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The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution

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ABSTRACT

The mobility of phosphate groups in aqueous solutions of casein micelles and sodium caseinate from bovine milk was determined with magic angle spinning (MAS) ^{31}P NMR as a function of the pH between pH 4 and pH 8. The chemical shifts and the relative amplitudes of the signals from mobile inorganic phosphate (orthophosphate) and mobile organic phosphate (phosphorylated serines) as well as that of immobile phosphate (colloidal calcium phosphate, and immobile phosphorylated serines) were determined. Sodium caseinate contained very little orthophosphate and all phosphates were mobile over the whole pH range. In micellar casein solutions most of the phosphate was immobile at pH > 6.0, but the fraction of mobile organic and inorganic phosphate increased sharply between pH 5.5 and pH 4.5, showing the disintegration of the CCP nanoclusters. Protonation of the phosphates with decreasing pH was determined from the chemical shift and was related to their mobility. The signal of mobile organic phosphate was different for micellar casein solutions and sodium caseinate demonstrating the influence of calcium phosphate in the former. The microscopic structure of protein solutions was investigated with confocal laser scanning microscopy. Large protein clusters were observed below pH 5.2 with a density that increased with decreasing pH down to pH 3.9. The mobility of either organic or inorganic phosphate at pH 6.8 was not significantly different after the pH had been reduced to 4.8 and subsequently increased to 6.8, but the microstructure was strongly influenced by the pH-cycling.

1. Introduction

Casein is the major protein component of cow's milk, representing about 80% of its protein content. Its structure and behaviour in aqueous solution have been investigated intensively for decades, see (Dalgleish, 2011; De Kruif, 2014; De Kruif, Huppertz, Urban and Petukhov, 2012; Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2009) for recent reviews, but are still not fully understood. Bovine casein consists of mainly four types: α_{s1}, α_{s2}, β and κ, and in milk the majority of casein is assembled into spherical aggregates, so-called casein micelles, with an average radius of about 100 nm. Currently, the generally accepted view is that the casein micelles contain nanoclusters of calcium orthophosphate, so-called colloidal calcium phosphate (CCP), that are distributed throughout the micelles. Phosphorylated serines (SerP) in the caseins bind to the nanoclusters or even become an integrated part of the CCP, which maintains the integrity of the micelles together with attractive interactions between the casein chains (Holt et al., 2013). The size of the casein micelles is limited by κ-casein that forms a polyelectrolyte brush at the surface of the micelle. Steric hindrance of the κ-casein layer together with electrostatic repulsion inhibits aggregation of the micelles in milk.

The net charge of the casein micelles decreases with decreasing pH from the value in milk (pH 6.7) towards its iso-electric point (pI ≈ 4.6). Acidification also leads to progressive protonation of organic and inorganic phosphate and causes progressive dissolution of the CCP until all phosphate is solubilized at pH < 5.3 (Dalgleish & Law, 1989; Famelart, Lepesant, Gaucheron, Le Graet, & Schuck, 1996; Le Graet & Brulé, 1993; Marchin, Putaux, Pignon, & Léonil, 2007). It was observed that the size of casein micelles in unheated milk decreases only very little with decreasing pH until pH 5.0 (Anema, Lowe, & Lee, 2004; Dalgleish, Alexander, & Corredig, 2004; De Kruif, 1997; Moitzi, Menzel, Schurtenberger, & Stradner, 2010). At lower pH, the proteins aggregate leading to precipitation or gelation, which is the basis for yoghurt formation. Even though the casein micelles remain largely intact, at least down to pH 5.0, their internal structure and the interaction between the

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caseins change during acidification as the CCP progressively dissolves. It is clear that in order to understand this process fully it is necessary to investigate the state of both the organic and inorganic phosphate as a function of the pH.

^{31}P NMR spectroscopy is a non-destructive technique that can be used to quantify the degree of protonation of the phosphoserine of casein as a function of the pH (Belton, Lyster, & Richards, 1985; Humphrey & Jolley, 1982; Sleigh, Mackinlay, & Pope, 1983). It was shown with this technique that the majority of organic (P_o) and inorganic (P_i) phosphorus in micellar casein solutions is immobile at pH 6.7 and that it can be characterized by magic angle spinning (MAS) NMR (Bak, Rasmussen, Petersen, & Nielsen, 2001; Rasmussen, Sørensen, Petersen, Nielsen, & Thomsen, 1997; Thomsen, Jakobsen, Nielsen, Petersen, & Rasmussen, 1995). However, as far as we are aware, no systematic investigation has been made so far of the mobility of P_o and P_i in aqueous solutions of casein micelles as a function of the pH.

Here we report on a MAS ^{31}P NMR investigation of aqueous solutions of casein micelles in the form of native phosphocaseinate over a wide pH-range (4–8). Our aim was to determine quantitatively the fraction of mobile P_o and P_i as a function of the pH in order to trace the dissolution of the CCP. We also determined the degree of protonation of the mobile phophoserine and inorganic phosphate and compared it with that of caseins without CCP, i.e. sodium caseinate (NaCas). The effect of decreasing the pH on the microscopic structure of the casein solutions was visualized by confocal laser scanning microscopy (CLSM). Finally, we investigated the effect of pH-cycling on the mobility of P_i and P_o and the microscopic structure for a micellar casein solution at pH 6.8 before and after acidification. In this investigation we have studied the effect of acidification on the state of organic and inorganic phosphate for casein in pure water. The influence of the presence of minerals on the effect of acidification will be addressed in a future investigation.

