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THE BINDING OF Ca^{2+} AND Mg^{2+} TO HUMAN SERUM ALBUMIN: A CALORIMETRIC STUDY

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ABSTRACT

The binding of calcium and magnesium to human serum albumin has been studied in the pH region 2.5–8.0 by a calorimetric procedure. Both metal ions bind to the carboxylate groups of albumin. 36 and 44 carboxylate groups appear to be involved in the binding of Ca^{2+} and Mg^{2+}, respectively. Based on previously reported results that twelve Ca^{2+} ions are the maximum which can bind to albumin, the results given here support previous X-ray crystallographic evidence that three carboxylate groups can be involved in the binding of a Ca^{2+} by a protein. The data also confirm that Ca^{2+} and Mg^{2+} binding is competitive. Binding of the cations to the carboxylate groups appears to involve the breaking of carboxylate–imidazole hydrogen bonds in the protein. Log K, ΔH and ΔS values obtained for the binding of metal ions to albumin in aqueous solution at 25°C are 2.72 ± 0.02, 0.0 ± 0.1 kcal/mole, and 12.4 ± 0.3 cal/mole K for Ca^{2+} and 1.12 ± 0.05, −0.2 ± 0.1 kcal/mole, and 4.5 ± 0.3 cal/mole K for Mg^{2+}, respectively.

INTRODUCTION

Calcium and magnesium binding to proteins in serum plays an integral role in homeostatic mechanisms for these two elements. A thorough understanding of the chemical nature of the binding of metal ions to plasma proteins and the factors which can influence it are therefore fundamental to the understanding of calcium and magnesium utilization by the body. Binding of these metal ions to albumin is governed by such factors as pH, ionic concentration, and temperature[1–9]. The binding of both Ca^{2+} and Mg^{2+} to albumin has been studied by various techniques and models have been proposed to explain the observed binding[4–6]. The majority of work has been done on the binding of Ca^{2+} to albumin. The reported[1, 2, 8–15] number of Ca^{2+} ions which can be bound by a molecule of albumin varies from 5 to 12 in the pH range 6.5 to 8.5, depending on the pH, temperature, calcium concentration, and

presence of impurities in the protein preparation, i.e. fatty acids, cholesterol, etc. The work of Pedersen[1, 8, 9] indicates 12 is the maximum number of Ca^{2+} ions bound over the temperature range 4–37°C and pH range 6.5–8.5. Reported dissociation constants vary from 10^{-2} to 7×10^{-4}, although most values lie between 10^{-2} and 10^{-3} (refs. 1, 3, 7–11, 13, 15–19). Most studies have indicated the binding involves carboxyl groups with at least two carboxyl groups involved for each Ca^{2+} bound. X-ray structural data on other Ca^{2+}–protein systems (i.e. thermolysin, α-amylase, etc.) suggest the number of carboxyl groups involved in the binding could vary from two to four[3]. Involvement of imidazole groups in the calcium binding in the neutral transition region[20] has been suggested by Zurawski and Foster[21], Harmsen, et al.[4], Saroff and Lewis[15] and Pedersen[1, 8, 9]. A model has been proposed by Harmsen et al.[4] and Moore[6] whereby the first calcium ion which is bound to the protein molecule initiates secondary and/or tertiary changes in the protein molecule allowing the other possible binding sites to become available for complexation. Harmsen et al.[4], Zurawski and Foster[21] and Eastman et al.[5] have proposed calcium binding is accompanied by conformational changes in the protein molecule involving the breaking of imidazole–carboxyl hydrogen bonds similar to those observed in the folded to non-folded tertiary structure change[22–25] (the N–F transition) in the pH region where imidazole ionization occurs.

The binding of Mg^{2+} to human albumin has been studied by equilibrium dialysis[12, 26] and by specific divalent cation electrodes[27, 28]. The results indicate the log K value for binding of Mg^{2+} to albumin is equal to[27] or smaller than[12] that for Ca^{2+}. The maximum number of Mg^{2+} ions bound per molecule of albumin reported[28] is 8. The binding of Mg^{2+} to human albumin appears to increase with increasing pH[27, 28] and to be temperature independent[28].

