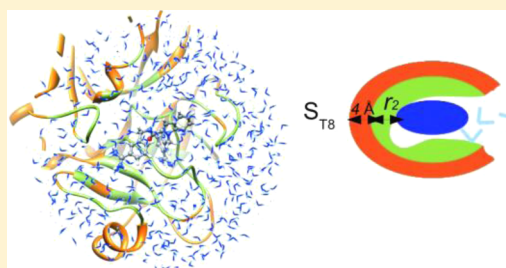


# The Normal-Mode Entropy in the MM/GBSA Method: Effect of System Truncation, Buffer Region, and Dielectric Constant

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**ABSTRACT:** We have performed a systematic study of the entropy term in the MM/GBSA (molecular mechanics combined with generalized Born and surface-area solvation) approach to calculate ligand-binding affinities. The entropies are calculated by a normal-mode analysis of harmonic frequencies from minimized snapshots of molecular dynamics simulations. For computational reasons, these calculations have normally been performed on truncated systems. We have studied the binding of eight inhibitors of blood clotting factor Xa, nine ligands of ferritin, and two ligands of HIV-1 protease and show that removing protein residues with distances larger than 8–16 Å to the ligand, including a 4 Å shell of fixed protein residues and water molecules, change the absolute entropies by 1–5 kJ/mol on average. However, the change is systematic, so relative entropies for different ligands change by only 0.7–1.6 kJ/mol on average. Consequently, entropies from truncated systems give relative binding affinities that are identical to those obtained for the whole protein within statistical uncertainty (1–2 kJ/mol). We have also tested to use a distance-dependent dielectric constant in the minimization and frequency calculation ( $\epsilon = 4r$ ), but it typically gives slightly different entropies and poorer binding affinities. Therefore, we recommend entropies calculated with the smallest truncation radius (8 Å) and  $\epsilon = 1$ . Such an approach also gives an improved precision for the calculated binding free energies.



## INTRODUCTION

MM/GBSA is an approximate method to estimate the absolute binding free energy,  $\Delta G_{\text{bind}}$ , of a ligand L to a biomacromolecule, e.g., a protein P, forming a complex PL. It estimates  $\Delta G_{\text{bind}}$  as the difference in free energy between PL, P, and L, viz.  $\Delta G_{\text{bind}} = G(\text{PL}) - G(\text{P}) - G(\text{L})$ . Each of these three free energies are estimated from the following sum<sup>1,2</sup>

$$G = \langle E_{\text{int}} + E_{\text{vdW}} + E_{\text{ele}} + G_{\text{solv}} + G_{\text{np}} - TS_{\text{MM}} \rangle \quad (1)$$

where the brackets indicate an average over snapshots from a molecular dynamics (MD) simulation. The first three terms on the right-hand side are the molecular mechanics (MM) internal (i.e., bonds, angles, and dihedral), van der Waals, and electrostatic energies,  $G_{\text{solv}}$  and  $G_{\text{np}}$  are the polar and nonpolar solvation free energies, and the last term is the absolute temperature multiplied by an entropy estimate. The latter estimate is usually taken as a combination of translational, rotational, and vibrational terms. All the terms are calculated on a system where water molecules from the simulation have been stripped off. Moreover, typically only the complex PL is simulated, and the free energies of P and L are obtained from the same simulation by simply deleting the coordinates of the other species.<sup>3,4</sup> Thereby, the  $E_{\text{int}}$  term cancels exactly, and the precision is improved by a factor of  $\sim 5$ .<sup>5,6</sup>

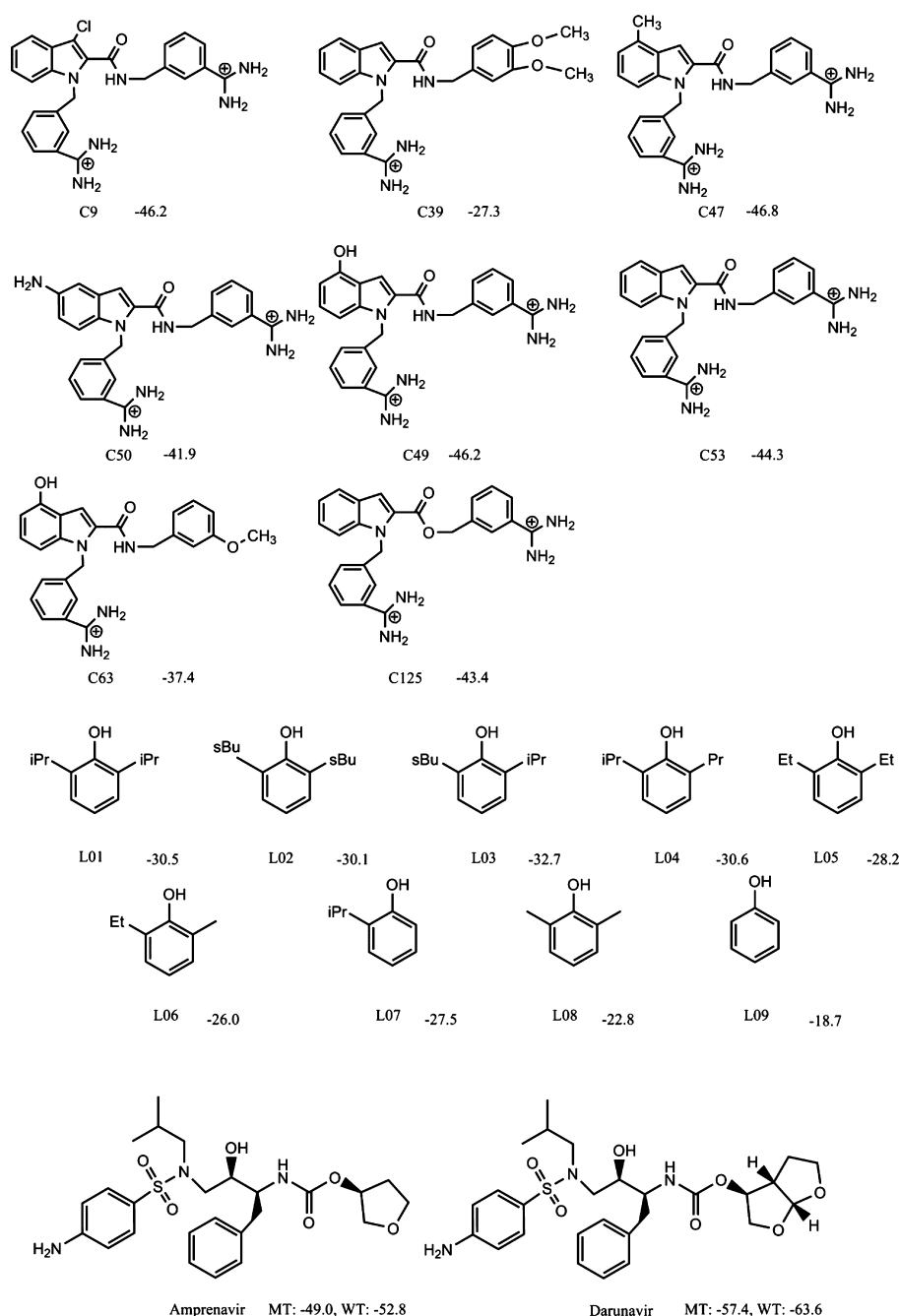
The most common method to estimate the vibrational entropy in the MM/GBSA method is to use frequencies from a normal-mode analysis (NMA)<sup>7</sup> performed at the MM level. In

