Binding of Ca^{2+} to Calbindin D_{9k}: Structural Stability and Function at High Salt Concentration

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ABSTRACT: Calcium binding constants of wild-type calbindin D_{9k} and mutant forms with one, two, and three neutralized negative charges in the vicinity of the Ca^{2+} binding sites are determined at varying KCl concentrations from 2 mM to 1 M. The results indicate that the added salt does not cause significant structural changes in calbindin D_{9k} and, along with site-directed mutagenesis, can be used as a well-controlled means for modulating electrostatic interactions. The lack of structural changes at high salt concentrations is also supported by two-dimensional ^1H NMR data. High salt concentrations are observed to substantially reduce the cooperativity of calcium binding to calbindin D_{9k}. This suggests that the cooperativity is strongly dependent on electrostatic interactions. The data have been used to test a dielectric continuum model for protein electrostatics using a macroscopic dielectric constant of water throughout the system. Excellent agreement between experiment and Monte Carlo simulations is observed for the whole set of data covering changes in the binding constant of more than 6 orders of magnitude. A simplified theoretical treatment using the Kirkwood−Tanford formula, based on the Debye−Hückel approximation, yields an almost equally good agreement with the experiment.

Calbindin D_{9k} is an intracellular globular protein of 75 amino acid residues and a molecular weight around 8500. It belongs to a class of structurally related Ca^{2+} binding proteins including both Ca^{2+} regulatory proteins such as troponin C and calmodulin and Ca^{2+} buffers like parvalbumin and calbindin D_{9k}. The class is commonly referred to as EF-hand proteins or the calmodulin superfamily, and the number of subfamilies is rapidly growing (Kretsinger, 1987; Nakayama *et al.*, 1992). Of particular interest are the many recent findings of EF-hand proteins in the mammalian central nervous system (Baimbridge *et al.*, 1992). In the human brain are found subpopulations of neurons characterized by differing levels of certain EF-hand proteins, for example, parvalbumin, calbindin D_{28k}, and calretinin (Andressen *et al.*, 1993). The Ca^{2+} binding sites of these proteins are composed of a characteristic helix−loop−helix motif, commonly referred to as an EF hand (Kretsinger & Nockolds, 1973). A pair of EF hands is capable of binding two Ca^{2+} ions with high affinity and positive cooperativity. The sites are surrounded by several negatively charged glutamate and aspartate residues, and the net charge of the apo form of the recombinant wild-type protein is −7 (−8 if the methionated N-terminal is formylated). An examination of the structure reveals that all the charged amino acids are close to the surface of the protein. These features, together with the extraordinary structural stability, make calbindin D_{9k} a fascinating and well-defined model object for theoretical and experimental studies on the role of ionic interactions in Ca^{2+} binding to proteins and on electrostatic interactions in proteins in general.

The theoretical approach to this large molecular system is based on statistical mechanics. The possibilities offered by Monte Carlo (MC) simulations are in this context particularly appealing, since one is not forced to adopt the problem to a simplified geometry; hence, it is possible to treat the protein in atomic detail. When focusing on electrostatic interactions, it is possible to avoid an explicit treatment of solvent molecules by invoking a dielectric continuum model where the solvent molecules are replaced by a continuum with an appropriate dielectric constant. An alternative to MC simulations is the Poisson−Boltzmann- (PB) approximation, which has been frequently used in the past (Warwicker & Watson, 1982; Gilson *et al.*, 1985; Klapper *et al.*, 1986; Gilson & Honig, 1987; Bacquet *et al.*, 1988; Harvey, 1989). The main advantage of the PB approximation, as compared to simulations, is that it is relatively easy to incorporate a dielectric discontinuity. However, the PB equation is solved on a three-dimensional grid, which requires substantial numerical efforts in order to avoid a dependence on the mesh size. A further disadvantage of the PB equation, beside the neglect of ion−ion correlations, is the difficulty in handling a finite protein concentration. Within the dielectric continuum model, it is possible to linearize the PB equation and apply a simple version of the Debye−Hückel theory with the protein treated as a hard sphere enclosing the charged amino acid residues (Tanford & Kirkwood, 1957).

