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Binding of bivalent cations to α-lactalbumin and β-lactoglobulin : effect of pH and ionic strength

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Summary

Interactions between α-lactalbumin and β-lactoglobulin and different cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}) were studied in solution at various pH (6.6, 5.5, 5.0) and ionic strengths ($\mu < 0.01$, $\mu = 0.05$, $\mu = 0.10$). The chelation was studied using binding ratios.

Both proteins are able to bind several cations at pH 6.6 when ionic strength is low. The affinity of α-lactalbumin for the first bound atom is high, then decreases for the following ions. In these physico-chemical conditions of the aqueous phase (pH 6.6 - $\mu < 0.01$), the maximal number of bound cations depends on the mineral. The binding ability of α-lactalbumin is 3.0 ions of Mn^{2+}, 3.5 of Mg^{2+}, 4.5 of Ca^{2+} and Zn^{2+}, 6.0 of Fe^{2+}, 10.6 of Cu^{2+} and more than 2.0 ions of Mn^{2+}, 2.5 of Zn^{2+}, 3.2 of Mg^{2+} and 3.5 of Ca^{2+}, Fe^{2+}, Cu^{2+} for β-lactoglobulin. The affinity of α-lactalbumin is high for Ca^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and reduced for Mg^{2+} and Fe^{2+}. For β-lactoglobulin the affinity is high for Zn^{2+}, Fe^{2+}, Cu^{2+}, and lower for Ca^{2+}, Mg^{2+} and Mn^{2+}.

The affinity becomes lower when pH decresases or when ionic strength increases, except for α-lactalbumin with calcium and for both proteins with copper. Except for Cu^{2+}, which is always strongly bound to both proteins, the binding ability is about one atom per protein molecule at pH 5.0 ($\mu < 0.01$) and at pH 6.6 ($\mu = 0.10$).

Key words : α-lactalbumin - β-lactoglobulin - Bivalent cation - Binding - pH - Ionic strength.

Résumé

Fixation des cations bivalents à l'α-lactalbumine et à la β-lactoglobuline : effet du pH et de la force ionique

Les interactions entre l'α-lactalbumine ainsi que la β-lactoglobuline et différents cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}) ont été étudiées en solution à différents pH (6,6 - 5,5 - 5,0) et différentes forces ioniques ($\mu < 0,01$ - $\mu = 0,05$ - $\mu = 0,10$).

Ces deux protéines sont capables de fixer plusieurs atomes à pH 6.6 et à une force ionique inférieure à 0,01. L'affinité de l'α-lactalbumine pour le premier cation fixé est élevée mais elle décroît pour les chélations d'ordre supérieur. Dans ces conditions physico-chimiques du milieu (pH 6,6 - $\mu < 0,01$) le nombre maximal d'ions fixés

dépend du type de minéral. Il est dans le cas de l'α-lactalbumine de 3,0 pour Mn^{2+}, de 3,5 pour Mg^{2+}, de 4,5 pour Ca^{2+} et Zn^{2+}, de 6,0 pour Fe^{2+} et de 10,6 pour Cu^{2+}. La β-lactoglobuline peut chélater plus de 2,0 atomes de Mn^{2+}, plus de 2,5 de Zn^{2+}, plus de 3,2 de Mg^{2+} et plus de 3,5 de Ca^{2+}, Fe^{2+} et Cu^{2+}. Dans le cas de l'α-lactalbumine, l'affinité est forte pour Ca^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} et plus réduite pour Mg^{2+} et Fe^{2+}. L'affinité de la β-lactoglobuline est élevée pour Zn^{2+}, Fe^{2+}, Cu^{2+} et plus faible pour Ca^{2+}, Mg^{2+}, Mn^{2+}.

L'affinité de ces protéines pour les minéraux diminue avec le pH ou avec une augmentation de la force ionique sauf dans le cas des complexes cupriques et de l'α-lactalbumine en présence de calcium. A l'exception du cuivre qui est toujours fortement lié aux protéines quelles que soient les conditions de milieu, la capacité de fixation est égale à un atome par molécule de protéine à pH 5,0 ($\mu < 0,01$) et à pH 6,6 ($\mu = 0,10$).

Mots clés : α-lactalbumine - β-lactoglobuline - Cation bivalent - Fixation - pH - Force ionique.

Introduction

α-lactalbumin and β-lactoglobulin represent the two major soluble proteins of cow's milk : the amount of each is 1 and 3 $g.l^{-1}$ respectively.

α-lactalbumin is a globular metalloprotein (Hiraoka *et al.*, 1980) with a molecular weight of 14 200, corresponding to 123 amino acids ; its structure is stabilized by 4 sulphur bridges. One atom of calcium, reversibly bound to this protein (Kronman, 1981), stabilizes the tertiary structure. α-lactalbumin, whose primary sequence is homologous to vertebrate lysozyme (Brew *et al.*, 1970), is involved in the synthesis of lactose. It binds to galactosyltransferase to form the lactose synthetase enzyme system which synthesizes lactose from UDP galactose and glucose. Affinity constant for the first binding site towards calcium is 2.7×10^6 M^{-1} (Kronman, 1981), $3\text{-}6 \times 10^8$ M^{-1} (Permyakov *et al.*, 1981), 10^{10} to 10^{12} M^{-1} (Murakami *et al.*, 1982) with different experimental conditions. According to Kronman and Bratcher (1984), α-lactalbumin has 4 chelating sites for minerals. Different cations can be bound to the calcium site : Manganese (II), Cadmium (II), Magnesium (II) and several lanthanides (Murakami *et al.*, 1982). Mn^{2+} can also be bound to a lower affinity site ; this chelation is physiologically important because galactosyltransferase needs Mn^{2+} to be active (O'Keeffe *et al.*, 1980). Murakami and Berliner (1983) have found two binding sites ; the first binds Ca^{2+} and the second, specific for Zn^{2+}, is able to bind Co^{2+} and Cu^{2+}. Zinc chelation inhibits the binding of calcium to the protein. Many assumptions for localization of these sites, involving glutamic and aspartic acids have been discussed (Gerken, 1984 ; Kita *et al.*, 1976 ; Stuart *et al.*, 1986).

