Using Multiconformation Continuum Electrostatics to Compare Chloride Binding Motifs in α-Amylase, Human Serum Albumin, and Omp32

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Ions are a ubiquitous component of the cellular environment, transferring into cells through membrane-embedded proteins. Ions bind to proteins to regulate their charge and function. Here, using multiconformation continuum electrostatics (MCCE), we show that the changes of chloride binding to α-amylase, human serum albumin (HSA) and Omp32 with pH, and of α-amylase with mutation agree well with experimental data. The three proteins represent three different types of binding. In α-amylase, chloride is bound in a specific buried site. Chloride binding is strongly coupled to the protonation state of a nearby lysine. MCCE calculates an 11-fold change in chloride affinity between the wild-type α-amylase and the K300R mutant, in good agreement with the measured 10-fold change. Without considering the coupled protonation reaction, the calculated affinity change would be more than 10^6-fold. In HSA, chlorides are distributed on the protein surface. Although HSA has a negative net charge, it binds more anions than cations. There are no highly occupied binding sites in HSA. Rather, there are many partially occupied sites near clusters of basic residues. The relative affinity of bound ions of different charges is shown to depend on the distribution of charged residues on the surface rather than the overall net charge of the protein. The calculated strong pH dependence of the number of chlorides bound and the anion selectivity agree with those of previous experiments. In Omp32, chlorides are stabilized in an anion-selective transmembrane channel in a pH-independent manner. The positive electrostatic potential in Omp32 results in about two chlorides and no cations bound in the transmembrane region of this anion-selective channel. The studies here show that with the ability to sample multiple binding sites and coupled protein protonation states, MCCE provides a powerful tool to analyze and predict ion binding. The calculations overestimate the affinity of surface chloride in HSA and Omp32 relative to the buried ion in amylase. Differences between ion–solvent interactions for buried and surface ions will be discussed.

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Introduction

The interior and exterior of living cells contain significant and different concentrations of salts. For example, the chloride concentration is 4 mM in the cell interior and 116 mM in blood.1 Thus, all cells have mechanisms to control ion transport across cell membranes and to sequester different ions. The function of many proteins is dependent on bound ions. Examples include the chloride channels, which are important in muscle function and signal transduction;2 hemoglobin, where oxygen-dependent anion binding helps regulate oxygen affinity;3,4 the bacterial chloride pump, halorhodopsin,5,6 which helps maintain the osmotic balance for halobacteria living in a saturated salt solution;7,8...
and the ArsA–ArsB anion ATPase complex, which mediates the resistance to arsenical and antimonials in bacteria.\textsuperscript{9,10} Computational methods to study anion binding help illuminate the mechanism of anion action in these proteins.

Ions can be bound in different ways to modulate protein structure and function. They can be bound internally, far from the surface and away from water. These ions interact strongly with the protein, allowing them to play important roles in stabilizing the structure\textsuperscript{11,12} or tuning the electrostatic environment at the catalytic site.\textsuperscript{13} Buried charged ions can induce large pK\textsubscript{a} shifts in nearby residues, leading to protonation changes and a strong pH dependence of ion binding. Ions can also play important roles by binding to the protein surface. These ions remain well solvated, leading to weak, diffuse binding. Surface ions are important for regulating the overall charge of the macromolecule, mediating salt bridges on the protein surface or between proteins and altering the electrostatic environment surrounding the protein. Diffuse binding of ions on the surface can collectively change the net charge of the protein. However, identifying individual surface binding sites explicitly may be difficult. Ions are transported in and out of cells via transmembrane channels. These ions must have sufficient affinity for the channels but cannot be bound so tightly that they are trapped inside. The ion–protein interactions can be regulated by the electrostatic potential gradient as in the voltage-gated ion channels,\textsuperscript{14} can be pH independent as in Omp32\textsuperscript{15,16} and can be highly selectively favoring one type of ion.\textsuperscript{17} In narrow regions of a channel, such as in the selective filter of potassium channel KcsA, where a narrow pore formed by backbone carbonyl groups allows passage of only single K\textsuperscript{+}\textsuperscript{18,19} ions will be highly desolvated; or they can remain solvated, as in the bacterial ion channels OmpF and Omp32, which are large enough to surround ions with water.\textsuperscript{20}

Ions have strong, favorable interactions with water, referred to as their solvation, Born energy, or reaction field energy.\textsuperscript{21} Ion binding sites need to replace the lost solvation energy with favorable interactions with the protein. The positively charged residues, Arg, Lys, and His, and hydrogen-bond donors, such as Ser and Thr, contribute to form the binding sites. For example, two Arg/Thr pairs and a Ser bind a chloride in the center of halorodopsin.\textsuperscript{22–24} Site-directed mutagenesis removing specific Arg\textsuperscript{25} or Lys\textsuperscript{26} reduces the chloride conductance in the chloride channel CLC-0. Several Lys residues are identified in the chloride binding site of hemoglobin.\textsuperscript{27} However, comparison of these binding sites does not reveal a specific binding motif for chloride. Rather, it seems that a pocket with a sufficiently positive electrostatic potential is enough, without any strong geometric constraints.

Quantum mechanical calculations using Hartree Fock theory have been used to study the volumes, coordination numbers, and hydration energies of simple ions.\textsuperscript{28} However, it has been suggested that spherically symmetric Lennard–Jones and electrostatic potentials are sufficient to model binding of closed-shell, spherical ions such as chloride.\textsuperscript{29} NMR measurements give a quadrupole coupling constant for a chloride bound to human serum albumin (HSA) that is consistent with protein–ion association being dominated by electrostatic interactions.\textsuperscript{30} Classical computational studies have been carried out on ions in solution using molecular dynamics (MD) and Monte Carlo approaches.\textsuperscript{31–38} Classical Born theory has been shown to do a good job of accounting for hydration (solvation) enthalpies.\textsuperscript{39} However, comparisons of different force fields for aqueous sodium chloride showed earlier Lennard–Jones parameters could not consistently model relative free energies and structures of ion association.\textsuperscript{38,40} The latest effort by Jensen and Jorgensen\textsuperscript{38} appears to provide a more self-consistent set of parameters for ions.

