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Electrostatic contributions to the binding of Ca^{2+} in calbindin mutants

A Monte Carlo study

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Monte Carlo simulation is used to calculate the free energy of binding of calcium ions to the native and several mutant forms of bovine calbindin D_{9K} in salt solution. The simulations are performed in the canonical ensemble wherein free energies are calculated with a modified Widom method. The protein is modelled as a set of fixed hard spheres of fractional or unit charge with the surrounding solution as a dielectric continuum containing counterions and added salt particles. The interior of the protein is assumed to have the same dielectric permittivity as the solvent, which turns out to be an excellent approximation. Indeed, this simple model is able to predict accurately experimentally measured shifts in the calcium binding constants of up to five orders of magnitude, due to mutations and added salt.

1. Introduction

Molecular dynamics simulation has become a major tool in theoretical biochemistry during the last decade [1]. With a priori knowledge of a biomolecule's molecular makeup, these simulations provide a detailed description of local dynamics, even in aqueous solution. Furthermore, structural information, for example, on interfacial water and the organization of side chains and other small groups [2], may also be extracted. The validity of these results obtained is dependent upon the accuracy of the intermolecular potentials used and also on the length of time the system is simulated. Indeed, it is often impractical to run these generally large simulations to complete convergence.

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Free energies associated, for example, with the binding of substrates to enzymes, are usually more interesting for the biochemist than local structures. However, they are also much more difficult to calculate in simulations. Perturbation methods have recently been applied to this problem with variable success [3] and not unexpected convergence problems appear when substrate binding induces large structural changes. The potential utility of these methods is enormous, but their future development is intrinsically tied to that of fast computers. On the other hand, one may expect that changes in binding free energies, upon variation in some property of the system, should be more readily calculable if structural changes induced by binding are relatively constant over the varied conditions.

In this communication we use Monte Carlo (rather than molecular dynamics) simulations to calculate free energy differences associated with

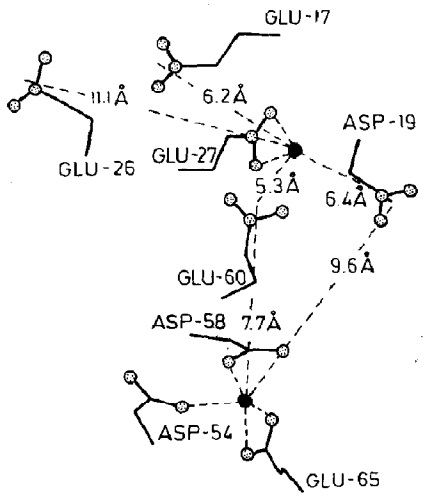

Fig. 1. Locations (from the X-ray structure of crystalline calbindin D_{9K}) of some of the charged amino acid residues relative to the bound Ca^{2+} (●). Distances are from the mid-point of the carboxylic oxygens to the Ca^{2+}.

the binding of Ca^{2+} to calbindin D_{9K}, while varying specific charged groups on the protein and the solution salinity. Calbindin is a globular protein of 75 amino acid residues. It contains a number of charged carboxylic residues as well as oppositely charged lysines. Calbindin belongs to a class of intracellular regulatory proteins and is capable of binding two Ca^{2+} with high affinity [4]. Each ion binds to a site in a helix-loop-helix arrangement, usually referred to as an 'EF-hand'. At neutral pH its bound form has a net charge of −4 (recombinant calbindin). Recent experiments have measured calcium binding constants of calbindin [5] — both for the native form and for several mutants, where charged glutamate (E) and aspartate (D) residues were replaced by their neutral counterparts, glutamine (Q) and asparagine (N) (see fig. 1 and table 1). The experiments [6] were performed at a protein concentration of 20–30 μM at 25 °C and at pH 7.5. Later experiments [7] have also considered the effect of adding up to 150 mM KCl.

