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# Effect of explicit water molecules for ligand-binding affinities calculated with the MM/GBSA approach

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**Abstract**

We have tested different approaches to include the effect of binding-site water molecules for ligand-binding affinities within the MM/GBSA approach (molecular mechanics combined with generalised Born and surface-area solvation). As a test case, we study the binding of nine phenol analogues to ferritin. The effect of water molecules mediating the interaction between the receptor and the ligand can be studied by considering a few water molecules as a part of the receptor. We extend previous methods by allowing for a variable number of water molecules in the binding site. The effect of displaced water molecules can also be considered within the MM/GBSA philosophy by calculating the affinities of binding-site water molecules, both before and after the binding of the ligand. To obtain proper energies, both the water molecules and the ligand need then to be converted to non-interacting ghost molecules and a single-average approach (i.e. the same structures are used for bound and unbound states) based on the simulations of both the complex and the free receptor can be used to improve the precision. The only problem is to estimate the free energy of an unbound water molecule. With an experimental estimate of this parameter, promising results are obtained for our test case.

**Key Words:** ligand-binding affinities, MM/GBSA, water displacement, water-mediated binding, molecular dynamics, entropy.

## Introduction

One of the largest challenges for computational chemistry is to develop methods to calculate the binding free energy ($\Delta G_{\text{bind}}$) of a ligand (L, e.g. a drug candidate) to its receptor (R, e.g. a protein or nucleic acid), forming a complex (RL).

$$\text{R} + \text{L} \rightarrow \text{RL} \tag{1}$$

Numerous methods have been suggested with this aim [1,2], ranging from simple scoring functions [3,4], which are fast but give quite uncertain results, to strict free-energy perturbations [5,6], which in principle should give correct results (with exhaustive sampling and a perfect force field), although they often are too time-consuming for routine use.

In the middle of this range are the so-called end-point methods, which employ molecular-dynamics (MD) simulation with a molecular mechanics (MM) potential, but only for the physical states of the reaction (RL, as well as possibly R and L). Examples of such methods are PDLD/s-LRA/$\beta$ (semi-macroscopic protein-dipoles Langevin-dipoles method within a linear-response approximation) [7,8], LIE [9,10,11] (linear interaction energy), and MM/PB(GB)SA [12,13] (molecular mechanics with Poisson–Boltzmann or generalised Born and surface-area solvation).

The latter method has received much interest, owing to its modular nature and because it does not contain any adjustable parameters. In MM/GBSA, it is assumed that the free energy of a species can be calculated from [12,13]

$$G = E_{\text{bnd}} + E_{\text{ele}} + E_{\text{vdw}} + G_{\text{solv}} + G_{\text{np}} - TS \tag{2}$$

where $E_{\text{bnd}}$, $E_{\text{ele}}$, and $E_{\text{vdW}}$ are the bonded (bonds, angles, and dihedrals), electrostatic, and van der Waals energy terms of the MM potential. When calculating these energy terms, water molecules are excluded and interactions with the surrounding solvent are instead estimated with a continuum-solvation approach, yielding a polar solvation energy, $G_{\text{solv}}$, e.g. estimated by the Poisson–Boltzmann or the generalised Born (GB) methods, and a non-polar solvation energy, $G_{\text{np}}$, typically estimated from the solvent-accessible surface area (SASA). Finally, the entropy ($S$ in Eqn. 2) is estimated by a normal-mode analysis of the harmonic frequencies, calculated at the MM level. Consequently, binding free energies can calculated from

$$\Delta G_{\text{bind}} = \langle G(\text{RL}) \rangle - \langle G(\text{R}) \rangle - \langle G(\text{L}) \rangle \tag{3}$$

where the brackets indicate ensemble averages over snapshots sampled from the MD simulations.

The MM/GBSA approach has been used to calculate $\Delta G_{\text{bind}}$ for many systems [14,15] and problems with the statistical precision [16] have been cured [17,18]. However, it has been showed that the results are not always satisfactory [19,20,21,22]. A possible reason for this is the use of the continuum-solvation approach, which ignores the explicit involvement of water molecules in the binding of ligands [23]. Water molecules may significantly affect the affinity of a ligand in at least two ways [24,25].

(a) The binding site of the receptor may contain water molecules before the ligand binds and different ligands may displace a varying number of water molecules. It is well-known that water molecules in protein clefts and cavities have differing properties compared to bulk water molecules [23] and it is believed that a significant part of the binding affinity is caused by the release of such binding-site waters upon binding [26,27,28,29].

(b) It is possible that the binding of ligands is mediated by water molecules, e.g. by bridging hydrogen bond. In fact, it has been observed that 85% of protein–ligand complexes involve water molecules in the binding interface [26]. It is unlikely that a continuum-solvation method would satisfactorily model such interactions.

In several cases, explicit water molecules have been included in MM/GBSA calculations as part of the receptor, often giving improved results [30,31,32,33,34,35,36,37,38]. For example, a study of the binding of six inhibitors to blood-clotting factor Xa showed that the MM/PBSA predictions were strongly improved if one water molecule that mediated the interaction between some of the ligands and the protein back-bone was explicitly considered (the correlation coefficient, *r*, increased from –0.8 to +0.8) [37]. Similar results were obtained if all water molecules that remained in a single position during the MD simulations were treated as a part of the protein. For the interaction of two proteins, McCammon and coworkers observed slightly improved results if two water molecules were explicitly considered, but much worse results if 200 water molecules were considered [35]. However, in a large test of 855 ligand complexes, inclusion of water molecules within 3.5 Å of the ligand deteriorated the results [39]. This shows, that the second (b) effect of water molecules often is significant and that it can be estimated by MM/PBSA, but it does is not always improve the results. Still another way to include the effect of water is to include a restricted number of explicit water molecules [40,41], combined with the COSMO-RS continuum solvation method [42], which has been shown to be insensitive to whether second-sphere water molecules are included explicitly in the calculations or not [43].

There have also been several attempts to estimate the free-energy gain of displacing water molecules during ligand binding. The binding energy of water molecules can be estimated by free-energy perturbation or grand canonical Monte Carlo methods [44,45,46,47,48,49]. Cheaper and more approximate methods have also been suggested [50,51,52,53,54,55,56]. It has been shown that prediction of relative binding affinities with free-energy perturbations are improved if water molecules in the binding site first are identified with the JAWS approach [57]. Moreover, the WaterMap water displacement energies have been shown to correlate well with ligand-binding free energies for several proteins [58,59,60,61]. Guimarães and Mathiowetz have tried to combine WaterMap with MM/GBSA to introduce explicitly the (a) water effect in MM/GBSA [62]. They observe small but consistent improvement of the correlation coefficient for two test cases, compared to the separate WaterMap and MM/GBSA methods. On the other hand, for the SRC kinase, WaterMap gave quite poor results and it did not improve results obtained with MM/GBSA [63]. Friesner and coworkers have also combined WaterMap with a MM/GBSA-like approach and obtained improved results for four out of five test systems [64].

