Modeling of calcium-induced solubility profiles of casein for biotechnology: influence of primary structure and post-translational modification

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SUMMARY

Molecular biology holds the promise of new tools for the food industry which include proteins with tailor-made functionality. Without a fundamental knowledge of the molecular bases of these properties, implementation will be strictly empirical. For example, the phenomena of salt-induced precipitation of proteins (salting-out) and their resolubilization (salting-in) has heretofore been discussed only qualitatively. A quantitative method, using Wyman's theory of thermodynamic linkage, has been developed and tested on the calcium-induced solubility profiles of the major milk proteins, the caseins. Salting-out was described by a salt-binding constant, k_1, and n, the number of moles of salt bound; salting-in was described by the corresponding terms k_2 and m. The magnitude of these parameters indicated involvement of protein phosphate groups in binding and precipitation, but enzymatic dephosphorylation showed significant increases in k_1 and k_2 indicating involvement of carboxylate groups as well. Studies on two genetic variants of α_{s1}-casein indicated the importance of a hydrophobically stabilized intramolecular ion pair in the functionality of the protein. These studies have led to a fuller understanding of the molecular basis for the solubility behavior of caseins and have laid the groundwork for future computer simulation of food protein functionality.

INTRODUCTION

In the food component area, functionality is an operational description of the behavior of a com-

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ponent under defined environmental conditions. Some examples of the functional properties of protein are solubility, emulsifying power and whippability, to name a few. A major limitation to this field of study may be our scientific approach. In attempting to accurately define any one property, we may lose sight of the objective, which is to be able to use this functional test in a predictive

fashion. Thus, in studying just one aspect of one protein we can lose sight of an important factor: the interactions which occur in a real food system. These interactions can render a one dimensional functional test inaccurate.

Many functional tests have been developed for proteins under a variety of conditions, yet the predictive value of these tests is often low for real processing situations. Rather than devising new and perhaps 'ultimate' functional property tests, research perhaps should focus on the basis for the behavior of proteins in these tests. More in-depth understanding of the basic chemical and physical properties which contribute to functionality is needed. There must also be a good interface between this basic work and its ultimate application in food systems, otherwise the applicable questions may never be asked or answered. Ovalbumin, the principal protein of egg white, is whippable. For this model it may not be important to conduct research to find the ultimate test conditions for whipping ovalbumin, but rather to uncover the molecular basis for its property of whippability and to be able to quantitate changes in such behavior with structural changes. Such information could allow us to understand why soy or whey proteins fail to function in the same way, and could suggest further modifications to increase their utilization.

Following the general approach outlined above, we have attempted to define and model one simple functionality test for the caseins. The property chosen is solubility as a function of calcium ion concentration. This system was selected for three reasons. From the point of view of the food industry caseinate is an important commodity and milk and dairy products are widely consumed for their calcium content. Secondly, the interactions occurring in this important colloidal-transport system are still not well defined. Finally, a wealth of information of a qualitative nature is available in the literature on calcium-induced casein solubility curves [1,10,15,16]. In order to better understand these calcium–protein interactions, the precipitation and resolubilization reactions of selected caseins were reinvestigated. The data were analyzed with respect to computer-generated models; pre-

liminary analysis of the data indicates that a thermodynamic linkage occurs between calcium binding and salting-out and salting-in reactions.

EXPERIMENTAL PROCEDURES

Materials. Purified caseins were prepared as described by Thompson et al. [15] using DEAE-cellulose chromatography. Enzymatic dephosphorylations were conducted as previously described [2]. Stock $CaCl_2$ solutions were prepared and their concentrations checked by atomic absorption analysis. Nomenclature of the genetic variants and abbreviations for casein are as recommended by the Milk Protein Nomenclature Committee [6].

Solubility of caseins. Solubility of caseins at each of two temperatures, 1 and 37°C, was carried out 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 15–20 min.

(2) To 2 ml of protein solution (in thick-walled centrifuge tubes), add 2 ml of $CaCl_2$ solutions, with or without buffer $\pm$ KCl. Invert the tube and let stand at desired temperature for 30 min.

(3) Centrifuge for 15 min at 43 800 $\times$ g_{max} at desired temperature in a Model L-8 Beckman ultracentrifuge.

(4) Transfer 500 μl of supernatant to a 5 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 pre-chilled to avoid precipitation of protein in the pipette. Read in 1-cm cuvettes at 280 nm. Absorption coefficient, ε, 1 cm 1% 280 nm for α_{s1}-caseins A and B is 10.0, and for β-casein C is 4.7 [6].