A methodology that can explicitly describe ion binding to proteins requires proper consideration of coupled protein protonation or conformational changes, efficient calculation of the Boltzmann distribution of weekly associated ions, and accurate estimation of ion hydration and ion–protein interactions. The Poisson–Boltzmann equation, which uses an implicit solvent and a Debye-Hückel-type ionic solution with a continuum ion density, is often used to describe the averaged, Boltzmann distribution of ions associated with the protein by electrostatic interactions.\textsuperscript{41} While this provides a good way to account for the ionic strength effect on the macromolecule electrostatic interactions, it cannot account for binding individual ions. Many studies have been devoted to the analysis of the ion–protein interactions in ion channels (for recent reviews, see Refs. 42 and 43). MD and Brownian dynamics simulations, with fixed residue protonation states, are the most commonly used for studying ion conductivity and selectivity in gramicidin A,\textsuperscript{44,45} OmpF,\textsuperscript{46–50} and potassium channels.\textsuperscript{51–53} Methods incorporating continuum electrostatics have been used to study the electrostatic basis of ion selectivity\textsuperscript{18,54} and the side-chain ionization states in channels.\textsuperscript{55,56} Other techniques including grand canonical Monte Carlo\textsuperscript{57} and free-energy perturbation and umbrella sampling\textsuperscript{57,58} have been integrated into these studies.

Multiconformation continuum electrostatics (MCCE) has been developed to calculate the Boltzmann distribution of ionization states of protein residues, cofactors, and substrates using Monte Carlo sampling.\textsuperscript{59–70} The approach introduced here samples ion binding within pK\textsubscript{a} calculations. This allows fast sampling of multiple ion binding sites, especially in cases where ion and proton binding are coupled. The chloride binding sites of three proteins are studied: α-amylase, with a single proton-coupled chloride binding site; human serum albumin (HSA), with a diffuse group of weakly pH dependent surface-associated ions; and Omp32, an anion-selective channel. The pH and chloride concentration dependence of chloride binding are calculated and compared to the available experimental data. The
calculated difference in chloride affinity between the wild-type α-amylase and the K300R mutant; the pH and chloride concentration dependence of α-amylase activity; the pH dependence of the amount of surface-bound chloride by HSA; and anion selectivity of ion binding in HSA and Omp32 all agree well with the experimental data. Thus, MCCE can provide an effective method for analysis of ion binding. As in standard continuum electrostatics simulations, an additive constant accounting for the ion concentration and its solvation energy is used.71

Results and Discussion

The distribution, occupancy, and pH dependence of chloride binding was calculated in α-amylase, HSA, and Omp32. Ions were added to cavities in all three proteins. In addition, a layer of surface chlorides was added to HSA and into the pore in the anion-selective channel Omp32. In each case, the ion positions were optimized with the standard MCCE minimization routines during conformer generation, moving them off grid to make more favorable interactions with the protein. Monte Carlo sampling generates a grand canonical ensemble, as each ion position in the protein has an alternate conformer in the solution that can be selected.

The term $kT \ln(\rho/\rho')$ in Eq. (5), where $\rho$ is the bulk ion density and $\rho'$ is the grid space density, is used to account for the concentration dependence of the free energy of binding. However, the absolute value of the chemical potential is difficult to estimate.38 While it has been shown that hydration enthalpies are inversely proportional to the radii based on the Born theory,39 the short-range, water–ion interactions in the solvation energy are not well modeled by the Born theory, which is the basis of the electrostatic energies used here.35,37 An additive constant ($\mu_{corr}$) is added to the energy of the ion in solution to connect the calculated and experimental chloride affinity at a particular chloride concentration in a given protein. Consistent but different values appear to be found for buried and surface-exposed ions.

The relative energy of the unbound chloride conformer is linearly related to the log of the solution chloride concentration. For all calculations here, ions are added at a $\rho'$ of 1 Å$^{-3}$. At 1 M, $\rho$ is 0.000602 ion/Å$^3$, so $kT \ln(\rho/\rho') = -4.38$ kcal/mol. In α-amylase, the $K_d$ at pH 7 is 4.4 mM, where $kT \ln(\rho/\rho') = -7.6$ kcal/mol. A $\mu_{corr}$ with a penalty of 7.1 kcal/mol added to a chloride in solution is needed to obtain 50% chloride occupancy at this concentration. This is within 0.5 kcal/mol of the $\mu_{corr}$ optimized in similar MCCE calculations of affinity of the buried chlorides in halorhodopsin (Song and Gunner, in preparation).

In contrast, for HSA, a $\mu_{corr} = 1.5$ kcal/mol gives ≈7 chlorides bound at pH 7 and 150 mM chloride as found in experiment.72 The concentration dependence of the number of chlorides bound to Omp32 has not been measured. However, the $\mu_{corr} = 1.5$ kcal/mol optimized for HSA gives values consistent with the chloride concentration used in experiments. The difference between $\mu_{corr}$ for different proteins is discussed below.

Fig. 1. (a and d) α-Amylase, (b and e) HSA, and (c and f) Omp32 with (a–c) sampled chloride ions, and (d–f) MCCE-calculated bound chloride ions. The sizes of the spheres are proportional to chloride occupancies at pH 7, with the largest size representing (d) 100% occupancy in α-amylase, (e) 81% in HSA, and (f) 43% in Omp32.
**α-Amylase**

α-Amylase is an enzyme that digests starch. One group of α-amylases found in animals and some bacteria can be activated by chloride binding, while most microbial and plant α-amylases are chloride independent. In the chloride-sensitive species, the ion dramatically enhances the protein activity and structural stability. The three ion ligands are a conserved Arg, Asn, and a Lys or Arg situated near the enzyme active site. In *Pseudoalteromonas haloplanktis*, these are R172, N262, and K300 (Fig. 2). The chloride-independent proteins lack the second basic residue. The optimum pH for the enzyme is in the acidic pH region for the chloride-independent variant and shifted toward more alkaline physiological pH in the chloride-dependent species.

MCCE initially adds 23 chlorides to the *P. haloplanktis* chloride-dependent wild-type α-amylase to fill all the cavities. Following Monte Carlo sampling, only the chloride found near the crystal structure position is bound (Figs. 1 and 2). Although the ion appears to be near the surface, it is not solvent-accessible. It is shielded by the surrounding residues, losing 16 kcal/mol of solvation energy (Table 1). Large pairwise electrostatic interactions with the protein compensate to stabilize the bound chloride. The positive potential from the backbone dipoles, mostly contributed by the helical segments, 175–179 and 222–233, and loop 262–264, attract the

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**Fig. 2.** Residues near the chloride binding site of α-amylase in the (gray) wild type and (green) K300R mutant. The bound chloride is shown. The essential catalytic residues are labeled in red. Protein surface is colored by the electrostatic potential calculated with the ionization states found in MCCE calculations at pH 7 in the wild-type Lys9Cl0 state. Positive potentials are blue and negative potentials are red. Colors are saturated at 3.0 kcal mol⁻¹ e⁻¹. The figure was prepared with PyMOL [http://www.pymol.org] with APBS plug-in by Michael Lerner [http://www-personal.umich.edu/~mlerner/PyMOL/].
The energy terms [Eq. (10)] contributing to chloride binding in wild-type given relative to neutral residue or empty binding site.

