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# Thermodynamic study of the binding of calcium and magnesium ions with myelin basic protein using the extended solvation theory

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**The interaction of myelin basic protein (MBP) from the bovine central nervous system with $Ca^{2+}$ and $Mg^{2+}$ ions, named as $M^{2+}$, was studied by isothermal titration calorimetry at 27 ºC in aqueous solution. The extended solvation model was used to reproduce the enthalpies of MBP+$M^{2+}$ interactions. The solvation parameters recovered from the extended solvation model were attributed to the structural change of MBP due to the metal ion interaction. It was found that there is a set of two identical and noninteracting binding sites for $Ca^{2+}$ and $Mg^{2+}$ ions.**

*Keywords* myelin basic protein; isothermal titration calorimetry; binding parameter

The energy of biochemical reactions or molecular interactions at constant temperature is measured by isothermal titration calorimetry (ITC) [1,2]. ITC gives invaluable information about biomacromolecule–ligand interaction. During the last two years, we attempted to study the metal ion binding on different proteins [3–5]. Metal ions change the conformational stability and formation of aggregates. The importance of metal ions such as $Ca^{2+}$ and $Mg^{2+}$ in regulating protein stability and function has been widely reported [5–10].

We have previously developed a theory to account for the solvation of solutes in mixed solvent systems. Studies within our group are aimed at developing an understanding of how the binding proteins of the metal ions and other ligands affect the stability of the biomolecules. One of the unique aspects of our approach is studying the stability of proteins by using the extended solvation model. Myelin basic protein (MBP) is one of the most abundant proteins of the myelin sheath of the central nervous system (CNS), and its primary role is generally considered to be maintenance of the stability of the sheath by holding together the apposing cytoplasmic leaflets of the oligodendrocyte membrane [11]. Previous investigations have revealed that the efficiency of MBP in preserving the compactness and the stability of the myelin membrane appeared to be enhanced in the presence of zinc (Zn) ions. There is also evidence that Zn stabilizes the *in vitro* self association of MBP dissolved in a phosphate buffer [12]. Since MBP addition to its associations with lipids, it has been shown to interact with calmodulin and cytoskeletal proteins such as actin and tubulin. Thus, functional roles for MBP in phosphoinositide-mediated signal transduction and other processes have been postulated [13]. The *MBP* gene encodes two families of paired formation of myelin sheets in primary cultures of cortical proteins: the classic MBPs and the Golli proteins, the function of which is less well understood. Previous work has suggested that Golli proteins may play a role in Ca homeostasis in oligodendrocytes and in T-cells [14].

Ca and Mg are of great physiologic importance by their intervention in many enzymatic systems and their function in neural excitability, muscle contraction blood coagulation, bone formation, hormone secretion, and cell adhesion [15]. The human body is equipped with an efficient negative feedback system that counteracts variations of Ca and Mg balance [16]. The maintenance of the Ca and Mg balance is controlled by the concerted action of intestinal absorption, renal excretion, and exchange with bone. After years of research, rapid progress has been made recently in identification and characteriza-

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tion of the Ca and Mg transport proteins that contribute to the delicate balance of divalent cations [15]. The interactions of MBP with $Ca^{2+}$-calmodulin have been previously investigated by fluorescence spectroscopy [17]. The apparent associated equilibrium binding constant for MBP interaction with $Ca^{2+}$-calmodulin was previously reported to be $2.1\pm0.1\ \mu M^{-1}$ [18]. MBP is an "intrinsically unstructured" or "natively unfolded" protein, therefore its three-dimensional structure might only be determined in its interaction with another protein [19–22]. As a clear understanding of operational stability constitutes an important goal in protein technology, our efforts aimed at elucidation of the structure-stability using the extended solvation model. This model is able to correlate the solvation parameters to the effect of metals on the stability of a protein in a very simple way.

The present paper reports some interesting experimental data for the heats of interaction of $Ca^{2+}$ and $Mg^{2+}$ ions with MBP, and analyzes these using the extended solvation theory for the first time. With regard to the importance of the presence of balance between Ca and Mg values in humans, and the role of MBP in Ca homeostasis in oligodendrocytes and T-cells, it seems that the present study has significant applications in pharmacology, neuroscience and drug delivery.

