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ARTICLE

Use of Surface Plasmon Resonance (SPR) To Study the Dissociation and Polysaccharide Binding of Casein Micelles and Caseins

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Tests were made to determine whether surface plasmon resonance (SPR) could be used as a technique to study the dissociation properties of bovine casein micelles or of sodium caseinate and the interactions between these protein particles and different polysaccharides. Surfaces of bound micelles or caseinate were made, and the changes in refractive index of these layers were used to define changes in the structures of the chemisorbed material. The technique appears to have some potential for studying details of the dissociation of casein micelles and of the binding of different polysaccharides to caseins.

KEYWORDS: Surface plasmon resonance; casein micelles; casein structure and interactions; protein−polysaccharide interactions

INTRODUCTION

The technique of surface plasmon resonance (SPR) has been developed to study the interactions of molecules attached to a surface. For example, it has been used to study antibody−antigen binding (*1*) and has also been used to quantify specific molecules present in a sample, by specific reaction with a monolayer of "acceptor" molecules such as antibodies (*2*). Interactions between molecules can also be studied, such as proteins and phospholipids (*3*, *4*), and between different proteins (*5*, *6*). This has been extended to the study of semi-intact organelles (*7*, *8*).

SPR is a phenomenon that occurs in thin conducting films deposited at the interface between two materials having different refractive indices. An example of this is a film of gold deposited on the surface of a glass plate, with the gold then in contact with an aqueous solution that has a lower refractive index than the glass. Illumination of the glass side of this sandwich at an angle of total internal reflection allows an evanescent wave to penetrate the solution to a depth of about half the wavelength of the incident light (approximately 300 nm). At appropriate conditions of angle of incidence of the light and refractive index difference between the glass and the solution, plasmons, which are electron density waves, are excited in the conductive gold film. This in turn causes a decrease in the intensity of the evanescent wave. SPR equipment measures the angle at which this decrease in intensity occurs, and for a given configuration this is directly related to the refractive index of the solution side of the gold film. The SPR equipment therefore measures the refractive index of the material on the solution side of the chip. This refractive index depends on the concentrations of materials present close to the surface, either in the solution or attached to the gold film (*9*).

Specific interactions between molecules can be measured in this way. In many uses of the technique, the surface of the gold can be modified by covalently linking specific molecules to it (*5*). Other interacting or potentially interacting molecules can be flowed over this bound self-assembled monolayer. Changes in the refractive index may arise from increases in the refractive index of the solution itself or from increasing amounts of material becoming immobilized on the gold surface (i.e., binding to the already chemisorbed molecules). This can indicate that interactions are occurring between the bound and free molecules. Under ideal conditions, quantitative binding details can be established, and in this way binding isotherms between molecules can be determined (*2*). SPR may offer the advantage over some other techniques that the complexes arising from interactions do not require separation from bulk solution.

We wished to examine the potential of this method for studying the interactions of milk proteins, specifically the caseins and casein micelles. Previous SPR studies on these proteins are limited to the quantification of the different types of caseins in milk (*10*−*12*), and studies of the interactions of the individual caseins with one another (*6*). Whereas the caseins tend to form small aggregates in solution (*13*), casein micelles are large aggregates of casein molecules, containing on average about 20000 individual protein molecules, held together by interactions with small domains of calcium phosphate (*14*) and by other noncovalent interactions, including hydrogen bonds and hydrophobic interactions (*15*). It is possible to covalently link the caseins, either in micellar or molecular form, to the gold substrate and then to attempt to react this chemisorbed layer with molecules in solution. In this paper we show that it is possible to use the SPR technique to study the progressive breakdown of casein micelles as well as the binding of different polysaccharides to micellar and to molecular layers of casein. We believe that this demonstrates the potential of the method for studying the basic interactions of these proteins.

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MATERIALS AND METHODS

Materials. Imidazole, NaCl $CaCl_2$, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 0.1 M *N*-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium caseinate (Alanate180) was provided by the Fonterra Co-operative Group Ltd. (New Zealand). The κ-casein was prepared from fresh milk in the laboratory in Guelph (Canada) using established methods (*16*). No attempts were made to separate either genetic variants or differently glycosylated forms of the protein. Carrageenans were commercial samples from Woods & Woods Pty Ltd., New South Wales, Australia; guar gum was purchased from H. B. Gum Industries Pvt Ltd., Kalol, Gujarat, India; and high-methoxyl pectin (HMP) and low-methoxyl pectin (LMP) were purchased from CP Kelco, Atlanta, GA.