Table 1. Experimental chloride binding affinities measured by Feller et al. and the experimental and calculated difference in binding energy between the wild type and the mutant

<table>
<thead>
<tr>
<th>Residue</th>
<th>$K_d$ (mM)</th>
<th>$\Delta \Delta G_{binding}$ (kcal/mol)</th>
<th>$\Delta \Delta G_{corr}$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K300R</td>
<td>44</td>
<td>1.36</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The averaged interaction of the chloride with the side chains of all residues other than residue 300 (Eq. 12).

Table 2. Boltzmann-averaged energy terms for all chlorides in HSA and Omp32 [Eqs. (9), (10) and (11)]

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\mu_{corr}$</th>
<th>$\Delta \Delta G_{rxn}$</th>
<th>$\Delta \Delta G_{binding}$</th>
<th>$\Delta \Delta G_{corr}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase WT (Cl)</td>
<td>-7.1</td>
<td>16.0</td>
<td>-8.9</td>
<td>-17.5</td>
</tr>
<tr>
<td>HSA (Cl)</td>
<td>-1.5</td>
<td>2.9</td>
<td>0.0</td>
<td>-5.5</td>
</tr>
<tr>
<td>HSA (X)</td>
<td>-1.5</td>
<td>1.9</td>
<td>-0.1</td>
<td>-3.0</td>
</tr>
<tr>
<td>Omp32 (Cl)</td>
<td>-1.5</td>
<td>1.4</td>
<td>0.1</td>
<td>-3.7</td>
</tr>
</tbody>
</table>

For X-amylase, $\sum_{j=1}^{N} \alpha_j \Delta G_{ij}$ is calculated with Lys' 100% occupied. $\Delta G_{ij}$ includes both electrostatic and Lennard-Jones interactions. X' has the same Lennard-Jones parameters and solvation energy free in solution as Cl but with a positive charge.

Fig. 3. Calculated relative equilibrium free energies for coupled binding of chloride and a proton in wild-type and R300K P. haloplanktis amylase.
chloride concentrations of 0.5, 2, 7, and 50 mM agrees remarkably well with the measured enzyme activity. This strongly suggests, in the three-pK_\text{a} model, that the high-pH pK_\text{a} monitors deprotonation of Lys300 coupled to chloride loss. Feller et al.\textsuperscript{83} have shown a chloride-dependent apparent pK_\text{a} of the acidic deprotonation (pK_2) (Fig. 4c). However, a chloride-independent pK_\text{a} of 6.3 reproduces the experimental pH and chloride dependence (Fig. 4b),\textsuperscript{83} showing only one chloride-dependent site is required.

The measured first two pK_\text{a}s cannot be assigned to any residue in the calculations here, which are based on a structure without substrate. Substitute binding is unlikely to alter the calculated coupled Lys pK_\text{a} and chloride K_\text{d}. Measurements show that the chloride K_\text{d} has a negligible dependence on substrate binding.\textsuperscript{83} Glu200, which has been proposed to be a proton donor in substrate hydrolysis,\textsuperscript{77-79} and Asp 174 and 264 are active-site residues proposed to contribute to two acidic pK_\text{a}s. MCCE calculations without substrate find the ionization states of these three residues coupled together, sharing one proton at pH 7. The cluster has pK_\text{a}s of 5 and 9. As these residues interact directly with the bound substrate, the cluster pK_\text{a}s are likely to shift with substrate binding, and so could better match the pH dependence of enzyme activity, with its pK_\text{a} near 6.3.

\textit{P. haloplanktis} amylase with Lys300 mutated to Arg has been found to retain chloride dependence, but the K_\text{d} increases from 4.4 to 44 mM, indicating a 1.36 kcal/mol reduction in chloride affinity (Table 1).\textsuperscript{13} The X-ray structure of the K300R mutant, 1JD7, shows that the chloride ligands in the mutant superimpose well onto the wild-type structure\textsuperscript{84} (Fig. 2). Therefore, the weaker affinity in the mutant is not caused by major changes in chloride ligation or protein structure.\textsuperscript{83} Twenty-five interior chlorides were added to the structure, and again after Monte Carlo sampling, only the position near the crystal structure is occupied. The calculated difference in chloride binding energy is 1.5 kcal/mol, leading to an 11-fold decrease in affinity between the wild type and the K300R mutant, in good agreement with the experimental data.\textsuperscript{83} Although Arg can make a bidentate hydrogen bond, the Lys stabilizes chloride binding by -4.8 kcal/mol more due to its more localized charge distribution (Table 1). In addition, the Arg side chain is bulkier than Lys, so the MCCE-optimized chloride position is about 0.5 Å further from Arg300 than found in the structure. In the crystal structure, Asn262 and His263 have also shifted (Fig. 2). The chloride is more exposed and better solvated in the mutant, favoring chloride binding. However, the interactions with Arg172, Thr221, and Asn262 are now ~5 kcal/mol less favorable. Overall, the mutant protein (with R300+) binds the chloride 9.1 kcal/mol more weakly than the wild-type amylase (with ionized K300+), equivalent to a 10\textsuperscript{6}-fold change in affinity. However, both experiment and calculation show only an ~10-fold change in K_\text{d}. In analogy to the wild-type protein Lys\textsuperscript{+}Cl\textsuperscript{−} state, Arg\textsuperscript{+}Cl\textsuperscript{−} is the predominant species without bound chloride. Therefore, the energy of Lys and Arg protonation must be included in the calculation of chloride affinity. Since Arg has a higher pK_\text{a,so} and smaller desolvation energy, Arg\textsuperscript{+} is more stable than Lys\textsuperscript{+}. Without chloride bound, Arg has a pK_\text{a} of 5, in contrast to Lys with a pK_\text{a} below 0 [Eq. (14)]. Since the protonated Arg\textsuperscript{+} is >7 kcal/mol more stable than the Lys\textsuperscript{+} in the absence of the anion, less energy is needed to bind the proton.
needed for chloride binding. It is thus necessary to account for the free energy of both proton and ion binding to be able to reproduce the experimental changes in chloride affinity on mutation.

