

Solubility Solvation, and Stabilization of α_{s1} - and β -CaseinsM. P. THOMPSON, W. G. GORDON, R. T. BOSWELL, and H. M. FARRELL, JR.¹**Abstract**

α_{s1} -Casein A has been observed to differ from α_{s1} -B by its solubility in CaCl_2 solutions at 1 and 37 C, and by its stabilization profile in the presence of κ -casein and calcium ions. The different characteristics of the protein α_{s1} -A have been attributed to the deletion of a segment of nonpolar amino acids resulting in decreased hydrophobic interactions among α_{s1} -A molecules. The deletion also has impaired the formation of α_{s1} - κ -casein micelles under conditions of normal formation.

Genetic polymorphism of milk proteins, primarily of *Bos taurus*, has been the object of intensive study during the past decade (1, 2, 4, 18). The soluble whey proteins, the bulk of which are β -lactoglobulin and α -lactalbumin have been extensively studied and it has been demonstrated that β -lactoglobulin polymorphs differ in degree of association, solubility, rate of heat denaturation, and immunochemical identity while differing only slightly in amino acid composition.

The colloidal phosphoprotein casein, separable from the soluble proteins by acidification, salting-out, or ultracentrifugation, has been demonstrated to be a mixture of different proteins and these, too, have been found to be polymorphic. Waugh and his colleagues (22) have shown that the casein micelle is stabilized by the protective colloid, κ -casein, against precipitation by calcium ions. Recent research on the components comprising the micelle, α_{s1} -, β -, and κ -caseins, has shown them to be genetically variable. Substantial evidence is available regarding the amino acid composition, elemental composition, end groups, and difference-peptide analysis of the genetic variants. Amino acid sequences of the α_{s1} -caseins are being determined in this laboratory. A paucity of information exists regarding the effect of genetic polymorphism of α_{s1} -caseins on their physical properties. However, Payens and Schmidt (12) indicated that the α_{s1} -B and

C variants (which differ only by a glu/gly substitution) associate to different degrees under identical experimental conditions. Variant A has been shown by Thompson and Gordon (15) to be the most peculiar variant in composition and in its unusual solubility in the presence of calcium ions.

In this paper we are reporting a number of physical characteristics of α_{s1} -casein A, the rarest of the caseins in this series, which has been shown to be devoid of eight amino acids (probably in sequence), seven of which are apolar. This protein is compared with the most abundant of the α_{s1} -variants, B, and with β -casein C.

Experimental Methods

Deionized water. Deionized water, prepared by passage of distilled water over a mixed bed cation-anion exchanger, was used throughout this study.

Materials. Crude α_{s1} -caseins A and B were prepared as described by Thompson and Kiddy (17); crude β -casein C was prepared by the method of Aschaffenburg (3). Final purification of these proteins was achieved by chromatography on DEAE-cellulose at pH 7.0, imidazole-HCl buffer, 3.3 M urea in the presence of mercaptoethanol (13), using a NaCl gradient of 0 to 0.25 M. Urea for column chromatography must be purified by filtration of an 8 M urea solution to remove debris and by passage over an Amberlite MB-1 (mixed bed resin) column to remove contaminating anions and cations. The 8 M urea solution was diluted to 3.3 M with the appropriate addition of buffer and water. The deionization step greatly improved resolution of protein components eluted from DEAE-cellulose and reduced the likelihood of carbamylation of lysine by cyanate ions.

Chromatographed proteins were dialyzed free of urea and eluant NaCl against several changes of water at 4 C, followed by lyophilization in the convenient form of soluble sodium caseinates. Dry proteins were stored at -20 C.

Deionized caseins. From 1 to 2 g of purified α_{s1} -caseins A and B, and β -casein C were dissolved in 100 ml water and slurried with a

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mixed bed resin (20 parts Amberlite MB-1 plus one additional part of IR-120H) essentially as described by Ho and Waugh (8). After at least 15 min the solution became colloidal and was stirred occasionally for an additional 75 min, when the isoionic point was generally reached. When the pH of the suspension did not change this was recorded as the isoionic point of the protein, which for α_{s1} -A, α_{s1} -B, and β -C was 5.15, 5.05, and 5.35, respectively. If allowed to stand for several hours, the suspension will settle. The suspension was filtered through glass wool to remove resin particles. The resin was washed three times with small volumes of water to remove additional protein. The deionized caseins were adjusted to pH 6.7 with 0.10 N NaOH and lyophilized as the soluble caseinates.