To avoid simulating large numbers of solvent particles, we use the primitive model of electrolyte theory [8] to describe the solution. In this model one replaces the solvent with a dielectric continuum, but maintains an explicit description of the small ions and the protein. It has proved very useful, in its application both to bulk electrolytes solutions and to more complicated electric double layer systems [9].

2. Model and methods

The protein molecule in salt solution was modelled by placing it at the center of a spherical cell containing charged hard spheres, the latter modelling the free ions. In salt-free systems, the cell

Table 1

Shift in $\Delta p(K_1K_2)$, the total binding constant calculated relative to the wild-type protein in salt-free solution

The experimental values are taken from ref. 7. Values from model B are given within parentheses. The last column shows the change in static field from the protein only, which is the same for both models. The standard deviation of the simulated numbers is estimated to be less than 0.05 and the estimated errors from the experiments are less than 0.1.

Mutant	0 M KCl		0.05 M KCl		0.15 M KCl		Stat.
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	
Wild-type	–	–	2.86	2.64 (2.79)	4.00	3.65 (3.83)	–
E17Q	1.21	0.96 (1.22)	3.38	3.48 (3.69)	4.24	4.45 (4.62)	1.43
D19N	1.31	1.10	3.49	3.62	4.36	4.54	1.61
E26Q	0.51	0.52	3.12	3.01	4.10	3.99	0.92
(E17Q + D19N)	2.40	2.37	4.10	4.48	4.94	5.31	3.05
(E17Q + E26Q)	2.26	1.69 (1.96)	3.79	3.87 (4.10)	4.61	4.76 (4.98)	2.35
(D19N + E26Q)	2.14	1.88	3.75	4.04	4.61	4.89	2.53
(E17Q + D19N + E26Q)	3.38	3.13 (3.43)	4.82	4.91 (5.20)	5.38	5.65 (5.83)	3.97
E60Q	0.32	1.43	2.98	3.99	4.00	4.92	1.98

radius was determined by the protein concentration. The number of counterions varied from five to eight (depending on mutant type) so as to maintain electroneutrality. For the salt concentrations considered here, keeping the same cell radius as for the salt-free case would have meant treating a large number of salt ions. Instead, we only added 50 counterions and 50 coions and adjusted the cell radius to give the appropriate salt concentration. Although this procedure is inconsistent, we always found that the cell was large enough to give insignificant boundary effects. During the simulation only free ions were moved, the protein, though containing no bound Ca^{2+} was assumed to be preformed into its bound configuration, taken to be the crystalline structure [10]. All atoms in the protein, except aliphatic hydrogens, were modelled as hard spheres. In the simplest protein model (model A), partial charges of -0.5 were assigned to carboxylic oxygens and $+1.0$ to the nitrogen atom in the amino group in lysine, all other protein atoms being uncharged. In a more elaborate version (model B) all the spheres in the protein were assigned a partial charge. These were taken from the molecular dynamics program package MUMOD [11]. Upon mutation, we assumed the protein structure to remain unchanged. For example, the replacement of a carboxylic group by an amide group was modelled by changing the charge on the oxygen atoms from -0.5 to zero. The dielectric continuum, modelling the solvent, was assumed to extend within the protein as well. Thus, the potential between any two charged species i and j is simply,

$$u_{ij} = q_i q_j e^2 / 4\pi\epsilon_0\epsilon_r r_{ij} \quad r_{ij} \geq (\sigma_i + \sigma_j)/2 \qquad (1a)$$

$$u_{ij} = \infty \qquad r_{ij} < (\sigma_i + \sigma_j)/2 \qquad (1b)$$

where q is a partial charge, e the elementary charge, ϵ_0 the permittivity of vacuum, σ the hard core diameter and r_{ij} the distance between particles i and j. The relative permittivity ϵ_r is set to 78.7, the value for water at 25 °C.

