Spectrofluorometric Study on Surface Hydrophobicity of Bovine Casein Micelles in Suspension and during Enzymic Coagulation

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The interactions of 1,8-anilinonaphthalenesulfonate (ANS) and Nile Red (NR) with bovine casein micelles (CM) were studied by fluorescence spectroscopy. Both fluorescent hydrophobic markers showed blue shifts of their fluorescence emission picks and fluorescence intensity enhancement, indicating their location in low-polarity regions of CM. Studies at two temperatures showed a weak interaction of high binding capacity ($K = 0.031\ \mu M^{-1}$ at 25 °C) for ANS (marker of anionic nature), probably involving hydrophobic and electrostatic components, and a strong one ($K = 36.0\ \mu M^{-1}$ at 25 °C) for NR (a noncharged molecule), clearly hydrophobic and of lower binding capacity. Comparison with the binding observed on CM disassembled in media without added Ca^{2+} pointed to the possibility that the markers permeate the porous CM structure acceding to casein molecules located into the micelles. Both markers inhibited CM enzymic coagulation, possibly by occupation of hydrophobic regions on renneted CM, thereby lowering the effectiveness of their collisions. The action of rennet in the first step of coagulation produced a decrease of about 10% of the fluorescence intensity of both bound markers, showing that a fraction of them could be located into the outer hydrophilic stabilizing layer of CM.

Keywords: *Casein micelles; enzymic coagulation; surface hydrophobicity; hydrophobic fluorescent markers*

INTRODUCTION

Hydrophobicity appears to be an important property of proteins in solution, highly related to their stability, conformation, and functionality (Kato and Nakai, 1980). The hydrophobicity of caseins plays an important role in the assembly of the complex supramolecular structure of casein micelles (CM). The models of CM currently accepted propose a core structure which is responsible for the maintenance of size and shape of the micelles (Walstra, 1990). This core involves mainly α_S-caseins held together by both colloidal calcium phosphate clusters and calcium bridges. An outer hydrophilic layer, the most important component of which is κ-casein, gives to the micelles their stability in aqueous media. β-Casein, bound into this structure mainly by hydrophobic interactions, is characterized by its mobility and could act as another kind of bridge by transference of a part of its hydrophobic moiety between submicelles (Van Dijk, 1992).

However, it is surface (effective) hydrophobicity that is considered to be one of the most important properties in connection with protein functionality. Surface hydrophobicity can be determined by several methods (Shimizu et al., 1986). Among them, the study of the interaction of proteins with fluorescent hydrophobic markers is one of the most widely used because of its simplicity and high sensitivity. Several authors have conducted studies on the changes in surface hydrophobicity of milk proteins upon thermal treatment and storage (Bonomi et al., 1988; Bonomi and Iametti, 1991) and on surface hydrophobicity of CM (Gatti et al., 1989) and renneted CM during milk coagulation (Peri et al., 1990; Iametti et al., 1993). It must be noted, however, that the markers used could show specificity for different hydrophobic regions of the protein, according to their molecular structures. Thus, comparison of the behavior of different probes, and further study of their binding characteristics, could be important to add to the knowledge of the structural and functional features of the protein studied.

The aim of this work was to study the surface hydrophobicity of bovine CM and smaller particles produced by dissociation, by interaction with two aromatic fluorescent hydrophobic markers: 1,8-anilinonaphthalenesulfonate (ANS), anionic, and Nile Red (NR), an uncharged phenoxazone dye of apolar nature (Sackett and Wolff, 1987). Information about the nature and characteristics of the binding processes was obtained for both markers by estimation of the apparent association constant (K) and the amount of marker moles bound at saturation per unit of protein concentration (n) at two temperatures. The hydrophobicity was also followed during renneting and coagulation of CM, and the kinetics of the flocculation step was studied in the presence of the markers to gain more insight into the participation of hydrophobic regions in such processes. A thorough understanding of the physicochemical bases of the coagulation process will be useful in relation to studies and design of industrial methods for the production of both traditional and new milk-based foodstuff.