## Materials and Methods

MBP from bovine CNS was obtained from Sigma (St. Louis, USA). Protein concentrations were determined from absorbance measurements at 277 nm in 1 cm quartz cuvette. All other materials and reagents were of analytical grade, and solutions were made in 50 mM NaCl using double-distilled water.

The isothermal titration microcalorimetric experiments were performed with the four-channel commercial microcalorimetric system, Thermal Activity Monitor 2277 (Thermometric, Jarfalla, Sweden). The titration vessel was made from stainless steel. The metal nitrate solution (2 mM) was injected, by use of a Hamilton syringe, into the calorimetric titration vessel, which contained 1.8 ml MBP (60 μM). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of metal nitrate solutions into the perfusion vessel was repeated 30 times, with 20 μl per injection. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the "Thermometric Digitam 3" software program. The heats of dilution of the metal ion solutions were measured as described above, except that MBP was excluded. The microcalorimeter was frequently calibrated electrically during the course of the study.

## Results

The enthalpies interaction of myelin basic protein (MBP) with $Ca^{2+}$ and $Mg^{2+}$ ions, were calculated in $kJ\cdot mol^{-1}$. The results of MBP+$Ca^{2+}$ and MBP+$Mg^{2+}$ were shown in **Table 1** and **2** respectively.

**Table 1 The enthalpies of MBP+$Ca^{2+}$ interactions**

| $[Ca]_T$ (μM) | $[MBP]_T$ (μM) | $\Delta H$ ($kJ\cdot mol^{-1}$) | $\Delta H_{dilut}$ ($kJ\cdot mol^{-1}$) |
|---|---|---|---|
| 8.1967213 | 13.2786890 | 3.0946502 | 4.0866667 |
| 16.1290320 | 13.0645160 | 5.7078189 | 3.7666667 |
| 23.8095240 | 12.8571430 | 7.9218107 | 3.4800000 |
| 31.2500000 | 12.6562500 | 9.7942387 | 3.2133333 |
| 38.4615380 | 12.4615380 | 11.3909470 | 2.9880000 |
| 45.4545450 | 12.2727270 | 12.7530860 | 2.7888889 |
| 52.2388060 | 12.0895520 | 13.9423870 | 2.6133333 |
| 58.8235290 | 11.9117650 | 14.9588480 | 2.4550000 |
| 65.2173910 | 11.7391300 | 15.8477370 | 2.3125926 |
| 71.4285710 | 11.5714290 | 16.6255140 | 2.1853333 |
| 77.4647890 | 11.4084510 | 17.3127570 | 2.0678788 |
| 83.3333330 | 11.2500000 | 17.9259260 | 1.9622222 |
| 89.0410960 | 11.0958900 | 18.4732510 | 1.8666667 |
| 94.5945950 | 10.9459460 | 18.9629630 | 1.7795238 |
| 100.0000000 | 10.8000000 | 19.4032920 | 1.6995556 |
| 105.2631600 | 10.6578950 | 19.8065840 | 1.6266667 |
| 110.3896100 | 10.5194810 | 20.1687240 | 1.5596078 |
| 115.3846200 | 10.3846150 | 20.5020580 | 1.4977778 |
| 120.2531600 | 10.2531650 | 20.8065840 | 1.4403509 |
| 125.0000000 | 10.1250000 | 21.0864200 | 1.3866667 |
| 129.6296300 | 10.0000000 | 21.3456790 | 1.3365079 |
| 134.1463400 | 9.8780488 | 21.5843620 | 1.2896970 |
| 138.5542200 | 9.7590361 | 21.8065840 | 1.2466667 |
| 142.8571400 | 9.6428571 | 22.0123460 | 1.2058333 |
| 147.0588200 | 9.5294118 | 22.2057610 | 1.1674667 |
| 151.1627900 | 9.4186047 | 22.3868310 | 1.1317949 |
| 155.1724100 | 9.3103448 | 22.5555560 | 1.0982716 |
| 159.0909100 | 9.2045455 | 22.7119340 | 1.0666667 |
| 162.9213500 | 9.1011236 | 22.8600820 | 1.0367816 |
| 166.6666700 | 9.0000000 | 23.0000000 | 1.0084444 |

$\Delta H$, and the enthalpies of dilution of $Ca(NO_3)_2$ solution in water; $\Delta H_{dilut}$, against $Ca^{2+}$ and MBP concentrations.

