Binding of Ca^{2+} to Calmodulin and Its Tryptic Fragments: Theory and Experiment

Bo Svensson, Bo Jönsson,* and E. Thulin

Physical Chemistry 2, Chemical Centre, POB 124 S-22100 Lund, Sweden

C. E. Woodward

Department of Chemistry, ADFA, Canberra, Australia

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ABSTRACT: The Ca^{2+}-binding constant of the protein calmodulin is determined experimentally at different pH and varying salt concentration. By comparison to statistical mechanical simulation results, it is shown that the shift in binding constant upon addition of salt is almost quantitatively due to electrostatic interactions. Specific interactions as well as effects due to structural rearrangements of the protein seem to be less important, indicating a structurally rather conserved protein upon addition of salt and changes in pH. The tryptic fragments of calmodulin also bind calcium with high affinity, and the electrostatic effects seem likewise to dominate the binding constant shifts in these systems. It is also shown that the chemical potential of free calcium ions, which is normally dominated by the salt, is strongly influenced by the highly charged calmodulin molecule. This complicates a detailed comparison at low salt concentration, since it requires very accurate information on the salt and protein concentrations, which normally are not available.

The existence of highly charged molecules in biological systems is well-known, it suffices to mention DNA as the prime example. However, the important role played by electrostatic interactions has in the past not been fully appreciated and in a few systems even completely neglected. This situation is now changing, and in recent years a considerable amount of experimental and theoretical studies focusing on charged biochemical system has been presented. One important class is highly charged proteins, in particular those responsible for the binding of different cationic species. To this group belong a large number of calcium-binding proteins, some of which have been thoroughly studied both spectroscopically and by kinetic and thermodynamic means (Rasmussen, 1986a,b). A recent comparison of measured and calculated binding constant shifts for the calcium-binding protein calbindin D_{9k} demonstrated the importance of electrostatic interactions (Svensson et al., 1990, 1991).

In this study we focus on calmodulin, which is capable of binding two pairs of calcium ions with high affinity, each pair in a separate globular domain. Due to its biological importance, calmodulin has been the subject of numerous studies (Forsén et al., 1986), most recently, one in which the binding constants of both the intact protein and of its tryptic fragments have been determined as a function of salt concentration (Linse et al., 1991). We have extended this experimental study to also incorporate a variation of pH. Recent experiments indicate that the binding of calcium ions is accompanied by the titration of some unidentified residue in calmodulin (Sellers et al., 1991). Together these experimental studies serve as an excellent starting point for a theoretical investigation. Calmodulin provides a severe test for theory as it binds four calcium ions, in contrast to calbindin, which binds only two. A comparison with the calcium-binding constants for the fragments may add further insight into the mechanism of binding. Due to the very high charge of intact calmodulin, one would expect that nonlinear effects become important, which makes this particular system a challenge for theory.

The obvious theoretical approach to these typically large molecular systems is based on statistical mechanics. While approximate statistical mechanical theories easily deal with systems of spherical or cylindrical symmetry, awkward numerical procedures are generally needed to solve for more complex geometries, such as that of a protein molecule. It is then easy to appreciate the possibility offered by the Monte Carlo (MC) simulation technique, since its applicability is not greatly affected by the symmetry of the system under study. We do not intend to give a detailed account of the Monte Carlo technique here. It suffices to mention that the Metropolis MC technique (Metropolis et al., 1953) is a numerical procedure for solving statistical mechanical averages, i.e., a method for solving many-dimensional integrals.

When attempting to calculate equilibrium properties of large biomolecules in solution, one is forced to turn to simple models. For instance, when focusing on electrostatic interactions, it is possible to avoid an explicit treatment of solvent molecules by invoking a dielectric continuum model. Here the solvent molecules are replaced by a uniform continuum with a suitable dielectric constant. A further commonly used simplification, which we will make use of, is to treat the protein as a rigid body. At first this may seem to be a drastic approximation—there are certainly structural rearrangements accompanying the binding of calcium. At the end, however, we calculate binding constant *shifts*, and the effect of structural changes in the protein then cancel. The real assumption used is that the structural changes upon binding are independent of added salt. Another point to stress is that the Coulomb potential is long-range and slowly varying, which makes it less sensitive to small structural rearrangements.

