Studies on the Interactions between Purified Bovine Caseins and Alkaline-Earth-Metal Ions

By I. R. DICKSON AND D. J. PERKINS

Department of Chemical Pathology, St George's Hospital Medical School, London S.W.1, U.K.

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1. Alkaline-earth-metal cations at low concentrations form soluble complexes with bovine caseins. The relative order of binding capacities is: $Mg^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+}$. 2. The cations interact with both free ionized carboxyl groups of aspartic acid and glutamic acid and with monoester phosphate groups covalently bound to serine and threonine; at low concentrations of the cations interactions are predominantly with the phosphate groups. 3. The order of binding capacities for purified components of the casein complex is: α_{s1} -casein $>$ β -casein $>$ κ -casein.

Casein is the major protein constituent of bovine milk and is a mixture of phosphoproteins. The most important of these are the α_{s1} -, β - and κ -fractions, which comprise 53%, 29% and 13% respectively of the casein complex; each fraction exists in several genetically variable forms that differ only slightly in their composition and properties. The fractions differ considerably in their phosphorus content (α_{s1} -casein, 1%; β -casein, 0.5%; κ -casein, 0.2%), but in each the phosphorus is present as monoester phosphates of serine and threonine residues (Hofman, 1958; Kalan & Telka, 1959; Anderson & Kelley, 1959; Osterberg, 1964). Ca^{2+} was shown to bind with the phosphate groups of α_{s1} -casein by i.r. spectroscopy (Ho & Waugh, 1965). The free carboxyl groups of the proteins are also potential metal-ion-binding sites. α_{s1} - and β -Casein are precipitated by relatively low concentrations of Ca^{2+} , but κ -casein remains soluble even at high concentrations of Ca^{2+} . In the presence of Ca^{2+} κ -casein, a glycoprotein, will form a micelle with either α_{s1} - or β -casein, stabilizing the latter against precipitation; this micelle-forming property of the caseins accounts for their stability in milk in the presence of high concentrations of alkaline-earth-metal cations.

Although studies have been carried out on the binding of Ca^{2+} by acid casein (McLean & Hastings, 1935; Weir & Hastings, 1936; Chanutin, Ludewig & Masket, 1942; Carr, 1953; Zittle, DellaMonica, Rudd & Custer, 1958), there is little information available about the binding properties of the pure fractions. Such studies are an essential preliminary to an investigation of the way in which Ca^{2+} participates in the formation of the casein micelle. The present paper is concerned with the interactions between Ca^{2+} and other alkaline-earth-metal

cations and purified samples of bovine casein fractions.

EXPERIMENTAL

Materials. Milk was obtained from a single cow of the pedigree Ayshire herd at the Unilever Ltd. Farm, Colworth, Beds., U.K. The genetic variants present in the caseins were α_{s1} -B, β -A and κ -A,B.

Acid casein was prepared from skim milk by isoelectric precipitation at pH 4.6 and 25°C with 1M-HCl. The precipitate was washed with water of pH 4.6, redissolved in water at pH 7.0 with the aid of 0.1M-NaOH and reprecipitated at pH 4.6. Casein was precipitated once more by this procedure and then stored at -20°C.

Crude α_{s1} -casein and β -casein were prepared from acid casein by chemical fractionation and purified by chromatography on DEAE-cellulose (Whatman DE11) in 3.3M-urea-0.01M-imidazole buffer, pH 7.0 (Thompson & Kiddy, 1964; Thompson & Pepper, 1964; Thompson, 1966). Crude β -casein was chromatographed in the presence of 2-mercaptoethanol. κ -Casein was prepared from acid casein by the method of McKenzie & Wake (1961). The final solution was dialysed against water at pH 7.0 and filtered through Whatman GF/C glass-fibre paper before being freeze-dried.