SPR Equipment and Preparation of the Chips. Two instruments were used in the course of this research. They were a Biacore model SPR 3000 at the University of Reading, U.K., and a Biacore Q at Fonterra Research, Palmerston North, New Zealand (Biacore International AB, Uppsala, Sweden). The CM-3 chip design was used: this chip contains a gold surface to which short (30 nm) polysaccharide chains, terminating in a reactive carbonyl group, are attached. The reactive sites may then be activated for the chemisorption of proteins, by a standard coupling reaction (see below). The CM-3 was used in preference to the CM-5 chip usually recommended for proteins because part of this study involved casein micelles, which are large particles, for which the use of CM-3 with shorter polysaccharide chains is recommended by the manufacturer (www.biacore.com/lifesciences/products, April 2010).

In a typical experiment, the chips were inserted into the instrument and then subjected to a series of injections and wash procedures, depending on the particular system being studied. After the initial activation of the chip, protein solutions, either a 0.1% w/w solution of sodium caseinate or κ-casein or a 2.5% w/w suspension of casein micelles, were injected, allowing immobilization of some of the protein. The chemisorbed layers arising from this process were then washed with buffer and subjected to further injections of different solutions, to study their interactions. A running buffer composed of 20 mM imidazole, 5 mM $CaCl_2$, and 50 mM NaCl, pH 6.7, was used in all experiments, unless otherwise stated. A standard flow rate of 20 μL/min was used for all experiments.

As described above, the instrument measures the refractive index of the material in the adsorbed layer. Thus, as increasing amounts of material bind to the surface (either during chemisorption or subsequent reaction with the chemisorbed layer), increases in the refractive index, or the observed signal from the instrument, are observed. Conversely, a decrease in the signal will imply some dissociation of the chemisorbed material. The record of an experimental run thus consisted of a series of changes in the refractive index as different amounts of material were associated with the surface of the chip.

The temperature was controlled at the fixed value of 25 °C within the instrument.

Activation of the SPR Chip. Solutions of 0.4 M EDC and 0.1 M NHS were prepared. Just before activation of the chip, 0.5 mL aliquots of the two reagents were mixed together. The surface of the SPR chip was then activated by passing 2 × 250 μL injections of the mixture over the surface. The chip was then washed with the running buffer. Because of the design of the instrument, running buffer was also passed through the system between injections. Generally, there was little change in the chip surface between the two injections of EDC/NHS, showing that activation was essentially complete after the first injection.

Preparation and Binding to the Surface of Casein Micellar Suspensions. To bind the maximum quantity of native casein micelles to the chip surface, it is not appropriate simply to inject skim milk over the activated chip. The milk contains not only the casein micelles but also whey proteins in nonaggregated form (*17*), and so injection of milk would result in a chemisorbed layer composed mainly of whey proteins because they are much smaller and diffuse more rapidly to the surface. Therefore, we prepared whey-protein-free micellar suspensions. Samples of fresh milk were obtained from the morning milking at the Fonterra dairy farm. After skimming by centrifugation at 5000*g* for 10 min and removing the fat layer, samples of the micellar fraction were harvested by centrifugation at 25000*g* using a Sorvall RC5C centrifuge and a FiberLite F21−8 × 50 mL rotor. The pellet of micellar material was collected and the supernatant serum discarded. Protein-free permeate from the same milk was prepared at room temperature using a laboratory ultrafilter (Prep-Scale-TFF 1 ft^2 cartridge PTGC, 10 kDa cutoff, Millipore Corporation, Billerica, MA, USA). The micelles were resuspended in this permeate to their original concentration in the milk, using gentle stirring. It has already been shown that casein micelles suspended in this way form chemisorbed layers on gold surfaces activated with EDC/NHS (*18*−*20*).

To bind casein micelles to the activated surfaces, 2 × 250 μL injections were made of the prepared suspension of casein micelles once the chip surface had been activated with EDC/NHS. The running buffer had been selected because it is one that is known to maintain some stability in suspensions of diluted casein micelles. Ideally, permeate should have been used as a running buffer, but because of its high lactose content, it has a high refractive index (as will be seen in **Figure 6**), and we preferred using the low refractive index imidazole buffer. In many experiments using SPR, it is recommended to wash the chip with ethanolamine solution after protein adsorption, to deactivate any unused EDC/NHS sites. However, we did not do this because the effects of ethanolamine on casein micelles are not known, and we did not wish to modify the bound material. It should be pointed out that we did not find any evidence that dissociated proteins did bind to unused EDC/NHS sites after desorption, nor was there any evidence, from specific experiments, to suggest that either imidazole or urea bound to the activated chip surface.

