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Co-adsorption of β-casein and calcium phosphate nanoclusters (CPN) at hydrophilic and hydrophobic solid–solution interfaces studied by neutron reflectometry

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ABSTRACT

Neutron reflectometry was used to study the co-adsorption of calcium phosphate nanoclusters (CPN) and β-casein at hydrophobized and hydrophilic silica–water interfaces. The structural characteristics of the adsorbed layer were determined from neutron reflectivity curves analysed with multi-layer optical models. We used a highly specific proteolytic enzyme, endoproteinase Asp-N in conjunction with a single neutron contrast to verify the model of the protein layer structure. The results showed that the calcium phosphate nanoclusters profoundly affected the rate of adsorption and structure of the interface compared to the adsorption of β-casein alone and for the hydrophobic interface the effects depended on the point at which the nanoclusters were added. It is proposed that the nanoclusters become surface active because whole β-casein molecules can replace one or more of the hydrophilic peptides in the shell of the nanoclusters.

1. Introduction

β-casein (together with α_{S1}-casein) is the most abundant protein among the four caseins present in cow's milk (Dickinson, 1999). Above 4 °C it readily forms surfactant micelle-like aggregates in aqueous solution starting at a concentration ~0.5 mg/ml (Leclerc & Calmettes, 1997; Payens & Vreeman, 1982). The biological role of casein molecules includes the sequestration of amorphous calcium phosphate to form stable complexes in milk (Holt, 1998; C. Holt & Sawyer, 1988; Holt, Wahlgren, & Drakenberg, 1996; Walstra & Jenness, 1984). Caseins are also frequently used as additives in food, paint, glue and coating colours for paper (Walstra & Jennes, 1984). Knowledge of the mechanisms by which caseins adsorb is therefore of great interest in many colloid-related industries.

The average hydrophobicity of the amino acid residues in β-casein is less than that of a typical globular protein and more than that of a typical natively unfolded protein. The N-terminal 21 residues contain most of the charged residues and sites of phosphorylation whereas the remainder is mostly uncharged and contains a few short hydrophobic sequences. To a degree, this gives the protein an amphiphilic character with a hydrophilic N-terminal head and a so-called "hydrophobic" tail, though it is only relatively hydrophobic compared to the head.

Studies of β-casein adsorption have mainly been performed at hydrophobic surfaces by a number of experimental techniques such as ellipsometry, surface force measurements, dynamic light scattering, neutron reflectivity, and wetting force measurements (Atkinson, Dickinson, Horne, & Richardson, 1995; Brooksbank, Davidson, Horne, & Leaver, 1993; Dalgleish & Leaver, 1991; Dickinson, Horne, Phipps, & Richardson, 1993; Fragneto, Thomas, Rennie, & Penfold, 1995; Kull, Nylander, Tiberg, & Wahlgren, 1997; Leaver & Dagleish, 1992; Mackie, Mingins, & North, 1991; Nylander & Tiberg, 1999; Nylander & Wahlgren, 1994, 1997). The common model emerging from these studies is that the protein forms a monolayer at hydrophobic surfaces, with the "hydrophobic" part of the protein sticking to the surface and the highly charged N-terminal portion protruding into the solution. The main driving force for adsorption at hydrophobic surfaces is entropic due to the de-solvation of adsorbed sequences. This model of adsorption at hydrophobic surfaces was also experimentally verified by sensibly studying the effects of a specific proteolytic enzyme (endoproteinase Asp-N) on the interfacial β-casein

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layer (Kull et al., 1997; Nylander & Wahlgren, 1994). Endoproteinase Asp-N can potentially cleave the β-casein molecule at four different sites where two are located close to the hydrophilic region (residues 43 and 47) and two in the "hydrophobic" region (residues 129 and 184) (Drapeau, 1980; Wahlgren, 1992). The results obtained at hydrophobic surfaces indicated that the enzyme could cleave the sites in the hydrophilic part of the protein and the rest of the adsorbed protein layer seemed to remain intact. This confirms that the protein adopts a brush-like structure when adsorbed onto hydrophobic surfaces with its hydrophilic sequence exposed to the solution.

Numerous studies indicate that the caseins interact with amorphous calcium phosphate primarily through short sequences containing three or more phosphorylated residues known as casein phosphate centres (Holt, Davies, & Law, 1986; Holt et al., 1989; Ono, Ohotawa, & Takagi, 1994). Indeed, short, phosphate centre-containing peptides can sequester amorphous calcium phosphate to form core-shell nanoparticles with the core comprising the calcium phosphate and the shell composed of 50 or so peptides (Holt, Timmins, Errington, & Leaver, 1998; Holt et al., 1996; Little & Holt, 2004). The particles are called calcium phosphate nanoclusters (CPN) and the best characterised of these is the one formed by the N-terminal tryptic peptide of β-casein, residues 1–25. This nanocluster is used here. Calcium phosphate nanoclusters are also formed or predicted to form by a number of recombinant phosphopeptides, osteopontin phosphopeptides and at least 11 other non-casein proteins (Clegg & Holt, 2009; Holt, Sørensen, & Clegg, 2009).

In spite of the importance of β-casein as a stabiliser of dispersions of amorphous calcium phosphate in milk and hydrophilic colloids in general, relatively few studies of the adsorbed layer properties at hydrophilic surfaces have been conducted.

