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The effect of protein concentration on ion binding

(electrostatic interactions/Monte Carlo simulations/^{1}H NMR/metal ion binding/Ca^{2+}-binding proteins)

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ABSTRACT **The concentration of protein in a solution has been found to have a significant effect on ion binding affinity. It is well known that an increase in ionic strength of the solvent medium by addition of salt modulates the ion-binding affinity of a charged protein due to electrostatic screening. In recent Monte Carlo simulations, a similar screening has been detected to arise from an increase in the concentration of the protein itself. Experimental results are presented here that verify the theoretical predictions; high concentrations of the negatively charged proteins calbindin D_{9k} and calmodulin are found to reduce their affinity for divalent cations. The Ca^{2+}-binding constant of the C-terminal site in the Asn-56 → Ala mutant of calbindin D_{9k} has been measured at seven different protein concentrations ranging from 27 μM to 7.35 mM by using ^{1}H NMR. A 94% reduction in affinity is observed when going from the lowest to the highest protein concentration. For calmodulin, we have measured the average Mg^{2+}-binding constant of sites I and II at 0.325, 1.08, and 3.25 mM protein and find a 13-fold difference between the two extremes. Monte Carlo calculations have been performed for the two cases described above to provide a direct comparison of the experimental and simulated effects of protein concentration on metal ion affinities. The overall agreement between theory and experiment is good. The results have important implications for all biological systems involving interactions between charged species.**

Electrostatic interactions are crucial for the function of many biological macromolecules. Charged residues on the surface of a protein can play an important role in attracting ionic ligands from the surrounding solvent (1–6). The net charge of a protein, as well as the distribution of negatively and positively charged side chains, is an essential factor in determining the strength of the protein–ion interaction. For example, in superoxide dismutase, the charged residues are organized to ensure efficient channeling of the superoxide radical (O_2^-) to the active site of the enzyme (7). It is possible to increase the rate of binding of O_2^- by engineering in extra positive charge, but only if the structural integrity of the network of charged residues in and around the active site is maintained (6). Negatively charged side chains around the calcium-binding sites have been shown to enhance the affinity of calbindin D_{9k} for the positive metal ion (4, 8). The importance of electrostatic interactions involving charged residues has also been demonstrated for the binding of cytochrome c_2 to the *Rhodobacter sphaeroides* reaction center (9), the pK_a values of titrable side chains, as well as the catalytic activity of subtilisin (10), and the assembly of calmodulin with its target enzymes (11). Since these effects depend on direct electrostatic interactions, they are screened by all other charged species in the surrounding solution.

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We have recently implemented Monte Carlo (MC) simulations by using a dielectric continuum model in calculations of electrostatic effects on calcium-binding affinities of proteins (12). These calculations have accurately reproduced experimentally determined shifts in calcium-binding constants up to six orders of magnitude that are produced in small organic chelators and proteins by screening with different salts and/or mutations of charged amino acids (13–15). Furthermore, the MC simulations predicted that the concentration of a charged protein will affect the affinity for an ionic ligand; an effect that can become significant and which to our knowledge has not yet been demonstrated experimentally. For a given protein, the magnitude of the effect is proposed to be strongly dependent on the net charge. For wild-type calbindin D_{9k} (net charge −7) the calculations predict that raising the protein concentration from 0.1 to 1.0 mM will lead to a 98% reduction in the product of the two macroscopic calcium-binding constants, whereas for a mutant with net charge of −4, the corresponding figure is a 92% reduction. The effect is smaller at lower protein concentrations but is predicted to be observable down to 0.1–1.0 μM (13).

Since the MC simulations were successful in reproducing general electrostatic effects, we were led to explore whether the proposed protein concentration effect on ion affinities can be verified experimentally. The validation of this phenomenon requires a system with binding constants that can be measured in a direct manner over a range of protein concentrations. Furthermore, it is imperative that the solution does not contain any other species that bind the particular ion during the measurements—e.g., when measuring binding constants by equilibration against a metal ion chelator—because variations in protein concentration would affect the calcium affinity of both the protein and chelator, and the effect would escape detection. This is a nontrivial conclusion, indicating that the binding constants determined in a direct and an indirect way—e.g., by using a chelator—will give different results depending on the protein concentration. To establish the existence of the protein concentration effect, all binding constants have been measured in solutions completely devoid of metal ion chelators other than the protein itself, and metal ion binding has been monitored by ^{1}H NMR. The experimental evidence presented here has been obtained for two small, well-characterized calcium-binding proteins calbindin D_{9k} and calmodulin.

