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Net proton charge of β- and κ-casein in concentrated aqueous electrolyte solutions

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Abstract

Titration experiments have been carried out in order to measure the net proton charge of β- and κ-casein in NaCl solutions at 0.1 M and 1 M salt concentrations, at 4 °C, in the pH range between 5.5 and 10.5. Experimental data are compared with model values calculated through pK_a's of titrable groups neglecting the electrostatic perturbation term (ΔpK_a) in order to evaluate the magnitude of the error caused by this approximation and to delimit its effectiveness. At both ionic strengths, the agreement is good for κ-casein in the pH range [5.5, 9.5], while errors of up to 2 charges are observed for β-casein in the same range. These deviations are likely to be caused by strong electrostatic effects induced by the high density of negative charges of β-casein 1–21 peptide. In order to account for these electrostatic effects, the net proton charge on this peptide is evaluated through a model based on the counterion condensation theory developed for the titration of polyelectrolytes with different types of ionizable groups.

Keywords: β-casein; κ-casein; Net proton charge; Potentiometric titration; Counterion condensation theory

1. Introduction

Experimental and theoretical information about hydrogen ion binding to protein molecules is very useful both from a fundamental and a practical point of view. Electrostatic interactions play a key role in determining the behavior of solutions containing large ionic biomolecules, such as proteins in aqueous electrolyte solutions. For example, the balance between repulsive electrostatic and attractive van der Waals forces controls numerous phenomena, such as protein aggregation and stability of aggregates or their precipitation. Furthermore, the net proton charge allows differences between like proteins to be emphasized or the occurrence of denaturation to be easily detected. Finally, reliable and useful molecular–thermodynamic models can be developed on the basis of interactions between salt ions and proteins [1] and between protein molecules. For example, the net proton charge at different ionic strengths is required to predict binding of other ions (i.e. chloride, sodium, etc.) to protein molecules in terms of the electric double layer theory [2].

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Table 1
Some properties of bovine milk caseins

	α_{s1}	α_{s2}	β	κ
Wt.% in the whole casein	36	10	34	13
Molecular weight	23600	25250	24000	19000
Number of aminoacidic residues	199	207	209	169
Number of genetic variants	4	3	6	2
Isoelectric point	4.1	4.1	4.5	4.1

Molecular–thermodymamics represents a bridge between fundamentals and applications; indeed, many of its models can be applied to predict perturbations on solution conditions (pH and ionic strength, for instance) needed to obtain a required effect. Protein solubility, salting effects, separations by electrophoresis and ion exchange HPLC are strongly influenced by electrostatic interactions and, therefore, by the number of charges on protein molecules, so that these phenomena could be predicted if electrostatic interactions could be properly represented. Unfortunately, the irregularity of the molecular surface, the non-uniform distribution of charges, the shift of p*K*'s of ionizable groups due to interactions with other charged groups in the molecule, etc., make the prediction of the protein charge and the calculation of electrostatic interactions not yet satisfactory. Many theoretical efforts are in progress in this field so it is very important to test the reliability of the proposed models in predicting the protein charge, as a function of different parameters (e.g. pH, ionic strength).

Table 2
Titrable residues in bovine β- and κ-casein

	β-casein						κ-casein	
Genetic variants	A_1	A_2	A_3	B	C	E	A	B
Acidic groups								
Glu	17	17	17	17	16	16	13	13
Asp	4	4	4	4	4	4	5	4
Ser-P[a]	5	5	5	5	4	5	1	1
Tyr	4	4	4	4	4	4	9	9
Cys	0	0	0	0	0	0	2	2
Basic groups								
Lys	11	11	11	11	12	12	9	9
Arg	4	4	4	5	4	4	5	5
His	6	5	5	6	6	6	3	3

[a] In phosphoserine (Ser-P), the phosphate group may lose two hydrogen ions and gain two negative charges.

Proton charge measurements can be carried out by quite simple methods. Potentiometric acid–base titration and electrophoretic experiments are relatively easy and commonly used techniques. Titration of protein solutions, in particular, appears to be an inexpensive and rather accurate method.

In this paper we report some data about the net proton charge of β- and κ-casein, at 4 °C, in the range between pH = 5.5 and pH = 10.5 at two different ionic strengths and we try to interpret the results through an analytic theory of polyelectrolyte ionization. These proteins, together with α_{s1} and α_{s2}-casein, make up the whole casein that is the main protein source of milk. They are attracting an increasing interest from the pharmaceutical industry [3] because of many potential applications of different fragments which can be obtained from these biomolecules. For example, the fragment 106–116 of κ-casein appears to be biologically active against thrombosis whereas the fragment 60–66 of β-casein exhibits analgesic properties [4]. In view of developing processes for the production of these compounds, research efforts have been devoted to separation and purification of different caseins, but the results so far obtained are not satisfactory from an industrial point of view. Table 1, adapted from data in the literature [4,5] reports some useful information about bovine milk caseins.

