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Casein Interactions Studied by the Surface Plasmon Resonance Technique

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‡The paper is dedicated to the memory of Dr. Jean-Claude Mani

ABSTRACT

Surface plasmon resonance technique was investigated for the first time to study the apparent hydrophobicity and association properties of the major bovine caseins: α_s-(α_{s1}- and α_{s2}-caseins in a 4:1 proportion), β-, and κ-caseins. The apparent hydrophobicities of the caseins were evaluated by a new method based on the binding level of casein on a hydrophobic sensor chip, and kinetic and equilibrium affinity constants were determined for the following casein interactions: α_s/α_s, α_s/β, α_s/κ, β/β, and β/κ, using a sensor chip modified with covalent immobilized caseins. The study by surface plasmon resonance technology of these casein interactions under different conditions (pH, ionic strength, calcium concentration, chemical modification) demonstrated that, at neutral pH, electrostatic repulsive forces play an important role since an increase in ionic strength of the medium resulted in a stronger interaction. When charge repulsions were reduced by either acidification, increase in ionic strength, or dephosphorylation, casein interactions were reinforced, presumably due to weak attractive forces. Moreover, in this molecular model, we showed that addition of calcium greatly increased the binding response between the most phosphorylated caseins and that the added calcium (2 m*M*) participated directly in the formation of bridges between the phosphate groups of the casein molecules.
(**Key words:** bovine casein, casein interaction, surface plasmon resonance, BIACORE)

Abbreviation key: $\mathbf{k_a}$ = association rate constant; $\mathbf{k_d}$ = dissociation rate constant; $\mathbf{K_D}$ = equilibrium dissociation constant; **P20** = polyoxyethylenesorbitan; **Ru** = resonance unit; **SPR** = surface plasmon resonance.

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INTRODUCTION

Caseins, which account for 76 to 86% of the total milk protein (30 to 35 g/L), are heterogeneous proteins represented by four principal proteins: α_{s1}-, α_{s2}-, β-, and κ-casein in the approximate proportion of 38, 10, 36, and 13% (Davies and Law, 1980). Additional heterogeneity arises from post-translational processing such as phosphorylation, glycosylation (only κ-casein), and limited proteolysis [yielding γ-caseins and proteose-peptones (3%) during the proteolytic action of plasmin on β-caseins] (Swaisgood, 1992). All the caseins are relatively small phosphoproteins (19 to 25 kDa) and strongly hydrophobic in the rank of β- > κ- > α_{s1}- > α_{s2}-caseins (Fox, 1989). The primary sequence indicates that the hydrophobic and polar or charged residus are not uniformly distributed (Swaisgood, 1992). The caseins have a particular amphiphilic nature arising from a separation between hydrophobic clusters and negatively-charged regions along the peptide chain, which explains the differences in their association properties. Caseins are not present in milk as individual molecular structures but rather as large protein complexes that also incorporate milk salts, particularly calcium salts. Self association of bovine caseins (Schmidt, 1970; Berry and Creamer, 1975; Buchheim and Schmidt, 1979; Snoeren et al., 1980; Slattery et al., 1989) and associations between different casein species (Payens, 1966; Schmidt, 1982; Rollema, 1992; Horne, 1998; De Kruif, 1999) have been investigated extensively because of their innate importance in the structure of the casein micelle particles (Schmidt and Payens, 1976) and in the nature of the forces maintaining their integrity. Casein molecules present also a strong affinity for bivalent and trivalent cations, attributable mainly to phosphoseryl residues (Baumy et al., 1989; Gaucheron et al., 1997). This property is important for the micellar integrity of caseins, which is greatly dependent on the presence of colloidal calcium phosphate (Walstra, 1990).

Casein interactions have been studied by sedimentation equilibrium patterns obtained by ultracentrifugation (Berry and Creamer, 1975; Buchheim and Schmidt,

1979) and by small-angle neutron scattering and light scattering experiments (Thurn et al., 1987). In the present study, the mechanism of casein interactions has been explored for the first time by surface plasmon resonance (**SPR**) technology. This new technology called biomolecular interaction analysis (BIA) is a label-free technology that monitors in real time molecular binding processes on the surface of a specially prepared biosensor chip. In this paper, we chose to analyze the interactions between three major categories of caseins, namely α_{s1}- and α_{s2}-caseins (the highly phosphorylated caseins), β-casein (the most hydrophobic casein), and κ-casein (the glycosylated casein), on a hydrophobic surface (HPA sensor chip) or bound to a particular casein immobilized on a hydrophilic sensor chip (CM5). The importance of various intermolecular binding forces and the influence of different parameters such as pH, ionic strength, calcium concentration, and chemical modification were evaluated to provide information on the mechanisms involved in the association of these complex molecules.

MATERIALS AND METHODS

Materials

The molecular interactions between the different caseins were investigated by surface plasmon resonance (**SPR**) using BIACORE 2000 (Biacore AB, Uppsala, Sweden). Commercial purified bovine caseins (α_s, β, κ, and dephosphorylated α_s) were purchased from Sigma Chemical Co., St. Louis, MO. CM5 and HPA sensor chips, N-hydroxysuccinimide (NHS), N-ethyl-N′-(dimethylaminopropyl)-carbodiimide (EDC), ethanolamine hydrochloride (1 *M*), pH 8.5, and HBS buffer (10 m*M* HEPES buffer at pH 7.4 containing 150 m*M* NaCl, 3 m*M* EDTA, and 0.005% of the nonionic surfactant polyoxyethylenesorbitan (**P20**) were obtained from Biacore AB. All other reagents were of analytical grade.

Gel Filtration Experiments

All experiments were performed on an ÄKTA explorer system using a Superdex 75 HR10/30 column (Amersham-Pharmacia, Inc.). The column was equilibrated at a flow rate of 0.5 ml/min with various buffers as described in the figure legend and at a temperature of either 4 or 25°C. Purified proteins (0.1 ml) were injected at a concentration of 0.1 mg/ml and detected by following the absorbance at 280 nm. The column was calibrated using a mixture of bovine serum albumin (M_t = 66 kDa, Stokes radius = 35.5 Å), ovalbumin (45 kDa, 30.5 Å), chymotrypsinogen A (25 kDa, 20.9 Å), and ribonuclease A (13.7 kDa, 16.4 Å).

SPR Detection Principle

Surface plasmon resonance is an optical phenomenon which is sensitive to changes in the optical properties of the medium close to the sensor surface. The detection system of the SPR monitor essentially consists of a monochromatic and polarized light source, a glass prism, a thin metal film in contact with the base of the prism, and a photodetector. Light traveling through an optically denser medium, e.g., a glass prism, is totally reflected back into the prism when reaching the interface to an optically less dense medium, e.g., buffer, provided that the angle of incidence is larger than the critical angle. This is known as total internal reflection. Although the light is totally reflected, a component of this incident light momentum called the evanescent wave penetrates a distance of the order of one wavelength into the buffer. The evanescent wave may be used to excite molecules close to the interface. If, however, the light is monochromatic and *p*-polarized and the interface between the media is coated with a thin metal film, the evanescent wave under certain conditions will interact with free-oscillating electrons (plasmons) in the metal thin surface. When SPR occurs, light energy is lost to the metal film and the reflected light intensity is, thus, decreased. The resonance phenomenon will only occur for light incident at a sharply defined angle which, when all else is kept constant, is dependent of the refractive index of the buffer close to the surface. Changes of the refractive index can be followed by continuous monitoring of the resonance angle (Jönsson et al., 1991).

