Molecular dynamics study of zinc binding to cysteines in a peptide mimic of the alcohol dehydrogenase structural zinc site†

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The binding of zinc (Zn) ions to proteins is important for many cellular events. The theoretical and computational description of this binding (as well as that of other transition metals) is a challenging task. In this paper the binding of the Zn ion to four cysteine residues in the structural site of horse liver alcohol dehydrogenase (HLADH) is studied using a synthetic peptide mimic of this site. The study includes experimental measurements of binding constants, classical free energy calculations from molecular dynamics (MD) simulations and quantum mechanical (QM) electron structure calculations. The classical MD results account for interactions at the molecular level and reproduce the absolute binding energy and the hydration free energy of the Zn ion with an accuracy of about 10%. This is insufficient to obtain correct free energy differences. QM correction terms were calculated from density functional theory (DFT) on small clusters of atoms to include electronic polarisation of the closest waters and covalent contributions to the Zn–S coordination bond. This results in reasonably good agreement with the experimentally measured binding constants and Zn ion hydration free energies in agreement with published experimental values. The study also includes the replacement of one cysteine residue to an alanine. Simulations as well as experiments showed only a small effect of this upon the binding free energy. A detailed analysis indicate that the sulfur is replaced by three water molecules, thereby changing the coordination number of Zn from four (as in the original peptide) to six (as in water).

I. Introduction

Zinc is one of the most common metal ions bound to proteins in living organisms.[1,2] In proteins, Zn ions are often coordinated to the amino acid side chains of aspartic acid, glutamic acid, cysteine and histidine.[3] So far, four different biological functions for zinc in proteins have been identified: catalytic, co-catalytic, interface binding and structurally stabilizing.[3] Altered zinc levels in cells can change protein expression levels and reversible binding of Zn to proteins plays a role in cell signaling.[4,5] Mammalian alcohol dehydrogenases (ADH, EC 1.1.1.1) are dimeric zinc metalloenzymes, with two Zn ions per subunit.[6,7] One of these Zn ions is a part of the catalytic site of the enzyme and has been the subject of several studies.[1,3,8] The other Zn ion plays a structural role and is crucial for protein stability.[1,3,8–11] The structures of the catalytic and structural zinc sites in horse liver alcohol dehydrogenase (HLADH) as revealed in crystallographic structures[7] have been studied computationally with quantum chemical as well as with classical molecular dynamics methods.[12–16] It is primarily the catalytic site that has been the subject of computational studies in attempts to determine the charge distribution and parametrise fractional charges. In the present study, we focus on the structural zinc site which is comprised of four closely spaced cysteine ligands (Cys97, Cys100, Cys103 and Cys111 in the amino acid sequence) positioned in an almost symmetric tetrahedron around the Zn ion[7] (Fig. 1 and 2). Zinc-binding repeats (see *e.g.* Brändén and Tooze[17]), named zinc fingers, play an important role for protein–nucleic acid interactions and one class share a four cysteine binding motif with the present system.

Zinc and other divalent ions that are embedded in peptides and proteins are far from trivial to model in an accurate way. Generally two different approaches are used: bonded[18] and non-bonded.[19] In the bonded models a stable site structure is ensured by the use of bond length restraints or appropriate harmonic potentials. This does not, however, allow a simulation of the zinc release from the peptide. For the non-bonded models, it is the use of proper van der Waals parameters that results in the right coordination of the Zn ion as well as stable site structure. Here, we choose a simple non-bonded representation, since in the structural site of HLADH, the binding between the Zn ion and the cysteine sulfurs has, due to its weakness, been suggested to be of non-covalent nature.[20] The Zn ion forms coordination bonds with the sulfurs and can bind/unbind reversibly, with a dissociation constant estimated to be in the nanomolar range.[10] If the interaction between the zinc and the sulfurs is due to electrostatics it may be represented by fixed fractional charges. Changes

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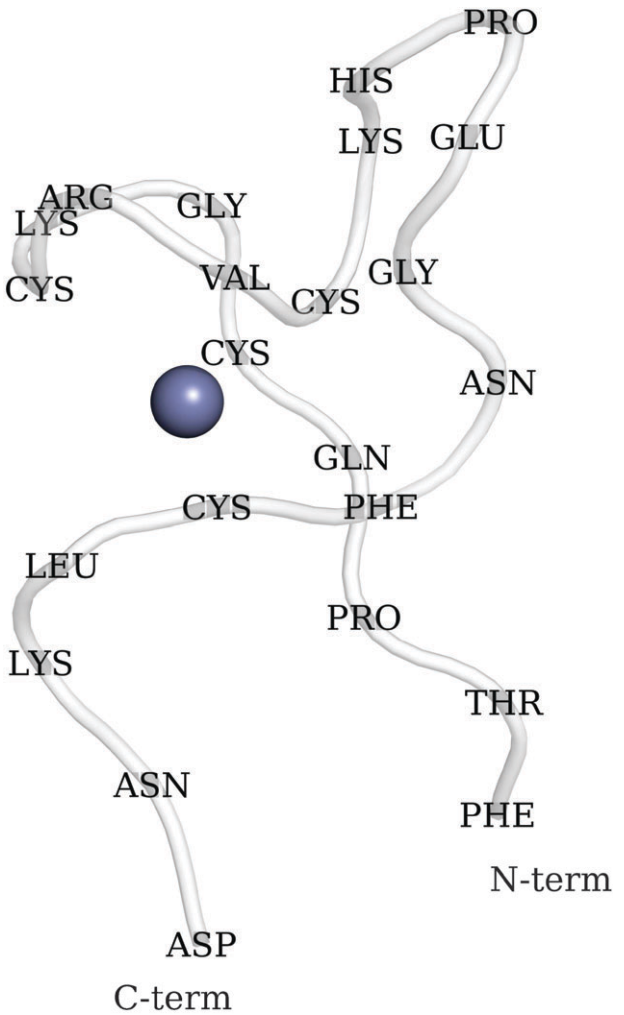