β-lactoglobulin is a 18 400 molecular weight protein, composed of 162 amino acids (Eigel *et al.*, 1984) ; it includes two sulphur bridges and one free SH group. This protein, which is a component of the milk of several mammals, is missing in human milk (Brignon *et al.*, 1985). The biological role of β-lactoglobulin is unknown, but it is able to polymerize and to complex other molecules. It binds retinol (Vit A1) (Fugate and Song, 1980) and also has a

similar structure to plasma retinol-binding protein (PAPIZ *et al.*, 1986) : β-lactoglobulin could thus enhance milk vitamin A1 absorption. This protein is also able to associate with the carcinogenic hydrocarbons (3,4 benzopyren) (ONO *et al.*, 1975). Only a few studies of mineral chelating ability have been made with β-lactoglobulin. NASSI *et al.* (1974), studying zinc content of various milk fractions, have concluded that this cation was not present in the « β-lactoglobulin » fraction. On the other hand, THOMPSON and BROWER (1985) have shown, using the « stains all » procedure, that β-lactoglobulin is able to bind calcium. KUWATA *et al.* (1985) have studied the elimination of this protein from cow's milk using a ferric precipitation at pH 3.0.

It seems useful to study the binding ability of α-lactalbumin and β-lactoglobulin in relation to some minerals (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}), within different physico-chemical characteristics of the aqueous phase (pH and ionic strength). The aim of such work was to acquire a better knowledge of the affinity of protein towards minerals and of the binding ability of these two proteins.

I. Materials and methods

A. *Purification of proteins*

α-lactalbumin and β-lactoglobulin were isolated from sweet cheese whey (Society PREVAL, Montauban de Bretagne). This whey was clarified through aggregation of lipoproteins under heat treatment and calcium addition (FAUQUANT *et al.*, 1985). It was then concentrated by ultrafiltration on a ROMICON (PM 10 − 1.4 m^2) cartridge at 30 °C ; the precipitation of α-lactalbumin and of some other components occured under a moderate heat treatment at pH 3.8 as described by PIERRE and FAUQUANT (1986) ; only β-lactoglobulin remained soluble in the supernatant. After resolubilisation of the pellet, Bovine Serum Albumin (BSA) and other proteins were removed from « α-lactalbumin » fraction by chromatography on SEPHADEX G100 (PHARMACIA) (column : length = 140 cm, diameter = 45 mm, flow rate = 70 $ml.h^{-1}$, phosphate buffer 0.2 M, pH 7.5). α-lactalbumin bound minerals were removed by dialysis at pH 1.7 and the protein was freeze-dried. β-lactoglobulin was diafiltered with distilled water, then concentrated up to 10 % protein by ultrafiltration and finally freeze-dried.

B. *Sample preparation*

α-lactalbumin and β-lactoglobulin were dissolved in bidistilled water, up to respectively 1 and 3 $g.l^{-1}$ total solids. In some preparations the ionic strength was modified by sodium chloride (MERCK) addition. Mineral chlorides ($CaCl_2,2H_2O$; $MgCl_2,6H_2O$; $ZnCl_2$; $FeCl_2,4H_2O$; $MnCl_2,2H_2O$; $CuCl_2,2H_2O$) (MERCK Purity > 99 %) were added to the protein solution. The cation added ranged from 0 to 5×10^{-4} M for α-lactalbumin and for β-lactoglobulin except for Ca^{2+} (up to 8×10^{-4} M) and for Mg^{2+} (up to 13×10^{-4} M). After careful mixing for a few minutes, the pH was adjusted to the values indicated,

with NaOH (N) or HCl (N), and samples were isolated to be analyzed. One hour was necessary to reach a complete binding equilibrium. Preparations and separations were performed at 20 °C.

C. *Phase separation*

Mineral separation between bound protein form and free form was performed with ultrafiltration. Preliminary ultrafiltration trials at different pHs and ionic strengths to ensure no protein in the permeate, led us to use a CENTRIFLO CF 25 cone (AMICON - cut off 25 000) for β-lactoglobulin and a CENTRICON 10 microconcentrator (AMICON - cut off 10 000) for α-lactalbumin. The ultrafiltration was performed on a centrifuge MISTRAL 6L (45 mn - 1 000 g) using 7 ml solution on CENTRIFLO CF 25 and 2 ml on CENTRICON 10 ; the amounts of permeate were respectively about 3 and 1 ml.

D. *Analytical procedures*

1. *Analysis of fractions during purification steps*

The different steps of protein purification were followed by electrophoresis according to the procedure of ANDREWS (1983).