Binding of Sodium Caseinate to the Activated Chip. Solutions (0.1%) of sodium caseinate were prepared in the imidazole/$CaCl_2$/NaCl buffer. Although it is known that the buffer will cause some aggregation of the caseinate, it was not sufficient to make it precipitate at the concentrations used. To make the chemisorbed layer of caseinate, three successive 250 μL injections of the solution were made over the activated chip surface.

Interactions of the Chemisorbed Protein Layers. Once the casein micelles or sodium caseinate had been bound to the surface, the chip was washed with running buffer, and then one or more reactive or nonreactive agents were injected in sequence. In most cases, 1 × 250 μL aliquots of different solutions, to be described later in the text, were injected, after which running buffer was again passed over the chip. In some cases, two injections were used to ensure that complete reaction had taken place. The solutions used were 6 M urea in 20 mM imidazole/$CaCl_2$/NaCl buffer, 10 mM EDTA in 20 mM imidazole, 50 mM NaCl, pH 6.7, and 0.5% sodium dodecyl sulfate (SDS) in water. Solutions (0.01%) of the polysaccharides κ-carrageenan, λ-carrageenan, and ι-carrageenan, guar gum, HMP, and LMP were made up by weighing out the appropriate amounts of polysaccharide, dispersing in the imidazole/$CaCl_2$/NaCl buffer, and then heating to 75 °C for 10 min in a water bath before cooling to room temperature.

RESULTS AND DISCUSSION

Binding of the Proteins to an Unactivated Chip. To ensure that we were seeing the reactions of truly chemisorbed material, a series of injections of protein was first made on an unactivated chip (i.e., not treated with the EDC/NHS mixture). This shows the amount of nonspecific adsorption of the protein on the chip surface. The results are shown in **Figure 1**. The first signal, with a value of about 14500 units, was the baseline value obtained when the interface was empty and running buffer was being passed over it. Each time casein micelles were injected, the observed refractive index became very high because of the high refractive index of the micellar suspension. However, after the addition of casein micelles and washing with the running buffer, the equilibrium signal increased to about 15500 units because of nonspecific adsorption of the casein micelles, an increase in signal strength of about 1000 units. Injection of 6 M urea partly disrupted this adsorbed material, but not fully, because 6 M urea does not completely disperse casein micelles (*21*); however, subsequent injection of SDS did clean all of the protein from the surface, showing that some hydrophobic effects were important in the adsorption of the protein.

The amount of nonspecifically bound sodium caseinate was seen to be very much less than that of the casein micelles, shown by the low values of the signal between 3000 and 4500 s of

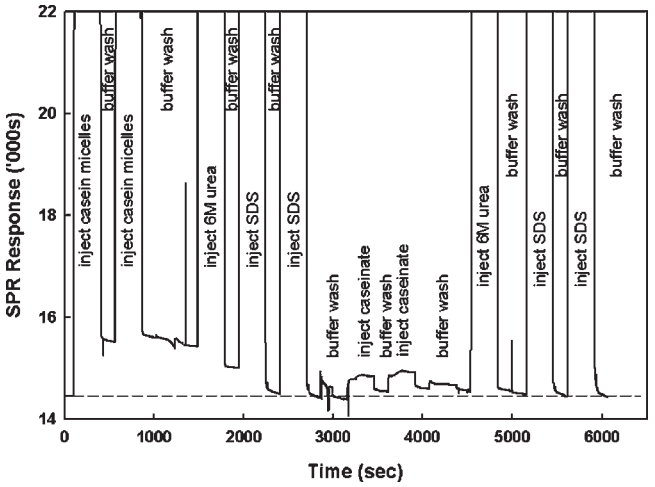

Figure 1. Test of nonspecific binding of casein micelles or sodium caseinate to the unactivated surface in a CM-3 chip. The figure shows the different injections of solutions on to the chip, and the response of the instrument after each injection. The height of the trace shows the refractive index; during injections, this shows the refractive index of the solution being injected. The value between injections relates to the amount of material. The broken line shows the original level of the untreated chip.

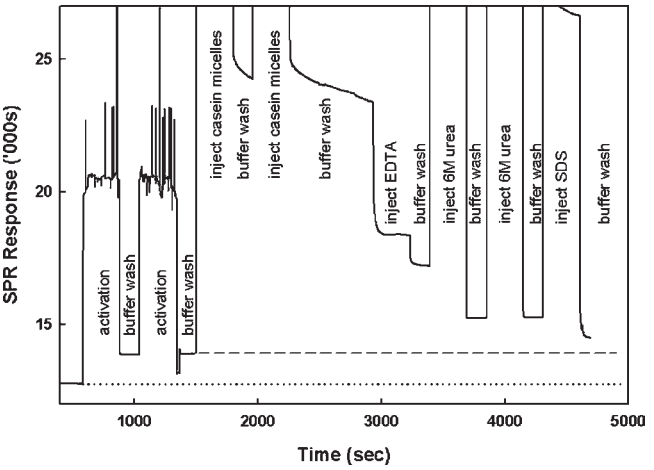

Figure 2. Binding of casein micelles to an activated Biacore CM-3 chip and their dissociation by EDTA, followed by 6 M urea, followed by SDS. The individual steps are labeled. The dotted line shows the original value for the unactivated chip, and the broken line shows the base value of the activated chip with no protein bound to it.