MATERIALS AND METHODS

Materials. ANS as ammonium salt and NR were purchased from Sigma Chemical Co. and used without further purification. Stock solutions of nearly 50 mM for ANS and nearly 0.5 mM for NR were prepared in water and methanol, respectively, and stored in the dark at 4 °C. The concentrations of the stock solutions were determined by absorbance

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measurement, using $\epsilon = 4950\ M^{-1}\ cm^{-1}$ at 350 nm for ANS and $\epsilon = 40\ 000\ M^{-1}\ cm^{-1}$ at 550 nm for NR.

Commercial liquid rennet was a gift of COTAR S.A. (Argentina) with a strength of 100 RU, where 1 RU is the activity required to coagulate 10 mL of substrate, i.e., reconstituted low-heat skim milk powder (~10.7% w/w) in 0.01 M calcium chloride at pH (nonadjusted) ~6.35, in 100 s under the conditions specified in the IDF Standard 110A (1987).

Absorbance measurements were made on a Beckman DU 640 spectrophotometer. For fluorescence intensity measurements a Jasco F P-770 spectrofluorometer was used.

Preparation of CM Samples. Suspensions of CM were prepared from bovine commercial nonfat dried milk (MOLICO, Société des Produits Nestlé S.A., Vevey, Switzerland) by dilution at 29.0 g/L with 5 mM $CaCl_2$, skimming, and filtration through glass fiber filters (Whatman GF/A) to retain fat globules (Horne, 1986). Protein concentration was determined according to the biuret method, in the presence of sodium deoxycholate for the dissolution of the micelles. Dilutions of the CM suspensions for binding or coagulation studies were prepared in 20 mM Tris-HCl/50 mM NaCl buffer, pH 7.0 for binding or pH 6.4 for coagulation, either with 5 mM $CaCl_2$ (buffer A, micelles conserved) or without Ca^{2+} (buffer B, micelles dissociated). The dilutions were allowed to equilibrate at the working temperature for 2 h before use to reach constant turbidity values.

State of CM. The state of CM in the dilutions was followed by the measurement of the wavelength (λ) dependence of turbidity (τ) in the 400−600 nm range. It has been shown that the values of $\alpha = (\mathrm{d} \log \tau / \mathrm{d} \log \lambda)$ are related to CM size (Horne, 1986), showing a decrease for increasing values of CM average radius.

τ was measured as absorbance, and residual τ values due to fat globules yet present were measured after dissociation of CM by addition of 0.11 M EDTA/0.15 M NaOH/0.1 (v/v) Tween 20 solution and were subtracted from the raw τ values of the dilutions.

Spectrofluorometric Studies of the Binding. Since high inner filter effects are present even in highly diluted CM suspensions because of turbidity, and quenching and filtering effects also appear at low concentrations for both ANS and NR, fluorescence measurements corrected for these effects are possible only in a narrow range of CM and ligand concentrations. These restrictive characteristics of the system make impossible the application of several of the methods currently used to analyze fluorescence binding data. Nevertheless, a combination of the methods proposed by Wang and Edelman (1971) and Leskovac et al. (1993) enabled us to estimate the values of the apparent association constant, K, and the amount of marker moles bound at saturation per protein milligram, n. Working at constant CM concentration and various ligand concentrations, and assuming a single-type binding site model, Wang and Edelman obtain

$$1/\Delta F = 1/\Delta F_M + [1/(K\Delta F_M l)] \quad (1)$$

where ΔF is the difference between the relative fluorescence intensity of the ligand−CM complex (FI) and the fluorescence intensity of the sole ligand (FI_0), at a given ligand concentration l, and ΔF_M is the value of ΔF when all protein molecules are saturated by the ligand. Equation 1 results are valid when l approaches the free ligand concentration, a condition that tends to be satisfied at high ligand concentrations. Thus, this relation can be used to determine ΔF_M from the intercept at the origin, by linear regression of the initial part of the curve.

The data treatment proposed by Leskovac et al., on the other hand, enables us to obtain, with the same assumptions as for eq 1, the equation

$$(l_1 - l_2)(B_1 - B_2) = 1/A + nmA[(l_1/B_1) - (l_2/B_2)](B_1 - B_2) \quad (2)$$

where $B = [(FI/FI_0) - 1]l$ was calculated for pairs of l values (l_1 and l_2), m is the CM concentration, and $A = (\beta/\alpha - 1)$, where β is the molar fluorescence coefficient of the ligand−CM complex and α is the molar fluorescence coefficient of the free ligand. This equation enables us to determine η without extrapolation of FI values to infinite ligand concentration, which is adequate for our case.