The BIACORE detects changes in refractive index of the solution close to the surface of the sensor chip. To perform interaction analysis, one reactant (the ligand) is immobilized in a dextran matrix on the sensor chip. The sample containing the other reactant (the analyte) is injected over the CM5 surface in a controlled flow. Any change in the surface concentration resulting from the interaction is detected as an SPR signal, expressed in resonance units (**Ru**). One Ru corresponds to approximately 1 pg/mm^2 of protein. The continuous display of Ru as a function of time gives a plot called a sensorgram, which provides a complete record of the progress of association and dissociation between the two interactants. After each analysis, the surface can be regenerated using an appropriate eluant solution that does not affect the immobilized ligand.

Adsorption on the HPA Sensor Chip

The HPA sensor chip is a flat hydrophobic gold surface covered with closely packed C14-alkanethiol molecules. The HPA chip was cleaned by an injection of octyl D-glucoside (10 min, 10 μl/min, 40 m*M*). Caseins (15

ml, 10 μl/min, 8 μM) were injected immediately after cleaning. After a dissociation time of 600 s, the surface was regenerated with octyl D-glucoside (10 min, 10 μl/min, 40 mM). The running buffer used for all experiments was HBS.

Covalent Immobilization of Casein on CM5 Sensor Chip

Covalent immobilization of casein to the CM5 (carboxymethyl dextran 500 kDa) sensor chip was carried out using the standard amine coupling procedure (Pharmacia Biosensor, 1994) at one pH unit under the protein pI, in acetate buffer (10 mM). Each casein (α_s, β, and κ) was independently immobilized at a low level to limit mass transport and rebinding on the measuring flowcell. The fourth flowcell did not contain immobilized casein and was taken as a control. Signals obtained from the control flowcell were subtracted from those of the other flowcells to correct the response for nonspecific binding.

Casein/Casein Interaction Analysis

SPR analysis allows the visualization in real time of the biomolecular interactions of a flowing analyte with an immobilized ligand, without the need to label the interacting partners. The response, measured in resonance unit (Ru), is a direct indication of the amount of bound analyte. The running and sample buffers were 10 mM HEPES, pH 7.4, containing 150 mM NaCl, except for special buffer compositions indicated in the figure legends. All buffers were filtered and degassed. Temperature was maintained at 25°C. Different concentrations of the caseins were tested, but the results are given for the concentration of 4 μM. The analyte was injected (300 s, 10 μl/min), followed by a dissociation phase (600 s) in running buffer and a regeneration step (120 s) with HCl (0.1 M). The binding levels were measured in all cases 10 s after the end of injection in order to avoid error due to differences in refractive index between the sample and the dissociation running buffer. These association levels (Ru value at 310 s) were directly obtained from the sensorgram after subtraction of the background signal recorded for the control flowcell. Each experiment was repeated at least twice. The values for association rate constant ($\mathbf{k_a}$), dissociation rate constant ($\mathbf{k_d}$), and equilibrium dissociation constant ($\mathbf{K_D}$) were obtained from each sensorgram by selecting a 1:1 Langmuir binding model using a global fitting method (Karlsson and Fält, 1997) from the BIAevaluation 3.1 software.

RESULTS

Multimerization State of Caseins

To study casein interactions using BIACORE, it is necessary to know the multimerization state of the proteins in the flow. Thus, we compared the retention volume of caseins by gel filtration chromatography in the buffers used for the BIACORE experiments. Casein molecules, because of their poorly structured conformations, behave on gel filtration columns as much larger proteins (Swaisgood, 1992). To take into account their abnormal retention volumes, we calibrated not only the column with globular protein standards (top chromatogram in Figure 1) but also with purified caseins under conditions known to give monomeric caseins in solution.

α_s-Caseins at high pH (pH 9.3) and low concentration (0.1 mg/ml) are monomeric (Swaisgood and Timasheff, 1968), and their retention volume (9.9 ml) corresponded to a Stokes radius of 32 Å (Figure 1a), in good agreement with values determined by viscometry measurements (Swaisgood, 1992). At neutral pH (pH 7.4), these proteins were mainly monomeric (70%) and eluted at a volume of 10.2 ml (Stokes radius of 30 Å). There was a second pic, representing 30% of the protein, at a volume (9.3 ml) which might correspond to a dimeric form of α_s-caseins (Figure 1a). At pH 7.4 in the presence of $CaCl_2$ (2 mM) and at acidic pH (pH 5.4), the proteins were also present as a monomer in solution (Stokes radius of 29 Å and 30 Å, respectively; Figure 1a). In most cases, there were also some high molecular weight multimers eluted in the void volume of the column (>100 kDa). Dephosphorylated α_s-caseins were eluted as a monomer (Stokes radius of 28 to 29 Å), and no dimer was detected at pH 7.4 or at pH 5.4 with the presence of calcium (Figure 1d). There were still, however, some high molecular weight multimers.

At 4°C, β-casein is a monomer and behaves as a protein with a Stokes radius of 37 Å (Andrews et al., 1979). In Figure 1b, β-casein exhibited a Stokes radius of 39 Å under those conditions. At neutral and acidic pH and at 25°C (conditions used for the BIACORE experiments), the β-casein was essentially monomeric (80 to 90%) as indicated by the retention volume (9.4–9.6 ml), and the Stokes radius (34 to 35 Å) were comparable to the values obtained at 4°C (Figure 1b).

When analyzed by gel filtration, κ-casein was eluted in the void volume of the column (M_t > 100 kDa), even at the lowest concentration tested in this experiment (0.1 mg/ml; Figure 1c). This demonstrated that the protein was multimeric and that no monomer was present in solution. This result confirmed that of Groves et al. (1992).