2. *Protein content*

Protein contents of the different solutions were estimated from HPL Chromatography (VARIAN 5000) on a TSK 3000 column using Tris buffer (0.01 M, pH 6.68 — Flow rate : 1 $ml.min^{-1}$ − 280 nm). The precision of this method was 5 %. The standars used were α-lactalbumin and β-lactoglobulin (SIGMA - CHEMICAL COMPANY), the protein content of which were determined using absorptivity at 280 nm respectively equal to 2.00 $cm^2.g^{-1}$ and 0.94 $cm^2.g^{-1}$.

3. *Mineral content*

Mineral content was determined by Atomic Absorption Spectrophotometry (VARIAN AA 1275). Ca and Mg were determined according to the procedure of BRULÉ *et al.* (1974) (Precision = 0.02 $mg.l^{-1}$). The determination of Zn, Cu and Mn was made using dilutions of the samples in order to obtain amounts of cation between 0.5 and 2.0 × $10^{-3} g.l^{-1}$. The absorption of the iron-protein complex at 248 nm hindered direct determination of total iron content of the samples : accurate amounts of iron were added to the solutions and the determination was only performed for free iron.

E. *Calculation*

The balance is : Protein + Mineral ⇆ Protein-Mineral
with molar ratio P A R

P = concentration of unoccupied binding sites (M)
A = concentration of free ligand (M)

R = concentration of occupied binding sites
(= concentration of bound ligand) (M)
X = total concentration of binding sites (M)
Y = total concentration of ligand (M)

The protein content of the solution was low so we can assume that

Mineral in the permeate = Free mineral in the solution

So : Bound mineral = Total mineral − Free mineral

The binding ratio (T) is equal to $T = R/Y$ and the binding constant to $K = R/PA$.

So K can be related to T :

$$1/K = (X - TY)(1 - T)/T$$

For X and Y constant, if T increases so K increases.

All the results were calculated for one protein mole in order to be able to compare samples with slight differences in protein amounts. The initial slope of the curves was calculated with linear regression on the first four points ; it gave an initial binding ratio. Binding ratios corresponding to different values of R (> 1) were estimated by linear interpolation.

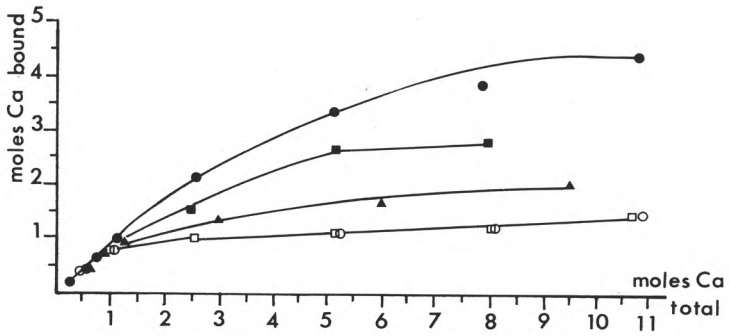
II. Results

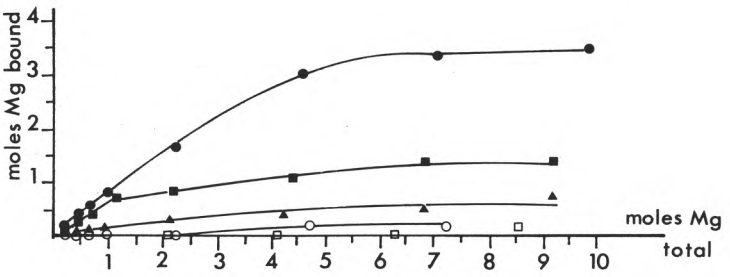
A. *α-lactalbumin*

1. *Effect of pH*

This study on the complexation of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+} by α-lactalbumin was made for pH 6.6, 5.5, 5.0 without ionic strength modifications (initial value was less than 0.01) ; this was a range from pH of milk to pH of milk acid clotting.

The curves (fig. 1) showing the amount of bound mineral (per α-lactalbumin mole) versus total mineral (per α-lactalbumin mole) were at first linear, then decreased to reach a final plateau. The maximal number of moles being able to be bound were at pH 6.6 higher than 4.5 for Ca, equal to 3.5 for Mg, 4.5 for Zn, 10.6 for Cu, 6.0 for Fe and 3.0 for Mn. The linear part of the curve was situated between 0 and 1 mole of bound mineral except for Cu where it went on up to 5 bound moles. The amount of chelated mineral depended on its nature : at pH 6.6 for 5.0 moles of total cation added to the solutions, we found about 3.1 bound moles of Mg, 3.2 of Mn, 3.4 of Ca, 3.6 of Zn, 4.2 of Fe and 4.9 of Cu. The linear part of the curves was common for the three pHs in the case of Ca and Cu, with slopes near 1, but for Mg, Zn, Fe, Mn it decreased with the lowering of pH. For higher amounts with the same total concentration of cation, the amount of bound mineral was smaller when pH decreased from 6.6 to 5.0. The maximal binding ability of α-lactalbumin took different values depending on the pH : in the case of Ca these values were higher than 4.5, 3.0 and 2.0 moles bound for pH 6.6, 5.5, 5.0.