a few studies, alternative methods have been tested, e.g., counting the number of rotatable bonds in the ligand, dihedral-distribution histogramming, or quasi-harmonic analysis.<sup>8–12</sup> The latter two estimates seem to be very hard to converge to any useful precision, whereas NMA estimates of entropies converge much better.<sup>10–12</sup> In practice, the NMA calculations are performed by first stripping off all water molecules. Typically, the protein is also truncated by removing all residues more than  $\sim 8$  Å from the ligand<sup>2</sup> because the frequency calculations are very demanding in terms of computer time and memory. Then, each truncated snapshot is minimized, and harmonic frequencies are calculated. In order to account for solvent effects in an approximate manner (i.e., to substitute for the deleted explicit water molecules), these calculations are often performed with a distance-dependent dielectric constant, viz.  $\epsilon = 4r$ .

However, it has been shown that such an approach gives large fluctuations in the calculated entropies and, therefore, a large statistical uncertainty, which typically limits the overall precision of the MM/GBSA method.<sup>13,14</sup> The reason for this is probably that the minimization of the truncated and unconstrained system with the approximate  $\epsilon = 4r$  may give rise to large and varying changes in the structure, compared to the snapshots of the full and explicitly solvated system. Kongsted

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**Figure 1.** Ligands used in this study. Et, iPr, and sBu indicate ethyl, isopropyl, and secondary butyl groups, respectively. The experimental affinities are shown in kJ/mol.<sup>18,19,49</sup>

and Ryde have suggested an approach to solve this problem by introducing a buffer region around the truncated system.<sup>8</sup> This buffer region typically contains all protein residues and water molecules within 4 Å of the truncated protein system. It is kept fixed during the minimization of the system, and it is excluded from the frequency calculation. The buffer ensures that there are no major changes in the structure during the minimization. Thereby, the questionable use of the distance-dependent dielectric constant can also be avoided. Test calculations showed that this simple modification improved the precision of the entropy term by a factor of 2–4, ensuring that it no longer limits the precision of MM/GBSA.<sup>8</sup>

In this article, we present a detailed analysis of the NMA entropy. We investigate the size-dependence of the truncation

and study how well truncated systems reproduce calculations with the full protein. Furthermore, we test whether a distance-dependent dielectric constant affects the results. As test cases, we use eight inhibitors of the blood-clotting factor Xa (fXa), nine inhibitors of ferritin, and two ligands of wild-type and a mutant HIV-1 protease. These systems have been used in previous studies of free energy methods.<sup>15–17</sup>

## METHODS

**Preparation of Proteins and Ligands.** In this paper, we study eight 3-amidinobenzyl-1*H*-indole-2-carboxamide inhibitors of fXa,<sup>18</sup> nine inhibitors to ferritin, as well as the two inhibitors amprenavir (APV) and darunavir (DRV) binding to

either wild-type (WT) or a double mutant (V82T/I84V, MT) HIV-1 protease (HIV-PR). All ligands are shown in Figure 1.

The preparation of the fXa and ferritin ligands has been previously described.<sup>15,16</sup> The fXa simulations were based on the crystal structure of fXa in complex with ligand C125 (PDB id. 1LPK),<sup>18</sup> and the preparation of the protein has been described previously.<sup>8</sup> The ferritin simulations were based on the crystal structure of ferritin in complex with phenol (L09; PDB id. 3f39),<sup>19</sup> and the preparation of the protein has been described previously.<sup>20</sup> The ligands were described by the general Amber force field<sup>21</sup> (GAFF) with charges from restrained electrostatic potentials (RESP)<sup>22</sup> calculations at the HF/6-31G\* level, and the proteins were described with the Amber99<sup>23</sup> (fXa) or Amber99SB<sup>24</sup> (ferritin) force field. All complexes were solvated in truncated octahedral boxes of TIP3P water molecules, extending at least 10 Å from the protein.<sup>25</sup>

The HIV-PR simulations of WT-APV, MT-APV, WT-DRV, and MT-DRV were based on the corresponding crystal structures with PDB ids. 1HPV, 1T7J, 1T3R, and 1T7I,<sup>26,27</sup> respectively. Crystal water molecules were removed, except one structurally important water molecule between the protease flaps and inhibitor. One of the catalytic Asp residues was protonated (Asp 25) on the basis of considerations made by Hou et al.,<sup>28</sup> whereas all the other Asp and Glu residues were negatively charged and all Arg and Lys residues were positively charged. The two His residues were protonated on the NE2 atom. The protein was described with the Amber03 force field<sup>29</sup> and the ligands with GAFF. The ligands were optimized at the HF/6-31G\*\* level using Gaussian09.<sup>30</sup> The electrostatic potential (ESP) was then calculated at the B3LYP/cc-pVTZ level with the polarizable continuum model<sup>31</sup> and a dielectric constant of  $\epsilon = 4$  at points sampled with the Merz–Kollman scheme.<sup>32</sup> Point charges were fitted to the ESP using the RESP<sup>22</sup> procedure with the antechamber program. All complexes were immersed in a truncated octahedral box of TIP3P water molecules<sup>25</sup> extending 10 Å from the protein. Finally, between five and seven chlorine ions were added to neutralize the system.

**MD Simulations.** The MD simulations of the fXa and ferritin complexes were conducted by the Amber10 software,<sup>33</sup> and they were taken from previous studies.<sup>15,16</sup> The simulations of the HIV-PR complexes were conducted either with the sander module of Amber 10 (equilibration) or the GPU version of the Amber 11 pmemd module (production). The temperature was kept constant at 300 K using a Langevin thermostat,<sup>34</sup> and the pressure was kept constant at 1 atm using a weak-coupling algorithm.<sup>35</sup> The nonbonded pair list was updated every 25th step, and the nonbonded cutoff was 8 Å (fXa or ferritin) or 9 Å (HIV-PR). Long-range electrostatics and van der Waals interactions were treated using particle-mesh Ewald summation<sup>36</sup> and a continuum approach, respectively. SHAKE constraints<sup>37</sup> were applied to all bonds involving hydrogen atoms, and the time step was 2 fs.