Figure 1; this small amount of adsorbed protein was also removed by 6 M urea followed by SDS. In comparison with the amounts of protein adsorbed to the activated surfaces (see later sections), the amount of nonspecific binding was not large.

Tests were also made of all of the polysaccharides used in the experiments to determine whether they bound to the chip surface (results not shown). No binding of any of the molecules was seen to either unactivated or activated chips. This is as expected, because the polysaccharides that were used have little hydrophobic character and lack amino groups by which to bind to the activated surfaces of the chips.

Binding and Dissociation of Casein Micelles. The chemisorption from a suspension of casein micelles to a surface that had been activated by EDC/NHS is shown in **Figure 2**. The changes in the surface during activation are shown, and the binding of the EDC/NHS product to the chip is shown by the increase in the signal after the injection of the mixed reagent. Subsequent injection of casein micelles caused a large increase of the signal strength, much larger than that found in the nonspecific binding. Washing with buffer caused a slow decrease of this signal, partly presumably because of the removal of small amounts of nonspecifically bound material trapped in the micellar layer. However, an increase of about 10400 units was observed as the casein micelles attached to the surface. This can be compared with the observation in **Figure 1**, where the amount of nonspecifically adsorbed casein micelles gave a signal of about 1000 units.

It is well-known that casein micelles are dissociated in the presence of EDTA, which chelates the calcium from the calcium phosphate that is an essential factor in holding the micellar structure together (*14*), and we used treatment with EDTA to confirm that it was indeed casein micelles, rather than simply casein molecules, that had chemisorbed to the chip surface. Washing of the bound layer of casein micelles by a solution of 10 mM EDTA caused an immediate decrease in the signal intensity of the protein particles by about 60%, from 10400 to 3300 units. This shows the removal of the calcium phosphate from the casein micelles by the chelating agent causes the micellar structure to break up. However, there was still a considerable amount of protein remaining attached to the chip surface, so that the EDTA did not completely dissociate the casein micelles. It is known that caseins in the micelles are held together by a mixture of forces (*15*, *22*), of which the interaction with calcium phosphate (*23*) is only one. The incomplete dissociation is therefore expected, because EDTA treatment will leave some aggregates linked to the chip.

Further dissociation of the remaining protein layer was caused by the injection of 6 M urea, which caused the signal intensity to decrease from 3300 to 1300 units. Again, this is to be expected, because it is known that high concentrations of urea disperse protein aggregates such as the complexes remaining after dissociation of the casein micelles with the EDTA. However, it seemed that even 6 M urea was not completely successful in dispersing the casein: treatment with SDS further decreased the signal attributable to the protein from 1300 to 670 units. This final signal showed that some molecular casein remained chemisorbed after the bound micelles had been completely dissociated. These molecules are presumably those by which the casein micelles were originally linked to the substrate. It is probable that the chemisorption of casein micelles does not give a close-packed monolayer, because the final signal is considerably less than the value of about 2000 units for a monolayer of casein (see below). Use of a similar activation and binding technique has been used to prepare samples for electron microscopy (*19*, *20*), and in these the casein micelles appear to be well-spread rather than tightly packed, either because other materials (small peptides) bind from the serum or because of intermicellar repulsive forces that prevent close packing of the particles. As the casein micelles bind to the chip, only a few protein molecules on the surfaces of the particles can be covalently attached to the surface. The remaining molecules in the particle are either in its interior or on its surface, but are prevented by its size from interacting with the surface of the chip. Therefore, the complete breakup of the micellar structure is likely to give a very thinly dispersed amount of casein on the chip surface, hence, the rather low signal at the end of the experiment.