Like ellipsometry, neutron reflection also allows the measurement of the refractive index profile at an interface, but in the form of the scattering length density (Holt, 1998; Lu & Thomas, 2000; Nylander et al., 2008; Penfold et al., 1997). The main benefit here is from the difference of scattering lengths between hydrogen and deuterium, which allows for contrast variation/optimisation by substituting H_2O with D_2O. Hence, the sensitivity of neutron reflection to the layer structure depends on the scattering length density of the components of the layer and the surrounding bulk media. The possibility of measuring at different contrasts allows for the application of more suitable multilayer models to interpret the adsorption data with a good degree of confidence. The inhomogeneity of the protein layer in the normal direction can be revealed by applying such models. Here we also show that one can use a highly specific proteolytic enzyme in conjunction with a single neutron contrast to explore the structure of a protein layer in more detail than with conventional contrast variation alone (Nylander, Tiberg, Su, Lu, & Thomas, 2001).

2. Materials and methods

The β-casein (genetic variant A^1, M_w = 24000 g/mole) was extracted from bovine milk and purified according to the procedure described by Nylander and Wahlgren (Nylander & Wahlgren, 1994). Endoproteinase Asp-N was purchased from Boehringer Mannheim Biochemica (now Roche Diagnostics) (Cat. 1054589, Lot 14184025). The water used was purified by a Milli-Q system from Millipore Corporation, Bedford, MA giving water with resistivity of 18 MΩcm and low bubble persistence. Deuterated water (99.9%, deuterated) was obtained from Fluorochem. All other chemicals used were of analytical grade. β-Casein (0.1 mg/ml) or endoproteinase Asp-N (0.04 μg/ml) were dissolved in 50 ml 0.02 M imidazole–HCl buffer (pH 7.0) containing 17 mM $CaCl_2$. All aqueous H_2O and D_2O solutions featured in this study were buffered similarly. Fresh solutions were always prepared immediately before the adsorption measurement was started. The calcium phosphate nanoclusters were prepared with β-casein 4P (f1-25) at a concentration of 5 mg/ml in either H_2O or D_2O by the method of Holt et al. (Holt et al., 1998). They are spherical in shape with an average core radius of 24 Å and a shell outer radius of 40 Å. Calculation of the equilibria in the nanocluster solution showed that 90% of the peptide was sequestering the calcium phosphate at pH 6.7 and 100% at pH 7.0 (Little & Holt, 2004). Samples were diluted to the required concentration and the adsorption surface washed with a calcium and phosphate-containing dilution buffer saturated in calcium phosphate so that the nanoclusters did not dissociate. This is important as dilution with water or calcium free buffer can destabilise the system.

The neutron reflection measurements were made on the 'white beam' time of flight reflectometer D17 at the Institut Laue-Langevin, Grenoble, France (Cubitt & Fragneto, 2002). Neutron wavelengths from 2.2 to 19 Å were used in these experiments. The sample cell consisted of a Teflon trough clamped against a silicon block of dimensions 12.50 × 5.08 × 2.54 cm (Fragneto, Lu, McDermott, & Thomas, 1996). The collimated beam enters the end of the silicon block at a fixed angle, is then reflected at a glancing angle from the solid–liquid interface, and exits from the opposite end of the silicon block. Each reflectivity profile was measured at two different glancing angles of 0.8° and 4°, respectively. The intensity of the reflected beam over the intensity of the incoming beam (reflectivity) as a function of the momentum (wave vector) transfer $Q = 4\pi/\lambda (\sin\theta)$, where λ is the wavelength and θ is the glancing angle of incidence, was determined and the results for the two angles were then combined. The background recorded simultaneously with the reflectivity measurements by the 2-dimensional ^{3}He gas detector to the side of the specular ridge was subtracted from the recorded data. The background for the D_2O runs was typically 2×10^{-6} given in terms of reflectivity (see next section).

The procedure for polishing the large face (111) of the silicon blocks has been described earlier (Fragneto et al., 1996). The large face was polished using an Engis polishing machine, on a mat using a 1-micron silica suspension followed by colloidal alumina. The blocks were then cleaned in a three-step process. They were first rinsed in water purified by a Milli-Q system from Millipore Corporation, Bedford, MA (water resistivity of 18 $M\Omega cm^{-1}$). Then, they were soaked in a solution consisting of water, sulphuric acid (98%) and hydrogen (27.5% peroxide solution in water) mixed in a volume ratio of 5:4:1 respectively at 80 °C for 40 min. The blocks were then removed from the cleaning solution and allowed to cool for a few minutes before being quenched by immersion in Milli-Q water. Finally, they were placed in a stream of oxygen and exposed to ultraviolet light for 30 min. This procedure rendered the surfaces hydrophilic and highly reproducible with respect both to the silicon oxide layer thickness and the β–casein adsorption. Before the adsorption measurements were started, the oxide layer at the surface of the silicon block was characterised in terms of structural parameters. The characterisation was done in different isotopic compositions of water in order to reveal features of the bare substrate such as roughness and oxide layer thickness. The water contrasts used were pure D_2O (scattering length density of 6.35×10^{-6} Å^{-2}), water CMSi (H_2O/D_2O weight ratio of 0.595/0.405 and scattering length density of 2.07×10^{-6} Å^{-2}), and finally pure H_2O (scattering length density of -0.56×10^{-6} Å^{-2}). Two batches of silicon blocks were used. The combined fitting gave an oxide layer thickness of 14 ± 2 Å for the batch used for silanisation 9 ± 1 Å for the other. The scattering length density for both blocks was 3.4×10^{-6} Å^{-2}. This scattering length density is the same value as that expected for amorphous silica, suggesting little penetration of water into the layer and hence a surface free of defects. The surface roughness was calculated to be ≤2–3 Å as the fit of the model could not be improved by using roughness values of 0 or 2–3 Å.