Electrostatic interactions in proteins can be modified experimentally using site-directed mutagenesis of ionic residues or changing the composition of the solvent. Suitable combination of these means may provide a unique set of experimental data [see, for example, Thomas *et al.* (1985) and Getzoff *et al.* (1992)] that can be used for successfully testing theoretical models of protein electrostatics. In the case of calbindin D_{9k}, several negatively charged amino acids, glutamate (E) and aspartate (D), in the vicinity of the binding sites have been replaced by their neutral counterparts, glutamine (Q) and asparagine (N), respectively. Since the exchanged amino acids are sterically very similar and located

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on the surface of the protein, this choice ensures a smallest possible structural perturbation as confirmed by NMR chemical shifts assignments (Linse *et al.*, 1991). Seven mutants with all possible combinations of the substitutions E17Q, D19N, and E26Q have recently been investigated with respect to their calcium binding properties (Linse *et al.*, 1991). The experimental binding constant shifts were significant and were reproduced by Monte Carlo simulations (Svensson *et al.*, 1991), indicating that they had a purely electrostatic origin.

It is thus established that added KCl causes substantial reduction in Ca^{2+} affinity of wild-type calbindin as well as of its charge neutralized mutants. This is mainly due to the screening of electrostatic interactions, since specific binding of K^+ ions seems to be insignificant (Linse *et al.*, 1991). Proceeding from these considerations, we present here a systematic study of electrostatic effects on the binding of two calcium ions to calbindin D_{9k}. Binding constants are determined for the wild-type protein and three charge neutralized mutants: D19N, E17Q + D19N, and E17Q + D19N + E26Q at different KCl concentrations ranging from 2 mM to 1 M. The present data also show the effect of high KCl concentrations on the cooperativity of Ca^{2+} binding to calbindin D_{9k}. We have also studied the degree of structural conservation of calbindin D_{9k} by measuring backbone chemical shifts in the protein at low (no added) and high (1 M) KCl.

BINDING FREE ENERGIES

Calcium binding to individual sites, labeled I and II, in a two-site protein is characterized by so-called *microscopic* binding constants: K_I, K_{II}, $K_{I,II}$, and $K_{II,I}$, one of which can be derived from the other three. For example, K_I and $K_{I,II}$ refer to Ca^{2+} binding to site I in the absence and presence, respectively, of Ca^{2+} in site II. The *macroscopic* binding constants K_1 and K_2 describe the binding of the first and second calcium ion, respectively, irrespective of which site is occupied. They are defined as

$$K_1 = \frac{[PrCa]}{[Pr][Ca^{2+}]} \quad \text{and} \quad K_2 = \frac{[PrCa_2]}{[PrCa][Ca^{2+}]} \tag{1}$$

where [Pr] and $[Ca^{2+}]$ are the concentrations of free protein and calcium, respectively, and [PrCa] and $[PrCa_2]$ are the concentrations of protein with one and two bound calcium ions, respectively. The binding is said to be cooperative if the binding free energy for the second calcium ion is more negative than for the first. In terms of the microscopic binding constants, the condition of positive cooperativity can be formulated as $K_{II,I}/K_{II} = KI_{,II}/K_I > 1$.

The following relations for the macroscopic binding constants K_1 and K_2 are easily derived:

$$K_1 = K_I + K_{II} \tag{2a}$$

$$K_2 = K_I K_{II,I}/(K_I + K_{II}) \tag{2b}$$

As stated above, the strong cooperativity of the binding of the two Ca^{2+} ions to the wild-type protein means that it is the product K_1K_2 which is best determined experimentally, and thus we shall concentrate our considerations to the total binding constant, K:

$$K = K_1K_2 = K_IK_{II,I} = K_{II}K_{I,II} \tag{3}$$

There are many contributions to the free energy change associated with Ca^{2+} binding. In the present 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, for example, hydration and direct interaction with calcium coordinating oxygens. Though the mutations involve changing both the charge and identity of specific amino acids, the fact that these groups are situated between 5 and 10 Å from the calcium sites means that the major influence on the binding arises via changes in the Coulombic interactions. Furthermore, the major role played by additional salt is the screening of electrostatic interactions. Therefore, we conjecture that only the changes in ΔG_{el} are sufficient to explain the observed shifts in K upon mutation and/or 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

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

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

$$\Delta G_{el} = \mu^{ex}(2Ca^{2+},B) - 2\mu^{ex}(Ca^{2+},F) \tag{5}$$

where μ^{ex} is an excess chemical potential, and B and F stand for bound and free calcium, respectively.

