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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

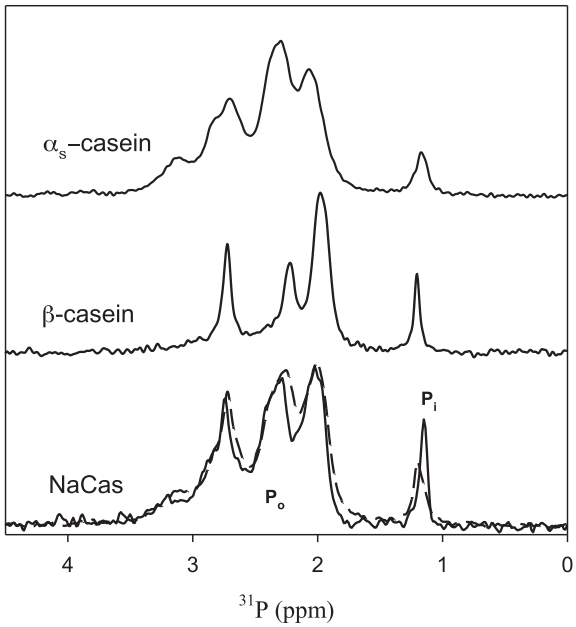

Fig. 1. ^{31}P MAS NMR spectra of aqueous solutions of NaCas and purified α_s- and β-casein at pH 6.8. 1 mM Na_2HPO_4 was added to the purified casein solutions. The dashed line in the spectrum of NaCas corresponds to the weighted average of the spectra of purified α_s- and β-casein.

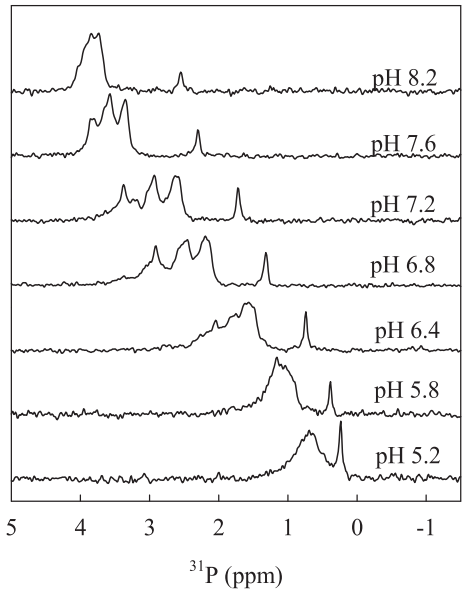

Fig. 2. ^{31}P MAS NMR spectra of aqueous solutions of NaCas at different pH indicated in the figure.

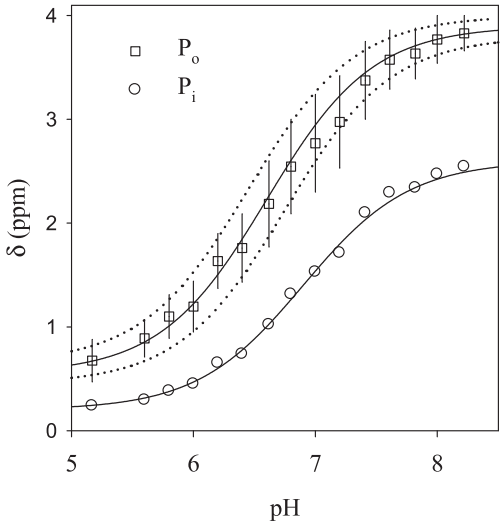

Fig. 3. pH-dependence of the chemical shift of organic and inorganic phosphorus. The bars indicate the width of the P_o signal. The solid and dotted lines represent fits with Eq. (1).

that was present in a small amount in the NaCas sample. The broad signal was due to organic phosphate from the phosphoserine residues (SerP). One can distinguish three peaks in the P_o signal as was earlier reported by (Kakalis, Kumosinski, & Farrell, 1990) for NaCas at pH 7.6 and by (Heber, Paasch, Partschefeld, Henle, & Brunner, 2012) for milk after addition of EDTA. For comparison we also show in Fig. 1 spectra of purified α_s- and β-casein solutions at pH 6.8. The signal of NaCas is very close to the average of α_s- and β-casein signals weighted in proportion to their content in the NaCas sample, see dashed line in Fig. 1. The contribution of κ-casein to the NMR signal is negligible (≈2%), because it contains only one SerP. α_{s1}-, α_{s2}-and β-casein contain 8–9, 11–13 and 5 SerP per chain, respectively, and it is clear that the peaks observed in the spectra are not to distinct signals from each phosphoserine. However, attempts have been made to assign the peaks to different groups of SerP with similar environment within the casein chain (Humphrey et al., 1982; Sleigh et al., 1983). We note that the narrow signal at 1.3 ppm was absent for the purified casein samples, but appeared when we added 1 mM Na_2HPO_4, which shows without ambiguity that this peak is caused by inorganic phosphate.