In the present paper, we explore the possibility of include both the (a) and (b) effects of water molecules within the MM/GBSA scheme in the hope of obtaining a more consistent method, without the risk of double-counting energy terms [62]. As a test case, we use the binding of nine phenol-derivatives to ferritin, which has been the subject of several previous theoretical studies [20,21,23,65].

## Methods

### *Preparation of the protein and ligands*

We have studied ferritin with nine phenol derivatives (L01–L09), which are shown in Figure 1. The preparation of the protein has been described before [21,23,66]. Ferritin is a multimer of 24 protein chains. However, to save computer time, only a dimer was simulated,

as has successfully been done before [21,23,65,67]. The simulations were based on crystal structures of horse spleen ferritin with ligand L09 (PDB code 3f39) [67]. The other ligands were built manually from this structure. All ionisable protein residues were assigned their standard protonation state at pH 7. In each subunit of ferritin, the histidine residues 49, 132, and 147 were assumed to be doubly protonated, residue 114 was protonated on the $N^{\delta 1}$ atom, and residue 124 on the $N^{\epsilon 2}$ atom [23]. The Amber99SB force field [68] was used to describe the protein, whereas the ligands were described by the general Amber force field [69]. Ligand charges were calculated with the restrained electrostatic potential method [70] using potentials calculated at the HF/6-31* level and sampled with the Merz–Kollman scheme [71]. The protein and ligand were immersed in an octahedral box of TIP3P [72] water molecules that extended at least 10 Å outside the protein.

*Simulation protocol*

The molecular dynamics (MD) simulations were run by the sander module in Amber 11 [73]. The temperature was kept at 300 K using Langevin dynamics [74] with a collision frequency of 2.0 $ps^{-1}$. The pressure was kept at 1 atm using a weak-coupling approach [75] with isotropic position rescaling and a relaxation time of 1 ps. Particle-mesh Ewald summation [76] with a fourth-order B-spline interpolation and a tolerance of $10^{-5}$ was used to treat long-range electrostatics and the long-range van der Waals interaction were treated with a continuum approach. The non-bonded cutoff was 8 Å and the non-bonded pair list was updated every 50 fs. The MD time step was 2 fs and the SHAKE algorithm [77] was used to constrain bond lengths involving hydrogen atoms.

The simulations were performed in the following way: First, the system was optimised by 100 steps of steepest descent minimization, keeping all atoms except those of the ligand restrained to their start positions with a force constant of 418 kJ/mol/$Å^2$ (this step was skipped for the protein without any ligand). Next, the system was minimised by 100 steps of steepest descent, keeping all atoms except those of water molecules and hydrogen atoms restrained with the same force constant. The minimisation was followed by 20 ps MD equilibration with a constant pressure and the restraint force constant reduced to 214 kJ/mol/$Å^2$. Finally, a 1000 ps simulation was performed at a constant pressure without any restrains, followed by a 200 ps production run, during which coordinates were sampled every 5 ps to be used for the energy calculations. We employed 40 independent simulations for each protein–ligand complex, by assigning different starting velocities to atoms at the start of the 1000-ps equilibration. Thus, the MM/GBSA estimates are based on 1600 energy calculations for each ligand. Reported uncertainties are standard errors over the 40 independent simulations (i.e. the standard deviation over these simulations divided by $\sqrt{40}$ ).

*Standard MM/GBSA calculations*

$\Delta G_{\text{bind}}$ was calculated according to Eqn. 2 using two different approaches: In the single-average (1av) approach, all three terms on the right-hand side of Eqn. 3 were obtained from the snapshots from the MD simulation of RL:

$$\Delta G_{\text{bind}}^{\text{1av}} = \langle G(\text{RL}) - G(\text{R}) - G(\text{L}) \rangle_{\text{RL}} \qquad (4)$$

(the subscript of the angle brackets show which simulations were used for the average). The coordinates of the other two states (R and L) were obtained from the RL simulation by simply removing the ligand or the receptor. Thereby, the $E_{\text{bnd}}$ term cancels and the precision is

improved. In the three-average approach (3av), the first term was obtained from the MD simulation of RL, the second from the MD simulation of R free in water, and the third from the MD simulation of L free in water:

$$\Delta G_{\text{bind}}^{\text{3av}} = \langle G(\text{RL}) \rangle_{\text{RL}} - \langle G(\text{R}) \rangle_{\text{R}} - \langle G(\text{L}) \rangle_{\text{L}} \quad (5).$$

The terms in Eqn. 2 were calculated using Amber [73] with water molecules stripped off and without any periodic boundary conditions, but with an infinite cutoff. The MM energies were estimated using the same force field as in the simulations. The polar solvation energy was calculated by the GB model of Onufriev et al., model I (α = 0.8, β = 0, and γ = 2.91) [78]. The non-polar solvation energy was estimated from the solvent-accessible surface area (SASA) according to $G_{\text{np}} = a$ SASA + $b$, with $a$ = 0.0227 kJ/mol/Å$^2$ and $b$ = 3.85 kJ/mol [79]. The calculations employed the second set of modified Bondi radii [78].

The entropy was estimated by a normal-mode analysis of the harmonic frequencies calculated at the MM level. For this calculation, we used our modification of MM/GBSA to improve the precision [17,80]: All residues more than 12 Å from any atom in the ligand were deleted and the remaining atoms were minimised, keeping all residues more than 8 Å from ligand fixed (including all water molecules), to ensure that the geometry is as close as possible to the original structure. In the frequency calculations, the fixed buffer region was omitted. In the three-average approach, we did nine entropy calculations for the free protein, because the protein residues included in the entropy calculation differed for the various ligands.

*MM/GBSA with explicit water molecules*

In this paper, we aim at estimating the effect of water molecules on the binding of a ligand to a receptor. We will consider the role of water in the binding site, both before and after the binding. Therefore, we consider a binding reaction that is more detailed than Eqn. 1, viz.

$$\text{RW}_m + \text{L} \rightarrow \text{RW}_n\text{L} + (m - n)\,\text{W} \quad (6)$$

where W denotes water, and $m$ and $n$ are the number of water molecules in the binding site before and after the ligand binds, respectively. The binding energies of the ligand, as well as of the binding-site water molecules can in principle be estimated by the MM/GBSA approach. However, when there is more than one binding molecule, there are several ways to perform the calculations.

If we are interested only in effect (b) above, i.e. the effect of water molecules mediating the interactions between the ligand and the receptor, we could simply consider those water molecules as a part of the receptor and perform standard MM/GBSA calculations with a few explicit water molecules that are kept explicit in the calculations. Thus, the binding energies are calculated for the reaction

$$\text{RW}_n + \text{L} \rightarrow \text{RW}_n\text{L} \quad (7)$$

This has been done before [30,31,32,33,34,35,36,37,38] and it is simple to implement. You only have to identify the water molecules of interest and change the scripts so that they are considered as a part of the receptor.