EXPERIMENTAL PROCEDURES

Materials. Wild-type calbindin D_{9k} and single, double, and triple mutants involving substitutions E17Q, D19N, and E26Q were produced in *Escherichia coli* using synthetic genes (Brodin *et al.*, 1986) and purified to homogeneity as previously described (Johansson *et al.*, 1990). The purity was confirmed by agarose gel electrophoresis, SDS−polyacrylamide gel electrophoresis, and ^1H NMR. Quin 2 was from Fluka (Buchs, Switzerland) and 5,5′-Br_2BAPTA was from Molecular Probes (Eugene, OR). Chelex-100 was from Bio-Rad. Inorganic salts and buffer were of analytical grade.

Macroscopic Ca^{2+} Binding Constants. The two macroscopic binding constants were determined from titrations of each protein with Ca^{2+} in the presence of a chromophoric chelator. The chelator was either Quin 2 or 5,5′-Br_2BAPTA, whose Ca^{2+} binding constants are known from separate experiments. All titrations were made in 2 mM Tris-HCl buffer at pH 7.5 and 25 °C with the desired KCl concentration. The free calcium concentration in each buffer was kept below 1 μM by continuous dialysis against Chelex-100. Protein and chelator (tetrapotassium salt) concentrations were in the 20−30 μM range. The total volume was 2.5 mL, and 5-μL aliquots of $CaCl_2$ stock solution (3.0 or 10.0 mM) were added. The absorbance at 263 nm was recorded at each titration point. The macroscopic binding constants, K_1 and K_2, were obtained by numerical fitting to the experimental points as described earlier (Linse *et al.*, 1991). Due to cooperative binding, the individual constants K_1 and K_2 were not as well determined as their product. Calcium binding constants for wild-type calbindin were determined in the

presence of Quin 2, and those for the single and triple mutants were determined in the presence of 5,5′-Br_2BAPTA. The binding constants to the double mutant were measured by using both chelators, the results of which were in good agreement. A further check was made at high KCl concentration where parallel measurements of the calcium binding to single and triple mutants were performed in the presence at both chelators yielding similar results.

^{1}H NMR. Two-dimensional ^{1}H NMR spectra were recorded for 2 mM protein solutions in 90% H_2O/10% D_2O at pH 7.5, 27 °C, on a GE-Omega 500 spectrometer operating at 500.13 MHz. 2D COSY spectra were recorded for the P43G mutant calbindin D_{9k} as well as the triple mutant (E17Q + D19N + E26Q) both in the presence and absence of 1 M KCl.

THEORY

The Model. The protein coordinates PDB3ICB from the Brookhaven Data Bank obtained from an X-ray diffraction study of the crystalline Ca^{2+} loaded protein (Szebenyi & Moffat, 1986) were used. Each protein atom was represented as a hard sphere, impenetrable to any solvent ions. Negatively charged carboxylic oxygens were given a charge of −0.5, while positively charged lysine residues carried a positive unit charge on the ζ-nitrogen. A mutation was done in a straightforward way by simply removing the −0.5 charge on each of the two carboxylic oxygen atoms in order to change the carboxylic group into an "amide group". The protein coordinates were kept fixed during the simulation, and 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. The interaction energy between charged species i and j is thus given by

$$u(r_{ij}) = q_i q_j e^2/4\pi\epsilon_0\epsilon r_{ij} \qquad r_{ij} \geq (\sigma_i + \sigma_j)/2 \quad (6a)$$

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

were q is a partial charge, e is the elementary charge, ϵ_0 is the permittivity of free space, σ_i is the hard core radius of particle i, and r_{ij} is the distance between the particles i and j. Only interactions with particles within the cell were taken into account. The dielectric constant and temperature were chosen to be 78.7 and 298 K, respectively, in order to correspond to water at room temperature. A schematic diagram of the system is given in Figure 1. Further simulation details are found in Svensson *et al.* (1991).

As the protein molecule will exclude solvent molecules from its interior, an interesting question arises as to what one should choose for the permittivity of the interior region. A seemingly viable choice is the electronic permittivity. However, this may lead to an underestimation as the assumption of a fixed protein structure precludes dielectric response from nuclear motions. Furthermore, it is not clear where one should locate the dielectric boundary between protein and solvent. 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. The penetration of water into the protein makes an unambiguous determination of the position of the dielectric discontinuity difficult.