^{31}P MAS NMR spectra of NaCas at different pH are shown in Fig. 2. An upfield shift of both the P_o and P_i signals was observed when the pH decreased. The P_i signal remained narrow over the whole pH-range, but the width of the broad P_o signal group taken at half its maximum amplitude decreased with increasing pH above pH 7.2 and with decreasing pH below pH 6.8. The three peaks of the P_o signal can be clearly distinguished only in a narrow range of the pH between 6.6 and 8.0. The dependence of δ on the pH of the narrow P_i signal and the average P_o signal is shown in Fig. 3. The width of the P_o signal is indicated by bars.

The upfield shift of δ with decreasing pH is caused by increased shielding of the P nuclei as consequence of protonation of the phosphates. The pH dependence of δ can thus be used to calculate the equilibrium constant of the second protonation of the phosphates. The chemical shift as a function of the pH was analysed assuming that protonation of each phosphate is characterized by a single pKa. In that case the relationship between the pH and the chemical shift is as follows:

$$\delta = (\delta_1 X + \delta_2)/(X + 1) \quad (1)$$

where δ_1 and δ_2 are the chemical shifts of protonated and deprotonated phosphate, respectively, and $X = 10^{pH\text{-}pKa}$ is the molar ratio between protonated and deprotonated phosphate. Only an average signal is observed because the exchange of protons between different phosphates is fast compared to the NMR time scale.

The narrow signal of P_i and the average P_o can be well described by Eq. (1), see solid lines in Fig. 3. For P_i we find pKa = 7.0 ($\delta_1 = 0.2$ and $\delta_2 = 2.6$ ppm) which is slightly smaller than the literature value for the second protonation of pure orthophosphate (pKa = 7.2). For the average chemical shift of the organic phosphate we find

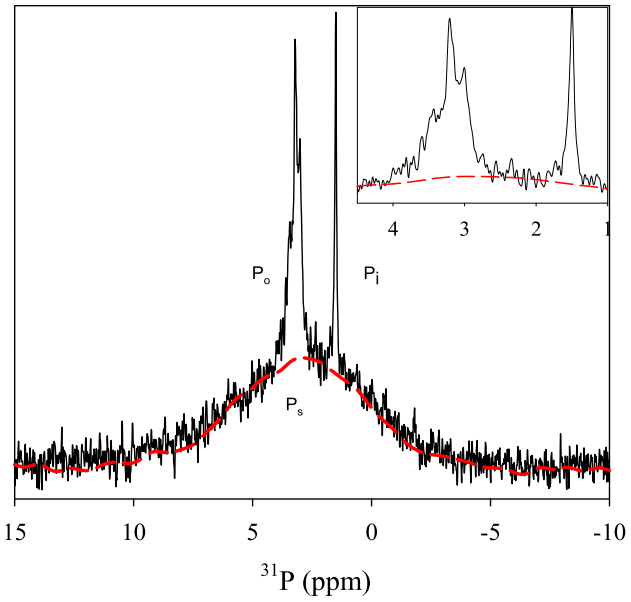

Fig. 4. ^{31}P MAS NMR spectrum of an aqueous solution of casein micelles at pH 6.8. The red dashed line represents the cross-polarization spectrum. The insert shows a close-up of the mobile P_o *and* P_i *signals*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pKa = 6.6 ($\delta_1 = 0.5$ and $\delta_2 = 3.9$). However, phosphoserines with different environments have slightly different pKa values, which caused the dispersion of the P_o signal. The variation of the width of the signal from the organic phosphate as a function of the pH can be explained by a pKa range from about 6.45 to 6.8, see dotted lines in Fig. 3. The pKa values obtained for P_o in this study are within the range (5.8–6.9) that has been reported for purified caseins in the literature (Belton et al., 1985; Humphrey et al., 1982).