moles Ca bound
moles Ca total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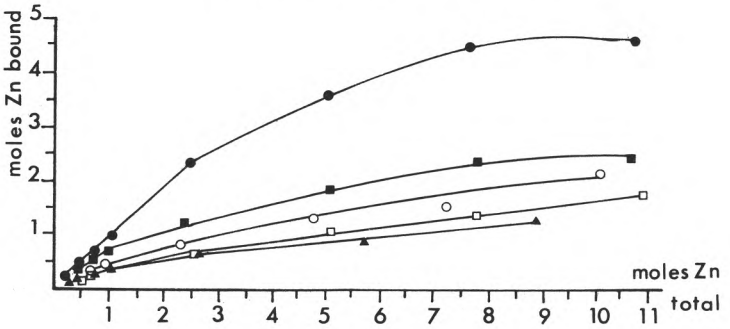
moles Mg total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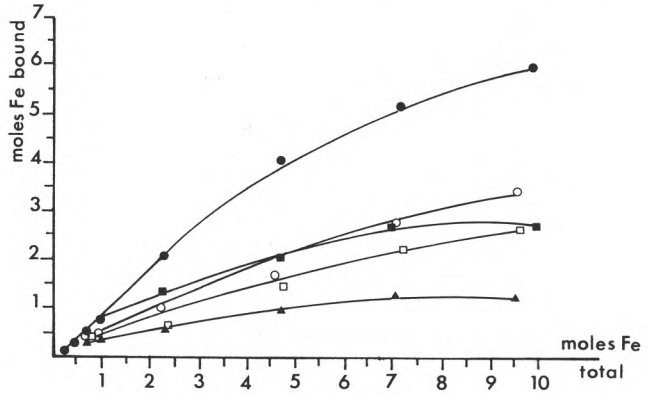
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moles Fe total

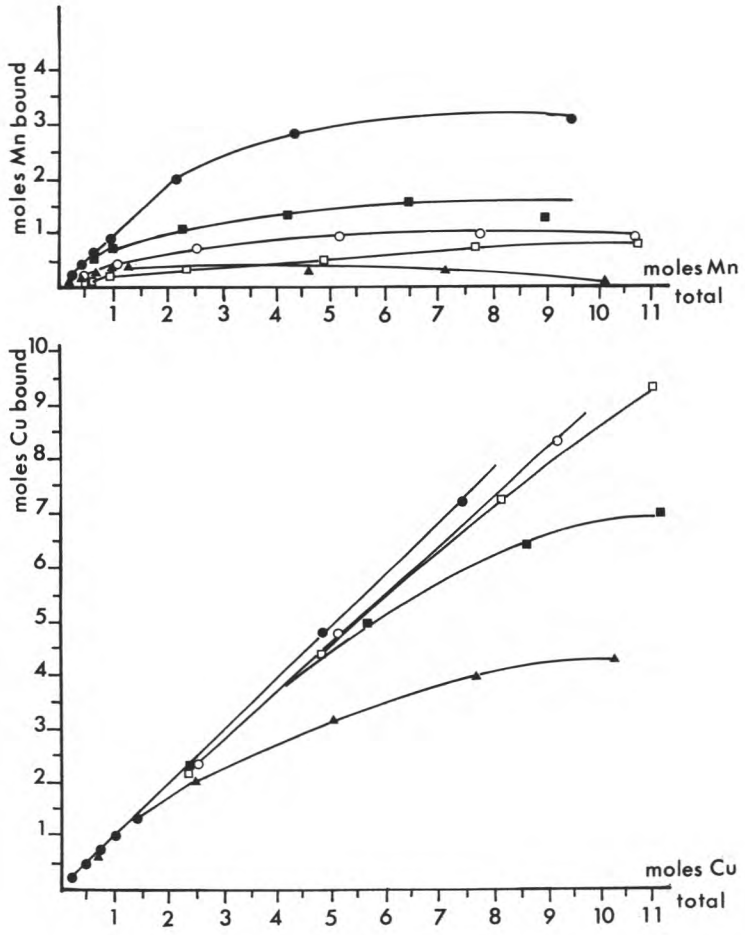

Fig. 1

Chelation of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} by α-lactalbumin : bound molarity ([Bound mineral^{2+}]/[α-La]) versus total molarity ([Total mineral^{2+}]/[α-La]) for pH 6.6, pH 5.5, pH 5.0 ($\mu < 0.01$) and for $\mu = 0.05$, $\mu = 0.10$ (pH 6.6).

Fixation de Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} sur l'α-lactalbumine : nombre de moles fixées ([Mineral^{2+} fixé]/[α-La]) en fonction du nombre de moles totales ([Mineral^{2+} total]/[α-La]) à pH 6,6, 5,5, 5,0 ($\mu < 0,01$) et pour $\mu = 0,05$ et 0,10 (pH 6,6).

● *pH 6.6* ○ $\mu = 0.05$
■ *pH 5.5* □ $\mu = 0.10$
▲ *pH 5.0*

The histograms showing the initial binding ratio for different minerals at various pHs are given in figure 2 ; they showed that if we consider high initial binding ratio (> 0.900), medium initial binding ratio (0.500 to 0.900) and low initial binding ratio (< 0.500) :