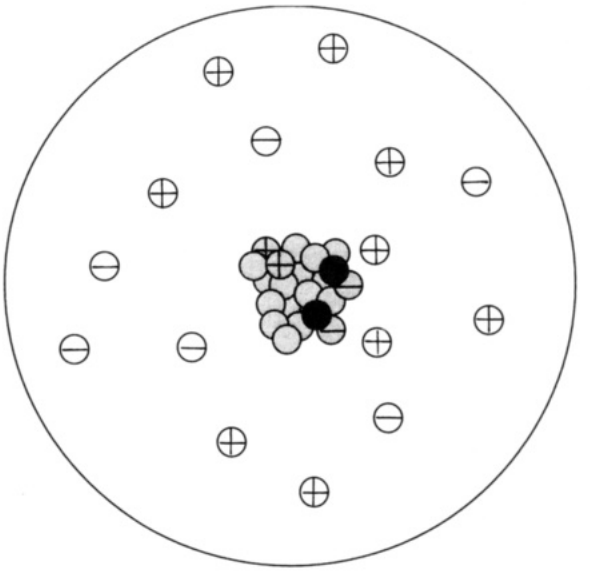

FIGURE 1: Schematic picture of the simulation cell with mobile ions and the protein. The free ions are shown as open circles, and the protein atoms are shaded circles with a sign indicating the charge. The two calcium binding sites are shown as filled circles.

Thus, in the present study the interior of the protein is assumed to have the same dielectric constant as the solvent. Though at first sight this may appear a rather drastic assumption, previous MC simulations of this model accurately reproduce observed shifts in the calcium binding affinity of several calcium binding proteins upon mutation of the protein and with varying ambient salt concentration and/or protein concentration (Svensson *et al.*, 1991; Linse *et al.*, 1994).

Monte Carlo Simulations. The simulations were performed in the canonical ensemble (Metropolis *et al.*, 1953), and the excess chemical potential of a bound ion was obtained by introducing the test particle at the binding site as specified in the crystal structure (Widom, 1963; Svensson & Woodward, 1988). The chemical potential of the free calcium was consistently evaluated by placing a test charge at random positions in the cell containing the protein and the appropriate amount of salt and counterions.

RESULTS AND DISCUSSION

Binding Constant Shifts. A series of Ca^{2+} titrations of calbindin mutants were performed at various KCl concentrations. Buffer and residual salt from the protein extraction procedure sets a lower limit for the salt concentration to be studied. We estimate these salt contributions to be of the order of 1 mM. The salt content reported in the following refers to directly added KCl. Besides the wild-type calbindin, we have also studied the salt effect on the calcium binding to three mutant forms of calbindin (D19N, E17Q + D19N, and E17Q + D19N + E26Q) with different steps of negative charge neutralization near the binding sites. Glu17 and Asp19 are coordinating a Ca^{2+} ion at site I, though not by their COO^- groups, and the COO^- oxygens of Glu26 are within the distance of 11 and 17 Å of sites I and II, respectively. The results are presented in Table 1. Calbindin D_{9k} retains a considerable calcium affinity even at very high salt concentration. This is also the case for the three mutants studied here. Note that the difference of 3 orders of magnitude between the wild-type protein and the triply

Table 1: Experimental Calcium Binding Constants (M^{-1}) for Wild-Type Calbindin and Three Mutants as a Function of KCl Concentration (mM)[a]

c_{KCl}	wild type	D19N	E17Q + D19N	E17Q + D19N + E26Q
2	16.44 (7.8)	15.05 (7.3)	14.05 (7.2)	13.06 (6.5)
5	16.01 (7.7)	14.69 (7.2)	13.76 (7.0)	12.67 (6.5)
10	15.44 (7.3)	14.34 (7.0)	13.39 (6.8)	12.44 (6.2)
25	14.67 (6.8)	13.68 (6.7)	12.93 (6.6)	12.28 (6.2)
50	13.96 (6.7)	13.05 (6.3)	12.45 (6.2)	11.83 (6.1)
100	13.21 (6.5)	12.50 (6.2)	11.80 (6.2)	11.50 (5.9)
150	12.78 (6.3)	12.24 (6.0)	11.76 (6.2)	11.28 (6.0)
300	12.16 (6.1)	11.75 (6.0)	11.18 (5.9)	11.13 (5.8)
500	11.78 (5.9)	11.53 (6.0)	10.75 (5.8)	10.56 (5.7)
1000	11.04 (5.6)	10.76 (5.9)	10.34 (5.7)	10.36 (5.7)

[a] The binding constant is given as $^{10}\log(K_1K_2)$ with $^{10}\log K_1$ in parentheses. The estimated error bar in $^{10}\log(K_1K_2)$ is ± 0.2 unit, while the separate binding constants K_1 or K_2 are subject to larger uncertainty.