A similar experiment is shown in **Figure 3**. Activation and chemisorption of casein micelles took place as before (in this and subsequent figures the activation step is not shown), but then 6 M urea was added as the first dissociating agent. This gave almost complete dissociation of the casein micelles, as shown by the considerable decrease of about 87% in the signal attributable to

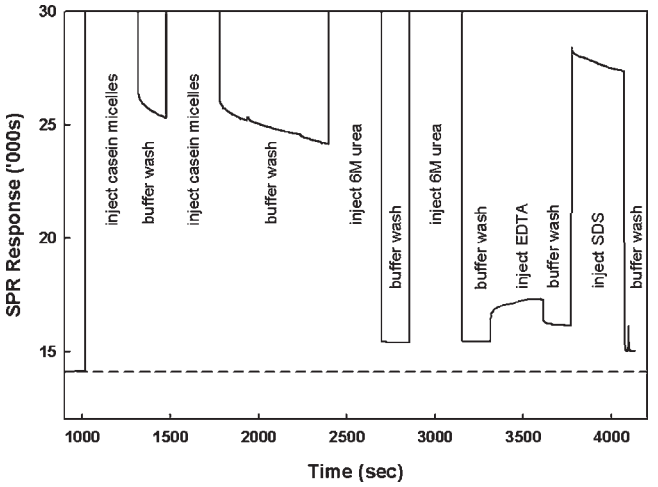

Figure 3. Binding of casein micelles to an activated chip and their dissociation with 6 M urea, followed by EDTA, followed by SDS. The individual steps are labeled. The broken line shows the base value of the activated chip. The activation process is not shown.

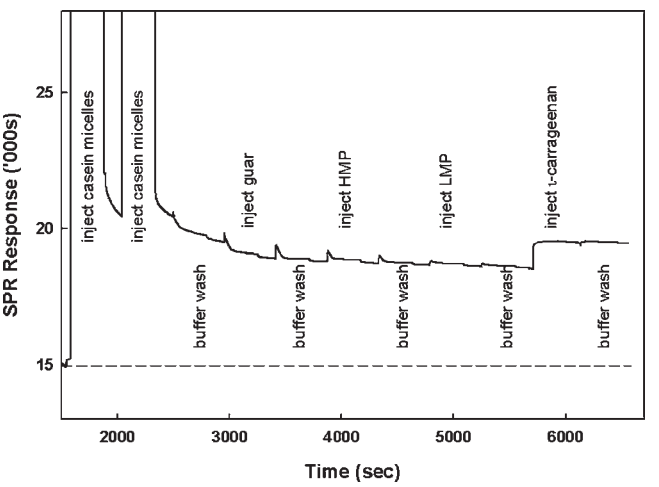

Figure 4. Effects of successive injections of 0.250 μL aliquots of 0.01% w/w solutions of guar gum, high-methoxyl pectin, low-methoxyl pectin, and ι-carrageenan to an immobilized layer of casein micelles. The broken line shows the base value of the activated chip.

the protein, from 10100 to 1300 units. The dissociation was not complete, however, because treatment with SDS lowered the signal still further. Urea does not dissolve the calcium phosphate of the casein micelles, but dissociates the micelles into their individual calcium phosphate nanoclusters (*21*), which will then be removed by washing. Thus, the material remaining after urea treatment is a layer of nanoclusters derived from the proteins close to the micellar surface, and not a layer of monomolecular casein. When EDTA was added after the urea; a permanent increase in signal was seen, suggesting that some material was binding to the surface. This could be the EDTA itself, binding either to the caseins or nonspecifically to the chip surface. It appeared to be removed by the SDS wash, which left a signal intensity of 910 units for the remaining protein. Separate experiments showed that EDTA itself did not bind or nonspecifically adsorb to the chip surface, so the effect was most likely due to a change in the conformation of the chemisorbed layer of protein. In this experiment, as in **Figure 2**, the residual amount of casein after the final dissociation of the remaining material with SDS was small.

Binding of Polysaccharides to Casein Micelles. Polysaccharides are important in many dairy foods, as texturants or to promote or minimize depletion flocculation effects. Of these polysaccharides, some (e.g., the carrageenans) are known to bind to casein micelles (*24*), whereas others (e.g., guar gum) are believed not to interact directly (*25*). To determine whether it was possible to look at the interactions in more detail, we studied the effect of the addition of different polysaccharides to a layer of casein micelles attached to the chip surface. Preliminary experiments showed that there was no binding of any of the polysaccharides used in this study to the chip surface, either before or after activation with NHS/EDC. **Figure 4** shows the effects of sequential additions of guar, HMP, LMP, and ι-carrageenan to the immobilized micelles. It is clear that neither guar, HMP, nor LMP had any tendency to bind to the micelles under the particular buffer conditions used in the experiment. Apart from a small change in signal at the time of injection, the buffer wash reduced the signal to its original value. It is interesting to note that the trace tended to go down only slowly, reflecting the relative stability of the layer of casein micelles under these conditions, so that the imidazole/$CaCl_2$/NaCl buffer is adequate to maintain micellar stability over the approximately 2 h span of the experiment.