We report here titration calorimetry studies of the binding of Ca^{2+} and Mg^{2+} to two samples of human serum albumin. The calorimetric technique used allows the direct study of the protein functional groups involved in metal ion binding. In addition, calorimetric vs. van 't Hoff ΔH values are compared and a complete thermodynamic description of the metal ion binding is given. The binding is found to involve carboxylate groups. Log K, ΔH, ΔS and $\bar{\nu}$ values at 25°C are reported for the interactions studied.

EXPERIMENTAL

Materials

Reagent grade HCl (Baker "analyzed" reagent), $CaCl_2$ (B and A reagent) and $MgCl_2$ (MCW, reagent) were used in the preparation of solutions. The HCl solutions were standardized by pH titration with Tris and the $CaCl_2$ and $MgCl_2$ solutions were standardized by Mohr titration with $AgNO_3$. The albumin samples, designated I and II (I-Miles, human albumin, Fraction V, fatty acid free, lot 31 and II-NBC human albumin, 4× crystalline, lot 9624), were used without further purification. Isoelectric focusing of I indicated only one fraction. A molecular weight of

69 000 was used for both albumin samples. All solutions were made using freshly distilled, deionized water and stored under N_2 until used.

Calorimetric studies

The calorimetric titrations were conducted using a previously described micro-isoperibol titration calorimeter[29]. The interaction of Ca^{2+} and Mg^{2+} with albumin was studied by titration of 0.2 ml of a 0.1 M aqueous metal ion solution into 2.7 ml of a 4 mg/ml aqueous solution of the albumin. The measured ΔH values for these interactions were close to zero. Therefore, the interaction was studied indirectly by titration of solutions of the albumin or albumin plus $CaCl_2$ and/or $MgCl_2$ with HCl. The metal ion concentrations used were high to force saturation of the metal binding sites. All titrations were made at 25°C. Four replicate runs were made for each experimental condition studied. Heat of dilution corrections were determined by addition of the titrant to H_2O. The methods used for data reduction[30, 31] and analysis[32] have been published. The pH titration data were obtained under conditions identical to those employed in the calorimetric studies.

RESULTS

The calorimetrically determined ΔH values for the interaction of Ca^{2+} and Mg^{2+} with I are 0.0 ± 0.1 and −0.2 ± 0.1 kcal/mole, respectively. The measured heat effects for the binding of both Ca^{2+} and Mg^{2+} were too small to allow calculation of the number of metal ions bound per albumin from the calorimetric titration data. Typical data for the titration of I and I plus $CaCl_2$ with HCl are given in Fig. 1. The titration curve covers the pH region from 6.0 to 2.5 and is associated

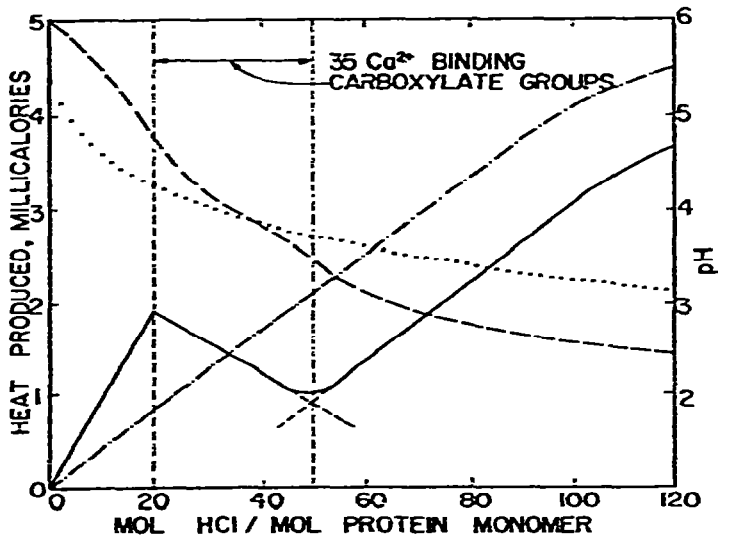


Fig. 1. Calorimetric titration curve for the addition of HCl to a 4 mg albumin I/ml solution, —·—, and a (4 mg albumin I ÷ 0.08 mmole $CaCl_2$)/ml solution, ——, and the corresponding pH titration curves in the presence, — —, and absence ..... of $CaCl_2$.