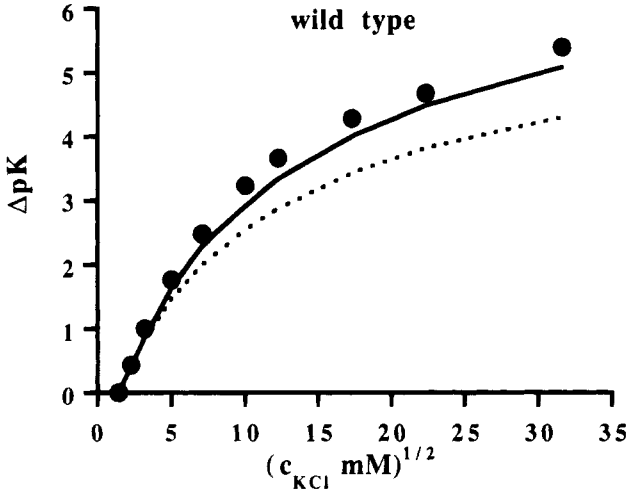

FIGURE 2: Experimental versus simulated ΔpK for wild-type calbindin D_{9k} in KCl solution. Symbols represent experimental data, and solid lines refer to MC simulations. The wild-type protein at 2 mM KCl has been taken as the reference. The dashed curve shows the results from the TK model.

mutated protein at 2 mM KCl has almost disappeared at 1 M KCl. Thus, with 1 M KCl we have effectively wiped out the long-range electrostatic contribution to the binding, and the remaining binding affinity is now dominated by short-range interactions, and hence, are similar for both wild-type calbindin and the three mutants. This similarity is also a strong indication that the protein retains its structure even at very high salt concentrations and that the structure is insensitive to the mutations performed.

Figure 2 shows the total shift for the binding of two calcium ions, $pK = -^{10}\log(K_1K_2)$, for wild-type calbindin. The total binding constant changes by more than 5 orders of magnitude when the salt concentration is increased from 0.002 to 1 M KCl. This shift is excellently described over the whole range by the MC simulations, and the largest ratio between theoretical and experimental shifts is approximately 2. Figures 3 and 4 show ΔpK for the singly and doubly mutated protein, and there is an equally good agreement between theory and experiment. The shift observed when the double mutant in 1 M KCl is compared to wild-type protein in 2 mM KCl exceeds 6 orders of magnitude. For the triple mutant at high salt concentration, there is a slightly less good agreement between theory and experiment—see Figure 5 where the largest ratio between theoretical and

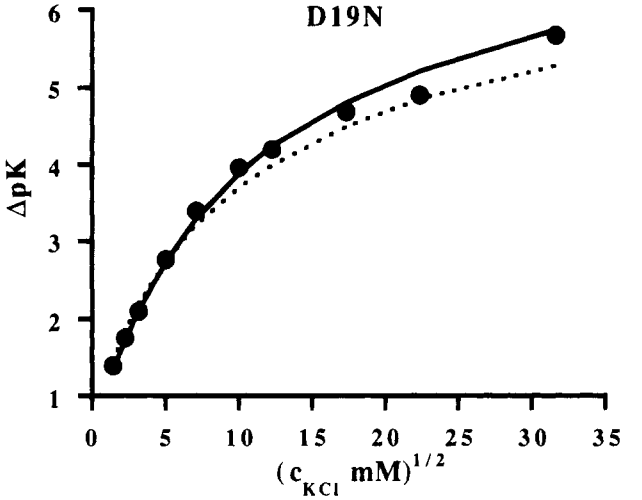

FIGURE 3: Experimental versus simulated ΔpK for the D19N mutant of calbindin D_{9k} KCl solution. Symbols are as in Figure 2.