METHODS

Protein Preparation. The Asn-56 → Ala, Pro-43 → Met mutant of bovine (minor A) calbindin D_{9k} (denoted the N56A mutant) and bovine calmodulin were each produced by overexpression of synthetic genes in *Escherichia coli* and purified as described (16, 17). The last step in the purification scheme was the desalting of the sample on a 200-ml Sephadex G-25 (Pharmacia) column. For this purpose, the protein was mixed with EGTA in excess over total calcium, and 20 ml of saturated NaCl was applied to the column immediately prior to the

Abbreviation: MC, Monte Carlo.

sample. The protein thus passed through the saturated NaCl zone. After this procedure the sample was analyzed by atomic absorption spectroscopy, showing that the residual Na^+ concentration was 5.2 mol per mol of protein and the Ca^{2+} content was below 0.05 mol per mol of protein. The protein concentration was determined by amino acid analysis after acid hydrolysis. The homogeneity of each protein was confirmed by agarose gel electrophoresis, SDS/gel electrophoresis, and isoelectric focusing. ^1H NMR analysis showed that the samples were free of EDTA, EGTA, Tris, and other small molecules. All chemicals were of the highest grade commercially available, and water was both deionized and distilled. A dialysis bag filled with Chelex 100 (Bio-Rad) was placed in the 2 mM Tris buffer stock solution (pH adjusted to 7.5 with HCl) to minimize the concentration of divalent metal ions. The dialysis bag was boiled and rinsed four times to remove soluble small molecules prior to being used. Protein solutions were made up in the 2 mM Tris buffer and the pH was adjusted to 7.5 with HCl. The Ca^{2+} and Mg^{2+} stock solutions were made in 2 mM Tris with the pH adjusted to 7.5 with HCl. Their metal ion concentrations were determined by atomic absorption spectroscopy.

^1H NMR Spectra. One-dimensional ^1H NMR spectra were recorded on a GE Omega 500 spectrometer operating at 500.13 MHz. The number of scans per spectrum was constant in each titration but varied between different protein concentrations. The number ranged from 16 scans per spectrum for 7.35 mM protein to 5000 scans per spectrum at a protein concentration of 27 μM.

Ca^{2+}-Binding Constants. All experiments were performed in 2 mM Tris·HCl, pH 7.5, in 90% H_2O/10% ^2H_2O at 25°C. The initial protein concentration was determined by amino acid analysis after acid hydrolysis. Each titration started with Ca^{2+}-free protein N56A calbindin D_{9k}, and $CaCl_2$ stock solution was added in steps of *ca.* 0.1 equivalent, followed by acquisition of ^1H NMR spectra. At Ca^{2+} additions below 1 equivalent, a slow exchange process was observed, corresponding to Ca^{2+} binding at site I. Ca^{2+} binding to site II is a fast exchange process, which is observed as chemical shift changes for several residues at Ca^{2+} additions above 1 equivalent. The Ca^{2+}-binding constant of site II in the presence of calcium in site I, $\log_{10} K_{II,I}$, was extracted from computer fits to the chemical shift of well resolved signals as a function of total calcium (as illustrated in Fig. 1*A*). The chemical shift δ_{calc} at each titration point was calculated as

$$\delta_{calc} = p\cdot\delta_{Ca2} + (1 - p)\cdot\delta_{Ca1},$$

where δ_{Ca2} and δ_{Ca1} are the chemical shifts in the $(Ca^{2+})_2$ and $(Ca^{2+})_1$ forms, respectively, and p is the fraction of protein in the $(Ca^{2+})_2$ form. p is determined from the total protein concentration (calculated from the initial protein concentration and the dilution due to calcium additions), total calcium concentration (calculated from the initial calcium plus the calcium additions and corrected for dilutions), and the equilibrium binding constant $K_{II,I}$. The parameters $K_{II,I}$, δ_{Ca2}, and δ_{Ca1} were allowed to adjust their values until an optimal fit to the measured chemical shifts was obtained. All points in the titration were given equal weights, except for the first one or two points, which were given the weight zero due to a slight overlap of the binding processes at the two sites. The reported average values and standard deviations are based on individual fits to the chemical shifts of three different amide protons [at 6.1, 9.5, and 9.8 ppm in the $(Ca^{2+})_2$ protein at pH 7.5]. Calbindin D_{9k} has no side chains with pK_a values in the range 6.3–10.7 (T. Kesvatera, B.J., and S.L., unpublished data). Therefore, 2 mM Tris buffer was sufficient to keep the pH constant during a titration at pH 7.5.