However, if we want to include also the effect (a) above, i.e. the fact that different ligands can displace a varying number of water molecules, we need to explicitly estimate the binding energy also of the binding-site water molecules. This can in principle also be done by

MM/GBSA. In the simplest case, we can consider the reaction

$$R + W \rightarrow RW \tag{8}$$

which is analogous with Eqn. 1 with a water molecule as a ligand.

However, a direct application of MM/GBSA (Eqn. 2) on this reaction will not give the desired results. The reason for this is that when the water molecule is removed from the receptor, it will be replaced by continuum solvent. Therefore, we will get the difference in the interaction energy of an explicit or implicit water molecule to the receptor. To get the desired result, we have to replace the water molecule with a non-interacting ghost molecule, i.e. a water molecule with zeroed charges and van der Waals parameters, which prohibits continuum water to occupy the volume of this molecule. It should also have the same radii as a normal water molecule, so that the SASA term does not change.

In principle, a similar approach could be used to successively calculate the binding affinity of several water molecules or of both the ligand and one or more water molecules. Unfortunately, such individual binding energies will depend on the order of the binding of the molecules (the interaction energy depends on what molecules are defined as a part of the receptor). However, if we are not interested in the binding energy of each water molecule, we can instead simply study the net reaction:

$$R + n\,W + L \rightarrow RW_nL \tag{9}$$

If the calculations are made consistently and with ghost molecules, the sum of the separate binding energies of the $n$ water molecules and the ligand in any order will give the same net results.

Consequently, we performed two simulations, one of the free receptor and one of the ligand-bound complex. With the latter, we studied the reaction in Eqn. 9 and with the former, we studied corresponding reaction without any ligand:

$$R + m\,W \rightarrow RW_m \tag{10}$$

The difference of these two reactions give our full binding reaction in Eqn. 6. By studying these two reactions separately, we can still use the one-trajectory approach of MM/GBSA, which has a much better precision than the three-trajectory approach [81].

In practice, we actually calculated individual binding energies for each water molecule according to their distance to the ligand (distance between any atom in the ligand and the water oxygen atom), the water molecule with the shortest distance was assumed by bind first. Already considered (bound) water molecules were treated as part of the receptor, whereas those not yet considered were treated as ghost molecules. For the complex, we let the ligand bind in a final step, after all water molecules. Thereby, we can separate the effects from cases (a) and (b) above. However, we want to emphasize that the final energies are independent of the order of the binding of molecules, because the intermediate energy terms cancel and the only terms remaining are those of R and $RW_nL$ or $RW_m$ (cf. Eqn. 11 below).

We defined the active site of ferritin as the volume within 5 Å from ligand L09 and all water molecules with at least one atom within this distance were considered to be in the active site. For the simulations with other ligands than L09, we overlayed the structure with the starting structure of the complex with L09, employing all C, CA, and N atoms of residues within 10 Å of the ligand in the fit.

With such a definition, there will be a varying number of water molecules in the binding in different snapshots. We solved this problem by grouping the snapshots after the number of

water molecules. Averages were calculated only over snapshots with the same number of water molecules ($i$ or $j$ ) and the final $\Delta G_{\text{bind}}$ was obtained by summing the averages, weighted by the probability of observing this number of water molecules in the snapshot ($p_i$ and $p_j$ ):

$$\Delta G_{bind}^{1avW} = \sum_{i=\min(n)}^{\max(n)} p_i \langle G(RW_i L) - G(Rw_i l) - G(L) \rangle_{RL} - \sum_{j=\min(m)}^{\max(m)} p_j \langle G(RW_j) - G(Rw_j l) \rangle_R - (m-n) G(W) \quad (11)$$

where lower-case $l$ and $w$ denote ghost ligand and water molecules, respectively. The individual free energies were obtained from Eqn. 2.

Standard errors were calculated for each ensemble average in Eqn. 11 by dividing the standard deviations by the square root of the number of snapshots with the same number of water molecules in the binding site, and the total uncertainty was obtained by error propagation, weighting each standard error by the probabilities $p_i$ and $p_j$. Such uncertainties are based on all the 1600 snapshots, instead of only the results of the 40 independent simulations, as for the single- and three-average approaches, and are therefore expected to underestimate the true uncertainty. Therefore, we calculated standard errors for the single-average approach also based on all 1600 snapshots and compared them to those based on the 40 independent simulations. The quotient was 1.6–2.6 and therefore, we multiplied all uncertainties based on all 1600 snapshots by the average of this quotient over the nine ligands, 2.2.

*Quality measures*

The accuracy of the results relative to the experimental affinities [67] was estimated using the mean absolute deviation (MAD), the MAD after the systematic error (i.e. the mean signed error) had been removed (MADtr), the correlation coefficient ($r^2$; a negative sign of $r^2$ is used to indicate that $r$ is negative), and Kendall's rank correlation coefficient, calculated for the pairs of ligands for which both the experimental and calculated differences in affinities are statistically significant at the 95% level ($\tau_{95}$) [82]. The number of such significant pairs is also given ($np_{95}$). The uncertainty of the quality measures was estimated using a parametric bootstrap (using 1000 random samples) [83] using the uncertainty of both the experiments and computational predictions.

## Result and Discussion

In this paper, we study the binding of the nine phenol derivatives in Figure 1 to a dimer of ferritin. Our focus is on the effect of water molecules on the binding. As detailed in the Methods section, we have tested several different approaches that will be described in separate sections below.

*Standard MM/GBSA*

We first performed standard MM/GBSA calculations in which all water molecules were replaced by the GBSA continuum solvent. We tested both the single-average approach, Eqn. 4, in which only the RL complexes were simulated, and the three-average approach, Eqn. 5, in which separate MD simulations were performed also for the free protein and the free ligand. All results are collected in Table 1.

It can be seen that the single-average approach gives good results: The calculated

affinities are –1 to –46 kJ/mol, whereas the experimental affinities are –19 to –33 kJ/mol [67]. As usual, MM/GBSA overestimates the range of the affinities (46 vs. 14 kJ/mol) [22], but in this case the absolute values of the calculated affinities are quite close to the experimental ones. In fact, the mean absolute deviation (MAD) is only 8.6±0.3 kJ/mol and it decreases only marginally if the average signed error (–1 kJ/mol) is subtracted (MADtr = 8.5±0.3 kJ/mol). Moreover, the correlation coefficient ($r^2$) between the calculated and experimental affinities is nearly perfect, 0.90±0.02 and Kendall's tau value for significant pairs ($\tau_{95}$) is 0.94±0.01 (based on 32 of the 36 possible pairs). The standard error of each of the individual estimates is 1 kJ/mol, owing to the use of 40 independent simulations with 40 snapshots in each [18]. Of course, the good results of the standard MM/GBSA method are somewhat problematic, leaving little room for improvements with explicit water molecules.