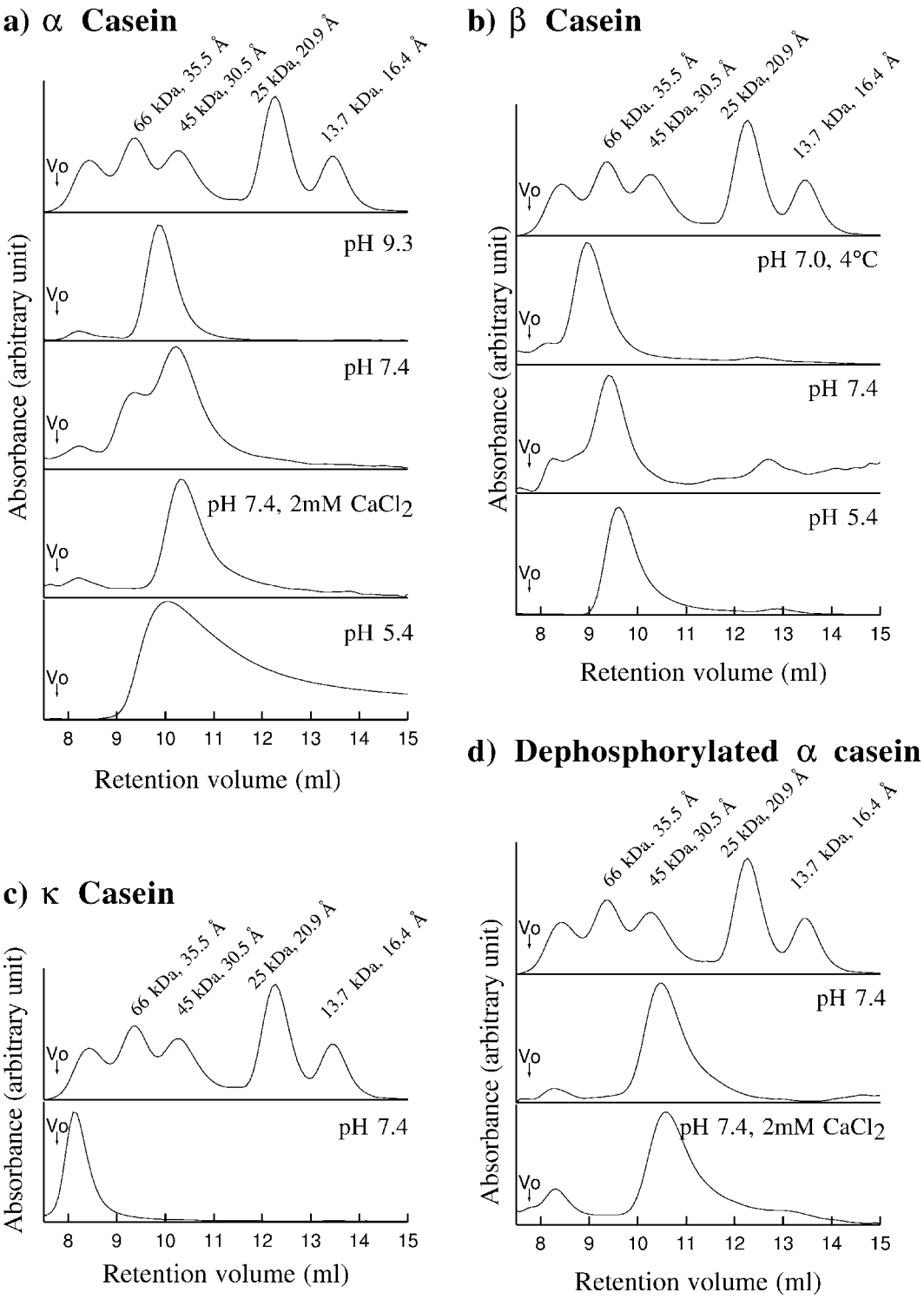

Figure 1. Oligomerization state of caseins. Analysis of purified caseins by gel filtration chromatography: (a) α_s-caseins; (b) β-casein; (c)κ-casein; (d) dephosphorylated α_s-caseins. Each protein was applied at a concentration of 0.1 mg/ml to a Superdex 75 HR10/30 column preequilibrated with the buffer. The buffers used were: 10 m*M* Tris, 150 m*M* NaCl, pH 9.3, at 25°C; 10 m*M* HEPES, 150 m*M* NaCl, pH 7.4, at 25°C; 10 m*M* HEPES, 150 m*M* NaCl, 2 m*M* $CaCl_2$, pH 7.4, at 25°C; 10 m*M* Na Acetate, 150 m*M* NaCl, pH 5.4, at 25°C; 10 m*M* Tris, 150 m*M* NaCl, pH 7.0, at 4°C. For each panel, the top chromatogram corresponds to the molecular weight standards (BSA, ovalbumin, chymotrypsinogen A, and ribonuclease A).

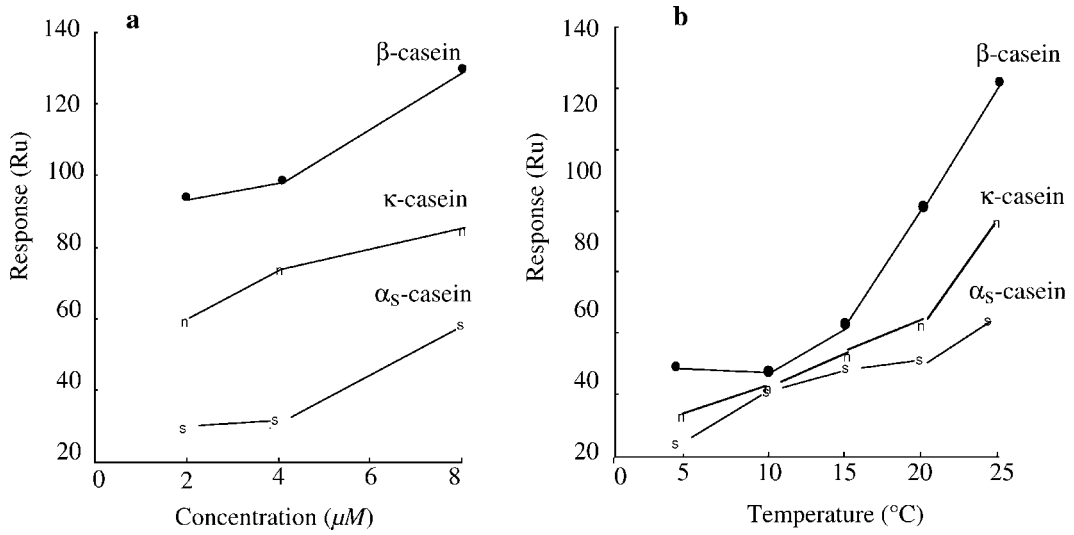

Figure 2. Binding response (Ru) of α_s-, β-, and κ-caseins on an HPA sensor chip. (a) Effect of α_s-, β-, and κ-casein concentrations on the binding response at 25°C. (b) Effect of temperature on the binding response of 8 μM α_s-, β-, or κ-casein.

Interaction of Caseins with an HPA Hydrophobic Surface: Determination of the Apparent Hydrophobicity

We used a new method to compare the apparent hydrophobicity of α_s-, β-, and κ-caseins. We determinated their binding level on the hydrophobic matrix of an HPA sensor chip using BIACORE. Because uncoated HPA chips are known to bind many proteins and especially bovine serum albumin (**BSA**; about 900 Ru were recorded after injection of 50 μl of 0.1 mg/ml BSA on the surface), we determined the minimal amount of detergent in the running buffer and in the sample buffer necessary to prevent BSA (taken as a test protein) adsorption. P20 (0.005%) was sufficient to suppress BSA association (data not shown) but allowed a significant degree of hydrophobic binding (30 to 150 Ru) of the three groups of caseins (Figure 2a); β-casein presented a higher binding level than κ-casein, which was itself higher than α_s-caseins at all concentrations tested. The SPR binding response as a function of temperature is reported in Figure 2b. At 10°C, the Ru values of each casein were lower than at 25°C and not significantly different from each other. When the temperature rose above 20°C, the differences in SPR responses between caseins increased with a maximal effect of temperature for β-casein.