The fXa and ferritin complexes were simulated as follows. First, the system was optimized by 500 steps of steepest descent minimization, keeping all atoms, except water molecules and hydrogen atoms, restrained to their starting positions with a force constant of 418 kJ/mol/Å<sup>2</sup>. The minimization was followed by 20 ps MD equilibration with a constant pressure, and the restraining force reduced to 214 kJ/mol/Å<sup>2</sup>. Finally, a 1200 ps simulation at a constant pressure but without any restraints was performed, and the final 200 ps were used for

energy calculations. For each complex, we ran 40 independent simulations by assigning different starting velocities to atoms and extracted snapshots every 5 ps so that in total we evaluated MM/GBSA energies on  $40 \times 40 = 1600$  snapshots. This has been shown to be a good protocol on several test cases.<sup>38</sup>

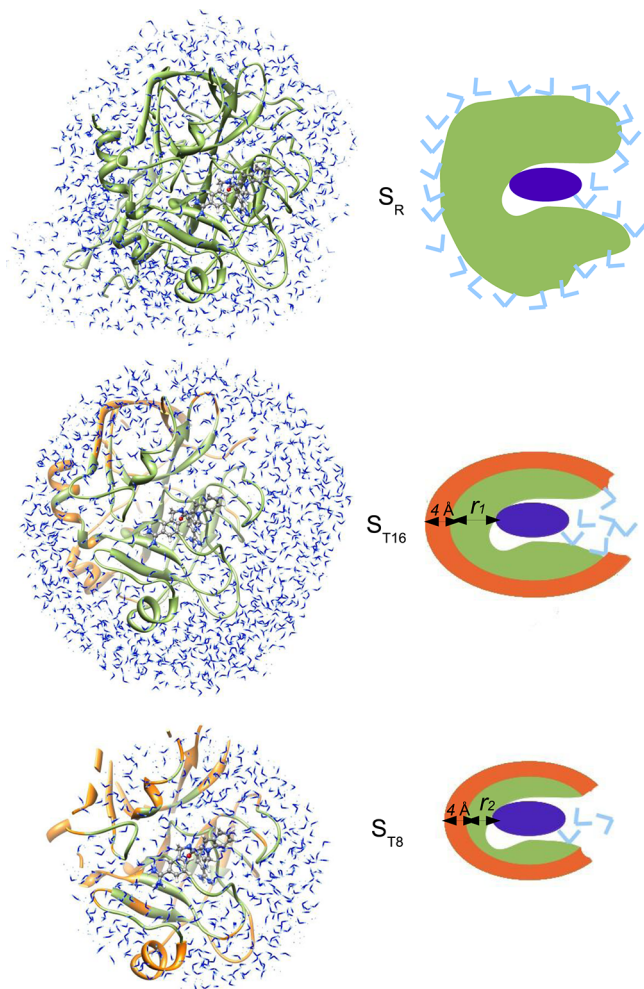
The HIV-PR complexes were simulated as follows. Water molecules and hydrogen atoms were relaxed first using 100 cycles steepest descent followed by 100 cycles conjugate gradient with all protein and ligand heavy atoms restrained with a force constant of 17 kJ/mol/Å<sup>2</sup>. The solute was relaxed by 1000 cycles steepest descent followed by 1000 cycles conjugate gradient without restraints. Initial velocities were randomly assigned from a Maxwell-Boltzmann distribution. The system was then gradually heated from 0 to 300 K over 50 ps and equilibrated for another 100 ps in the NVT ensemble. Finally, 1 ns production simulations were carried out in the NPT ensemble. For each system, equilibration and production steps were repeated 20 times with both different random seeds and different initial water boxes.<sup>39</sup> Snapshots were extracted every fifth picosecond, so that in total we evaluated the MM/GBSA energies on  $200 \times 20 = 4000$  snapshots.

**MM/GBSA Calculations.**  $\Delta G_{\text{bind}}$  was calculated according to eq 1. The terms were calculated with Amber 10 with all 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 generalized Born (GB) model of Onufriev et al.,<sup>40</sup> model I ( $\alpha = 0.8$ ,  $\beta = 0$ , and  $\gamma = 2.91$ ). The nonpolar solvation energy was estimated from the solvent-accessible surface area (SASA) according to  $G_{\text{np}} = 0.0227 \text{ SASA } (\text{\AA}^2) + 3.85 \text{ kJ/mol}$ .<sup>41</sup>

The translational and rotational entropy was calculated with standard statistical mechanics formulas.<sup>2,7</sup> The vibrational entropies were estimated from the vibrational frequencies using the ideal-gas rigid-rotor harmonic-oscillator approximation.<sup>7</sup> As mentioned in the introduction, the systems were minimized before the frequencies were calculated. Both the minimization and the frequency calculations were performed in a vacuum.

Several approaches were employed for the entropy calculations, differing in the considered system and the dielectric constant. In one set of calculations, called  $S_{\text{F}}$  (full) in the following, the entire complex was considered but no water molecules. The second set of calculations included the full complex but also a 4 Å shell of explicit water molecules surrounding the complex (Figure 2). This buffer was kept fixed during the minimization and the frequency calculations, and no frequencies and entropies were calculated for this buffer.<sup>8</sup> This approach will be denoted  $S_{\text{R}}$  (reference). In the third approach, all protein residues (but no water molecules) within a radius  $R$  of the ligand (including the ligand itself) were included in the calculations together with a buffer that consisted of all residues within 4 Å of the former residues, as well as all water molecules within  $R + 4$  Å of the ligand (Figure 2). Again, the buffer region was kept fixed during the minimization and the frequency calculation. Radii of  $R = 8, 12, 14$ , and  $16$  Å were tested (the radii of the protein–ligand complexes are 35, 40, and 25 Å for fXa, ferritin, and HIV-PR, respectively). These calculations will be denoted by  $S_{\text{TR}}$ , e.g.,  $S_{\text{T8}}$ . All three types of calculations were performed in two variants viz. with a unit dielectric constant ( $\epsilon = 1$ ) and with a distance-dependent dielectric constant,  $\epsilon = 4r$ , where  $r$  is the distance between the involved atoms. The latter calculations will be suffixed by  $r$ , e.g.,  $S_{\text{FR}}$ ,  $S_{\text{RR}}$ , and  $S_{\text{TR}}$ . The six