Finally, ΔF can be expressed as a function of l

$$\Delta F = (\Delta F_M/2Kmn)\{1 + Kl + Kmn - [(1 + Kl + Kmn)^2 - 4K^2lmn]^{1/2}\} \quad (3)$$

and the values of K can be calculated by fitting the experimental data with this equation, using the ΔF_M and n values obtained by eqs 1 and 2, respectively.

Samples for spectra determination or FI measurement were prepared by adding to 3 mL of a 1/1000 (v/v) CM dilution (0.029 g/L) (buffer A or B) either the required volume of 1.66 mM ANS solution or 10 μL of NR solution of adequate concentration. After vortexing, the mixtures were allowed to equilibrate for at least 2 h at constant temperature. This equilibration step is very important to assure the total diffusion of the ligands into the micellar structure and to reach constant and reproducible FI values. Samples of the supernatant obtained after 1 h of centrifugation at 100000g of a CM suspension in buffer A were used to examine the possibility of binding of the markers to residual fat globules and soluble caseins. A 1.66 mM ANS solution was prepared by diluting ANS stock solution in the desired buffer, while NR solutions were prepared by diluting NR stock solution in methanol. FI of CM−ANS or CM−NR complexes were obtained by subtracting FI of CM dilution or CM dilution added to 10 μL of methanol, from FI of CM−ANS or CM−NR mixtures, respectively. All FI values were corrected, when necessary, for the inner filter effect by applying the relation (Rendell, 1987)

$$FI_{corr} = FI_{obs} \times 10^{0.5(A_{exc} + A_{em})} \quad (4)$$

where FI_{corr} is the corrected FI value and FI_{obs} the observed one and A_{exc} and A_{em} are the absorbances at excitation and emission wavelengths, respectively. The excitation and emission wavelengths used were, respectively, 380 and 484 nm for ANS and 550 and 623 nm for NR.

Coagulation Kinetics. The first step in enzymic milk coagulation involves the proteolysis of κ-casein to produce para-κ-casein and soluble casein macropeptides. Hydrolyzed CM in suspension become unstable with respect to precipitation, and a second step, flocculation, occurs rapidly. Working at high enzyme concentrations, the first step can be almost completed in a short time at the start of the process (Carlson et al., 1987a,b), and the overall rate of agglomeration will depend on aggregation kinetics. Since it has been shown that this process reaches limiting agglomeration states, it is accepted that the aggregation phase progresses via a mechanism whose kinetics appears to be more complex than suggested by von Smoluchowski for colloidal flocculation (Payens, 1979; Carlson et al., 1987b). However, the disappearance of primary particles to form doublets will initially follow a second-order kinetics

$$\mathrm{d}N/\mathrm{d}t = -k_2N^2 \quad (5)$$

where N is the number density of primary particles and k_2, a second-order rate constant, is determined by the diffusion rate of the particles and the efficiency of their collisions. At initial times, when $N \approx N_0$ (initial number density of paracasein micelles):

$$(\mathrm{d}N/\mathrm{d}t)_0 \approx -k_2N_0^2 \quad (6)$$

Working at constant N_0, the initial flocculation rate $(\mathrm{d}N/\mathrm{d}t)_0$ can be estimated by the initial rate of increase of the turbidity $(\mathrm{d}\tau/\mathrm{d}t)_0$ (Dalgleish, 1979; Russel et al., 1991). However, comparison between flocculation rates of samples in different conditions must take into account that $(\mathrm{d}\tau/\mathrm{d}t)_0$ involves scattering parameters of primary particles and doublets, which may changes with size variations of the particles. Although

the model here proposed for CM coagulation is only strictly valid for dilute sols, the results obtained about the basic physicochemical characteristics of the process can still be applied to more concentrated systems, as milk, provided the numerical values of the parameters are not taken as absolute.

