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Calcium Binding to Dipeptides of Aspartate and Glutamate in Comparison with Orthophosphoserine

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ABSTRACT: Aspartate binds calcium(II) better than glutamate with $K_a = 7.0 \pm 0.9$ L mol^{-1} for Asp and $K_a = 3.0 \pm 0.8$ L mol^{-1} for Glu, respectively, as determined using calcium-selective electrodes for aqueous solutions of ionic strength 0.20 at 25 °C at pH of relevance for milk products. For the mixed peptides, the affinity seems additive with $K_a = 27 \pm 3$ L mol^{-1} for Asp-Glu and 22.7 ± 0.1 for Glu-Asp as compared to the expected 21 L mol^{-1}. In contrast, for Asp-Asp, the affinity is less than additive with $K_a = 23 \pm 5$ L mol^{-1} as compared to the expected 49 L mol^{-1}, whereas for Glu-Glu, the affinity is more than additive with $K_a = 26 \pm 4$ L mol^{-1} as compared to the expected 9.0 L mol^{-1}, indicating specific structural effects for Glu-Glu. Ionic strength effects, 1.0 versus 0.20 studied, are similar for Asp and Glu with decreasing affinity for higher ionic strength, whereas the dipeptides with Glu as C-terminus are more sensitive to increasing ionic strength than with Asp as C-terminus. Despite little affinity of calcium to serine with $K_a = 0.9 \pm 0.2$ L mol^{-1}, Glu has increasing affinity for calcium in the serine dipeptide Ser-Glu with $K_a = 10 \pm 3$ L mol^{-1}, which becomes comparable to phosphorylated serine with $K_a = 22 \pm 5$ L mol^{-1}.

KEYWORDS: *calcium binding, aspartate, glutamate, phosphorylated serine*

INTRODUCTION

Calcium is important in cellular signaling and in regulating enzyme activity through site-specific binding in proteins and is an essential part of the human diet.[1,2] Calcium bioavailability from various foods, however, often becomes critical, especially for bone and tooth health in certain population segments.[3] Dairy products are one of the main sources of calcium in the human diet with good bioavailability due to a strong protein binding of calcium.[4] Calcium binding to proteins, also important for in vivo calcium transport and storage and for the many calcium regulatory functions, depends on complex formation between calcium(II) ions and most often negatively charged amino acid residues.[5] Such binding of calcium also prevents precipitation of insoluble calcium phosphates and calcium soaps during digestion of food and facilitates absorption of calcium in the intestine.[6−10]

In milk calcium is associated with the caseins of the micellar phase and to a lesser degree with the smaller serum proteins.[11] The caseins are highly phosphorylated with clusters of phosphoseryl residues, whereas α-lactalbumin and β-lactoglobulin, as the main whey proteins, are not phosphorylated.[4,11] The binding of calcium to caseins and to the casein-ophosphopeptides (CPP) formed through enzymatic hydrolysis during digestion occurs mostly through the negatively charged phosphate groups, whereas for the whey proteins, binding depends on the side-chain carboxylates as in α-lactalbumin.[8,12]

Calcium binding to phosphorylated serine in the caseins and CPP and to the highly phosphorylated egg protein phosvitin is relatively well characterized.[13−15] Binding to nonphosphorylated food proteins seems to depend on specific peptide motifs in the protein amino acid sequence. Notably, specific amino acid motifs seem capable of binding calcium even better than amino acid phosphate esters.[16,17] Better knowledge of such amino acid sequences should be of relevance for the design of both dairy and nondairy functional foods for optimal calcium uptake. Accordingly, we have determined the affinity for calcium binding to glutamate and aspartate as important nonphosphorylated amino acids involved in calcium binding and further for their four dipeptides to provide a better understanding of the structural requirement for calcium binding in nonphosphorylated food proteins. Our investigation is also prompted by conflicting reports of the ordering of affinity for binding of calcium to aspartate and glutamate.[18−20]

MATERIALS AND METHODS

Chemicals. $CaCl_2 \cdot 2H_2O$, NaCl, NaOH, and P_4O_{10} were from Merck (Darmstadt, Germany). Sodium azide, L-glutamic acid monosodium salt monohydrate (≥98.0%), L-aspartic acid monosodium salt monohydrate (≥98%), L-serine, *O*-phospho-L-serine of analytical grade, and the dipeptides Asp-Glu (≥95%), Glu-Glu (≥95%), and Ser-Glu of analytical grade were from Sigma-Aldrich (Steinheim, Germany). The dipeptides Glu-Asp (>95%) and Asp-Asp (>95%) were from ChinaPeptides Co., Ltd. (Shanghai, China). All aqueous solutions were made from purified water using a Milli-Q Plus system from Millipore Corp. (Bedford, MA, USA).