**Figure 2.** Illustration of the truncation approaches. Water molecules are shown in blue, protein residues that are included in the frequency calculations are shown in green, and protein residues that are in the buffer region are shown in orange. The ligand is shown in ball-and-stick representation. The protein is fXa with C53.

approaches are summarized in Table 1. The protein entropy was calculated with the same methods as for the complex but with the ligand omitted. The ligand entropy was always calculated for the isolated ligand but with either  $\epsilon = 1$  or  $\epsilon = 4r$ . Throughout this article, all entropies are reported in energy

**Table 1. Summary of Entropy Methods Employed in This Study.<sup>a</sup>**

Abbreviation	Residues	Residue buffer	Water buffer	Dielectric constant
$S_F$	All	No	No	1
$S_R$	All	No	Yes	1
$S_{TR}$	All within $R$ , Å	All between $R$ and $R + 4$	Yes	1
$S_{Fr}$	All	No	No	$4r$
$S_{Rr}$	All	No	Yes	$4r$
$S_{TRr}$	All within $R$ , Å	All between $R$ and $R + 4$	Yes	$4r$

<sup>a</sup> $R = 8, 10, 12, 14$ , and  $16$  Å. The residue and water buffer is kept fixed during the minimization and the frequency calculation. No frequencies or entropies are calculated for the buffer atoms.

units, i.e., as  $-T\Delta S_{\text{bind}}$  at 300 K, where  $\Delta S_{\text{bind}} = S(\text{PL}) - S(\text{P}) - S(\text{L})$ .

**Uncertainties and Quality Metrics.** The reported uncertainties for the entropy and free-energy estimates are standard deviation of the averages over the 20 or 40 independent simulations, ignoring the variance within each simulation (i.e., the standard deviation over the independent simulations divided by  $(20)^{1/2}$  or  $(40)^{1/2}$ ).

The quality of the calculated binding affinities compared to experiments is described using the mean unsigned error (MUEtr) after removal of the systematic error (i.e., the mean signed deviation), the correlation coefficient ( $r^2$ ), and Kendall's rank correlation coefficient. The latter was calculated after removing pairs of ligands that have either an estimated or experimental difference that is not statistically significant at the 90% confidence level ( $\tau_{90}$ ) because we cannot require that the calculations should reproduce random variations correctly.<sup>42</sup> The number of pairs included in  $\tau_{90}$  will be denoted by  $np_{90}$ . The uncertainties of these metrics were obtained by a simple parametric bootstrap, employing 1000 random samples and assuming normal distribution of the calculated affinities.<sup>37</sup> Uncertainties in both the calculated and experimental values were included in the calculation. As no uncertainty of the experimental binding affinities has been reported, we assumed a typical uncertainty of 1.7 kJ/mol for all three test cases.<sup>43</sup>

## RESULTS

**Entropies of the fXa Complexes.** We have computed the MM/GBSA entropy in several different ways (as described in Table 1) for the eight inhibitors of fXa shown in Figure 1. The calculated entropies for this test system are given in Table 2. As a reference, we choose the calculation of the full protein–ligand complex, including a 4 Å shell of explicit water molecules that were kept fixed during the minimization and the frequency calculations ( $S_R$ ). These are time-consuming calculations, taking  $\sim 2$  h per snapshot (i.e., calculations of the complex, protein, and ligand entropies) on a single 3.0 GHz Intel Xenon processor (i.e., 133 CPU days for the results of each ligand in Table 2). Moreover, for larger proteins, the memory requirements may become prohibitively large for typical batch computers ( $>4$  GB). Therefore, the complex is usually truncated. For instance, the  $S_{T8}$  calculation takes only  $\sim 3$  min per snapshot.

The results in Table 2 show that  $S_{T8}$  deviates on average by 5 kJ/mol from  $S_R$ . Ligand C49 shows the largest deviation of  $8 \pm 2$  kJ/mol, and most ligands have deviations of  $\sim 4$  kJ/mol. Interestingly,  $S_{T8}$  is always more positive than  $S_R$ , showing that the complex and the free protein are affected by the truncation somewhat differently (the ligand entropy is not affected by the truncation). This systematic deviation means that relative entropies (i.e., the difference in entropies among the various ligands) are more accurate than the absolute entropies. In fact, the error in the relative entropies is only 1.3 kJ/mol on average ( $-1$  to 3 kJ/mol). The correlation coefficient ( $r^2$ ) between  $S_{T8}$  and  $S_R$  is 0.7 (note that the range of the entropies is only 6–9 kJ/mol).

For all ligands except C50, the deviation decreases going from  $S_{T8}$  to  $S_{T10}$  (and the difference for C50 is not statistically significant). The mean absolute deviation (MAD) between  $S_R$  and  $S_{T10}$  is only 3 kJ/mol. With even larger systems, the deviation decreases further, so that the MAD for  $S_{T12}$ ,  $S_{T14}$ , and  $S_{T16}$ , the MAD decreases from 2 to 1 kJ/mol, indicating that the calculations converge toward  $S_R$ . The maximum deviation

**Table 2.** Total Entropy ( $-T\Delta S_{\text{bind}}$ ) for the Binding of the Eight Inhibitors to fXa (kJ/mol) Calculated with 14 Different Approaches (explained in Table 1)