with the protonation of the carboxylate groups[33] in the albumin molecule. The calorimetric titration curve in the absence of Ca^{2+} shows no anomalies. Under the conditions of these experiments (4 mg protein/ml) no additional heat effects are observed through the Foster N–F transition region[22–25] when titrating with acid in the absence of metal ion. However, heat effects due to this transition are observed as a function of albumin concentration and are influenced by the presence of Ca^{2+} and Mg^{2+}.

The new endothermic region present in the titration of albumin plus $CaCl_2$ with HCl, Fig. 1, can be attributed to the titration of those carboxylate groups bound to Ca^{2+}. Titration of the albumin–$CaCl_2$ solutions with NaOH indicates addition of the metal ion does not induce a similar anomaly in the regions where imidazole, phenolic or amino groups are titrated. For example, the titration of the 17 imidazole and 1 α-amino groups[33] in solutions containing 0.10 or 0.0050 M $CaCl_2$ gives identical end points with 18.6 ± 0.6 total groups titrated and ΔH_i values of →7.0 ± 0.2 and →6.4 ± 0.1 kcal/mole, respectively, for the two Ca^{2+} concentrations. The endothermic region shown in Fig. 1 is only present where carboxylate groups are being titrated and only when Ca^{2+} is present in the solution. Further, the length of the region is directly proportional to the metal ion concentration. The length of this region is a measurement of the equivalents of acid required to protonate those carboxylate groups bound to Ca^{2+}, and, therefore, is a direct measure of $\bar{\nu}$, the number of carboxylate groups bound to Ca^{2+} per molecule of albumin. Similar results are obtained for different concentrations of $CaCl_2$ and $MgCl_2$ and for titration of solutions containing albumin plus both $CaCl_2$ and $MgCl_2$. Similar results were also obtained for II. Data for II are somewhat more complex since all carboxylate groups in II did not titrate with the same ΔH value. An endothermic region similar to that seen in Fig. 1 was also evident for titrations of II plus $CaCl_2$ and/or $MgCl_2$ with HCl. The apparent ΔH values for protonation of the metal binding carboxyl groups in the albumins studied in the absence and presence of metal ions are given in Table 1. The measured $\bar{\nu}$ values are given in Table 2.

TABLE I

CALORIMETRICALLY DETERMINED ΔH VALUES AT 25°C FOR THE PROTONATION OF ALKALINE EARTH BINDING CARBOXYL GROUPS IN ALBUMIN IN THE PRESENCE AND ABSENCE OF Ca^{2+} OR Mg^{2+}

Albumin	*Metal*	ΔH_i *(kcal/mole* H^+*)*
I		−0.24 ± 0.04
	Ca^{2+}	0.18 ± 0.20
	Mg^{2+}	0.06 ± 0.11
II		−0.66 ± 0.14
	Ca^{2+}	−0.35 ± 0.12
	Mg^{2+}	−0.38 ± 0.19

TABLE 2

CALORIMETRICALLY OBTAINED VALUES OF $\bar{\nu}$ FOR ALKALINE EARTH BINDING CARBOXYL GROUPS IN AQUEOUS ALBUMIN SOLUTIONS AT 25°C