Interestingly, the results become much worse if the three-average approach is instead used. As can be seen in Table 1, the calculated affinities become 109–197 kJ/mol more positive, making all predicted affinities positive and all quality measures bad. In particular, MADtr increases to 18±5 kJ/mol and the correlation becomes negative ($r$ = –0.5). This large effect comes almost entirely from the protein – the difference between average energies for the ligand obtained from the complex or free-ligand simulations differ by less than 5 kJ/mol. In particular, the van der Waals energy changes when the protein is allowed to relax – it is lowered by 57–154 kJ/mol. The electrostatic energy decreases even more, by ~600 kJ/mol, but this is mostly compensated by the GB solvation energy so that the net effect of the sum is only 50 kJ/mol on average. The other terms show smaller effects 7–8 kJ/mol on average, but the SASA energy decreases always, showing that the solvent-exposed surface area decreases for free protein. The standard errors of the three-average estimates are very large, 19–21 kJ/mol, explaining why the results are close to random. In fact, only one of the pair-wise differences between the nine ligands is statistically significant, but it has an incorrect sign ($\tau_{95}$ = –1) .

*Three-average MM/GBSA with 3–5 explicit water molecules as part of the receptor*

To include water molecules in the MM/GBSA calculations, we first studied the number of water molecules in the binding site in the various simulations. As is detailed in the Methods section, the number of water molecules was determined by overlaying each snapshot with the starting crystal structure of the smallest ligand, L09, and counting all water molecules within 5 Å of any atom in L09. The results are shown in Figure 2. For the protein without any bound ligand, there were 1–13 water molecules in the binding site, with three as the most common value, and five as the average value. However, the distribution is skewed towards higher values, indicating that the structure frequently opens up significantly.

For the complexes, the number is lower, 0–9. On average, all complexes have three water molecules in the binding site, except those with ligands L07 and L09, which have four water molecules. The number of water molecules follows quite systematically the size of the ligand, with the results for L07 indicating that the water molecules bind preferentially on one side of the ligand.

As a first approximation, we used the average number of water molecules in the calculations: We performed three-average MM/GBSA calculations based on the RL, R, and L simulations, but including five water molecules as a part of the free receptor and four (L07 and L09) or three water molecules as a part of the receptor in the calculations with the complex. The water molecules were always those closest to the ligand. Consequently, the binding energies were estimated from:

$$\Delta G_{\mathrm{bind}}^{\mathrm{3avW}} = \langle G(\mathrm{RW}_n\mathrm{L})\rangle_{\mathrm{RL}} - \langle G(\mathrm{RW}_5)\rangle_{\mathrm{R}} - \langle G(\mathrm{L})\rangle_{\mathrm{L}} + (5-n)G(\mathrm{W}) \qquad (12)$$

with $n$ = 3 or 4. No ghost ligands were used because the water molecules are replaced by the ligand.

It can be seen in Eqn. 12 that the estimates involve the $G$(W) term. This term was estimated from a standard MM/GBSA calculation. Since the geometry of a TIP3P water molecule is rigid, any water molecule in any snapshot will give the same energy, –103 kJ/mol, which comes from the GB solvation energy (–48 kJ/mol), the SASA term (4 kJ/mol), and the entropy ($-TS$ = –58 kJ/mol). The latter consists of translational (–43 kJ/mol) and rotational (–15 kJ/mol) contributions, whereas the vibrational part is negligible.

Using this value for $G$(W), we obtain the binding affinities included in Table 1, column 3avW. It can be seen that the results are slightly improved compared to the three-average approach. However, the results are still far from satisfactory, with positive affinities, MADtr = 17±5 kJ/mol, and $r^2$ = 0.0. The standard errors are slightly larger than those of the three-average approach, 20–22 kJ/mol (ignoring the uncertainties in $G$(W) and the number of water molecules), and the range of the calculated affinities is 4.5 times larger than for the experimental data, 62 kJ/mol.

*Single-average MM/PBSA with explicit water molecules*

Apparently, it is hard to obtain statistically converged results with the three-average approach, with or without explicit water molecules, in particular for this test case (~400 times more simulations are needed to bring the standard errors down to ~1 kJ/mol). Therefore, we instead tested an approach in which we calculated individual binding energies for each water molecule and ligand in the binding site. These were estimated by the single-average approach, based on simulations of either only RL or of both RL and R, ensuring more reasonable standard errors. In addition, we decided to consider only water molecules in the binding site, the number of which varies in the different snapshots with the frequencies shown in Figure 2.

In the first step, we concentrate on effect (b) discussed in the introduction (water molecules mediating the binding to the receptor). Therefore, we estimated the binding affinity of each ligand, keeping the 0–9 water molecules in the binding site as part of the receptor.

The free energies were calculated from

$$\Delta G_{bind}^{1\text{avng}} = \sum_{i=1}^{\max(n)} p_i \langle G(RW_iL) - G(RW_i) - G(L) \rangle_{RL} \qquad (13)$$

As detailed in the Methods section, snapshots with the same number of water molecules were grouped together ($i$ in Eqn. 13). Therefore, we got 6–9 different results for each complex (max(n) in Eqn. 13), depending on the number of water molecules in the complex.

There was often a quite large variation between the estimated binding affinities for the different number of water molecules, with ranges of 2–49 kJ/mol for the nine ligands. The snapshots with the smallest and especially the largest number of water molecules gave the most extreme values (because they are based on averages over only few snapshots). However, when weighting by the probability of finding a certain number of water molecules in the binding site ($p_i$ in Eqn. 13), stable averages were obtained (which actually do not differ from plain averages by more than 6 kJ/mol). These weighted averages are included in Table 1, column 1avng. It can be seen that these affinities are remarkably similarly to those obtained with the single-average approach, with differences of less than 6 kJ/mol. However, the calculated affinities reproduce the experimental data slightly better than the single-average approach, with a significantly better MADtr = 6.5±0.3 kJ/mol and a smaller range of 38

kJ/mol. The correlation and ranking is the same as for the single-average approach ($r^2$ = 0.89±0.03 and $\tau_{95}$ = 0.95±0.01), as is the uncertainty, 1 kJ/mol. Thus, the effect of water bridges between the ligand and the protein (effect (b) above) is quite small, but significant.

As a first step to include the (a) effect also (displacement of water), we can repeat the same calculations, but convert the ligand to a ghost molecule:

$$\Delta G_{bind}^{1avgh} = \sum_{i=\min(n)}^{\max(n)} p_i \langle G(RW_i L) - G(RW_i l) - G(L) \rangle_{RL} \quad (14)$$

Again, we performed 6–10 calculations per ligand, depending of the number of water molecules in the snapshots. The variation in the calculated binding affinity between the various snapshots was similar to what was found without the ghost ligand, 5–51 kJ/mol. The calculated affinities, averaged over the snapshots are listed in Table 1, column 1avgh. It can be seen that the effect of the ghost ligands is significant, although it is mainly a constant shift of all affinities by –15 to –29 kJ/mol. Therefore, the predictions are of a similar quality (but slightly worse), with a MADtr of 10.6±0.3 kJ/mol, $r^2$ = 0.86±0.03, and $\tau_{95}$ = 0.94±0.01. The uncertainty remains at 1 kJ/mol.