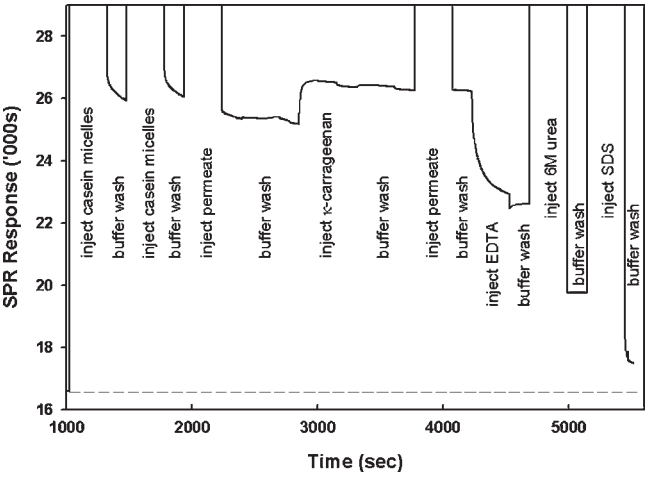

Figure 5. Binding of κ-carrageenan to immobilized casein micelles and subsequent dissociation with EDTA, followed by 6 M urea, followed by SDS. The broken line shows the base value of the activated chip.

Injection of 250 μL of 0.01% ι-carrageenan increased the signal permanently, by about 1000 units, demonstrating that the polysaccharide binds to the chemisorbed casein micelles. The first injection was enough to saturate the casein, because a second injection of polysaccharide did not further increase the signal. However, this shows that the binding of polysaccharide to the casein micelles can be detected and, in principle, quantified.

Similar results were observed for the binding of λ-carrageenan (not shown) and κ-carrageenan (**Figure 5**) to the immobilized casein micelles. In all cases, the carrageenans caused an increase in the signal of about 1000 units that was not removed by washing with buffer, indicating strong binding of the polysaccharide to the casein micelles. It should be noted that calcium was present in all of the solutions of polysaccharide, so that, for example, the κ-carrageenan would be in its helical form (*26*). The binding of carrageenan did not seem to greatly affect the dissociation of the micelle by EDTA followed by 6 M urea and SDS (**Figure 5**). The injection of EDTA onto the casein/carrageenan complexes reduced the signal of the protein/polysaccharide complex by 38%, substantially less than was seen with the uncomplexed micelles (**Figure 2**). Treatment with 6 M urea also gave less of a decrease

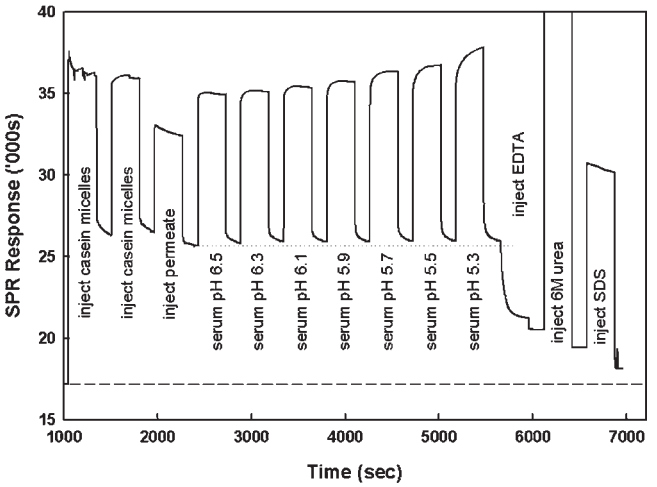

Figure 6. Treatment of bound casein micelles with milk sera of different pH values, with a final dissociation using EDTA, followed by 6 M urea, followed by SDS. The broken line shows the base value of the activated chip.

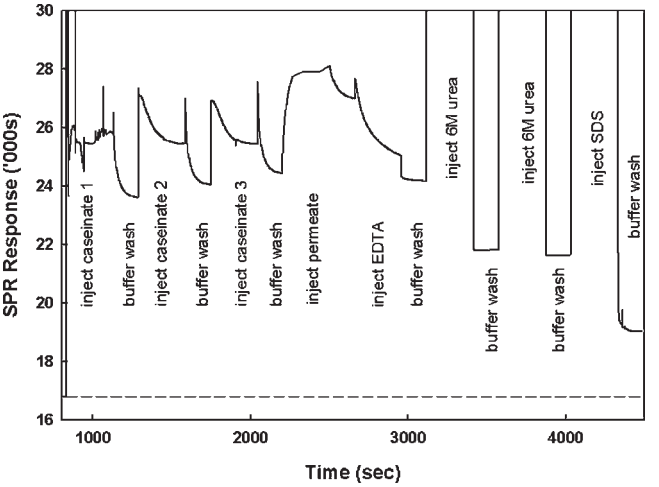

Figure 7. Binding of sodium caseinate to the activated chip surface and effects of dissociating agents. The broken line shows the base value of the activated chip.