Albumin sample	*Initial conc. of salt (mole/l)*		*Initial conc. of albumin ($\times 10^6$ mole/l)*	$\bar{\nu}$[a]
	$CaCl_2$	$MgCl_2$		
I	0.0983	—	67.4	35.3 ± 1.9
	0.0804	—	57.9	35.2 ± 1.4
	0.00804	—	60.6	29.7 ± 4.1
	0.00161	—	58.8	14.8 ± 5.7
	—	0.2000	58.0	31.1 ± 2.3
	—	0.1000	74.2	25.7 ± 3.7
	—	0.0195	60.4	8.8 ± 1.2
	0.1000	0.1000	58.1	37.3 ± 2.5
II	0.0804	—	58.3	21.7 ± 2.9
	0.00804	—	58.0	13.1 ± 2.1
	—	0.1000	58.1	20.2 ± 2.7
	—	0.0100	58.0	13.1 ± 2.4
	0.0402	0.0500	57.9	23.3 ± 3.8

[a] $\bar{\nu}$ = number of Ca^{2+} and/or Mg^{2+} binding carboxyl groups per albumin molecule.

DISCUSSION

The results summarized in Fig. 1 and Tables 1 and 2 indicate Ca^{2+} and Mg^{2+} complexes with the carboxylate groups of albumin as only carboxylate groups are perturbed by the addition of Ca^{2+} and/or Mg^{2+}. If binding involves independent and equivalent sites then[34]

$$\frac{\bar{\nu}}{n - \bar{\nu}} = K[M^{2+}] \tag{1}$$

or

$$\bar{\nu} = (n - \bar{\nu})/K[M^{2+}] \tag{2}$$

where n is the total number of carboxylate groups which can bind the metal ion, $[M^{2+}]$ is the concentration of unbound metal ion in the solution and K is the equilibrium constant for association of the metal ion with the protein. If a value is assumed for the number of carboxyl groups bound per metal ion, n', then $[M^{2+}]$ may be calculated for each condition studied from eqn. (3) where $[M^{2+}_{total}]$ is the total metal ion concentration and [Pr] is the total concentration of the protein. Assuming the maximum number of bound Ca^{2+} ions is 12 as reported by Pedersen[1, 8, 9], we

$$[M^{2+}] = [M^{2+}_{total}] - \frac{\bar{\nu}}{n'}[Pr] \tag{3}$$

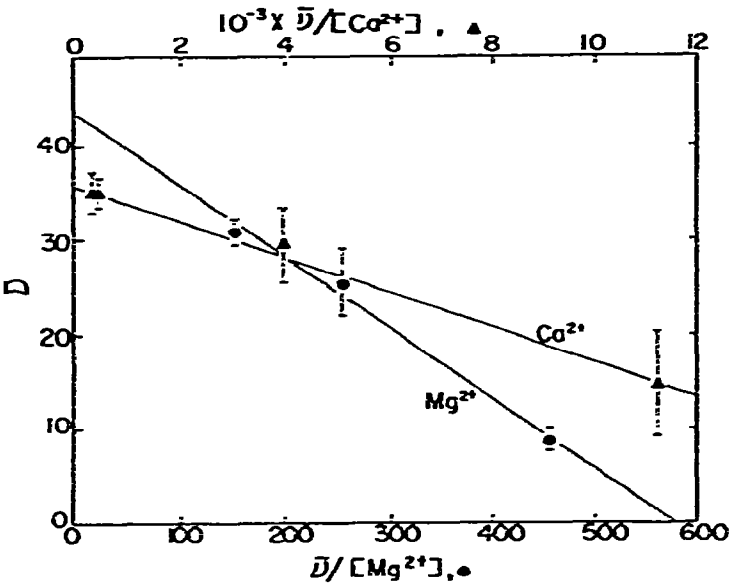


Fig. 2. A plot of $\bar{v}$ vs $\bar{v}/[M^{2+}]$, l/mole, for the data obtained for albumin sample I.