Fig. 1 Amino acid sequence and structure of the 23-residue synthetic peptide corresponding to the HLADH structural zinc site. The folding shown is based on the structure of the corresponding protein segment (residues 93–115) in HLADH[7] which was used as a structural scaffold in the simulations.

in polarisation or covalent contributions require a quantum mechanical treatment.[21,22]

To model the interactions between cysteine residues and zinc, we used synthesised peptides and computational methods constructed to mimic the zinc binding properties at the structural site of HLADH. In the experiments, the dissociation constant for zinc was determined.[23] This relates to a binding free energy of the Zn ion that is calculated from the simulations. In this manner, we can use experimental values to validate the theory and elucidate details in the binding/unbinding process. The binding free energy of the Zn ion is the difference between the solvation free energy of the ion in the peptide and in bulk water, which are both very large in number. Therefore, small relative errors in their absolute values have a major impact on their difference. Experimental data are also available for comparison to the solvation free energy of the Zn ion in water. However, determining hydration energies of ions from simulations is problematic even for monovalent ions, because of finite size artifacts. For the sodium ion, a scheme of correction terms has been proposed that has showed consistent results for a variety of treatments of electrostatics as well as simulation box sizes.[24,25] In this paper, we show that using such a scheme, the hydration energy of the Zn ion can be calculated from the force field parameters in agreement with experiments, using both cutoff and PME to treat long-range electrostatic interactions.

Further, since the thermodynamic stability of a protein can be altered by single point mutations, as shown in experiments where each cysteine in the structural site of human class-I and class-III alcohol dehydrogenase was mutated to alanine or serine, resulting in unstable proteins,[11] a substitution of a cysteine to an alanine was examined by both experimental and computational work.

To ensure reliable results the MD free energy calculations were performed using different methods, to find out the influence of the methodology on the results. The classical results were corrected by employing DFT calculations on smaller clusters of atoms to determine covalent contributions and polarisation effects.

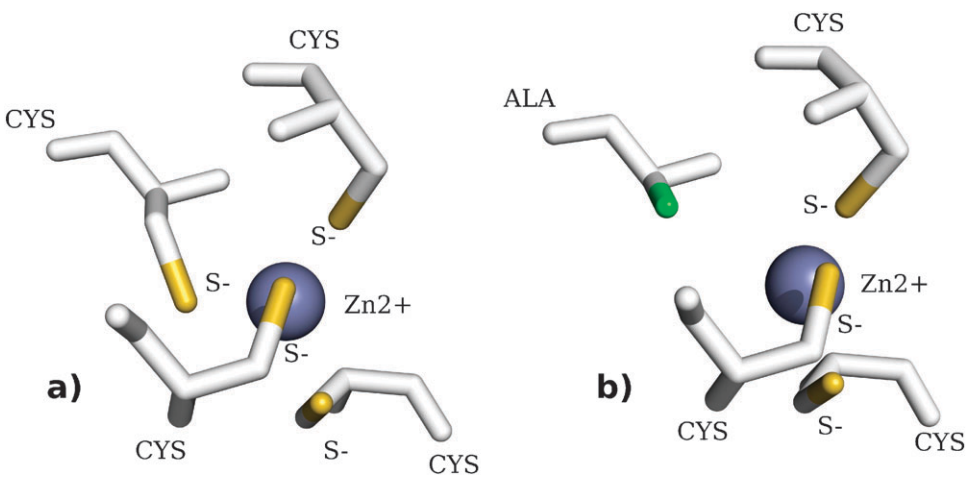

Fig. 2 Geometry of the structural zinc site in the simulations, all cysteines are considered to be deprotonated. (a) The peptide with four cysteines, Peptide(4Cys). (b) The peptide with three cysteines, Peptide(3Cys). One cysteine is replaced with an alanine, breaking the site symmetry.

II. Theory

We consider the reversible chemical reaction

$$\text{Peptide(sol)} + \text{Zn(sol)} \rightleftharpoons \text{Peptide–Zn(sol)}, \qquad (2.1)$$

where the right hand side denotes the dissolved peptide in its native state with a bound Zn ion in the structural site, while the left hand side shows the peptide without bound zinc and with the Zn ion in solution. The equilibrium constant can either be defined in terms of molar fractions

$$K_X = \frac{X(\text{Peptide(sol)})X(\text{Zn(sol)})}{X(\text{Peptide} - \text{Zn(sol)})} = e^{-\Delta G^0/RT}, \qquad (2.2)$$

where R is the gas constant and T the absolute temperature, or in terms of concentrations

$$K_D = \frac{[\text{Peptide(sol)}][\text{Zn(sol)}]}{[\text{Peptide} - \text{Zn(sol)}]}. \qquad (2.3)$$

Thus, the free energy of binding, ΔG^0 is:

$$\Delta G^0 = -RT \ln K_X = -RT \ln K_D/C^0. \qquad (2.4)$$

with C^0 being 1 M if ΔG^0 is defined for the standard states of the pure substances. In this case, the measured values for K_D are in the low nanomolar range.[10,23] A dissociation constant of 1 nM corresponds to a free energy of binding of 52 kJ mol^{-1}. Since the experimental solvation energy of zinc in water is $\sim$2000 kJ mol^{-1},[26–30] this means that we should have a binding of zinc to the peptide of $\sim$2050 kJ mol^{-1}. We therefore need to resolve both these energies with precision better than a few percent to get the actual dissociation constant accurately.