The kinetics of CM coagulation in the presence of hydrophobic markers was studied following the turbidity of the system at 600 nm. Adequate amounts of either ANS or NR solutions were added to 3 mL samples of a 1/100 (v/v) CM dilution (0.29 g/L) in buffer A, and, after vortexing, the mixtures were allowed to equilibrate at 35 °C for at least 2 h. The equilibrated mixtures were then transferred to a 1 cm cuvette in a jacketed cuvette holder thermostated at the same temperature. To start the coagulation, 100 μL of a 1/8 (v/v) dilution of rennet was added to the cuvette. The mixture was gently stirred with a Teflon stirrer for 5 s, and the absorbance of the micelles suspension at 600 nm was recorded before rennet was added and until a maximum in the absorbance value was reached.

The concentration of rennet used was sufficient to hydrolyze casein maximally in a short time at the starting of the process, as was shown following the release of peptides soluble in 30 g/L trichloroacetic acid (TCA) (Queiroz Macedo et al., 1993) according to the Lowry–Peterson method (Peterson, 1977). A spectrophotometer was used for turbidity measurements using rectangular optical glass cuvettes with frosted lateral walls. Initial turbidity values were subtracted from turbidity values at different times, the differences ($\Delta\tau$) were plotted as a function of time, and the initial rate of increase of turbidity was graphically calculated.

The FI of the fluorescent markers was also followed during the renneting and coagulation processes to detect changes in their binding. In these cases, a 1/800 (v/v) CM dilution (0.036 g/L) mixed with the desired concentrations of the markers solutions was used. After equilibration for 2 h at 35 °C, the mixtures were transferred to a fluorescence cuvette placed in the spectrofluorometer into a jacketed cuvette holder thermostated at the same temperature, and coagulation was started by addition of 100 μL of a 1/64 (v/v) dilution of rennet. FI was recorded during the time necessary to achieve agglomeration, using the excitation and emission wavelength corresponding to each of the markers. The kinetics of coagulation in the conditions used in these cases was followed by turbidity measurements in parallel experiments.

RESULTS AND DISCUSSION

State of CM in the Presence or Absence of Ca^{2+}. τ and α values of CM dilutions showed the presence of a CM dissociation equilibrium, strongly dependent on Ca^{2+} concentration. At 5 mM Ca^{2+}, decreasing CM concentrations produced a low increment of α values (Figure 1) and an almost linear decrease of τ values. A slight dissociation process, leading to lower values of CM average diameter, could be the explanation of such results. According to Horne (1986), the average diameter diminution can be estimated as not higher than 10% of its original value.

Conversely, when CM were diluted in buffer solution without Ca^{2+}, τ values (corrected for fat globules contribution) tended rapidly to zero with decreasing CM concentration, suggesting the presence of a marked disassembly of the micellar structure at CM concentrations lower than 0.264 g/L. Dilutions of CM in the absence of Ca^{2+} were then used as samples containing soluble caseins and small aggregates.

Binding of ANS and NR to CM. Emission spectra (not shown) of ANS and NR in the presence of CM, either in media containing 5 mM Ca^{2+} or not, showed a blue shift of the emission picks of the markers and exalteration of their FI. These results are currently accepted as evidence of the insertion of the markers into low-polarity environments showing hydrophobic characteristics. Taking into account that the addition of ANS and NR at several concentrations to the supernatant obtained after 1 h of centrifugation at 100000g of a CM suspension produced negligible FI increments, the exaltation of FI observed in the presence of CM can be attributed to markers–CM binding and used to calculate binding parameters, as explained under Materials and Methods. ΔF vs l curves for ANS (Figures 2A and 3A) and for NR (Figures 2B and 3B) were well fitted by eq 3, using the values of ΔF_M and n previously calcu-