Human serum albumin

HSA is a highly charged protein that accounts for 55% of plasma proteins. HSA helps maintain proper cellular osmotic pressure and transports hydrophobic substrates such as hormones and fatty acids. In an average protein, 24% of the residues are Asp, Glu, Lys, and Arg, while these constitute 31% of HSA. There are 98 acidic residues (Asp and Glu), 83 basic residues (Lys and Arg), and 16 His. The pI of HSA calculated by MCCE with 150 mM implicit ionic strength via the Poisson–Boltzmann equation and no explicit ions is 5.5, close to the experimentally determined 5.387 or 5.472 (Fig. 5). The resultant negative net charge helps keep the protein from leaking into urine through the negatively charged glomerular capillary wall. Chloride binding has a strong pH dependence and it competes with the binding of other substrates. Experimental measurements of the residual liquid junction potential at physiological ionic strength show about seven chlorides and no calcium or sodium bound. One outstanding question is why this anionic protein has a higher affinity for anions than for cations.

MCCE calculations highlight the differences between amylase, with its catalytic binding to a unique, saturatable binding site, and HSA, which shows weak, diffuse ion binding to the surface. With

Fig. 5. The calculated pH dependence (lines) of the number of bound Cl\(^-\) (red), X\(^+\) (blue), and resultant protein net charge (black) in HSA. Dashed lines represent the net charge in a calculation without explicit ions. The experimental data (points) were adopted from Fogh-Andersen et al.\textsuperscript{72} $\mu_{corr}$ was fixed to reproduce the number of chlorides bound at pH 7.

Fig. 6. Calculated chloride distribution in HSA at pH 7. All chlorides with >1% occupancy are shown in magenta. The sizes of the spheres are proportional to chloride occupancies with the largest calculated occupancy of 81%. Important residues that stabilize chloride binding are shown.
μcorr of 1.5 kcal/mol at pH 7, an average of 7.4 surface chlorides are now bound out of 5887 added, matching the experimental total at 150 mM ionic strength.72 None of the chlorides added to the cavities in HSA are bound. Rather, binding is diffuse, with 186 positions accepted more than 1% of the time in Monte Carlo sampling, and only one, near Arg 222, is occupied over 50% of the time (Fig. 6). Approximately six chlorides are found near the three clusters of basic residues, shown in Fig. 6. The remaining diffuse chloride is bound more weakly. However, since the surface area contributing to weak binding is more than 10 times as large as the positive patches, the weakly bound ions, which are infrequently seen in Monte Carlo sampling, add approximately one ion to the total.

The interactions stabilizing the chloride binding in HSA are also different from those found in α-amylase (Table 2). Because binding occurs mostly on the HSA surface, the desolvation penalty is much smaller. Overall, nearly 60% of the bound chlorides have a desolvation energy less than 2 kcal/mol and only 10% lose more than 4 kcal/mol (Fig. 7). However, the screening by water diminishes electrostatic interactions between chloride and any single charged residue on the protein so they are at most 2–4 kcal/mol. More than 80% of the bound chlorides have more than –4 kcal/mol total favorable electrostatic interactions contributed by clusters of positively charged residues. The entropy loss moving from solution to the binding site, which reduces the affinity, is accounted for by the term kT ln(ρ′/ρ).

The pH dependence of chloride binding to HSA calculated by MCCE is in good agreement with the experimental results72 (Fig. 5). The number of bound chlorides increases rapidly at acidic pH, reaching over 20 at pH 4. Now over 630 added chloride positions are >1% occupied, and another chloride position near Arg117 and 145 is occupied in >50% of the accepted sites in Monte Carlo sampling. The number of bound chlorides decreases slowly with increasing pH, with about five still bound at pH 9.

Chloride binding to HSA is coupled to smaller changes in protein protonation states than in α-amylase. Calculations with and without explicit chlorides are compared. In each case the implicit ionic strength used in the DelPhi Poisson–Boltzmann calculation is 150 mM. At pH 7, 7.4 chlorides and 2.1 protons are bound with explicit anions present, and the pI shifts up to 5.8. Thus, adding explicit chlorides increases the net negative charge, with more chloride than protons bound. As the chloride binding is diffuse, so are the changes in protonation. Only four residues, Lys190 and 199, His288, and Tyr411, change protonation states by >0.2 H⁺, while there are smaller shifts in protonation of other residues.

Physiologically, HSA needs a larger capacity for binding anions than cations so that the protein charge is enhanced instead of neutralized by the bound ions.72 Calculations with a monovalent cation X⁺, with the same radius as Cl⁻, and with Na⁺ are carried out with MCCE. The ions used for Monte Carlo sampling are in the same positions as the chlorides. With X⁺, 3.5 cations are bound at pH 7. The same μcorr is used for both calculations so that the affinity of the protein for the two ion types can be directly compared. This number is significantly smaller than for anion binding, but larger than found experimentally.72 The contrast between the calculated Cl⁻ and X⁺ shows how the negatively charged protein can attract anions. Figure 7 shows the distribution of interactions at all chloride sites and the distribution weighted by Boltzmann occupancies. The net negative charge yields a protein surface with an overall negative potential. However, there are local regions at positive potential that stabilize anion binding by >4 kcal/mol, but there are no regions that stabilize cations by this much (Fig. 7). The largest X⁺ occupancy at any site is 4%, and 2.5 out of the total 3.5 bound cations represent the summation of sites with average occupancies of less than 1%. Only 1 of the 7.4 chlorides results from distributed sites with such small average occupancy. Thus, the overall protein negative charge yields weak binding, with many sites having small cation affinity. In contrast, the localized positively charged sites are more effective in attracting and holding anions.

Calculations using parameters optimized for Na⁺ have less favorable electrostatic energies than the
cationic chloride analog, $X^+$. With the smaller, correct Na$^+$ radius, the loss of reaction field energy destabilizes the buried cation more. There is some uncertainty in the Na$^+$ Lennard–Jones parameters.40 The Jensen and Jorgensen parameters, which have a much less negative minimum potential for Na$^+$ than for Cl$^-$, yield only 2.1 Na$^+$ bound at pH 7. Even with the Smith and Dang91 parameters, with a more favorable Lennard–Jones potential for Na$^+$ than for Cl$^-$, results in only 5.8 Na$^+$ bound, fewer than found for Cl$^-$. Thus, independent of the parameters used, the negatively charged HSA is calculated to have a greater affinity for anions than for cations.

**Omp32**

Omp32 is one of the bacterial porins that form channels in the outer membrane to pass water, ions, and other substrates in and out of cells.92 In contrast to most porins, Omp32 is strongly anion selective.92 The calculated binding affinities of Cl$^-$ and Na$^+$ are compared in the transmembrane pore region of Omp32. With the HSA $\mu_{\text{corr}} = 1.5$ kcal/mol and the Jensen and Jorgensen Lennard–Jones parameters,40 2.1 Cl$^-$ and $< 0.1$ Na$^+$ are found on average in each channel at 150 mM chloride (Fig. 8). The occupied positions compare well with earlier potential of mean force simulations,49 showing regions near Arg38, 75, and 133 are most favorable for chloride binding (Fig. 9c). As in HSA, binding is diffuse. Of 1651 chlorides added in the pore, 184 are more than 1% occupied, but no individual site has a probability $> 50\%$ of being occupied. Shifting $\mu_{\text{corr}}$ by $\pm 0.5$ kcal/mol (equivalent to changing concentration by 2.3-fold) changes the total number of chloride for each monomer by $\approx 0.4$ with little change in the number of bound Na$^+$ (Fig. 8). Calculations with the $\mu_{\text{corr}}$ used for buried chlorides in amylase lead to 8.9 chlorides being bound, significantly more than expected.