Theory and data analysis. Here, we assume that there are essentially two classes of binding sites for ligands responsible for the sequential salting-out and salting-in processes. Therefore, the concept of Wyman's linked functions [18] can be used to treat these processes according to the following reaction:

$$p + nx \overset{k_1^n}{\underset{}{\rightleftharpoons}} PX_n + mx \overset{k_2^m}{\rightleftharpoons} PX_nX_m \tag{1}$$
$$(S_0) \qquad (S_1) \qquad (S_2)$$
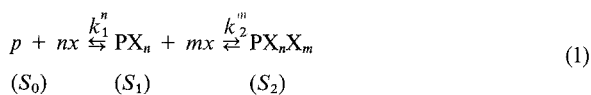
where p is the unbound protein, x is the free salt, n and m are the number of moles bound to species PX_n and $PX_n X_m$, and S_0, S_1 and S_2 are the solubilities of the species indicated. For this study S_1 and S_2 will be relative to S_0. The mathematical relationship representing the above stoichiometry can be represented according to the following:

$$S_{app} = S_0 f(p) + S_1 f(PX_n) + S_2 f(PX_n X_m) \tag{2}$$

where S_{app} is the apparent protein solubility at a given salt concentration (S_t), $f(i)$ are the protein fractional components of species i and the S terms are as previously defined. Incorporation of the salt binding equilibrium constants as defined by (1) into (2) leads to the following:

$$S_{app} = \frac{S_0 p}{p + k_1^n\, p\, x^n} + \frac{S_1\, k_1^n\, p\, x^n}{p + k_1^n\, p\, x^n} + \frac{(S_2 - S_1)\, k_2^m\, p\, x^m}{p + k_2^m\, p\, x^m} \tag{3}$$

where p is the concentration in percent of the unbound protein and x is the concentration of free salt. Cancellation of common terms yields:

$$S_{app} = \frac{S_0}{1 + k_1^n\, x^n} + \frac{S_1\, k_1^n\, x^n}{1 + k_1^n\, x^n} + \frac{(S_2 - S_1)\, k_2^m\, x^m}{1 + k_2^m\, x^m} \tag{4}$$

It should be stressed here that the latter expression is valid only for sequential binding, i.e. $k_1 > k_2$, and where n sites saturate prior to the binding of m sites on the protein. Also, for n or m values greater than one, k_1 and k_2 represent an average value for each of the n or m binding sites. In reality, n or m moles of salt will bind with only one equilibrium constant (K_1), i.e. $K_1 = k_1^n$ and $K_2 = k_2^m$.

Now, since the total salt concentration, X_T, is the sum of x, the free salt concentration, and the concentration of the bound salt of both species PX_n and $PX_n X_m$, it can be shown that

$$X_T = x \left(1 + \frac{n k_1^n\, P_T\, x^{(n-1)}}{1 + k_1^n\, x^n} + \frac{m k_2^m\, P_T\, x^{(m-1)}}{1 + k_2^m\, x^m} \right) \tag{5}$$

where P_T is the total concentration of protein. From equation (5) it can be seen that X_T approaches x when P_T is small relative to x. In our experiments this assumption is reasonable because the concentration range of the total salt is large, while the molecular weight of the caseinates reduces their molar concentration. Therefore, since the total salt concentration could be substituted into (4) for the free concentration; salt-induced solubility profiles can be directly analyzed using Gauss-Newton non-linear regression analysis (program developed at this laboratory by Dr. William Damert). All solubility profiles were analyzed by fixing the values of n and m and calculating the best least-squares fit for the optimum evaluated k_1 and k_2 values. The n and m values were then fixed to new integer values and the whole procedure was repeated. The n and m values which yielded the minimum root-mean-square value for the analysis with the minimum error in k_1 and k_2 were then reported.

RESULTS

In 1929 Linderstrom-Lang [9], as a result of his studies on casein, postulated that the colloidal milk complex (the casein micelle) is composed of a mixture of calcium-insoluble proteins which are stabilized by a calcium-soluble protein. The latter protein would be readily split by the milk-clotting enzyme chymosin, destabilizing the colloid and allowing coagulation to occur. In the intervening years, individual caseins have been isolated and purified and the α_{s1}-, α_{s2}- and β-caseins have been shown to be calcium-insoluble, while κ-casein is not only soluble in the presence of calcium ions, but is readily split by the enzyme chymosin [7].

Solubility at 37°C. Solubility determinations of α_{s1}-caseins A and B (α_{s1}-A, α_{s1}-B) and β-casein C (β-C) had previously been reported at 37°C in 10 mM imidazole-HCl pH 7.0, 0.07 M KCl at initial protein concentrations of 10 mg/ml [15]. KCl was chosen as the electrolyte because it predominates in the milk salt system [7]. As in the experiments of Noble and Waugh [10,16] the proteins become increasingly less soluble at about 5 mM CaCl$_2$ (Fig.