As the molecule will exclude solvent molecules from its interior, the interesting question arises as to what one should choose for the permittivity of the interior region. A seemingly viable choice is the electronic permittivity (Harvey, 1989). However, this may lead to an underestimation, as the assumption of a fixed protein structure precludes dielectric response from nuclear motions. Furthermore, the crucial issue

* Author to whom correspondence should be addressed.

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is where to locate the dielectric boundary between protein and solvent. In reality, the surface of a protein in solution is rather permeable to water, especially in view of the fact that most of the charged species are present there. Thus, there will be a transition zone between the high dielectric solvent and the protein interior. To place one dielectric boundary at the surface of the protein then seems to be a crude and ill-supported choice. It is also important to note that a low dielectric interior will only affect the electrostatic interactions significantly if it has a permittivity which is an order of magnitude different from that of the solvent. In real numbers it means that a protein in aqueous solution should have a dielectric permittivity of 10 or less in order to play a noticeable role.

With these considerations in mind, we assumed a uniform dielectric permittivity in our model. That is, the interior of the protein is assumed to have the same dielectric constant as the solvent. Recent MC simulations, based on this assumption, accurately reproduce observed shifts in the calcium-binding affinity of calbindin, upon mutation of the protein and with varying ambient salt concentration (Svensson et al., 1990, 1991). Furthermore, linearized Poisson–Boltzmann calculations using a low dielectric interior, for the same system, are unable to reproduce the experimental shifts (Juffer, unpublished data). However, gradually increasing the permittivity of the protein in these calculations improves the agreement with experimental data.

EXPERIMENTAL PROCEDURES

Materials. Bovine testes calmodulin and its tryptic fragments were prepared as previously described (Andersson et al., 1983; Vogel et al., 1983), and the purity was checked by sodium dodecyl sulfate gel electrophoresis, agarose gel electrophoresis, and ^1H NMR. The residual Ca^{2+} content was determined by atomic absorption spectroscopy. Calmodulin was dissolved in distilled water to a concentration of 71 μM and the tetrapotassium salt of 5,5′-Br_2BAPTA to a concentration of 95 μM. Two different buffers were used: 4.0 mM Tris plus 4.0 mM PIPES with a pH of 6.5, 7.5, or 8.5. The concentration in the original KCl solutions varied between 0 and 600 mM. The final sample was obtained by mixing equal volumes of the four solutions described above giving a calmodulin concentration of 18 μM, 2 mM buffer solution, 0–150 mM KCl, and a pH between 6.4 and 8.3. The pH was also measured after the titration and found to change less than 0.1 of a pH unit. The Ca^{2+} titration was performed with $CaCl_2$ of either 1.47 or 2.94 mM concentration in a 2 mM Tris-HCl solution of pH 7.5. Buffer and KCl solutions were treated with Chelex 100 to avoid Ca^{2+}. All chemicals used were of pro analysi quality.

The binding constant of the protein was obtained from least-square fits directly to the absorbance at 263 nm as a function of the total Ca^{2+} concentration (Linse et al., 1991). The binding constant of the chelator in a solution containing KCl at pH 7.5 has previously been determined (Linse et al., 1991). The binding constant of the chelator was assumed to be independent of the pH in the range 6.5–8.5. The absorbance of BAPTA was corrected for the presence of K^+ ions. The estimated experimental uncertainty is given in Table I.

FREE ENERGY OF BINDING

Several calcium-binding proteins show a cooperative binding of calcium ions (Linse et al., 1987). A controversial issue has been whether this is also true for calmodulin or not. The existence of a cooperative mechanism for the binding of two ^{1}valent ions is of course an interesting phenomena, but it is at present probably beyond our electrostatic model to describe. As we will not concern ourselves with the cooperativity, real or not, we leave this controversary for the moment.

Table I: Experimental Shifts in the Calcium-Binding Constant of Calmodulin at Varying pH and KCl Concentration[a]

KCl (mM)	pH 6.4 ± 0.1	pH 7.5 ± 0.1	pH 8.3 ± 0.1
150[b]	7.1	6.2 (7.1)	7.0
100	6.0	6.0 (5.9)	6.4
50	5.4	5.1 (5.2)	5.5
25	3.6	3.6 (3.6)	3.4
10	2.2	1.9 (2.0)	1.7
2[c]	0.0	0.0 (0.0)	0.0

[a] The binding constants at 2 mM are taken as references and the shifts are given in units of ΔpK. Values in parentheses are from Linse et al. The uncertainty in pK values is less than ±0.6. [b] The pK shifts at high salt concentration are subject to larger uncertainties than those at lower KCl concentration. [c] The lowest salt concentration is not accurately known in the experiments, because of the contributions from buffer and residual salt in the protein solution.