These energies are not intended to be used alone, but they should be combined with calculated affinities of the water molecules in the binding site, both for the complex and the free protein. In the free protein, each water molecule had a negative interaction energy ($G(\mathrm{RW}_m) - G(\mathrm{RW}_{m-1}\mathrm{w})$) of –10 to –77 kJ/mol, with an average of –48 kJ/mol (most negative in the snapshots with many waters and closest to zero in those with few waters). The interaction energies roughly become less favourable (negative) the more water molecules are removed (because they are removed according to their distance to the ligand). However, the total interaction energy of all water molecules is independent of the order they are removed. The weighted sum to remove all water molecules is –268.9±1.5 kJ/mol, which is close to the product of the average number of water molecules (5.5) and the average binding energy of each water, –261 kJ/mol.

Similar results are obtained for the water molecules in the protein–ligand complexes: The interaction energy of all water molecules is negative with a single exception, 5 to –89 kJ/mol with an average of –48 kJ/mol (i.e. the same as in the free protein). The weighted sum to remove all snapshot is 139–209 kJ/mol for the complexes with the nine ligands.

We can now subtract these water interaction energies for the complexes and the free protein and add to the binding energy of the ligand to the complex with $n$ explicit water molecules, according to Eqn. 11. To this sum, we should also add $(m - n)*G(\mathrm{W})$ for the various snapshots.

With the MM/GBSA value for $G$(W) (–103 kJ/mol), we get a decent correlation and ranking ($r^2$ = 0.82±0.02 and $\tau_{95}$ = 0.87±0.01), but the affinities are much too negative and with a too large range (–108 to –232 kJ/mol), and therefore MADtr is also large (28±1 kJ/mol; Table 1, column 1avW).

This indicates that the MM/GBSA estimate of $G$(W) is too negative. This is supported by the experimental hydration free energy of water, which is –26 kJ/mol [84], i.e. 18 kJ/mol less negative than the GBSA estimate. Likewise, the standard entropy of liquid water at 298 K is 70 J/mol/K [85], corresponding to $-TS$ = –21 kJ/mol. Thus, an experimental estimate of the $G$(W) term is –47 kJ/mol, i.e. very close to the average interaction energy of water molecules to both the free protein and the complexes.

Using this value of $G$(W) instead, we get a similar $r^2$ (0.81±0.04), a slightly better $\tau_{95}$ (0.92±0.01), and a much improved MADtr (9.0±0.8 kJ/mol). Although all three results are

slightly worse than for the single-average approach, none of the differences are statistically significant at the 95% level. The statistical uncertainties of the binding affinities are 2–3 times larger than for the single-average approach, 2.2–2.4 kJ/mol, coming mainly from the water binding energies and the fact that two estimates with a similar uncertainty (~1.5 kJ/mol) are combined.

Alternatively, we could use $G$(W) as a fitting parameter, although this would make the approach more empirical and it will loose its advantage of not containing any adjustable parameters. Figure 3 shows how the range, MAD, MADtr, and $r^2$ depend on the value of $G$(W). The best correlation, $r^2 = 0.82$, is obtained for $G$(W) in the range of –62 to –108 kJ/mol. On the other hand, the best MADtr (3 kJ/mol) is obtained for $G$(W) = –21 kJ/mol and the smallest range is found for $G$(W) = –16 kJ/mol (9 kJ/mol , i.e. slightly smaller than the experimental range of 14 kJ/mol). The smallest MAD (4 kJ/mol) is obtained for $G$(W) = –32 kJ/mol. Thus, it is not possible to optimise all quality measures simultaneously. Moreover, it is likely that the optimum value depends on the system and the methods used. For example, for the 3avW approach, all optimum results are obtained for appreciably more negative values of $G$(W) as can be seen in Figure 3b.

## Conclusions

In this article, we have introduced, tested, and discussed different methods to include explicit water molecules within the MM/GBSA framework. As a test case, we used the binding of nine phenol analogues to ferritin, because it has been shown that these ligands displace water molecules upon binding [65,67].

In a first step, we tried to include a number of explicit water molecules in the calculations as a part of the receptor. In variance to previous attempts with the same aim [30,31,32,33,34, 35,36,37,38], we did not use a fixed number of water molecules, but instead included all water molecules in the binding site, which varied in the different snapshots. Therefore, we grouped the snapshots after the number of water molecules and treated them separately, obtaining the final affinity as a weighted mean over the various numbers of water molecules in the binding site (Eqn. 13). The effect of the binding affinities was modest, up to 6 kJ/mol, but both the MADtr and the range of the affinities were improved. This seems to be a reasonable approach to include the effect of water molecules mediating the binding of a ligand to the receptor.

Next, we tried to include also the effect of displaced water molecules. Our hypothesis was that the MM/GBSA should be able to estimate the binding of a water molecule to the receptor in the same way as a ligand. The only difference is that the water molecules need to be converted to a non-interacting ghost molecules to avoid that the continuum solvation calculation includes their effects when not bound. A first attempt to include this effect with the three-average MM/GBSA approach and fixed number of water molecules failed, primarily owing to the very large uncertainty of the three-average approach, especially for this protein (20–22 kJ/mol).

Therefore, we instead suggested that these calculations should be based on two single-average calculations, one for the complex and one for the free receptor. In both cases, the binding affinity of all water molecules in the binding site is estimated, together with the affinity of the ligand in the complex calculation. Both the ligand and the water molecules are converted to ghost molecules when unbound. Again, we allowed for a different number of water molecules in the various snapshots by grouping the snapshots and calculating the results as a weighted average over the snapshots (Eqn. 11). We show that this approach works well and gives a reasonable uncertainty.

The only problem with the approach is the free energy of an unbound water molecule,

*G*(W), which enters all approaches with displaced water molecules. It seems that MM/GBSA estimate, –103 kJ/mol is too negative. The reason for this is partly the poor estimate of the solvation energy of a water molecule by the present GB method [78], giving an error of 18 kJ/mol, which is unusually large for a GB method, which typically give mean absolute errors of 4–6 kJ/mol for small neutral molecules [86]. Of course, this estimate will change if the continuum solvation method is changed. However, the main reason for the overestimate is the entropy, which is estimated by Sackur–Tetrode equation, valid for an ideal gas, but probably not for water in aqueous solution. This problem has been thoroughly discussed in other contexts [85,87,88,89], and the MM/GBSA entropy is 37 kJ/mol more negative than the standard entropy of liquid water. Further studies with much larger test sets are needed to settle the appropriate value of *G*(W); for time being, we recommend the experimental estimate, –47 kJ/mol. For this value, we obtain good results for our test case, showing that our method is a promising approach to include the effect of water molecules in MM/GBSA.

