Surface Hydrophobicity Changes and Heat-Induced Modifications of α-Lactalbumin

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A study on the thermal modification of the structure of α-lactalbumin was carried out on the calcium-containing and the calcium-free forms of the protein using different techniques, namely differential scanning calorimetry, intrinsic fluorescence, and spectrofluorometric determination of changes in the binding properties of the fluorophor 1,8-anilinonaphthalenesulfonate. A modification of this latter technique was used to study surface hydrophobicity changes while the proteins were heated at a given temperature and allowed the detection and the analysis of molecular modifications in the course of the heat treatment. Some of these modifications were found to differ from those made evident by other experimental approaches. Comparison of the E_a values determined for the individual phases of the surface hydrophobicity changes in kinetic studies with those obtained by other methodologies suggests that the overall denaturation process—including the formation of aggregates and the precipitation of the protein in whey and skim milk—is related to the swelling of the protein structure in the early phases of exposure to heat.

INTRODUCTION

Bovine α-lactalbumin is a relatively small, calcium-containing protein which has been the subject of much basic and applied research. Basic research on this protein was focused on its role as a regulatory component of the lactose synthase complex and on its structural and metal-binding properties and was extensively covered in a recent review (Kronman, 1989). Applied research took advantage of the heat denaturation and/or insolubilization of α-lactalbumin for quantitating the heat damage ensuing from thermal treatment of milk (Manji and Kakuda, 1986; Mulvihill and Donovan, 1987; Dannenberg and Kessler, 1988; Duranti et al., 1991). Current knowledge states that thermal insolubilization of proteins is possibly the last step in a sequence of structural alterations, beginning with the breakdown of intramolecular bonds and the rearrangement of hydrophobic regions in the protein molecule (Kella et al., 1989).

Protein surface hydrophobicity is known to be closely related with technologically relevant properties of food proteins (Nakai, 1983; Kinsella, 1984). Recently, methods based on the use of the time-proven fluorescent hydrophobic probe 1,8-anilinonaphthalenesulfonate (ANS) were used to estimate the average surface hydrophobicity of food proteins and the changes of this parameter upon processing. Among the processes investigated by using ANS were the heat treatment of milk (Bonomi et al., 1988; Pagliarini et al., 1990) or of milk fractions (Bonomi and Iametti, 1991; Saulnier et al., 1991) as well as curd formation (Peri et al., 1990) and cheese ripening (Iametti et al., 1991).

In all of these studies, a clear understanding of the molecular basis of the observed changes in the ANS-binding properties of the complex protein systems during processing was not achieved, and investigation of a simpler, better known model seemed necessary. In this paper we compare equilibrium and kinetic data obtained by measuring protein surface hydrophobicity by using ANS with the structural and thermodynamic data obtained by using more established methodologies, such as differential scanning calorimetry (DSC) and intrinsic fluorescence on an individual whey protein, namely α-lactalbumin. In particular, heat-induced modifications of this protein were addressed. For the purpose of a possible direct transfer of this comparison to milk and whey samples, the experiments were carried out in conditions (i.e., pH and composition of the buffer) as close as possible to those typical of milk. Calcium-containing and calcium-free α-lactalbumins were used in the present study, since the equilibrium between the two forms of the protein varies as a function of the different mineral content in whey samples obtained from different cheese-making processes.

MATERIALS AND METHODS

Chemicals and Proteins. Chemicals were of reagent or HPLC grade. Buffer was 50 mM sodium phosphate, pH 6.8, containing 0.02% sodium azide. Proteins were obtained from Sigma. Calcium-containing α-lactalbumin (holo-α-lactalbumin, H-ALA, Sigma L-6010, 1–2 mol of Ca^{2+}/mol of protein) was used as such. Unless otherwise indicated, the nominally calcium-free α-lactalbumin commercially available (Sigma L-5835) was made free of variable amounts of residual metal (typically, 0.3 mol of Ca^{2+}/mol of protein) by incubating the protein with 100 mM EDTA in buffer for 30 min at room temperature, followed by removal of excess EDTA by gel filtration. The treated protein is referred to as apo-α-lactalbumin (A-ALA).