In the following, for simplicity we will not make any distinction between Helmholtz' and Gibbs' free energies. Since the systems are condensed and not very compressible, this is a good approximation that simplifies the treatment. ΔF_{bind} is the free energy difference between the state where the Zn ion is bound to the peptide and the state where the Zn ion is free in the solvent:

$$\Delta F_{\text{bind}} = F_{\text{bound}} - F_{\text{free}}. \qquad (2.5)$$

There are various ways to calculate such free energy differences.[31] Most of them rely on an integration over intermediate states and correspond to an equilibrium simulation of the work performed in the binding/unbinding process. These methods originate from the work of Zwanzig.[32] To calculate this free energy difference the Hamiltonian H of the intermediate states are written as a function of a coupling parameter λ, with $H(\lambda = 1) = H_{\text{bound}}$ and $H(\lambda = 0) = H_{\text{free}}$. It can be rigorously shown that the binding free energy may be calculated as

$$\Delta F_{\text{bind}} = F_{\text{bound}} - F_{\text{free}} = \int_0^1 \left\langle \frac{\mathrm{d}H}{\mathrm{d}\lambda} \right\rangle_{\text{NVT};\,\lambda} \mathrm{d}\lambda. \qquad (2.6)$$

III. Materials and methods

A Computational details

The molecular dynamics simulations were carried out using the GROMACS package.[33] Both the simulation system and the experimental system were comprised of the same two 23-residue peptides corresponding to the protein segment forming the structural zinc site in HLADH (see Experimental details for more information).[10,23] The 3D structure of the peptides in the molecular dynamics simulations was taken from crystallographic studies at high resolution,[34] labelled 1n8k in the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (http://www.rcsb.org). The simulation system was equilibrated in a cubic box with side length 5 nm containing 3975 water molecules, equivalent to a peptide and zinc concentration of 13 mM. Periodic boundary conditions were used in all directions. The temperature was kept constant at 300 K using the Berendsen thermostat.[35] The system was initially equilibrated for 1 ns with the Berendsen barostat at 1 atm while the final simulations were performed at constant volume. The N- and C-termini of the peptide were kept in protonation states corresponding to positive and negative charge, respectively. The net charge of the Zn ion was set to $+2e$ in the simulations, corresponding to a completely filled d-shell with 10 d-electrons. We used the van der Waals-parameters for the non-bonded Zn ion from Stote and Karplus[19] which are widely used together with different force fields. The OPLS all-atom (OPLS-AA) force field[36,37] was used for the peptide with bonds constrained using the LINCS algorithm.[38] The water molecules were represented with the TIP4P model.[39] For the electrostatic interactions either a neighbour-list-based twin-range cutoff with which interactions were updated every time-step up to 1.4 nm and every 10th time-step (when the neighbour list was updated) between 1.4 nm and half the box length (2.5 nm) or PME (particle mesh Ewald)[40,41] was used. The Lennard-Jones interactions were truncated at 1.4 nm. The integration was performed using a leap frog algorithm with a time-step of 2 fs.

B MD free energy calculations

The binding free energies of the Zn ion to the peptides were calculated with three methods using equilibrium MD simulations. One method was a thermodynamic integration (TI), in which the binding free energy of the Zn ion, ΔF_{bind}, was calculated as the difference in free energy between deleting the Zn ion in the solvated peptide–Zn complex and in bulk water. ΔF_{bind} was obtained by numerical integration of eqn (2.6) from a series of independent equilibrium simulations. The change of net charge was compensated by a smeared out continuum charge in both cases.

In the second method the Zn ion was pulled out of the binding site into the bulk water, using overlapping umbrella potentials to bias the energy landscape. By using a slow pulling process the system was maintained close to equilibrium, and the binding free energy was obtained from the reversible work performed on the system.[42]

Finally, the linear interaction energy (LIE) method[43,44] was used. It is based on linear response theory[45] and estimates the electrostatic part of the binding free energy as half the electrostatic potential energy of binding. In addition to the electrostatic contributions, the LIE method includes a Lennard-Jones term from an empirical expression. Here, that part is only a few percent of the total binding free energy.

For details about the usage of these methods, ESI is available.†

C Quantum mechanical corrections to the calculated classical binding energies

Quantum mechanical binding energies have been computed by means of Kohn–Sham density functional theory as implemented in the Gaussian 03 suite of programs.[46] Geometry optimisations were performed for the following systems: the Zn^{2+} and $(S–CH_3)^-$ ions, a water molecule (H_2O) and the $(Zn–(S–CH_3)_4)^{2-}$ and the $(Zn–(H_2O)_6)^{2+}$ clusters. The binding energies of the two clusters were computed using the supermolecule approach, *i.e.* by subtracting the energies of the separate molecules from the energies of the clusters.

The geometry optimisations were performed using the B3LYP exchange–correlation functional with a range of basis sets; 6-31++G(d,p), 6-31+G(2d,p), 6-31++G(2d,p) and 6-31++G(3d,p), to investigate the effect of the basis set on the binding energy. We have not found it necessary to apply the counterpoise correction for the basis set superposition error (BSSE) since large diffuse basis sets were used.[47] Zero point corrections to the binding energies (ZPEs) were computed from harmonic vibrational frequencies obtained at the B3LYP/6-31++G(d,p) level (no scaling of the ZPEs was applied since the optimum scaling factor is 0.98[48,49]). These positive energy contributions were added to the negative binding energies to obtain a final estimate of those.