**Table 2 The enthalpies of MBP+$Mg^{2+}$ interactions**

| $[Mg]_T$ (μM) | $[MBP]_T$ (μM) | $\Delta H$ (kJ·mol$^{-1}$) | $\Delta H_{dilut}$ (kJ·mol$^{-1}$) |
|---|---|---|---|
| 8.1967213 | 13.2786890 | 3.4197531 | 3.9400000 |
| 16.1290320 | 13.0645160 | 6.2962963 | 3.9400000 |
| 23.8095240 | 12.8571430 | 8.7037037 | 3.5511111 |
| 31.2500000 | 12.6562500 | 10.7242800 | 3.2516667 |
| 38.4615380 | 12.4615380 | 12.4279840 | 3.0053333 |
| 45.4545450 | 12.2727270 | 13.8683130 | 2.7788889 |
| 52.2388060 | 12.0895520 | 15.0987650 | 2.5866667 |
| 58.8235290 | 11.9117650 | 16.1563790 | 2.4175000 |
| 65.2173910 | 11.7391300 | 17.0699590 | 2.2711111 |
| 71.4285710 | 11.5714290 | 17.8641980 | 2.1333333 |
| 77.4647890 | 11.4084510 | 18.5637860 | 2.0139394 |
| 83.3333330 | 11.2500000 | 19.1810700 | 1.9061111 |
| 89.0410960 | 11.0958900 | 19.7283950 | 1.8097436 |
| 94.5945950 | 10.9459460 | 20.2181070 | 1.7200000 |
| 100.0000000 | 10.8000000 | 20.6543210 | 1.6395556 |
| 105.2631600 | 10.6578950 | 21.0493830 | 1.5654167 |
| 110.3896100 | 10.5194810 | 21.4115230 | 1.4980392 |
| 115.3846200 | 10.3846150 | 21.7366260 | 1.4359259 |
| 120.2531600 | 10.2531650 | 22.0329220 | 1.3785965 |
| 125.0000000 | 10.1250000 | 22.3086420 | 1.3256667 |
| 129.6296300 | 10.0000000 | 22.5596710 | 1.2765079 |
| 134.1463400 | 9.8780488 | 22.7901230 | 1.2309091 |
| 138.5542200 | 9.7590361 | 23.0041150 | 1.1889855 |
| 142.8571400 | 9.6428571 | 23.2057610 | 1.1491667 |
| 147.0588200 | 9.5294118 | 23.3909470 | 1.1120000 |
| 151.1627900 | 9.4186047 | 23.5637860 | 1.0771795 |
| 155.1724100 | 9.3103448 | 23.7242800 | 1.0444444 |
| 159.0909100 | 9.2045455 | 23.8765430 | 1.0130952 |
| 162.9213500 | 9.1011236 | 24.0164610 | 0.9836782 |
| 166.6666700 | 9.0000000 | 24.1522630 | 0.9555556 |

$\Delta H$, and the enthalpies of dilution of $Ca(NO_3)_2$ solution in water; $\Delta H_{dilut}$, against $Ca^{2+}$ and MBP concentrations.

## Discussion

We have shown previously that the enthalpies of the solute-solvent ($M^{2+}$+MBP+water in this case) interactions in the aqueous solvent ($M^{2+}$+water in the present case) system, can be accounted for quantitatively in terms of three factors: preferential solvation by the components of a mixed solvent, weakening or strengthening of solvent-solvent bonds by the solute and the change in the enthalpy of the solute-solvent interactions [23–31]. This treatment leads to:

$$\Delta H = \Delta H_{max} x'_B - \delta^{\theta}_A (x'_A L_A + x'_B L_B) - (\delta^{\theta}_B - \delta^{\theta}_A)(x'_A L_A + x'_B L_B) x'_B \quad 1$$