Methods. Heat-treated proteins were analyzed for the formation of aggregates by HPLC on a Superose 12 column (HR 10/30) run in 50 mM phosphate and 100 mM NaCl, pH 7.5, on a Waters LC 625 instrument.

Calorimetric measurements were performed in a Perkin-Elmer DSC7 microcalorimeter, calibrated with indium and standardized with denatured β-lactoglobulin. Samples were heated at 5 or 10 °C/min. They were cooled either at 10 or at 250 °C/min and reheated as before, when an estimate of the reversibility of the heat modifications was required.

Spectrofluorometric measurements were performed in a Perkin-Elmer MPF-2A fluorometer. Progressive heating during the measurement of intrinsic fluorescence was achieved with a regular thermostated cell holder and a circulating water bath. Equilibrium titration of protein samples with the hydrophobic flu-

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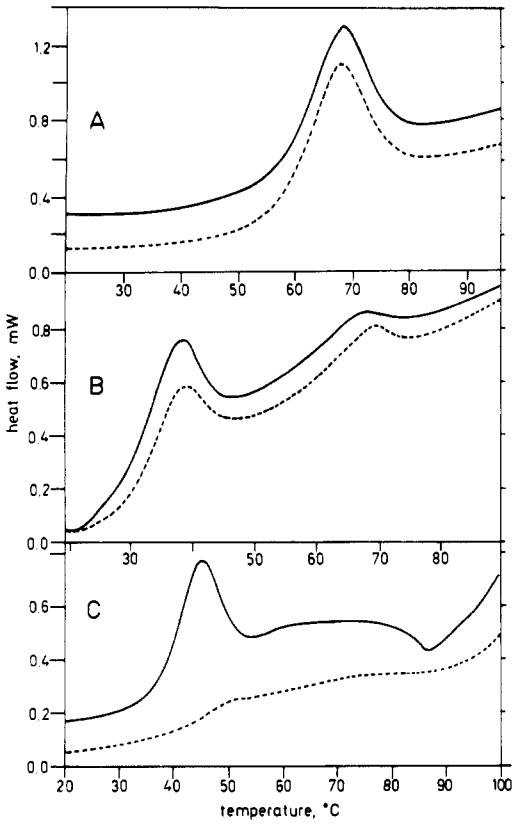

Figure 1. Differential scanning calorimetry tracings of H-ALA and A-ALA. The heating thermogram is given as the solid line and was obtained with a heating rate of 10 °C/min. The sample was cooled at 250 °C/min and reheated as before. These second thermograms are given as the dotted lines. (A) H-ALA, 4.6 mM in 50 mM phosphate buffer, pH 6.5. (B) Commercial A-ALA, 6.8 mM in water. (C) H-ALA, 4.6 mM in 50 mM phosphate buffer, pH 6.5, containing 0.2 M EDTA.

orophor 1,8-anilinonaphthalenesulfonate (ANS) was performed as described previously (Bonomi et al., 1988). Binding equilibria were analyzed with the Ruzic algorithm (Ruzic, 1982).

Measurement of fluorescence changes upon isothermal heating of mixtures of proteins and excess ANS was performed in an improved version of the home-made, electronically thermostated microcell holder already described elsewhere (Bonomi and Iametti, 1991). Either by using the clouding point of detergent solutions as an indirect test or by monitoring the sample temperature with a modified electronic device, we found that the sample reached a temperature within 2–4 °C of that of the oil bath in the microcell holder in no more than 5–8 s.

Kinetic analysis of the changes in fluorescence observed in these experiments required, in some cases, determination of the initial and final fluorescence values, which were not available from the recorded tracings. Starting fluorescence values were calculated from plots of ln (fluorescence) vs time, while fluorescence at equilibrium was extrapolated by plotting ln (fluorescence) vs the reciprocal of time. When required, these values were used to calculate fractional fluorescence variation. The apparent rate constant for fluorescence changes, k'_{app}, was calculated from standard semilog values of the fractional fluorescence variation vs time, which were derived from a set of several replicate experiments and were found to be linear for at least two reaction half-times.