1a and b). Comparison of the solubility profiles of α_{s1}-A and B indicates that at 37°C α_{s1}-A is more soluble than α_{s1}-B, while β-C is the most soluble. In order to quantify the data, non-linear regression analyses were performed. The data of Fig. 1a were fitted by equation (4) using iterative analysis. Values of k_1 were obtained at fixed integer values of n (range of $n = 1$–32); the correct value of n was taken to be the fit with the lowest root-mean-square deviation (RMS). Fig. 1a shows the fit to $n = 2, 4$ and 8 for α_{s1}-A; values for $n = 8$ gave the minimum RMS. In each case the parameters S_1, S_2, S_i were also determined by the analyses. Analysis of the solubility profiles of α_{s1}-A, α_{s1}-B and β-C at 37°C,

Table 1

Calcium-induced insolubility of casein at 37°C[a]

Casein	k_1[b]	n	S_1[c]
α_{s1}-A	157 ± 3	8	0.9 ± 0.2
α_{s1}-B	186 ± 3	8	0.1 ± 0.1
β-C	156 ± 12	4	2.0 ± 0.3

[a] Solutions buffered at pH 7.0, 10 mM imidazole-HCl, 0.07 M KCl [15].

[b] 1/mol.

[c] S_1 denotes the soluble protein (mg/ml) at elevated Ca^{2+} concentrations as defined in equation (1).

where protein aggregations due to hydrophobic interactions are maximized with respect to temperature, showed no salting-in behavior, so that k_2 and m were essentially zero. Values obtained for k_1 (salting-out) and n are given in Table 1. Since the n values appear to correspond to the number of phosphate groups per mole of protein (8 and 4, respectively, for α_{s1}- and β-caseins [6]), it may be hypothesized that calcium binds to the serine phosphates of casein, and that this binding in turn is linked to changes in the solubilities of the various protein species.

Solubility at 1°C. β-C does not precipitate at 1°C at concentrations of Ca^{2+} of up to 400 mM. It is known that hydrophobic forces are dominant in the association reactions of β-caseins [12], and that β-casein binds Ca^{2+} at 1°C. In this case binding is not
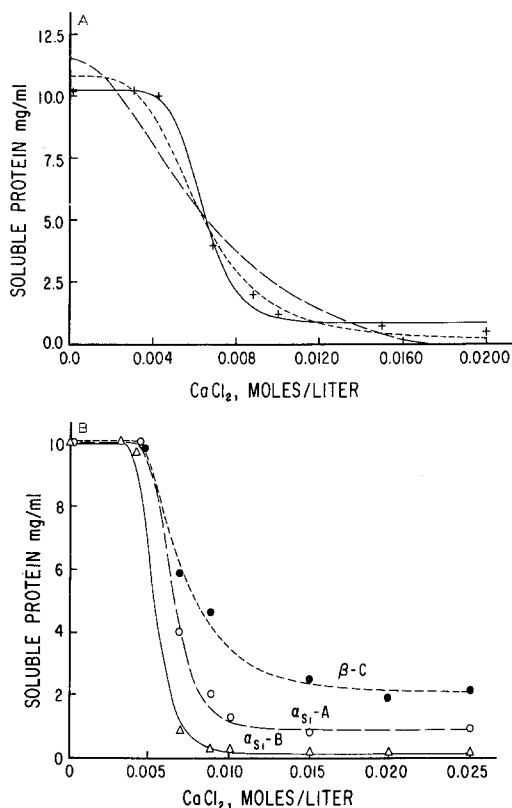

Fig. 1. Solubility at 37°C of the calcium salts of α_{s1}-A and B, and β-C as a function of increasing CaCl$_2$ concentrations. Solutions buffered at pH 7.0, 10 mM imidazole-HCl with 0.07 M KCl. (a) The experimental data for α_{s1}-A (+) were fitted by equation (4) by non-linear regression analysis with values of 2, 4 and 8 assigned to n. The best fit was obtained for $n = 8$. (b) Similar fits for α_{s1}-B and β-C, α_{s1}-A repeated. Results of analyses are given in Table 1.
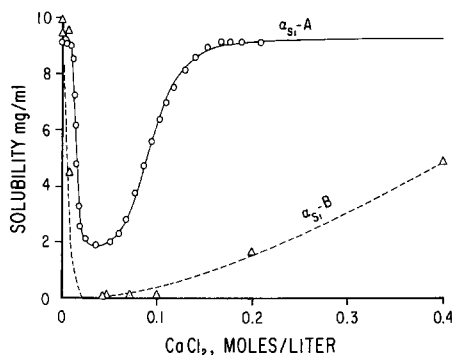

Fig. 2. Solubility at 1°C of calcium α_{s1}-B caseinate and calcium α_{s1}-A caseinate as a function of increasing CaCl$_2$ concentration at 1°C. Data were fitted by equation (4). Results of analyses are given in Table 2.

linked to decreased solubility. Addition of calcium chloride to α_{s1}-casein solutions results in a rapid decrease in solubility; at 50 mM $CaCl_2$ the protein is nearly totally precipitated (Fig. 2). When the calcium chloride concentration exceeds 100 mM a gradual salting-in of the protein occurs at 1°C. The data for α_{s1}-B were fitted to equation (4) and the salting-out parameters k_1 and n, as well as the salting-in parameters k_2 and m, were determined (Table 2).