3.2. Casein micelles

The direct excitation ^{31}P MAS NMR spectrum of a micellar casein solution at pH 6.8 shows one narrow peak at $\delta = 1.5$ ppm, a broader peak at 3.2 ppm and a very broad peak centred at 2.5 ppm, see Fig. 4. Similar spectra have been reported for micellar casein solutions in the literature (Andreotti, Trivellone, & Motta, 2006; Bak et al., 2001; Belton et al., 1985; Heber et al., 2012; Rasmussen et al., 1997; Thomsen et al., 1995). The narrow peak at 1.5 ppm is due to free orthophosphate in solution, while the peak at 3.2 ppm can be assigned to mobile SerP. The latter is broadened by the different chemical environments of the SerP as was discussed above for NaCas. However, the shape of this peak is different from that of NaCas which means that the environment of the mobile SerP was different. The very broad peak at 2.5 ppm originated from immobile phosphate (Bak et al., 2001; Rasmussen et al., 1997; Thomsen et al., 1995) both organic and inorganic. This can be confirmed by cross polarisation NMR that probes specifically the signal from immobile phosphate, see dashed line in Fig. 4. A few authors have analysed this signal in more detail and distinguished different types of immobile phosphate (Bak et al., 2001; Rasmussen et al., 1997; Thomsen et al., 1995), but we will not attempt this here. The relative amplitude of the signals was obtained by deconvolution of the spectrum, yielding 2%, 7.5% and 90.5% for mobile P_i, mobile P_o, and immobile P, respectively. The fraction of P_i and P_o in the casein micelle powder used for this study was 0.4 and 0.6, respectively, see Materials and Methods. This means that 97% of P_i and 81% of P_o is immobile. Most likely both the immobile organic and the inorganic phosphate form the CCP nanoclusters. We note that no significant difference was observed between spectra obtained for freshly prepared samples and after standing for 1 month at 4 °C implying that the casein micelles were stable at the protein concentration used in the study.

Ultracentrifugation (5×10^4 g, 1 h) leads to sedimentation of casein micelles, while NaCas remains in the supernatant. When an aqueous NPCP solution at pH 6.8 was centrifugated only 85–90% of the casein sedimented, which means that 10–15% of the casein was not incorporated into the micelles. This may in part explain why 20% of P_o was mobile. However, the fraction of casein in the supernatant was found to decrease with decreasing pH in accordance with results obtained by Famelart et al. (Famelart et al., 1996) for a different batch of NPCP, while the fraction of mobile P_o increased as we will show below. Therefore one cannot relate the fraction of mobile P_o directly to the fraction of non-sedimentable casein.

Fig. 5 shows the ^{31}P MAS NMR and cross-polarization NMR spectra of aqueous micellar casein solutions at different pH. As was observed for NaCas, the chemical shift of the mobile P_i and P_o signals moved upfield when the pH was decreased. In addition, the

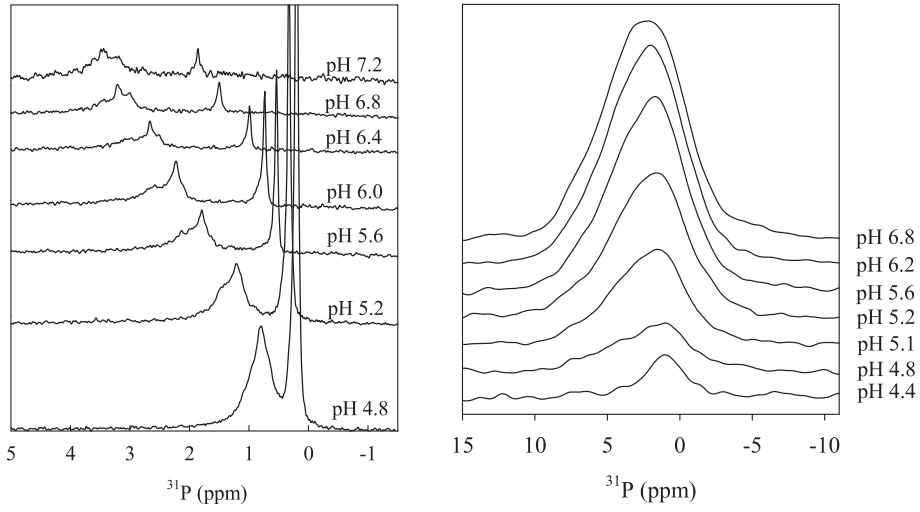

Fig. 5. ^{31}P MAS NMR (left panel) and cross-polarization NMR (right panel) spectra of aqueous solutions of casein micelles at different pH. For clarity the spectra at different pH have been shifted vertically.