Interaction of Soluble Caseins with Immobilized Casein

To study casein/casein interactions, each group of casein (α_s-, β-, and κ-casein) was immobilized by the ε-amino group of their lysine residues on three different flowcells of a CM5 sensor chip, the fourth being used as a control. The immobilization level was the same (2000 Ru) for each casein, indicating that the protein density on each test flowcell surface was the same. The interaction of each soluble injected casein with each immobilized casein was measured. The sensorgrams of α_s-, β-, or κ-casein binding to the covalently-bound caseins, after subtraction of the appropriate controls, are shown in Figures 3a, b, and c. The soluble α_s-, β-, and κ-caseins showed different binding responses on the three immobilized caseins: the α_s-casein binding levels (Figure 3a) were lower than the β-casein binding responses (Figure 3b) which themselves were lower than the κ-casein levels (Figure 3c). In Figure 3d, the same results are presented differently to compare the binding levels of each casein on the same immobilized casein. When α_s-caseins were immobilized, there was more κ-casein bound than β-casein (132 Ru vs 35 Ru) and more β-casein than α_s-caseins (35 Ru vs 15 Ru). The same rank order was found for immobilized β- and κ-caseins. These experiments also served to study the reversibility of the binding response: Nearly the same Ru level was obtained (Figure 3d) for the binding of α_s-caseins on β-casein (30 Ru) as that of β-casein on α_s-caseins (35 Ru), whereas the binding level of κ-casein on immobilized α_s-caseins (132 Ru) was superior to that of α_s-caseins on immobilized κ-casein (26 Ru). This phenomenon was also observed for κ-casein on immobilized β-casein and is presumably due to the fact that κ-casein under these conditions was present as multimer in the flow (Figure 1c). Kinetic data and affinity constants

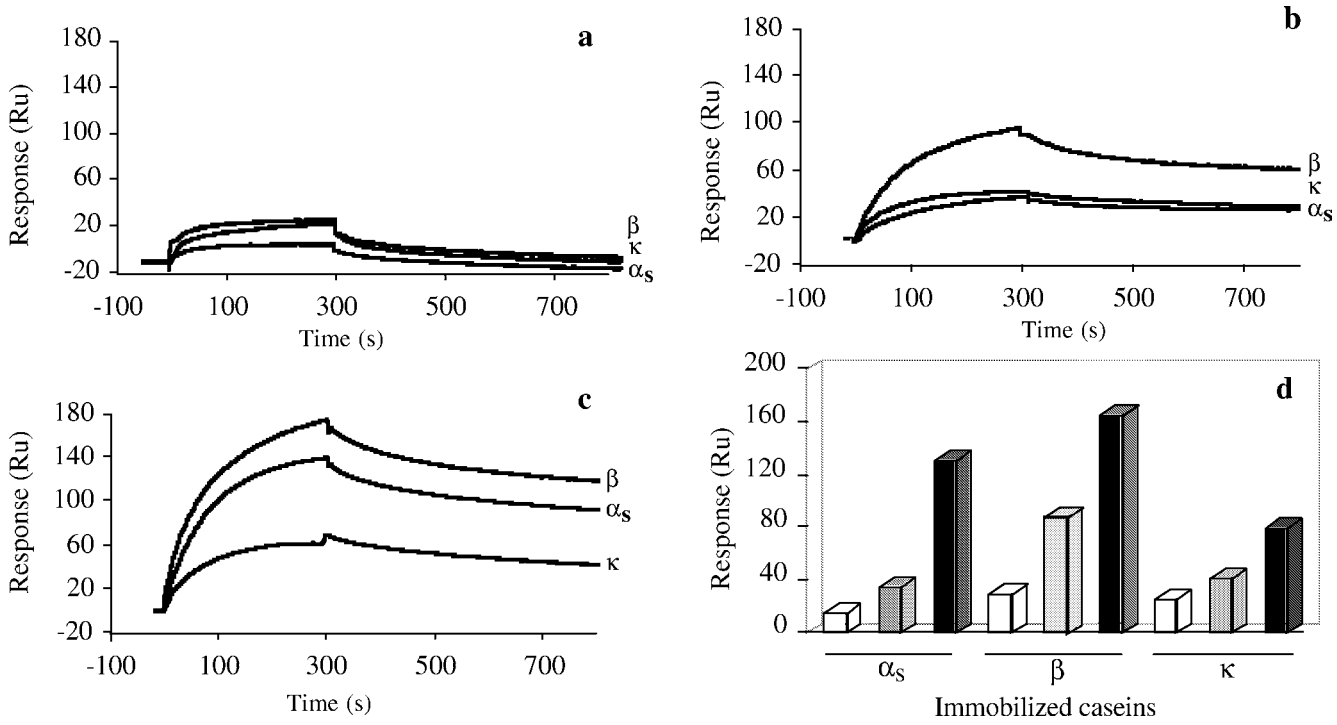

Figure 3. SPR analysis of the interaction between soluble caseins and caseins immobilized on a CM5 sensor chip at 25°C. Buffer: 10 m*M* HEPES, pH 7.4, containing 150 m*M* NaCl. (a) Sensorgrams of soluble α_s-caseins binding to immobilized α_s-, β-, and κ-caseins. (b) Sensorgrams of soluble β-casein binding to immobilized α_s-, β-, and κ-caseins. (c) Sensorgrams of soluble κ-casein binding to immobilized α_s-, β-, and κ-caseins. (d) Binding responses (Ru) at 25°C of soluble α_s-, β-, and κ-caseins on immobilized α_s-, β-, and κ-caseins on the CM5 sensor chip, α_s-caseins (white), β-casein (gray), κ-casein (black). Buffer: 10 m*M* HEPES pH 7.4, 150 m*M* NaCl.

were calculated only for α_s- and β-caseins, which are essentially present as monomers in the flow (Figures 1a and b). These constants, calculated from sensorgrams a and b (Figure 3) using a 1:1 Langmuir binding model are listed in Table 1. Globally, the K_D values were in the same range (10^{-7} *M*): The greatest association rates were obtained for the binding of soluble α_s-casein, but this binding displayed also high dissociation rates. The binding levels ($\beta > \alpha_s$) were influenced more so by the dissociation rates than by the association rates. However, the difference in the rate constants when α_s-caseins (or β-casein) were tested in soluble form or coated onto the sensor chip may indicate that coupling of protein to the chip modifies in part its conformation and, therefore, its interaction with β-casein (or α_s-casein) partner.