Imidazole-HCl buffer. A stock buffer solution of imidazole-HCl, 0.10 M, pH 7.0 was used where appropriate.

Calcium chloride solutions. Calcium chloride solutions at twice the desired concentrations needed for casein solubility studies and containing imidazole-HCl buffer (0.02 M) + KCl, each at twice the desired concentrations, were used as stock solutions. One set of calcium chloride solutions contains no buffer or KCl. Stock solutions ranged from 0.004 M to 0.80 M CaCl_2 .

Other salt solutions. The chlorides of Mg^{++} , Co^{++} , Cu^{++} , and Zn^{++} were prepared the same as the CaCl_2 solutions, buffered at pH 7.0 with imidazole-HCl, but with no KCl added. Eight solutions of each chloride were prepared so that the final cation concentration, upon addition to protein solution, would be 0.005, 0.008, 0.012, 0.018, 0.040, 0.070, 0.10, and 0.20 M. Solubility of casein solutions was determined as below for CaCl_2 solutions, except for CuCl_2 solutions which, at 2,800 Å, absorb strongly, so that a blank of CuCl_2 is of no value. In this case centrifuged pellets were recovered, weighed, and soluble casein determined by difference.

Solubility of caseins. Solubility of caseins at each of two temperatures, 1 and 37 C, is reported in detail, although intermediate temperatures at 10, 20, and 30 C also have been examined. The steps for the procedure are as follows:

1) Dissolve caseins (about 20 mg/ml) in water and adjust pH to 7.0 with 0.1 N KOH or NaOH. Equilibrate in water bath at desired temperature for 15 to 20 min.

2) To 2 ml of protein solution (in cellulose nitrate centrifuge tubes), blow in 2 ml of CaCl_2 solutions, with or without buffer + KCl,

invert the tube, let stand at desired temperature for 30 min.

3) Centrifuge for 15 min at 20,000 rpm (43,800 g max) at desired temperature, (see "solvation" for operation at 37 C) in SW-39 rotor, Model L-4 Beckman³ ultracentrifuge.

4) Transfer 1 ml of supernatant to a 10-ml volumetric flask containing 1 ml 1 N sodium citrate plus a few milliliters of water; make up to volume with water. When solubility is determined at 1 C, pipettes must be prechilled to avoid precipitation of protein in the pipette. Read in 1-cm cuvettes at 2,800 Å in a Beckman DU³ Spectrophotometer using a blank containing 0.01 M imidazole-HCl buffer. Extinction coefficients, ϵ , 1 cm 1%, 2,800 Å for α_{s1} -A and B are 10.0, β -casein C, 4.7. Assuming complete solubility of α_{s1} -caseins A and B, an absorbancy reading of 1.0 would be observed for each.

Casein pellet solvation. All solvation studies were made at 37 C. At this temperature it is impossible to operate the Beckman Model L-4 ultracentrifuge. However, following a suggestion of Dr. D. F. Waugh, Massachusetts Institute of Technology, and the designs of J. A. Connelly and P. D. Hoagland of this Laboratory, the instrument was modified for satisfactory operation at 37 C by installing a 100-w squirrel cage heater (12) in the chamber so that the SW-39 rotor was surrounded by heating filaments. Temperature of the rotor was determined by an air temperature thermistor which, by an adjustable shaft, was lowered into the swinging bucket rotor radiation handle. The adjustable shaft, vacuum sealed, was mounted through an easily removable lid cover. Heat was supplied by a Model 72, Yellow Springs Instrument² Proportional Temperature Controller. The controller was calibrated against actual sample temperature in the centrifuge tubes and temperature variation of the sample did not exceed ± 0.10 C at 37 C.

Close temperature control was best accomplished if the vacuum (less than 10 μ) was achieved before acceleration. To obtain such vacuum, a toggle switch was installed to actuate the diffusion pump 10 min before starting. Without this switch the diffusion pump was not actuated until the starting button was pushed.