The α_{s1}-A genetic variant, in contrast to the α_{s1}-B, exhibits solubility behavior over a range of calcium chloride concentrations. At 1°C (Fig. 2) α_{s1}-A, like α_{s1}-B, is precipitated with calcium at about 8 mM; in the presence of 0.07 M KCl after aliquot addition of $CaCl_2$, the protein is driven back into solution at 90 mM with $n = m = 8$. The protein is now positively charged, acting as a cation. This conclusion was previously verified by free-boundary electrophoresis at pH 7.0, in 10 mM imidazole, 150 mM $CaCl_2$, where the protein is soluble at 1°C; it migrates ($+1.36 \times 10^{-5} cm^2 \cdot V^{-1} \cdot s^{-1}$) toward the cathode [15].

Influence of electrolyte on salting-out at 37°C. When the 0.07 M KCl was omitted from these experiments and solubility was tested in $CaCl_2$ alone, somewhat different results were obtained (Fig. 3). The two genetic variants of α_{s1}-casein (A and B) still showed decreased solubility at 5 mM $CaCl_2$ and gave salting-out constants, k_1, of 180 and 204 $l \cdot mol^{-1}$ at 4 mg/ml; these values are significantly different, but more surprisingly, the number of calcium-binding sites (n) was 8 and 16, respectively. This difference was of interest, since in

Fig. 3. Solubility at 37°C of calcium α_{s1}-A and B caseinates as a function of increasing $CaCl_2$ without KCl at 5 mg/ml. Data were fitted by equation (4).

the presence of KCl (Tables 1 and 2) the apparent number of calcium-binding sites was equal to 8 for both genetic variants of the casein. Effects of KCl on the solubility of α_{s1}-B were tested at two different protein concentrations; data are summarized in Table 3. With no KCl present k_1 is smaller for α_{s1}-B but n, the number of limiting sites, is 16. In the presence of 0.07 M KCl the number of sites is reduced to $n = 8$ for both concentrations, but k_1 is larger. Apparently K^+ will compete with Ca^{2+} for a group of binding sites present in the α_{s1}-B genetic variant, but not in the α_{s1}-A. The α_{s1}-A genetic variant is the result of the deletion of 13 amino acid residues from α_{s1}-B (Fig. 4). The losses of glutamate residues 14 and 17 as well as arginine 22, which are surrounded by hydrophobic side chains, may cause different physical properties for this protein. One explanation could be the loss of a hydrophobically stabilized intra-molecular ion-pair [14]. Increased K^+ concentrations may break this ion pair, leading to increased Ca^{2+} binding and altered equilibria among species present for α_{s1}-B. Indeed, α_{s1}-A, as noted above, is readily salted-in at 1°C whereas the B variant is not.

Influence of phosphate groups on salting-out and salting-in. As shown in Fig. 1, α_{s1}-A and B and β-C readily precipitate at 37°C in 0.07 M KCl. Since under these conditions n was correlated with the number of phosphate residues in the native casein, the importance of these residues in the precipitation

Table 2

Calcium-induced insolubility and solubility of caseins at 1°C[a]

Casein	k_1[b]	n	k_2[b]	m
α_{s1}-B	123 ± 5	8	2.5 ± 0.2	4
α_{s1}-A	68 ± 1	8	10.6 ± 0.3	8
β-C	totally soluble			

[a] Conditions as in Table 1.

[b] l/mol.

Table 3

Calcium-induced insolubility of α_{1s}-B at 37°C

Protein concentration[a]	k_1[b]	S_0[c]	S_1[c]	n
5 + KCl	225 ± 2	5.31 ± 0.07	0.12 ± 0.04	8
10 + KCl	182 ± 4	11.11 ± 0.41	0.30 ± 0.20	8
4 − KCl	204 ± 4	4.31 ± 0.04	0.49 ± 0.04	16
10 − KCl	151 ± 1	9.42 ± 0.04	0.49 ± 0.04	16

[a] Protein initial concentration in g/l ± 0.07 M KCl.
[b] 1/mol.
[c] Concentration in g/l.