Method	$S_R$	$S_F$	$S_{T8}$	$S_{T10}$	$S_{T12}$	$S_{T14}$	$S_{T16}$
C9	113.3 ± 1.9	116.1 ± 2.1	117.7 ± 0.6	113.0 ± 0.6	110.9 ± 0.6	110.9 ± 0.7	110.3 ± 0.6
C9	113.3 ± 1.9	116.1 ± 2.1	117.7 ± 0.6	113.0 ± 0.6	110.9 ± 0.6	110.9 ± 0.7	110.3 ± 0.6
C39	112.5 ± 1.4	107.7 ± 2.3	116.5 ± 0.5	114.1 ± 0.7	115.0 ± 0.7	114.4 ± 0.5	114.5 ± 0.6
C47	113.7 ± 1.6	116.2 ± 2.0	117.5 ± 0.7	115.6 ± 0.6	113.2 ± 0.6	113.2 ± 0.5	112.9 ± 0.6
C49	112.9 ± 1.7	119.7 ± 2.4	121.0 ± 0.6	117.7 ± 0.6	114.1 ± 0.6	116.1 ± 0.6	115.0 ± 0.6
C50	113.1 ± 1.5	115.9 ± 2.4	117.1 ± 0.7	117.8 ± 0.6	115.3 ± 0.6	114.2 ± 0.7	114.2 ± 0.7
C53	110.4 ± 1.7	116.5 ± 2.1	116.4 ± 0.5	111.4 ± 0.6	111.2 ± 0.6	110.3 ± 0.6	109.6 ± 0.6
C63	107.6 ± 1.8	109.2 ± 2.2	111.6 ± 0.5	110.5 ± 0.6	111.1 ± 0.6	110.2 ± 0.6	108.9 ± 0.7
C125	107.4 ± 1.6	115.8 ± 2.7	113.5 ± 0.6	111.6 ± 0.6	109.2 ± 0.7	109.0 ± 0.6	108.2 ± 0.6
MAD <sup>a</sup>		4.5	5.0	2.7	1.9	1.7	1.5
Method	$S_{Rr}$	$S_{Fr}$	$S_{T8r}$	$S_{T10r}$	$S_{T12r}$	$S_{T14r}$	$S_{T16r}$
C9	111.6 ± 1.4	124.5 ± 2.3	113.5 ± 0.7	110.0 ± 0.8	110.0 ± 0.8	110.9 ± 0.8	111.7 ± 0.8
C39	110.9 ± 1.6	126.4 ± 2.3	117.7 ± 0.7	117.6 ± 0.8	118.7 ± 0.7	118.9 ± 0.7	119.1 ± 0.8
C47	112.1 ± 1.7	128.3 ± 2.7	115.4 ± 0.8	114.1 ± 0.8	113.0 ± 0.7	113.3 ± 0.8	114.2 ± 0.8
C49	112.2 ± 1.6	122.0 ± 2.3	120.1 ± 0.8	117.4 ± 0.8	110.5 ± 0.7	118.0 ± 0.9	118.3 ± 0.9
C50	113.8 ± 1.4	125.4 ± 2.3	115.3 ± 0.6	116.8 ± 0.9	116.4 ± 0.8	116.4 ± 0.7	117.0 ± 0.7
C53	109.2 ± 1.6	119.9 ± 2.3	111.4 ± 0.7	109.7 ± 0.7	110.4 ± 0.7	110.0 ± 0.7	110.5 ± 0.8
C63	106.8 ± 1.3	121.5 ± 2.2	111.0 ± 0.6	111.7 ± 0.7	112.8 ± 0.7	114.3	113.1 ± 0.8
C125	107.6 ± 1.5	106.8 ± 1.3	109.3 ± 0.7	107.7 ± 0.7	106.3 ± 0.7	108.0	108.1 ± 0.8
MAD <sup>b</sup>		11.5	3.7	3.0	2.9	3.4	3.5

<sup>a</sup>Mean absolute deviation compared to  $S_R$  <sup>b</sup>Mean absolute deviation compared to  $S_{Rr}$ **Table 3.** MM/GBSA Free Energies for the Binding of the Eight Inhibitors to fXa ( $\Delta G_{\text{bind}}$  in kJ/mol) Calculated with the Various Entropy Estimates<sup>a</sup>

Entropy	$S_R$	$S_F$	$S_{T8}$	$S_{T10}$	$S_{T12}$	$S_{T14}$	$S_{T16}$	Exp <sup>18</sup>
C9	-68.6 ± 2.1	-65.8 ± 2.2	-64.2 ± 1.0	-68.9 ± 1.0	-71.0 ± 0.9	-71.0 ± 1.0	-71.6 ± 0.9	-46.2
C39	-53.3 ± 1.7	-58.1 ± 2.0	-49.3 ± 1.0	-51.7 ± 1.0	-50.8 ± 1.0	-51.5 ± 1.0	-51.3 ± 1.1	-27.3
C47	-63.0 ± 2.0	-60.5 ± 2.3	-59.2 ± 1.1	-61.1 ± 1.0	-63.6 ± 1.0	-63.5 ± 0.9	-63.8 ± 1.0	-46.8
C49	-65.5 ± 2.0	-58.7 ± 2.5	-57.4 ± 1.1	-60.7 ± 1.0	-64.3 ± 1.1	-62.2 ± 1.1	-63.3 ± 1.0	-41.9
C50	-63.7 ± 1.5	-60.9 ± 2.5	-59.7 ± 0.9	-58.9 ± 0.8	-61.5 ± 0.9	-62.6 ± 0.9	-62.6 ± 0.9	-46.2
C53	-67.3 ± 1.9	-61.1 ± 2.0	-61.2 ± 1.0	-66.3 ± 1.1	-66.4 ± 1.1	-67.4 ± 1.0	-68.0 ± 1.1	-44.3
C63	-57.7 ± 2.2	-56.2 ± 2.5	-53.7 ± 1.6	-54.8 ± 1.5	-54.3 ± 1.5	-55.1 ± 1.6	-56.4 ± 1.6	-37.4
C125	-69.9 ± 1.9	-61.5 ± 2.8	-63.8 ± 1.2	-65.7 ± 1.1	-68.1 ± 1.1	-68.3 ± 1.0	-69.1 ± 1.1	-43.4
MUEtr	2.9 ± 0.7	3.3 ± 0.8	2.4 ± 0.6	3.5 ± 0.6	3.4 ± 0.7	3.2 ± 0.7	3.1 ± 0.7	
$r^2$	0.68 ± 0.14	0.39 ± 0.18	0.77 ± 0.10	0.60 ± 0.11	0.70 ± 0.19	0.71 ± 0.10	0.71 ± 0.10	
$\tau_{90}$	1.00 ± 0.06	1.00 ± 0.16	1.00 ± 0.06	0.87 ± 0.05	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.04	
$np_{90}$	12	3	15	14	15	14	14	
Entropy	$S_{Rr}$	$S_{Fr}$	$S_{T8r}$	$S_{T10r}$	$S_{T12r}$	$S_{T14r}$	$S_{T16r}$	Exp <sup>18</sup>
C9	-70.3 ± 1.5	-57.4 ± 2.3	-68.4 ± 0.9	-71.9 ± 1.0	-71.9 ± 1.0	-71.0 ± 1.0	-70.2 ± 1.0	-46.2
C39	-54.9 ± 1.7	-39.4 ± 2.2	-48.2 ± 1.0	-48.2 ± 1.2	-47.2 ± 1.1	-46.9 ± 1.1	-46.8 ± 1.1	-27.3
C47	-64.6 ± 1.7	-48.5 ± 2.6	-61.3 ± 1.2	-62.6 ± 1.1	-63.7 ± 1.1	-63.5 ± 1.2	-62.5 ± 1.2	-46.8
C49	-66.2 ± 1.9	-56.4 ± 2.4	-58.2 ± 1.1	-61.0 ± 1.1	-67.9 ± 1.1	-60.4 ± 1.2	-60.1 ± 1.1	-41.9
C50	-63.0 ± 1.5	-51.4 ± 2.1	-61.5 ± 0.9	-60.0 ± 1.1	-60.4 ± 1.1	-60.4 ± 1.0	-59.8 ± 1.1	-46.2
C53	-68.4 ± 1.7	-57.7 ± 2.4	-66.3 ± 1.1	-68.0 ± 1.1	-67.3 ± 1.2	-67.6 ± 1.0	-67.1 ± 1.1	-44.3
C63	-58.5 ± 1.9	-43.8 ± 2.2	-54.3 ± 1.4	-53.7 ± 1.5	-52.5 ± 1.5	-51.1 ± 1.6	-52.2 ± 1.6	-37.4
C125	-69.7 ± 1.8	-56.6 ± 2.0	-68.0 ± 1.0	-69.6 ± 1.0	-71.0 ± 1.0	-69.3 ± 1.1	-69.2 ± 1.0	-43.4
MUEtr	3.1 ± 0.7	4.0 ± 0.9	3.3 ± 0.7	3.9 ± 0.7	4.5 ± 0.7	3.8 ± 0.7	3.7 ± 0.7	
$r^2$	0.65 ± 0.13	0.58 ± 0.14	0.72 ± 0.10	0.66 ± 0.10	0.65 ± 0.10	0.71 ± 0.10	0.70 ± 0.10	
$\tau_{90}$	1.00 ± 0.06	0.83 ± 0.05	1.00 ± 0.06	1.00 ± 0.04	0.75 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	
$np_{90}$	13	12	16	14	16	15	14	