Aminoacid sequences, also reported in the literature [4], can show some minor differences, for each protein, depending on the genetic variant. Table 2 reports the number of acid and basic titrable groups for each genetic variant of bovine milk β- and κ-casein. The high fraction of acid

residues indicates an acid behavior of both proteins.

Starting from the N-terminus, the first part of the aminoacid chain (sequence 1–50) of β-casein is mainly hydrophilic and rich in acid residues, whereas the rest of the chain is highly hydrophobic. As for κ-casein, the sequence 106–169 (κ-casein macropeptide) exhibits hydrophilic features whereas the remaining part (1–105: para-κ-casein) is hydrophobic, especially in the range 20–80. Therefore, in both cases, and especially above the isoelectric point, one end of the chain is negatively charged and polar whereas the other part is nearly uncharged and strongly hydrophobic. This feature is responsible for micelle formation in aqueous solutions. Both these proteins exist in solution as a monomer and as a multimer, with a well-defined aggregation degree (dependent on chemical and physical conditions), when critical micellar concentration (CMC) is exceeded. The formation of casein micellar aggregates is due to hydrophobic interactions; according to the endothermic nature of these interactions, β- and κ-casein exhibit a CMC and solubility increase when temperature is lowered. It was observed that β-casein in NaCl aqueous solutions does not form any micellar aggregates at 4 °C or lower temperatures [6]. As for κ-casein, a CMC of 0.53 g/l at 20 °C in 0.1 M NaCl solutions it is reported and 0.24 g/l in 1 M NaCl solutions at the same temperature [7]. According to the endothermic nature of casein aggregations κ-casein is also expected to be in a monomeric form at 4 °C when concentration is lower than 0.24 g/l.

About the structure of these proteins, recent Raman optical activity studies [8] have shown a loose and non-cooperative character in aqueous solution with relatively mobile or reomorphic conformations. The major secondary structure elements present in β- and κ-casein are left-handed polyproline-II helix and β strand.

Some preliminary tests have shown that both β- and κ-casein are very poorly soluble in NaCl aqueous solutions at pH below 5. According to the literature data reported and the results of the preliminary tests, we decided to explore the range of pH's higher than 5.5 at 4 °C using a low protein concentration (0.1 g/l) in order to be sure of a complete dissolution of the protein in a well-defined state (monomeric form) over the entire pH range. Lower concentration values would allow the pH range to be extended to values lower than 5.5 but this procedure is not suitable because of a poorer precision in the measurements.

2. Materials and methods

2.1. Materials

β-casein (C-6905, claimed purity minimum 90%) and κ-casein (C-0406, claimed purity minimum 80%) from bovine milk were purchased from Sigma–Aldrich and used without any further purification. Sodium chloride was a Fluka product (claimed purity $>99.5\%$). Titrant solutions were prepared by diluting, with doubly distilled water, stock solutions of hydrochloric acid and sodium hydroxide (Farmitalia Carlo Erba) to 0.1 N.

2.2. Titration experiments

β- and κ-casein solutions were prepared, at two ionic strengths, by dissolving 0.01 g of the protein in 100 ml of 0.1 N and 1 N sodium chloride solutions, respectively. Salt solutions were prepared by using doubly distilled water made CO_2-free through stripping by nitrogen for approximately 30 min.

Protein dissolution lasted approximately 20 h in a glass vessel filled with nitrogen and hermetically sealed. Dissolution and subsequent potentiometric titration were carried out in the same vessel maintained at 4 °C ± 0.1 °C by a thermostated bath. Solutions were agitated by magnetic stirrers in a gentle way in order to avoid the formation of foam. Accuracy of the measurements strongly depends on the absence of carbon dioxide. Therefore, also during titration measurements, the headspace of the vessel was swept with nitrogen at very small positive pressure to remove air contained carbon dioxide.

The pH of the solutions was first adjusted to 5.5 by hydrochloric acid solution and then sodium hydroxide titrant solution aliquots were injected until pH 10.50 was reached, allowing sufficient time to elapse for electrode equilibration between two subsequent injections. Hydrogen ion activity was measured by a digital pH-meter (Accumet Model 50-Denver Instrument Company) equipped with a HI-1131 combination electrode (Hanna Instruments) and able to show three decimal figures. Prior to each titration experiment, the electrode response was calibrated by using three standard buffers (Hanna Instruments) with pH values 4.01, 7.01, 10.01. Standardization was performed at the same temperature and under the same stirring conditions as the titration measurements. Blank experiments, i.e. titration of solutions without protein but under otherwise identical conditions, were also carried out in order to provide the means of calculation of the net proton charge. Since the added titrant was always less than 0.5 ml, ionic strength can be considered constant at every pH value.