Sample Preparation. Solutions for determination of calcium binding to amino acid and peptides as ligand were prepared at two ionic strengths, 0.2 and 1.0 mol L^{-1}. Ionic strength in the samples was adjusted with NaCl when needed. For each ligand, two concentrations were investigated, both with a calcium concentration of 1.0×10^{-3} mol L^{-1}. Ligand concentrations were for the amino acids 1.0×10^{-1} and 2.0×10^{-1} mol L^{-1}, and the samples were prepared in duplicates. For dipeptides and phosphorylated serine the concentrations were 1.0×10^{-2} and 5.0×10^{-2} mol L^{-1}, except for the dipeptide Glu-Asp, for which the concentrations 1.0×10^{-2} and 5.0×10^{-3} mol L^{-1} were used with several electrode measurements for each solutions.

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Potentiometric Determination of Free Calcium. Free calcium ion concentration [Ca^{2+}] was measured with a calcium-sensitive electrode ISE25Ca together with an REF 251 electrode from Radiometer, Copenhagen, Denmark. The electrode was calibrated using calcium standard solutions of 1.00×10^{-4}, 1.00×10^{-3}, and 1.00×10^{-2} mol L^{-1} prepared from a 1.00 M stock solution of dried $CaCl_2$ with the same ionic strengths as for the sample. $CaCl_2 \cdot 2H_2O$ was dried and stored in a closed glass container over P_4O_{10} prior to use. Ionic strengths of 0.20 and 1.0 were adjusted with NaCl in all calibration solutions. Calcium concentration in the samples was determined from the linear relationship (derived from the Nernst equation) between the electrode potential (mV) and the corresponding pCa of the calibration solutions as previously described.[10] All measurements were performed at 25.0 ± 0.1 °C.

pH Measurements. A pH meter (713 pH Meter, Metrohm, Denmark) with a glass electrode (602 Combined Metrosensor glass electrode, Metrohm, Denmark), standardized before each measurement against NBS international activity pH-standard with pH 2.000, 4.000, 7.000, and 9.240, was used for pH measurements according to pH = −log a_{H^+}. pH was adjusted to 5.0 in dipeptides and phosphorylated serine samples by a dropwise addition of aqueous NaOH.

Calculation of Association Constants. For the calculation of the association constants, it was assumed that 1:1 complexes were formed, and only the most predominant forms of the respective amino acid or peptide as ligands at the actual pH values were used for the calculations. The reaction of eq 1 describes complex formation between calcium and ligand, where L^- is aspartate and glutamate, dipeptides, or phosphorylated serine:

$$Ca^{2+} + L^- \rightleftharpoons CaL^+ \quad (1)$$

From [Ca^{2+}], the free calcium ion concentration as measured by the calcium ion-selective electrode, [L^-], the free ligand concentration, is calculated using

$$[L^-] = [L^-]_0 - [CaL^+] = [L^-]_0 - ([Ca^{2+}]_0 - [Ca^{2+}]) \quad (2)$$

where $[L^-]_0$ denotes initial ligand concentration and $[Ca^{2+}]_0$ initial calcium concentration. [CaL^+] is the complex formed between calcium and ligand and was calculated as the difference between initial calcium concentration $[Ca^{2+}]_0$ and measured free calcium ion concentration as denoted in the last part of eq 2.

RESULTS AND DISCUSSION

Calcium is known to bind to amino acids, peptides, and proteins in aqueous solution with increasing affinity for increasing pH.[18,19,21,22] For conditions relevant to milk and milk products with pH slightly below 7, protonation of amino groups in amino acids and amino acid side chains makes binding weak as mainly carboxylate groups are available for complex formation. Such weak binding was confirmed for aspartate (Asp) and glutamate (Glu) for the pH of their zwitterions and to a lesser degree for serine (see Table 1). The binding is concluded to be dominated by formation of binary complexes in agreement with the reaction of eq 1, because the association constant K_a

$$K_a = \frac{[CaL^+]}{[Ca^{2+}][L^-]} \quad (3)$$

does not depend on the total ligand concentration, as is evident from Table 1. Aspartate binds calcium more strongly than glutamate, in agreement with the results reported by Lumb and Martell:[18] K_a = 40 L mol^{-1} for Asp and K_a = 27 L mol^{-1} for Glu at ionic strength of 0.1 at 25 °C to be compared with K_a = 7.0 and 3.0 L mol^{-1}, respectively, from the present study for ionic strength of 0.20 (see Table 1). The stronger binding for both Asp and Glu previously reported is understandable,[18] because