RESULTS

Calorimetric Studies. The thermograms of H-ALA in the presence and in the absence of excess EDTA are shown in Figure 1 along with those of commercial apo-α-lactalbumin. A single fully reversible transition was observed for H-ALA, with T_p between 67.3 and 68.4 °C and ΔH_{app} between 283 and 383 kJ/mol. These figures

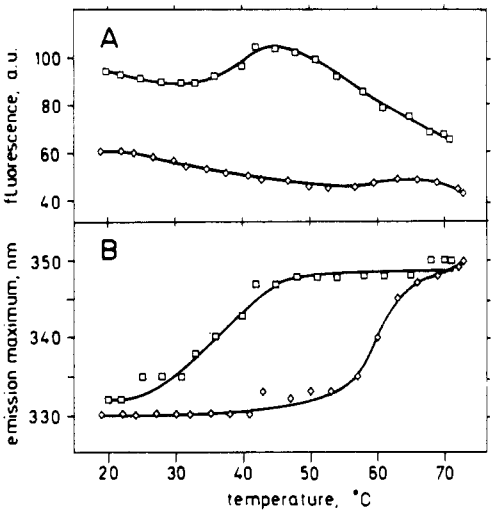

Figure 2. Temperature dependence of fluorescence parameters in α-lactalbumin solutions. Emission spectra were taken on a protein solution (1 mg/mL in buffer) progressively heated to the given temperatures and held at that temperature during the acquisition of the spectrum. Excitation wavelength was 298 nm. (Squares) A-ALA; (diamonds) H-ALA. (A) Temperature dependence of the maximum fluorescence intensity. (B) Temperature dependence of the maximum emission wavelength.

were not modified by the addition of Ca^{2+} up to a 10-fold molar excess to the protein, thus confirming previous observations about the differences in the Ca^{2+} binding sites of the protein and the significance of the binding of the metal to the higher affinity site (Lindahl and Vogel, 1984).

In the presence of a large excess of EDTA (0.2 M), H-ALA gave a single irreversible transition at 45 °C, with ΔH_{app} = 109 kJ/mol.

Commercial apo-α-lactalbumin, containing 0.3 mol of Ca^{2+}/mol of protein and dissolved in water, gave two reversible peaks, at 37–38 and 67–68 °C. Titration of commercial apo-α-lactalbumin with increasing amounts of Ca^{2+} resulted in the progressive disappearance of the low-temperature transition, so that in the presence of a stoichiometric amount of Ca^{2+} only the transition at 68 °C, which can be regarded as typical of H-ALA, was evident in the thermograms (not shown). While the disappearance of the transition at 68 °C upon addition of 0.2 M EDTA to H-ALA (or to commercial apo-α-lactalbumin, not shown) can be attributed to the disappearance of the contribution of residual Ca^{2+} ions, the lack of reversibility of the low-temperature transition and its subtle shift in T_p in the presence of the chelator are likely a consequence of a saturating concentration of sodium ions (Segawa and Sugai, 1983) or of interaction between the large excess of EDTA and the protein in the possible presence of residual Ca^{2+} (Kronman and Bratcher, 1983; Permyakov et al., 1987).

For these reasons, subsequent experiments on A-ALA were carried out with the EDTA-treated commercial protein but after the removal of EDTA by gel filtration. These samples gave thermograms identical to the ones shown in Figure 1C, which are reported elsewhere (Relkin et al., 1992a,b).

Intrinsic Fluorescence. Changes in the intrinsic fluorescence of the proteins were monitored during progressive heating from 25 to 72 °C, in a fashion similar to the heating phase in DSC experiments but at a much slower heating rate.

As shown in Figure 2A, the intrinsic fluorescence of A-ALA was higher than that of H-ALA over the tempera-

ture range considered in this study, despite its linear decrease at temperatures higher than 40–45 °C. Changes in fluorescence intensity were much less evident in the case of H-ALA, for which an increase in temperature resulted in a monotonic decrease in fluorescence.