reaction could be tested. In previously conducted research, the phosphate groups of α_{s1}-B were removed enzymatically [1] and the effects of KCl on the precipitation of native (N) dephosphorylated (O-P) caseins had been compared (Fig. 5a–c) but never quantitated. Analysis of these data by use of equation (4) is summarized in Table 4. With no KCl present, dephosphorylation increases k_1 and some salting-in occurs for the O-P-form; surprisingly, for both proteins (N and O-P) $n = 16$. In the presence of 0.07 M KCl, n is again reduced to 8 for both N and O-P forms of α_{s1}-B; the k_1 values are similar to those found in the absence of KCl and salting-in occurs only for the dephosphorylated casein.
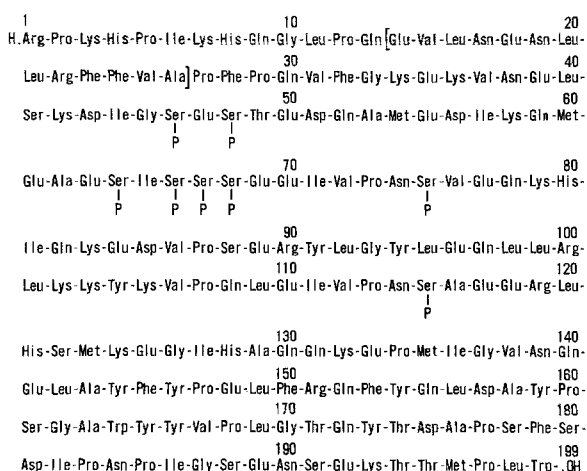
When α_{s1}-A is dephosphorylated, it becomes

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1                          10                            20
H.Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln[Glu-Val-Leu-Asn-Glu-Asn-Leu-
                                 30                            40
        Leu-Arg-Phe-Phe-Val-Ala]Pro-Phe-Pro-Gln-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-
                                 50                            60
        Ser-Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met-
                      |         |
                      P         P
                                 70                            80
        Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Glu-Gln-Lys-His-
                      |         | | |                         |
                      P         P P P                         P
                                 90                            100
        Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-
                                 110                           120
        Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-Ser-Ala-Glu-Glu-Arg-Leu-
                                                           |
                                                           P
                                 130                          140
        His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-
                                 150                          160
        Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-
                                 170                          180
        Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-
                                 190                          199
        Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-.OH
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Fig. 4. Sequence of α_{s1}-B showing amino acids deleted to yield the α_{s1}-A variant. The deleted segment, residues 14–26, is enclosed in brackets.

nearly completely soluble at 1°C and is salted-in even at 36°C (Fig. 6). In contrast (Fig. 1), the native α_{s1}-A is not appreciably salted-in at 37°C. Results are compared in Table 5. For dephosphorylated α_{s1}-A without KCl $n = 8$, mirroring the numbers found for the native protein, but for the O-P form at 1°C $n = 2$ and $n = 4$. The k_2 values observed for both native and O-P α_{s1}-A at both 1 and 36°C are similar to each other and to that of α_{s1}-B at 1°C. However the small degree of salting-in which occurs for O-P of α_{s1}-B does so with an elevated k_2 (Table 4), showing another significant difference between the A and B variants.

Effect of various cations at 1°C. Fig. 7 illustrates the solubility of α_{s1}-A in the presence of various cations. Cu^{2+} and Zn^{2+} are the most effective precipitants, as might be expected from their atomic numbers. Coordinate complexes may be formed between α_{s1}-A molecules with Co^{2+}, Zn^{2+} and Cu^{2+}. Ca^{2+} is effective, as a precipitant, to a lesser extent than Cu^{2+} or Zn^{2+}, whereas Mg^{2+} is the least effective of the five cations studied. The salting-out and salting-in constants were estimated from equation (4) for each cation and are given in Table 6. Cation variation (i.e., use of magnesium, calcium, cobalt, copper and zinc) of these profiles showed k_1 and k_2 behavior consistent with concepts of phosphate- and carboxylate-ligand coordination, respectively. Clearly, an inverse relationship exists between casein solubility (as quantified by changes in k_1) and the atomic number of the divalent cations studied. The salting-in constant k_2 appears to decrease and then increase with atomic
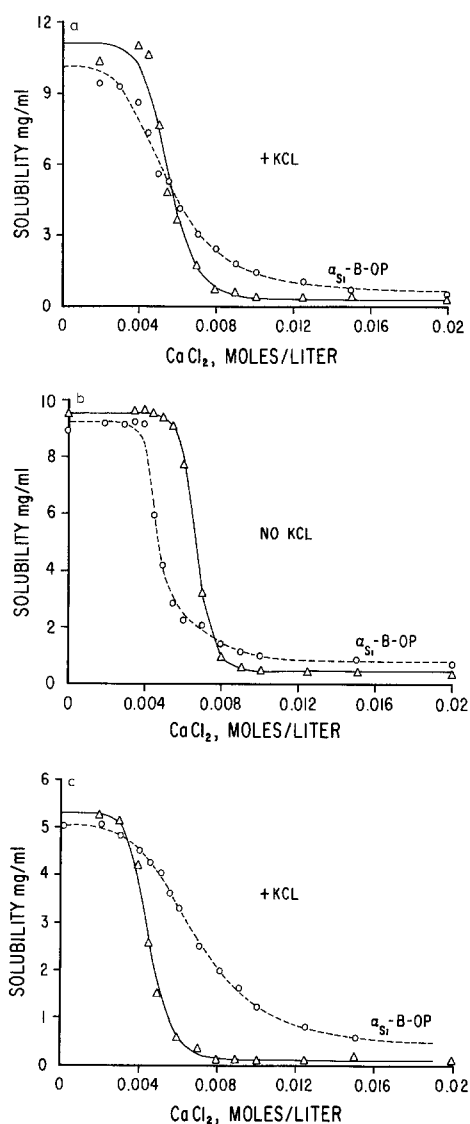
Fig. 5. Solubility at 37°C of native and dephosphorylated α_{s1}-B as a function of $CaCl_2$ concentration (a) with 0.07 M KCl at 10 mg/ml, (b) without KCl at 10 mg/ml and (c) with 0.07 M KCl at 5 mg/ml. Results of analyses are given in Tables 3 and 4.