<sup>a</sup>The results are compared to experimental data<sup>18</sup> using three quality estimates, MUEtr,  $r^2$ , and  $\tau_{90}$ .

decreases somewhat less and is 3 kJ/mol for the three largest truncations. On the other hand, the relative error toward  $S_R$  is the same for all truncated systems, 1.3–1.6 kJ/mol.

It is also of interest to compare the reference calculations with the calculations of the full complex without the water buffer ( $S_F$ ). From Table 2, it can be seen that the MAD is 4

kJ/mol with a maximum deviation of 8 kJ/mol for C125. This indicates that the water buffer stabilizes the energies quite strongly: If the buffer is omitted, the entropies change almost as much as for the most truncated system. However, due to the large uncertainties, the difference between  $S_F$  and  $S_R$  is only significant for three of the ligands at 95% confidence. The

standard error is always larger for  $S_F$  than for  $S_R$ , viz. 2.3 compared to 1.7 kJ/mol on average, and the standard errors for the truncated calculations are even lower, only 0.6 kJ/mol on average for all the truncated calculations irrespective of the radius. This shows that the truncations stabilize the calculations (decrease the fluctuations among the snapshots), probably because the minimized system is smaller, so there is a smaller number of possible conformations.

Next, we repeated all the calculations using  $\epsilon = 4r$  rather than  $\epsilon = 1$ . This can be seen as a primitive but cheap way to add solvation effects to the system. The  $S_{Rr}$  entropy does not differ much from the  $S_R$  entropy, 1 kJ/mol on average and with a maximum deviation of 2 kJ/mol ( $r^2 = 0.9$ ), showing that for a full protein with an explicit water buffer,  $\epsilon = 4r$  is a redundant but harmless approximation. The entropies obtained by truncating the protein are more erratic with  $\epsilon = 4r$  than with  $\epsilon = 1$ . For three of the ligands, it seems that the entropies converge to within 2 kJ/mol of the  $S_{Rr}$  entropy already with  $S_{T8r}$ . But for other ligands, the deviations compared to  $S_{Rr}$  are 4 to 8 kJ/mol, and for some ligands, it increases with increasing truncation radius. Consequently, the MAD is almost constant, 3–4 kJ/mol for all truncation radii.

Interestingly,  $S_{Fr}$  gives a MAD from  $S_{Rr}$  of 12 kJ/mol, which shows that without the buffer the minimized system can change extensively. Likewise, the MAD between  $S_F$  and  $S_{Fr}$  is 9 kJ/mol, indicating that using  $\epsilon = 4r$  for a calculation of the full protein changes the structures and hence the entropies significantly. This shows that the commonly used  $S_{Fr}$  approach is quite suspicious.

The standard errors using  $\epsilon = 4r$  are slightly larger than with  $\epsilon = 1$ , 0.7–0.8 kJ/mol on average for the truncated systems and slightly increasing with the truncation radius. However, for the full systems, the standard errors are similar or slightly smaller with  $\epsilon = 4r$  than with  $\epsilon = 1$ , 1.5 kJ/mol for  $S_{Rr}$  and 2.2 kJ/mol for  $S_{Fr}$  on average.

**Free Energies of the fXa Complexes.** Next, we combined the entropies with estimates of interaction energies and solvation free energies to obtain binding free energies according to eq 1. These binding free energies are shown in Table 3. The trends due to entropy estimates have already been discussed in the previous section, so here the interesting thing is to compare the calculated binding affinities with the experimental data (shown in Figure 1).<sup>18</sup> It can be seen that the reference entropy estimates ( $S_R$ ) give reasonably good results: The MUEtr is  $3 \pm 1$  kJ/mol, which is slightly better than the null hypothesis that all estimates are equal, with MUEtr = 5 kJ/mol (reflecting that all the experimental binding affinities are quite similar; in fact, six of them are within 5 kJ/mol). The correlation coefficient is rather good,  $r^2 = 0.7 \pm 0.1$ , and Kendall's rank correlation coefficient, based on the pairs that have statistically significant differences in both the experiments and calculations (12 out of 28 possible pairs), is perfect,  $\tau_{90} = 1.0 \pm 0.1$ .

From Table 3, it can be seen that all the other entropy methods give similar results: MUEtr = 2–4 kJ/mol, with differences that are not statistically significant at the 95% level. However, all calculations with  $\epsilon = 4r$  give a higher MUEtr than the corresponding calculations with  $\epsilon = 1$ . Likewise, differences in  $r^2$  (0.4–0.8) and  $\tau_{90}$  (0.8–1.0) are not statistically significant. The latter two measures show no clear difference between the two dielectric constants. The reason why we do not see any clear differences between the binding affinities obtained with the various entropies is that the relative entropies vary by only ~1.5 kJ/mol for  $S_{TR}$  and by 2–3 kJ/mol for  $S_{TRr}$ . This variation

is within the uncertainty of the method (note we use quality measures that are sensitive only to the relative affinities not to the absolute ones because the absolute MM/GBSA affinities depend strongly on the continuum-solvation model and rarely give accurate absolute affinities;<sup>44,45</sup> moreover, absolute affinities are seldom of interest in drug development). However, it is notable that  $S_F$  gives the poorest correlation coefficient ( $r^2 = 0.4$ ) and  $S_{Fr}$  gives the poorest  $\tau_{90} = 0.8$  among all tested entropy methods—these two approaches also gave entropies that differed most from  $S_R$ .