To further investigate the nature of the interactions during casein-casein association in relation to casein charges, the effect of pH on the binding level of α_s- and β-caseins to immobilized α_s-, β-, and κ-caseins was studied (Figure 4). Three pH values were chosen (namely, 7.4, 6.4, and 5.4) above the casein isoelectric point of pH 4.6 to avoid precipitation. The results show that decreasing the pH increased the binding of α_s-

Table 1. Kinetic data obtained by the sensorgrams of soluble α_s- and β-caseins binding to immobilized α_s-, β-, and κ-caseins.[a]

Immobilized casein	Soluble casein	$k_a \times 10^{-3}$ (M^{-1} s^{-1})	$k_d \times 10^{3}$ (s^{-1})	$K_D \times 10^{7}$ (*M*)
α_s	α_s	9.17 ± 0.20	4.61 ± 0.03	5.00 ± 0.14
	β	1.62 ± 0.04	1.03 ± 0.01	6.36 ± 0.22
β	α_s	8.42 ± 0.23	1.96 ± 0.02	2.33 ± 0.09
	β	2.09 ± 0.04	1.20 ± 0.01	5.74 ± 0.16
κ	α_s	8.92 ± 0.16	1.01 ± 0.01	1.12 ± 0.03
	β	3.15 ± 0.02	0.67 ± 0.01	2.12 ± 0.04

[a]The values in this table were calculated from the sensorgrams in Figure 3.

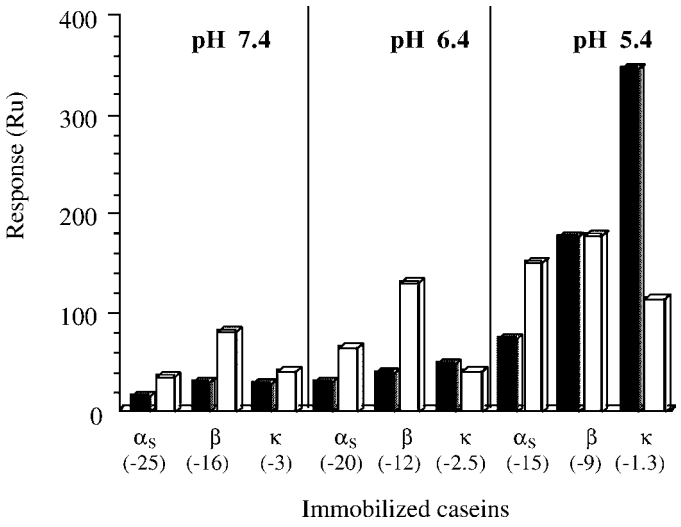

Figure 4. Effect of pH on the binding responses (Ru) of soluble α_s-caseins (■) and β-casein (□) on immobilized α_s-, β- and κ-caseins immobilized on the CM5 sensor chip. The calculated net charges of the caseins at the given pH values are presented in parentheses. The buffers used were 10 m*M* HEPES, pH 7.4; 10 m*M* HEPES, pH 6.4; 10 m*M* Acetate, pH 5.4; all buffers contained 150 m*M* NaCl. HEPES and acetate buffers gave identical results at each pH.

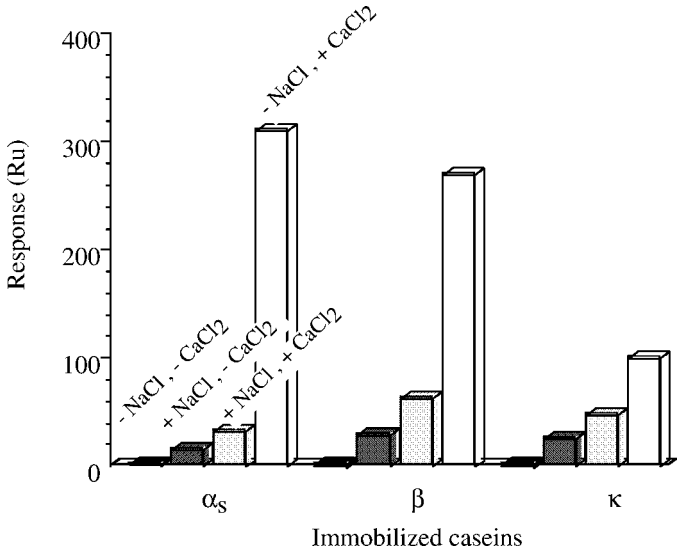

Figure 5. Effect of $CaCl_2$ (2 m*M*) and NaCl (150 m*M*) on the binding response of soluble α_s-caseins on immobilized α_s-, β- and κ-caseins on the CM5 sensor chip. Buffer: 10 m*M* HEPES, pH 7.4, without NaCl and without $CaCl_2$ (black), with NaCl and without $CaCl_2$ (dark gray), with NaCl and with $CaCl_2$ (gray), without NaCl and with $CaCl_2$ (white).

and β-casein on the three immobilized caseins, with a greater effect observed between pH 6.4 and 5.4 (Figure 4). At pH 7.4, the binding values were globally low and similar, whereas at pH 6.4 and especially at pH 5.4, the binding level of α_s-caseins on immobilized κ-casein was higher than on β-casein and α_s-caseins. These results can be correlated with the electronegative net charge of each casein calculated at the three pH values studied: α_s-caseins are more electronegative than β-casein, which are more electronegative than κ-casein; the decrease in pH decreased the global negative charge of the caseins. Between pH 6.4 and 5.4, the pH effect on the binding levels obtained for soluble α_s-caseins was generally higher than for soluble β-casein, in accordance with their global negative charges (indicated in Figure 4). The β-casein binding levels were always higher on immobilized β-casein than on α_s- and κ-caseins, whatever the pH value; this is probably due to a contributing hydrophobic effect.

The effect of calcium (2 m*M*) on the binding of soluble α_s-caseins to immobilized α_s-, β-, and κ-caseins at pH 7.4 (running and sample buffers contained 10 m*M* HEPES, 150 m*M* NaCl) is shown in Figure 5. Only a small increase (< 35 Ru) in the α_s-caseins binding levels was observed in the presence of calcium (Figure 5); but no significant effect of calcium was observed for binding of the soluble β- and κ-caseins (data not shown). Similar experiments were also carried out without NaCl in the running and sample buffers. The calcium effect on the association was notably enhanced: the addition of calcium (2 m*M*) in the absence of NaCl led to higher binding levels, whereas in the absence of calcium, the association level fell to zero. In the absence of NaCl, calcium had a greater activating effect on the binding of α_s-caseins to immobilized α_s-caseins (309 Ru) than to immobilized β-casein (271 Ru) or immobilized κ-casein (102 Ru; Figure 5). The same behavior was observed for soluble β-casein (data not shown). Since this activating effect seems to be correlated with the presence of phosphoseryl groups on casein molecules, we decided to determine the role of phosphoserine residues on casein interactions in the presence of calcium in the reaction medium. To this end, we compared the binding of dephosphorylated α_s-caseins and native α_s-caseins to immobilized α_s-, β-, and κ-caseins in a HEPES buffer, pH 7.4, with NaCl (150 m*M*) but without calcium (Figure 6). Dephosphorylated α_s-caseins gave a greater association level than did native α_s-caseins, whereas the K_D values were not significantly different (in the range of 10^{-7} *M*). In fact, dephosphorylation of α_s-caseins, which preserves the positive charge on the amino groups but abolishes the negative charges on the phosphoryl groups, led to a rise in the isoelectric point of the proteins with a decreased global negative charge. Comparatively, these experiments indicate that dephosphorylated α_s-caseins displayed an increased binding capacity which was greater for immobilized α_s-caseins (13-fold) than for β-caseins (7-fold) or κ-caseins (5-fold).