The macroscopic binding constants K_1–K_4 for calmodulin (CaM) are defined in terms of concentrations as

$$K_1 = \frac{[CaMCa]}{[CaM][Ca^{2+}]} \quad K_2 = \frac{[CaMCa_2]}{[CaMCa][Ca^{2+}]}$$

$$K_3 = \frac{[CaMCa_3]}{[CaMCa_2][Ca^{2+}]} \quad K_4 = \frac{[CaMCa_4]}{[CaMCa_3][Ca^{2+}]} \quad (1)$$

where [CaM] and $[Ca^{2+}]$ are the concentrations of free protein and calcium, respectively, and $[CaMCa_n]$ is the concentration of protein at varying degree of calcium binding. The total binding constant is given by

$$K = K_1K_2K_3K_4 \quad (2)$$

which is also represented here as

$$pK = -\log K \quad (3)$$

The total and the macroscopic binding constants for the tryptic fragments are defined in an analogous way.

In the following we will restrict the comparison to the total binding constants, although all the macroscopic binding constants are accessible experimentally. The reason for this limitation is that in order to compare shifts in the macroscopic binding constants one needs to know the individual site binding constants (Svensson et al., 1991) which have not been determined experimentally; in addition, the total binding constants are more accurately determined than their components in the experiments.

Consider then the free energy changes upon binding Ca^{2+} ions to each of the sites. It is convenient to imagine this as a two-step process. In the first step we envisage constraining the unbound protein so that it adopts configurations consistent with there being Ca^{2+} ions present in the binding sites. The free energy cost associated with such a constraint will always be positive. In the second process, Ca^{2+} ions are transferred from solution, to the (pre-formed) sites in the constrained protein. There are many contributions to the free energy change associated with this process. In this work we are concerned only with the electrostatic contribution, denoted as ΔG_{el}. It is essentially the difference in *electrostatic* free energy of the systems corresponding to the bound and free calcium states. The rest of the free energy contributions are due to, e.g., hydration and ligand binding energies. The major role played by additional salt is the screening of electrostatic

interactions. Therefore, we conjecture that the changes in ΔG_{el} are sufficient to explain the major part of observed shifts in K, upon changes in the surrounding salt concentration. This permits us to calculate relative changes in the binding constants with respect to a chosen reference state using

$$K/K^{ref} = \exp[-\beta(\Delta G_{el} - \Delta G_{el}^{ref})] \quad (4)$$

Among other things, this assumption implies that the structural changes in the protein that occur on addition of salt are too subtle to be felt by the corresponding changes in the electrostatic interactions of the protein.

Thus our aim is to calculate ΔG_{el} for a range of salt concentrations. We can write this quantity as

$$\Delta G_{el} = \mu^{ex}(B) - N\mu^{ex}(F) \quad (5)$$

where $\mu^{ex}(F)$ is the excess chemical potential of N free calcium in a solution of given salt and protein concentration, and $\mu^{ex}(B)$ is the excess chemical potential for the N bound calcium ions ($N = 4$ in the case of calmodulin and $N = 2$ for the fragments) under the same conditions. As is described in more detail below, we use Monte Carlo simulations to obtain these excess chemical potentials, assuming a fixed protein structure, that of the crystalline state.

THEORETICAL PROCEDURES

We used the coordinates PDB3CLN from the Brookhaven Data Bank, which were obtained from an X-ray diffraction study of the crystalline protein (Babu et al., 1985, 1988; Kretsinger & Weissman, 1986). The coordinate list does not included hydrogen atoms. These may be added using geometrical constraints. However, it has been shown that the hydrogen atoms only have minor effects on the simulated results, and consequently they were neglected (Svensson et al., 1991). A few terminal amino acids were also missing in the X-ray structure. These were added using the molecular dynamics program MUMOD (Teleman et al., 1991), which allows an approximate refinement of these extra residues. The intact protein contained 148 amino acid residues, while the two fragments contained residues 1–74 and 78–148, respectively. The total number of protein atoms included in the MC simulations were 1166, 571, and 569 for the intact protein and the two fragments, respectively. The X-ray coordinates of the fragments were taken from the intact protein. Each protein atom was represented as a hard sphere of 4-Å diameter, impenetrable to any solvent ions. The protein coordinates were kept fixed during the simulation.