Solvation studies were conducted as follows:

1) α_{s1} -Caseins A and B and β -casein C were

² It is not implied that the U.S. Department of Agriculture recommends the above company or its products to the possible exclusion of others in the same business.

dissolved in water to give solutions of 50 mg/ml at pH 7.0.

2) To 2 ml of the casein solutions, in cellulose nitrate tubes, at 37 C, was added (by blowing in) 2 ml of the appropriate CaCl_2 buffer-KCl solution. The tubes were inverted and allowed to equilibrate at 37 C for 30 min.

3) The samples were centrifuged in an SW-39 rotor at 25,000 rpm ($68,000 \times g$ max) for 35 min at 37 C.

4) Supernatant was poured off, tubes allowed to drain inverted for 5 min, and the cellulose nitrate tubes cut off with a razor blade 2 to 3 mm above the pellets. Tubes and pellets were weighed (w_1).

5) Tubes + pellets were lyophilized for 20 hr and the dry weight recorded (w_2). The pellet is carefully scraped from the tube and weighed (w_3). $w_1 - w_2/w_3 =$ solvation, grams water/gram protein.

An alternate method to determine the amount of casein in the pellet is to carefully extract the undried pellet with dilute sodium citrate, dilute to a given volume, and read absorption at 2,800 Å. This method is not feasible, however, for solvation studies on skim milks because absorbancies vary due to a) differing ratios of α_{s1} -, β -, and κ -caseins in the pellet and b) variations in amount of whey protein and lactose in the pellet.

Stabilization of α_{s1} -caseins. Stabilization of α_{s1} -caseins by κ -casein in the presence of Ca^{++} was performed by the method of Zittle (23) with several modifications. First, stabilization was performed at constant ratios of α_{s1}/κ (40:1, 20:1, 10:1) with increasing calcium chloride concentrations (0.003 to 0.015 M). Zittle maintained the calcium level at 0.010 M and the temperature at 30 C, whereas our studies were performed at 37 C. Our procedure was as follows:

1) One milliliter of stock α_{s1} -casein solution (40 mg/ml) was added to a 20-ml conical graduated centrifuge tube. The appropriate amounts of κ -casein were added to a series of tubes to achieve ratios of α_{s1}/κ of 40:1, 20:1, and 10:1 and the mixtures diluted to 5 ml with water.

2) Solutions were warmed to 37 C and 5 ml of desired calcium chloride-imidazole-HCl buffered solution blown into the solution. The tubes were inverted twice and allowed to stand in a 37 C water bath for 30 min.

3) Tubes were centrifuged at $1,500 \times g$ for 10 min in an A. H. Thomas³ Model HN centrifuge.

4) Two milliliters of the supernatant were added to a 10-ml volumetric flask containing

1 ml of 1 N sodium citrate plus 3 ml water and the mixture diluted to 10 ml. Turbid solutions were cleared by the addition of one drop of 0.1 M Na_2EDTA and the supernatant protein concentration determined at 2,800 Å.

Results and Discussion

Solubility of caseins. β -Caseins (A, B, and C) are normally soluble in the presence of calcium ions over a broad concentration range and remain soluble up to 18 C, where the protein abruptly precipitates (19). The insolubility is entirely reversible, however; by cooling the suspension to 15 to 16 C, β -caseins are again dissolved. Figure 1 shows that β -casein C is not precipitated by Ca^{++} at a concentration of 10 mg/ml. Solubility of β -casein at 1 C has clearly distinguished it from the α_{s1} -caseins (α_{s1} -) which, by definition, are insoluble at 0 to 4 C in calcium chloride solution. Figure 1 also shows the solubility profile of α_{s1} -B at 1 C. Aliquot addition of calcium chloride solutions to α_{s1} -casein solutions results in a rapid decrease in solubility from 0.008 to 0.05 M, where the protein is quantitatively precipitated. The protein remains insoluble at elevated temperatures and unlike β -caseins the insolubility is not reversible when the temperature is lowered. When the calcium chloride concentration exceeds 0.10 M a gradual "salting-in" of the protein ensues, such that the net charge on the molecule is positive. It is increasingly clear that preparation of α_{s1} -caseins by precipitation at 1 C and 0.40 M CaCl_2 , which has been standard practice, results in preparative losses; 0.10 to 0.20 M would be more desirable concentrations for precipitation.

