immobilized and uranyl oxalate-stained casein micelle.

ness of the sample traversed by the electron beam is greater. When viewed by using stereo-pairs of images (Figure 4), this dimensional compression artifact was eliminated and the supramolecular structure was observed to be uniform throughout the casein micelle. Another advantage of using stereo-pairs is that because multiple images of the same casein micelle are obtained at different angles, only objects recorded at both angles will converge, giving greater confidence that what is being observed in the micrograph represents a real electron-dense entity and is not an artifact related to imaging at a very high magnification.

It was possible, by viewing stereo-pair images of casein micelles (Figure 4) or anaglyphic images by using red-blue 3-dimensional glasses (Figure 5A), to visualize

the electron-dense locations (i.e., the dark pixels) as individual entities ranging in size from 1×1.5 to 4×4 nm. This is close to the size range of 4.8 nm in diameter calculated for calcium phosphate nanoclusters by Holt et al. (1998) but is less than the Stokes radius of approximately 4 nm calculated for casein monomers (Swaisgood, 2003).

The tertiary structure of the caseins is such that some portions of the protein molecules would be compact (and therefore electron-dense) and other portions would be more open (and therefore less electron-dense). Those regions of the proteins that carry negative charges would also bind the uranyl ion more, so hydrophobic regions devoid of negatively charged side groups would be less heavily stained. Thus, it needs to be recognized

Figure 5. Anaglyphic version of transmission electron micrographs of (A) a freeze-dried immobilized and uranyl oxalate-stained casein micelle, and (B) a digitally magnified peripheral region of a casein micelle.

that only portions of the casein molecules were being imaged. In addition, as shown by McMahon and McManus (1998), conditions under which the image is recorded and printed influence the observed size of objects. As contrast is increased, the observed size of the electron-dense locations decreases. We used minimal contrast even though this produces images with less difference in grayscale between electron-dense locations and the background. Even with these limitations, when viewing stereo-pair TEM images, the predominant appearance of casein micelles was that of electron-dense locations present as interlocked chains. Open spaces between the electron-dense locations would represent regions devoid of matter (i.e., occupied by serum in the native casein micelle) as well as regions of the proteins that are unstained or that have a more open conformation.

Because portions of the individual proteins are not imaged, our TEM images do seem to underestimate the volume being occupied by the proteins. A model structure for casein micelles needs to reflect the expected sizes of the proteins, and even so, it was apparent that casein micelles still have the open, porous structure expected for a colloidal particle with a high voluminosity, in which proteins account for only approximately 20% of the supramolecule volume (Farrell et al., 2003). The freeze-etched replica images of Heertje et al. (1985) and the freeze-fractured replica images of Karlsson et al. (2007) also showed such an open structure. Cryo-TEM images of casein micelles without replica formation have also been published (Marchin et al., 2007), but the same structural patterns were present in the vitrified ice portion of their images as well as in the casein micelles.

Casein Micelle Structure

Structural Arrangements. When viewing an entire casein micelle, the large number of individual components being visualized (approximately 10^4 , depending on the casein micelle size) makes it difficult to view the central region of the casein micelle. There are too many overlapping planes of electron-dense locations to isolate individual planes visually. Even so, it was evident that there were no major differences among structural arrangements between the central portions of casein micelles (Figure 5A) and their peripheral regions (Figure 5B). This allowed us to determine structural arrangements in peripheral regions where individual planes of components could be examined and use this information to make general conclusions about the overall supramolecular structure of the casein micelles.

A region on the periphery of a casein micelle was selected for close examination of electron-dense entities

(Figure 6A) and a digitally magnified image of this region was then visually examined stereoscopically. At this stage of our investigation, we had not yet assigned a presumed identity to the electron-dense entities as protein or calcium phosphate nanoclusters, so they were simply color-coded according to their functionality (f) as shown by their number of near neighbors (Figure 6B). That is, were they adjacent to 1, 2, 3, or 4 or more other dark (i.e., electron-dense) spots? Electron-dense spots that were observed to be not in the same plane were erased from the digital image. As shown in Figure 6B, chain-terminating entities ($f = 1$) were colored red, entities associated with 2 other particles ($f = 2$) were colored green, those with $f = 3$ were colored blue, and those with $f \geq 4$ were colored black.