Preparation of $(Ca^{2+})_2$·Calmodulin. A slight excess of calcium (over two equivalents) was added before the magnesium titration to inhibit Mg^{2+} binding at sites III and IV. Calcium is bound eight times stronger to sites III and IV than to sites I and II. The Ca^{2+} affinity of sites III and IV is *ca.* five orders of magnitude higher than the Mg^{2+} affinity. Furthermore, these sites bind magnesium with only one-eighth the affinity of sites I and II. Lyophilized apocalmodulin was dissolved in 2 mM Tris·HCl buffer, pH 7.5, in ^2H_2O at 25°C. To obtain the $(Ca^{2+})_2$ protein, Ca^{2+} was added stepwise to the ion-free protein and followed by ^1H NMR to well above 2 equivalents. Concentrated calcium-free protein solution was then added stepwise until the ^1H NMR spectrum agreed with that at 2.1 equivalents of calcium. This procedure was simplified by the fact that binding of the first two Ca^{2+} ions is a slow exchange process and binding of the last two is a fast exchange process. The protein concentration of this solution was 3.25 mM as determined by amino acid analysis after acid hydrolysis. The 1.08 and 0.325 mM $(Ca^{2+})_2$·calmodulin solutions were prepared by diluting aliquots from the 3.25 mM solution in 2 mM Tris·HCl buffer, pH 7.5, in ^2H_2O at 25°C.

Mg^{2+}-Binding Constants. $MgCl_2$ stock solution (in 2 mM Tris·HCl, pH 7.5) was added in a stepwise manner to the $(Ca^{2+})_2$·calmodulin solution and the binding process was monitored by ^1H NMR. The binding constant was obtained from computer fits, in a similar manner as described above for calcium-binding constants, to the chemical shift as a function of total magnesium concentration for one selected signal (at 6.6 ppm) by using the function

$$\delta_{calc} = p\cdot\delta_{Ca2Mg2} + (1 - p)\cdot\delta_{Ca2}$$

and from fits to the difference in chemical shift between two signals (close to 7.2 ppm) by using the function

$$\Delta\delta_{calc} = p\cdot\Delta\delta_{Ca2Mg2} + (1 - p)\cdot\Delta\delta_{Ca2}.$$

In this case p is the fraction of protein in the $(Ca^{2+})_2(Mg^{2+})_2$ state. Only data points up to 4 mM free Mg^{2+} were taken into account in the analysis, since above 4 mM, the electrostatic screening from the free Mg^{2+} ions will lead to lower affinity for each addition.

MC Simulations. The MC simulations, which are described in greater detail in ref. 13, are performed in the canonical ensemble where the temperature, volume, and number of particles are kept constant. The protein is placed at the center of a solvent sphere, the radius of which is determined by the protein concentration. The sphere also contains counterions, buffer, and additional salt to match the experimental conditions, and the whole sphere is treated as a dielectric continuum. The protein is described at atomic detail using an available three-dimensional structure—e.g., determined by x-ray crystallography, NMR, or molecular modeling—with charges on the glutamate, aspartate, lysine, and other charged side chains set according to pH. The protein is held fixed in the MC simulations, while all ions and buffer molecules, treated as charged hard spheres, are thermally averaged. The change in binding constant(s), as a result of increased protein and/or salt concentration, is calculated relative to a chosen reference state as

$$\Delta pK = pK - pK_{ref} = (\Delta G_{el} - \Delta G_{el,ref})/k_B T \ln 10,$$

where k_B is the Boltzmann constant and T the temperature in Kelvin. ΔG_{el} is the electrostatic free energy changes on ion binding and for the case of two binding sites is given as the difference in excess chemical potentials, μ_{ex}, for bound and free ions,

$$\Delta G_{el} = \mu_{ex}(\text{bound, site I}) + \mu_{ex}(\text{bound, site II}) - 2\mu_{ex}(\text{free}),$$

where the excess chemical potentials are calculated by using a modified Widom technique (18, 19). In this procedure, the calcium ion (valency of +2) is introduced as a test particle at

some point **r**, without disturbing the underlying simulation. The excess chemical potential is obtained as