2. Materials and methods

Commercial NaCas powder (Lactonat EN) was provided by Lactoprot Deutschland GmbH (Kaltenkirchen, Germany). It contained 90% (w/w) protein (TNC, Kjeldahl) and 1.3% (w/w) sodium and 0.7wt% phophorus. Pure α_s and β-casein powders were purchased from Sigma–Aldrich (St. Louis, USA). Micellar casein in the form of native phosphocaseinate powder (NPCP) was obtained by micro- and diafiltration and was provided by INRA-STLO, (Rennes, France). The powder contained 83% (w/w) of protein (TNC, Kjeldahl), 2.6% (w/w) calcium and 1.7% (w/w) phosphorus. The casein composition of the samples was obtained using reverse phase high pressure liquid chromatography. The fractions of α_{s1}-, α_{s2}-, β-, and κ-casein in NaCas were 34, 3, 44, 9%, respectively. In addition, the sample contained 9% unidentified protein, most likely partially degraded casein. The casein composition of caseins in NPCP was approximately the same. Considering that the amount of P_i in NaCas is very small it follows from the phosphorus content that the fraction of P_i and P_o in the casein micelle powder used for this study was 0.6 and 0.4 respectively, in agreement with that reported by Famelart et al. (Famelart et al., 1996).

2.1. Sample preparation

NaCas and NPCP were dissolved while stirring in deionised water (Millipore) containing 3 mM sodium azide as a bacteriostatic agent. NaCas solutions were heated at 80 °C for 30 min and NPCP solutions were heated at 50 °C for 16 h in order to obtain fully hydrated homogeneous suspensions. The protein concentration was determined by absorption of UV-light with wavelength 280 nm (Varian Cary-50 Bio, Les Ulis, France) assuming an extinction coefficient of 0.81 L/g cm. The pH was adjusted by dropwise addition of concentrated 0.1–1 M NaOH or HCl solutions while vigorously stirring. All experiments shown here were done at a fixed casein concentration after pH adjustment of C = 100 g/L, except the pure α-casein solution for which C = 75 g/L. The fraction of proteins in the form of micelles was determined by centrifugation at $5.10^4 g$ during 2 h at 20 °C using an ultracentrifuge (Beckman Coulter, Allegra 64R, Villepinte, France). About 15% of the proteins did not sediment in these conditions. Further dissolution of casein micelles was extremely slow at this high protein concentration and was negligible over the duration of the experiments.

2.2. Nuclear magnetic resonance experiments

The NMR experiments were conducted on a Bruker Avance III 300 MHz WB spectrometer equipped with a 4 mm MAS VTN type probe head with two channels. An HR/MAS rotor was filled with about 50 μL of a casein or caseinate solution at C = 100 g/L and spun at 3 kHz. ^{31}P direct excitation spectra were accumulated over between 128 and 1024 repetitions with a relaxation delay of 30 s, an acquisition time of 0.2 s, and a ^{1}H decoupling with reduced power (8.5 kHz) in order to avoid probe head damage during the relatively long acquisition needed for adequate sampling of relatively narrow signals. In addition, ^{1}H–^{31}P cross-polarization (CP) spectra have been acquired with 6144 repetitions with a relaxation delay of 5 s in order to characterize the broad signal from immobile phosphorus. All spectra have been normalized with respect to the H_2O ^{1}H signal in order to account for the variation in the sample volume in the HR/MAS rotor. The line width and position of the broad signal from immobile P were determined from the complementary cross-polarization spectra. The mobile signals from inorganic and organic phosphate were quantified by deconvolution and integration of the corresponding peaks in the ^{31}P direct excitation spectra after subtraction of the immobile P signal. The spectra have been referenced to an aqueous solution of H_3PO_4.

2.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) images were made with a Leica TCS-SP2 (Leica Microsystems, Heidelberg, Germany) using a water immersion objective lens HCx PL APO 63x NA = 1.2. The caseins were labelled with the fluorochrome rhodamine B by adding a small amount of a concentrated rhodamine solution for a final concentration of 5 ppm in the solutions. The rhodamine was excited using a helium–neon laser with wavelength 543 nm and the fluorescence was detected with a photomultiplier. Care was taken not to saturate the fluorescence signal and it was verified that the amplitude of the signal was proportional to the protein concentration.

3. Results and discussion

We will first discuss the effect of acidification of aqueous solutions of NaCas at C = 100 g/L that contains only trace amounts of calcium and inorganic phosphate. Then we will show the results for acidification of micellar casein solutions at the same protein concentration and compare them with those obtained for NaCas.

3.1. Sodium caseinate

Fig. 1 shows the direct excitation ^{31}P MAS NMR spectrum of NaCas at pH 6.8. It contains a relatively small narrow peak at δ = 1.2 ppm and a broader signal with larger amplitude at δ ≈ 2.5 ppm. The narrow peak was due to free orthophosphate