It became apparent after making these assignments that the plane of electron-dense locations consisted of short linear and branched polymer chains that were interlocked together on a regular basis. At these interlocking sites, there was a grouping of electron-dense locations with many near neighbors and the polymer chains appeared to radiate outward until they encountered another interlocking site. This gave the appearance of the electron-dense material forming into a lattice-type structure consisting of interlocked orbs with chains of material that encompassed areas devoid of any electron-dense material. Once this skeletal structure of the casein micelle supramolecule had been determined, it was then necessary to make assignments to the various locations based on the known size and functionalities of the components that make up the casein micelle.

Casein Polymerization. The polymerization (aggregation) behavior of the various casein molecules has been described based on chemical structure. Their functionality depends on the possibility of calcium-mediated interactions via clusters of phosphoserine groups (Dalglish and Parker, 1979), interactions via their hydrophobic regions, interactions with water via their hydrophilic regions (Yoshikawa et al., 1981), as well as hydrogen bonding and the various electrostatic interactions (such as calcium bridging between negatively charged sites and ion pairing) that are common to all proteins. Because of the varied ways in which the caseins can interact—with themselves (homopolymers and aggregates), with each other (heteropolymers and aggregates), and with minerals (e.g., ionic Ca^{++} and calcium phosphate nanoclusters)—there is a range of functionalities over which they can interact, depending on their surrounding environment.

Dalglish and Parker (1979) assigned an average $f = 2$ for the calcium-induced aggregation of α_{s1} -casein based on 4 to 10 calcium ions being bound per α_{s1} -casein molecule. Horne et al. (1988) assigned chain-

terminating ($f = 1$), bifunctional ($f = 2$), and trifunctional ($f = 3$) roles to κ -, β -, and α_s -caseins, respectively, although it may be more appropriate to assign α_{s1} -casein as $f = 2$, and α_{s2} -casein as $f = 3$. Within casein micelles, there are opportunities for both specific phosphoserine-mediated interactions with calcium phosphate nanoclusters, and hydrophobic and ionic interactions with other proteins. These interactions can also be viewed as the caseins being block copolymers consisting of blocks with high levels of hydrophobic or high levels of hydrophilic amino acid residues (Horne, 2002; Euston and Horne, 2005).

β -Casein can act as a duo-block polymer that can interact with other proteins via calcium bridging because of its cluster of phosphoserine residues as well through its hydrophobic region. α_{s1} -Casein could interact as a triblock polymer through predominantly hydrophobic regions at its C- and N-terminal ends, as well as through a hydrophilic-rich region that contains its phosphoserine clusters. α_{s2} -Casein can be considered a hybrid of α_{s1} -casein and β -casein and it can interact through 2 sets of hydrophobic-rich and hydrophilic-rich domains. Its N-terminal is a hydrophilic domain with anionic clusters, followed by a hydrophobic domain, then another hydrophilic domain with anionic clusters, and finally a C-terminal positively charged hydrophobic domain (Farrell et al., 2004). κ -Casein would act primarily as a monoblock unit because it lacks a cluster of phosphoserine residues and the glycosylation of its hydrophilic C-terminal prevents any strong electrostatic interactions with other proteins except via its N-terminal hydrophobic region. It is known to be present in milk as disulfide-linked oligomers with other κ -casein molecules (Rasmussen et al., 1999), and either alone or as an aggregate, it can be considered as a polymerization terminator.

The observed redundancy in functionality of caseins in bovine milk suggests that the range of functionalities is more important than which casein actually performs a particular function. In addition, a molecule such as α_{s2} -casein, with its 4 potential interaction regions, may

Figure 6. (A) Transmission electron micrograph of a freeze-dried immobilized and uranyl oxalate-stained casein micelle: (box) a peripheral portion of a casein micelle was digitally magnified as a stereopair and a single plane of electron-dense spots was identified and then (B) color-coded based on the number of immediately adjacent spots (red = 1 neighbor, green = 2 neighbors, blue = 3 neighbors, black = 4 or more neighbors), with those not in the same plane deleted from the digital image; then on the same image (C), the intersecting locations were overlaid with a dark gray circle of 4.8 nm in diameter representing a calcium phosphate nanocluster, and (D) the remainder of the electron-dense spots were overlaid with a light gray circle of 8 nm in diameter representing casein molecules.

not always associate with the other components to its maximum functionality because of steric hindrance between its binding partners.