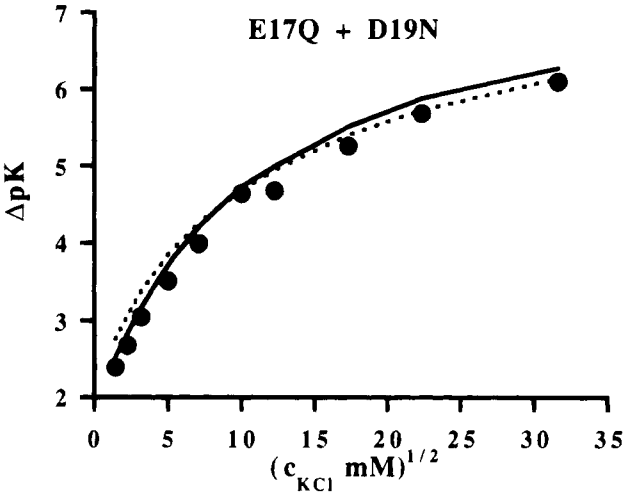

FIGURE 4: Experimental versus simulated ΔpK for the E17Q + D19N mutant of calbindin D_{9k} KCl solution. Symbols are as in Figure 2.

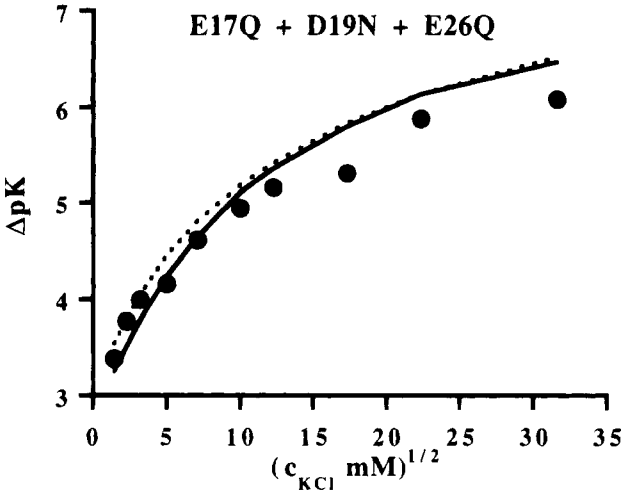

FIGURE 5: Experimental versus simulated ΔpK for the E17Q + D19N + E26Q mutant of calbindin D_{9k} KCl solution. Symbols are as in Figure 2.

experimental shifts is found to be approximately 3. One can also see that the experimental shifts as a function of added salt become more scattered. A reason for this are the low values of the binding constants under these extreme condi-

tions. The binding constants therefore fall in the lower limit of what is accessible with the current method. It is also possible that salting-out/salting-in properties of the protein have been changed upon triple mutation. Such effects of nonelectrostatic origin have been observed on the binding of substrate to enzymes at high salt concentration (Kesvatera, 1990; Kesvatera *et al.*, 1990). Here, it is important to point out that the reference in the theoretical calculations is that of wild-type calbindin at 2 mM KCl for all ΔpK values in Figures 2−5. The agreement between theory and experiment is extremely good for all protein forms at low salt concentration (<10 mM). Thus, we would not interpret the differences for the triple mutant in Figure 5 as a breakdown of the theory.

The high degree of structural similarity observed for calcium-loaded calbindin D_{9k} in the crystal state and solution (Kördel *et al.*, 1993) justifies the use of X-ray coordinates in the theoretical calculations. It is also established that, in solution, the structural changes when going from calcium-loaded to calcium-free protein are much attenuated in comparison with the current models for calmodulin and troponin C (Skelton *et al.*, 1994). It would seem more appropriate to use the solution structure from NMR studies (Kördel *et al.*, 1993) as a basis for the simulations. To perform a correct computation, however, based on the solution structure would require separate MC simulations on each of the 33 structures in the ensemble (since the average NMR structure has no physical meaning) and to average over the results. Although in principle possible assuming all structures to be equally probable, it is still a significant computational task. A further complication with the NMR-derived solution structure is that it is least well-defined in the calcium binding loops due to a relative scarcity of NMR-derived constraints in this part of the protein (Kördel *et al.*, 1993).

It is interesting to note that the change in salt concentration from 2 mM to 1 M KCl, apparently without any significant change in the protein structure, reduces the electrostatic contribution to calcium binding by 5.4 pK units. This is a much more dramatic change than a triple charge neutralization gives rise to $-\Delta pK = 3.4$ for wild-type vs triple mutant at 2 mM KCl. Thus, simple inorganic salt is a powerful and well-controlled tool in experimental modification of electrostatic interactions in biological systems.