The pH dependence of the number of bound chlorides is small (Fig. 8). Compared to HSA where many surface ionizable residues change ionization states at neutral pH with ion binding, most of the residues in the transmembrane region of Omp32 are basic with high $pK_a$s. In addition, these residues are
sufficiently far apart that their $pK_a$s do not significantly perturb each other. The lack of pH dependence is consistent with earlier calculations and with the experimental observation that the channel conductance is $pH$ independent.\(^{93}\)

The Boltzmann-averaged energies of the chlorides occupied in Monte Carlo sampling versus the coordinates along the channel axis ($z$-axis) are shown in Fig. 9. Chloride binding is favored throughout the channel, while cations have a small attraction to half of the channel and unfavorable interactions in the other half. The desolvation energy for each ion increases as it moves into the channel. The largest desolvation penalty is found in the constriction zone, where the cross section of the channel is narrowed by a loop folded into the channel found near $z=0$ Å in Fig. 9.\(^{94}\) The positive potential that repels cations continues into this narrow region.\(^{95}\) Interactions with the protein backbone dipoles are small, while interactions with side chains favor anions in the channel and repel cations. It should be noted that the interaction profiles of bound anions and cations are not a simple mirror of each other because anions are favored by positive surface potential and cations by negative potential, leading to different Boltzmann-weighted distributions.

Three positions in the channel favor chloride binding (Fig. 9). Region 1, near the edge of the channel, includes Lys52, Arg89 and 76, and Thr44 and Ser82. Region 2 is near the center of the transmembrane region, surrounded by Arg38 and 75, Lys218, and Ser35, 108, and 211. This is near the $SO_4^{2-}$ found in the crystal structure that is removed from the calculations.\(^{94}\) Region 3 is near Arg133, Lys74, and Asn130. A previous MD study calculated the energy profile of a chloride pulled in the $z$ direction with constant force.\(^{49}\) They identified region 2 as having the highest affinity, a different site near Lys308, and region 3 as also favoring the presence of chloride (Fig. 9). However, region 1 was not seen. This area is off the transmembrane axis relative to position 2 so it may not have been well sampled in the earlier steered MD studies. The calculated chloride distribution here thus suggests the chloride pathway might zigzag through the channel rather than follow a straight path defined by the $z$-axis (Fig. 9c).

**Solvent energy correction**

The solvent energy correction, $\mu_{corr}$, is adjusted in $\alpha$-amylase to match the experimentally measured $K_d$ at 150 mM chloride\(^{43}\) and in HSA to match the number of bound chlorides at pH 7.72. Then for a given protein, the same correction is used to calculate the pH dependence, influence of mutation, and ion concentration. The difference in the $\mu_{corr}$ used for $\alpha$-amylase and in HSA is 5.6 kcal/mol. With the HSA $\mu_{corr}$, the calculated $K_d$ of chloride in amylase would be 10,000-fold weaker. However, using the $\mu_{corr}$ for Omp32 optimized for HSA leads to a reasonable number of chlorides calculated to be bound in the channel, while the amylase value leads to too many chlorides bound. Thus, MCCE underestimates the affinity of the chloride binding to $\alpha$-amylase relative to HSA.

Chloride in $\alpha$-amylase is deeply buried with a large desolvation energy, which is balanced by large favorable interactions with the protein, while in HSA and Omp32, chloride is only partially dehydrated (Table 2). Similar chloride binding calculations in halorhodopsin with a deeply buried binding site use the same $\mu_{corr}$ as that of $\alpha$-amylase.\(^{93}\) Thus, the difference in $\mu_{corr}$ for HSA and Omp32 and for $\alpha$-amylase and halorhodopsin may reflect the difference in the analysis of surface and buried ions. Here, the larger $\mu_{corr}$ needed to bind a buried chloride indicates the calculations either overestimate the desolvation penalty for buried ions or the favorable interactions of surface chlorides with the solvent. Earlier calculations also found difficulties in calculating the absolute affinity of ions.\(^{44,96-100}\)

Calculations were carried out modifying the MCCE parameters to help identify the source of the difference in $\mu_{corr}$. The spacing between added chlorides was reduced to 0.3 Å to improve sampling; the depth of the van der Waals potential for chloride was increased and the coefficient for the implicit van der Waals attraction of the chloride was increased and the coefficient for the implicit van der Waals attraction of the chloride was varied from 0.04 to 0.10 kcal/mol/Å$^2$ (default value is 0.06). None of these significantly changed the needed offset in $\mu_{corr}$. Increasing the solvent-accessible radius used to calculate the reaction field energy from 1.937 Å, which was derived from calculation of ion hydration energies,\(^{39}\) to 3.3 Å does allow one $\mu_{corr}$ to be used. The ability to reconcile $\mu_{corr}$ with this unphysically large radius suggests that the calculations may overestimate the desolvation penalty for the buried ions. Other missing terms could arise while combining the implicit ions incorporated in the Poisson–Boltzmann equation with the explicit anions bound to the surface or from the lack of explicit counterions in the calculations.

**Conclusion**

The calculated chloride binding in the three proteins presented here highlights different ways proteins can bind ions. In $\alpha$-amylase, the binding site is deeply buried in the protein, leading to a large desolvation penalty and large compensatory interactions with the protein, while in HSA, chlorides are weakly bound on the surface of the protein, and in Omp32, chlorides are localized in three sites in the transmembrane channel. Interactions of the surface-bound chlorides with the protein are much smaller than they are in $\alpha$-amylase. The same electrostatic factors that shift $pK_a$s for buried and surface-exposed residues act to bind ions to proteins.\(^{66,101}\) However, exchangeable protons are covalently bound to specific acidic and basic residues, while chlorides are associated by nonbonded electrostatic and van der Waals interactions. Thus, while a tight-binding chloride is well localized in a binding site, weakly
bound chloride can use a delocalized binding motif by interacting weakly with many residues. These proteins also highlight potential differences in the coupling between ion and proton binding. In α-amylase, chloride binding is electroneutral, tightly coupled to proton uptake by Lys300. Thus, the chloride binding affinity is pH dependent and the pKₐ of Lys300 is chloride dependent. In HSA, chloride binding is weakly coupled to changes in protonation of individual residues. When 7.4 chlorides are bound at pH 7, only two protons are taken up and no residue changes ionization by more than 20%. However, the protein net charge is highly sensitive to pH, leading to a strong pH dependence of the number of bound chlorides. In Omp32, the basic residues interacting with bound chloride have high pKₐs, so chloride binding is pH independent. Similar interactions between proteins and added charges have been well studied for oxidation–reduction reactions, which have strong pH dependence in only some proteins. Previous studies have shown that the pH dependence of these electron transfer reactions are properly calculated when the redox states and protein ionization states are sampled together. Here, with the same methodology, MCCE is able to determine different coupling modes between ion binding and protonation.