Reproducibility of the measurements was checked, replicating three times each single titration.

2.3. Experimental titration curves of proteins

Proteins are able to release or bind covalently a large number of hydrogen ions to the side chains of acid and basic aminoacid residues and two hydrogen ions to the end amino- and carboxyl-group of the polypeptide chain. The net proton charge Z_p is a relative quantity defined as the number of hydrogen ions exchanged by the protein when the pH of the solution is changed from a reference value (pH*) to any other value [9]. A titration curve is a plot of Z_p vs. pH at constant ionic strength and temperature. Experimental determination of the function Z_p(pH) can be done by measuring the moles of strong acid (or strong base) per mole of protein to be added to the protein solution and to the blank solution to vary their pH from an arbitrary reference starting value to a new value. By blank solution we mean a solution which does not contain protein but has the same volume, ionic strength, temperature, etc. The difference between the former amount, which is always greater, and the latter one, is the mole number of H^+ bound (or dissociated) to the protein.

$$Z_p(\mathrm{pH}) = \frac{[\Delta V^p_{add}(\mathrm{pH}) - \Delta V^b_{add}(\mathrm{pH})]\cdot M}{n_p} \quad (1)$$

In Eq. (1), n_p represents the moles of protein in solution, M is the molarity of titrant solution and ΔV^p_{add}, ΔV^b_{add} are the volumes of titrant solution added to the protein and to the blank solution, respectively, to change pH from the reference value to the desired value. Z_p(pH) is, thus, the mole number of H^+ ions bound or released from one mole of protein in the range [pH*, pH].

This result strictly holds if the number of free H^+ ions is the same both in the protein and blank solution at each pH value. Therefore, the volume of the protein solution must always be equal to that of the blank solution during the titration and the activity coefficient of hydrogen ion must be the same in both solutions at each pH value. The latter condition is equivalent to omitting the effect of the protein on activity coefficient, i.e. to assuming that the presence of the protein does not affect the ionic strength of the solution. Both the previous conditions are reliable at 0.1 g/l protein concentration and 0.1 N titrant solutions.

3. Theoretical evaluation of net proton charge

Net proton charge of protein molecules can be calculated if the state of protonation of each site, A_i, is known at the protein chain conditions. This property is ruled by the dissociation constant $K_{a,i}$:

$$K_{\mathrm{a},i} = \frac{a_{H^+}\cdot a_{A_i}}{a_{HA_i}} \quad (2)$$

where $a_{H+} = C_{H+\gamma H+}$ is the hydrogen ion activity, $a_{Ai} = C_{Ai\gamma Ai}$ and $a_{HAi} = C_{HAi\gamma HAi}$ are the activities of the deprotonated and protonated state, respectively; γ stands for activity coefficient and C for concentration. Eq. (2) yields:

$$\mathrm{p}K_{\mathrm{a},i} = \mathrm{pH} - \log\left(\frac{\alpha_i}{1-\alpha_i}\right) - \log\left(\frac{\gamma_{A_i}}{\gamma_{HA_i}}\right) \quad (3)$$

where α_i is the dissociated fraction of A_i sites.

The values of γ depend, mainly, on the ionic strength of the solution because of attenuation effects of ions on electrostatic interactions. Therefore, we can assume $\gamma_{Ai}=\gamma_{HAi}$ (and, moreover, in dilute solutions $\gamma_{Ai}=\gamma_{HAi}=1$) so that the last term at the r.h.s. of Eq. (3) can be neglected. Therefore, the titration behavior of proteins can be analyzed by the following equation:

$$\log\left(\frac{\alpha_i}{1-\alpha_i}\right)=\mathrm{pH}-\mathrm{p}K_{\mathrm{a},i} \tag{4}$$

When dissociation degrees are calculated for all the sites in the protein, both at pH and pH*, the net proton charge is given by:

$$Z_p(\mathrm{pH})=-\sum_{i=1}^{n}(\alpha_i-\alpha_i^*) \tag{5}$$

where the summation is extended to all *n* titrable groups.

However, some problems arise with the values of $\mathrm{p}K_{\mathrm{a},i}$ to use in Eq. (4). The ionization of the residues in the environment of the native protein molecule is, in principle, quite different from the one observed for free aminoacids, mainly because of electrostatic interactions with spatially neighboring side chains. Therefore, in protein conditions, $\mathrm{pK}_{a,i}$'s undergo a shift, with respect to their intrinsic values, depending on the protein charge. In order to account for these effects, $\mathrm{p}K_{\mathrm{a},i}$'s are usually expressed as the sum of two terms [1]:

$$pK_{\mathrm{a}}=pK_{\mathrm{a,model}}+\Delta pK_{\mathrm{a}} \tag{6}$$

where $\mathrm{p}K_{\mathrm{a,model}}$ indicates the hypothetical $\mathrm{p}K_{\mathrm{a}}$ value in absence of any intramolecular electrostatic interactions and $\Delta\mathrm{p}K_{\mathrm{a}}$ represents the perturbation due to electrostatic effects introduced by placing the titrating group in the environment of the native protein molecule.