Moreover, it is clear that entropies calculated with the full protein had a higher uncertainty than those obtained for the truncated systems and that this uncertainty dominates the total binding affinities; The standard error of  $\Delta G_{\text{bind}}$  obtained with  $S_R$  is 2 kJ/mol, whereas it is only 1 kJ/mol when obtained with  $S_{TR}$  for any radius. This is an important advantage of the entropies obtained with truncated systems.

Owing to the high cost of the entropy calculations in MM/GBSA, the entropy term has in several cases been completely ignored.<sup>46–49</sup> We have also tested such an approach for fXa and present the corresponding affinity estimates in Table 4. It can

**Table 4. MM/GBSA Free Energies for the Binding of the Eight Inhibitors to fXa ( $\Delta G_{\text{bind}}$  in kJ/mol) Calculated without Any Entropy Estimate**

	$\Delta G_{\text{bind}}$	Exp <sup>18</sup>
C9	$-181.9 \pm 0.7$	-46.2
C39	$-165.8 \pm 1.0$	-27.3
C47	$-176.7 \pm 0.9$	-46.8
C49	$-178.4 \pm 0.8$	-41.9
C50	$-176.8 \pm 0.9$	-46.2
C53	$-177.6 \pm 0.8$	-44.3
C63	$-165.3 \pm 1.5$	-37.4
C125	$-177.3 \pm 0.9$	-43.4
MUEtr	$2.9 \pm 0.6$	
$r^2$	$0.71 \pm 0.11$	
$\tau_{90}$	$1.00 \pm 0.04$	
np <sub>90</sub>	13	

be seen that the omission of the entropy term makes all binding affinities much too negative (−165 to −182 kJ/mol compared to the experimental estimates of −27 to −47 kJ/mol). However, it can be seen that the MUEtr (3 kJ/mol),  $r^2$  (0.7), and  $\tau_{90}$  (1.0) are similar to what is obtained for MM/GBSA with the various entropy estimates. Hence, for this test case, the entropy estimates do not significantly change the relative affinities.

**Ferritin and HIV-1 Protease.** From the calculations on the fXa complexes, it seems sufficient to calculate the entropies with  $S_{T8}$  if we are only interested in relative energies or entropies. To test this hypothesis we performed  $S_R$  and  $S_{T8}$  calculations also for the binding of nine inhibitors to ferritin and for the binding of two ligands to two different forms of HIV-PR. For ferritin, it can be seen in Table 5 that  $S_{T8}$  is an excellent approximation to  $S_R$  with deviations of 0.1 to 1.7 kJ/mol, giving a MAD of only 0.9 kJ/mol for absolute entropies and 0.7 kJ/mol for relative entropies. Consequently, there are no significant differences in the binding free energies calculated with the two entropy approaches. They both give a MUEtr of 9 kJ/mol compared to the experimental estimates.<sup>19</sup> This is appreciably worse than the null hypothesis that all ligands have the same affinity (giving a MAD of 3 kJ/mol), which reflects

**Table 5. Entropies and Free Energies for the Binding of Nine Inhibitors to Ferritin Calculated with the  $S_R$  and  $S_{T8}$  Approaches, as well as without Any Entropy Estimate (kJ/mol)**

	$-T\Delta S_{\text{bind}}$		$\Delta G_{\text{bind}}$			Exp <sup>19</sup>
	$S_R$	$S_{T8}$	$S_R$	$S_{T8}$	No S	
L01	67.5 ± 1.3	66.7 ± 0.6	−37.2 ± 1.5	−38.0 ± 0.9	−104.7 ± 0.6	−30.5
L02	72.4 ± 1.4	73.5 ± 0.7	−47.5 ± 1.7	−46.5 ± 1.2	−119.9 ± 0.9	−30.1
L03	70.0 ± 1.4	69.8 ± 0.8	−42.6 ± 1.5	−42.7 ± 1.0	−112.6 ± 0.6	−32.7
L04	68.8 ± 1.5	68.4 ± 0.6	−37.4 ± 1.7	−37.8 ± 0.8	−106.2 ± 0.6	−30.6
L05	63.1 ± 1.2	63.0 ± 0.6	−30.4 ± 1.4	−30.5 ± 0.8	−93.5 ± 0.8	−28.2
L06	61.4 ± 1.3	60.0 ± 0.4	−20.5 ± 1.4	−21.9 ± 0.6	−81.9 ± 0.5	−26.0
L07	60.5 ± 1.3	59.5 ± 0.5	−20.1 ± 1.4	−21.1 ± 0.8	−80.6 ± 0.6	−27.5
L08	57.4 ± 1.2	56.1 ± 0.5	−16.0 ± 1.2	−17.3 ± 0.7	−73.4 ± 0.6	−22.8
L09	52.8 ± 1.2	51.1 ± 0.4	0.9 ± 1.4	−0.8 ± 1.0	−51.9 ± 1.0	−18.7
MUEtr		0.9 <sup>a</sup>	9.1 ± 0.5	8.5 ± 0.3	14.2 ± 0.2	
$r^2$			0.89 ± 0.02	0.90 ± 0.01	0.89 ± 0.01	
$\tau_{90}$			1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.05	
np <sub>90</sub>			20	20	20	

<sup>a</sup>Mean absolute difference from the  $S_R$  estimates.**Table 6. Entropies and Free Energies for the Binding of Two Ligands to WT and MT-HIV-PR, Calculated with the  $S_R$  and  $S_{T8}$  Approaches, as well as without Any Entropy Estimate (kJ/mol)**

	$-T\Delta S_{\text{bind}}$		$\Delta G_{\text{bind}}$			Exp <sup>49</sup>
	$S_R$	$S_{T8}$	$S_R$	$S_{T8}$	No S	
MT-APV	101.3 ± 0.7	104.8 ± 0.6	−151.6 ± 2.7	−148.1 ± 2.7	−252.9 ± 2.6	−49.0
WT-APV	102.3 ± 1.1	107.4 ± 0.5	−144.1 ± 2.0	−139.0 ± 1.7	−246.4 ± 1.6	−52.8
MT-DRV	113.6 ± 1.1	116.7 ± 1.0	−158.5 ± 2.4	−155.3 ± 2.3	−272.0 ± 2.1	−57.4
WT-DRV	111.6 ± 0.9	116.3 ± 0.9	−160.5 ± 2.1	−155.8 ± 2.1	−272.1 ± 1.9	−63.6
MUEtr		4.1 <sup>a</sup>	3.9 ± 1.0	4.7 ± 1.0	6.4 ± 1.2	
$r^2$			0.55 ± 0.18	0.45 ± 0.16	0.66 ± 0.15	
$\tau_{90}$			1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.09	
np <sub>90</sub>			4	4	4	