$$\mu_{ex}(\mathbf{r}) = -k_B T \ln\langle \exp\{-2e\Phi(\mathbf{r})/k_B T\}\rangle_o,$$

where $\Phi(\mathbf{r})$ is the instantaneous electrostatic potential at **r**, and e the elementary charge. The brackets denote a canonical average over the unperturbed system. For a bound calcium ion, **r** is taken as either of the calcium sites defined in the x-ray structure, while for a free ion it is averaged over all possible positions within the cell. If there is a hard-core overlap between the inserted ghost particle and any other atom, it will give a zero contribution to the average. In simulations of calbindin D_{9K}, we used the crystal structure (20) but with Asn-56 replaced by Ala and Pro-43 replaced by Met. The simulations of calmodulin were based on the "common vertebrate" crystal structure of Babu *et al.* (21) but with Asp-129 instead of Asn-129.

RESULTS AND DISCUSSION

The existence of "the protein concentration effect" on ion-binding affinities is demonstrated for calbindin D_{9k} and calmodulin. The calcium-binding constants for wild-type calbindin D_{9k} measured previously with a small chromophoric chelator (8) are too high for accurate measurements by direct methods. Hence, binding constants have been measured for a mutant with the substitution Asn-56 → Ala (N56A). An additional Pro-43 → Met mutation is included to facilitate NMR analysis (22), with only a minor effect on calcium binding. The Asn-56 substitution was designed to reduce the Ca^{2+} affinity of the C-terminal site (site II) in which the Asn-56 side chain provides one oxygen atom to calcium coordination in the wild-type protein. Although a reduced Ca^{2+} affinity is observed for both sites, the reduction in site II is much more substantial (*ca.* 2.5 orders of magnitude relative to the wild-type protein versus a factor of 10 for site I). In this mutant, the calcium-binding constant of site II when calcium is already bound to site I ($K_{II,I}$) is therefore of a suitable magnitude for the present study. It has been measured both as a function of protein concentration at low ionic strength and as a function of KCl concentration at both high and low protein concentration. A typical titration curve is shown in Fig. 1*A*. The experimental results are summarized in Table 1 and clearly demonstrate that the calcium-binding constants of calbindin D_{9k} depend on protein concentration. These data thus confirm the protein concentration effect, which was predicted on the basis of MC simulations (13, 14).

To test the reliability of the electrostatic predictions, a new series of MC simulations have been performed for the N56A mutant. The mechanism of the protein concentration effect should in principle be described by simple Debye–Hückel theory, although for numerical reasons MC simulations turn out to be more efficient when treating a realistic protein model. The validity of the MC simulations in calculating these screening effects relies on the conformational response to ion binding being invariant over the range of protein and salt concentrations examined. Although such a general assumption is intuitively reasonable for most proteins, there is little available experimental support, since conformational changes upon ion binding have usually been characterized at one protein and salt concentration. However, in the case of calbindin D_{9k}, the global structural response to calcium binding is modest (23), and the conformation of both the apo- and holoproteins are highly resistant to salt addition (24).

The experimental and simulated shifts in the binding constant of the calbindin D_{9k} mutant due to differences in protein concentration over the range from 7 mM to 30 μM are compared in Fig. 1*B*. The overall agreement between theory and experiment is very good. Although the comparison is less