The model allows the use of partial charges on each and every atom. However, we have in a previous study (Svensson et al., 1990) shown that models with partial charges give results virtually indistinguishable from those obtained with net charges. The net charge model used here assigns a charge of –0.5 to negatively charged carboxylic oxygens, which are located on glutamate and aspartate residues as well as the C-terminal residue. The positively charged residues are lysines, arginines, and the N-terminal amino group. Lysines carry a positive unit charge on the ζ-nitrogen while the arginines carry $^1/_3$ unit charge on each of the ϵ, η_1, and η_2 nitrogens. All other atoms are kept neutral. The net charge of calmodulin was -23 to be compared to the net charge of calbindin D_{9k}, which is only –8. The tryptic fragments had net charges of –12 and –13, respectively (two positively charged residues are lost when cleaving calmodulin).

The protein was placed in the center of a spherical cell, to which counterions and salt ions were added. These were treated as mobile charged hard spheres confined in the cell.

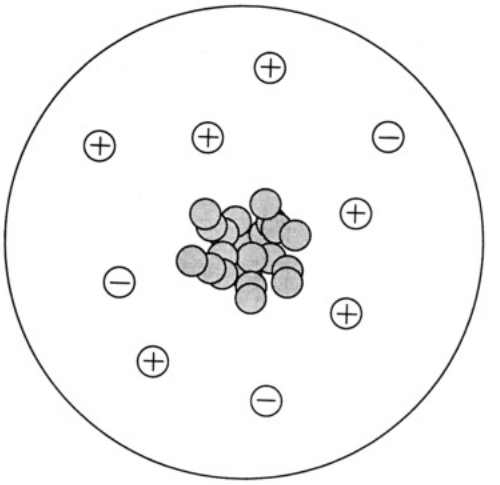


FIGURE 1: Schematic picture of the model system. The protein is placed in the center of a spherical cell with its atoms represented as shaded spheres. The mobile K^+ and Cl^- ions are shown as open spheres.

The hard core diameter of the ions, including calcium, was 4 Å. The calculated ΔpKs are rather insensitive to the ionic radii, and changing these ±1 Å has virtually no effect. Furthermore, the calcium radius only plays a role for the free ion, since the bound calcium ions are placed in pre-formed sites and there only interact electrostatically with the protein atoms. The cell radius was chosen so as to correspond to the actual protein concentration, and the solvent was treated as a uniform dielectric continuum throughout the cell. The only way it enters the calculations is through the relative dielectric constant, ϵ. The interaction energy between charged species i and j is thus given by

$$u(r_{ij}) = q_i q_j e^2/4\pi\epsilon_o\epsilon r_{ij} \qquad r_{ij} \geq (\sigma_i + \sigma_j)/2 \quad (6a)$$

$$u(r_{ij}) = \infty \qquad r_{ij} < (\sigma_i + \sigma_j)/2 \quad (6b)$$

where q is a partial charge, e is the elementary charge, ϵ_o is the permittivity of free space, and r_{ij} is the distance between the particles i and j. Only interactions with particles within the cell were taken into account. These approximations constitute the so-called primitive model for electrolyte solutions (Friedman, 1962). The dielectric constant and temperature were chosen to be 78.7 and 298 K, respectively, in order to correspond to water at the experimental conditions. A schematic diagram of the system is given in Figure 1.

The model described above was used both for calmodulin and its tryptic fragments. In an additional set of simulations, we also tested the effect of a high aggregate net charge on the excess chemical potential of free calcium ions. In these simulations we used a simplified model describing the aggregate as a sphere with a net charge at its center (see Figure 4).

The Monte Carlo simulations were performed in the canonical ensemble, where, in addition to the temperature, the number of particles and the cell volume are constant (Metropolis et al., 1953). At the start of a simulation a few thousand configurations per ion were generated to allow the system to equilibrate. After this, at least 10 000 configurations per ion were collected to generate the average properties.

The excess chemical potentials of free and bound ions, appearing in eq 5, were calculated with a modified Widom technique, recently developed for electrolyte solutions (Widom, 1963; Svensson & Woodward, 1988). The traditional Widom

procedure is to introduce a test particle at a position r which does not affect the "real" particles of the simulation. The interaction energy, $v(\mathbf{r})$, of the test particle with all other particles in the solution is calculated and the excess chemical potential of the test particle at r, is then obtained as

$$\beta\mu^{ex}(\mathbf{r}) = -\ln \langle \exp[-\beta v(\mathbf{r})] \rangle \quad (7)$$

The angular brackets stand for an ensemble average over the unperturbed system. The modified Widom technique as well as details of the MC procedure have been described at length in earlier publications (Svensson & Woodward, 1988; Teleman et al., 1991), and we do not repeat it here.