Perhaps more informative were the shifts in the position of the fluorescence emission maximum (Figure 2B), showing for either protein a sigmoidal temperature dependence with midpoints at 35–37 (A-ALA) or 60–62 °C (H-ALA). For both of these proteins, and for H-ALA in the presence of a 10-fold molar excess of Ca^{2+}, the fluorescence emission maximum and the intensity of fluorescence returned to the original, room temperature value upon cooling. The results obtained in our experiments were found to be in close agreement to those obtained previously, respectively, for H-ALA in the presence of a 1.5-fold molar excess of Ca^{2+} and for A-ALA in the so-called Na^+ form (Permyakov et al., 1985).

Titration with ANS. Changes in the environment of aromatic residues evidenced by intrinsic fluorescence spectroscopy may modify the overall surface hydrophobicity of the proteins. To verify the occurrence and the extent of such modifications, proteins were titrated at fixed temperatures with ANS. Protein solutions were left for 15 min at the required temperature, so that structural modifications could be reasonably complete, and then titrated while hot. In a separate set of experiments, to evaluate the reversibility of any heat-induced modification in protein surface hydrophobicity, protein solutions were kept at the required temperature for 30 min, cooled, and titrated at room temperature.

Even after 15 min at 80 °C the proteins did not show evidence for the formation of aggregates when analyzed in size exclusion chromatography on an HPLC column under nondenaturing conditions at room temperature.

Results of titrations were expressed as a function of the temperature in terms of n, i.e., the number of ANS-binding sites on the surface of the protein, and of the average affinity of the protein itself toward the probe, which is quantitated by the association constant K_a. This latter parameter was found to be in the range 60–80 mM^{-1} for both proteins and did not change with temperature.

The temperature dependence of n is reported for both proteins in Figure 3. At room temperature, A-ALA showed a higher number of binding sites for ANS than H-ALA. When the temperature of titration was increased, the number of binding sites on A-ALA remained unchanged, while new sites were apparently made accessible on H-ALA. For this latter protein, prolonged heating above 80 °C resulted in a marked decrease in the number of available sites. The heat-induced modifications resulting in changes in n for H-ALA were reversible, since for this protein n returned to room temperature values upon cooling, whereas heating and cooling of A-ALA solutions resulted in a net decrease of n with respect to the untreated protein. This latter result may indicate that the unstable (but native-like) protein folding acquired at high temperature converts to a less accessible (or more compact) one upon cooling but that in this process A-ALA does not refold into the original conformation.

Isothermal Heating of Mixtures of the Proteins and ANS. In the experiments reported in Figure 4, individual mixtures of the proteins and of excess ANS were heated at constant temperature, and the fluorescence changes ensuing from altered interaction between the protein and the probe as a consequence of exposure to heat were monitored continuously from 5 to 120 s from the start of the heat treatment. DSC tracings of the ANS/protein

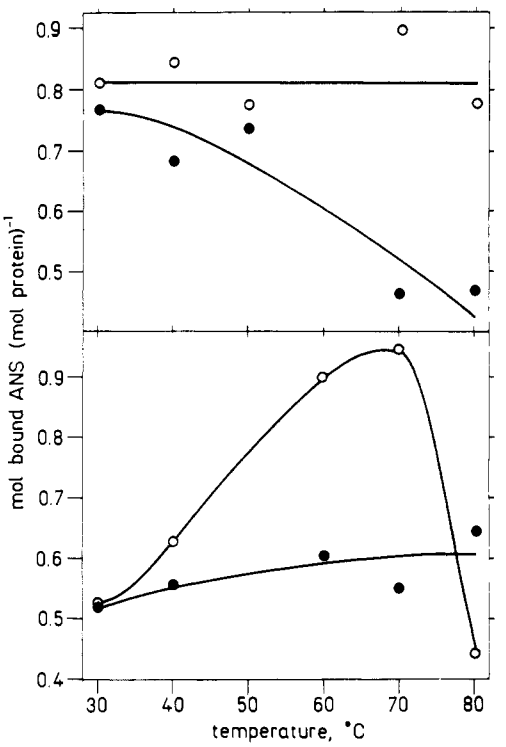

Figure 3. Temperature dependence of the number of sites available for the binding of ANS to α-lactalbumins. A solution of the appropriate protein (1 mg/mL in buffer) was heated for 15 min at the given temperature, and then it was spectrofluorometrically titrated with ANS either at the same temperature (open symbols) or after cooling at room temperature (solid symbols). Upper panel: A-ALA; lower panel: H-ALA.