Calcium Phosphate Nanoclusters. The most likely candidate for the grouping of electron-dense locations observed in Figure 6B, which appear to form interlocking sites in the supramolecular structure of the casein micelles, is the calcium phosphate nanoclusters. Holt et al. (1996) proposed a functionality ≥ 4 for calcium phosphate nanoclusters because of their ability to simultaneously bind multiple phosphoproteins (i.e., α_{s1} -, α_{s2} -, and β -casein). In Figure 6C, the calcium phosphate nanoclusters are depicted as dark gray spheres of 4.8 nm in diameter. The rapid binding of 4 to 5 caseins to the calcium phosphate nanoclusters would then act as the structure-forming points during casein micelle synthesis as proposed by Holt et al. (2003). This would produce a casein-calcium phosphate aggregate in the size range of 7 to 13 nm observed by Hojou et al. (1977).

With the phosphoserine cluster domains of the proteins (α_{s1} -, α_{s2} -, or β -casein) being oriented toward, and bound to, the calcium phosphate nanocluster, it is likely that hydrophobic regions would be oriented toward neighboring proteins. Such lateral binding of caseins would be analogous to the protein orientation and monolayer in situ polymerization that occurs when proteins are adsorbed onto an oil-water interface (Dickinson and Matsumura, 1991). Thus, in addition to the caseins being bound via their phosphoserines to the calcium phosphate nanocluster, they would be linked together through hydrophobic interactions and other electrostatic interactions. Then, if the calcium phosphate nanocluster was dissolved, much of the surrounding protein organization would stay intact unless the hydrophobic and electrostatic interactions were also disrupted.

Supramolecule Structure. Because it is not possible to differentiate among proteins based on TEM images alone, the remainder of the electron-dense locations in the micrographs were simply assigned as being protein with an average diameter of 8 nm, shown as light gray spheres in Figure 6D. These casein molecules can bind to the casein-calcium phosphate aggregates as either monomers, oligomers, or even aggregates bound to a different calcium phosphate nanocluster. Interactions can occur via hydrophobic regions or calcium bridging through their carboxylate or phosphoserine side chains, which are not part of the phosphoserine clusters bound to the calcium phosphate nanoclusters (Swaisgood, 2003). In the volume assigned to each protein molecule, multiple electron-dense locations were often observed, so this assignment is based on what we considered the most likely arrangement, and other interpretations of our TEM images are also possible.

One should also realize that the conformational shape of the proteins is not spherical and would, to some extent, depend on interactions with their neighbors. Even though some of the original assignments of functionality of the electron-dense locations were lost, one can see that the proteins have a similar range of functionalities with linear and branched chains.

In examining TEM images of casein micelles, we observed various structural arrangements, including long linear chains and even double-stranded chains. Short branches on chains were common throughout the entire casein micelle. This was expected because κ -casein, which acts as a chain terminator, is known to be present throughout the entire casein micelle and not just on its surface. Overall, it is still a very open structure that, although allowing considerable variation within the casein micelle, is self-reinforcing through the interlocking of protein strands at the calcium phosphate nanoclusters. The occluded spaces within the supramolecule matrix structure would be occupied by the serum phase of milk comprising water along with dissolved lactose, ions, and other soluble substances.

Interlocking Lattice Model. On the basis of our observations and the assumptions described above, a cross-sectional schematic of the casein micelle supramolecule as an interlocked lattice is presented in Figure 7. Proteins are represented as spheres of 8 nm in diameter that both surround the calcium phosphate nanoclusters (represented as spheres of 4.8 nm in diameter) and extend as short chains between the interlocking points and out from the supramolecule periphery.

The supramolecular structure is irregular and allows for a large diversity of linkages among the proteins, including chain extenders (β -casein or α_{s1} -casein), chain branch points (α_{s1} -casein or α_{s2} -casein), chain terminators (κ -casein), and interlocking points (calcium phosphate nanoclusters). On the periphery of the casein micelle, some chains of proteins can extend outward, placing κ -casein (either individually or as disulfide-linked polymers) well out from the bulk of the casein micelle. The number of these protuberances on the casein micelle surface is less than postulated in early depictions of the casein micelle surface as a hairy layer (Horne, 1984) or polymeric brush (de Kruif and Holt, 2003). We observed them to be up to approximately 30 nm in length, which is similar in length to that observed by Dalgleish et al. (2004) by scanning electron microscopy. Some of them may be as an individual chain and thus be thinner than the protuberances observed by scanning electron microscopy, whereas others would be of similar diameter and result from termination of a loop of proteins (as shown for a single 2-dimensional plane in Figure 6D) or, when considered in 3 dimen-

Figure 7. Schematic diagram of an interlocking lattice model of the casein micelle with casein-calcium phosphate aggregates throughout the entire supramolecule and chains of proteins extending between them. Drawn as cross-sectional scaled views of (A) the complete supramolecule, and (B) a portion of the supramolecule periphery. Calcium phosphate nanoclusters are shown with a diameter of 4.8 nm and approximately 18 nm apart, and caseins are shown with a hydrodynamic diameter of 8 nm.

sions, the chains of proteins would form an orb on the supramolecule periphery.