Fig. 6. Solubility of α_{s1}-A O-P as a function of calcium ion concentrations at 1°C (———) and at 36°C (– – –). Data were fitted by equation (4). Results of analyses are given in Table 5.

yses of the solubility profiles of two genetic variants of the major bovine casein (α_{s1}-) have been accomplished using Wyman's theory of thermodynamic linkage. These analyses have yielded parameters which quantify the precipitation reactions. A notable feature of this analysis is that values of k_1 and k_2 obtained from the data of a variety of investigators [1,10,15,16] are in excellent agreement (e.g., compare k_1 of Tables 1 and 4 for α_{s1}-β + KCl at 10 mg/ml with a value of 190 l · mol^{-1} calculated from Noble and Waugh [10]). The parameters k_1 and k_2 are taken to be related to the average association constants for the binding of calcium to casein. The logic behind this assumption is that as the casein binds Ca^{2+} ions a charge neutralization occurs, the complex approaches its iso-electric

number; no apparent correlation with ionic radius is evident.

DISCUSSION

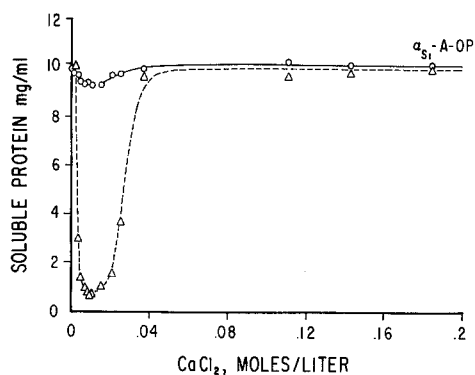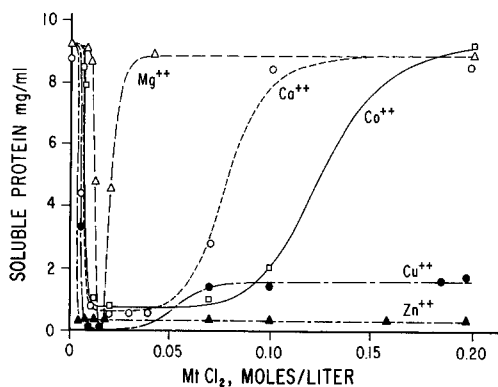The caseins of milk can be subdivided on the basis of their solubility in Ca^{2+} solutions [7]. Anal-

Fig. 7. Solubility at 1°C of various salts of α_{s1}-A as a function of increasing cation concentration. Data were fitted by equation (4). Results are given in Table 6.

Table 4

Calcium-induced insolubility of native (N) and dephosphorylated (O-P) α_{s1}-B at 37°C

Protein	$k_1{}^a$	$k_2{}^a$	$S_0{}^b$	$S_1{}^b$	$S_2{}^b$	n	m
$N_{10} - KCl^c$	151 ± 1	–	9.42 ± 0.04	0.49 ± 0.04	–	16	0
$O-P_{10} - KCl^d$	219 ± 2	135 ± 12	9.2 ± 0.1	2.6 ± 0.3	0.8 ± 0.6	16	8
$N_{10} + KCl$	182 ± 4	–	11.1 ± 0.4	0.3 ± 0.2	–	8	0
$O-P_{10} + KCl$	218 ± 10	154 ± 22	9.9 ± 0.2	6.4 ± 1.8	0.6 ± 0.6	8	4

[a] l/mol.
[b] S_i denotes the soluble protein species defined in equation (1).
[c] N_x = protein (native) and its initial concentration in g/l ± 0.07 M KCl.
[d] O-P = dephosphorylated protein and its initial concentration in g/l ± 0.07 M KCl [1].