The quantum mechanical binding energies were then compared to the classical binding energies of the same clusters calculated after energy minimisation using the same force field as in the free energy integrations. Thus, a quantum mechanical correction to the classical binding energy of both clusters was obtained. The difference between the QM correction terms to the two clusters was finally applied to the classical binding free energy for the Zn ion to the peptide. We also assigned the correction term for the $(Zn–(H_2O)_6)^{2+}$ cluster directly to the free energy integration for the hydration energy of the Zn ion.

In order to facilitate an interpretation of the difference between the quantum mechanical and classical binding energies, atomic partial charges of the molecular clusters have been computed from surface electrostatic potentials. The Gaussian 03 implementation of the charge derivation procedure of Besler, Merz and Kollman was utilised for this analysis.[50]

D Experimental details

Two synthetic 23-residue peptides corresponding to the segment in HLADH responsible for binding the structural zinc (residues 93–115) were employed in the experiments. One peptide is the replica of HLADH residues 93–115 (Peptide(4Cys)), while for the other, one cysteine residue (Cys103) was replaced by an alanine (Peptide(3Cys)). In the zinc binding studies, Hepes buffer (20 mM, pH 7.5) was used and the peptides were reduced with dithiothreitol (DTT).[10] Experiments were performed at μM peptide concentrations. Before zinc incubation, the DTT was removed with exclusion chromatography. The zinc binding stoichiometry was evaluated by atomic absorption spectrophotometry and amino acid analysis.[10] The metallochromic chelator 4-(2-pyridylazo)resorcinol (PAR) was used for determination of the zinc binding constants *via* extraction of zinc from the metal-saturated peptides and by measuring the absorbance at 500 nm for the PAR_2–Zn complex.[23]

IV. Results and discussion

The results of the classical free energy calculations of the Zn ion to the peptides are presented in Table 1. The table lists the calculated free energy for the peptide with four cysteines (Peptide(4Cys)), the peptide with three cysteines (Peptide(3Cys)) and the free energy difference between them. The results are sorted according to method and treatment of the long-range electrostatics (cutoff or PME). All calculations were carried out using both cutoff and PME to treat the long-range electrostatic interactions, except for the pulling of the Zn ion out of the site, where only cutoffs were employed. A general estimate of the statistical errors has been made for all methods and is presented along with the results (*cf.* appendix of ref. 51). We define $\Delta\Delta F \equiv \Delta F_{\mathrm{Peptide(3Cys)}}–\Delta F_{\mathrm{Peptide(4Cys)}}$, so that $\Delta\Delta F > 0$ implies that the peptide with three cysteines binds zinc less strongly than the peptide with four cysteines.

The quantum mechanical corrections calculated for Peptide(4Cys) are shown in Table 2 and the net correction term obtained for the largest basis set (B3LYP/6-31++G(3d,p)) was applied to the values in Table 1. The final energies of binding are listed in Table 3, including a column with the protonation contribution for the cysteines, added according to the discussion in the next paragraph.

A Setup of the surrounding environment in the MD simulations

In the experimental work, the peptides were in an aqueous environment containing buffer molecules. In the molecular dynamics simulations, neither buffer molecules nor free protons (H^+) were present. The protonation of all amino acid residues, except for cysteine, was set according to experimentally determined pK_A values for the respective amino acid side chains at a pH of 7.5, equal to that in the experimental work. The cysteine residues were set deprotonated due to their coordination to the Zn ion, along the lines of computational work by Dudev and Lim[52] and Ryde.[15] The protonation states were kept during the integration procedure. Because of two negatively charged

Table 1 Calculated classical binding free energies for the Zn ion to the peptides with four and three cysteines with sequences corresponding to the structural zinc site of HLADH. The statistical errors for all computational methods were estimated to ±20 kJ mol^{-1} (at the most), and to ±5 kJ mol^{-1} for the experimental data. All units are in kJ mol^{-1}

Method	Electrostatics	$\Delta F_{\mathrm{Peptide(4Cys)}}$	$\Delta F_{\mathrm{Peptide(3Cys)}}$	$\Delta\Delta F^e$
Method I[a]	Cutoff	−158	−137	21
	PME	−186	−145	41
Method II[b]	Cutoff	−195	−176	19
Method III[c]	Cutoff	−220	−212	8
	PME	−131	−123	8
Experiment[d]		−58	−54	4

[a] Growing/deleting the Zn ion in the peptide and in the bulk water. [b] Pulling the Zn ion out of the peptide zinc binding site. [c] Linear response and the linear interaction energy (LIE) methods [d] Data obtained from experimental work. [e] $\Delta\Delta F \equiv \Delta F_{\mathrm{Peptide(3Cys)}} - \Delta F_{\mathrm{Peptide(4Cys)}}$.

Table 2 Summary of the quantum mechanical correction terms to the classical binding free energy of the Zn ion to the peptide with four cysteines (Peptide(4Cys). Units in kJ mol^{-1}

Method	$Zn(H_2O)_6^{2+}$ [a]	$Zn(S–CH_3)_4^{2-}$ [a]	QMC Zn–water[b]	QMC Zn–sulfur[b]	QMC net[c]
B3LYP/6-31++G(d,p)	−1481.9	−2740.9	−217.6	−88.4	129.2
B3LYP/6-31+G(2d,p)	−1450.1	−2746.6	−185.8	−94.1	91.7
B3LYP/6-31++G(2d,p)	−1450.9	−2746.3	−186.6	−93.8	92.8
B3LYP/6-31++G(3d,p)	−1442.6	−2745.8	−178.3	−93.3	85.0
QM zero point energy	55.5	21.5			
Classical energy	−1208.8	−2631.0			

[a] Binding energy of the Zn–water and the Zn–sulfur clusters. [b] Quantum mechanical corrections to the classical binding free energies of the Zn–water and the Zn–sulfur clusters, obtained as the difference between the quantum mechanical B3LYP binding energy (with the zero point energy added) and the classical energy. [c] Net QM correction to the binding free energy of the Zn ion to Peptide(4Cys), calculated as the difference between the two preceding columns.