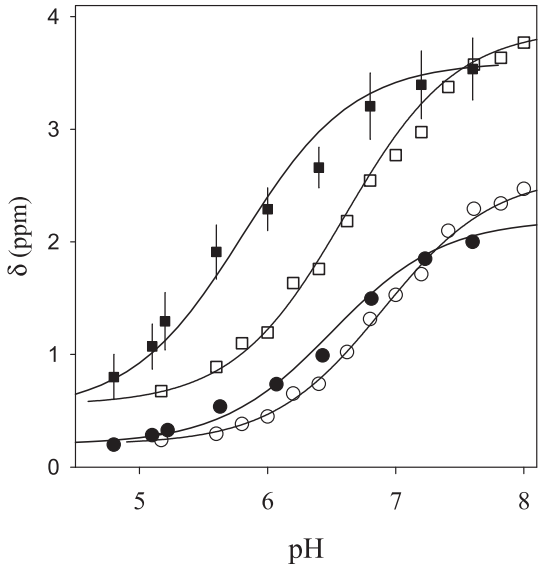

Fig. 6. pH-dependence of the chemical shift of organic (squares) and inorganic (circles) phosphorus in aqueous solutions of casein micelles (filled symbols) and NaCas (open symbols). The bars indicate the width of the P_o signal for the casein micelles. The solid lines represent fits corresponding to Eq. (1).

amplitude of these signals increased strongly below pH 6.0. The chemical shift of mobile P_o and P_i in micellar casein solutions as a function of the pH is compared to that in NaCas solutions in Fig. 6. It is clear that compared to NaCas the sharp upfield shift with decreasing pH occurs at lower pH for the casein micelles especially for P_o. Models assuming a single equilibrium constant yield pKa = 5.8 ($\delta_1 = 0.5$, $\delta_2 = 3.6$ ppm) for P_o and pKa = 6.5 ($\delta_1 = 0.2$, $\delta_2 = 2.2$ ppm) for P_i. However, the assumption of a single pKa describes the data less well for the casein micelles than for NaCas. The shift of the protonation of the mobile phosphate groups to lower pH entails that their environment was different as is also evident from the different shape of the NMR signal. In addition, the environment changes with decreasing pH, which may explain why the model assuming a single pKa was not as good as for NaCas. The most likely reason for the different pH-dependence of the chemical shift is that the micellar casein solutions contain calcium and orthophosphate in the ionic form in increasing quantities as the pH is decreased as a consequence of the CCP being dissolved. These ions can screen electrostatic interactions and interact specifically with SerP.

The relative amplitudes of the different phosphorus signals were determined by deconvolution of the single pulse spectra and from the peak integral in the CP spectra shown in Fig. 5. Fig. 7a shows that the fraction of immobile phosphate decreased sharply for pH < 6.0 and became very small for pH < 4.5. The fraction of mobile organic and inorganic phosphate is plotted as a function of the pH down to pH 4.8 in the inset of Fig. 7a. At lower pH, the P_i and P_o signals overlapped strongly and could not be reliably separated. In Fig. 7b we have plotted the variation of the fraction of mobile P_i and P_o with respect to the total amount of each. At pH > 6.0 almost 20% of the organic phosphate was mobile whereas less than 10% of the inorganic phosphate was mobile. The fraction of mobile phosphate increased rapidly below pH 5.5 and reached 75% at pH 4.8 for both P_i and P_o.

Le Graët & Gaucheron (1999) determined the fraction of 'soluble' inorganic phosphate in solutions of NPCP in milk ultrafiltrate as a function of the pH. Soluble phosphate was defined as the phosphate that passed a filter with a 25 kDa cut-off membrane. They showed that the fraction of soluble phosphate increased rapidly for pH < 5.5 and depended weakly on the casein concentration. The