The parameters $\delta^{\theta}_A$ and $\delta^{\theta}_B$ are the indexes of MBP stability in the low $M^{2+}$ ion concentrations and in the maximum concentration of the $M^{2+}$ upon saturation of all MBP respectively. Cooperative binding requires that the macromolecules have more than one binding site, since cooperativity results from the interactions between binding sites with ligands. If the binding of a ligand at one site increases the affinity for the ligand at another site, the macromolecule exhibits positive cooperativity. Conversely, if the binding of a ligand at one site lowers the affinity for a ligand at another site, the protein exhibits negative cooperativity. If the ligand binds at each site independently, the binding is non-cooperative: $p<1$ or $p>1$ indicate positive or negative cooperativity of the macromolecule for binding with the ligand, respectively; $p=1$ indicates that the binding is non-cooperative. $x'_B$ can be expressed as follow:

$$x'_B = \frac{px_B}{x_A + px_B} = \frac{\nu}{g} \quad 2$$

$x_B$ is the fraction of the metal ion needed for saturation of the binding sites, and $x_A = 1 - x_B$ is the fraction of unbounded $M^{2+}$ ions. Now the model is a simple mass action treatment, with metal ions replacing water molecules, at the binding sites in the present case. We can express $x_B$ fractions, as the total $M^{2+}$ concentrations divided by the maximum concentration of the $M^{2+}$ upon saturation of all MBP as follows:

$$x_B = \frac{[M^{2+}]_T}{[M^{2+}]_{max}};\ x_A = 1 - x_B \quad 3$$

$[M^{2+}]_T$ is the total concentration of metal ions and $[M^{2+}]_{max}$ is the maximum concentration of the $M^{2+}$ upon saturation of all MBP. In general, there will be "$g$" sites for binding of $M^{2+}$ per MBP molecule and $\nu$ is defined as the average moles of bound $M^{2+}$ per mole of total MBP. $L_A$ and $L_B$ are the relative contributions of unbounded and bounded metal ions to the enthalpies of dilution in the absence of MBP and can be calculated from the enthalpies of dilution of $M^{2+}$ in buffer, $\Delta H_{dilut}$, as follow:

$$L_A = \Delta H_{dilut} + x_A \left( \frac{\partial \Delta H_{dilut}}{\partial x_B} \right);$$

$$L_B = \Delta H_{dilut} - x_A \left( \frac{\partial \Delta H_{dilut}}{\partial x_B} \right) \quad 4$$

The enthalpies of $M^{2+}$+MBP interactions, $\Delta H$, were fitted to **Equation 1** over the whole $M^{2+}$ compositions. In the procedure the only adjustable parameter ($p$) was changed until the best agreement between the experimen-

tal and calculated data was approached (**Figs. 1–3**). $\delta_A^\theta$ and $\delta_B^\theta$ parameters have been also optimized to fit the data. The optimized $\delta_A^\theta$ and $\delta_B^\theta$ values are recovered from the coefficients of the second and third terms of **Equation 1**. The small relative standard coefficient errors and the high $r^2$ values (0.99999) support the method. The agreement between the calculated and experimental results (**Fig. 1**) is striking, and gives considerable support to the use of **Equation 1**.

$\Phi$ is the fraction of the MBP molecule undergoing complexation with $M^{2+}$ ions which can be expressed as follows:

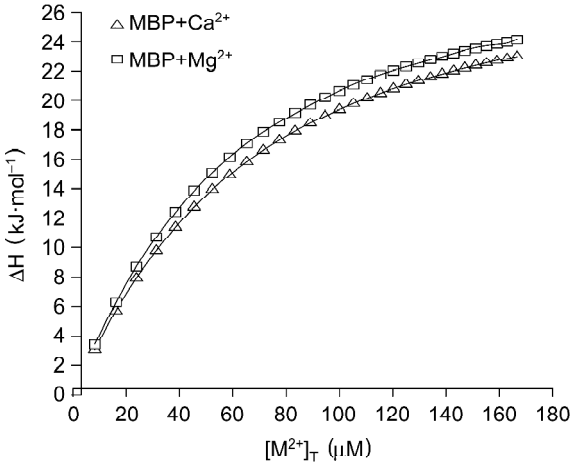


**Fig. 1 Comparison between the experimental enthalpy values via Equation 1** $[M^{2+}]_T$ is the total concentration of $Ca^{2+}$ or $Mg^{2+}$ ions in μM.