<sup>a</sup>Mean absolute difference from the  $S_R$  estimates.

that the MM/GBSA method overestimates the difference in affinity between the various ligands. On the other hand, MM/GBSA gives excellent  $r^2 = 0.9$  and  $\tau_{90} = 1.0$  (based on 20 out of the 36 possible pairs). Omitting the entropy estimate completely gives a significantly worse MUEtr (14 kJ/mol) but similar  $r^2$  and  $\tau_{90}$ . The precision of the two entropy estimates and the binding free energies is improved for the truncated systems, e.g., from 1.3 to 0.6 kJ/mol on average for the entropy. It should be mentioned that for this test case the molecular weights correlates well with the experimental affinities ( $r^2 = 0.86$ ). However, this is due to the large range of weights, from 206.3 g/mol for L02 to 94.1 g/mol for L09 (phenol), and that the interaction between drug and receptor is mainly dictated by van der Waals forces. The large range of weights leads to a MADtr of 33.3 kJ/mol, i.e., much worse than any of the MM/GBSA estimates.

For the HIV-PR complexes, the difference between  $S_{T8}$  and  $S_R$  ranges from 3 to 5 kJ/mol, as can be seen in Table 6, giving a MAD of 4 kJ/mol. However, the difference is systematic so the difference in relative entropies is only 0.8 kJ/mol, which shows that relative entropies can accurately be calculated with  $S_{T8}$  also for this test case. Consequently, the two entropy approaches give similar binding affinities. The MUEtr of  $\Delta G_{\text{bind}}$  compared to experimental data<sup>50</sup> is 4–5 kJ/mol, which is similar to the null hypothesis (5 kJ/mol). The  $r^2$  is rather low (0.45–0.55) but  $\tau_{90} = 1.0$ . However, the calculated entropy differences do not reproduce the isothermal calorimetry values.<sup>49</sup> Omitting the entropy gives a somewhat worse MUEtr, the same  $\tau_{90}$ , and a

slightly better  $r^2$ , but none of the differences is statistically significant at the 90% level. Interestingly, the precision of the entropy is not so much improved for the truncated protein as for the other test cases.

## CONCLUSIONS

In this article, we have investigated the effect of truncation and the dielectric constant on NMA entropy estimates employed in the MM/GBSA method. As the reference, we use the full protein surrounded with a shell of fixed water molecules ( $S_R$ ). As test cases, we study the binding of eight inhibitors to fXa, nine ligands to ferritin, and two ligands to wild-type and mutant HIV-PR. Several interesting results are obtained.

- Truncation of the protein is not innocent. Truncating the protein outside a radius of 8–16 Å from the ligand gives rise to average deviations of 1–5 kJ/mol, and the deviations increase when the radius is decreased. Fortunately, the deviations are systematic, so for relative entropies, the average deviation is only 0.7–1.6 kJ/mol for all radii. Consequently, we do not see any statistically significant difference for the total ligand-binding affinities with any of our quality measures (which compare only relative affinities) when the systems are truncated. On the other hand, the precision is nearly always improved for the truncated systems.
- Calculations with a distance-dependent dielectric constant ( $\epsilon = 4r$ , a primitive model to account for solvent



effects when water molecules are excluded) give a rather constant MAD from  $S_{R_r}$  of 3–4 kJ/mol, independent from the truncation radius. This is similar to the results with  $\epsilon = 1$  for the smallest radii. However, the deviations are less systematic, so for relative entropies, the average deviations are larger than with  $\epsilon = 1$ , 2–3 kJ/mol. Entropies calculated with  $\epsilon = 4r$  differ by 1–2 kJ/mol on average from those calculated with  $\epsilon = 1$ .

- A shell of fixed water molecules and protein residues for the truncated systems is important to stabilize the energies. Without this shell, the entropies change by 4 kJ/mol on average with  $\epsilon = 1$  and by 12 kJ/mol with  $\epsilon = 4r$ . Moreover, the standard error increases by 0.6 kJ/mol on average.
- We have tested to ignore the entropy term completely in MM/GBSA. Such an approach has been shown to improve the results in several studies,<sup>45–48</sup> although the results become worse in other cases.<sup>16,46</sup> For the fXa and HIV-PR test cases, it does not lead to any statistically significant changes in our quality measures, but for ferritin, the results become slightly worse. Thus, omission of the entropy term is not always favorable or innocent.<sup>46</sup>

Of course, our results depend on the selection on  $S_R$  as the reference entropy. Other references are conceivable but not necessarily better. The most natural would be the full complex with all water molecules treated the same way as in the MD simulations, i.e., with periodic boundary conditions and Ewald summation. Unfortunately, this leads to too large systems (the memory requirements become prohibitive) to treat with today's computers. Moreover, the water molecules need to be excluded from the frequency calculations (the water entropy is included in the two solvation terms), and a minimization of unconstrained systems may lead to extensive changes in the structure. For the latter reason, we believe that a fixed shell of water molecules and protein residues is the best solution to keep the structure close to the MD snapshot. It also leads to an improved precision. Once such a buffer is used, the need of solvation effects becomes minor, as the similarity of the results with  $\epsilon = 1$  and  $\epsilon = 4r$  show. Therefore, we do not expect large improvement by using more sophisticated continuum-solvation methods as, e.g., generalized Born, as has recently been suggested.<sup>12,51</sup> We favor the approach proposed here as it retains a quite consistent energy model between the underlying MD simulations and the subsequent estimation of free energy changes and is computationally more efficient due to the smaller size of the truncated system.

Thus, we can conclude that entropies calculated with a protein truncated beyond a distance of 8 Å from the ligand and with a 4 Å buffer of fixed protein residues and water molecules together with  $\epsilon = 1^8$  provide a proper compromise between accuracy and computational efficiency, albeit only for relative binding affinities. However, it has been repeatedly pointed out that MM/GBSA cannot be expected to give accurate absolute affinities, only relative affinities.<sup>43,44,52</sup> Finally, it should also be remembered that NMA is a crude approximation because it assumes that phase space is Gaussian-shaped with a single well, although it is clear that many degrees of freedom, in particular many dihedral angles, have multiple minima. Therefore, other methods to calculate the entropy may give significantly different results.<sup>9,11</sup>

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### Notes

The authors declare no competing financial interest.

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