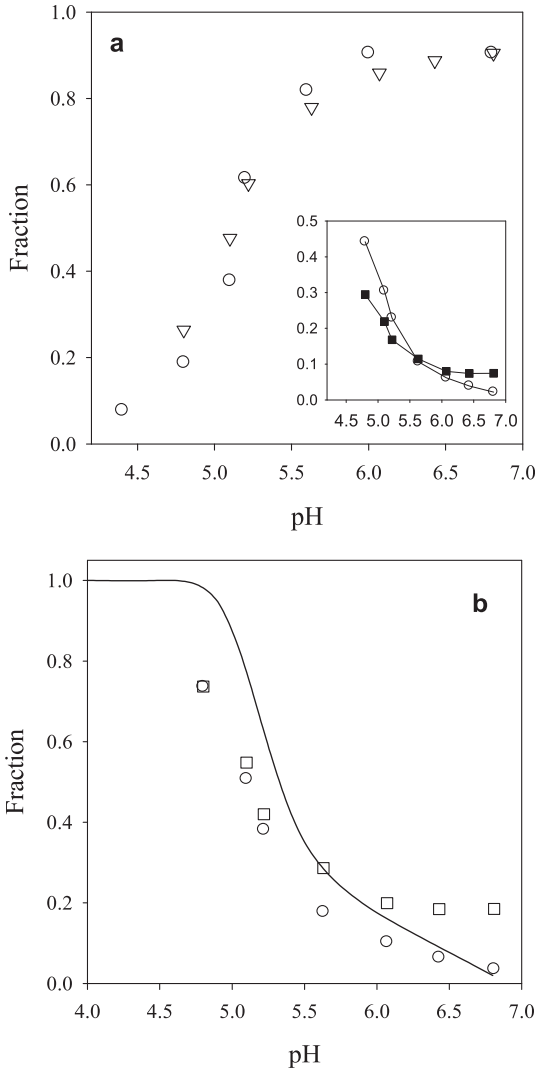

Fig. 7. (a) Fraction of immobile phosphate as a function of the pH in aqueous solutions of casein micelles obtained from single pulse (triangles) and cross-polarization experiments (circles). The in-set shows the fraction of mobile organic (filled symbols) and inorganic (open symbols) phosphate. (b) Fraction of mobile organic (squares) and inorganic (circles) phosphate with respect to the total organic and inorganic phosphate, respectively. The solid line represents the fraction of soluble inorganic phosphate taken from ref. Le Graët & Gaucheron (1999).

fraction of solubilized inorganic phosphate obtained in this manner at the casein concentration used here is compared with the fraction of mobile P_i, see solid line Fig. 7b. Qualitatively the pH-dependence is the same, but the sharp increase of the fraction of soluble P_i reported by Le Graët & Gaucheron (1999) started at slightly lower pH than the increase of mobile P_i observed here. This may be due to the fact that they prepared the casein micelles solutions in milk ultrafiltrate while we have prepared them in pure water. Alternatively, it may mean that a fraction of the soluble P_i was immobile at these pH values.

Visual inspection of the samples showed that a significant fraction of the proteins in casein micelles solutions below pH 5.0 formed large flocs that sedimented slowly under gravity. This observation is consistent with results by Famelart et al. (1996) who reported for NPCP solutions in pure water a sharp increase of the fraction of casein that sedimented after centrifugation for 1 h at $10^3 g$ from less than 5% for pH ≥ 5.2 to about 90% for pH ≤ 5.0. The CLSM images in Fig. 8 show the formation of microscopic casein

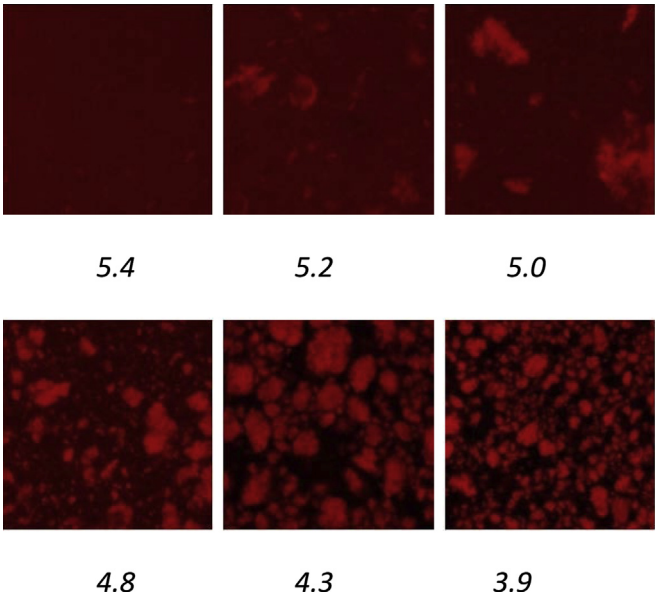

Fig. 8. CLSM images (150 × 150 μm) of micellar casein solutions at different pH indicated in the figure. The proteins were labelled (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flocs after acidification starting at pH 5.2 with a density that increased sharply with decreasing pH. Remarkably, large scale aggregation of the caseins did not render the SerP groups immobile, but, on the contrary, the fraction of mobile phosphate continued to increase progressively below pH 5.2. The large scale flocculation of the caseins does not seem to be directly related to the state of the phosphate. We notice that NaCas also precipitates or gels below pH 5.2 (Braga, Menossi, & Cunha, 2006; HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008; Lucey, Van Vliet, Grolle, Geurts, & Walstra, 1997; O'Kennedy, Mounsey, Murphy, Duggan, & Kelly, 2006; Ruis, Venema, & van der Linden, 2007).