mixtures used in kinetic studies were identical to those of the isolated proteins.

For both proteins, heat treatment resulted in an initial increase in fluorescence. Since excess ANS was present, this increase should mainly correspond to an increase in the number of ANS-binding sites on the protein surface, and the contribution from possible changes in the affinity of the protein for ANS should be minimized. This primary event, denoting an increased hydrophobicity of the protein surface, occurred at a lower temperature and to a larger extent for A-ALA than for H-ALA.

As the temperature was increased, a second phase in the fluorescence changes became progressively evident and was characterized by a decrease in fluorescence. Similarly to what was observed for the primary phase of heat-induced modifications, the decrease in fluorescence occurred at a much lower temperature and to a much greater extent for A-ALA than for H-ALA.

Activation energies (E_a) and transition temperatures for the different events observed in these kinetic studies were calculated from the Arrhenius plots presented in Figures 5 (primary phase, fluorescence increase) and 6 (secondary phase, fluorescence decrease). In these experimental conditions, the values of E_a derived from kinetic analysis represent an estimate of the energy associated with structural modifications. Discontinuities in the Arrhenius plots indicate the appearance of a different type of modification, which can be related to the involvement of different molecular species.

As summarized in Table I, low-temperature structural modifications, leading to an increase in fluorescence and hypothetically corresponding to the formation of a more relaxed (accessible) protein structure, showed a different value of E_a for each protein. When the temperature was increased, the relaxation process for both proteins showed

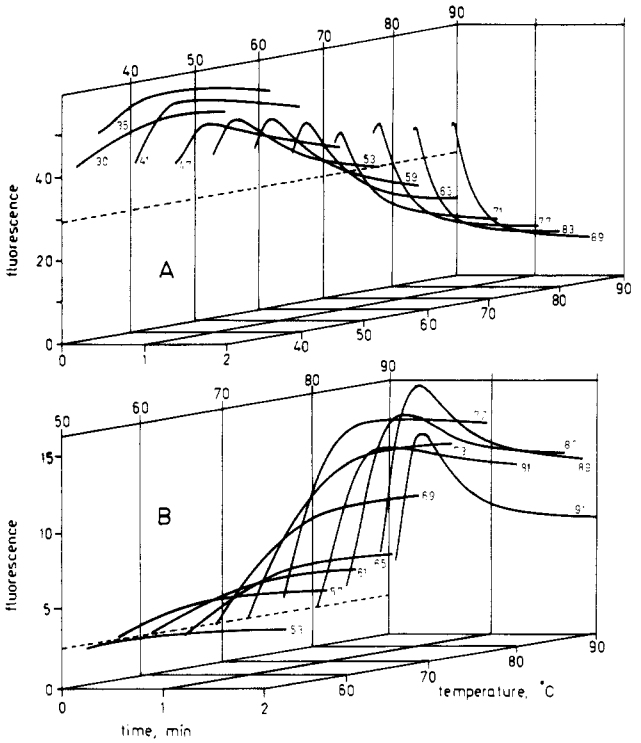

Figure 4. Time course of the fluorescence changes observed upon exposure at fixed temperatures of mixtures of α-lactalbumin and ANS. Mixtures of the protein (1 mg/mL) and 0.667 mM ANS in buffer were prepared at room temperature. Their fluorescence was measured and is indicated by the thin dotted line in the *yz* plane. Aliquots (0.2–0.25 mL) of the mixture in a 3 mm i.d. cylindrical microcuvette were then placed in the thermostated cell holder, kept at the given temperature, and the fluorescence changes were monitored over a 2-min time span. Recording started 5–10 s after insertion of the cell. (A) A-ALA. (B) H-LA.