Chemical modification of casein fractions. Purified caseins were acetylated in 50%-saturated sodium acetate at 2°C with acetic anhydride (Hoagland, 1968) and 2-carboxypropionylated at pH 8.0 and 25°C with succinic anhydride (Habeeb, 1967). Histidine residues were modified with α -bromoacetate at pH 7.0 (Banaszak, Andrews, Burgner, Eylar & Gurd, 1963). Free amino groups were determined with ninhydrin reagent (Moore & Stein, 1944) with unmodified protein as a control. Free histidine residues were determined by using diazonium 1H-tetrazole (Sokolovsky & Vallee, 1966). Caseins were phosphorylated for 48 h with a solution of phosphorus pentoxide in phosphoric acid (Ferrel, Olcott & Fraenkel-Conrat, 1948).

The dried proteins were esterified with anhydrous methanolic 0.1M-HCl (Wilcox, 1967). α_{s1} -Casein was dephosphorylated by dissolving 200mg of protein in 15ml of water at pH 6.5 and adding 0.5ml of acid phosphatase (EC 3.1.3.2) [grade II, 1 mg/ml; Boehringer Corp. (London) Ltd., London W.5, U.K.]. The mixture was dialysed against 1 litre of citrate buffer (0.5M-citric acid adjusted to pH 6.0 with 0.1M-NaOH) at 37°C overnight. The non-diffusible material was adjusted to pH 4.6 with 0.1M-HCl and the solution centrifuged at 1000g for 10min. The residue was dissolved in water at pH 7.0 and dialysed against water before being freeze-dried.

Phosphorus was determined by the method of Allen (1940) and nitrogen by a semi-micro-Kjeldahl technique. Factors used to convert nitrogen values into protein values were: α_{s1} -casein, 6.52; β -casein, 6.58; κ -casein, 6.58.

Electrophoresis. Caseins were examined by starch-gel electrophoresis in 7M-urea-tris-citrate gels at pH 8.6 (Wake & Baldwin, 1961) and by disc electrophoresis in 10% polyacrylamide gels with a discontinuous buffer system (Tombs & Akroyd, 1967).

Equilibrium dialysis. Protein (1.5 ml; 0.5% solution) was placed in dialysis tubing (Visking cellulose tubing, $\frac{8}{32}$ in) and dialysed on an automatic dialyser (Stewart, Perkins & Greening, 1962) against 10 ml of solution containing buffer and radioactive alkaline-earth-metal salt diluted with carrier; the radioactive isotopes used were ^{45}Ca , ^{85}Sr and ^{133}Ba (obtained from The Radiochemical Centre, Amersham, Bucks., U.K.). Samples (1 ml) of the protein and outer-compartment solutions were assayed for radioactivity on a Gamma Spectrometer. Magnesium was determined directly by using an atomic-absorption spectrophotometer. The pH of the outer-compartment solution was measured at the temperature of dialysis. Protein was determined by measurements of E_{280} by using values of 9.6 (α_{s1} -casein), 4.7 (β -casein) and 9.8 (κ -casein) for the extinction coefficients of a 1% (w/v) solution.

Preliminary experiments showed that binding of alkaline-earth-metal cations to caseins varied considerably with changes of pH, ionic strength and free cation concentration. Two of these parameters were maintained constant while the variation of the third was studied. The ionic strength of the buffer systems used was 0.1; anion-distribution studies with $^{131}\text{I}^-$ showed that at this ionic strength the correction for the Donnan membrane effect was insignificant. The high volume ratio of outer-compartment solution to protein solution used in the experiments was adequate to maintain the free-cation concentration at a constant value. Most of the binding studies at constant pH were carried out at pH 7.4, where the second hydroxyl group of monoester phosphate groups is fully ionized.

RESULTS AND DISCUSSION

Binding of Ca^{2+} to α_{s1} -casein increases as the free metal-ion concentration is raised; the protein is precipitated at about 10mM- Ca^{2+} . β - and κ -Casein behave in a similar fashion except that κ -casein is not precipitated, even at much higher Ca^{2+} concentrations. The relative order of binding capacities for Ca^{2+} to casein fractions is: α_{s1} -casein > β -casein > κ -casein (Fig. 1).