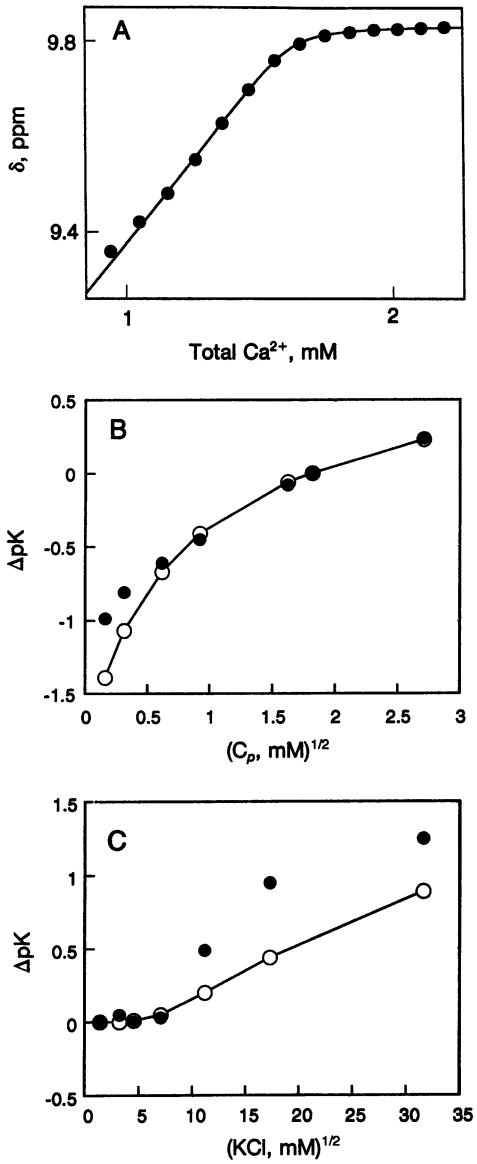

FIG. 1. Data for the N56A calbindin D_{9k} mutant. (*A*) Typical titration experiment: ^1H NMR chemical shift of one backbone amide proton as a function of total calcium concentration for 0.85 mM calbindin D_{9k} mutant in 2 mM Tris·HCl, pH 7.5, in 90% H_2O/10% ^2H_2O at 25°C. ●, Experimental data points, —, curve of optimal fit calculated for $\log_{10} K_{II,I} = 5.18$. (*B* and *C*) Comparison of theoretically and experimentally derived shifts in binding constants as a function of protein concentration at no added salt (*B*) and as a function of salt at 3.3 mM protein (*C*). ○, Theoretical values; ●, experimental values plotted as $\Delta pK_{II,I}$ (i.e., change in $-\log_{10} K_{II,I}$). The binding constant at 3.30 mM protein with no added salt was used as a reference. The uncertainties in the simulated pK shifts are less than 0.1 of a pK unit. The uncertainties in the experimental values are given in Table 1.

favorable at lower protein concentrations, the calculated values are all within the error limits of the experimental values. The level of agreement is surprising given that the theoretical model neglects the variation of the dielectric permittivity near and within the protein. In other more complex models this is approximated by a dielectric discontinuity near the protein surface. The MC simulations also predict that dielectric screening upon addition of salt is less significant at high protein concentration. This result is indeed born out by the data in Fig. 1*C*; at 3.3 mM calbindin D_{9k}, the calcium affinity is invariant with salt up to at least 50 mM KCl. There are significant differences between calculated and experimental shifts at the

Table 1. Experimental results for calbindin D_{9k}

Protein, mM	Na^+, mM	KCl, mM	$\log_{10} K_{II,I}$
0.027	0.14	—	5.65 ± 0.17
0.097	0.5	—	5.47 ± 0.10
0.380	2.0	—	5.27 ± 0.02
0.850	4.4	—	5.11 ± 0.04
2.63	13.7	—	4.74 ± 0.10
7.35	38.2	—	4.42 ± 0.11
3.30	17.2	—	4.66 ± 0.12
3.30	17.2	10.4	4.61 ± 0.09
3.30	17.2	20.8	4.67 ± 0.09
3.30	17.2	50.0	4.63 ± 0.06
3.30	17.2	125	4.17 ± 0.05
3.30	17.2	300	3.73 ± 0.06
3.30	17.2	1000	3.40 ± 0.15

The calcium-binding constant $\log_{10} K_{II,I}$ of the C-terminal EF-hand site in N96A calbindin D_{9k} was experimentally derived when calcium is already bound to the N-terminal site.

extreme conditions of both high salt and high protein concentration, in contrast to the condition of low (25–30 μM) protein concentration, where the calculated and experimental shifts due to salt addition agree in the entire range of 2 mM to 1M KCl (13, 24).

The protein-concentration effect is not expected to be particular to calbindin D_{9k}, as this phenomenon is the result of general screening by charged species. To verify the general validity of this observation, binding-constant measurements and MC calculations have been carried out for calmodulin. The calcium-binding constants of this protein (25, 26) are too high for accurate determination in a direct manner, therefore, the effect of protein concentration on the geometric mean ($\log_{10} K_{av}$) of the magnesium-binding constants of sites I and II in the N-terminal domain, when calcium is already bound to sites III and IV in the C-terminal domain, has been determined (Fig. 2 *A* and *B*). Again the experimental data show a strong influence of protein concentration on the metal-ion affinities. The comparison with the MC simulations is less straightforward in this case, as the structures of calmodulin in the $(Ca^{2+})_2$ and $(Ca^{2+})_2(Mg^{2+})_2$ states are not known and the $(Ca^{2+})_4$ structure is used as a model. However, we stress that the fundamental assumption for the calculations is that only the conformational response to Mg^{2+} binding is independent of protein concentration in the range studied. Despite these uncertainties, the agreement between experiment and the MC simulations is very good. This can be attributed to the protein-concentration effect being a general screening phenomenon caused by slowly varying long-range electrostatic forces, which implies that the calculations would not be critically dependent on the fine structure of the protein. Structures inferred from homology modeling should therefore be useful for simulations designed to estimate the magnitude of the effect.