than was found for the casein micelles on their own. However, injection of SDS again removed the bulk of the remaining casein, to give a signal of 960 units. In view of the increase found when the polysaccharide bound to the casein micelles, these results may be interpreted by assuming that even as the casein micelles partially dissociate under the influence of EDTA and urea, some carrageenan remains bound to the residual particles. Only when they are disrupted by SDS is all of the carrageenan removed.

These results are important because they give a direct demonstration that there are interactions between casein micelles and all types of the carrageenan molecules but that there are no interactions either with noncharged polysaccharides or with negatively charged polysaccharides (HMP and LMP) at neutral pH.

Acidification of Casein Micelles. The final experiment involving casein micelles bound to the SPR chip was to attempt to study the extent of their dissociation as a function of pH. For this, a range of milk sera at appropriate pH values in the range of 6.5−5.2 were prepared. Samples of skim milk were adjusted to the defined pH values by slow addition of HCl. They were then centrifuged at 25000*g* for 1 h, and the supernatant sera were collected. It is important to use sera of this type, because acidification causes the progressive release of the micellar calcium phosphate as the pH decreases. Casein micelles were prepared as normal and bound to the SPR chip using a running buffer of imidazole/$CaCl_2$/NaCl. Successive injections of the different sera were made, in order of decreasing pH, with washes with imidazole buffer between injections (these intermediate washes are obligatory in the operating protocol of the instrument, so that it is not possible to make successive injections with no intervening wash step).

The results are shown in **Figure 6**. It can be seen that successive washing with the sera in order of decreasing pH had very little effect on the amounts of micellar casein bound, because the signal between injections returned to almost the same value, showing that the casein micelles did not tend to dissociate as the pH was decreased. This constancy of the base value also showed that there was no secondary binding of the whey proteins from the ultracentrifugal sera. Interestingly, the refractive indices of the sera themselves were seen to increase as the pH dropped (as shown by the maximum values attained during the injection period). A possible explanation of this increase is that the refractive indices of the sera are modified by the release of calcium and phosphate from the casein micelles in the milks during the acidification used to produce the sera. Therefore, each serum is enriched in soluble calcium and phosphate as the pH drops (*27*). Whether this is the entire reason for the increase in the refractive index of the sera remains to be demonstrated.

Even after being subjected to serum at pH 5.3, the bound micelles were intact, although they must have been depleted in calcium phosphate. Calcium still played a part in maintaining the structure of the particles, however, because treatment with EDTA at the end of the experiment once again caused significant disruption. The interim washes with the neutral calcium-containing buffer presumably caused the formation of a layer of calcium-linked casein, which was then disrupted by this final treatment with EDTA. As normal, urea followed by SDS caused final dissociation of this layer. The results on the apparent stability of the casein micelles to acid treatment are in agreement with observations of acidified milks, that little dissociation of casein takes place during acidification as long as the temperature is maintained at or above 20 °C (*28*).

Binding of Sodium Caseinate to an Activated Chip. The binding of sodium caseinate dispersed in the imidazole/Ca/NaCl buffer to the NHS/EDC activated surface is shown in **Figure 7**, and it shows important differences from the behavior of the suspension of casein micelles. The injection of the solution of 0.1% sodium caseinate gave a large increase in signal, almost as large as that given by the casein micelles, demonstrating that there were considerable amounts of protein bound to the chip surface. This material must be covalently bound, because the signal is much higher than the very small signal obtained from nonspecifically bound caseinate (**Figure 1**). Additional injections of caseinate show small increases in the amount bound. Injections of EDTA solution (to break up any calcium-induced complexes of the casein) did not cause a large diminution of the signal, so that it seems that the layer of casein was not held together by calcium, despite Ca being present in the running buffer. Conversely, approximately half of the material was lost when urea was injected as a dissociating agent. This showed that the caseinate was not attached as a monolayer, but as a multilayer; this is in accordance with the known tendency of caseins to form aggregates (*13*). It required SDS to reduce the signal to its final value, so even urea was not capable of disrupting some of the hydrophobically linked aggregates. The signal remaining after binding and dissociation of the casein layer was considerably higher (2200 units) than that resulting from the binding and subsequent dissociation of the casein micelles (700 units): this demonstrates

that the caseinate, being in much smaller particles than the casein micelles during the adsorption process, can form a layer in which more casein molecules are in contact with the surface and can bind to the activated groups there. It may also suggest that there is no secondary attachment of material dissociated from casein micelles during their treatment with urea or EDTA; otherwise, the final response in that experiment would be closer to that of monolayers of caseinate.