A further simplification of the theoretical calculations can be achieved via the classical Tanford−Kirkwood(TK) model. It amounts to replacing the protein with an equivalent sphere enclosing all charged atoms and placing this sphere in a salt solution. For this particular system, it is possible to solve the Debye−Hückel equation even in the presence of a dielectric discontinuity. The relevant expressions for the free energy of the protein, albeit rather complex, can be obtained in closed form and can be found in the original paper (Tanford & Kirkwood, 1957). The chemical potential for a free ion can easily be calculated within the Debye−Hückel approximation, which happens to be surprisingly accurate for the particular case of a *divalent* ion in a 1:1 salt (Svensson *et al.*, 1992). Thus, we can use eqs 3 and 4 to evaluate the binding constant shifts. Figures 3−5 show that the TK model is an excellent approximation for all the mutated calbindin molecules. With a sufficiently highly charged protein, one would expect the TK model to break down. The difference seen for the wild-type protein in Figure 2 could be a sign of this. We have also tried to use the TK model, choosing a low dielectric permittivity for the interior of the protein. The outcome of such a calculation is in complete disagreement with the experimental shifts between the wild-type protein and the mutants. For a particular protein and within the TK model, however, we find that the salt shifts are essentially independent of the relative permittivity of the protein.

^{1}H NMR Chemical Shift. Comparisons. To address the question of possible structural changes of calbindin D_{9k} at high KCl concentration, 2D ^{1}H NMR COSY spectra were recorded for the apo form of two mutants—P43G and E17Q + D19N + E26Q—at 1 M KCl and without added salt. P43G was selected as a substitute for the wild type since it has simpler NMR spectra due to the presence of Gly instead of Pro in position 43 (Chazin *et al.*, 1989). For both proteins, KCl addition appeared to cause a general drift of all backbone resonances toward higher chemical shifts. The apparent average displacement for P43G was 0.076 ppm for NH and 0.073 for $C^{\alpha}H$. For the triple mutant, it was 0.085 for NH and 0.063 for $C^{\alpha}H$. This apparent drift most likely reflects the salt effect on the water signal. The chemical shift displacement of the water resonance in aqueous alkali halide solutions have in fact been measured for a range of electrolytes using coaxial glass tubing, and the reported shift displacement in 1 M KCl is −0.063 ppm (Bergqvist & Forslind, 1962). Thus, after correcting the chemical shift of the water resonance by this value, there is within the error limits no salt-induced change in the average backbone chemical shifts of the two mutants. If we then calculate the average *absolute* change upon the addition of 1 M KCl, for P43G it is 0.05 ppm for NH and 0.03 for $C^{\alpha}H$, the largest individual displacements being 0.17 and 0.19 ppm, respectively. For the triple mutant, the averages are 0.06 and 0.03, and the largest effects are 0.23 and 0.14, for NH and $C^{\alpha}H$, respectively. These findings point to a very high degree of structural conservation of the apoproteins in 1 M KCl.

Cooperativity. An important property of EF-hand proteins is their ability to bind calcium ions with positive cooperativity. A large number of experimental studies (Akke *et al.*, 1991; Linse *et al.*, 1991; Pearlstone *et al.*, 1992a,b; Waltersson *et al.*, 1993; Carlström & Chazin, 1993) as well as more theoretically oriented papers (Reid & Hodges, 1980; Wesolowski *et al.*, 1990) have been devoted to this phenomenon. Still, a conclusive molecular mechanism for the cooperativity remains to be specified. Previous studies (Linse *et al.*, 1991) of the eight charge substitution mutants at salt concentrations from less than 2 mM to 0.15 M showed the following: (i) that the cooperativity is reduced in some charge deletion mutants, and (ii) that it is also attenuated by salt addition. The present study gives the possibility to extended the analysis of the cooperativity to the salt concentration range from 150 mM to 1 M. The cooperativity parameter that can be deduced from macroscopic binding constants is denoted as $-\Delta\Delta G_{\eta=1}$, and it is equal to $RT \ln(4K_2/K_1)$. It is a lower limit to $-\Delta\Delta G$ ($=RT \ln(K_{I,II}/K_I)$. Calcium titrations monitored by ^{1}H NMR have shown that $\eta = (=K_{II}/K_I)$ is indeed close to unity (Linse *et al.*, 1991), and therefore, changes in $-\Delta\Delta G_{\eta=1}$ primarily reflect changes in $-\Delta\Delta G$. These findings are also supported by simulations, which show that η only changes by approximately a factor of 1.5 when increasing the salt concentration from 2 mM to 1 M KCl.