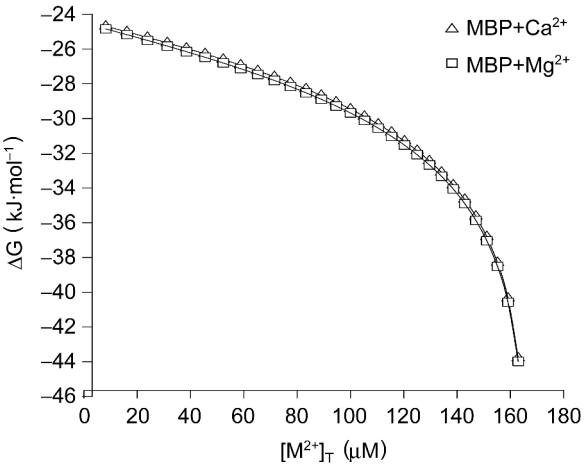


**Fig. 2 Comparison between the experimental Gibbs free energies via Equation 1** $[M^{2+}]_T$ is the total concentration of $Ca^{2+}$ or $Mg^{2+}$ ions in μM.

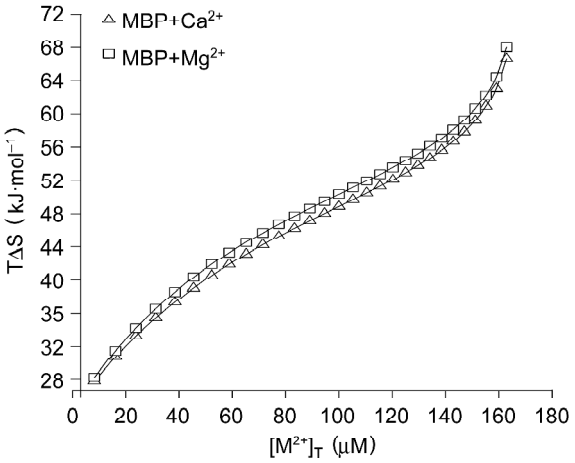


**Fig. 3 Comparison between the experimental TΔS values via Equation 1** $[M^{2+}]_T$ is the total concentration of $Ca^{2+}$ or $Mg^{2+}$ ions in μM.

$$\Phi = \frac{\Delta H}{\Delta H_{\max}} \quad 5$$

where $\Delta H_{\text{dilut}}$ represents the heat value upon saturation of all MBP. The appearance association equilibrium constant values, $K_a$, as a function of $M^{2+}$ concentration can be calculated as follows:

$$K_a = \frac{\Phi}{(1-\Phi)[M^{2+}]_F} \quad 6$$

$[M^{2+}]_F = (1-x_B)[M^{2+}]_T$ is the unbounded or the free $M^{2+}$ ion concentrations. The variable $\Phi$ represents the fraction of binding sites that are occupied on the peptide molecule. Therefore, $1-\Phi$ represents the fraction of binding sites that are not occupied. The appearance association equilibrium constants, $K_a$, for successive replacement of water molecules by $M^{2+}$ ions are as follow:

$$K_a = x_A^{\,g} - \sum_{i=1}^{g} K_i \frac{x_B^i}{x_A^{i-g}} \quad 7$$

where the $K_i$ is the macroscopic association equilibrium constants which are the equilibrium constants for every successive replacement of water molecules by $M^{2+}$ ions in the equilibria:

$$MBP(H_2O)_g + i\,(M^{2+}) \Leftrightarrow MBP(H_2O)_{g-i}(M^{2+})_i \quad \text{E1}$$

$K_a$ values obtained from **Equation 6**, have been fitted to **Equation 7** using a computer program for nonlinear least-