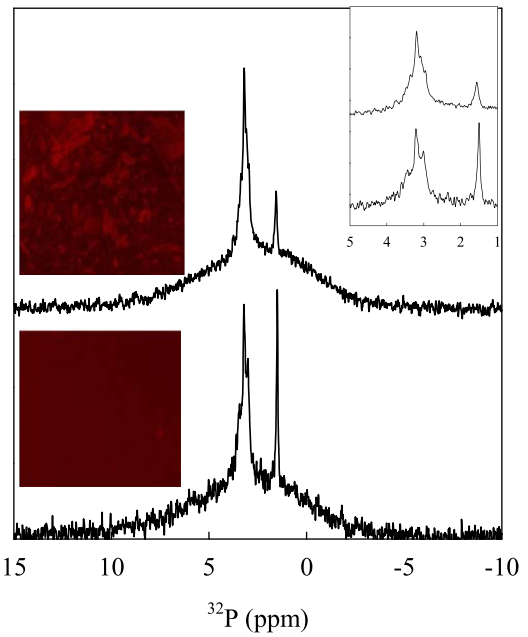

Fig. 9. ^{31}P MAS NMR spectra of aqueous solutions of casein micelles at pH 6.8 before (bottom) and after (top) pH-cycling to pH 4.8. The insert shows a close-up of the signals of mobile P_o and P_i. CLSM images of the corresponding solutions are also shown.

It is clear that dissolution of the CCP by lowering the pH releases inorganic phosphate and calcium from the CCP and renders SerP mobile. Silva et al. (2013) dialysed micellar casein solutions at different pH between 6.7 and 5.5 against milk ultrafiltrate in order to remove the released calcium phosphate. They subsequently increased the pH back to 6.8 and determined the fraction of non-sedimentable casein after ultracentrifugation. The fraction of non-sedimentable casein was found to increase from 25% at pH 6.7 to 70% at pH 5.5. It was also found that the fraction of inorganic phosphate that was removed by dialysis increased with decreasing pH up to 60% at pH 5.5. The fraction of dissociated casein and solubilized P_i at pH 5.5 was significantly larger than the fraction of mobile P_o and P_i at this pH found in the present study, which may be due to the extended dialysis. Nevertheless, these experiments show that demineralization of casein micelles by acidification leads to dissociation of a fraction of the casein micelles at neutral pH where the charge density of the caseins is higher. However, as we mentioned above, if the system is maintained at lower pH the caseins remain aggregated and sediment during ultracentrifugation. This means that the reduced stabilizing effect of CCP nanoclusters is compensated at lower pH by other types of attractive interactions between the casein chains (Holt et al., 2013). When the charge density of the caseins is increased by increasing the pH these interactions are not sufficient to maintain the integrity of the micelles in the absence of a large fraction of the calcium phosphate.

3.3. pH cycling

An interesting question is whether the mobilization of phosphates by acidification is reversible upon increasing the pH. Fig. 9 shows the ^{31}P MAS NMR spectrum of a micellar casein solution of which the pH was first reduced from 6.8 to 4.8, which rendered most of the phosphates mobile (compare Fig. 5), and was subsequently increased back to 6.8. Comparison of the spectra of the solution at pH 6.8 before and after pH-cycling, shows that the signals were almost the same except that the amplitude of the mobile P_i signal was slightly larger before cycling. The signal of residual mobile P_o was similar before and after pH-cycling, but not identical. Clearly most of the phosphate was immobilized when the pH was raised, but no calcium phosphate particles were visible on length scales accessible to CLSM. Most likely growth of the calcium phosphate clusters was limited by incorporation of SerP, which rendered the latter immobile. Holt, Wahlgren, & Drakenberg (1996) observed the formation of amorphous dicalcium phosphate nanoclusters from an undersaturated solution of salts and the phosphopeptide (β 1–25) by raising the pH slowly from 5.5 to 6.7. The ^{31}P NMR spectrum at pH 6.7 in that study contained a very broad signal that represented 75% of the total signal implying that 75% of the phosphate had been immobilized by the formation of the nanoclusters. In addition to the broad signal they observed a narrow signal from mobile inorganic phosphate and a broader three peaked signal from mobile organic phosphate.