The effects of pH on the binding of Ca^{2+} to caseins were studied in the absence of a buffer, since many alkaline amine buffer systems form complexes with Ca^{2+} (Fig. 2). Binding of Ca^{2+} cannot be studied near pH 5 because all caseins are precipitated at their isoelectric points. Binding of Ca^{2+} is low at pH 6, but increases markedly between pH 6 and pH 7 owing to the ionization of the second hydroxyl group of the monoester phosphate groups and to deprotonation of histidine residues. There is little alteration of the ionizing groups on amino acid residues between pH 7 and pH 9, and this is reflected by a flattening of the binding curves in this region. At more alkaline pH values binding increases owing to deprotonation of the lysine and guanidine residues and to ionization of tyrosine hydroxyl groups. Binding of Ca^{2+} decreases markedly as the ionic strength of the medium is raised (Fig. 3). This may be due in part to competition by Na^+ . There is no experimental evidence for the binding of alkali-metal ions to serum proteins, but Ho & Waugh (1965) showed binding to α_{s1} -casein by using i.r. spectroscopy. We found, by using $^{22}\text{Na}^+$ as tracer, that Na^+ was bound to a α_{s1} -casein in a solution that had previously been deionized by passing it through a column of Bio-Deminrolit resin. However, binding is very low compared with binding of Ca^{2+} , and the main effect of high concentration of Na^+ on binding of Ca^{2+} will be on the activity of the Ca^{2+} rather than by competition for binding sites. As Na^+ is bound to caseins, other univalent ions (including buffer ions) will probably also form complexes. Our findings provide indirect evidence of this, for under identical conditions of pH, ionic strength and Ca^{2+} concentration the amount of Ca^{2+} bound to α_{s1} -casein is higher in cacodylate buffer than in tris buffer. Tris cations do not interact with alkaline-earth-metal cations and the effect of the formation of a Ca^{2+} -cacodylate

Fig. 1. Effect of increasing Ca^{2+} concentration on the binding of Ca^{2+} to α_{s1} -casein (○), β -casein (●) and κ -casein (□) at pH 7.4 (tris-HCl buffer, I 0.1).

Fig. 2. Effect of pH on the binding of Ca^{2+} to α_1 -casein (O), β -casein (●) and κ -casein (□). There were no buffering ions. All media had an initial concentration of 2 mM- Ca^{2+} and contained 0.1 M-NaCl.

complex would be to reverse this order, so the phenomenon is a result of either tris binding to anionic protein sites or cacodylate binding to cationic sites, or both of these effects.

The effects produced on binding of Ca^{2+} to caseins by changes in ionic strength, free- Ca^{2+} concentration and pH indicate that binding is fully reversible according to the empirical definition given by Hughes & Klotz (1956). Our tests of binding reversibility have confirmed that the amount of Ca^{2+} bound to these proteins at equilibrium is dependent only on the free Ca^{2+} concentration and is not affected by Ca^{2+} bound to the protein initially.

The relationship between the reciprocals of the concentrations of the bound and the free cations can yield information about the nature of the binding sites. Linear relationships are obtained for each distinct class of sites if the system conforms to the theory of multiple equilibria. In caseins, the possible binding sites are monoester phosphate groups contributed by serine and threonine residues and carboxyl groups of aspartic acid and glutamic acid. Experimentally observations are limited to a narrow range of Ca^{2+} concentrations. Therefore it is impossible to analyse the interactions in full detail according to the theory. We observed a linear relationship (Fig. 4) at low Ca^{2+} concentrations, but deviations were becoming obvious above 3–4 mM. By extrapolating the linear portion of the reciprocal plot to the y axis we estimated the number of binding sites, n , comprising the linear portions of the graphs. These experiments show that there is more than one class of binding sites but that one is preferentially filled. The values obtained for

Fig. 3. Effect of varying the ionic strength on the binding of Ca^{2+} to α_1 -casein at pH 7.4 (tris-HCl buffer, I 0.04). Ionic strength was varied by the addition of NaCl. The initial Ca^{2+} concentration was 2.5 mM.