The value of $\mathrm{p}K_{\mathrm{a,model}}$ can be set equal to the $\mathrm{p}K_{\mathrm{a}}$ of a compound containing polar groups similarly located as the given site or can be evaluated from titration of fully denatured proteins or of end-blocked polypeptides. However, the simplest way to obtain quite a good approximation of $\mathrm{p}K_{\mathrm{a,model}}$ is to use the $\mathrm{p}K_{\mathrm{a}}$ value of the side chain of the corresponding aminoacid.

The perturbation term, $\Delta\mathrm{p}K_{\mathrm{a}}$, is expected to be negative for groups located in positively charged zones of the macromolecule, and positive in negatively charged ones. Since charge distribution depends on pH, $\Delta\mathrm{p}K_{\mathrm{a}}$ is a function of pH. Furthermore, the expected magnitude of $\Delta\mathrm{p}K_{\mathrm{a}}$ decreases with increasing ionic strength, because the presence of salt attenuates electrostatic interactions in the local microenvironment of each titrable group.

The first theoretical attempt to evaluate $\Delta\mathrm{p}K_{\mathrm{a}}$ is due to Linderstrom–Lang, who deduced a simple expression holding for compact globular proteins [9]. In this model, all charges are assumed to be spread on the protein surface and the non-uniform distribution of electric charges is not accounted for. This model was applied to describe the titration curve of β-casein [10] although this protein is not globular and, when it aggregates, it forms micelles that are not compact [11,12]; furthermore, caseins show a strong disuniformity of charges along the sequence. In spite of this, the results obtained through a fitting approach were good but it has to be pointed out that the values of some physical parameters, used as adjustable ones, appear to be unrealistic with respect to more recent data. Moreover, also $\mathrm{p}K_{\mathrm{model}}$ terms, that are theoretically independent from electrostatic interactions, and so they should not depend on the model chosen to represent them, were used as adjustable parameters. Some authors [13,14] showed that the Linderstrom–Lang and other distributed charge models proved to be inadequate to describe electrostatic interactions of caseins with realistic values of physical parameters. These authors proposed to use discrete, instead of distributed, charge models based on the location of charges as indicated by the primary structure. Electrostatic interactions due to nearby charges along the peptide chain were taken into account, whereas interactions between distant charges (in terms of primary structure), that may eventually be in proximity by bending or coiling of the peptide chain, are neglected. In particular, it was pointed out that the major electrostatic effects on β-casein are due to the N-

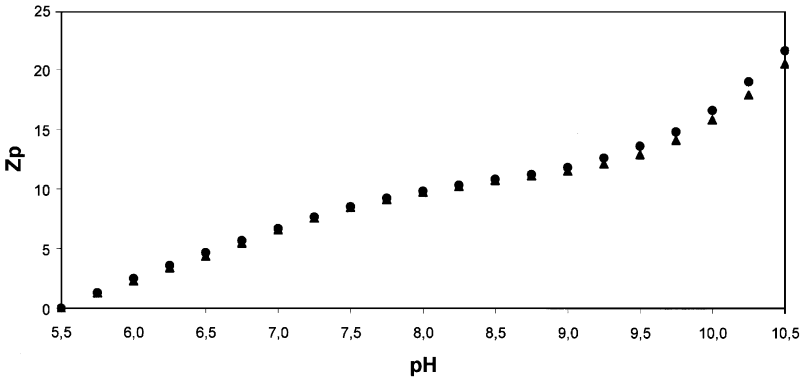

Fig. 1. Experimental data of the net proton charge (absolute values) of β-casein in NaCl aqueous solutions (▲: 0.1 M; ●: 1 M). Reference pH 5.5.

terminal region that carries, in a wide range of pH, essentially all of the net charge.

A more attractive molecular theory for the prediction of titration curves for polyelectrolytes was proposed by Manning [15]. With respect to other models based on the Poisson–Boltzmann (PB) equation, Manning's counterion condensation theory (CCT) does not require to solve by machine the non-linear second-order differential equation describing the electrostatic potential around the polyion in the presence of a salt in solution. The original expression for ΔpK_a, developed in the context of CCT [15], concerned homopolymers of monoprotic titrable groups. The polyelectrolyte chain is modeled as an infinite length rigid line of discrete charges, equally spaced at distance *b*, where *b* is a function of the degree of deprotonation α. An interesting discussion about the two main models of a charged polymer, cylinder with uniform charge and a linear array of equally spaced discrete charges, has appeared recently [16]. Manning's expressions allow ΔpK_a be calculated as a function of the charge density dimensionless parameter (ξ), that is equal to $\alpha\xi^0$, where ξ^0 is defined as