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**References**


1 Gohlke H, Klebe G (2002) Angew Chem Int 41:2644-2676
2 Sharp KA in Protein–ligand interactions, H Gohlke, ed Wiley-VCH Verlag GmbH & Co, 2012, pp 3-22
3 Rajmani R, Good AC (2007) Curr Opin Drug Discov Develop10:308-315
4 Sotriffer C in Protein–ligand interactions, H Gohlke, ed Wiley-VCH Verlag GmbH & Co, 2012, pp 237-263
5 Michel J, Essex JW (2010) J Comput-Aided Mol Design 24:639-658
6 Steinbrecher T in Protein–ligand interactions, H Gohlke, ed Wiley-VCH Verlag GmbH & Co, 2012, pp 207-236
7 Lee FS, Chu ZT, Bolger MB, Warshel A (1992) Protein Eng 5:215-228
8 Singh N, Warshel A (2010) Proteins 78:1705-1723
9 Åqvist J, Medina C, Samuelsson J-E (1994) Protein Eng 7:385-391
10 Hansson T, Marelius J, Åqvist J (1998) J Comput-Aided Mol Design 12:27-35
11 Brandsdal BO, Östberg FM, Almlöf M, Feierberg I, Luzhkov VB, Åqvist J (2003) Adv Prot Chem 66:123-158
12 Srinivasan J, Cheatham TE, Cieplak P, Kollman PA, Case DA (1998) J Am Chem Soc 37:9401-9409
13 Kollman PA, Massova I, Reyes I, Kuhn B, Huo S, Chong L, Lee M, Lee T, Duan Y, Wang W, Donini O, Cieplak P, Srinivasan J, Case DA, Cheatham TE (2000) Acc Chem Res 33:889-897
14 Foloppe N, Hubbard R (2006) Curr Med Chem 13:3583-3608
15 Huang N, Jacobson MP (2007) Curr Opin Drug Discov Develop 10:325-331
16 Weis A, Katebzadeh K, Söderhjelm P, Nilsson I, Ryde U (2006) J Med Chem 49:6596-6606
17 Kongsted J, Ryde U (2009) J Comput Aided Mol Design 23:63-71
18 Genheden S, Ryde U (2010) J Comput Chem 31:837-846
19 Pearlman DA, (2005) J Med Chem 48:7796-7807
20 Kawatkar S, Wang H, Czerminski R, Joseph-McCathy D (2009) J Comp-Aided Mol Des 23:527-539
21 Mikulskis P, Genheden S, Wichmann K, Ryde U (2012) J Comput Chem 33:1179-1189
22 Mikulskis P, Genheden S, Rydberg P, Sandberg L, Olsen L, Ryde U (2012) J Comput-Aided Mol Design 26:527-541
23 Genheden S, P Mikulskis, L Hu, Kongsted J, Söderhjelm P, Ryde U (2011) J Am Chem Soc 133:13081-13092
24 de Beer SBA, Vermeulen NPE Oostenbrink C (2010) Curr Top Med Chem 10:55-66
25 Wong SE, Lightstone FC (2011) Expert Opinion Drug Discov 6:65
26 Baron R, Setny P, McCammon JA in Protein–ligand interactions, H Gohlke, ed Wiley-VCH Verlag GmbH & Co, 2012, pp 145-170
27 Chiba S, Harano Y, Roth R, Knoshita M, Sakurai M (2012) J Comput Chem 33:550-560
28 Snyder PW, Mecinović J, Sherman W, Moustakas DT, Thomas SW, Harder M, Mack ET, Lockett MR, Héroux A, Sherman W, Whitesides GM (2011) Proc Natl Ac Sci USA 44:17889-17894
29 Breiten B, Lockett MR, Sherman W, Fujita S, Al-Sayah M, Lange H, Bowers CM, Héroux A, Krilov G, Whitesides GM (2011) J Am Chem Soc 135:15579-15584
30 Spackova N, Cheatham TE, Ryjacek F, Lankas F, van Meervelt L, Hobza P, Sponer J (2003) J Am Chem Soc 125:1759-1769
31 Masukawa KM, Kollman PA, Kuntz ID (2003) J Mol Catal 46:5628-5637
32 Lepsik M, Kriz Z, Havlas Z (2004) Proteins 57:279-293

33 Treesuwan W, Hannongbua S (2009) J Mol Graph Model 27:921-929
34 Das D, Koh Y, Tojo Y, Ghosh AK, Mitsuya H (2009) J Chem Inf Model 49:2851-2862
35 Wong S, Amaro RE, McCammon JA (2009) J Chem Theory Comput 5:422-429
36 Nurisso A, Blanchard B, Audfray A, Rydner L, Oscarson S, Varrot A, Imberty A (2010) 285:20316-20327
37 Wallnoefer HG, Liedl KR, Fox T, (2011) J Comput Chem 32:1743-1752
38 Huang Y, Rizzo RC (2012) Biochem 51:2390-2406
39 Greenidge PA, Kramer C, Mozziconacci J-C, Wolf RM (2012) J Chem Inf Model 53:201-209
40 Grimme S (2012) Chem Eur J 18:9955
41 Mikulskis P, Cioloboc D, Andrejic M, Khare S, Brorsson J, Genheden S, Mata R, Söderhjelm P, Ryde U (2014) J. Comp.-Aided Mol. Design, in press.
42 Klamt A, J Phys Chem 1995, 99, 2224
43 Kozisek M, Bray J, Rezacova P, Saskov K, Brynda J, Pokorna J, Mammano F, Rulisek L, Konvalinka J (2007) J Mol Biol 374:1005-1016
44 Wade RC, Mazor MH, McCammon JA, Quiocho FA (1990) J Am Chem Soc 112:7057–7059
45 Hamelberg D, McCammon JA (2004) J Am Chem Soc 126:7683–7689
46 BarillariC , TaylorJ , Viner R, Essex JW (2007) J Am Chem Soc 129:2577-2587
47 Marrone T, Resat H, Hodge C, Chang C-W, McCammon JA (1998) Proteins 7:573–579
48 Woo H-J, Dinner AR, Roux B (2004) J Chem Phys 121:6392–6400
49 Michel J, Tirado-Rives J, Jorgensen WL (2009) J Am Chem Soc 131:15403-15411
50 Ross GA, Morris GM, Biggin PC (2012) PLOS One 7:e32036
51 Michel J, Tirado-Rives J, Jorgensen WL (2009) J Phys Bhem B 113:13337-13346
52 Abel R, Young T, Farid R, Berne B J, Friesner R A (2008) J Am Chem Soc 130:2817–2831
53 Clausen RP, Naur P, Kristensen AS, Greenwood JR, Strange M, Brauner-Osborne H, Jensen AA, Nielsen AST, Geneser U, Ringgaard LM, Nielsen B, Pickering DS, Brehm L, Gajhede M, Krogsgaard-Larsen P, Kastrup J S (2009) J Med Chem 52:4911–4922
54 Lazardis T (1998) J Phys Chem B 102:3542-3550
55 Huggins DJ (2012) Phys Chem Chem Phys 14:15106-15117
56 Riniker S, Barandun LJ, Diederich F, Krämer O, Steffen A, van Gunsteren WF (2012) J Comput-Aided Mol Des 26:1293-1309
57 Luccarelli J, Michel J, Tirado-Rives J, Jorgensen WL (2010) J Chem Theory Comput 6:3850-3856
58 Beuming T, Farid R, Sherman W (2009) Prot Sci 18:1609-1619
59 Pearlstein RA, Hu Q-Y Zhou J, Yowe D, Levell J, Dale B, Kaushik VK, Daniels D, Hanrahn SD, Sherman W, Abel R (2010) Proteins 78:2571-2586
60 Wang L, Berne BJ, Friesner RA (2011) Proc Natl Acad Sci 108:1326-1330
61 Robinson DD, Sherman W, Farid R (2010) Chem Med Chem 5:618
62 Guimarães CR, Mathiowetz AM (2010) J Chem Inf Model 50:547-559
63 Kohlmann A, Zhu X, Dalgarno D (2012) ACS Med Chem Letters 3:94-99
64 Abel R, Salam NK, Shelley JK, Farid R, Friesner RA, Shermay W (2011) Chem Med Chem 6:1049-1066
65 Vedula L S, Brannigan G, Economou N J, Xi J, Hall M A, Liu R, Rossi M J, Dailey W P, Grasty K C, Klein ML, Eckenhoff RG, Loll P J (2009) J Biol Chem 284:24176-24184
66 Mikulskis P, Genheden S, Wichmann K, Ryde U (2012) J Comput Chem 33:1179-1189
67 Vedula, L. S.; Brannigan, G.; Economou, N. J.; Xi, J.; Hall, M. A.; Liu, R.; Rossi, M. J.; Dailey, W. P.; Grasty, K. C.; Klein, M. L; Eckenhoff, R. G.; Loll, P. J. J. Biol. Chem.