Fig. 4. Plots of $1/v$ [(g-atoms of Ca^{2+} bound/10⁵ g of protein)⁻¹] against $1/c$ [(free Ca^{2+}) (mM)⁻¹] for α_1 -casein (O) and phosphorylated α_1 -casein (●) at pH 7.4 (tris-HCl buffer, I 0.1).

n (Table 1) are greater than the maximum number of available phosphate sites, as determined by phosphate analysis. Experiments with chemically modified caseins (Table 1 and Fig. 5) show that the ester phosphate groups are the binding sites but that there may be other active sites in this class. These may be carboxyl groups with binding affinities similar to those of the phosphate groups.

Since the formation of the Ca^{2+} -casein complex is fully reversible, the amount of protein-bound Ca^{2+}

will be altered by changes in the net negative charge on the protein. Thus binding of Ca^{2+} is increased by acetylation, 2-carboxypropionylation, carboxymethylation and phosphorylation of the protein and decreased by esterification or dephosphorylation (Fig. 5). Estimates of the number of binding sites from reciprocal-plot analyses (Table 1) showed that acetylation or carboxymethylation had no effect, 2-carboxypropionylation or phosphorylation increased the number of sites, and esterifica-

Fig. 5. Effect of increasing Ca^{2+} concentration on the binding of Ca^{2+} to chemically modified caseins (○, phosphorylated; ●, 2-carboxypropionylated; □, carboxymethylated; △, esterified; ▲, dephosphorylated; ■, native). All experiments were done at pH 7.4 (tris-HCl buffer, *I* 0.1).

tion or dephosphorylation had the reverse effect. It is probable that the effect of methanolic hydrochloric acid is to esterify monoester phosphate groups as well as carboxyl groups, thus decreasing the number of phosphate groups available for binding of Ca^{2+} . The increase in the number of sites after 2-carboxypropionylation indicates that the carboxyl groups introduced by this modification have higher association constants with Ca^{2+} than those of the native carboxyl groups and those introduced by carboxymethylation. The properties of the N^{ϵ} -(2-carboxypropionyl)lysyl carboxyl group may be distinctive as it is at the extremity of a long chain ($\text{C}_{\alpha\alpha}$ -[CH₂]₄-NH-CO-[CH₂]₂-CO₂H) and will be sterically freer to take part in a dicarboxylic salt formation with Ca^{2+} and another carboxyl group. The association constants for Ca^{2+} interactions with dicarboxylic acids are greater than for those with monocarboxylic acids.

The apparent $\log K_1$ values reported for simple Ca^{2+} complexes (Table 2) are small compared with those for bovine serum albumin. Saroff & Lewis (1963) showed that the intramolecular interactions of the ionizing groups of albumin led to binding properties different from those predicted from simple model compounds. Only the use of chemically modified albumin gives an indication of the reactive groups involved. The presence of a large number of phosphate groups in caseins as well as those due to the usual amino acid residues complicates the identification of the reacting groups. In Table 2 the $\log K_1$ values are also shown for Ca^{2+} complexes with α_{s1} -casein and two model

Table 1. Number of Ca^{2+} -binding sites, determined by reciprocal-plot analysis, found in native and chemically modified caseins compared with the number of phosphate groups present in each preparation

See Fig. 4 for examples of this determination. The numbers of phosphate groups were calculated from the observed phosphate analyses. The numbers of histidine residues are those found on analyses with diazonium *IH*-tetrazole reagent. Other authors found 21 residues from complete analyses of amino acid hydrolysates of α_{s1} -casein (Koning & de Van Rooijen, 1965). The residues shown are the number of lysine and terminal amino residues obtained by using the ninhydrin method (Jollés, Alais & Jollés, 1962). N.D., Not determined.