The excess chemical potential of the bound calcium ions was obtained by introducing the divalent test particles in the binding sites, as specified in the crystal structure. Since the positions of the bound ions and the rest of the protein were fixed, the bound ion–protein interaction had to be calculated only once at the start of a simulation. With increasing salt concentration, an accurate estimate of the *bound* ion excess chemical potential can be obtained with a much smaller cell size than specified by the actual protein concentration. This is due to the efficient screening of the protein charge and leads to reduced computation times. In general, at concentrations above 10 mM KCl, it was sufficient to include 50–100 salt pairs in a simulation. For dilute protein concentrations and for the salt concentrations considered here, a *free* calcium ion would see mainly a uniform salt solution. Thus using a reduced cell radius could lead to errors in free ion chemical potentials. In such cases a better estimate can be obtained from a simulation of the isotropic salt solution at the appropriate concentration. When no additional salt is present, however, or when the protein concentration becomes comparable with that of the salt, the chemical potential of the free calcium is more consistently evaluated by randomly placing the test particle in the cell, containing the protein and the appropriate amount of salt and counterions. This is an intriguing point, which will be discussed more in the next section.

RESULTS AND DISCUSSION

Calmodulin with a net charge of −23 at neutral pH is a highly charged aggregate even if its surface charge density is far from that of, for example, surfactant aggregates. Still it is not unreasonable to expect some carboxylic residues to show an increased pK. As mentioned above, there exists experimental evidence from calorimetric studies that the binding of calcium at low salt concentration is accompanied not by the *release* of protons but by the *addition* of protons to the calmodulin molecule (Sellers et al., 1991). From a theoretical point of view, it is straightforward to calculate the shift due to a charge change in the protein, *provided* one knows which residue is titrating. Since this is unknown, one can only make rough estimates based on previous calculations on calbindin (Svensson et al., 1990) or on simplified models. On this basis we argue that a titrating residue in calmodulin would cause a pK shift of 0.5–1 unit. That is, the pK shift when going from 2 to 150 mM KCl at pH 6.4 and at 8.3 should differ by this amount. In order to investigate this aspect, we have extended the measurements made by Linse et al. at pH 7.5 to both lower and higher pH. Table I shows that within the pH regime 6.4–8.3 there does not seem to be any titrating groups active, and within the experimental errors we record identical binding constant shifts at pH 6.4 and 8.3. Our data also agree with those of Linse et al. at pH 7.5. Thus, we find it difficult to reconcile both our experimental and theoretical findings with the calorimetric studies by Sellers et al.

Table II: Comparison of Experimental and Theoretical Shifts in the Calcium-Binding Constant (ΔpK) of the Two Calmodulin Fragments TR_1C and TR_2C at a Concentration of 20 μM[a]

	TR_1C		TR_2C	
KCl (mM)	theory	exp	theory	exp
150	4.0	3.5	3.9	3.7
100	3.5	3.3	3.5	3.2
50	2.7	2.9	2.7	2.6
10	1.0		1.1	
5	0.6		0.4	
2[b]	0.0	0.0	0.0	0.0
1	−0.2		−0.2	
0	−0.4		−0.3	

[a] The binding constants at 2 mM are taken as references. The experimental uncertainty is ±0.6 and ±0.2 for TR_1C and TR_2C, respectively. The theoretical numbers are significant to within 0.1 of a pK unit. [b] The lowest salt concentration is not accurately known in the experiments, because of the contributions from buffer and residual salt in the protein solution.

Table III: Comparison of Experimental and Theoretical Shifts in the Calcium-Binding Constant (ΔpK) of Calmodulin at a Concentration of 20 μM[a]

KCl (mM)	theory	exp
150	5.9	4.8
100	4.9	4.2
50	3.1	3.4
25	1.6	1.6
10	0.0	0.0
5	−0.9	
2[b]	−1.2	−1.9
1	−1.2	
0	−0.8	

[a] The binding constant at 10 mM is taken as a reference. The experimental numbers are average values from Table I, and the experimental uncertainty is approximately ±0.4. The theoretical numbers are significant to within 0.1 of a pK unit. [b] The lowest salt concentration is not accurately known in the experiments, because of the contributions from buffer and residual salt in the protein solution.