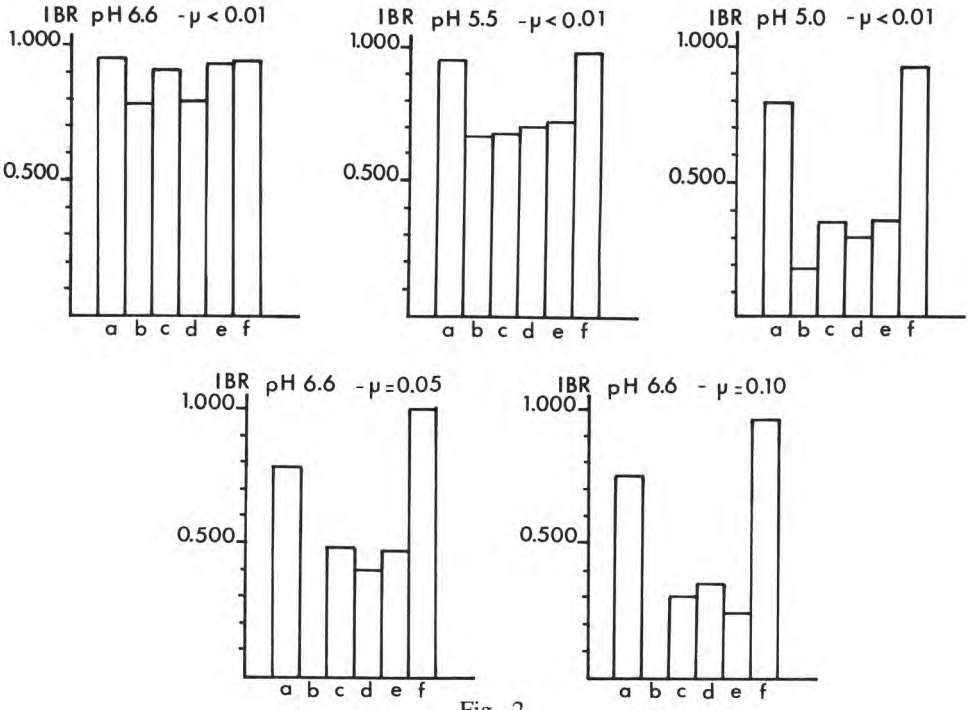

Fig. 2

Initial binding ratio (IBR) of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} by α-lactalbumin at pH 6.6, 5.5, 5.0 ($\mu < 0.01$) and $\mu = 0.05$, $\mu = 0.10$ (pH 6.6). (IBR = [Bound cation]/[Total cation], calculated with linear regression on the first 4 points of the binding curve).

Taux de fixation initial (IBR) de Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} sur l'α-lactalbumine à pH 6,6, 5,5, 5,0 ($\mu < 0,01$) et pour $\mu = 0,05$ et 0,10 (pH 6,6). (IBR = [Cation fixé]/[Cation total], rapport déterminé par régression linéaire sur les 4 premiers points de la courbe de fixation).

a = Ca^{2+} *d* = Fe^{2+}
b = Mg^{2+} *e* = Mn^{2+}
c = Zn^{2+} *f* = Cu^{2+}

— Ca had high initial binding ratios at pH 6.6 and 5.5 and medium at pH 5.0.

— Mg and Fe had medium initial binding ratios at pH 6.6 and 5.5 and low at pH 5.0.

— Zn and Mn had high initial binding ratios at pH 6.6, medium at pH 5.5 and low at pH 5.0.

— Cu had high initial binding ratios at all pHs.

The histograms concerning the binding ratios for 4 bound moles (R = 4) at pH 6.6 or 2 (R = 2) at pH 5.5 (fig. 3) showed that, at pH 6.6, Cu was bound strongly to α-lactalbumin (T = 0.978) ; the ratios were lower for Fe,

Mn, Zn and were only equal to 0.472 for Ca and 0.224 for Mg. At pH 5.5 (R = 2), the affinity order was :

Cu > Ca > Fe > Zn > Mn > Mg.

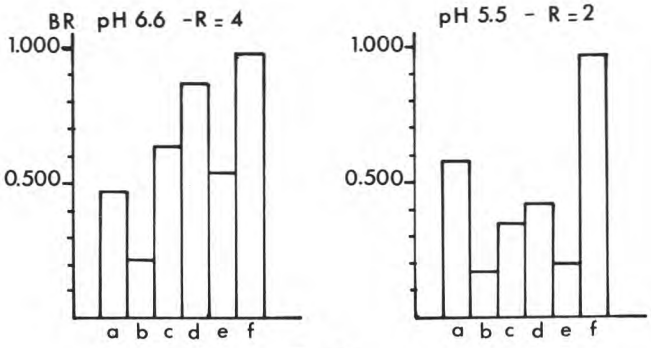

Fig. 3

Binding ratio (BR = [Bound cation]/[Total cation]) of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} on α-lactalbumin for 4 bound moles (R = 4) at pH 6.6 and 2 bound moles (R = 2) at pH 5.5.

Taux de fixation (BR = [Cation fixé]/[Cation total]) de Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} sur l'α-lactalbumine pour 4 moles de cation fixées (R = 4) à pH 6,6 et 2 moles (R = 2) à pH 5,5.

a = Ca^{2+} *d* = Fe^{2+}
b = Mg^{2+} *e* = Mn^{2+}
c = Zn^{2+} *f* = Cu^{2+}

2. *Effect of ionic strength*

This experiment was made at pH 6.6 with three starting ionic strengths (μ) : < 0.01, 0.05 and 0.10. The curves (fig. 1) showed differences between μ < 0.01 and μ = 0.05 or μ = 0.10. The complexed mineral amount decreased when ionic strength increased. α-lactalbumin always had a high affinity for Cu but on the contrary Mg was not bound at all (μ = 0.05 and 0.10). Initial binding ratios, shown on figure 2 are high, for Cu (μ = 0.05 and μ = 0.10) respectively equal to 0.778 and 0.746 ; they are low for all other minerals. This initial binding ratio was always higher when ionic strength was approximately zero ; it decreased when μ increased.