Table 3 Calculated binding free energy of the Zn ion to Peptide(4Cys) including the quantum mechanical correction term obtained using the largest basis set (B3LYP/6-31++G(3d,p)) and the free energy of protonation of the cysteines. Units in kJ mol^{-1}

Method	Electrostatics	$\Delta F_{\text{Peptide(4Cys)}}$	$\Delta F_{\text{Peptide(4Cys)}} + \Delta F_{4H^+}$
Method I[a]	Cutoff	−73	−57
	PME	−101	−85
Method II[b]	Cutoff	−110	−94
Method III[c]	Cutoff	−135	−119
	PME	−46	−30
Average method[d]		−93	−77
Experiment[e]		−58	−58

[a] Growing/deleting the Zn ion in the peptide and in the bulk water. [b] Pulling the Zn ion out of the peptide zinc binding site. [c] Linear response and the linear interaction energy (LIE) methods. [d] Average over all methods and electrostatics with a standard deviation calculated to ±31 kJ mol^{-1}. [e] Data obtained from experimental work.

residues of the peptide this meant that the system with four cysteines was neutral in all simulations. The mutated system with one cysteine replaced by an alanine had the fixed net charge $+e$ in all simulations. In the PME simulations this had to be compensated by one net charge smeared out over the entire system to avoid divergent electrostatic energies. In the cutoff case a smeared out charge gives the extra electrostatic energy of $-e^2r_c^2/2\varepsilon_0L^3$ in all simulations. This could be added afterwards but will not change the binding energies. The size of this term would be about −40 kJ mol^{-1} with L = 5 nm and r_c = 2.5 nm. Alternatively, one may include one or several explicit counter ion(s), which might seem more realistic but will be more problematic due to the need of excessive equilibration and sampling times.[53]

The free energy differences are thus properly calculated assuming that the cysteines remain deprotonated in water solution after the release of the Zn ion. We might, however, gain free energy for the system with the Zn ion released by protonating the cysteines if their pK_A values are larger than the pH of the system. A simple estimate of this contribution to the free energy is obtained (for a single cysteine) using the known pK_A^{Cys} = 8.2 (ref. 54) for cysteine in water, and pH = 7.5:

$$\Delta F^{Cys}_{protonation} \approx -2.303\ RT\ (pK_A^{Cys} - pH) = -4\ \text{kJ mol}^{-1}. \quad (4.7)$$

We have added four times this contribution to the final estimate of the calculated binding free energies in Table 3. Admittedly, this correction could be different since the actual pK_A values of the cysteines in the peptide might be shifted due to the local electrostatic surroundings. This could in principle be calculated from electrostatic programs but would require extensive sampling over MD trajectories simulated at different protonation states. We have refrained from trying to calculate such an improved protonation correction.

B A comparison between the classical free energy calculation methods

Table 1 shows calculated and experimental binding free energies of the Zn ion to the peptides. It is clear that the binding energies calculated with the classical methods are consistently 50–150 kJ mol^{-1} more negative than the experimental values. Based on the fluctuations of the integrand in eqn (2.6), we estimate the statistical error to be of the order of 20 kJ mol^{-1}. Longer simulations at each λ-value would reduce the statistical error (but only slowly since this varies as the inverse square root of the total number of simulation steps). A denser sampling in λ would better capture rapid changes of the Hamiltonian with λ and thus reduce the error. Simulations of the unbound peptides for ~100 ns indicate similar conformational changes taking place for both peptides, but the electrostatic energies remained practically unchanged. The free energy change from slow conformational transitions can thus be expected to play a minor role.

The small weakening effect of the replacement of one cysteine to an alanine is reproduced, within the relatively large statistical error. The calculations for pulling the Zn ion out of the site were only carried out using cutoff, but the method should be independent of the treatment of long-range interactions since the contributions to the pull force are local. The linear response binding free energy was calculated from the same simulations as those used for growing the zinc with an additional simulation of a single Zn ion free in water solvent. With cutoff the obtained binding free energy from the LIE method was larger than for the other methods, but with PME it was slightly smaller. The Lennard-Jones contribution in the LIE method is negligible and less than 3% of the electrostatic contribution. We observe that there are substantial non-linear contributions to the free energy (Fig. 3).

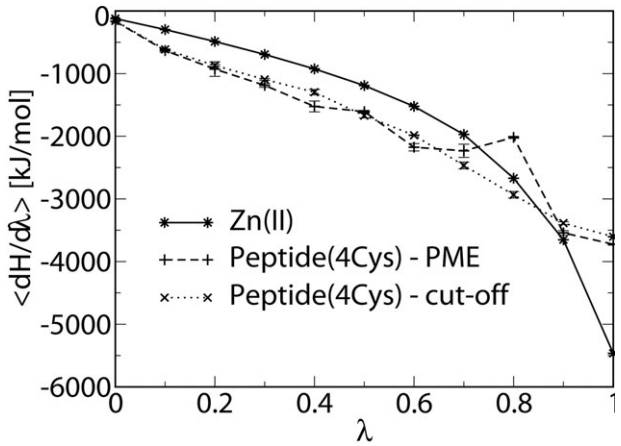

Fig. 3 $\langle dH/d\lambda \rangle = dF/d\lambda$ for different λ when growing/deleting the Zn ion in the peptide with four cysteines and in bulk water. Data points are from the simulations used for the calculations in Method I. $\lambda = 0$ corresponds to the condition when the charge and van der Waals parameters for the Zn ion are turned off and in the case of $\lambda = 1$ they are fully turned on. The curves for Zn in water are identical for PME and cut-off.