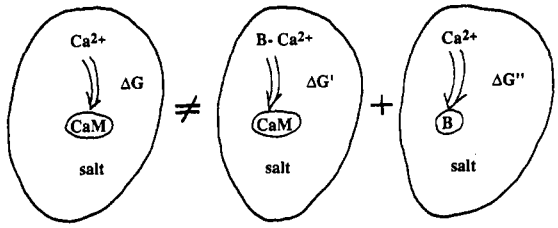
The experimental calcium-binding constants for the two tryptic fragments of calmodulin have been determined by Linse et al. (1991) using the same technique as in the present study. Both fragments show a high affinity for calcium ions, and the binding constants are sensitive to addition of KCl. Table II shows a comparison of experimental and theoretical binding constant shifts using the 2 mM KCl solution as reference. There is an excellent agreement between experiment and theory for the second fragment, which happens to be where the experimental results are most accurate. The comparison for the first fragment is slightly inferior, although there is agreement within the combined experimental and theoretical uncertainties. The lowest salt concentration given by Linse et al. is only specified as "low". The actual concentration is most likely less than 10 mM (Linse, personal communication). Thus, the overall agreement going from "low" to 150 mM KCl concentration is good, being off by a few tenths of a pK unit. To specify the salt concentration at the millimolar level in a biomolecular solution is a notorious problem, which hampers the comparison between theory and experiment. There are also other problems at low salt content, which we will discuss in more detail below.

Table III contains a similar comparison for calmodulin, but where we have chosen the 10 mM solution as reference point. There is a good agreement, and a few points are in very good agreement, but the comparison is not as favorable as for

$$CaM + 4\,B\text{-}Ca \rightarrow CaM\text{-}Ca_4 + 4\,B \qquad K = \frac{[CaM\text{-}Ca_4]\,[B]^4}{[CaM]\,[B\text{-}Ca]^4}$$

$$B + Ca \rightarrow B\text{-}Ca \qquad K_B = \frac{[B\text{-}Ca]}{[B]\,[Ca]}$$

Hence the final binding constant is: $K_{CaM} = K\,K_B^4$



$\Delta G \neq \Delta G' + \Delta G''$ since $\mu_{Ca,free} \neq \mu''_{Ca,free}$

FIGURE 2: Binding process of calcium ions to calmodulin and the chelator as analyzed in the experiment and in theory. Experimentally, one measures the competition between a chromophore (B) and calmodulin (CaM). The calcium-binding constant of the chromophore is measured separately in a salt solution *without* calmodulin. The process in the left sphere corresponds to the theoretical treatment, while the experimental procedure is described by the spheres to the right.

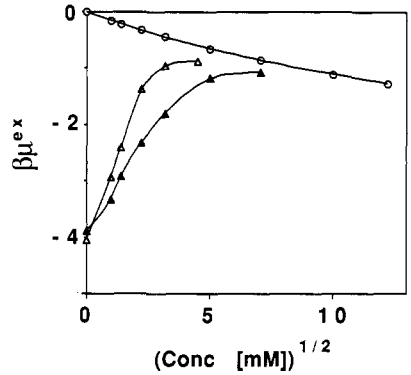


FIGURE 3: Excess chemical potential of a free calcium ion at various concentration of KCl. (○) Isotropic salt solution, (△) 30 μM calmodulin, and (▲) 100 μM calmodulin.

the fragments. In particular, one notes that the pK shift to "low" concentration, which is approximately 2 mM, is off by almost one pK unit, while the shift from 10 to 100 mM is relatively speaking much better described. This is probably due to an artifact in the analysis of the experimental data, which shows up at low salt concentrations. Consider the events schematically drawn in Figure 2. The experimental binding constant is obtained in a two-step process as described above, where the first step in the process is the transfer of calcium ions from the chelator to the protein. The second step is the transfer of free calcium ions at the same salt concentration to the chelator—this experiment is of course performed in the absence of protein. The process we ideally want to study is the transfer of free calcium ions to the protein. These two processes would be identical if the chemical potential of free calcium ions in the chelator and protein solution were the same. This is to a good approximation true at high salt concentrations but not at low salt content or high protein concentration as can be seen from Figure 3. A low net charge on the protein will of course reduce the effect. Figure 4 shows how μ^{ex}(F) for a divalent ion varies in a 30 μM hypothetical protein solution at a given salt concentration, but where the protein charge is allowed to vary (experimentally one could realize such a situation by changing the pH). The protein is modeled as a sphere of 40-Å diameter, with one single charge