Table 5

Calcium-induced insolubility of native (N) and dephosphorylated (O-P) α_{s1}-A at 1°C

Protein	$k_1{}^a$	$k_2{}^a$	$S_1{}^b$	$S_2{}^b$	n	m
$N_{10} + 36°C^c$	140 ± 3	–	0.90 ± 0.20	–	8	0
$O-P_{10} + 36°C^d$	326 ± 7	36 ± 1	0.6 ± 0.3	10.0 ± 0.6	8	8
$N_{10} + 1°C$	130 ± 4	13 ± 1	0.4 ± 0.2	–	8	8
$O-P_{10} + 1°C$	223 ± 59	46 ± 4	8.8 ± 1.8	10.0 ± 0.6	2	4

[a] l/mol.
[b] S_i denotes the soluble protein species defined in equation (1).
[c] N_x = protein and its initial concentration in g/l with no KCl.
[d] O-P = dephosphorylated protein and its initial concentration in g/l with no KCl and at the indicated temperature.

Table 6

Cation-induced solubility of α_{s1}-A at 1°C^a

Cation	$k_1{}^b$	$k_2{}^b$	$S_2 - S_1{}^c$ (mg/ml)	Atomic No.	R^d (Å)
Mg^{2+}	76 ± 9	56 ± 7	8.4 ± 1.3	12	0.66
Ca^{2+}	150 ± 27	13 ± 2	8.2 ± 1.4	20	0.99
Co^{2+}	166 ± 4	8.1 ± 0.2	8.5 ± 0.2	27	0.72
Cu^{2+}	229 ± 2	18 ± 2	1.5 ± 0.1	29	0.72
Zn^{2+}	373 ± 27	202 ± 35	0.20 ± 0.03	30	0.74

[a] $n = m = 8$ for all calculations; no KCl present.
[b] l/mol.
[c] $S_2 - S_1$ = concentration of soluble α_{s1}-A in mg/ml (equation 1).
[d] Cation atomic radius in Å.

Table 7

Comparison of association constants for various calcium-complexes with values of k_1 and k_2

Complex of Ca^{2+}	$\log k_a{}^a$	$\log k_1{}^b$	$\log k_2{}^b$
α_{s1}-B	2.6	2.26	0.39
α_{s1}-A	–	2.20	1.02
β-C	2.6	2.26	–
O-Phosphoserine	2.2		
Glutamate	0.8		
Acetate	0.6		

[a] From the data of Dickson and Perkins [5].

[b] This study.

point, and decreases in solubility. Previous research by Waugh and coworkers [3,17] has indicated that all of the various ionic species present are in true equilibrium and, as developed in the experimental section, analysis of the soluble protein as a function of total calcium ion present should yield parameters (k) akin to association constants for calcium binding if the observed solubility changes are thermodynamically linked to the binding. The fits of the data here indicate this to be so. Table 7 shows a comparison of experimental binding constants compiled by Dickson and Perkins [5] with both values ($\log k_1$, k_2) obtained in this study, both in the presence of added electrolyte. The k_1 values are in quite good agreement with literature values for calcium binding from dialysis experiments for the caseinates and for model phosphate compounds (Table 7). Salting-in constants (k_2), however, appear to be more in agreement with association constants related to calcium binding by carboxylate groups. Thus, salting-in may be the result of further Ca^{2+} binding to carboxyl groups resulting in increased positive charge of the salt-protein complex resulting in increased solubility.

The second parameter obtained by this method of analysis is n (or m). In the case of n, the values obtained in KCl (8 and 4 for α_{s1}- and β-caseins) appear to correspond to the number of moles of calcium bound as calculated from the data of Dickson and Perkins [5] obtained in 0.1 M NaCl. These values also correspond to the number of phosphate

residues found by sequence analyses for the respective caseinates. (Note that β-C has only four groups as opposed to five found on A and B forms [6]). α_{s1}-A contains a total of 29 carboxylate groups and the net charge at neutral pH can be calculated to be -24; binding of eight divalent calcium ions would decrease this number to -8, thus considerably reducing the net charge of the molecule but not yielding an isoelectric precipitate. In a similar fashion the net charge on β-C is -12 and binding of four divalent cations reduces this number to -4. These numbers (-8 and -4) may thus reflect that such binding then leads to decreased solubility. Other binding, not linked to solubility, could first occur at higher-affinity sites, and thus would not be 'seen' in this analysis. Only that binding directly linked to solubility is disclosed. It is interesting to note that in the case of α_{s1}-A, which is totally resolubilized at 1°C, $n + m = 16$, reflecting a charge reversal to a cationic species with a net charge of $+8$. In fact, free boundary electrophoresis in calcium showed the molecule to have a cathodic mobility, and equilibrium dialysis experiments gave a value of 17 mol of Ca^{2+} bound at 1°C in 0.07 M KCl [15]. It thus appears as though the parameters n and m correspond to the number of moles of Ca^{2+} bound per monomer which leads to solubility changes, while the values of k_1 and k_2 may relate to the association constants for phosphate and/or carboxylate binding of Ca^{2+}. Initially the values of k_1 and n calculated for α_{s1}-B in 0.07 M KCl seemed to agree with literature values for binding of calcium to casein phosphate groups. This being the case, one would expect a decrease in k_1 and n with dephosphorylation so that its values would become more like those of k_2. In point of fact, k_1 increased upon dephosphorylation (Tables 4 and 5) and n remained the same; instead of abating calcium binding, dephosphorylation apparently enhanced it. Experiments conducted in the absence of KCl also showed similar trends: a class of tighter-binding sites for the dephosphorylated form of α_{s1}-B, with $n = 16$ for both. For α_{s1}-A, $n = 8$ for native and dephosphorylated caseins and the dephosphorylated form had the highest value of k_1 observed (326 $1 \cdot mol^{-1}$). These observations are not contradic-

tory; if altering the protein or its environment leads to decreased solubility, then the equations disclose those binding sites linked to this phenomenon under the specific conditions used.