$$\xi^0 = \frac{q^2}{4\pi_{\varepsilon 0}\,\varepsilon_r kTb^0} \tag{7}$$

In Eq. (7), q is the elementary charge, ε_0 and ε_r are the vacuum and the relative dielectric constant, respectively, k is the Boltzmann constant, T the absolute temperature and b^0 is the lowest possible distance between two charges, that is obtained, for acid groups, when $\alpha = 1$. As a consequence, ξ^0 is the maximum possible charge density for the macromolecule. ξ is the crucial parameter of the model: when its value exceeds a critical value ($\xi_{cr} = 1$), corresponding to a critical value of $\alpha(\alpha_{cr} = 1/\xi^0)$, counterions 'condense' on the polyelectrolyte (for monovalent counterions). The functional relation for ΔpK_a proposed by Manning shows a discontinuity at the critical value.

Subsequently, the theory has been improved and extended to different contexts [17–20]. In particular, Cesàro et al. [18] have obtained an expression for ΔpK_a that does not show any discontinuity at the critical point. In order to account for the presence of two or more different titrable groups, an extension of the theory was presented [19,20]. Manning's CCT is now widely used, in different contexts, for estimating the degree of ionization of polyelectrolytes (see for example [21]) in saline solutions because of reliable results that CCT allows to be obtained in a simpler way than other approaches.

In this work, we present an attempt to model experimental titration measurements through CCT and we analyze the improvements that this approach enables in the prediction of the net proton charge, with respect to the oversimplified calcula-

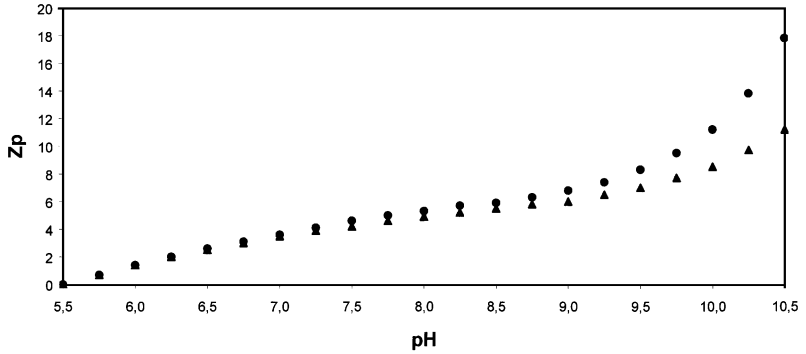

Fig. 2. Experimental data of the net proton charge (absolute values) of κ-casein in NaCl aqueous solutions (▲: 0.1 M; ●: 1 M). Reference pH 5.5.

tion based on pK_{model} values of single titrable groups.

4. Results and discussion

Figs. 1 and 2 report titration curves for β- and κ-casein, respectively, at 4 °C and two ionic strengths. Standard deviation is at most ± 0.6 charges in the neutral pH range [5.5, 9.5] while is at most ± 1.0 in the pH range [9.5, 10.5] for β-casein and ± 1.1 for κ-casein in the same range. As it was to be expected according to the number of acid and basic residues, the net proton charge is higher for β- than for κ-casein. The shape of the curves show a quite flat region between pH 7.5 and pH 9. This behavior depends on the scarcity of residues able to undergo dissociation in this range. Our experimental data for β-casein are in agreement with the data reported by Creamer at 25 °C [10]. In fact, we have found approximately the same number of titrable groups in the whole range that we have studied but with a delayed deprotonation in the central part of the curve: this is likely caused by the higher p*K*'s of phosphoserine and histidine at 4 °C with respect to 25 °C.

In order to compare experimental and calculated results, $pK_{a,model}$ of side chain of aminoacid residues and $pK_{a,model}$ of the amino and carboxyl termini of the polypeptide chain are needed. Data reported in the literature usually refer to 25 °C; furthermore, ionic strength and the type of salt were not always specified. For all the side chain residues, except phosphoserine, we used data at 25 °C reported at salt concentrations equal to those ones used here, although referred to KCl solutions, as a basis [1]. Data at 4 °C were obtained through Gibbs–Helmoltz equation using three ΔH values (5 °C, 25 °C, 50 °C) correlated by a quadratic polynomial, or just one ΔH value (at 25 °C) when other values were not found. The dependence of ΔH from ionic strength was omitted. Enthalpy data were collected from [22]. About phosphoserine, we assumed $pK_1 = 2.2$ [4]. As for pK_2, other sources [9] indicate values approximately 6,5-6,6 at 25 °C for model compounds resembling this residue. Since we have not found deprotonation

Table 3
p*K* values used in calculation of model titration curve at two salt concentrations