The calculated (as well as experimental) $\Delta\Delta F$ between the binding of zinc to Peptide(4Cys) and Peptide(3Cys) is of about the same size as the statistical error in the simulations ($\sim$20 kJ mol^{-1}). We note that even if the sign and approximate size of our result are in agreement with experiment, we would need more than one order of magnitude longer simulations to reach the precision of the experimental value.

C Contributions to the classical binding free energy of the Zn ion

The calculated binding free energy ΔF_{bind} is the difference between two separate free energy integrations; from simulations of the Zn ion in the solvated Peptide–Zn complex and simulations of a single Zn ion in water solution. For both of these cases, the long-range electrostatic interactions state a problem regardless of treatment. Cutoff methods neglect the polarisation of water at long distances from the ion. This contribution is substantial but can be accurately estimated from a simple Born model. Additionally there is a back effect from the water outside the Zn ion cutoff (interacting with the water inside the cutoff) that will be neglected with the Born correction. This effect can be estimated using an integral equation approach.[25] Lattice summation methods like PME do not perform substantially better since they are more sensitive to system size. These artifacts can be corrected for and in the end Kastenholz and Hühnenberger[24,25] obtained a hydration free energy for a sodium ion with an accuracy of a few kJ mol^{-1} (0.5%).

When the system contains a peptide it is less obvious how to calculate these corrections accurately. The hope is that the long-range contributions for the Peptide–Zn complex and the single ion in water are very similar. Hence we assume that the corrections described above cancel out when integrating the binding free energy of the Zn ion in the complex (since the net charge is preserved).

The experimental binding energy of zinc to the peptide with four cysteines (Peptide(4Cys)) is -58 ± 4 kJ mol^{-1}. We can compare this value to the literature values for the hydration free energy of a single Zn ion in water, which ranges from -1955 to -2030 kJ mol^{-1}.[26–30] These data imply that we need the solvation and binding energies of the Zn ion separately with an accuracy of 0.1% to reach experimental accuracy. A reduction of the statistical errors down to such levels would call for simulations approaching μs time scales instead of the present ones of $\sim$10 ns. However, there are also systematic errors in the classical simulations resulting in too strong binding free energy of the Zn ion in the structural site.

D Quantum corrections to the classical binding free energy

The quantum mechanical binding energies of the $(Zn–(S–CH_3)_4)^{2-}$ and $(Zn–(H_2O)_6)^{2+}$ clusters calculated with density functional theory using different basis sets are summarised together with the classical binding energies in Table 2. It is seen that the classical treatment underestimates the binding of the water molecules to the Zn ion with 178 kJ mol^{-1} as well as the binding of the S–CH_3 to zinc but then only with 93 kJ mol^{-1}. In the water case the positively charged Zn ion polarises the closest water molecules. A fractional charge of $-1.12e$ on the nearest water oxygens indicates an increased dipole moment with about 35% compared to bulk water. That increased dipole moment also strengthens the classical electrostatic binding. In the sulfur case the reason is more subtle. The positive charge on the Zn ion is reduced to about $1.4e$ implying that the negatively charged sulfurs donate or share a part of their electrons with the Zn ion. This would weaken a classical electrostatic bond and can only be understood as a covalent contribution to the bond. It is also important to note that the distance between the Zn ion and the water oxygens increases from 2.02 Å in the classical treatment to 2.12 Å in the quantum mechanical treatment. For the sulfurs the same effect is even stronger; the distance increases from 2.10 Å in the classical case to 2.44 Å using quantum mechanics (Fig. 4). This is in better agreement with the Zn–S bond lengths in the protein X-ray structures, which tend to be about 2.35 Å,[7,34] and matching results from earlier QM studies on the structural zinc site.[15]

The solvation free energy of the Zn ion in water obtained from the classical simulations becomes -1783 kJ mol^{-1} for cutoff and -1791 kJ mol^{-1} for PME, after adding corrections in the same way as proposed by Kastenholz and Hünenberger.[25] If the quantum mechanical correction of -178 kJ mol^{-1} from Table 2 (which is due to the zinc ion polarising the neighbouring water molecules) is added, we obtain calculated solvation energies of -1961 and -1969 kJ mol^{-1}. A final estimate of -1965 ± 10 kJ mol^{-1} is in agreement with the experimental figures which are in the interval -1955 to -2030 kJ mol^{-1}, as presented above.

By adding the QM corrections to the free energy of the Zn ion binding to Peptide(4Cys), the binding is weakened with 85 kJ mol^{-1} due to that the quantum mechanical effects strengthening the Zn–water attraction more (178 kJ mol^{-1}) than the Zn–S interactions (93 kJ mol^{-1}). After this adjustment, and including protonation of the cysteines (16 kJ mol^{-1}), the calculated free energies end up in the interval -30 to -120 kJ mol^{-1} depending on the method for free energy integration. The method