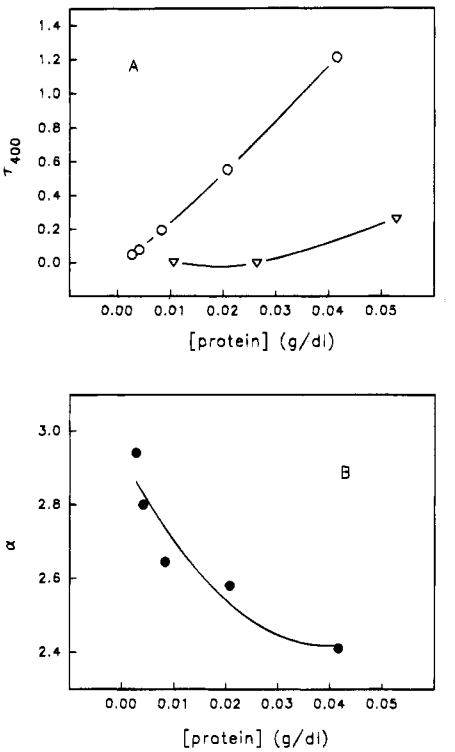

Figure 1. τ values of CM suspensions (A) and α, absolute value of the slope of log τ vs log λ plot (B), as function of CM concentration, in media with or without 5 mM added Ca^{2+}. The medium used was a 50 mM NaCl/20 mM Tris-HCl buffer system, pH 7.0, T = 25 °C. (●) α, 5 mM Ca^{2+}; (▽) τ, 0 mM Ca^{2+}; (○) τ, 5 mM Ca^{2+}.

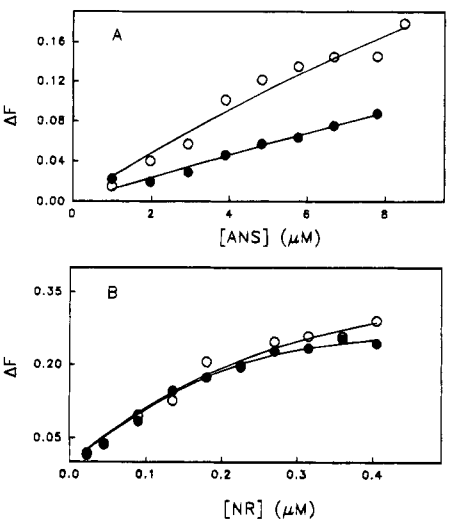

Figure 2. Fluorescence intensity increment of ANS (A) or NR (B) in the presence of CM, as functions of marker concentration. The medium used was a 5 mM Ca^{2+}/50 mM NaCl/20 mM Tris-HCl buffer system, pH 7.0. (○) 25 °C; (●) 35 °C. Points are the average of at least three determinations. Lines are fits of the experimental points by eq 3 under Materials and Methods.

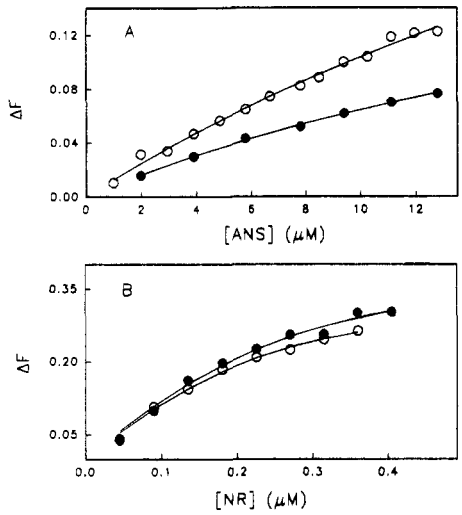

Figure 3. Fluorescence intensity increment of ANS (A) or NR (B) in the presence of disassembled CM, as functions of marker concentration. The medium used was a 50 mM NaCl/20 mM Tris-HCl buffer system, pH 7.0. (○) 25 °C; (●) 35 °C. Points are the average of at least three determinations. Lines are fits of the experimental points by eq 3 under Materials and Methods.

Table 1. Apparent Association Constant K and Micromoles of Ligand Bound at Saturation per Casein Concentration Unit Determined by Fluorescence Intensity Measurements (n and n'), at 25 and 35 °C for ANS–CM and NR–CM Interactions, either in the Presence or in the Absence of 5 mM Ca^{2+} in 50 mM NaCl/20 mM Tris Buffer Solution, pH 7.0, Protein Concentration 0.029 g/L

	temp (°C)	K (μM^{-1})	n (μmol/mg)	n' (mol/mol)
		With 5 mM Ca^{2+}		
ANS	25	0.031 ± 0.001[a]	47 ± 2	2.0 ± 0.1
	35	0.011 ± 0.001	47 ± 2	2.0 ± 1
NR	25	8.7 ± 0.7	4.0 ± 0.2	0.17 ± 0.01
	35	30 ± 3	4.0 ± 0.2	0.17 ± 0.01
		Without Added Ca^{2+}		
ANS	25	0.028 ± 0.001	31 ± 1	1.3 ± 0.1
	35	0.039 ± 0.001	28 ± 1	1.2 ± 0.1
NR	25	16.6 ± 0.8	4.0 ± 0.2	0.17 ± 0.01
	35	11.5 ± 0.7	4.2 ± 0.2	0.18 ± 0.01