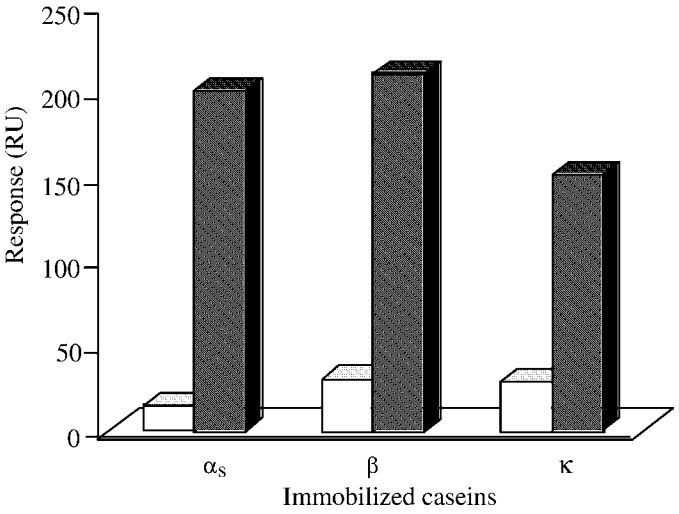

Figure 6. Binding response of soluble α_s-caseins (□) and dephosphorylated α_s-caseins (■) on α_s-, β- and κ-caseins immobilized on the CM5 sensor chip. Buffer: 10 m*M* HEPES, pH 7.4, 150 m*M* NaCl.

Concerning the calcium effect in the absence of NaCl, the sensorgrams obtained for α_s-caseins confirmed that calcium was necessary for measurable binding of α_s-caseins to immobilized α_s-, β-, and κ-caseins (Figures 7a, b, and c). In contrast, the binding of dephosphorylated α_s-caseins to α_s- and β-caseins was independent of the presence of calcium (Figures 8a and b). Only the binding of dephosphorylated α_s-caseins to κ-casein was enhanced in the presence of calcium (Figure 8c). The lack of a calcium effect on the binding of dephosphorylated α_s-caseins to immobilized α_s- and β-caseins rules out the possibility of the formation of calcium bridges between a carboxyl group of dephosphorylated α_s-caseins and a phosphoryl or a carboxyl group of the α_s- and β-caseins in this model at this calcium concentration.

DISCUSSION

The casein molecules have different degrees of hydrophobicity: α_s-caseins, although being the most hydrophilic, present hydrophobic C-terminal parts; β-casein has a predominantly hydrophobic neutral C-terminal region and a charged N-terminal peptide; κ-casein, like β-casein but slightly more hydrophilic, presents a hydrophobic neutral N-terminal region and a highly charged C-terminal peptide with only one phosphoseryl residue but several glycosylated sites (Swaisgood, 1992). It is important to quantify the hydrophobic potential of interaction between the different caseins in solution, because it is essential in the maintenance of micellar structure (Slattery et al., 1989) and represents the main driving forces involved in network formation during the enzymatic coagulation of milk (Bringe and

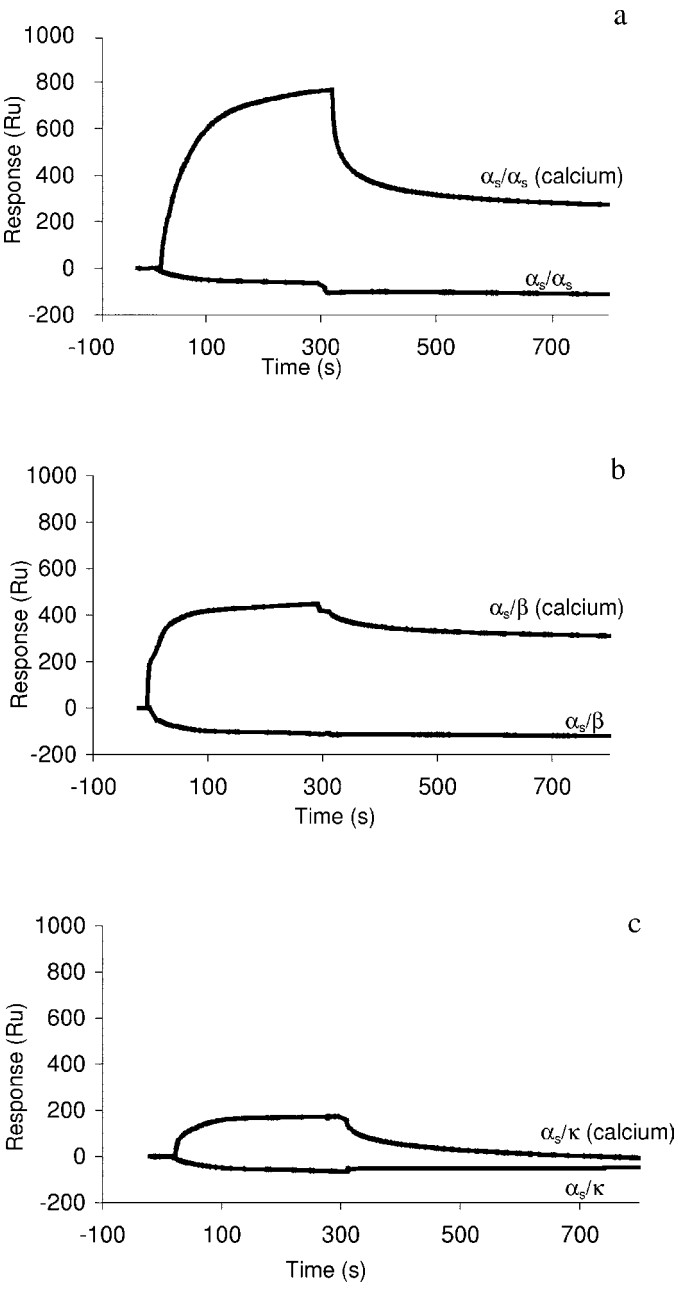

Figure 7. Effect of 2 m*M* $CaCl_2$ on the interaction of soluble α_s-caseins with immobilized α_s-, β- and κ-caseins on the CM5 sensor chip. Buffer: 150 m*M* HEPES, pH 7.4. (a) Sensorgrams of soluble α_s-caseins binding to immobilized α_s-caseins (a), to immobilized β-casein (b), and to immobilized κ-casein (c).