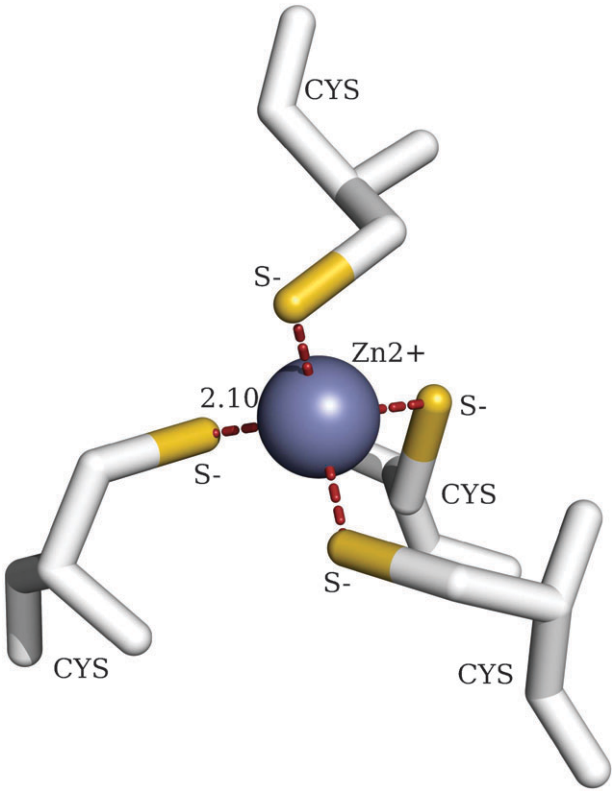

Fig. 4 Coordination of the Zn ion in the Peptide(4Cys) binding site after 1 ns of molecular dynamics simulation. The average length for the Zn–S coordination bond was 2.10 Å during the MD simulations (shown in the figure) and 2.44 Å including the QM calculations.

that we believe is most reliable (method I), which is not subject to the linear response approximation (method III) and without the more serious non-equilibrium problems of method II, gives -57 kJ mol^{-1} with cutoff and -85 kJ mol^{-1} using PME for the electrostatics. The experimental figure (-58 kJ mol^{-1}) falls within the error bars of the cutoff figure and slightly outside the error bars of the PME figure.

E Implications for the parametrisation of a classical model

At first glance, one may argue that the deviations in bond length and free energy could be corrected classically merely by adjusting the Lennard-Jones repulsion-parameter for Zn and/or S. For the Zn ion such a tuning could be achieved by weakening the Lennard-Jones repulsion enough to increase the hydration energy with about 10%, but this would also strengthen the Zn–S attraction and shorten the Zn–S bonds even further. In the peptide, this could be adjusted by changing either the Lennard-Jones parameters of S or the fractional charges of Zn or S. In both cases one would nevertheless need a simultaneous lengthening of the bond and strengthening of the attraction (to compensate for the larger hydration energy of Zn) which is not achievable. In addition, this makes the Zn ion coordinated by more than six water molecules in water solution. A classical polarisable water model might solve the problem with Zn ion hydration free energy, without any adjustment of the Zn parameters or problems with the water coordination.

For the sulfur Lennard-Jones parameter σ_S, a successful adjustment would directly effect the binding free energy of the Zn ion to the peptides. In the OPLS-AA force field, $\sigma_S = 0.355$ nm, which originates from a parametrisation *versus* a number of sulfur containing compounds[37] with a slight spread in their parameters. We found that a 4% larger value ($\sigma_S = 0.370$ nm) was needed to reproduce the properties of liquid hydrogen sulfide (H_2S). The use of σ_S would correspond to a 2% increase in the Zn–S distance, which is an average of the Zn and S parameters. This would (classically) result in a reduction of the binding free energy of the Zn ion with $\sim$40 kJ mol^{-1}, about the right amount to account for the discrepancy between the MD simulations and the experimental values (keeping the wrong classically computed hydration energy of the Zn ion). This would also only increase the Zn–S distance from 2.10 Å to 2.15 Å, which is far from sufficient.

These arguments show that a parameter adjustment in a classical model cannot at the same time achieve the three objectives: a reduced binding free energy of the Zn ion, an increased Zn–S distance and an increased solvation energy of the Zn ion in water. Despite that this may seem counter-intuitive, we conclude that the quantum mechanical effect on the Zn–S bond is strengthening of the binding energy accompanied by lengthening of the bond distance.

F The effect of the Cys-to-Ala replacement in the peptide zinc binding site

We also studied the effect of replacing one cysteine in the peptide zinc binding site with alanine. This means that we make one of the four cysteine residues neutral, and we would expect to lose 25% of the predominantly electrostatic binding free energy. Experimentally we observe weakening of the binding by only 7%. The different classical simulations give weakening in the range 4–22% with an average of 10%. We propose that this is due to the specific details of the coordination of the Zn ion in the structural site. In the native state, the Zn ion is coordinated almost symmetrically in a tetrahedral shape. In this shape, the structural site shields the Zn–water interactions so that no water molecules can reach the Zn ion. In the simulations with the peptide with three cysteines, the symmetry collapses, the alanine disappears and the cysteines reorganise around the Zn ion. Subsequently, three water molecules appear to coordinate to the Zn ion in the voids in the hydration sphere. We suggest that the Zn–water interactions account for some of the free energy in the peptide with three cysteines, which gives the small $\Delta\Delta F$ compared to if only Zn–cysteine interactions are considered.