**Table 3 Binding parameters for MBP+$Ca^{2+}$ and MBP+$Mg^{2+}$ interactions calculated by Equations 1 and 7**

| | $Ca^{2+}$+MBP | $Mg^{2+}$+MBP |
|---|---|---|
| $\delta_A^o$ | 0.053±0.016 | 0.968±0.100 |
| $\delta_B^o$ | −12.322±0.046 | −11.395±0.060 |
| $K_1/\mu M^{-1}$ | 1.664±0.076 | 1.504±0.112 |
| $K_2/\mu M^{-1}$ | 1.500±0.027 | 1.617±0.035 |
| $\Delta H_{max}$/kJ·mol$^{-1}$ | 23.000±0.007 | 24.152±0.038 |
| $g$ | 2 | 2 |
| $p$ | 1.040 | 1.040 |

$p$=1 indicates that the $Ca^{2+}$and $Mg^{2+}$ ions occupy the identical binding sites non-cooperatively.

square fitting. Therefore, we can approach the "$g$" value and the macroscopic association equilibrium constant in the first, $K_1$, and the second, $K_2$, binding site (**Table 3**), which correspond to $\nu = 1$, $\nu = 2$ and $\nu = 3$ respectively. values can be calculated at any concentration of $M^{2+}$ via **Equation 2**. The binding parameters obtained from this method are listed in **Table 3**. The Gibbs energies as a function of $M^{2+}$ concentrations can be obtained as follow:

$$\Delta G = -RTLnK_a \qquad 8$$

Gibbs energies, $\Delta G$, calculated from **Equation 8** are shown graphically in **Fig. 2**. $T\Delta S$ values were calculated using $\Delta G$ values and have shown in **Fig. 3**. The less negative Gibbs free energies in the low $M^{2+}$ concentrations (**Fig. 2**) indicate the lower affinity in this region.

A nonpolar residue dissolved in water induces a solvation shell in which water molecules are highly ordered. When two nonpolar groups come together on the folding of a polypeptide chain, the surface area exposed to the solvent is reduced, and part of the highly ordered water in the solvation shell is released to the bulk solvent, which results in an increase in the entropy. It is possible to introduce a correlation between change in $\delta_A^\theta$ and increase in the stability of proteins. The $\delta_A^\theta$ value reflects the hydrophobic property of MBP, leading to the enhancement of water structure. The greater the extent of this enhancement, the greater the stabilization of the MBP structure and the greater the value of $\delta_A^\theta . \delta_A^\theta$ value (**Table 3**) for MBP+$M^{2+}$interactions are small and positive (**Table 3**), indicating that in the low concentration of $M^{2+}$ the MBP structure is stabilized, resulting in an increase in its biological activity. $\delta_B^\theta$ value in high $M^{2+}$ concentration are big and negative, indicating that the MBP structure is destabilized by these cations in this region.

The $p$ values are very close to one ($p$=1.04), indicating that there are a set of two identical non-operative binding sites for MBP+$Ca^{2+}$and MBP+$Mg^{2+}$. In practice it turns out that even for good data it is usually very difficult to distinguish between heterogeneity and cooperativity, if more than two sites are present. Furthermore, it is almost impossible to derive accurate binding parameters from real data, if binding is cooperative and the binding sites are not equivalent.

A value of $p = 1$ would mean that $K_1 = K_2$, indicates that the binding of the second site occurs non-cooperatively as compared to binding of the first site, whereas a value of $p>1$ would indicate that binding of the second site is facilitated and a value of $p<1$ indicates that binding of the second site is inhibited (anti-cooperativity or negative cooperativity).

The same conclusion is reached via **Equation 7** because the macroscopic association equilibrium constants recovered from this equation are roughly the same ($K_1 \approx K_2$ in **Table 3**).

Using fluorescence studies, Barylko *et al* have represented that there are two binding sites on MBP for $Ca^{2+}$-calmodulin, but they could not determine the exact values of dissociation constants of every site [17]. Whereas, we can calculate all thermodynamic functions, cooperativity parameters, equilibrium constants and stability prediction as a result of ligand interaction with a biopolymer, just using **Equations 1** and **7**, and it is the most useful method in the ligand+macromolecule interactions.

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