The preferential bindings were :

μ < 0.01	Ca > Cu > Mn > Zn > Fe > Mg
μ = 0.05	Cu > Ca > Zn > Mn > Fe
μ = 0.10	Cu > Ca > Fe > Zn > Mn

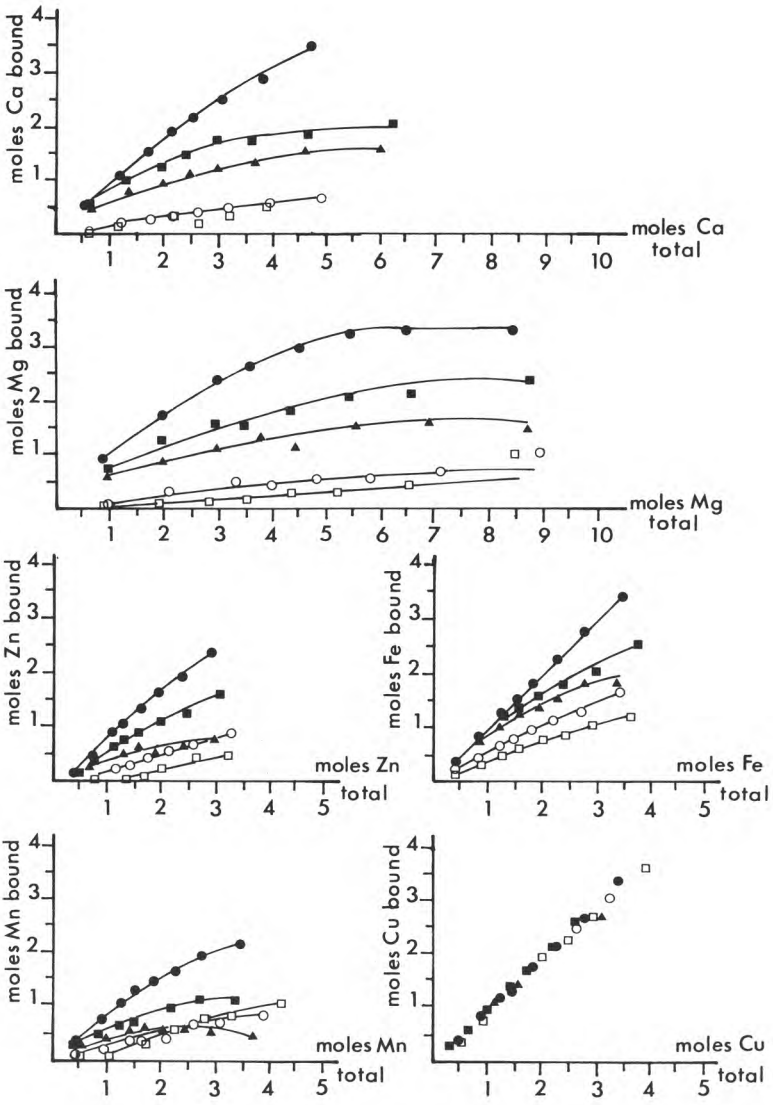

Fig. 4

Chelation of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} by β-lactoglobulin : bound molarity ([Bound mineral^{2+}]/[β-Lo]) versus total molarity ([Total mineral^{2+}]/[β-Lo]) at pH 6.6, pH 5.5, pH 5.0 ($\mu < 0.01$) and for $\mu = 0.05$, $\mu = 0.10$ (pH 6.6).

Fixation de Ca^{2+}; Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} sur la β-lactoglobuline : nombre de moles fixées ([Mineral^{2+} fixé]/[β-Lo]) en fonction du nombre de moles totales ([Mineral^{2+} total]/[β-Lo]) à pH 6,6, 5,5, 5,0 ($\mu < 0,01$) et pour $\mu = 0,05$ et 0,10 (pH 6,6).

● *pH 6.6* ○ $\mu = 0.05$
■ *pH 5.5* □ $\mu = 0.10$
▲ *pH 5.0*

B. β-*lactoglobulin*

1. *Effect of pH*

β-lactoglobulin contained less than 0.01 mg per kg of protein of each mineral studied. The complexing experiments of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+} on β-lactoglobulin have been made at pH 6.6, 5.5, 5.0 without any change in ionic strength ; it was always lower than 0.01 after salts were added.

Figure 4 corresponding to the mineral bound molarity (per β-lactoglobulin mole) versus total mineral (per β-lactoglobulin mole) showed various bound amounts for the different pHs. For the 6 minerals, the binding curves were at