α_{s1} -Casein A exhibits extraordinary solubility behavior over a broad range of calcium

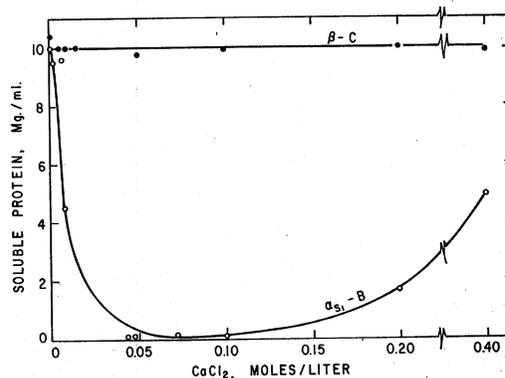


FIG. 1. Solubility at 1 C of calcium α_{s1} -B caseinate and calcium β -caseinate as a function of increasing CaCl_2 concentration at 1 C.

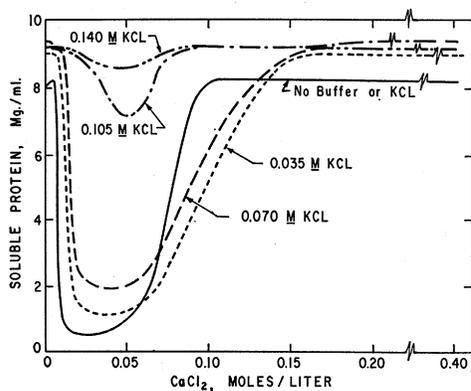


FIG. 2. Solubility at 1 C of calcium α_{s1} -A caseinates as a function of increasing CaCl_2 and KCl concentrations.

chloride concentrations and temperatures. At 1 C (Fig. 2) α_{s1} -A, like α_{s1} -B (Fig. 1), is precipitated with calcium at 0.008 M, whereupon the net electrical charge on the protein is close to zero. In the absence of electrolyte (KCl) or buffer, and after aliquot addition of CaCl_2 , the protein is driven into solution at 0.09 M. The protein is now positively charged, acting as a cation. This conclusion has been verified by free-boundary electrophoresis at pH 7.0, 0.01 M imidazole, 0.15 M CaCl_2 , where the protein is soluble. The protein migrates ($+1.36 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \times 10^{-5}$) toward the cathode. Aliquot addition of CaCl_2 in the presence of various concentrations of KCl results in (at 0.035 M and 0.070 M) a shift in the solubility profile where additional amounts of calcium are required to achieve complete solubility—about 0.15 M CaCl_2 . At 0.105 M KCl and 0.140 M KCl a shift in the insolubility dip is apparent from 0.008 M (at no KCl) to about 0.050 M CaCl_2 . These changes in solubility profiles are attributed to the competitive effect of K^+ for phosphate and carboxyl group binding sites.

The effect of temperature and CaCl_2 concentration on the solubility of α_{s1} -A is pronounced (16). At 30 C, for example, α_{s1} -A is essentially soluble; warming to 33 C results in an abrupt precipitation which, as with the β -caseins, may be totally reversed upon cooling to 28 C. When equal quantities of α_{s1} -A and α_{s1} -B (at 1 C) are mixed at 0.40 M CaCl_2 , the A variant solubilizes the normally insoluble B variant. Warming to 25 C results in formation of a precipitate which, for the most part, is α_{s1} -B, while the supernatant is α_{s1} -A. This observation is valuable because cows homozygous for α_{s1} -A are rare and other methods of frac-

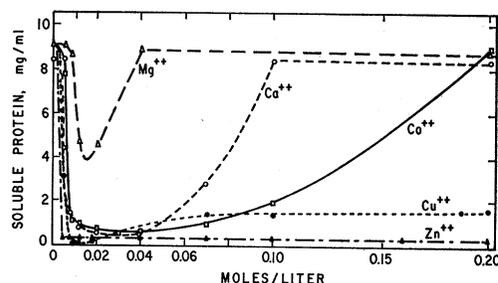


FIG. 3. Solubility at 1 C of salts of α_{s1} -A caseinates as a function of increasing salt concentration.

tation (column chromatography, in particular) have met with failure in the separation of A from AB or AC.