The simulations used the standard Metropolis algorithm [12] in the canonical ensemble. After an initial equilibration each mobile ion was subject to at least 20 000 moves. This was probably excessive and reliable averages could have been obtained from much shorter runs. The CPU time required was modest, ranging from a few minutes to half an hour on an IBM 3090-170S. Model B required somewhat more computer time than model A, as the number of charged protein atoms is increased from 46 to 722.

The excess chemical potentials of bound and free calcium were measured, using a modified Widom technique recently developed for electrolyte solutions [13]. In this procedure, the calcium ion (valency $+2$) was introduced as a test particle at some point x, without disturbing the underlying simulation. The excess chemical potential was obtained as,

$$\beta\Delta\mu = -\ln\langle\exp\{-2\beta\phi_x\}\rangle \qquad (2)$$

where ϕ_x is the instantaneous electrostatic potential at x and $\beta = (k_B T)^{-1}$, where k_B denotes Boltzmann's constant and T the temperature. The angular brackets denote the simulation average. For a uniform system $\Delta\mu$ is independent of x.

Consider now the binding of two Ca^{2+} to the originally unbound protein. Simulations allow one to calculate a part of this free energy given by,

$$\Delta G = \Delta\mu(\mathrm{B}) - 2\Delta\mu(\mathrm{F}) \qquad (3)$$

$\Delta\mu(\mathrm{B})$ is the excess chemical potential for two bound Ca^{2+}. It was obtained by applying eq. 2 to two test particles, one inserted at each of the binding sites; it also includes their mutual interaction energy. $\Delta\mu(\mathrm{F})$ is the excess chemical potential of a free Ca^{2+}. In salt-free solutions, both $\Delta\mu(\mathrm{B})$ and $\Delta\mu(\mathrm{F})$ were obtained from a single simulation, wherein $\Delta\mu(\mathrm{F})$ was calculated by properly averaging over random placements of a test particle in the simulation cell. For the case of finite salt concentrations $\Delta\mu(\mathrm{F})$ was obtained from a separate simulation of the bulk salt solution. This was mimicked using a cubic box with periodic boundary conditions, containing only salt particles. In both cases $\Delta\mu(\mathrm{F})$ gave only a relatively small contribution to the total free energy change. The measured chemical potentials turned out to be rather insensitive to the choice of ionic radii and we used a radius of 2.125 Å for all the ionic species.

The remaining contributions, to the free energy of binding, are not considered in our analysis. They include the change in translational entropy of Ca^{2+} and in configurational free energy of the protein in the implicit preformation of its bound structure. In addition, one may expect some loss in solvation energy of Ca^{2+} though our simple model predicts this to be zero. Nevertheless, whatever the magnitude of these other free energy changes, the implicit assumption in this work is that they are, to a large extent, independent of minor mutations of the protein and of the ambient salt concentration.

3. Results and discussion

Our model cannot be used to calculate equilibrium binding constants for specific mutant types at given salt concentrations. However, the assumptions do allow us to predict shifts upon variations in these conditions. We use, as a reference, the wild-type protein in salt-free solution and compare our results with the experimentally measured shifts in the total equilibrium constant — or its negative logarithm, $\Delta p(K_1K_2)$ — where,

$$\Delta p(K_1K_2) = \beta\{\Delta G - \Delta G_{ref}\}/\ln 10 \qquad (4)$$

ΔG is defined by eq. 3 and ΔG_{ref} corresponds to its value in the reference solution.

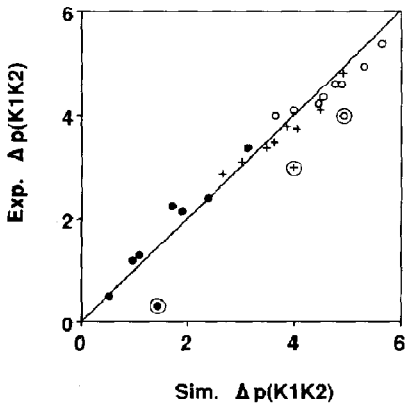

Fig. 2. Experimental vs simulated $\Delta p(K_1K_2)$ for the mutants in salt solution. (●) 0 M KCl, (+) 0.05 M KCl and (○) 0.15 M KCl. Results from mutant E60Q are encircled. The simulated values are obtained with model A.