2009, 284, 24176-24184.
68 Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C (2006) Proteins 65:712
69 Wang JM, Wolf RM, Caldwell KW, Kollman PA, Case DA (2004) J Comput Chem 25:1157
70 Bayly CI, Cieplak P, Cornell WD, Kollman P A (1993) J Phys Chem 97:10269
71 Besler BH , Merz KM, Kollman PA (1990) J Comput Chem 11:431
72 Jorgensen WL, Chandrasekhar J, Madura JD, Impley RW, Klein ML (1983) J Chem Phys 79:926
73 Case DA, Darden TA, Cheatham TE, Simmerling CL, Wang J, Duke RE, Lou R, Walker RC, Zhang W, Merz KM, Roberts B, Wang S, Hayik B, Roitberg A, Seabra G, Kolossváry I, Wong KF, Paesani F, Vanicek J, Wu X, Brozell SR, Steinbrecher T, Gohlke H, Cai Q, Ye X, Wang J, Hsieh M-J, Cui G, Roe DR, Mathews DH, Seetin M G, Sagui C, Babin V, Luchko T, Gusarov S, Kovalenko A, Kollman PA AMBER 11, University of California, San Francisco, 2010
74 Wu X, Brooks BR (2003) Chem Phys Lett 381:512
75 Berendsen HJC, Postman JPM, van Gunsteren WF, DiNola A, Haak JR (1984) J Chem Phys 81:3684
76 Darden T, York D, Pedersen L (1993) J Chem Phys 98:10089
77 Ryckaert JP, Ciccotti G, Berendsen HJC (1977) J Comput Phys 23:327
78 Onufriev A, Bashford D, Case DA (2004) Proteins 55:383–394
79 Kuhn B, Kollman P A (2000) J Med Chem 43:3786
80 Genheden S, Kuhn O, Mikulskis P, Hoffmann D, Ryde U (2012) J Chem Inf Model 52:2079-2088
81 Genheden S, Ryde U (2012) Proteins 80:1326-1342
82 Mikulskis P, Genheden S, Rydberg P, Sandberg L, Olsen L, Ryde U (2012) J Comput-Aided Mol Design 26:527-541
83 Genheden S, Ryde U (2010) J Comput Chem 31:837
84 Marenich AV, Olson RM, Kelly CP, Cramer CJ, Truhlar DG (2007) J Chem Theory Comput 3:2011-2033
85 Dunitz JD (1994) Science 264:670
86 Kongsted J, Söderhjelm P, Ryde U (2009) J Comp-Aided Mol Design 23:395-409
87 Amzel LM Proteins (1997) Struct Funct Genet 28:144
88 Rulíšek L, Jensen KP, Lundgren K, Ryde U (2006) J Comput Chem 27:1398-1414
89 Irudayam SJ, Henchman RH, (2009) J Phys Chem B 113:5871-5884

**Table 1.** Calculated binding affinities and standard errors (kJ/mol) for the various variants of MM/GBSA, the standard single-average (1av) and three-average (3av) approaches, the 3av approach with 3–5 explicit water molecules (3avW), the 1av approach based on only the RL simulations with 0–9 water molecules treated as part of the receptor and without (1avng) or with (1avng) converting the ligand to a ghost molecule, and the full explicit-water 1av approach, based on both the RL and R simulations (1avW). The master equation for each method is given in the Eqn. row. In applicable cases, the value of $G$(W) used (kJ/mol) is also noted. In addition, the experimental data [67] are also included (Exp). The second part of the table contains the quality measures of the various results.