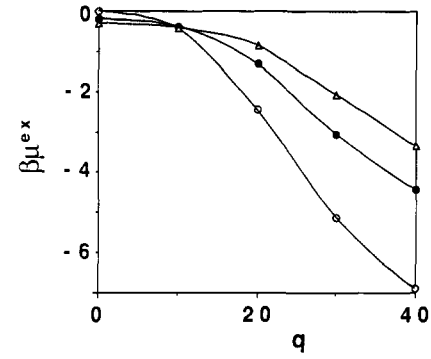


FIGURE 4: Dependence of the excess chemical potential of a free calcium ion on the protein charge. The protein concentration is 30 μM, and the protein is modeled as a sphere of 20-Å radius containing a charge in its center. (○) Counterions only, (●) 2 mM KCl, and (△) 5 mM KCl.

in its center. When we let the charge increase, there is a significant influence on the excess chemical potential of the free ion. We also note that for a high protein charge, addition of small amounts of salt also give rise to large changes in the excess chemical potential.

Figures 3 and 4 tell us that extracting the true binding constant from the two-step process described above sometimes introduces artifacts. Table III indicates that this might be the case for calmodulin. The artifact appears at low salt concentration, which means that only the shifts relative to low salt content are affected. One could in principle correct for this effect using the data in Figure 3. However, Figure 3 also shows that the chemical potential of free calcium ions is sensitive to both salt and protein concentration at low salt content. Thus, in order to perform an adequate correction, one requires an accurate knowledge of the salt as well as the protein concentration. We emphasize that this is an effect that appears only with highly charged protein at low salt concentration and/or high protein concentration. For example, this effect is of much less importance for the two fragments of calmodulin (Table II) or calbindin (Svensson et al., 1991).

When going from 10 to 150 mM KCl, we see an experimental shift of 4.8 units, while theory predicts it to be one unit larger, 5.9. Part of this discrepancy can probably be attributed to the experimental difficulties encountered at high salt concentration, but it can also be due to structural changes in calmodulin.

In the above analysis, we have assumed that K^+ ions only interact electrostatically with calmodulin and its fragments and that any specific binding is negligible. There exists conflicting evidence in the literature on this point, but it seems as if most experimental studies support the idea of a very small specific binding of K^+ ions. If a strong specificity did exist, it ought to differ between the sites. Hence, we would expect a less favorable comparison for one of the fragments, which does not seem to be the case. A more extensive study of calcium-binding constant shifts in calbindin D_{9k} also supports the notion of a weak specificity (Svensson et al., 1991).

The sum of the theoretical binding constant shifts for the fragments in the range 2–150 mM KCl is 7.9, while the shift is only around 7 for calmodulin. This can be understood by considering that the free energy of binding is a difference between the chemical potential of bound and free calcium (see eq 5). What one then finds is that the shift in μ^{ex}(B) for the fragments and the intact calmodulin are almost the same, while the shift in the free calcium chemical potential, $N\mu^{ex}$-