Overall, this supramolecular structure would produce a very stable colloidal particle constituting thousands of protein molecules (or tens of thousands, depending on the casein micelle diameter) and hundreds of calcium phosphate nanoclusters. The distance between interlocking sites appeared similar to the 18-nm interval predicted by de Kruif and Holt (2003) for calcium phosphate nanoclusters. Some were further apart, whereas others were closer together such that there could be from 2 to 6 protein molecules between the interlocking sites (Figure 6). According to Smith et al. (2004), calcium phosphate accounts for approximately 7% of the dry mass of casein micelles, and casein micelles of 200 nm in diameter and 10^6 kDa contain approximately 800 calcium phosphate nanoclusters. This equates to a ratio of approximately 60 protein molecules per calcium phosphate nanocluster, which is more than we observed in our electron micrographs if it was assumed that each interlocking point in the lattice structure was a calcium phosphate nanocluster. Some of the interlocking points may also result from branches in the protein chain, possibly by α_{s2} -casein.

In bovine milk, there is some redundancy in the set of casein proteins produced, as shown in the overlap in functionality of α_{s1} -casein with both β -casein and α_{s2} -casein. In other mammalian species, it is not essential to have all of these proteins or to produce them in the same ratio. For example, in caprine milk, α_{s1} -casein F (which lacks a phosphoserine cluster) is thought to act as a chain terminator and have an apparent surface location in the casein micelle supramolecule (Tziboula and Horne, 1999), like κ -casein does in bovine milk. Provided the proteins that are synthesized by the mammary glands can fulfill the necessary functions, a stable colloidal supramolecule for transporting calcium phosphate to the neonate will be produced. We observed similar structures in casein micelles from human, mare, and pygmy goat milk (Figure 8) as were seen in the bovine casein micelles and as has been reported previously (Rollema, 1992). This makes the casein micelle supramolecule a prime example of the self-aggregation, interdependency, and diversity that Swimme and Berry (1992) proposed as essential parts of natural systems.

CONCLUSIONS

An interlocking lattice model of the casein micelle supramolecule is proposed from our interpretation of high-resolution TEM micrographs of freeze-dried, immobilized, uranyl oxalate-stained casein micelles. Calcium phosphate nanoclusters play an integral role in casein micelle synthesis and in maintaining supramole-

Figure 8. Transmission electron micrographs of freeze-dried immobilized and uranyl oxalate-stained casein micelles from (A) human milk, (B) mare milk, and (C) pygmy goat milk.

cule integrity and were presumed to be located at the interlocking sites as calcium phosphate nanoclusters stabilized by phosphoserine-containing casein molecules. These calcium phosphate-casein aggregates serve as structure-forming sites to which other caseins can be bound, forming short chains between them. Hydrophobic interactions as well as calcium bridging are thought to be the predominant interactions between the caseins; they are involved in chain formation and occur between caseins bound to the calcium phosphate nanoclusters. Hydrogen bonding and other electrostatic and entropic interactions common to proteins probably also play a role in maintaining supramolecule integrity. This multiplicity of binding interactions and the interlocking structure give casein micelles a level of elasticity and make them resistant to complete dissociation if only a single interaction is eliminated. The interlocking of the structure also implies that chemical changes to milk, such as by adding alcohol, will result in global changes in the supramolecule and not just influence surface casein molecules.

The model allows for a predominance of κ -casein on the supramolecule periphery as terminal molecules (or disulfide-linked polymers) with protuberances that can extend into the surrounding environment. Apart from a degree of periodicity provided by the interlocking sites throughout the supramolecule, the lattice structure is irregular in nature and supports an open structure of the casein micelle. Different caseins can perform a variety of functions during casein micelle synthesis, such as attaching to calcium phosphate nanoclusters preventing calcification in the mammary gland, forming linear or branched chains, and terminating chain growth, and similar supramolecule structures were observed across species.

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