Consider the Zn–water oxygen coordination number in the three cases: A single Zn ion in bulk water, and the Zn ion in the dissolved Peptide(4Cys) and Peptide(3Cys). Here the coordination number is defined according to

$$\zeta(r) = 4\pi n_0 \int_0^r r'^2 g_{\mathrm{Zn-O}}(r')\mathrm{d}r', \qquad (4.8)$$

where the integral is calculated from the centre of the Zn ion ($r' = 0$) to a point in space ($r' = r$). n_0 is the bulk number density of water and $g_{\mathrm{Zn-O}}(r)$ is the standard pair-correlation function between the Zn ion and the oxygens of the water. Fig. 5 illustrates the shielding of the native site in terms of ζ. A Zn ion in water solution is coordinated by six water

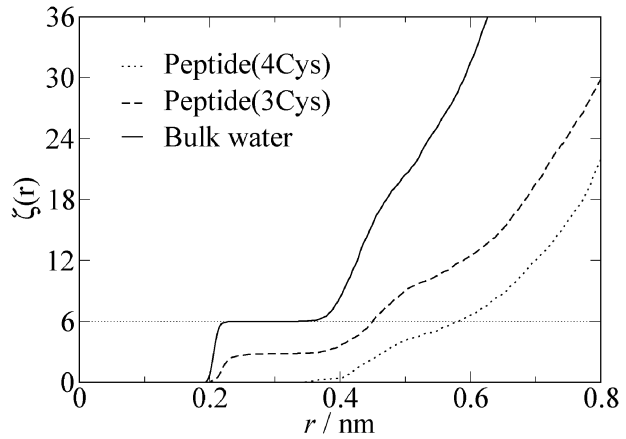

Fig. 5 Zn–O (water oxygen) coordination number, ζ, as a function of the distance from the Zn ion centre. The thick lines are for the Zn ion in the dissolved peptide with four cysteines (dotted), for the Zn ion in the dissolved peptide with three cysteines (dashed), and for the Zn ion in bulk water (solid). The thin dotted line is a guide to the eye to mark the first solvation shell of the Zn ion in bulk water.

molecules, by four cysteines in the native peptide and by three cysteines and three waters in the modified Peptide(3Cys). In addition we performed simulations where Cys97, Cys100 and Cys111 were replaced by alanine which showed a consistent pattern with three cysteine residues binding to the Zn ion.

We consider the quantum mechanical corrections in the case of the Zn ion binding to Peptide(3Cys) similar to that of four cysteines since the two quantum contributions are the same: polarisation of water molecules and covalency between zinc and sulfur. The balance between them might be somewhat shifted but not changing the nature of the corrections in a considerable way.

V. Summary

The free energy of zinc binding to a peptide with a sequence corresponding to the structural zinc site of HLADH has been calculated using classical molecular dynamics simulations applying three different methods for the free energy calculations. Quantum mechanical corrections to this energy have also been calculated, and the free energy has been determined experimentally from the binding constant. The study also includes the effect of replacing one of the zinc binding cysteines by an alanine, thus altering the coordination of the Zn ion in the peptide from four to three cysteines. The experimental data were used as a reference for the computational work.

The purely classical simulations overestimated the free energy of binding, ΔF, with about 100 kJ mol^{-1}, with a statistical error of 20 kJ mol^{-1}. In addition, the Zn–S bond lengths seen in the classical MD simulations were about 10% shorter than in protein crystals. On the other hand, the small experimental difference (4 kJ mol^{-1}) between the two peptides (Peptide(4Cys) and Peptide(3Cys)) was reproduced within the large statistical uncertainty of the simulations.

The quantum corrections, as calculated from small clusters of atoms in vacuum, indicate that the binding of water to zinc in solution is 178 kJ mol^{-1} (10%) stronger than what would be expected from classical simulations. This is due to the closest neighbour water molecules being strongly polarised by the Zn ion, and having a 35% larger dipole moment than the bulk waters. All in all, this increases the absolute free energy of solvation for the Zn ion to -1965 ± 10 kJ mol^{-1}, a figure which agrees with experimental literature values. Inclusion of additional solvent shells by a polarisable continuum model (PCM)[55,56] only slightly increases the polarisation (6%) of the first solvation layer.

However, the binding of the Zn ion to the sulfurs is also strengthened. In this case it is due to the negatively charged sulfurs sharing some of their electron density with the positively charged Zn ion, which leads to a small covalent contribution to the predominately electrostatic bond. This strengthens the Zn–S bond with 93 kJ mol^{-1}. At the same time the Zn–S distance increases compared to the classical simulations with about 10% needed to fit the experimental figures. The net result is that quantum corrections weaken zinc binding to the peptide with $178–93 = 85$ kJ mol^{-1}. This gives a net free energy difference that agrees with the results obtained from experimental binding constants and also the Zn–S bond lengths are in satisfactory agreement to protein X-ray crystal data.

The binding free energy of zinc is dominated by electrostatic interactions. The treatment of long-range electrostatics is in general a subtle problem, due to several simulation artifacts having to be corrected for. In the present study we do, however, calculate a binding free energy that is the difference between the energy in a state with the ion bound to the peptide surrounded by water and the ion in pure bulk water. Hence, there is a reason to believe that long distance- and finite size artifacts to a large extent cancel out, or at least are of much less importance, than the absolute ion solvation free energies. We conclude that the binding is governed by short-range electrostatic interactions, specifically how the Zn ion coordinates to the charged cysteine sulfurs.

When compared with the mutated peptide, experimental as well as computational data show a small and positive $\Delta\Delta F$, indicating that zinc binds slightly less strongly to Peptide(3Cys) compared to Peptide(4Cys). From a simple theoretical argument, one would expect that the difference in free energy of zinc binding between the two peptides corresponds to the binding of one cysteine to the Zn ion, *i.e.* 25% of the absolute binding free energy for the peptide with four cysteines. The observed difference is, however, smaller since three water molecules replace the cysteine in the coordination sphere around the Zn ion.

Increased knowledge on Zn binding motifs, such as in Zn fingers or the structural site in ADH, opens up possibilities to create new synthetic peptides with a potential to target specific genomic sites.

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