Fig. 2 and table 1 show that, apart from mutant E60Q, the agreement with experiment is excellent. This includes the mutant where three charges have been neutralized and the shift in the binding constant is five orders of magnitude! That is, we are able to describe equally well the effect of a particular mutation and its salt dependence. In general, the calculated shifts differ from the experimental values by less than a factor of two. This difference should be compared with the total shifts, which are in the range of 10^3–10^5.

The mutation of a charged residue into a neutral one affects the potential at a binding site in two ways: (i) by changing the static field from the protein and (ii) by altering the response of the ions in the solution. Our results show that for no added salt the former causes the major part of the binding constant shift. The observed discrepancies with mutant E60Q are most probably linked to the uncertainty in the position of the mutated group, even in crystallographic measurements. Furthermore, molecular dynamics simulations have shown that the position of this group in the solvated protein changes significantly from its reported crystalline value [14].

For comparison, we also tried a simple Debye-Hückel approach, which solves the linearized Poisson-Boltzmann approximation with the assumption that the screening salt is able to penetrate into the protein. For no added salt, the predicted shifts are the same as those reported in table 1. At finite salt concentrations, however, we found the shifts were far too large compared to the experiments. For instance, for the wild-type protein in 0.05 M KCl solution, the Debye-Hückel theory gave a shift in pK of around 4.4. It is clear that this approximation overestimates the screening of salt by neglecting the protein excluded volume.

It is noteworthy that models A and B give virtually the same results. This is due to the potential of a fixed charge distribution, at long range, being rather insensitive to its local structural details. More remarkable is the fact that such good agreement is achieved with a uniform dielectric permittivity. This is despite recent claims that the presence of a dielectric discontinuity is necessary to understand the properties of proteins [15]. It is clear that the interior of a protein has a polariza-

bility which is different to the surrounding water solvent. However, attempts to model possibly complex electrostatic effects with simple dielectric continua may mean that the position of the dielectric boundaries and the value of the protein's dielectric constant are rather uncertain quantities [16]. For instance, the discontinuity may not coincide with what may be intuitively ascribed to the protein surface. In any case, our results indicate that a dielectric discontinuity is not necessary to provide sufficiently accurate predictions for the binding constants of Ca^{2+} in calbindin. It may indicate that the effective position of the expected dielectric discontinuity at the protein solvent interface is somewhat below the sites of the bound calciums and mutated groups (all of which lie close to the containing surface of the protein), thus making the effect of the discontinuity on electrostatic interactions negligible. Another possibility is that, in reality, significant charge correlation effects (screening) occur within the protein, not accounted for by our stiff model.

Experimentally it has been observed that binding of Ca^{2+} at one site increases the affinity for binding at the other site [6]. Our simple model is unable to reproduce this cooperativity and it seems likely that structural changes in the protein have to be invoked in order to describe this phenomenon.

4. Conclusions

We have shown that a simple electrostatic continuum picture of calbindin is capable of accurately reproducing shifts in the total calcium binding constants upon mutations of protein or upon addition of salt. The excellent agreement between theoretical and experimental data implies that the change in calbindin structure and other contributions to the total free energy, not considered explicitly in our analysis, remain largely unaffected by the mutations as well as the added salt. The results also imply that the possibly low dielectric interior of the protein plays a minor role in these cases and that the assumption of a uniform continuum is justified, if a postiori. Future work will address the question of whether this is true for other proteins as well. This is important to know, considering the much larger computational effort required to handle dielectric discontinuities. On the technical side, the Monte Carlo method including the modified Widom technique is an extremely efficient approach to this type of problem.

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