Figure 3 illustrates the solubility of α_{s1} -casein A in the presence of various cations. Cupric ions and Zn^{++} are the most effective precipitants, as might be expected from their atomic numbers. Coordinate complexes may be formed between α_{s1} -A molecules with Cu^{++} and Zn^{++} and Co^{++} . Co^{++} is effective, as a precipitant, to a lesser extent than Cu^{++} or Zn^{++} , whereas Mg^{++} is the least effective of the five cations studied. Clearly, an inverse relationship exists between casein solubility and the atomic number of all the divalent cations studied.

Solubility determinations of α_{s1} -caseins A and B and β -casein C were also performed at 37 C, 0.07 M KCl, and 0.01 M imidazole-HCl, pH 7.0, at initial protein concentrations of 10 mg/ml. And, as in the experiments of Noble and Waugh (11) with α_{s1} -B, the protein

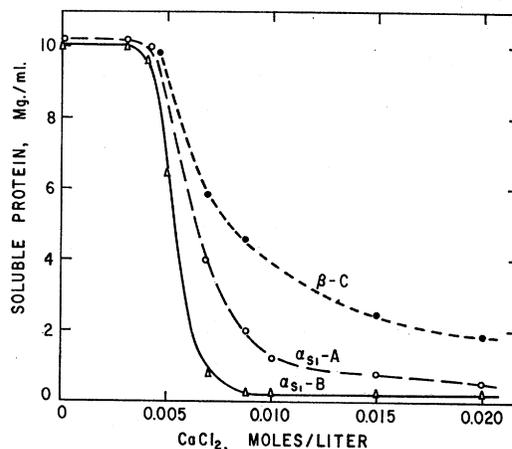


FIG. 4. Solubility at 37 C of the calcium salts of α_{s1} -caseins A and B, and β -casein C as a function of increasing CaCl_2 concentration. Solutions buffered at pH 7.0, 0.01 M imidazole-HCl.

and 0.140 M is solvated at levels of 6.0 and 7.7 g water/gram protein, respectively, or about 4.5 times as solvated as the α_{s1} -caseins. Highly solvated pellets of β -casein are completely transparent, whereas the less solvated pellets of α_{s1} -B are opalescent. Solvation levels of β -casein as a function of CaCl_2 and KCl concentrations are shown in Table 1. When equal amounts of α_{s1} -B and β -casein are mixed, solvation levels are close to those of α_{s1} -B (20).

Examination of several hundred individual cows' milks for casein pellet solvation disclosed an average of 1.9 g water/gram protein (14). However, this average varies with breed and individual cows and pellet solvations vary from 1.25 to 2.3. κ -Casein concentration in the pellets as determined by the DTNB method of Cavallini et al. (5) for total -SH, modified by Heinselman, Phillips, and Jenness (7), appears to be relatively constant at 14 to 16%. Total calcium in the pellets ranges from 2.1 to 3.0%, with a mean value of 2.5%. An average weight ratio of 1.5 (1.16 on a molar basis) exists for Ca/P; if this ratio is decreased solvation of the pellets increases, while the converse is true. Therefore, in the normal milk system, inorganic and organic phosphate (and perhaps citrate), as well as total calcium concentration in casein micelles, dictate the solvation level and ultimately, therefore, the colloidal stability of the system to heat (14).

Furthermore, *in vivo* and *in vitro* formation of casein micelles differ markedly, largely because of the incorporation of phosphate into the micelle. We have attempted to arrive at solvation levels of micelles reconstituted from first-cycle soluble caseins by dialysis against skim milk, reconstituted low-heat skim milk powder, or the simulated milk sera of Jenness and Koops (9). Milks of two known casein solvation levels, 1.4 and 2.0, were centrifuged and the casein prepared as first-cycle soluble caseins. Dialysis of either of these two at 2.5% casein concentration, pH 7.0 and 37 C for 20 hr (antibiotic used to retard bacterial growth) against any of the three systems revealed a) less than 50% of the casein is driven into micelles, b) the solvation levels restored for both first-cycle soluble casein samples were close to 1.5 and never reached the average of normal milk (1.9), and c) micelles formed by dialysis were more uniform (and usually larger) than naturally occurring micelles as observed by electron microscopy. Micelle formation can be driven to completion by addition of calcium ions, but solvation is not restored to that of the original milk.