Residue	Glu	Asp	Ser-P	Tyr	Cys	Lys	Arg	His	Carboxyl terminus	Amino terminus
0.1 M	4.21	3.80	2.2 7.0	10.50	8.71	11.55	13.46	6.51	4.3	7.4
1 M	4.15	3.76	2.2 7.0	10.35	8.72	11.59	13.56	6.72	4.3	7.4

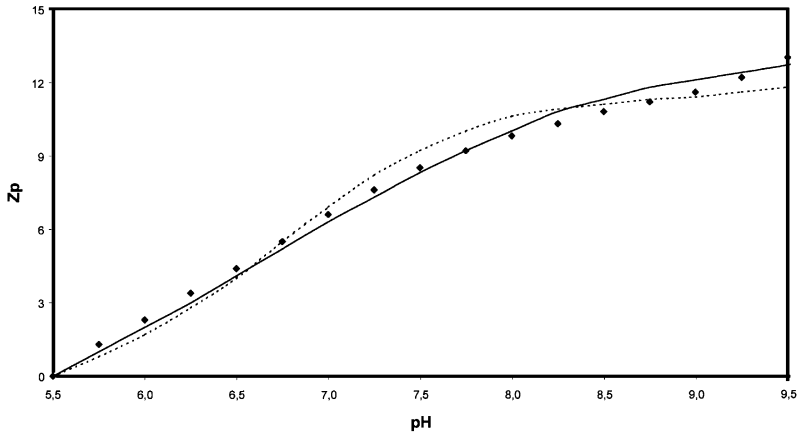

Fig. 3. Absolute values of the net proton charge of β-casein in 0.1 M NaCl solutions. Comparison between experimental and model values (♦ experimental, dashed line refers to predictions neglecting electrostatic effects, solid line refers to predictions based on CCT).

enthalpy data, we have analyzed ΔH experimental data referred to β-casein, reported in [10]; they indicate that an increment of some tenth of pK units is reliable. On the whole, $pK_2 = 7.0$ was assumed as a sensible guess. Since both carboxyl and ammo termini account for just 1 charge and they are titrated in a range where several other residues are titrated, the temperature dependence of their pK was omitted. About bovine κ-casein, which is a phosphoglycoprotein, binding one molecule of *N*-acetylneuraminic acid, the ionization of this group was not taken into account since it is expected to undergo deprotonation outside the pH range we have studied. Furthermore, even though a low degree of deprotonation occurred above 5.5, its effect would be completely hidden by the large number of acid aminoacid that are titrated in the same pH range. Table 3 reports all the pK values that were used to calculate; the model titration curve.

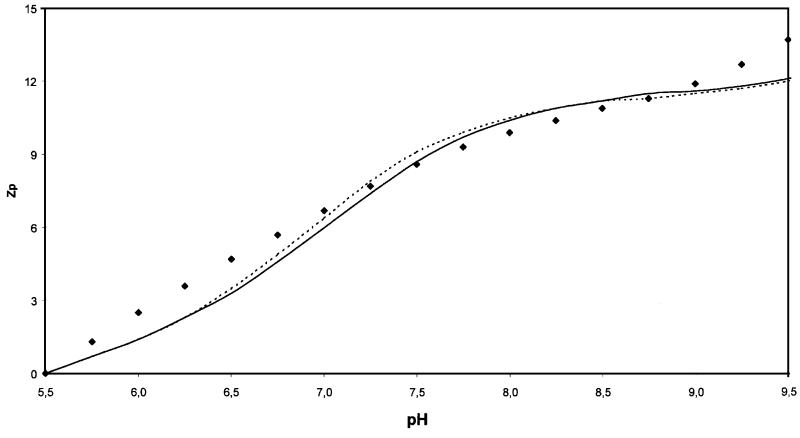

Fig. 4. Absolute values of the net proton charge of β-casein in 1 M NaCl solutions. Comparison between experimental and model values (♦ experimental, dashed line refers to predictions neglecting electrostatic effects, solid line refers to predictions based on CCT).

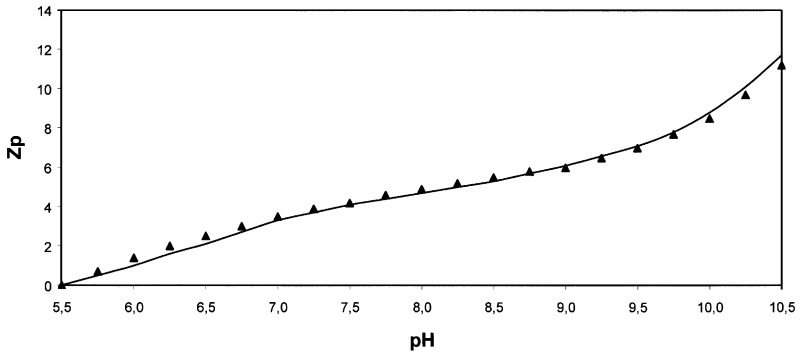

Fig. 5. Absolute values of the net proton charge of κ-casein in 0.1 M NaCl solutions. Comparison between experimental and model values.