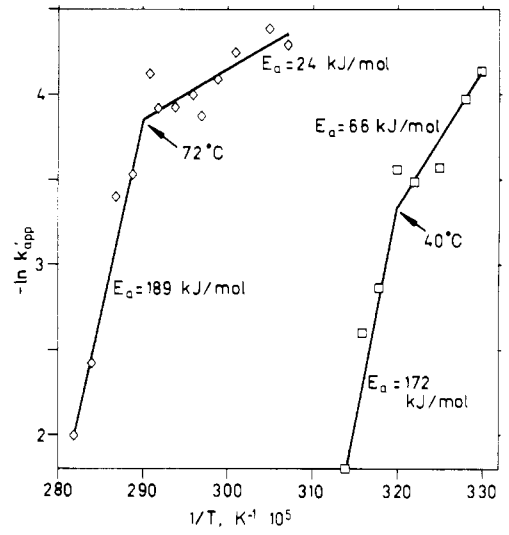

Figure 5. Arrhenius plot for the primary phase of the fluorescence changes observed upon heating of mixtures of α-lactalbumin and ANS. Data were collected from curves similar to those presented in Figure 4. Transition temperatures and E_a values are given for each protein. (Squares) A-ALA; (diamonds) H-ALA.

a marked change in E_a, and the values of this latter parameter became closely comparable. As already indicated by DSC experiments, the transition temperature at which this change occurs for A-ALA (40 °C) is considerably lower than that observed for H-ALA (72 °C).

High-temperature structural modifications, leading to a decreased fluorescence and ensuing either from the disappearance of hydrophobic patches or from their acquired inaccessibility to the probe, showed a marked difference in E_a between the two proteins. At temperatures where this event became observable also for H-ALA (i.e., above 70–75 °C), the value of E_a for this latter protein was at least twice that measured for A-ALA. The decrease in fluorescence for this latter protein shows an unexpected change in E_a around 65 °C, indicating either an alteration of the mechanism of the observed changes or the involvement of different structural species of the protein in processes occurring at this temperature.

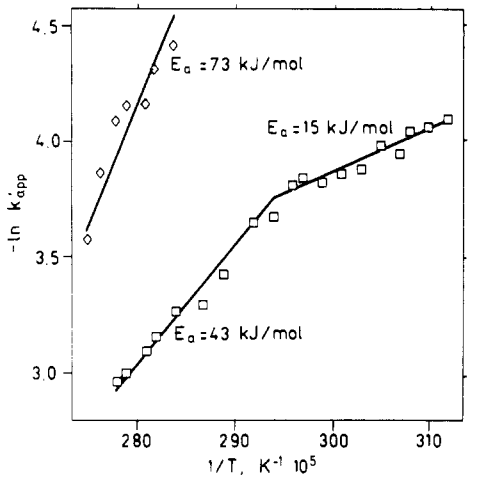

Figure 6. Arrhenius plot for the secondary phase of the fluorescence changes observed upon heating of a mixture of α-lactalbumin and ANS. Data were collected from curves similar to those presented in Figure 4 and corrected for any residual contribution from the primary phase. Transition temperatures and E_a values are given where appropriate. (Squares) A-ALA; (diamonds) H-ALA.

Table I. Comparison of Thermodynamic Parameters for Thermal Modification of the Structure of H-ALA and A-ALA, As Determined with Different Methodologies

(A) Transition Temperatures, °C

methodology	parameter	A-ALA	H-ALA
DSC	peak position	38	68
intrinsic fluorescence	semivariation of emission wavelength	36	60
titration with ANS	change in *n*		70
fluorescence kinetics	change in E_a, primary phase	40	72
fluorescence kinetics	change in E_a, secondary phase	65	
insolubilization kinetics	change in E_a		80–95[a–c]

(B) Activation Energies, kJ/mol

methodology	temp, °C	A-ALA	temp, °C	H-ALA
DSC[d]	20–100	280	20–100	234
insolubilization kinetics			70–80	268[a]
			80–150	68[a]
			70–90	176[b]
			70–95	161[c]
			95–150	62[c]
fluorescence kinetics				
primary phase	30–40	66	30–72	24
	40–45	172	72–80	189
secondary phase	45–65	15	80–90	73
	65–90	43		

[a] Dannenberg and Kessler (1988). [b] Manji and Kakuda (1986). [c] Hillier and Lyster (1979). [d] Relkin et al. (1992).