Table 1. Association Constants K_a for the Binary Complexes of Calcium with Amino Acids in Aqueous Solution at 25.0 °C, Ionic Strengths of 0.20 and 1.0, and pH of the Amino Acid Zwitterions[a]

			K_a (L mol^{-1})		
ligand	pH	ionic strength	c_{Ligand} = 0.10 M	c_{Ligand} = 0.20 M	av
aspartate	6.8	0.20	6.20 ± 0.04	7.8 ± 0.2	7.0 ± 0.9
	6.6	1.0	3.58 ± 0.06	4.10 ± 0.04	3.8 ± 0.3
glutamate	6.9	0.20	3.6 ± 0.4	2.4 ± 0.7	3.0 ± 0.8
	6.7	1.0	1.50 ± 0.04	1.73 ± 0.07	1.6 ± 0.1
serine	5.9	0.20	0.8 ± 0.2	1.0 ± 0.1	0.9 ± 0.2
	5.8	1.0	0.58 ± 0.04	0.9 ± 0.1	0.7 ± 0.2

[a]Total calcium concentration is 0.0010 mol L^{-1}, and ligand concentration is 0.10 or 0.20 mol L^{-1}.

the pH titration technique used for determination of association constants in the previous study involves alkaline pH conditions, where amino groups are nonprotonated and contributing to binding of calcium, and for these conditions, a chelate structure involving the α-amino groups was suggested.[18] In contrast, the constants of Table 1 relate to the pH of milk products, where binding becomes weaker, because only less stable chelate structures involving two carboxylates are possible under the conditions relevant for milk products (see Figure 1).

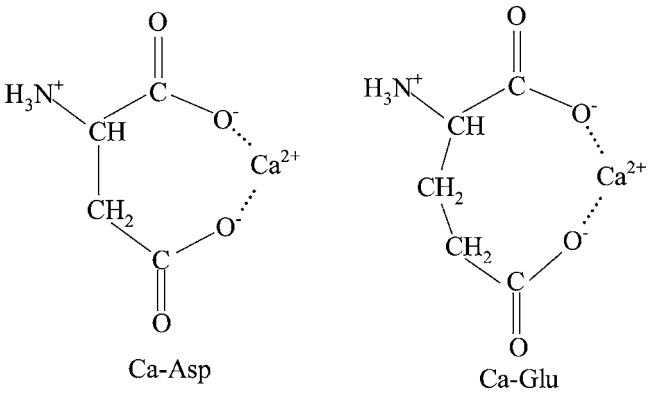

Figure 1. Proposed binding of calcium to aspartate and glutamate at the pH of milk.

Still, the ratio between K_a for binding of calcium to Asp and to Glu seems hardly dependent on pH. This common ratio of approximately 2 between K_a for binding of calcium to Asp and Glu is, however, clearly different from the ratio of 0.7 between K_a = 18 L mol^{-1} for Asp and K_a = 26 L mol^{-1} for Glu for an ionic strength of 0.10 as reported from another study also based on pH titration.[20] Serine binds calcium with less affinity corresponding to K_a = 0.9 L mol^{-1}, which could indicate that it is mainly the side-chain carboxylate which is contributing to binding of calcium in Asp and Glu. The α-carboxylate common for all amino acids has at the pH of milk a less effective negative charge due to the neighboring positively charged amonium group.

The association constant, K_a, based on concentrations, as defined in eq 3, depends for Asp and Glu on the activity coefficients, γ, according eq 4:

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Table 2. Association Constants K_a for the Binary Complexes of Calcium with Dipeptides and Phosphorylated Serine (o-p-Ser) in Aqueous Solution at 25.0 °C and Ionic Strengths of 0.20 and 1.0 at pH 5.0[a]

			K_a (L mol^{-1})			
ligand	pH	ionic strength	c_{Ligand} = 0.010 M	c_{Ligand} = 0.050 M	av	calcd[b]
Asp-Glu	5.0	0.20	25	29	27 ± 3	21
		1.0	10	8	9 ± 1	6.1
Glu-Glu	5.0	0.20	28	23	26 ± 4	9.0
		1.0	11	8	10 ± 2	2.6
Glu-Asp	5.0	0.20	22.8	22.6[c]	22.7 ± 0.1	21
		1.0	12	13[c]	13 ± 1	6.1
Asp-Asp	5.0	0.20	26	19	23 ± 5	49
		1.0	15	8	12 ± 5	14.4
Ser-Glu	5.0	0.20	12	8	10 ± 3	2.7
		1.0	7	5	6 ± 1	1.1
o-p-Ser	5.0	0.20	24 ± 6	20 ± 1	22 ± 5	
		1.0	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.6	