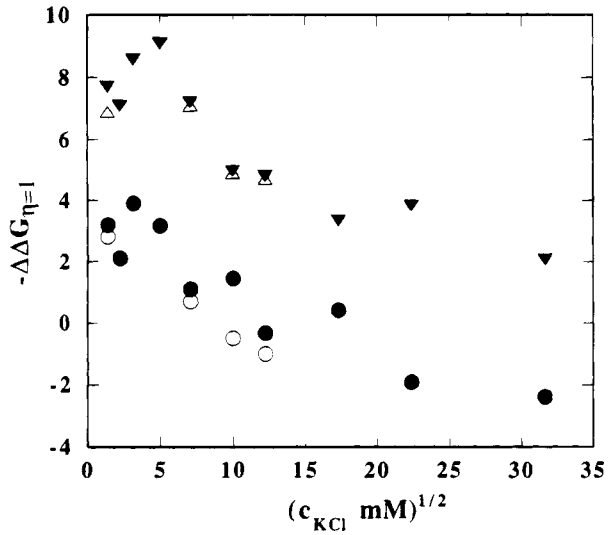

FIGURE 6: Effect of KCl concentration on the cooperativity of calcium binding in the calbindin D_{9k} wild-type (circles) and E17Q + D19N + E26Q mutant (triangles). Filled symbols represent the data of the present study, and open symbols are used for data from a Linse *et al.* (1991) study, $-\Delta\Delta G_{\eta=1} = RT \ln(4K_2/K_1)$.

For the wild-type and the three mutants of the present study, we see a further reduction in $-\Delta\Delta G_{\eta=1}$ when increasing the KCl concentration from 0.15 to 1.00 M, but it is not as steep as in the lower salt concentration range. The data for the wild-type and the triple mutant are shown in Figure 6. For the wild-type protein, $-\Delta\Delta G_{\eta=1}$ is as low as 2 kJ mol^{-1} at 1 M KCl as compared to roughly 7−8 kJ mol^{-1} at low ionic strength. This shows that electrostatic interactions have a profound effect on the cooperativity.

In this context, it is interesting to recall that simple repetitive systems can bind divalent cations with positive cooperativity to an array or sheet of negative charges (Lee & Veis, 1980; Sorokin *et al.*, 1983; Waalkes & Poirier, 1984). In addition, the N-terminal module of several blood coagulation factors has been observed to bind calcium ions with positive cooperativity to a cluster of 8−12 γ-carboxyglutamic acid (Gla) residues (Stenflo & Ganrot, 1973; Henriksson & Jacksson, 1975; Bajaj, 1982; Lawler & Simons, 1983). The cooperativity in this type of protein module appears to be mediated by the array of Gla residues, since it is lost when normal Glu residues substitute for Gla (Malhotra *et al.*, 1991). Hence, the importance of electrostatic interactions in the cooperative Ca^{2+} binding is not confined only to EF-hand proteins.

CONCLUSIONS

Calbindin D_{9k} largely retains its structure and calcium binding function even at very high salt concentration and subject to several charge neutralizing mutations, which implies that simple inorganic salt is a powerful and versatile means for modulation of the calcium binding function. The cooperativity is markedly affected by added salt and has almost disappeared in 1 M KCl.

The excellent agreement obtained between theory and experiment shows that a simple dielectric continuum model, with a uniform dielectric permittivity and fixed protein structure, is a realistic model that is capable of reproducing an experimental binding constant over a wide range of salt concentrations and mutations. The basic structural assumption, i.e., that the change in structure upon calcium binding is independent of salt concentrations and mutations, seems to be valid. Certainly the electrostatics are not significantly affected by it.

The good agreement found between the Tanford−Kirkwood model and both experiment and simulated numbers is to some extent fortuitous. It would, for example, not be the case for a more highly charged protein, and it will certainly not be the case at high protein concentrations (Linse *et al.* 1994). Nonetheless, for the present case, it is impressive considering the mathematical simplicity of the model.

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