Kinsella, 1987). Usually, the apparent hydrophobicity of native proteins is determined by using a fluorescent probe such as 1, 8-anilinonaphthalenesulfonic acid (ANS; Hayakama and Nakai, 1985) or by using a detergent such as sodium dodecyl sulfate (Kato et al., 1984). In this work, our original approach consisted of measuring the interaction of different caseins on hydrophobic

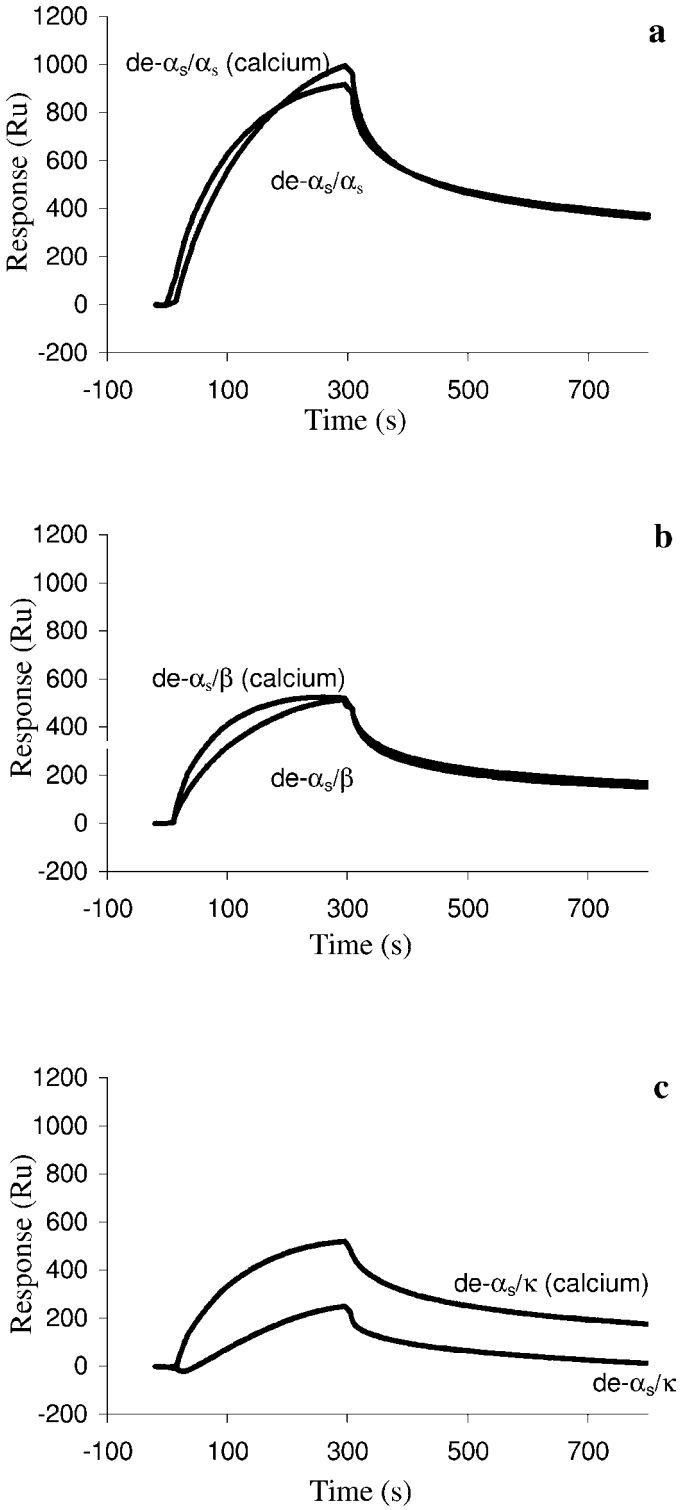

Figure 8. Effect of 2 m*M* $CaCl_2$ on the interaction of soluble dephosphorylated α_s-caseins (de-α_s) with immobilized α_s-, β- and κ-caseins on the CM5 sensor chip. Buffer: 150 m*M* HEPES, pH 7.4. Sensorgrams of soluble dephosphorylated α_s-caseins binding to immobilized α_s-caseins (a), to immobilized β-casein (b), and to immobilized κ-casein (c).

surfaces in the presence of a nonionic polyoxyethylene-sorbitan surfactant by using SPR technology.

The SPR binding levels obtained for each of the caseins were in good agreement with the range of hydrophobicity reported in the literature, i.e., 5.58, 5.12, 4.87, and 4.64 kJ per residue for β-, κ-, α_{s1}-, and α_{s2}-casein, respectively, (Fox, 1989) and with the extent of apolar amino acids in the primary sequence of each casein, namely, 72/209 for β-casein (34.4%), 54/169 for κ-casein (31.9%), 63/199 for α_{s1}-casein (31.6%), and 58/207 for α_{s2}-casein (28%). Moreover, the SPR response on the hydrophobic matrix increased with increasing temperature, confirming the hydrophobic nature of these interactions. Since the SPR analysis on HPA sensor chips was conclusive enough to discriminate between α_s-, β-, and κ-caseins, this technique might represent a new and simple procedure for a quantitative evaluation of apparent protein hydrophobicity in an aqueous environment.

Although hydrophobic interactions are important in casein/casein interactions, other forces such as electrostatic ones should also be taken into account. The differences in the sensorgrams of casein/casein interactions at neutral pH could be attributed, to a large extent, to charge differences. In fact, the sensorgrams in Figure 3 show that the highest SPR binding response at pH 7.4 was given by the least electronegative soluble casein (κ-casein > β-casein > α_s-caseins). The high binding level observed for soluble κ-casein may be due in part to the multimeric state of soluble κ-casein. For the α_s- and β-caseins, the increase of binding level was mainly related to the decrease in global negative charge and, thus, to the decrease in dissociation rates.

The decrease in pH to 5.4 probably led to neutralization of the numerous charged phosphoryl groups, enabling non-ionic interactions to occur, as shown by the greatly increased binding levels at acidic pH. This effect could be explained by the fact that the pKa value of the charged phosphoryl groups of phosphoserine is around 6 (Baumy et al., 1989). We also demonstrated that the reduction of ionic strength inhibited casein/casein interactions at neutral pH, showing that electrostatic repulsions were important at this pH. The presence of 150 m*M* NaCl slightly increased the casein/casein interactions, suggesting that the positive monovalent sodium ion at this concentration neutralized the negatively charged groups. This reduction in charge repulsions could favor nonionic forces. The replacement of NaCl by 2 m*M* $CaCl_2$ considerably increased the binding response. In fact, the divalent calcium ion acted not only as a neutralizing agent of negatively charged groups but also as a cross-linking agent between each casein. We observed that the attraction between α_s-caseins and either α_s- or β-caseins was stronger than between the

α_s-caseins and κ-casein. This could be explained by the fact that α_s- and β-caseins, which possess a Ser(P)-Ser(P)-Ser(P)-Glu-Glu cluster (Aoki et al., 1987; Baumy et al., 1989), present a site of high affinity for calcium ions; whereas κ-casein with only one Ser(P) residue, only presents a site of weak affinity.