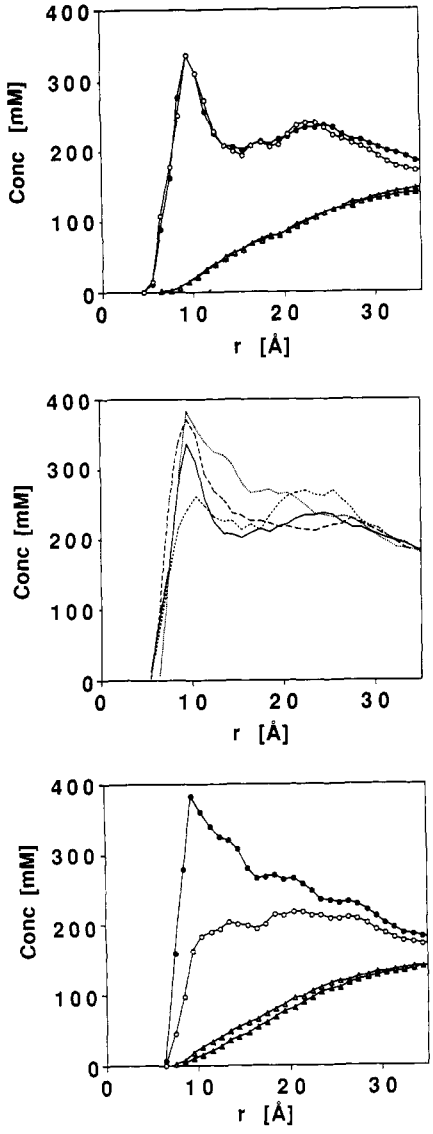


FIGURE 5: (a, top) Spherically averaged ion distributions around site 1 of the apo forms of calmodulin and TR_1C. The solution contains 150 mM KCl. Calmodulin, (○) K^+ and (△) Cl^-; TR_1C, (●) K^+ and (▲) Cl^-. (b, middle) Spherically averaged counterion distributions around the four binding sites of apocalmodulin. The solution contains 150 mM KCl. (—) Site 1, (– – –) site 2, (- - -) site 3, and (· · ·) site 4. (c, bottom) Spherically averaged ion distributions around site 4 in the apo and the fully calcium-loaded calmodulin. The solution contains 150 mM KCl. Apocalmodulin, (●) K^+ and (▲) Cl^-; calcium-loaded calmodulin, (○) K^+ and (△) Cl^-.

(F), for the fragment and the calmodulin solutions differs by approximately one pK unit when going from 2 to 150 mM KCl. This result can be contrasted to the experiments where the sum of the binding constant shifts for the fragments and the pK shift for intact calmodulin are in much better agreement. This is, however, a result of the improper treatment of the free calcium ions at low salt concentration in the calmodulin system (see Figure 2).

The distribution of co- and counterions around the proteins are shown in Figure 5a–c. The distributions are obtained as spherical averages around the different calcium-binding sites. Figure 5a shows a comparison between the ionic distribution around site 1 in calmodulin and the same site in TR_1C in a solution containing 0.15 M KCl. Surprisingly enough, the curves are virtually identical, which indicates that at this salt concentration the protein charge distribution around the site dominates the profiles at least up to 20 Å from the site. Further out one can notice a small difference, due to the different net charge of calmodulin and TR_1C, before the curves approach the bulk value. Figure 5b gives an indication of how different the four sites are from an electrostatic point of view. TR_1C contains sites I and II while TR_2C contains sites III and IV. The net charge of the apo and calcium-loaded form of calmodulin differs by eight units, and this has a profound effect on the counterion distribution as can be seen from Figure 5c. The co-ion profile, however, is less affected by the calcium binding.

In the present comparison we have assumed a uniform dielectric permittivity which is obviously incorrect—the protein interior does not have the same dielectric response as has pure water. Still we see a good agreement between theory and experiment in systems where we substantially change the electrostatic interactions; e.g., in calmodulin the binding process involves a charge change of 8e and in calbindin a change of 4e. This agreement might be fortuitous, but in the case of calbindin the comparison has been extended to a number of mutants and salt of different valency with the same result. The model with a uniform dielectric permittivity does also seem to work for small chelators (Svensson et al., 1992). In a model with a dielectric boundary, the results will be extremely sensitive to the location of the boundary between the low and high permittivity region. Experimentally, we have no guidance as to where to place this boundary. It is also obvious that the low dielectric region will only play a role as long as its permittivity is much smaller than that of water, i.e., $\ll 80$. Our present standpoint is that the major part of the protein shows a reasonably high dielectric response, and it is only the core of the protein that may have a low permittivity, which then explains the success of a uniform dielectric model.

CONCLUSIONS

We have demonstrated that the calcium-binding constant of calmodulin is independent of pH within the range 6.4–8.3, which indicates that the binding of calcium is not accompanied with a release or uptake of protons. The experimental binding constant shifts for calmodulin as a function of salt concentration are reasonably well described within a dielectric continuum model. An excellent agreement between experiment and theory is seen for the two calmodulin fragments, TR_1C and TR_2C, where the experimental and theoretical binding constant shifts seem to be in quantitative agreement. At low salt and/or high calmodulin concentration, the chemical potential of free calcium ions becomes strongly affected by the presence of calmodulin. This is a phenomenon that appears wherever the external potential, i.e., the aggregate charge, is large and only weakly screened. The effect is difficult to describe in a traditional Poisson–Boltzmann approach but is readily incorporated in a Monte Carlo simulation. The present simulations are performed with a uniform dielectric permittivity, and it is difficult to envisage that the inclusion of a low dielectric interior of the protein would improve the results.

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