The overall behavior of the adsorbed caseinate is consistent with its being originally in the form of a multilayer, held together by hydrogen bonds (disrupted by urea) and hydrophobic interactions (disrupted by SDS). This is in accord with the known tendency of the different casein molecules to form aggregates (*6*).

Binding of Polysaccharides to Adsorbed Sodium Caseinate. Similarly to the experiments described in **Figures 4** and **5**, the chemisorbed layer of sodium caseinate was treated with solutions of different polysaccharides. In general, less evidence for interaction between the adsorbed layer of protein and the polysaccharides was observed, although some small and consistent increases in the signal occurred when any of the carrageenan molecules were present, showing that small amounts of the polysaccharides were binding to the casein surface. The small size of the effect can probably be attributed to the small amount of κ-casein present in the adsorbed layer of caseins. Casein micelles have a surface rich in κ-casein, to which the carrageenans bind specifically (*29*), but in a layer of sodium caseinate, assuming that the individual caseins form a monolayer whose composition is that of the total caseinate, the κ-casein represents only about 12−15% of the total (*30*), so the number of available binding sites for the carrageenans will be much less. As with the casein micelles, other polysaccharides (guar, LMP) gave no evidence of interaction with the casein. However, the HMP gave much evidence of binding to the chip surface and could not be washed off again either by urea or by SDS. Because the HMP does not bind significantly to a nonactivated chip, it seems either that the HMP bound to the casein, which is unlikely, or that it contained some small amount of protein impurity which caused significant binding to the chip surface. This also seems unlikely, because it is expected that the caseinate would form a fairly complete layer on the surface of the chip, leaving little space for the binding of other molecules. The explanation for this observation remains unclear.

The results with the carrageenans suggest that it is not necessary for the caseins to be in their micellar form to interact with the polysaccharides. Nor does the conformation of the carrageenan seem to matter, because approximately the same response was obtained for all three carrageenans used, which are known to have different conformations (helical in the case of κ- and ι-carrageenans and coiled in the case of λ-carageenan) (*24*).

Studies with Pure κ-Casein. It is known that in its form as extracted from milk, κ-casein exists as oligomers of the basic protein, stabilized by intermolecular disulfide bridges (*31*). It will chemisorb therefore in a different way from the sodium caseinate, which may be aggregated, but in which the molecules in the aggregates are not permanently linked. The disulfide bridges in the aggregated κ-casein can be broken by treatment with mercaptoethanol.

In experiments with untreated κ-casein, considerable chemisorption was found, and the signal was only slightly diminished when 0.01% mercaptoethanol was injected to try to dissociate the adsorbed protein aggregates. Mercaptoethanol was then added to the running buffer, and this was pumped over the surface for extended periods of time. This showed that a slow dissociation of the κ-casein complexes occurred, over a period of 48 h. Conversely, direct addition of mercaptoethanol to the solution of κ-casein and to the running buffer before the chemisorption of the protein showed that the material was partly dissociated (the response on chemisorption was less than with the untreated κ-casein) but that there was still some slow dissociation over a period of 48 h.

However, both before and after mercaptoethanol treatment, the adsorbed layer of κ-casein was capable of binding all of the carrageenans, as with the casein micelles and sodium caseinate. The increase in signal intensity, however, was considerably larger than what was found using sodium caseinate, indicating more extensive binding of the polysaccharide to the protein. This could be because there is specific binding of the carrageenans to the κ-casein (*29*), so that a layer of κ-casein gives more of a reactive surface than caseinate. Alternatively, the polymeric nature of the κ-casein may give more scope for protein−polysaccharide interactions, although the similarity in the amount of polysaccharide bound in the presence and absence of mercaptoethanol suggests that the specificity of the protein−polysaccharide reaction may be more important.

The experiments overall show that the technique of SPR is well-adapted to studying the interactions of not only individual caseins but also small casein complexes (sodium caseinate) and large casein complexes (casein micelles). Previous studies on caseins using this technique are few. The most important study has been that of Marchesseau et al. (*6*), who studied the interactions of the different individual caseins using the technique. We have shown the broader application of the technique to the potential study of the interactions of casein micelles as well as of the individual proteins, and this could allow more detailed knowledge of the interactions of casein micelles with other molecules (e.g., denatured whey proteins) to be studied. We conclude that the technique has important applications which will increase our understanding of the interactions of caseins in general and casein micelles in particular.

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