It appears that also for micellar casein solutions studied here, nanoclusters were formed when the pH was increased, but we have no information on the size of the clusters nor on their structure. Therefore we do not know if they are similar to the ones in native casein micelles. In any case, CLSM images of the solution before and after pH-cycling showed that the structure on larger length scales was different, see Fig. 9. Before pH-cycling the system was homogeneous on microscopic length scales, while after pH-cycling we observed large flocs, albeit with a much lower density than at pH 4.8. The acid-induced aggregation of micellar caseins is thus not reversible upon increasing the pH contrary to the acid-induced

aggregation of NaCas. We note that the presence of large flocs after pH-cycling causes some uncertainty in the amount of casein of this sample in the NMR probe and thus the total amplitude of the signal. We may speculate that the internal structure of the large flocs is similar to that of the casein micelles. Calcium phosphate nanoclusters incorporating phosphoserines of α- and β-caseins are bound to other caseins via attractive interactions between the proteins. The main difference with the structure of native casein micelles is the role of κ-casein that did not form a stabilizing layer that inhibits growth of the casein/nanocluster aggregates during formation of the native micelles. This may be due to the fact that casein was already aggregated at pH 4.8 and that the protein concentration used in this study was relatively high. Further investigation employing scattering techniques and electron microscopy are needed to establish to what extent the local structure of the casein assemblies after pH-cycling resembles that of native casein micelles.

4. Conclusions

Magic angle spinning ^{31}P NMR allows one to determine quantitatively the fraction and the protonation of mobile organic and inorganic phosphate in casein solutions as a function of the pH in the range 4.8–8.0. The organic phosphate of sodium caseinate in aqueous solution is mobile over the whole pH range even for pH < 5.2 where the caseinate precipitates during centrifugation and microscopic flocs are observed in CLSM image. The ^{31}P NMR signal of sodium caseinate at pH 6.8 is equal to the weighted average of purified α- and β-caseins implying that the local environment of the phosphoserines is not very different from that in the pure α- and β-casein aggregates. The average pKa value of NaCas was 6.6, but varied slightly for different SerP on the caseins, which caused the appearance of distinct peaks around pH 7.

For casein micelles in aqueous solution at the pH of milk, 97% of the inorganic phosphate and 81% of the organic phosphate is immobile and most likely incorporated in calcium phosphate nanoclusters. The fraction of mobile organic and inorganic phosphate increased weakly with decreasing pH down to pH 5.5 and then increased sharply to reach 75% at pH 4.8. Protonation of the inorganic and organic phosphates was shifted to lower pH and for both it was less well described by Eq. (1) than for NaCas solutions, demonstrating the influence of the minerals present in micellar casein solutions. In spite of the release of the phosphates from the nanoclusters by lowering the pH, most of the proteins sedimented during ultracentrifugation at all pH and large flocs were observed for pH < 5.2. There is no correlation between the mobility of the phosphates and the solubility of the caseins.

Calcium phosphate nanoclusters that were dissolved by lowering the pH to 4.8 reformed when the pH was increased again to 6.8 and the fraction of immobile phosphate was almost the same before and after pH-cycling. However, the microscopic structure of the micellar casein solutions was different as after pH-cycling microscopic casein clusters were visible with CLSM. Possibly the local organisation of the caseins and the calcium phosphate is similar before and after pH-cycling, but the stabilizing role of κ-casein that limits growth during formation of the native micelles is absent after pH-cycling.

References

Andreotti, G., Trivellone, E., & Motta, A. (2006). Characterization of buffalo milk by ^{31}P-nuclear magnetic resonance spectroscopy. *Journal of Food Composition and Analysis, 19*, 843–849.

Anema, S. G., Lowe, E. K., & Lee, S. K. (2004). Effect of pH at heating on the acid-induced aggregation of casein micelles in reconstituted skim milk. *LWT – Food Science and Technology, 37*, 779–787.

Bak, M., Rasmussen, L. K., Petersen, T. E., & Nielsen, N. C. (2001). Colloidal calcium phosphates in casein micelles studied by slow-speed-spinning ^{31}P magic angle spinning solid-state nuclear magnetic resonance. *Journal of Dairy Science, 84*, 1310–1309.

Belton, P. S., Lyster, R. L., & Richards, C. P. (1985). The ^{31}P nuclear magnetic resonance spectrum of cows' milk. *Journal of dairy research, 52*, 47–54.

Braga, A., Menossi, M., & Cunha, R. (2006). The effect of the glucono-δ-lactone/caseinate ratio on sodium caseinate gelation. *International Dairy Journal, 16*, 389–398.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft Matter, 7*, 2265–2272.

Dalgleish, D., Alexander, M., & Corredig, M. (2004). Studies of the acid gelation of milk using ultrasonic spectroscopy and diffusing wave spectroscopy. *Food Hydrocolloids, 18*, 747–755.

Dalgleish, D. G., & Law, A. J. R. (1989). pH-induced dissociation of bovine casein micelles. II Mineral solubilization and its relation to casein release. *Journal of Dairy Research, 56*, 727–735.