[a]Total calcium concentration is 0.0010 mol L^{-1}, and ligand concentration is 0.010 or 0.050 mol L^{-1}. [b]Calculated from values for individual amino acids (see Table 1). [c]0.0050 mol L^{-1} for Glu-Asp.

$$K^{\circ}_{a} = \frac{a_{CaL^+}}{a_{Ca^{2+}} \times a_{L^-}} = \frac{[CaL^+]}{[Ca^{2+}][L^-]} \times \frac{\gamma^+}{\gamma^{2+}\gamma^-} \approx K_a/\gamma^{2+} \quad (4)$$

In this equation, K°_{a} is the thermodynamic association constant based on ionic activities.[23] The values for K_a at the higher ionic strength of 1.0 investigated are smaller than those for ionic strength of 0.20 for both Asp and Glu, in agreement with a decreasing value for γ^{2+}, the activity coefficient of calcium, for increased ionic strength. The ratio between K_a at ionic strengths of 1.0 and 0.20 is 0.53 for both Asp and Glu, providing further evidence for the validity of the measurements. The net charge of Ser under the actual conditions is close to zero; therefore, the complex will have the same charge as the calcium ion. K_a is accordingly expected to show little if any dependence on ionic strength, as confirmed by the present measurement (see Table 1).

The two acidic amino acids may combine into four dipeptides for all of which the association constant K_a indicates higher affinity for calcium binding as compared to the individual amino acids at both ionic strengths (see Table 2). A similar effect is seen for the dipeptide Ser-Glu and phosphorylated serine. For the four aspartate-glutamate dipeptides additivity of affinity, ΔG°_{dipep}, for binding of calcium at the two carboxylate side chains of the dipeptide would result in a binding constant for the dipeptide equal to the product of individual stability constants. Such a simple relationship is confirmed for the two mixed dipeptides at the ionic strength of 0.20 with K_a(dipep) = 21 L mol^{-1} to be compared to 27 ± 3 L mol^{-1} for Asp-Glu and 22.7 ± 0.1 L mol^{-1} for Glu-Asp, confirming that the side-chain carboxylates are most important for calcium binding. This simple additivity seems, however, accidential, because the carbonyl of the amide is known to bind calcium only with less affinity than the carboxylate side chain.[24] In contrast, for Asp-Asp, the affinity is less than additive with K_a(dipep) = 23 ± 5 L mol^{-1} as compared to 49 L mol^{-1}. For Glu-Glu the affinity is more than additive with K_a = 26 ± 4 L mol^{-1} as compared to 9.0 L mol^{-1}, a difference that may indicate specific structural effects in Glu-Glu. A similar pattern is seen for the higher ionic strength of 1.0 (see Table 2). Notably, the synergism in binding of calcium to Glu-Glu is even more pronounced at the higher ionic strength, in agreement with a more significant effect of ionic strength on calcium binding to the aspartate-glutamate dipeptides with Glu as the C-terminus. The Ser-Glu dipeptide also shows a strong synergistic effect in calcium binding, especially at the high ionic strength, at which calcium binding becomes comparable to binding of calcium to phosphorylated serine. Phosphorylation of serine is known to provide caseins and casein-ophosphopeptides with their efficient calcium binding capabilities,[25,26] and it is notable that Ser-Glu binds almost as efficiently as the phosphorylated serine, especially at high ionic strength (see Table 2). Phosphorylated serine is negatively charged for pH values of milk products with an approximate net charge of 1.5,[13] and again a significant dependence of ionic strength is expected for binding of calcium to phosphorylated serine in contrast to binding to Ser, as is confirmed by the experimental values for K_a (see Table 2).