Materials and Methods

Structures of the wild-type α-amylase (1AQH) and the K300R mutant (1JD7) from P. haloplanktis (formerly Alk helpomonas haloplanktis) and HSA (1BMO) were taken from the Protein Data Bank. The trimeric biological unit of Omp32 from Comamonas acidovorans (1E54) was obtained from the Macromolecular Structure Database at the European Bioinformatics Institute, which uses the crystallographic symmetry information to assemble biological quaternary states. MCCE was used to sample residue ionization states, rotamer positions, and chloride occupancy as a function of pH and chloride concentration. The details of MCCE and the recent improvements have been previously described. Previous studies have shown that a dielectric constant (ε) of 4 is assigned to the protein and chloride ions, while the surrounding water has an ε of 80 with a continuum salt concentration of 150 mM. PARSE charges and radii for the amino acids are used for input to DelPhi, which solves the Poisson–Boltzmann equation. Amber94 van der Waals parameters are used to calculate the Lennard-Jones and torsion energies. All crystal water molecules were removed and cavities were filled with continuum dielectric material with an ε of 80. Previous studies have shown that this simple model provides results that are quite close to those found by putting explicit water molecules with conformational degrees of freedom into cavities. For Omp32, a 30 Å slab of low-dielectric material was used with the use of the IPECE subroutine to mimic the membrane region and given a dielectric constant of 4. Low-dielectric material was excluded from the transmembrane pore region, which is therefore filled with an ε of 80. Chloride has a charge of −1 and a solvent-accessible radius of 1.937, which gives it a Born solvation energy of −20.32 kcal/mol in water with an intraion dielectric constant of 4. The Lennard–Jones chloride parameters are taken from the consistent OPLS-style parameter set of Jensen and Jorgensen. To compare cation and anion binding, an ion (X⁺) with the same Born radius and Lennard–Jones parameters as chloride and a +1 charge is used. Calculations are also carried out with Na⁺ Lennard–Jones parameters and a Born radius of 1.680. Other Lennard–Jones parameter sets give similar values for chloride but differ in their values for sodium. The sensitivity of ion binding to the choice of parameter set is evaluated in the HSA calculations comparing the results found with Jensen and Jorgensen and Smith and Dang parameters.

One chloride ion is found buried in the active site in both the wild-type α-amylase and the K300R mutant structure and it is included in the calculations. An additional surface chloride is found in the α-amylase mutant that is stripped off by the standard preparation routine in MCCE. One Ca²⁺ is found in the crystal structure of α-amylase wild type and mutant. This is included and forced to be occupied during the Monte Carlo sampling. It plays an essential role in stabilizing the structure. Interactions between this Ca²⁺ and charges in the chloride binding site are between 0.5 and 1 kcal/mol, so it has only a small impact on calculated residue pKₐs and chloride binding energies. Additional ions are added to the interior using IPECE with a 2.0 Å probe radius to fill all the cavities (Fig. 1). In the final Monte Carlo calculations, α-amylase has 23 additional chloride positions in the wild type and 25 in the K300R mutant. Ion site occupancies are then sampled together with acidic and basic residue ionization.

No chlorides are found resolved in the HSA crystal structure. Here, chloride ions are added to fill all cavities and chloride ions are placed on all grid points 2.0 to 4.0 Å from the surface with a grid spacing of 1 Å, adding 5778 possible binding sites. Omp32 is an anion-selective porin. There are no chlorides found in the crystal structure. Ions are added to fill the transmembrane pore region with the same strategy as used for the HSA surface, providing 1651 possible binding sites.

The standard MCCE routines for heavy-atom relaxation and hydrogen-bond optimization are used to relax the initial grid-based ions in the context of the nearby protein conformers. This process can add additional charges. The force field includes standard AMBER Lennard-Jones and torsion energies. The electrostatic interactions are calculated with Coulomb’s law using ε = 1 and charges from the residue topology files. SHAKE fixes all bond lengths and bond angles. As the conformers are isolated, constraints are added to keep the new positions close to the original. Only a short, 50-step minimization is used with a femtosecond step. Following each step, all velocities are set to zero. A harmonic restraint, E = 0.5κ (|r - r₀| - d)², is added to all heavy atoms, where r is a spring constant of 10 kcal mol⁻¹ Å⁻², x is the current position, xo is the original position before any relaxation, and d is the distance within which no penalty is applied (1 Å is default). Five independent runs are carried out with initial chloride grid randomly shifted. The standard deviation of the calculated total occupancy of bound ions is <5%. Only a 2 Å thick layer of ions is added. However, for ions further away from the protein surface, the interactions between ion and protein decreases more rapidly than 1/r due to the increased solvent screening. The impact of the total solution ionic strength is included in the 150 mM salt used in the Poisson–Boltzmann analysis of the electrostatic energies for the system.

† http://pqsb.ebi.ac.uk/pqsb-doc.shtml
In MCCE, each choice of a charge state, proton, or heavy-atom rotamer position is called a conformer. All protonatable side chains and amino and carboxyl termini have ionized and neutral conformers. Ionizable, polar, and neutral amino acids all sample various positions. Each chloride is given an additional conformation that represents the ion moved from the protein into the bulk solution. This chloride has no interaction with the protein and is given a concentration-dependent self-energy and a \(\mu_{\text{solvent}}\) to match one piece of experimental data for each system. Monte Carlo sampling then finds the Boltzmann distribution of microstates where one microstate has a single conformer of each side chain and ion.

For each conformer in the system, two sets of DelPhi calculations solving the Poisson–Boltzmann equation\(^{114}\) are carried out. In one, all possible side-chain conformations are included (multiconformation DelPhi), allowing all pairwise interactions to be calculated.\(^9\) However, compared to a real protein, this has a larger region of low-dielectric material because of the presence of the unoccupied dielectric material.\(^{114}\) For the surface ion binding calculations here, all possible conformers of residue \(j\) are used to sample the interactions between conformer \(i\) and all conformers of residue \(j\).