[a] Standard deviations were obtained by fitting experimental data with eq 3.

lated from eqs 1 and 2, respectively. Table 1 shows the K and n values obtained. To gain more information about their stoichiometric meaning, n figures were also expressed as average bound marker/casein molar ratios (n') using for caseins an average molecular mass of 23 500 Da calculated from the molecular mass of each of the main components of the mixture (α_{S1}, α_{S2}, β, and κ), and the mixture mass composition.

The values obtained for K and n showed clear differences between the binding processes of the two markers. While CM (media with 5 mM Ca^{2+}) presented a relatively low apparent association constant and a high binding capacity for ANS, the binding of NR appeared with opposite characteristics, showing higher K values and a lower capacity of CM to bind the marker. K values for ANS–CM binding were of the same order of those reported, as dissociations constant, by Bonomi et al. (1988), despite the fact that the authors worked with raw milk and ANS concentration ranges higher than ours. When the ANS–CM equilibrium was submitted to a temperature increment of 10 °C, K showed a slight decrease (Table 1), a behavior that could be associated with weak hydrophobic interactions or with the possibility that this anionic marker, besides its hydrophobic binding, could be interacting with positive groups of the caseins by electrostatic interactions of exothermic nature. In the case of n, its constancy shows that the temperature variation used did not produce any conformational change able to modify the hydrophobic regions or binding sites detected by ANS. Increasing temperature raised K values for the NR–CM binding (Table 1), pointing to the presence of an endothermic process, a characteristic that agrees well with the assumed hydrophobic nature of the NR–CM interaction. On the other hand, the values of n remained constant at both temperatures used, confirming the results obtained for ANS.

The results displayed in Table 1 for n' indicate that ANS bound to CM at saturation amounts near two marker molecules per casein molecule. The extension of the binding could indicate that ANS permeates into the open CM structure, reaching casein molecules located inside the micelles, as has been suggested for other ionic ligands (Green, 1982). In the case of NR a lower amount, of the order of one marker molecule per five casein molecules, was bound. This difference with respect to ANS could be the result of the absence of net charge on the NR molecule, which is thus unable to produce electrostatic interactions, whether or not it has been able to permeate into CM.

The results obtained for ANS binding in the absence of added Ca^{2+} (Table 1) showed n' values that were lower than the values obtained for ANS–CM binding, suggesting the presence of binding sites associated with the micellar structure. The values of n' for NR in absence of added Ca^{2+} were the same as for NR–CM binding, showing that neither creation nor hiding of binding sites for NR was associated with CM assembly. Practically no variations were observed in K values by the variation of temperature for ANS as well as for NR.

Effect of ANS and NR on CM Coagulation Kinetics. Figure 4 shows several of the curves obtained for CM coagulation kinetics in the presence of different concentrations of either ANS (Figure 4A) or NR (Figure 4B). $(d\tau/dt)_0$ was calculated for each of the curves obtained as explained under Materials and Methods, and the values were plotted as functions of ANS (Figure 5A) or NR (Figure 5B) concentration.

Figure 5 permits one to appreciate the presence of a similar inhibitory action of both markers on the rate of the aggregation step of enzymic coagulation, as shown by a similar decrease of $(d\tau/dt)_0$. Although CM charge is one of the most important factors in CM stability (Walstra, 1990), the decrease of $(d\tau/dt)_0$ observed was similar for both markers used: ANS, of anionic nature and extensively bound to CM, and NR, an uncharged molecule. This observation could indicate that the inhibitory effect is not especially related to a CM charge variation. Since CM stability is strongly related also to voluminosity and to the thickness of the micelles' outer hydrophilic layer (Walstra, 1990), α and τ values were determined as functions of ANS and NR concentration to detect changes produced in CM average radius by the binding of the markers. The values obtained showed no variations either for ANS or for NR, indicating that the inhibitory effect is no longer related either to voluminosity or to outer layer thickness changes. The decrease of $(d\tau/dt)_0$ values could then be associated with the insertion of the markers into hydrophobic regions of the paracasein micelles surface, able to participate