The degree of solvation somewhat predicts

the aggregate size of casein micelles as indicated by electron microscopy. Poorly solvated milks (1.25 to 1.6) show micelles as large as 2,500 Å (the average is 1,000 to 1,200 Å); micelle size ordinarily decreases as the level of solvation increases to 1.9 and above.

Stabilization of α_{s1} -caseins. Figures 5 and 6 show the stabilization of α_{s1} -caseins B and A by κ -casein (pH 7.0 imidazole-HCl buffer, 37 C) as a function of increasing calcium ion concentration. While these experiments were performed in the absence of KCl, the dip at 0.005 M CaCl_2 reported by Noble and Waugh (11), and not seen by others, was apparent, but not as pronounced as that previously observed. The dip represents (as proposed by Noble and Waugh) α_{s1} —not coated with κ -casein on the descending limb, and α_{s1} —coated with κ -casein on the ascending limb. We have not observed the great degree of micelle stability between 0.01 and 0.02 M CaCl_2 reported by Noble and Waugh. A partial explanation is that our centrifugal force ($1,500 \times g$ for 10 min) far exceeded that used ($500 g$ for 1 min) by Noble and Waugh. Maximum stability of micelles at weight ratios of 40:1 and 20:1 (α_{s1} -/ κ -) occurred at 0.007 M, whereas at 10:1 the system was much more stable over a broader (0.007 to 0.012 M) range of calcium

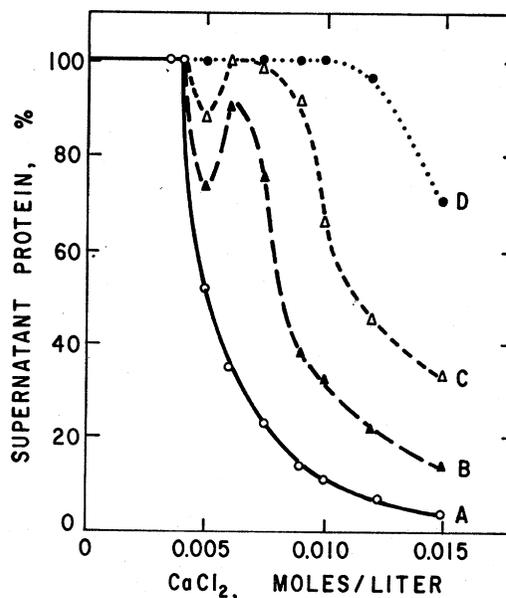


FIG. 5. Supernatant protein at 37 C resulting from the increment addition of CaCl_2 to A, α_{s1} -casein B, no κ -casein; B, α_{s1} -B + κ -casein, 40:1; C, α_{s1} -B + κ -casein, 20:1; and D, α_{s1} -B + κ -casein, 10:1. Solutions buffered at pH 7.0, 0.01 M imidazole-HCl. Initial protein = 4 mg/ml.