One can visualize three classes of calcium binding sites on α_{s1}-casein, all with k_1 of the order of magnitude for calcium phosphocaseinate, and which are thermodynamically linked to solubility changes under the selected test conditions. With the assumption that the protein is monomeric, these classes include:

(1) A class of sites with the highest calcium affinity observed (k_1) which are unmasked by dephosphorylation; $n = 8$ for this class.

(2) A class of sites with k_1 between 160 and 180 for which $n = 8$ and which may correspond to phosphate groups (for β-casein $n = 4$ for this class).

(3) A weaker set of sites which lower the average k_1 in the absence of KCl and which are expressed in α_{s1}-B with n equal to 8 but which are not expressed in α_{s1}-A. ((n without KCl) − (n with KCl) = 16 − 8.)

It is tempting to speculate that the calcium-binding sites unmasked by dephosphorylation arise from changes in structure due to conversion of P-serine to serine. This point is controversial as to whether or not P-serine may disrupt secondary structures or promote their formation. In theoretical calculations based on primary structures, Bloomfield and Mead considered P-serine to be a structure breaker, while Creamer et al. [4] considered it to be neutral. In either event most of the phosphate falls in areas of no regular structure or α-helix depending on the stance taken. Here a change from 8 to O-P could induce a conformational change leading to tighter binding of calcium. For α_{s1}-A this change is most dramatic; the deleted residues (which occur in another area of uncertain structure) plus the deleted phosphate leads to even tighter binding of Ca^{2+} (Table 4). Here the deleted segment may allow more carboxyl groups to act in concert and display the greatest affinity for calcium ($k_1 = 326$). In the α_{s1}-A variant there are a total of 16 glutamic and aspartic acid residues in the sequence bounded by residues 35–85. In the native molecule, P-serines are interspersed with carboxyl-

ates and dephosphorylation could allow structural changes which would significantly increase k_1. This is in contrast to dentin and salivary phosphoproteins where higher than expected association constants for calcium are dramatically reduced by dephosphorylation [8]. Dephosphorylation increased k_1 at 10 mg/ml and the amount of soluble casein also increased. Native α_{s1}-A can be salted-in at 1°C, but dephosphorylation of α_{s1}-A caused the molecule to be salted-in at 36°C as well, presumably through stronger Ca^{2+} protein interactions with carboxylate groups. Comparison of the k_2 values for the dephosphorylated forms of α_{s1}-A and B shows an important difference between the two genetic variants. Salting in of the O-P A variant is readily accomplished at 37 and 1°C and k_2 is of the order of magnitude expected for carboxylate interaction as noted (Table 5). The limited salting-in observed for α_{s1}-B O-P, however, showed a k_2 nearly equivalent to k_1 (Table 4).

The curves for the α_{s1}-A calcium complexes, both native and O-P forms, show a strong tendency toward resolubilization at 1°C whereas α_{s1}-B does not. α_{s1}-A represents a sequential deletion of 13 amino acid residues from α_{s1}-B (Fig. 4). Note that residues 14 and 17 are glutamic acids while residue 22 is an arginine, the remainder of the amino acid residues deleted being hydrophobic or non-charged. It is possible that a strong hydrophobically stabilized intra-molecular ion-pair [14] occurs in α_{s1}-B. Upon addition of Ca^{2+} this bond is broken and when precipitation occurs, further binding of calcium leads to greater insolubility for the B variant. In the absence of this bond, α_{s1}-A can be readily salted-in at 1°C whereas the B-variant cannot. The genetic alteration of these electrostatic interaction sites produces a major change of the physical properties of the α_{s1}-A protein. Similar calcium-sensitive ion-pairs may be of importance in other phosphoprotein systems such as dentin formation and plaque deposition [8]. Examination of the sequences of salivary phosphopeptides, which have been implicated in the latter reaction, shows repeating sequences of Arg and Glu interspersed with hydrophobic residues [11]. Future research on site-specific changes in the caseins and their result-

ing changes in physical properties may be possible since Stewart et al. [13] have recently constructed the cDNA of the α_{s1}-B gene, and indications are that Richardson and co-workers have succeeded in obtaining a cDNA clone of the rare α_{s1}-A gene (T. Richardson, University of California at Davis, personal communication).

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