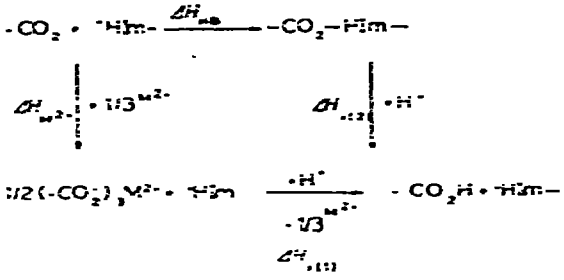
conclude three carboxyl groups (a total of 36) are involved in the binding of each Ca^{2+} ion. This is consistent with results reported in X-ray structure studies of calcium–protein crystals[3] where three or four carboxyl groups are found associated with the calcium ion. The binding of Mg^{2+} to albumin involved fewer carboxyl groups as compared to that for Ca^{2+} for any given total metal ion concentration. The data with sample I, however, indicate that over 30 carboxyl groups can be involved in the binding of Mg^{2+}. The studies on solutions with both Ca^{2+} and Mg^{2+} present indicate the binding is competitive. Therefore, we conclude that three carboxylate groups are also bound to each Mg^{2+}. With these assumptions, a plot of $\bar{v}$ vs. $\bar{v}/[M^{2+}]$ should be linear with a slope of $-1/K$ and an intercept of n. Such a plot is given in Fig. 2 for the data obtained with Albumin I assuming $n' = 3$. Least squares fit of the data gives $n = 36.3 \pm 1.0$ and $\log K = 2.72 \pm 0.02$ for the binding of Ca^{2+} and $n = 43.9 \pm 0.6$ and $\log K = 1.12 \pm 0.05$ for the binding of Mg^{2+}. Because of the large excess of metal ion used in these experiments, the results obtained for n and $\log K$ are not sensitive to the value of n' used and the calculated n and $\log K$ values obtained with $n' = 2$ or 4 are not significantly different from those obtained with $n' = 3$. The single data point for albumin sample I with both $MgCl_2$ and $CaCl_2$ indicates the binding of the two cations is highly competitive. The calculated $\bar{v}$ value using the $\log K$ values obtained with only Mg^{2+} or Ca^{2+} in solution, and assuming complete competition for all binding sites, is 35.3 compared to the experimentally obtained value of 37.3 ± 3.5, see Table 2. If the binding for Mg^{2+} and Ca^{2+} was independent the calculated $\bar{v}$ value would be 60.6. The data obtained for albumin sample I are then consistent with the model where 36 carboxyl groups in albumin can competitively bind 12 Ca^{2+} ($\log K = 2.72$) or, presumably, 12 Mg^{2+} ($\log K = 1.12$) ions.

Human serum albumin contains 89 carboxylate groups and 13 histidyl residues[35]. In the amino acid sequence of human serum albumin kindly provided by Professor J. R. Brown (Department of Chemistry, University of Texas, Austin[35, 36]) one finds that of the 89 carboxylate groups (36 asparatyl and 53 glutamyl) only 30 occur as doublets (i.e. -glu–glu-, -asp–asp-, and -asp–glu-). If all these sites bind calcium then 15 calciums would be bound at saturation.

The data by Pedersen[1, 8, 9] indicating a maximum of 12 Ca^{2+} ions bind and our data indicate a total of 36 carboxylate groups are involved in this binding suggest that 12 of these doublets plus 12 other carboxylate groups may be involved in the binding of Ca^{2+} to albumin. If all doublets plus fifteen other carboxylate groups were involved in binding a metal ion the total number of carboxylate groups measured would be 45. Interestingly, this is similar to what is measured for Mg^{2+}

The binding of both Mg^{2+} and Ca^{2+} to sample II is significantly different from that for I. At equivalent levels of total metal ion the $\bar{\nu}$ values are significantly lower for sample II than for I. Least squares fit of the data for sample II to eqn. (2) assuming an n' value of 3 results in $n = 23.4$ and $\log K = 2.22$ for Ca^{2+} binding and $n = 21.7$ and $\log K = 2.20$ for Mg^{2+} binding. These values would predict a $\bar{\nu}$ of 25.4 for the solution with both $CaCl_2$ and $MgCl_2$ if the binding is competitive, in good agreement with the experimental value of 23.2 ± 3.8. In contrast, the $\bar{\nu}$ value predicted if Ca^{2+} and Mg^{2+} bind independently is 41.0. The data, therefore, suggest that sample II has only 2/3 the sites of sample I available for binding and presumably will bind only 2/3 the metal ion. Sample II is the less pure of the two samples, the difference being the presence of sodium caprylate and/or acetyl tryptophonate as a contaminant of the commercial preparation process. Heat denaturation occurs rapidly in boiling water for sample I but no evidence of denaturation of sample II was detected after such treatment for 10 min.