| Method | 1av | 3av | 3avW | 1avng | 1avgh | 1avW | | Exp. |
|---|---|---|---|---|---|---|---|---|
| Eqn. | 4 | 5 | 12 | 13 | 14 | 11 | | |
| $G$(W) | | | –103 | | | –103 | –47 | |
| L01 | -38.0±0.9 | 106±21 | 52±21 | -39.2±0.9 | -61.8±0.9 | -217.5±2.3 | -73.9±2.3 | -30.5±0.1 |
| L02 | -46.5±1.2 | 134±21 | 73±22 | -45.8±1.0 | -75.3±0.9 | -231.5±2.2 | -77.2±2.2 | -30.1±0.5 |
| L03 | -42.7±1.0 | 154±19 | 95±20 | -42.4±0.9 | -68.6±0.9 | -231.8±2.2 | -75.3±2.2 | -32.7±0.4 |
| L04 | -37.8±0.8 | 135±19 | 75±20 | -37.6±0.9 | -62.0±0.9 | -211.2±2.2 | -70.5±2.2 | -30.6±0.2 |
| L05 | -30.5±0.8 | 95±20 | 33±21 | -31.5±0.9 | -51.5±0.9 | -190.7±2.3 | -59.5±2.3 | -28.2±0.1 |
| L06 | -21.7±0.7 | 117±21 | 57±22 | -23.8±0.9 | -40.3±0.9 | -172.5±2.3 | -54.4±2.3 | -25.9±0.1 |
| L07 | -21.1±0.8 | 102±21 | 74±21 | -24.4±0.9 | -43.4±0.9 | -153.7±2.3 | -52.1±2.3 | -27.4±0.1 |
| L08 | -17.3±0.7 | 107±21 | 49±21 | -22.1±0.9 | -38.2±0.9 | -176.3±2.3 | -59.4±2.3 | -22.8±0.1 |
| L09 | -0.8±1.0 | 109±20 | 90±21 | -7.3±0.8 | -22.3±0.8 | -107.5±2.4 | -29.3±2.4 | -18.7±0.1 |
| Range | 45.7 | 56 | 62 | 38.5 | 52.9 | 124.3 | 47.9 | 14.0 |
| MAD | 8.6±0.3 | 145±7 | 94±7 | 6.8±0.3 | 24.1±0.3 | 160.7±0.7 | 33.8±0.7 | |
| MADtr | 8.5±0.3 | 18±5 | 17±5 | 6.5±0.3 | 10.6±0.3 | 28.3±0.8 | 9.0±0.8 | |
| $r^2$ | 0.90±0.02 | 0.3±0.2 | 0.0±0.1 | 0.89±0.03 | 0.86±0.03 | 0.82±0.02 | 0.81±0.04 | |
| $\tau_{95}$ | 0.94±0.01 | -1.0±0.3 | -1.0±0.2 | 0.93±0.01 | 0.94±0.01 | 0.87±0.01 | 0.92±0.01 | |
| $np_{95}$ | 32 | 1 | 1 | 30 | 32 | 31 | 26 | |

**Figure 1.** The nine phenol derivatives L1–L9 used in this study (Et, Pr, iPr and sBu denote ethyl, *n*-propyl, isopropyl and secondary butyl groups, respectively).

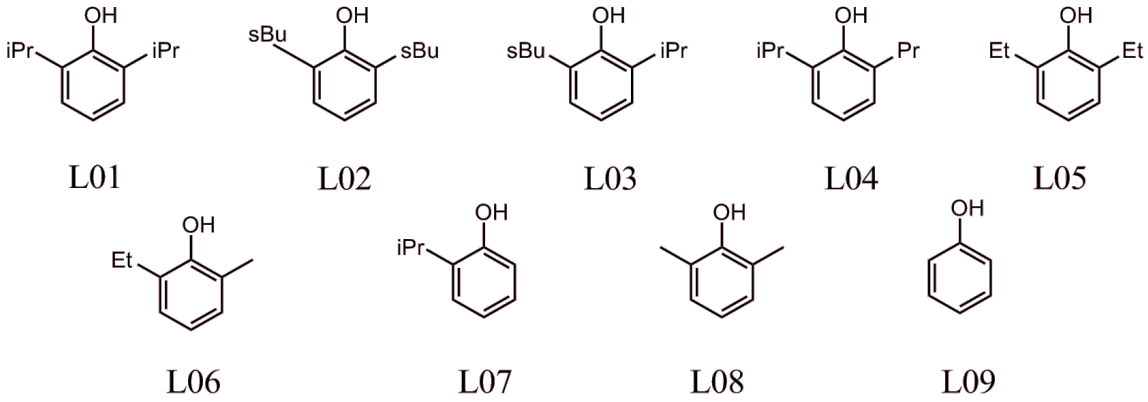

**Figure 2.** Probability of finding different numbers of water molecules in the binding site of ferritin for the simulations without (No) or with the various ligands. The number in the legend shows the average number of water molecules over the 1600 snapshots.

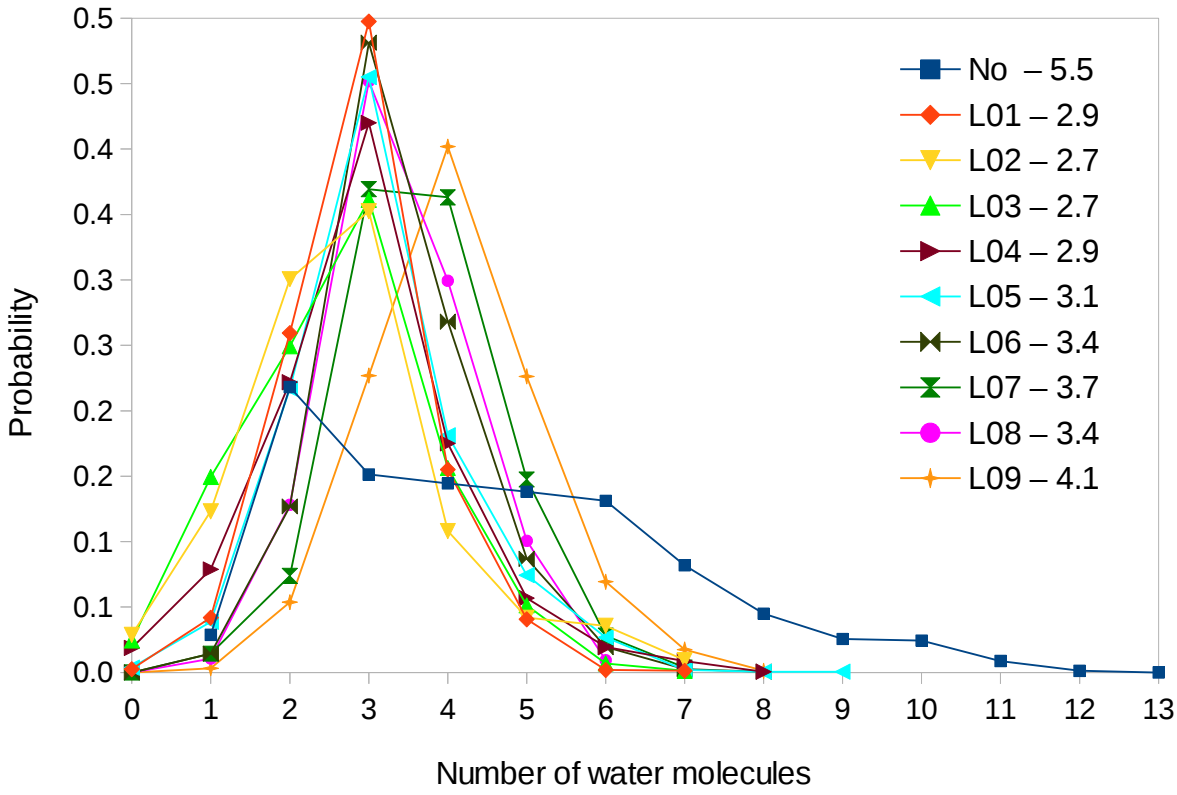

**Figure 3.** Dependence of the range, MAD, MADtr (left axis) and $r^2$ (right axis) of the calculated affinities obtained with the 1avW (top) and 3avW (bottom) approaches as a function of $G$(W). Note the different scales of $G$(W) in the two figures. The MM/GBSA estimate of $G$(W) is –103 kJ/mol and the experimental estimate is –47 kJ/mol.