The phosphoserine residues can also be neutralized by chemically converting them to serine residues. Dephosphorylation of α_s-caseins diminished the electrostatic repulsions at pH 7.4 and greatly enhanced their binding to the immobilized caseins, particularly to the most phosphorylated proteins (the α_s- and β-caseins). Chemical modification experiments showed that calcium participates directly in the formation of calcium bridges between the phosphate esters on casein molecules. Different authors (Byler and Farrel, 1989; Swaisgood, 1993) have proposed that the carboxyl groups of glutamic or aspartic acid could form calcium bridges between caseins within a micellar structure but at concentrations up to 2 m*M*. Without completely denying a role for the carboxyl groups, it seems that in our experimental molecular model the insertion of calcium (2 m*M*) was principally located between the phosphate groups of α_s- and β-caseins.

CONCLUSIONS

In this paper, we have demonstrated that the apparent hydrophobicities of caseins determined by a new approach using SPR technology with hydrophobic sensor chips are in good agreement with the results reported by others. Using this technology, we also characterized the casein-casein interactions at the molecular level and the factors influencing these interactions. The K_D values obtained for all the casein-casein interactions studied were in the range of 10^{-7} *M*. At neutral pH, the highest SPR binding response on the immobilized caseins was obtained for the least electronegative soluble casein ($\kappa > \beta > \alpha_s$). The decrease in protein charge, induced either by acidification, an increase in ionic strength, or phosphorylation minimized the charge repulsion and increased globally the casein-casein interactions. In the presence of 2 m*M* $CaCl_2$, casein-casein interactions were strong for the most phosphorylated caseins (α_s and β), which possess a Ser(P) cluster, the site of high affinity for the calcium ion. Experiments with dephosphorylated α_s-caseins seemed also to confirm the predominant affinity of calcium for the phosphate groups of the α_s- and β-caseins.

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REFERENCES

Andrews, A. L., D. Atkinson, M. T. A. Evans, E. G. Finer, J. P. Green, M. C. Phillips, and R. N. Robertson. 1979. The conformation and aggregation of bovine β-casein A. I Molecular aspects of thermal aggregation. Biopolymers 18:1105–1121.

Aoki, T., N. Yamada, I. Tomita, Y. Kako, and T. Imamura. 1987. Caseins are cross-linked through their ester phosphate groups by colloidal calcium phosphate. Biochim. Biophys. Acta 911:238–243.

Baumy, J. J., P. Guenot, S. Sinbandhit, and G. Brulé. 1989. Study of calcium binding to phosphoserine residues of β-casein and its phosphopeptide (1–25) by ^{31}P NMR. J. Dairy Res. 56:403–409.

Berry, G. P., and L. K. Creamer. 1975. The association of bovine β-casein. The importance of the C-terminal region. Biochemistry. 14:3542–3545.

Bringe, N. A., and J. E. Kinsella. 1987. Forces involved in the enzymatic and the acidic coagulation of casein micelles. Pages 159–194 in Development in Food Protein. B. J. F. Hudson, ed., Elsevier Applied Sciences, London, United Kingdom.

Buchheim, W., and D. G. Schmidt. 1979. On the size of monomers and polymers of β-casein. J. Dairy Res. 46:277–280.

Byler, D. M., and H. M. Farrel Jr. 1989. Infrared spectroscopic evidence for calcium ion interaction with carboxylate groups of casein. J. Dairy Sci. 72:1719–1723.

Davies, D. T., and A. J. R. Law. 1980. The content and composition of protein in creamery milks in southwest Scotland. J. Dairy Res. 47:83–90.

De Kruif, C. G. 1999. Casein micelle interactions. Int. Dairy J. 9:183–188.

Fox, P. F. 1989. The milk protein system. Pages 1–53 in Developments in Dairy Chemistry. P. F. Fox, ed., Elsevier Applied Sciences, London, United Kingdom.

Gaucheron, F., Y. Le Graet, E. Boyaval, and M. Piot. 1997. Binding of cations to casein molecules: importance of physicochemical conditions. Milchwissenschaft. 52:322–327.

Groves, M. L., H. J. Dower, and H. M. Farrell Jr. 1992. Reexamination of the polymeric distributions of bovine κ-casein. J. Protein Chem. 11:21–26.

Hayakama, S., and A. Nakai. 1985. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. J. Food Sci. 50:486–491.

Horne, D. S. 1998. Casein interactions: casting light on the black boxes, the structure in dairy products. Int. Dairy J. 8:171–177.

Jönsson, U., L. Fägerstam, B. Ivarsson, B. Johnsson, R. Karlsson, K. Lundh, S. Löfäs, B. Persson, H. Roos, I. Rönnberg, S. Sjölander, E. Stenberg, R. Ståhlberg, C. Urbaniczky, H. Östlin, and M. Malmqvist. (1991). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. BioTechniques 11, 620–627.

Karlsson, R., and A. Fält. 1997. Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. J. Immunol. Methods 200:121–133.

Kato, A., T. Matsuda, N. Matsudomi, and K. Kobayashi. 1984. Determination of protein hydrophobicity using a sodium dodecyl sulfate binding method. J. Agric. Food Chem. 32:284–287.

Payens, T. A. J. 1966. Association of caseins and their possible relation to structure of casein micelle. J. Dairy Sci. 49:1317–1324.

Pharmacia Biosensor, AB. 1994. in BIAapplications Handbook. Biosensor, ed. Pharmacia Biosensor AB Uppsala, Sweden.

Rollema, H. S. 1992. Casein association and micelle formation. Pages 111–140 in Advanced Dairy Chemistry P. F. Fox, ed., Elsevier Science Publishers., Barking, United Kingdom.

Schmidt, D. G. 1970. The association of α_{s1}-casein B at pH 6.6. Biochem. Biophys. Acta. 207:130–138.

Schmidt, D. G., and T. A. J. Payens. 1976. Micellar aspects of casein. Pages 165–229 in Surface and Colloid Science. Vol. 9. E. Matijevic, ed. John Wiley and Sons, New York, NY.

Schmidt, D. G. 1982. Association of caseins and casein micelle structure. Pages 61–86 in Developments in Dairy Chemistry—1. P. F. Fox, ed., Elsevier Science Publishers, London, United Kingdom.
Slattery, C. W., S. M. Sood, and P. Chang. 1989. Hydrophobic interactions in human casein micelle formation: β-casein aggregation. J. Dairy Res. 56:427–433.
Snoeren, T. H. M., B. Van Markwij, and R. Van Montfort. 1980. Some physicochemical properties of bovine α_{s2}-casein. Biochem. Biophys. Acta. 622:268–276.
Swaisgood, H. E., and S. N. Timasheff. 1968. Association of α_s-casein C in the alkaline pH range. Arch. Biochem. Biophys. 125:344–361.
Swaisgood, H. E. 1992. Chemistry of the caseins. Pages 63–110 in Advanced Dairy Chemistry—1: protein 2nd ed. Elsevier Applied Science, London, United Kingdom.
Swaisgood, H. E. 1993. Review and update of casein chemistry. J. Dairy Sci. 76:3054–3061.
Thurn, A., W. Burchard, and R. Niki. 1987. Structure of casein micelle. I. Small angle neutron scattering and light scattering from β- and κ-casein. Colloids Polymer Sci. 265:653–666.
Walstra, P. 1990. On the stability of casein micelles. J. Dairy Sci. 73:1965–1977.