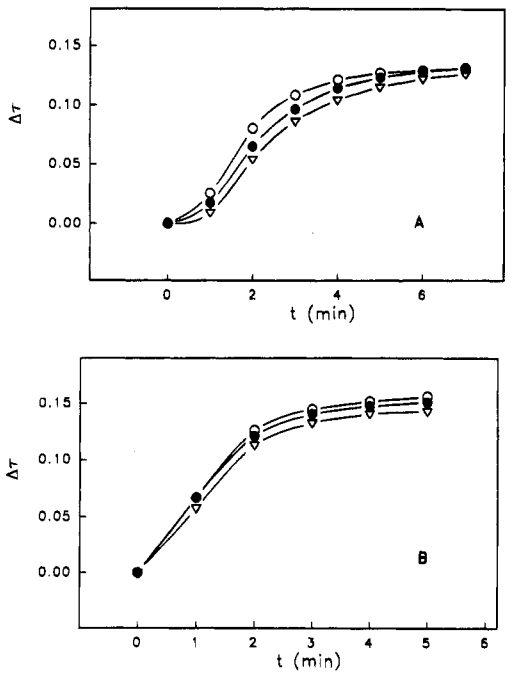

Figure 4. $\Delta\tau$ values as a function of time for enzymic CM coagulation in the presence of ANS (A) or NR (B). Casein concentration was 0.208 mg/mL. The medium used was 5 mM Ca^{2+}/10 mM Tris-HCl buffer system, pH 6.4, T = 35 °C. (A) (○) Without added ANS; (●) 4.69 μM ANS; (▽) 9.39 μM ANS. (B) (○) Without added NR; (●) 0.83 μM NR; (▽) 3.30 μM NR (0.007 v/v methanol).

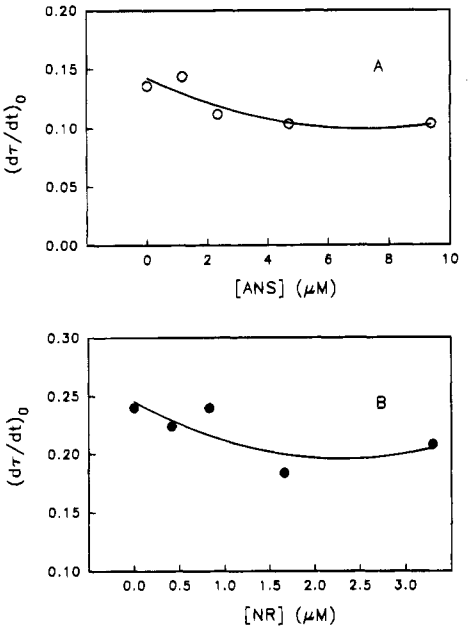

Figure 5. $(d\tau/dt)_0$ values as functions of added markers concentration. (A) ANS; (B) NR. Media and conditions are the same as in Figure 4.

in micelle–micelle interactions, decreasing in that way the probability or effective collisions between renneted micelles.

When FI of the markers was followed during coagulation (Figure 6A), an initial fall was observed for both markers upon rennet addition. This decrease could not be attributed to a release of marker adventitiously adsorbed onto the CM, as long as the marker/CM concentration ratios used are in the range of almost total binding of the marker, and an excess of the ligand to produce another kind of binding appears as improbable.