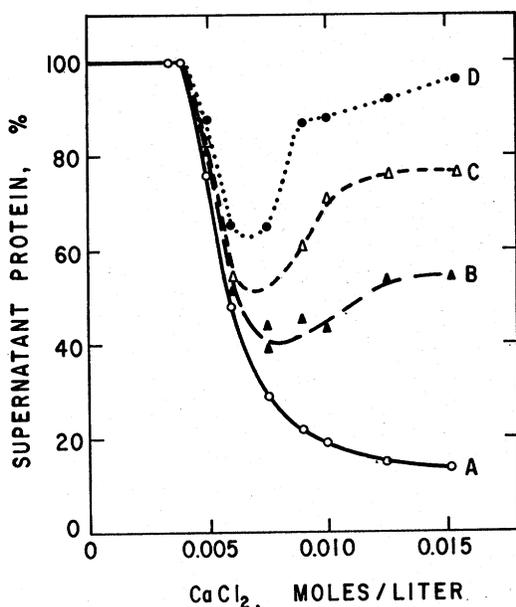


Fig. 6. Supernatant protein at 37 C resulting from the increment addition of CaCl_2 to A, $\alpha_{s1}\text{-A}$, no κ -casein; B, $\alpha_{s1}\text{-A} + \kappa$ -casein, 40:1; C, $\alpha_{s1}\text{-A} + \kappa$ -casein, 20:1; and D, $\alpha_{s1}\text{-A} + \kappa$ -casein, 10:1. Solutions buffered at pH 7.0, 0.01 M imidazole-HCl. Initial protein = 4 mg/ml.

ions. Destabilization of micelles is evident at 0.010 M CaCl_2 , except when the $\alpha_{s1}\text{-}\kappa$ -ratio is 10:1.

α_{s1} -Casein A once again exhibits unusual behavior; several points can be drawn from Figure 6: 1) The dip is pronounced and depends upon the weight ratio of $\alpha_{s1}\text{-}\kappa$; increasing κ -casein concentration shifts the minimum of the dip to lower CaCl_2 concentration. 2) Unlike $\alpha_{s1}\text{-B}$, an increase in calcium concentration increases the "degree of stability" at all levels of κ -casein concentration, but the solutions never appear opalescent until the weight ratio of $\alpha_{s1}\text{-}\kappa$ is 10:1 and CaCl_2 exceeds 0.010 M. The deletion of seven nonpolar residues probably reduces the chance for intermolecular hydrophobic interaction with κ -casein; hence, the $\alpha_{s1}\text{-A}$ complex with κ -casein would be weakened. Electron microscopy bears out the observation that micelles do not form (particles less than 300 Å exist) until these conditions are achieved. Therefore, with α_{s1} -casein A we are observing another property of the variant—difficulty of stabilization by κ -casein against precipitation with Ca^{++} . When $\alpha_{s1}\text{-A}/\kappa$ -solutions are dialyzed against milk or artificial buffers, micelles form only at 37 C at higher calcium concentrations.

General considerations. The foregoing re-

sults indicate that α_{s1} -casein A behaves differently from $\alpha_{s1}\text{-B}$. The reason for this must certainly be involved in a change in protein conformation brought about by the deletion of hydrophobic amino acids (two phenylalanine, three leucine, one valine, and one alanine residue) which appear to be absent from within the polypeptide chain [as suggested by Kalan et al. (10)] and not from the N-terminal or C-terminal sequence. Furthermore, Thompson, Farrell, and Greenberg (16) have suggested the deletion of these amino acids to be sequential and in a region which does not contain the phosphorus-rich portion of the molecule. While deletions of this type are not common, one such deletion has been reported to occur in the hemoglobin variant, "Gun Hill." $\alpha_{s1}\text{-A}$ offers, as far as we can tell, the best example of a major sequential deletion of amino acids within the polypeptide chain.

The effect of the multi-amino acid deletion in $\alpha_{s1}\text{-A}$ has greatly affected its solubility in calcium, such that the protein behaves more like a β -casein than an α_{s1} -casein. Its classification as an α_{s1} -casein is strengthened more by genetic information than by physical behavior. In addition, $\alpha_{s1}\text{-A}$ probably binds more calcium per mole than does $\alpha_{s1}\text{-B}$.

The presence of $\alpha_{s1}\text{-A}$ in milk leads to an unusually low solvation level, yet purified $\alpha_{s1}\text{-A}$ is more highly solvated than $\alpha_{s1}\text{-B}$. The increased calcium binding of the $\alpha_{s1}\text{-A}$ variant, coupled with its weaker interaction with κ -casein, may be the reason for the unusually low solvation levels in milks containing the variant. All of the unusual properties of the $\alpha_{s1}\text{-A}$ variant, which in all likelihood are due to a decrease in hydrophobic bonding (caused by the deletion of seven nonpolar amino acids) serve to illustrate the point that hydrophobic bonding is of great importance in the milk system in the formation of colloidal casein micelles.

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