The interpretation of the binding constant data relies upon the proteins remaining monomeric over the entire range of protein and salt concentrations examined. This property is confirmed by several lines of evidence. The most direct evidence is the absence of changes in the ^1H NMR linewidths of both calbindin D_{9k} and calmodulin as either the protein concentration or KCl concentration (calbindin D_{9k} only) is varied. The values of the rotational correlation time measured by ^{15}N NMR relaxation for apo- and $(Ca^{2+})_2$·calbindin D_{9k} at 4 mM (27, 28) and for $(Ca^{2+})_4$·calmodulin at 1.5 mM (29) correspond to values expected for protein monomers in solution. The value of the rotational correlation time measured by fluorescence spectroscopy for $(Ca^{2+})_2$·calbindin D_{9k} is nearly identical within experimental error and has been found to be invariant over the concentration range of 40 μM to 4 mM (G. Carlström, W.J.C., and D. P. Millar, unpublished data).

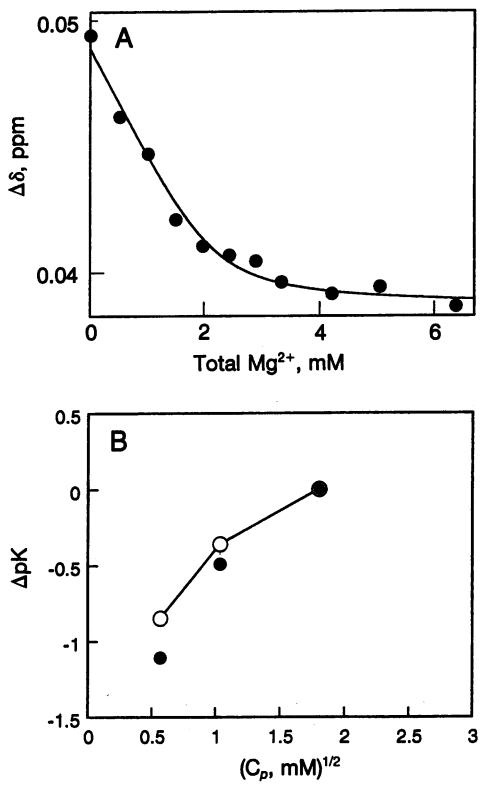

FIG. 2. Data for calmodulin. (*A*) Typical titration experiment. Difference in ^1H NMR chemical shift of two aromatic protons as a function of total Mg^{2+} concentration for 1.08 mM $(Ca^{2+})_2$·calmodulin in 2 mM Tris·HCl buffer, pH 7.5, in ^2H_2O at 25°C. ●, Experimental data points; —, curve of optimal fit calculated for $\log_{10} K_{av} = 3.65$. $\log_{10} K_{av}$ is the geometric mean of the Mg^{2+}-binding constants of the two sites in the N-terminal globular domain (sites I and II) of calmodulin when calcium is already bound to the two sites in the C-terminal lobe (sites III and IV). (*B*) Protein concentration effects. ○, Theoretical values; and ●, experimental values plotted as ΔpK_{av} (i.e., change in $-\log_{10} K_{av}$). The binding constant at 3.25 mM protein was used as a reference. The uncertainties in the simulated pK shifts are less than 0.1 pK unit. The uncertainties in the experimental values are between 0.2 and 0.3 pK units.

CONCLUDING REMARKS

The present work clearly demonstrates that the ion-binding affinities of charged proteins depend on the protein concentration. This result emphasizes that for a proper comparison between theory and experiment, as well as between different experiments, it is essential that solvent conditions are explicitly stated. This statement extends not only to pH, temperature, and the concentrations of buffer and salt but also to the exact concentration of protein, as well as all other charged species. The results also raise important questions with respect to the validity of binding constants measured *in vitro* for metalloproteins and their interpretation in terms of *in vivo* activities. We suggest that similar effects are present in all biological systems involving interactions between charged molecules.

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