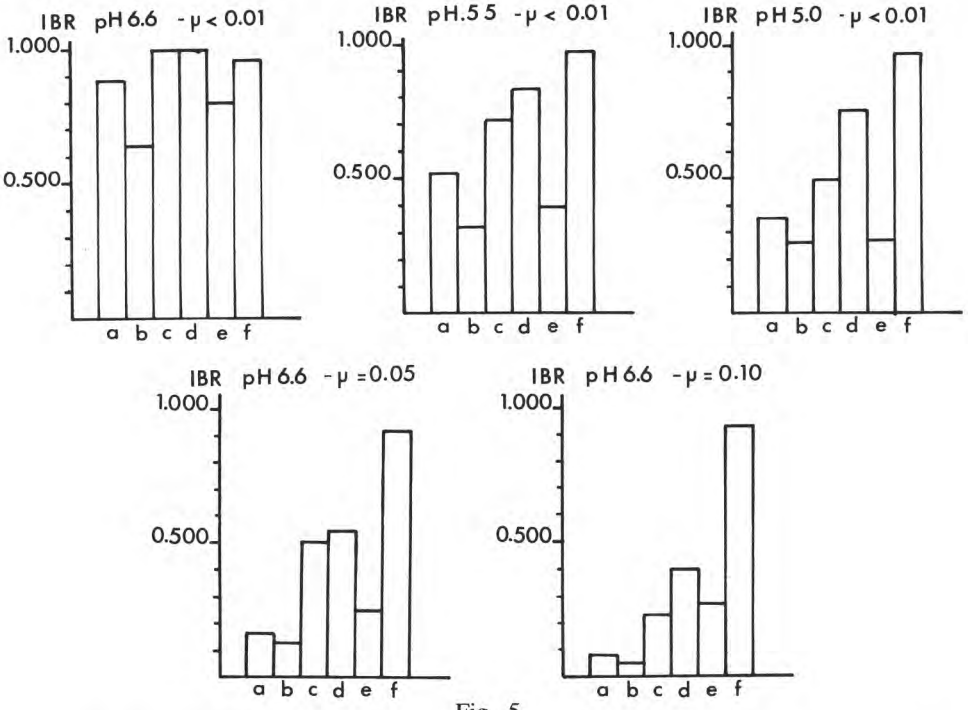

Fig. 5

Initial binding ratio (IBR) of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} by β-lactoglobulin at pH 6.6, 5.5, 5.0 ($\mu < 0.01$) and $\mu = 0.05$, $\mu = 0.10$ (pH 6.6). (IBR = [Bound cation]/[Total cation], calculated with linear regression on the first 4 points of the binding curve).

Taux de fixation initial (IBR) de Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} sur la β-lactoglobuline à pH 6,6, 5,5, 5,0 ($\mu < 0,01$) et pour $\mu = 0,05$ et 0,10 (pH 6,6). (IBR = [Cation fixé]/[Cation total], rapport déterminé par régression linéaire sur les 4 premiers points de la courbe de fixation).

a = Ca^{2+} *d* = Fe^{2+}
b = Mg^{2+} *e* = Mn^{2+}
c = Zn^{2+} *f* = Cu^{2+}

first linear then decreased to reach a plateau, as for example, for 1.0 mole of total Mg at pH 6.6, we found 1.0 bound mole, for 3.0 we had 2.3 bound moles and for 5.0 and 6.0 respectively 3.1 and 3.2 moles were bound. For the others minerals, at pH 6.6, the maximal number of bound cations was higher than 3.5 for Ca, Fe, Cu, 2.0 for Mn, 2.5 for Zn. In every case, except for Cu, when the pH decreased, the bound amount decreased. When 4.0 moles of Ca were added to the solutions at pH 6.6, 5.5 and 5.0, 3.0, 1.8 and 1.4 moles were respectively bound to the protein. Unlike other minerals, the chelation of Cu on the protein was complete for all pHs.

The histograms showing the initial binding ratios for different cations at various pHs are given in figure 5 ; they showed that the initial binding ratio depended on the nature of mineral.

— Ca had a high initial binding ratio at pH 6.6, medium at pH 5.5 and low at pH 5.0.

— Mg and Mn had medium initial binding ratios at pH 6.6 and low at pH 5.5 and 5.0.

— Fe and Zn had high initial binding ratios at pH 6.6 and medium at pH 5.5 and 5.0.

— Cu had high initial binding ratios for all pHs.

Although these initial binding ratios were higher at pH 6.6 and lower at pH 5.0, the sensitivity of β-lactoglobulin-mineral complex towards pH was variable for the different minerals, since for a pH lowering from 6.6 to 5.0, this ratio decreased by 61 % for Ca and only by 26 % for Fe. At pH 6.6 the affinity order was :

Fe = Zn > Cu > Ca > Mn > Mg

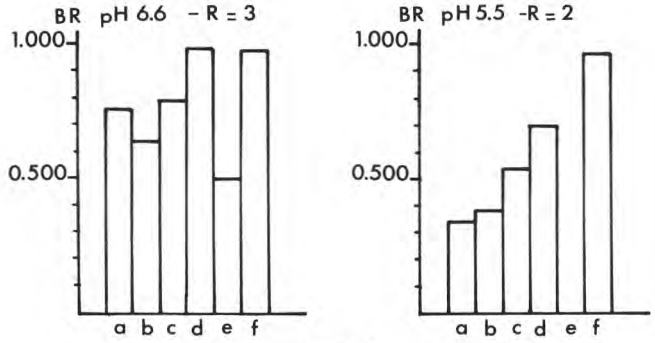

Fig. 6

Binding ratio (BR = [Bound cation]/[Total cation]) of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} to β-lactoglobulin for 3 bound moles (R = 3) at pH 6.6 and 2 bound moles (R = 2) at pH 5.5.

Taux de fixation (BR = [Cation fixé]/[Cation total]) de Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} sur la β-lactoglobuline pour 3 moles de cation fixées (R = 3) à pH 6,6 et 2 moles (R = 2) à pH 5,5.

a = Ca^{2+} *d* = Fe^{2+}
b = Mg^{2+} *e* = Mn^{2+}
c = Zn^{2+} *f* = Cu^{2+}

The histograms (fig. 6) showing the binding ratios for 3 (R = 3) at pH 6.6 and 2 bound moles (R = 2) at pH 5.5 gave the following affinity :

pH 6.6 (R = 3) : Fe > Cu > Zn > Ca > Mg > Mn
pH 5.5 (R = 2) : Cu > Fe > Zn > Mg > Ca

2. *Effect of ionic strength*

Different experiments, made at pH 6.6 for the ionic strengths < 0.01, 0.05 and 0.10, gave the curves of figure 4. It showed that an increasing ionic strength involved a great decrease on bound cation amount : in the case of calcium, after a rapid decrease between $\mu < 0.01$ and $\mu = 0.05$ the initial binding ratio was constant (fig. 5). It was low for Ca, Mg, Mn, medium for Fe, Zn at $\mu = 0.05$ and low at $\mu = 0.10$; for Cu it was always high (> 0.900).