For the surface ion binding calculations here, all possible ion positions are included in the multiconformation DelPhi calculations (Supplementary Fig. S1a). For each bound ion, a second ion with the same charge being bound nearby is unlikely due to the electrostatic repulsion. Therefore, in the single-conformation DelPhi calculations for each ion, the rest of the ions are excluded; and for the calculations of interactions between protein side-chain conformations, all ions are excluded (Supplementary Fig. S1b and c). Calculations of HSA and Omp32 are carried out without additional heavy-atom rotamers with standard MCCE polar proton conformer selection and the side-chain pairwise minimization included.\(^3\) For \(a\)-amylase, MCCE calculations with and without heavy-atom rotamers were compared and no significant difference in chloride binding affinity was found. This is not uncommon in MCCE, where much of the improvement is generated by multiple available conformations of polar protons, isosteric interchange of the Asn and Gln termini and His tautomers, which are always included here.

### Theory of ion binding in Monte Carlo simulations

The energy cost for adding an ion to water can be expressed as\({}^{10,115-118}\)

\[
\mu_{\text{solvent}} = -kT \left\{ \ln \frac{V}{N+1} + \frac{3}{2} \ln \left( \frac{2\pi nkT}{h^2} \right) 
+ \ln \left[ \frac{d\sigma^N e^{-\mu_i(\bar{\sigma}^N)}}{d\sigma e^{-\mu_i(\bar{\sigma})}} \right] \right\}
\]

where \(k\) is the Boltzmann constant, \(T\) is the temperature, \(V\) is the volume, \(N\) is the total number of the ions in the ensemble before the ion is added, \(h\) is the Planck constant, \(\mu_i(\bar{\sigma})\) is the microstate energy for \(N\) ions in solution, \(d\sigma^N\) integrates this over all coordinates for the \(N\) ions, where \(s\) is the normalized coordinate of an ion.

The system energy change on adding an ion into a given binding site of a protein is:

\[
\Delta_{\text{binding site}} = -kT \left\{ \ln \frac{V'}{N+1} + \frac{3}{2} \ln \left( \frac{2\pi nkT}{h^2} \right) 
+ \ln \left[ \frac{d\sigma^{N+1} e^{-\mu_i(\bar{\sigma}^{N+1})}}{d\sigma e^{-\mu_i(\bar{\sigma})}} \right] \right\}
\]

Now \(r\) describes normalized coordinates of the atoms in the protein. Thus, \(d\sigma^{N+1}\) integrates over all coordinates of \(N\) atoms of the apoprotein and \(d\sigma^N\) integrates over all coordinates of the protein and of the bound ion. The integration over the bound ion is limited to the binding site; therefore, the normalization factor, \(V'\), is the size of the cavity. \(\Psi(\bar{r}^N, s)\) is the microstate energy of the protein with an ion in the binding site and \(\Psi(\bar{r})\) is the microstate energy of the protein in the apo state. The flexibility of the protein is modeled with the different conformers in MCCE and the integrals are calculated by summation over sampled microstates.

The binding energy of an ion is the energy of moving an ion from the solution to the binding site:

\[
\Delta G_{\text{binding}} = \Delta_{\text{binding site}} - \Delta_{\text{solvent}}
\]

\[
= -kT \left\{ \ln \frac{V'}{N+1} - \ln \frac{V}{N+1} + \ln \left[ \frac{d\sigma^{N+1} e^{-\mu_i(\bar{\sigma}^{N+1})}}{d\sigma e^{-\mu_i(\bar{\sigma})}} \right] 
- \ln \left[ \frac{d\sigma^N e^{-\mu_i(\bar{\sigma})}}{d\sigma^N e^{-\mu_i(\bar{\sigma})}} \right] \right\}
\]

By reorganizing Eq. (3), \(\Delta G_{\text{binding}}\) can be written as

\[
\Delta G_{\text{binding}} = -kT \ln \left( \frac{V'}{V} \right) - kT \left[ \ln \left( \frac{d\sigma^{N+1} e^{-\mu_i(\bar{\sigma}^{N+1})}}{d\sigma^N e^{-\mu_i(\bar{\sigma})}} \right) + \ln \left( \frac{d\sigma^N e^{-\mu_i(\bar{\sigma})}}{d\sigma^N e^{-\mu_i(\bar{\sigma})}} \right) \right]
\]

Here, the first term is the entropy change of an ion moving from the bulk concentration in solution to a binding site. A 10-fold increase in ion concentration leads to a 2.303 \(kT\) (1.36 kcal/mol) shift, favoring binding. The second term is the free energy of the protein with a bound ion and \(N\) ions in the bulk solution, and the third term subtracts the free energy of the apo protein with \(N+1\) ions in the solution.

In MCCE calculations, ions are added on a grid. Therefore, each grid point is treated as a microbinding site with a size, \(V'\), of \(a^3\), where \(a\) is the grid spacing. Assuming the ion density in the bulk solution approaches 1, the ideal gas approximation, Eq. (4) can be simplified as:

\[
\Delta G_{\text{binding}} = -kT \ln \left( \frac{\rho}{\rho'} \right) + \langle \Psi(\text{holo}) \rangle - \langle \Psi(\text{apo}) \rangle - \langle U \rangle
\]

where \(\rho\) is the bulk ion density, \(\rho' = 1/a^3\) is the grid space density, \(\langle \Psi(\text{holo}) \rangle\) is the free energy of the protein and bound ion in the holo state and \(\langle \Psi(\text{apo}) \rangle\) is the free energy of the apoprotein, and \(\langle U \rangle\) is the free energy of adding an ion to solution. In MCCE, \(\langle U \rangle\) includes the reaction field energy and implicit van der Waals interaction of the ion with the...
The absolute binding energy is found to be difficult to estimate, requiring knowledge of conformational flexibility of the protein and substrate, conformational changes coupled to binding, and hydration free energies.\textsuperscript{84,96–100} Rather, for each protein, an additional energy correction term, \( \mu_{corr} \), is added to the bulk solution conformer and adjusted to match the measured chloride occupancy\([\text{Cl}^-]/[\text{Cl}^0] \) at one pH and chloride concentration.

\[
\frac{[\text{Cl}^-]}{[\text{Cl}^0]} = e^{-(\mu_{corr}/kT)} \tag{7}
\]

and no interaction with the protein. The difference of \( \mu_{corr} \) is the total number of conformers.