The stronger binding of calcium to Asp than to Glu is found not to correlate with basicity of Asp and Glu, because the carboxylate groups of Asp are significantly more acidic than the carboxylate groups of Glu, and accordingly the Asp carboxylates are less basic than the Glu carboxylates.[18,22] Factors other than electrostatic attraction for binding of calcium ions relative to binding of hydrogen ions clearly are important. Involvement of the α-carboxylate group as shown in Figure 1 could contribute to the binding of calcium, resulting in better stabilization for Asp than for Glu due to a more stable ring structure of the calcium Asp complex than for the larger calcium Glu complex. Theoretical calculation for calcium binding to dipeptides supports simultaneous binding to three oxygens at neutral pH,[27] whereas NMR spectroscopy seems to confirm the presence of seven-membered rings.[28] Calculation of excess free energy ($\Delta G^{\circ}_{excess}$) for binding of calcium to dipeptides defined according to

$$\Delta G^{\circ}_{excess} = \Delta G^{\circ}(\text{dipeptide}) - (\Delta G^{\circ}(\text{as1}) + \Delta G^{\circ}(\text{as2})) \quad (5)$$

where ΔG°(dipeptide) is the free energy for binding calcium to the dipeptide and ΔG°(as1) and ΔG°(as2) are free energies for binding to each of the amino acids, provides the results shown in Table 3 for the four aspartate-glutamate dipeptides. For the two mixed dipeptides, Asp-Glu and Glu-Asp, binding is almost additive, and $\Delta G^{\circ}_{excess}$ is close to zero. For Asp-Asp, $\Delta G^{\circ}_{excess}$ is positive, indicating that the structural stabilization in Asp disappears and the binding becomes controlled by electrostatic forces. For Glu-Glu electrostatic control now makes binding to this dipeptide stronger than to Asp-Asp, in agreement with the relative basicity of Glu and Asp (see Figure 2).

Table 3. Excess Binding Affinity, $-\Delta G^{\circ}_{excess}$, for Binding of Calcium to Aspartate−Glutamate Dipeptides

ligand	ΔG°(dipeptide) (kJ mol^{-1})	$\Delta G^{\circ}_{excess} = \Delta G^{\circ}(\text{dipeptide}) - (\Delta G^{\circ}(\text{as1}) + (\Delta G^{\circ}(\text{as1}))$ (kJ mol^{-1})
Asp-Glu	−8.17	−0.63
Glu-Glu	−8.07	−2.63
Glu-Asp	−7.74	−0.20
Asp-Asp	−7.77	+1.87

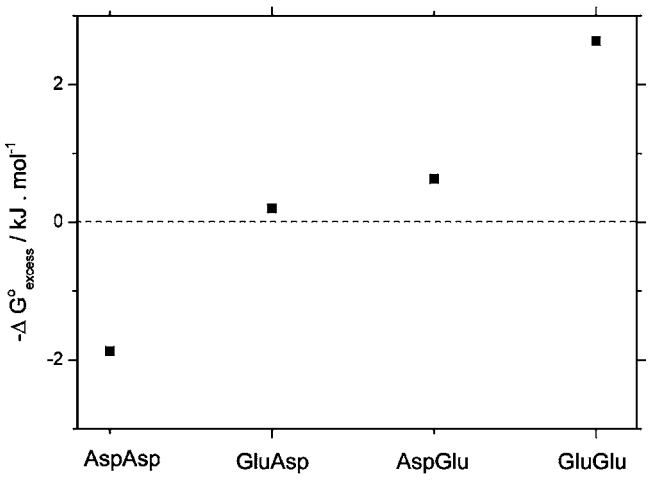

Figure 2. Excess binding affinity, $-\Delta G^{\circ}_{excess}$, for calcium complexes of the four aspartate-glutamate dipeptides. The two dipeptides to the left have aspartate as C-terminus.

For a series of phosphopeptides it was recently found that calcium binding capacity was not paralleled by binding affinity expressed as an association constant.[16] A series of pentaphosphopeptides were synthetized to mimic the core sequence of caseinophosphopeptide Ser(P)-Ser(P)-Ser(P)-Glu-Glu. The binding capacity ranged from 1 to 21 and the association constant from 30 to 131 L mol^{-1} for aqueous solutions of pH 7 at 25 °C. Notably, the strongest binding was found for the nonphosphorylated pentapeptide Ser-Ser-Ser-Glu-Glu included in the series.[16] Likewise, in a fish protein hydrolysate, a nonphosphorylated heptapeptide has been isolated for which Glu seems important for calcium binding.[17] The calcium binding motif in the peptide Ser-Ser-Ser-Glu-Glu, which binds only one calcium, was not identified further. However, according to the results of Table 2, both Ser-Glu and Glu-Glu show a significant enhancement of affinity for calcium as compared to the individual amino acids. Binding of one calcium to such nonphosphorylated oligopeptides apparently results in saturation of the binding capacity. Accordingly, some specific motifs of nonphosphorylated dipeptides seem to have significantly higher affinity for calcium than others. Such basic knowledge may help in the design of food products and nondairy beverages with enhanced calcium bioavailability.

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