Preparation	No. of Ca^{2+} -binding sites/ 10^5 g of protein	Calc. no. of phosphate sites/ 10^5 g of protein	No. of histidine residues/ 10^5 g of protein	No. of lysine residues/ 10^5 g of protein
α_{s1} -Casein				
Native	36	28	17	61
Phosphorylated	53	55	N.D.	N.D.
Dephosphorylated	5	0	N.D.	N.D.
Acetylated	39	28	N.D.	11
Carboxymethylated	36	28	3	N.D.
2-Carboxypropionylated	50	28	N.D.	7
Esterified	21	N.D.	N.D.	N.D.
β -Casein				
Native	20	19	N.D.	N.D.
κ -Casein				
Native	11	6	N.D.	51
2-Carboxypropionylated	33	6	N.D.	7

Table 2. Comparison of the apparent $\log K_1$ values for the complexes of Ca^{2+} with α_1 -casein and some model compounds

The values quoted for the model compounds were obtained from Sillén & Martel (1964).

Ca^{2+} complex with	$\log K_1$
α_1 -Casein	2.6
Serine <i>O</i> -(dihydrogen phosphate)	2.3
Orthophosphoric acid	2.2
Bovine serum albumin	2.4
Acetic acid	0.6
Glycine	1.4
Glutamic acid	0.8
Citric acid	3.2

compounds. There is no distinctive difference between the values for bovine albumin and casein, so the similarity between α_1 -casein and the model compounds cannot be taken as a satisfactory method of identifying the active groups.

The $\log K_1$ values previously reported range around our value of 2.6. Their precision varies according to the method of measurement. Also, there are no data on the quality of the different caseins used. The criteria of protein purity and integrity have been improved over the years. Zittle *et al.* (1958) discussed the differences in the methods, conditions and protein preparations used. They noted that the number of Ca^{2+} -binding sites observed varied widely among the preparations used for each reported investigation.

There are differences in the amounts of individual alkaline-earth-metal cations bound to α_1 - or β -casein fractions; the relative order of binding capacities to either is: $\text{Mg}^{2+} > \text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+}$ (Fig. 6). The corresponding order for κ -casein was similar, except that there was no significant difference between binding of Sr^{2+} and Ba^{2+} . One might expect the order to be: $\text{Mg}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+}$ (Irons & Perkins, 1962); we have studied the binding of Sr^{2+} and Ba^{2+} to this protein and find the order: $\text{Sr}^{2+} > \text{Ba}^{2+}$; the difference is not statistically significant ($0.25 < P < 0.30$). Studies with phosphatidylserine from hen's-egg yolk, a protein containing approx. 10% of phosphorus, all present as monoester phosphates of serine residues, showed that the binding order for alkaline-earth-metal cations was similar to that in caseins (I. R. Dickson & D. J. Perkins, unpublished work).

The effect of pH and of ionic strength on the binding of the other alkaline-earth-metal cations to caseins are similar to those of Ca^{2+} , and reciprocal-plot analyses show that the same number of sites on the proteins is available to any of those cations. Thus each of the alkaline-earth-metal cations forms complexes of a similar nature with caseins. The

Fig. 6. Effect of increasing metal-ion concentration on the binding of Mg^{2+} (●), Ca^{2+} (○), Sr^{2+} (□) and Ba^{2+} (■) at pH 7.4 (tris-HCl buffer, I 0.1).

differences observed are in the association constants of the complexes formed between them and monoester phosphate groups. It is therefore probable that the anomalous positions of Sr^{2+} and Ba^{2+} in the binding order for caseins are attributable to a property of the organically linked monoester phosphate groups.

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