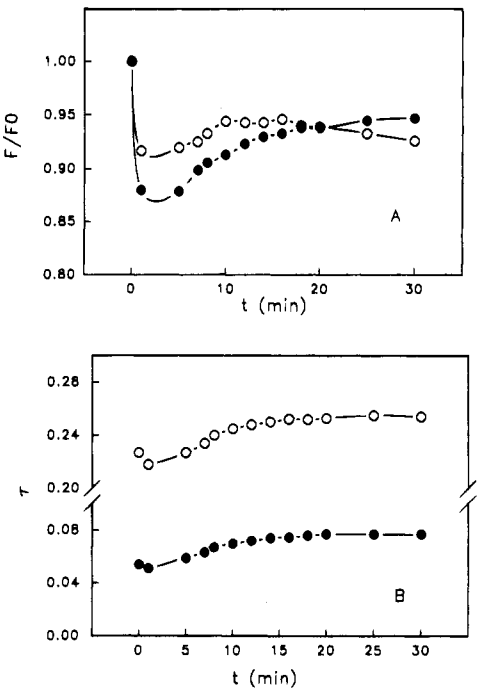

Figure 6. Relative FI (A) and turbidity values (B) as functions of time for enzymic CM coagulation in the presence of ANS or NR. Casein concentration was 0.042 mg/mL. The medium used was a 5 mM Ca^{2+}/10 mM Tris-HCl buffer system, pH 6.4, T = 35 °C. (○) 9.37 mM ANS; (●) 0.226 μM NR (0.007 v/v methanol).

On the other hand, a competition between rennet and CM for the markers seems also unlikely since no increments were observed in the FI of the markers in the presence of rennet in the concentrations used.

Comparison of parts A and B of Figure 6 shows that the observed decrease, in the order of 10% of the initial FI value for both markers, appeared during the first step of proteolysis, simultaneously with the initial decrease of τ. It could be the result of the splitting of hydrophilic moieties of κ-casein by the action of the enzyme, with abolition of part of the structure of the CM outer "hairy" layer, and thereby possible disassembly of hydrophobic structures able to locate the markers. This structural change can lead to abolition of hydrophobic binding sites with release of the bound markers or, at least, to decrease in their fluorescence quantum yield by opening of these sites and exposure to more polar environments. A rise of FI was observed during the second step of coagulation, a fact that could be caused either by an increase of the binding of the markers because of the creation of new hydrophobic regions or by increment of the fluorescence quantum yield when bound marker molecules were buried into clusters of CM of increasing structural complexity. In the case of ANS, however, there was a further decrease of FI, pointing to a probable release of bound marker, possibly by competition of the ANS–CM interaction with micelle–micelle (or cluster–cluster) interactions, during the end of the second step of coagulation. The results described above seem not to be in accord with those reported by Peri et al. (1990), pointing to the creation of hydrophobic sites during the first step of rennet coagulation. It must be taken into account, however, that the system used in each case was different. While Peri et al. studied enzymic coagulation of raw milk, we worked with CM previously isolated by heating and skimming, and in conditions in which the first step appears as practically separated from the

second one. Nevertheless, Peri et al. applied to follow ANS binding a method based on partition of the marker between the phases formed in coagulation. This procedure does not permit one to specify if ANS was bound to CM or to fat globules or merely trapped into the solid phase during coagulation and precipitation. Conversely, the method used in our work measures, through the FI enhancement, only the bound marker related to low-polarity environments.

Conclusions. Although the FI exaltation observed for both the ANS–CM and the NR–CM binding indicates the relation of the bound ligands to less polar regions of CM, the former process seems to involve an electrostatic contribution, while the second one appears as stronger, less extended, and more clearly hydrophobic. The FI of both bound markers was partially modified by the action of rennet, a fact that points to their possible partial location in the CM outer layer, in relation with κ-caseins. Despite this initial action of rennet, the inhibition of the second step of coagulation by the markers could be related to an occupancy of hydrophobic regions of the renneted CM outer layer, able to act as active sites for CM aggregation. Therefore, the results here obtained for the two markers support the participation of hydrophobic interactions in the clustering of renneted CM, a hypothesis generally proposed but with little experimental evidence in its favor. The high fluorescence remaining upon proteolysis by rennet, however, is indicative of the presence, in either the case of charged molecules as ANS or noncharged as NR, of marker molecules bound to other regions of CM. Taking into account the high CM porosity and, in the case of ANS, the extension of the binding, the idea that these regions are not necessarily in the paracasein micelles' outer surface should not be neglected. The results obtained point to the importance of knowledge about the nature and characteristic of marker binding processes in relation to their usefulness for the study of surface hydrophobicity, especially in the case of complex structures such as CM.

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