III. Discussion

Mineral determination on α-lactalbumin solutions purified according to the method of PIERRE and FAUQUANT (1986), have shown that one molecule of calcium was bound to α-lactalbumin ; this result which confirms the chelation of this mineral by the protein (HIRAOKA *et al.*, 1980) have led us to use a demineralization at pH 1.7 to remove this cation. The binding of the first calcium atom on α-lactalbumin is neither pH nor ionic strength dependent, which is in agreement with the fact that α-lactalbumin is a metalloprotein.

We cannot use SCATCHARD's plot (R/A versus R) in this study because the affinity constant was too high and the accuracy of the mineral determination was too weak to obtain precise results in free cation amount.

For every cation the binding curves are different for the various pHs and ionic strengths. The initial binding ratios of the two proteins are not easily comparable because the ranges of measurement are different, respectively from 0 to 1 and from 0 to 2 moles of added mineral for α-lactalbumin and β-lactoglobulin. The rapid change in the slope of the binding curves, as well as the decreasing binding ratio, at pH 6.6, show that these proteins have many chelating sites with different affinities for minerals. The binding plateau reached by α-lactalbumin, corresponds to a saturation of the sites with cations. For β-lactoglobulin the added mineral amount was too low to reach this stage except for Mg ; the binding potentialities given there are minimal values.

The binding sites seem to have specificities because the maximal numbers of bound mineral are variable. At pH 6.6 α-lactalbumin can respectively bind 3.0, 3.5, 4.5, 4.5, 6.0 and 10.6 ions of Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}. β-lactoglobulin can complex more than 2.0 ions of Mn^{2+}, 2.5 of Zn^{2+}, 3.2 of Mg^{2+} and 3.5 of Ca^{2+}, Fe^{2+}, Cu^{2+}. The binding capacities of α-lactalbumin are greater than the single site for Ca, the 2 sites for Zn and the 3 sites for Mn, described by BRATCHER and KRONMAN (1984) : this may be due to the use of different buffer solutions (pHs and ionic strengths). α-lactalbumin, at pH 6.6 and $\mu < 0.01$, has a high affinity for Ca, Mn, Zn, Cu and a slightly lower one

for Fe and Mg ; the highest binding ratios for β-lactoglobulin correspond to Cu, Fe, Zn, the lowest to Ca, Mn, Mg.

Except for Cu the number of sites is pH and ionic strength dependent : the lower the pH or the higher the ionic strength, the lower is the number of binding sites. Both proteins have a high affinity for Cu at all the pHs and ionic strengths : the initial binding ratio was always higher than 0.920.

The decrease in ionization which takes place at pH 5.0, near the pK of carboxylic residues of amino acids, causes an important decrease of mineral binding ability except for Ca and Cu with α-lactalbumin and for Fe and Cu with β-lactoglobulin.

The variable results concerning different ionic strengths show the importance of the physico-chemical characteristics of the aqueous phase on mineral chelation. KUWAJIMA *et al.* (1986) give an affinity constant for α-lactalbumin with Na equal to 1000 M^{-1} : when NaCl content is high, Na may bind to α-lactalbumin at the expense of the studied mineral. The effect of ionic strength which may be detected for small NaCl content, is constant for higher values. The lower number of bound cations may also be due to a change of tertiary structure of the protein when ionic strength is high. At pH 6.6 for $\mu = 0.10$ the proteins can only chelate one cation per molecule, but Mg is not bound at all on α-lactalbumin.

The binding sites for divalent cations consist of a group of ionic amino acids (acids for the pH range studied). The carboxylic groups, located on the side chain of glumatic and aspartic acids are more or less dissociated with pH, because their pK (about 3.9) depends on their chemical surroundings. Coordination links with O and N are also involved (COOH - NH_2 - CONH), especially for Cu^{2+}, the binding of which is less pH and ionic strength dependent than the other cations.

The other properties being involved in the bonds of cations are ionic radius and electronegativity (PAULING's scale). Cu and Fe have a high electronegativity, medium for Zn and Mn, low for Ca and Mg. Among these 8 atoms, Ca has the largest ionic radius and Mg the smallest. These facts agree with a higher affinity for Cu and Fe, medium for Zn and Mn and lower for Ca and Mg.

These results shows that the affinity of protein depends on the mineral, on the binding site and decreases with decreasing pH or increasing ionic strength. Inner binding abilities of these two proteins are important, but the characteristics of the solution (pH, ionic strength) can reduce them. The feature of α-lactalbumin is the presence of a calcium binding site with a high affinity.

This work shows the interest that these proteins should have as mineral transfer means. Peptides from α-lactalbumin and β-lactoglobulin hydrolysis may be able to bind minerals. Our experiences do not make it possible to determine neither the localization nor the possibility of binding different minerals of the sites. An extension of this work will be to study the competitive binding of these cations to determine the common and the particular sites.

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