DISCUSSION

A comparison of the transition temperatures obtained with different methods and listed in Table IA, shows that

overall structural changes measured by DSC occur at the same temperatures at which structural transitions involving hydrophobic patches on the protein surface occur. One exception is the appearance of a transition at 65 °C in kinetic fluorescence studies on mixtures of A-ALA and ANS. As stated above, this novel transition can be consequent to the formation of a mixed protein population during progressive heating of A-ALA. Also, the transition temperature observed for tryptophan exposure in H-ALA does not match the values found with other methodologies. The structural modification resulting in tryptophan exposure upon heating is reversible for both proteins, whereas the modifications detected by other methodologies on A-ALA are irreversible. Thus, in some cases, different methodological approaches allow the observation at different molecular events.

The transition temperatures obtained with the methodologies used in the present study differ markedly from those calculated for H-ALA in kinetic insolubilization studies carried out on skim milk or on cheese whey. The difference can be explained on the basis of different considerations: (1) Insolubilization of a protein represents a process different from, and likely consequent to, its actual thermal denaturation. (2) The other proteins present in whey or milk may exert some form of protection against the denaturation of H-ALA or some sort of cooperative effect in its insolubilization. Indeed, no loss of soluble material or formation of aggregates was found in samples of either H-ALA or A-ALA when each isolated protein was heated at 80 °C for 15 min.

The occurrence of multiple events in the overall denaturation process is made evident by kinetic studies on the fluorescence changes in mixtures of protein and ANS, which allowed the detection and a preliminary quantitative analysis of transitions for which no match was found, at least in terms of E_a, with those determined by other methodologies.

Both proteins gave an increase in surface hydrophobicity in the early phases of the heat treatment. When intrinsic fluorescence experiments are also considered, this could be a consequence of the "swelling" or relaxation of the protein structure, which facilitates the access of ANS to previously inaccessible hydrophobic regions of the protein structure.

As the temperature was increased, the fluorescence increase became very rapid and was followed by a decrease in fluorescence. The loss of ANS binding sites on the protein could be a consequence either of the "shrinking" of the protein to a more compact and inaccessible structure or of a dramatic protein unfolding resulting in the disruption of organized hydrophobic patches recognizable by ANS on the protein surface. A more complete understanding of this point could derive from hydrodynamic and proteolytic studies, which are currently under way in our laboratory.

Taking into account the close relationship between functional properties and surface hydrophobicity of proteins (Nakai, 1983; Kinsella, 1984), the transient increase of surface hydrophobicity upon heating could be relevant to the use of whey and/or whey proteins in the stabilization of foams and emulsions.

Comparison of the E_a values for the individual phases of the surface hydrophobicity changes in mixtures of H-ALA and ANS with those obtained with other methodologies (Table IB) suggests that the overall denaturation process—including the formation of aggregates and the eventual precipitation of the protein in whey and skim milk—is conditioned by the swelling of the protein structure in the early phases of exposure to heat, at least in the temperature range typical of mild sanitization procedures. At higher temperatures, structural collapse of the isolated protein and formation of insoluble aggregates in milk and whey samples show comparable E_a values, suggesting a close relationship between the two phenomena.

The detailed mechanism of the rapid structural modifications made evident by kinetic fluorescence studies and the structural features of any short-lived intermediate formed upon heating remain to be established. In this context, correlation of the fluorescence kinetic data with the kinetics of changes detectable by DSC and by intrinsic fluorescence will be helpful and will be the subject of future work.

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