The same \( \mu_{corr} \) is used for calculations of the pH influence and mutation of mixture and added ions for that protein. Thus, the chloride moved to the bulk solution has a self-energy:

\[
\Delta G_{self}(\text{Cl}^0) = \mu_{corr} + (\langle U \rangle + kT \ln \left( \frac{\rho}{\rho^0} \right) \tag{8}
\]

and no interaction with the protein. The difference of \( \mu_{corr} \) needed for the different proteins to correlate with the solvent exposure of the ion binding site with a relatively larger value needed to increase the calculated binding of buried chloride.

MCCE uses Metropolis sampling to determine acceptance given the energy \( \Delta G_i \) of microstate \( x \).\textsuperscript{59,62}

\[
\Delta G^i = \sum_{i=1}^{M} \delta_j \left[ 2.3m_i T \left( \rho - pK_{sol} \right) + n_i \left( -\mu_{corr} - kT \ln \left( \frac{\rho}{\rho^0} \right) \right) \right]
+ \left( \Delta \Delta G_{\text{ran},i} + \Delta \Delta G_{\text{bakbn},i} + \Delta G_{\text{nonsense},i} \right)
+ \Delta \Delta G_{\text{ASAS},i} \tag{9}
\]

\( M \) is the total number of conformers. \( \delta_j \) is 1 if conformer \( i \) is present in the microstate or 0 otherwise. \( n_i \) is 1 if conformer \( i \) represents a bound ion, which has a self-energy term \( -\mu_{corr} - kT \ln (\rho/\rho^0) \); \( n_i = 0 \) for all residue conformers and bulk solution ion conformers. The loss of interactions between ion and water, \( \langle U \rangle \) [Eq. (8)], is included in the loss of reaction field energy for the bound ion conformer \( \Delta \Delta G_{\text{ran},i} \), and implicit van der Waals energy term \( \Delta \Delta G_{\text{ASAS},i} \). \( m_i \) is 1 for bases, \( -1 \) for acids, and 0 for neutral conformers. \( b_k T \) is 0.59 kcal/mol (0.43 pH units) at 298 K, the default temperature. The pH describes the ability of the solvent to donate protons. The \( pK_{sol} \), is the reference solution \( pK_i \) of groups involved in acid–base reactions. These are properties of the residue, not the conformer.\textsuperscript{71} The second line of the equation describes the conformer self-energies, which are independent of the other conformers in the microstate. The third line gives the electrostatic and Lennard–Jones pairwise interactions, which depend on the conformers selected in the microstate. In all cases, the reference state is assumed to be the neutral acids and bases and unbound ion.

### Analysis

For proteins without ionization changes strongly coupled to chloride binding, such as HSA and Omp32, the ion binding energy can be expressed as:

\[
\Delta G_{\text{binding}} = - \left( \mu_{corr} + kT \ln \left( \frac{\rho}{\rho^0} \right) \right) + \Delta \Delta G_{\text{ran}} + \Delta \Delta G_{\text{bakbn}} + \sum_{j=1}^{N_{corr}} \alpha_j \Delta G_j \tag{10}
\]

\( \Delta \Delta G_{\text{ran}} \) is the loss of the ion–solvent interactions when the chloride is bound and is the difference between the self-energy of the ion in water \( \langle U \rangle \) and in protein, \( \Delta \Delta G_{\text{bakbn}} \) is the Lennard–Jones and electrostatic interactions of the ion with the backbone and \( \sum_{j=1}^{N_{corr}} \alpha_j \Delta G_j \) is the mean-field average of its interactions with all side chains, where \( \alpha_j \) is the occupancy of the \( j \)th conformer in the rest of the protein and \( \Delta G_j \) is the interaction of this conformer with the ion.

For multiple binding sites, the Boltzmann-averaged binding energy is:

\[
\langle \Delta G_{\text{binding}} \rangle = \frac{\sum_i \alpha_i \Delta G_{\text{binding}}}{\sum_i \alpha_i} \tag{11}
\]

where \( \alpha_i \) is the occupancy of chloride binding site \( i \) and \( \Delta G_{\text{binding}} \) is the binding energy of this site. The sum of \( M \) binding sites is limited to those matching a given criterion and excluding all other chlorides. For example, in Omp32, the criterion is the position of chloride sites between \( z = (z_i - 1) \text{Å} \) and \( z = (z_i + 1) \text{Å} \), where \( z_i \) is along the \( z \)-axis, normal to the membrane surface.

When chloride and proton binding are strongly coupled, such as in \( \alpha \)-amylase, analysis used a reduced model allowing calculations of all microstates of a small number of groups.\textsuperscript{63} Within the strongly coupled region, all ionization states are explicitly included. The energies of the rest of the protein are included by a mean field approximation. Microstates are formed by the combination of the chloride state and the coupled residue ionization states. The energy of a given microstate is:

\[
\Delta G = \delta_C \left[ - (\mu_{corr} + kT \ln \left( \frac{\rho}{\rho^0} \right) ) + \Delta \Delta G_{\text{ran}} + \Delta \Delta G_{\text{bakbn}} + \sum_{j=1}^{N_{corr}} \alpha_j \Delta G_j \right]
+ \delta_{\text{ionization}} \cdot \delta_C \cdot \Delta G_{\text{CT}} (R, \delta) \tag{12}
\]

where \( \delta_C \) is 1 for occupied Cl and 0 for empty cavity, \( \delta_{\text{ionization}} \) is 1 for the ionized state of the \( k \)th coupled residue and 0 for the neutral state, \( \Delta G_{\text{CT}} (R, \delta) \) is the interaction between the occupied Cl and the ionized residue. Residue \( k \) is omitted from the summation over \( N \).

To analyze the energy of protonation coupled to chloride binding, \( pK_\gamma \), the energy for changing the protonation state of a residue at pH 7 is used:\textsuperscript{120}

\[
\Delta G_{\text{ionization}} = \Delta G_7 = 1.36m(7 - pK_\gamma) \text{ kcal/mol} \tag{13}
\]

where \( m \) is \( -1 \) for an acid and \( +1 \) for a base; and \( pK_\gamma \) describes the pH where the protonation free energy would
be zero with the rest of the protein retaining the same ionization and conformational states as at pH 7.

\[ pK_T = pK_\text{K, sol} - m \left( \Delta G_{\text{ion}} + \Delta G_{\text{sol}} + \sum_{i=1}^{N_{\text{ion}}} \delta_i \Delta G_i \right) / (2.303RT) \]

(14)

\[ pK_\text{K, sol} \] is the solution pK of the ionizable residue. The pK\(_T\) rather than the residue pK\(_i\) is used here because the residue of interest is changing its protonation at pH 7 on chloride binding. In contrast, the pK\(_i\) measures the ionization cost at a different pH, and thus includes the effects of pH changes on ionization and conformation in the rest of the protein.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.01.038

References

Calculated Chloride Binding