In Figs. 3 and 4 experimental data are compared with calculated results for A2 genetic variant of β-casein, in the range between pH 5.5 and pH 9.5. The dashed line represents the titration curve calculated with the pK_{model}, i.e. without any electrostatic effect. This oversimplified data interpretation leads to appreciable differences (1–2 electric charges), both at 0.1 M and 1 M NaCl concentration. Ser-P is titrated in this pH range. A possible incorrect value of pK_a for Ser-P at 4 °C does not account for the observed behavior as it can be checked by calculations with varied values of this parameter. Therefore, we attribute this behavior to not negligible values of ΔpK_a terms. It is well known that caseins are proteins in which only short range interactions along the sequence are likely, so the explanation of this discrepancy is the high density of negative charges in the N-terminal region. Actually, β-casein exhibits a surprisingly high concentration of Ser-P and other acid residues (i.e. Glu) in the fragment 1–21. The progressive titration of these groups produces electrostatic effects delaying the deprotonation of the subsequent titrable groups. This interpretation is supported by the absence of this behavior in the case of κ-casein (Figs. 5 and 6) which does not hold such a high number of neighboring acid groups in its chain. At approximately pH 9–9.5, both experimental and calculated values show approximately 12 net charges for β-casein. These charges correspond to the deprotonation of histidine, phosphoserine, amino terminus and the residual deprotonation of some acid group. Actually, all the expected groups have been titrated in this range.

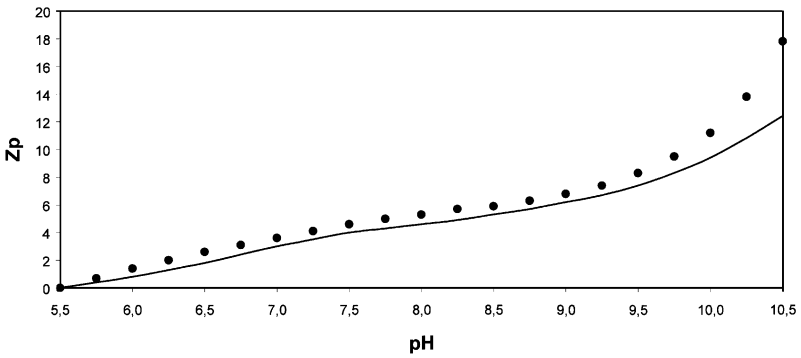

Fig. 6. Absolute values of the net proton charge of κ-casein in 1 M NaCl solutions. Comparison between experimental and model values.

Electrostatic effects do not change the number of groups titrated in the neutral region but affect the timing of the deprotonation. As for κ-casein, 7 charges are expected by calculation in the range between pH 5.5 and pH 9.5 and 7–8 charges are found by measurements. These charges correspond to the deprotonation of histidine, phosphoserine, cysteine and one charge is to be accounted to the acid titration that has not been completed below pH 5.5 (the amino terminus of κ-casein is a pyrrolidon carboxylic acid that is not going to ionize in this range). No electrostatic effect, as mentioned before, is noted.

In the attempt to predict the behavior of the titration curve of β-casein in the pH range 5.5–9.5, we have applied a model based on CCT as developed in [18], to calculate ΔpK_a of the groups in the N-terminal region of the protein. The linear structure at the basis of the model appears to be a suitable assumption because of the secondary structure assessed in [8]. Since we deal with a solution that is very dilute with respect to the protein, we have used ΔpK_a expressions reported in [18] letting the polyelectrolyte concentration tend to zero. Therefore, we have obtained the following equations:

for $\alpha < \alpha_{cr}$

$$\Delta pK_a = -0.434\left\{2\alpha\xi^0 \ln\left[1-\exp\left(\frac{-Ac_s^{0.5}}{\alpha\xi^0}\right)\right] - \frac{Ac_s^{0.5}}{\exp\left(\frac{Ac_s^{0.5}}{\alpha\xi^0}\right)-1}\right\} \quad (8)$$

while for $\alpha > \alpha_{cr}$ is

$$\Delta pK_a = -0.434\left\{2\ln\left[1-\exp\left(\frac{-Ac_s^{0.5}}{\alpha\xi^0}\right)\right] - \frac{Ac_s^{0.5}}{(\alpha\xi^0)^2\left[\exp\left(\frac{Ac_s^{0.5}}{\alpha\xi^0}\right)-1\right]}\right\} \quad (9)$$

where A is a constant defined by

$$A^2 = (8\pi)10^3 N_{av}\left(\frac{q^2}{4\pi\varepsilon_0\varepsilon_r kT}\right)^3 \quad (10)$$