The measured heats of protonation of the carboxylate groups in the presence and absence of metal ions provide insight to environmental changes which occur in the protein as metal ions are complexed. The ΔH value for protonation of the carboxylate groups involved in Ca^{2+} and Mg^{2+} binding is more endothermic in the presence of the metal ions than would be predicted by the ΔH values for complexation of the metal ions with albumin. This suggests that the environment of the carboxylate groups that are protonated is not the same for the initial solution (pH = 6.0) as at the pH at which they are protonated in the presence of Ca^{2+} or Mg^{2+} (pH = 4.5). This difference may be attributed to dielectric changes in the region of the carboxylate groups resulting from the N–F transition or to specific changes in other carboxylate–protein bonds. Harmsen et al.[4], and Eastman et al.[5] have proposed that binding of Ca^{2+} to the protein involves the breaking of a carboxylate–imidazole hydrogen bond. At pH = 6.0 the hydrogen bond can be assumed to exist since essentially all carboxylate groups will be ionized and all imidazole groups protonated. At pH = 4.5, presumably the hydrogen bond would not exist due to protonation of the carboxylate groups. The reactions occurring can be represented by the cycle



ΔH_{HB} is the enthalpy change associated with forming the carboxylate-protonated imidazole hydrogen bond in the protein. $\Delta H_{M^{2+}}$ is the enthalpy change associated with formation of the carboxylate–metal ion bond. The ΔH value measured by titration of the protein with metal ion would be a sum of $\Delta H_{M^{2+}}$ and some fraction of $-\Delta H_{HB}$, depending on the magnitude of the equilibrium constant for the hydrogen bonding reaction. Addition of the metal ions to the protein then induces the breaking of this hydrogen bond and complexation of the metal ion with the carboxylate groups occurs. Addition of hydrogen ion to the protein solution in the absence of metal ions involves the breaking of the hydrogen bond and formation of protonated carboxylate groups, $\Delta H_{i(2)}$. In contrast, addition of hydrogen ion to the metal ion–carboxylate complex involves only the breaking of the metal ion–carboxylate bond and protonation of the carboxylate anion, but not the breaking of the hydrogen bond and $\Delta H_{i(1)}$ is measured. If we assume that in the protein solution the hydrogen bond is stoichiometrically formed, then the difference in ΔH_i values in the absence or presence of metal ion is the heat due to formation of the hydrogen bond. Albumin contains only 17 imidazole groups per monomer[33] and the number of carboxylate groups involved in hydrogen bonding per bound metal ion would not exceed one, i.e. one hydrogen bond for every three carboxylate groups involved in metal binding. With these assumptions, we calculated the apparent ΔH value for formation of the carboxylate anion–protonated imidazole hydrogen bond in albumin to be $\rightarrow$0.9 kcal/mole of metal ion for both samples of albumin studied. The calculated values of log K for metal binding as a function of pH will also reflect the formation or breaking of this hydrogen bond. Based on literature results[1, 9, 17] for log K of Ca^{2+}–albumin interaction in the pH range from 4 to 8 the apparent free energy for formation of the carboxylate anion–protonated imidazole hydrogen bond can be estimated to be -1.2 kcal/mole and the ΔS value to be -1 e.u. These values are consistent with those expected for hydrogen bond formation, suggesting the indicated mechanism may be correct. It should be emphasized, however, that agreement between expected and calculated thermodynamic values does not confirm the proposed mechanism but only indicates its plausibility.

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