De Kruif, C. (1997). Skim milk acidification. *Journal of Colloid and Interface Science, 185*, 19–25.

De Kruif, C. (2014). The structure of casein micelles: a review of small-angle scattering data. *Applied Crystallography, 47*.

De Kruif, C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V. (2012). Casein micelles and their internal structure. *Advances in Colloid and Interface Science, 171*, 36–52.

Famelart, M. H., Lepesant, F., Gaucheron, F., Le Graet, Y., & Schuck, P. (1996). pH-Induced physicochemical modifications of native phosphocaseinate suspensions: influence of aqueous phase. *Le Lait, 76*, 445–460.

HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids, 22*, 1460–1466.

Heber, A., Paasch, S., Partschefeld, C., Henle, T., & Brunner, E. (2012). ^{31}P NMR spectroscopic investigations of caseins treated with microbial transglutaminase. *Food Hydrocolloids, 28*, 36–45.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Caseins and the casein micelle: their biological functions, structures, and behavior in foods. *Journal of Dairy Science, 96*, 6127–6146.

Holt, C., Wahlgren, N. M., & Drakenberg, T. (1996). Ability of a beta-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *The Biochemical Journal, 314*, 1035–1039.

Horne, D. S. (2009). Casein micelle structure and stability. *Milk Proteins: From Expression to Food*, 133–162.

Humphrey, R. S., & Jolley, K. W. (1982). ^{31}P-NMR studies of bovine beta-casein. *Biochimica et Biophysica Acta, 708*, 294–299.

Kakalis, L. T., Kumosinski, T. F., & Farrell, H. M. (1990). A multinuclear, high-resolution NMR study of bovine casein micelles and submicelles. *Biophysical Chemistry, 38*, 87–98.

Le Graet, Y., & Brulé, G. (1993). Les équilibres minéraux du lait: influence du pH et de la force ionique. *Le Lait, 73*, 51–60.

Le Graët, Y., & Gaucheron, F. (1999). pH-induced solubilization of minerals from casein micelles: influence of casein concentration and ionic strength. *Journal of Dairy Research, 66*, 215–224.

Lucey, J., Van Vliet, T., Grolle, K., Geurts, T., & Walstra, P. (1997). Properties of acid casein gels made by acidification with glucono-δ-lactone. 1. Rheological properties. *International Dairy Journal, 7*, 381–388.

Marchin, S., Putaux, J.-L., Pignon, F., & Léonil, J. (2007). Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small-angle x-ray scattering/ultrasmall-angle x-ray scattering. *The Journal of Chemical Physics, 126*, 045101.

Moitzi, C., Menzel, A., Schurtenberger, P., & Stradner, A. (2010). The pH induced sol–gel transition in skim milk revisited. A detailed study using time-resolved light and X-ray scattering experiments. *Langmuir, 27*, 2195–2203.

O'Kennedy, B. T., Mounsey, J. S., Murphy, F., Duggan, E., & Kelly, P. M. (2006). Factors affecting the acid gelation of sodium caseinate. *International Dairy Journal, 16*, 1132–1141.

Rasmussen, L. K., Sørensen, E. S., Petersen, T. E., Nielsen, N. C., & Thomsen, J. K. (1997). Characterization of phosphate sites in native ovine, caprine, and bovine casein micelles and their caseinomacropeptides: a solid-state phosphorus-31 nuclear magnetic resonance and sequence and mass spectrometric study. *Journal of Dairy Science, 80*, 607–614.

Ruis, H. G., Venema, P., & van der Linden, E. (2007). Relation between pH-induced stickiness and gelation behaviour of sodium caseinate aggregates as determined by light scattering and rheology. *Food Hydrocolloids, 21*, 545–554.

Silva, N. N., Piot, M., de Carvalho, A. F., Violleau, F., Fameau, A.-L., et al. (2013). pH-induced demineralization of casein micelles modifies their physico-chemical and foaming properties. *Food Hydrocolloids, 32*, 322–330.

Sleigh, R. W., Mackinlay, A. G., & Pope, J. M. (1983). NMR studies of the phosphoserine regions of bovine αs1- and β-casein. *Biochimica et Biophysica Acta (BBA) – Protein Structure and Molecular Enzymology, 742*, 175–183.

Thomsen, J. K., Jakobsen, H. J., Nielsen, N. C., Petersen, T. E., & Rasmussen, L. K. (1995). Solid-state magic-angle spinning 31P-NMR studies of native casein micelles. *European Journal of Biochemistry/FEBS, 230*, 454–459.