where N_{av} is Avogadro's number and c_s is the molarity of the salt. All the parameters in Eq. (10) must be inserted in the MKS system. In order to use Eqs. (8) and (9) for the acid peptide, that is a copolymer, α has to be calculated according to [19,20]:

$$\alpha = \sum_{i=1}^{t} X_i\alpha_i \quad (11)$$

where X_i is the number of titrable species i divided for the total number of titrable groups, α_i is the dissociation fraction of species i and t is the number of different titrable groups. Dissociation fractions of different species (i and j) are linked by the equation:

$$\alpha_j = \frac{10^{(pK_i^0 - pK_j^0)}\alpha_i}{1-\alpha_i+10^{(pK_i^0 - pK_j^0)}\alpha_i} \quad (12)$$

where pK^0 indicates a pK_{model} term. Since this model can be applied to a region of the polymer with charges of the same sign, we have applied it to the peptide 3–21, so to exclude Arg-1. We have excluded also Glu-2 because, being so close to Arg-1, is likely to behave differently than the other groups of the peptide. The peptide 3–21 contains 6 residues of glutamic acid and 5 residues of phosphoserine and so it can bear a maximum of 14 charges. The monomer length in a peptide chain is 3.62 angstrom so the total length of the peptide is 68.8 angstrom and so $b^0 = 4.91$ angstrom. Assuming $\varepsilon_r(4\ ^\circ C) = 86.3$ [23], $\xi^0 = 1.42$ and $\alpha_{cr} = 0.70$ are evaluated. The constant A, for solvent water at 4 °C, is equal to 2.29. The peptide was modeled as a copolymer of 3 different groups, considering the diprotic phosphoserine as it consisted of 2 distinct groups, the first with $pK_{model} = 2.2$ and the other with $pK_{model} = 7.0$. As a consequence, α has been evaluated from the equation:

$$\alpha = 0.286\alpha_1 + 0.429\alpha_2 + 0.286\alpha_3 \quad (13)$$

where the subscript 1 refers to the first ionization of the phosphoserine, 3 to the second ionization and 2 indicates the glutamic acid.

The solid line in Fig. 3 represents the titration curve calculated with the model based on CCT. This figure shows that, at NaCl concentration 0.1 M, predicted results and experimental data are in

very good agreement (errors are not larger than standard deviation). It may be useful to emphasize that the prediction is obtained without any adjustable parameter. In Fig. 4 calculated results and experimental data (at NaCl 1 M) are compared. In this case, the difference between the dashed line and the solid line is slight. This means that the model overestimates the screening of electrostatic effects on the charged peptide at this high value of ionic strength. We have tried also to not consider the presence of neutral groups on the peptide, imposing $b^0 = 3.62$ angstrom, so to regard the titrable groups as they were consecutive. The results (data not reported) show a slight overestimation of the charge for the 0.1 M solutions while no sensible improvement are noticed for the 1 M.

Above pH 9.5, calculated results underestimate experimental data. This feature could be interpreted as a consequence of not negligible electrostatic effects if groups capable of deprotonating in this range, i.e. tyrosine and lysine, were located in positively charged zones of the protein, so to cause an anticipated dissociation. However, since positive groups are distributed and, at alkalyne pH, there is a predominance of negative and neutral group, it is difficult to assess that electrostatic interactions are the only explanation of this anticipated deprotonation. It has to be emphasized that the choice of pK_{model} has a strong influence on the titration curve, whatever electrostatic model is utilized. It is likely that the pK_{model} referred to free aminoacid overestimate the value of the pK_{model} of tyrosine and lysine residue in the protein chain condition. This interpretation is reinforced by the analysis of the results obtained by Creamer [10]. He evaluated $pK_{model} = 10.0$ for lysine while 10.4 was expected. In other words, at alkalyne pH the author found an anticipated deprotonation, with respect to the expectations. However, it is not possible to give any comprehensive explanation about the alkalyne range because it has been just partially explored and data are less accurate than those ones of the neutral zone.

5. Conclusions

The comparison between experimental data and calculated results shows that, for κ-casein, perturbation terms ΔpK_a can be neglected in the range $5.5 < pH < 9.5$ at 0.1 M and 1 M NaCl concentration. The prediction of the proton charge can be made very simply by just considering $pK_{a,i}$ of the side chains of the corresponding aminoacids. The same operation leads, for β-casein, to errors of up to 2 charges, because of strong electrostatic effects induced by the high density of negative charges of 1–21 peptide; thus, this calculation procedure is suitable just for first approximation calculations. To interpret these electrostatic effects, a predictive model, without any adjustable parameter, based on CCT was applied. Predictions and experimental data are in very good agreement at 0.1 M NaCl while the model underestimates electrostatic effects at 1 M NaCl. Further exploration is needed for the alkalyne range.

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