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Osmotic Pressure Simulations of Amino Acids and Peptides Highlight Potential Routes to Protein Force Field Parameterization

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Abstract

Recent molecular dynamics (MD) simulations of proteins have suggested that common force fields overestimate the strength of amino acid interactions in aqueous solution. In an attempt to determine the causes of these effects, we have measured the osmotic coefficients of a number of amino acids using the AMBER ff99SB-ILDN force field with two popular water models, and compared the results with available experimental data. With TIP4P-Ew water, interactions between aliphatic residues agree well with experiment, but interactions of the polar residues serine and threonine are found to be excessively attractive. For all tested amino acids, the osmotic coefficients are lower when the TIP3P water model is used. Additional simulations performed on charged amino acids indicate that the osmotic coefficients are strongly dependent on the parameters assigned to the salt ions, with a reparameterization of the sodium:carboxylate interaction reported by the Aksimentiev group significantly improving description of the osmotic coefficient for glutamate. For five neutral amino acids, we also demonstrate a decrease in solute-solute attractions using the recently reported TIP4P-D water model and using the KBFF force field. Finally, we show that for four two-residue peptides improved agreement with experiment can be achieved by re-deriving the partial charges for each peptide.

Introduction

Although molecular dynamics (MD) simulations provide atomically detailed views of the conformational dynamics of protein systems, their usefulness depends critically on their ability to reproduce experimental behavior. One common way to validate the protein force fields used in MD simulations is by comparison with structural data obtained from NMR experiments on peptide systems. For example, a benchmark comparison of protein force fields and water models carried out by the Pande group demonstrated that a number of popular force field combinations performed surprisingly well at reproducing NMR

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Supporting Information

Figures pertaining to system setup; experimental controls; osmotic coefficients using TIP3P water; comparisons of standard deviations between water models; additional comparisons between different concentrations from experimental data, systems of mixed charges, and different force fields (TIP4P-D and KBFF); GAMESS and RESP input files; and GROMACS topology files for the re-derived partial charges for two-residue peptides can be found in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

observables such as scalar couplings and chemical shifts.¹ More recently, our group showed² that the experimental $^3J_{HNH\alpha}$ scalar couplings of hundreds of two-residue peptides measured by the Cho group³ could be reproduced quite well by variants of the AMBER force fields, with especially good results being obtained with the newly developed RSFF2 variant.⁴⁻⁵ A second approach to validating protein force fields is to compare solution properties such as densities, viscosities, and dielectric constants with experimental values for amino acids (as in one of our previous studies⁶) or for large collections of small molecules (see for example Coleman et. al.⁷). A third way to validate protein force fields is by comparison with thermodynamic data for peptides or analogous systems. A number of groups, for example, have reported comparisons of computed hydration free energies of side chain analogs of amino acids with experimental data;⁸⁻¹¹ the Chong group¹² has compared computed and experimental association free energies of salt bridging side chain analogs with a variety of different force field combinations, and the Nerenberg¹³ and Head-Gordon¹⁴ groups have used comparisons with the hydration free energies and vaporization enthalpies of a wide range of small molecules to propose changes to the van der Waals terms of the AMBER force field. A somewhat different approach has been taken by the Smith group, which has pioneered the use of Kirkwood Buff Integrals¹⁵ (KBIs) as a means of connecting simulated and experimental thermodynamics over a wide range of solute concentrations, and has used KBIs calculated for a number of sidechain analogs as a basis for developing a general protein force field, KBFF.¹⁶⁻¹⁸

While the above studies have examined peptide systems, a number of recent MD studies of proteins have highlighted some potentially serious challenges facing current force fields. First, simulations by the Zagrovic group using a variety of different force fields have shown that the small protein Villin Headpiece rapidly aggregates at concentrations at which it is known to be soluble experimentally.¹⁹ Second, several groups have carried out simulations in which intrinsically disordered proteins (IDPs) collapse into compact conformations that are inconsistent with experimental data;^{9, 20-25} interestingly, however, the Smith group's KBFF has been shown not to suffer from this problem, at least for one such IDP, the 82-residue Nup153.²¹

Attempts to rectify one or both of the above issues have already led to the development and testing of at least two new variants of the AMBER force fields. One comes from the Best and Mittal groups who have focused on strengthening protein-water interactions in the AMBER ff03w force field by the relatively straightforward approach of scaling the ϵ values for solute-water Lennard-Jones interactions by a factor of 1.1; they showed that this adjustment improved agreement between simulation and experiment for the radius of gyration (R_g) of the 34-residue IDP, Csp M34, at increasing temperatures.⁹ Encouragingly, a recent simulation study carried out by the Skepö group using this modified force field on a second small IDP, the 24-residue Histatin 5, resulted in R_g values and Kratky plots (used to distinguish globular from disordered states) that were also both in excellent agreement with experiment.²³ A second force field variant that has recently been reported comes from the Shaw group, which has focused on re-parameterizing water-water interactions, again by adjusting the strengths of their Lennard-Jones interactions. Their work has resulted in a new water model, TIP4P-D, that has been shown to lead to increased R_g values (i.e. closer to

experiment) for five IDPs, ranging from the 55-residue N-terminal zinc-binding domain of HIV-1 integrase to the 140-residue α -synuclein.²⁰

Despite these advances, the extent to which force field variants that accurately describe IDPs can also describe natively structured proteins remains unclear. KBFF, for example, which performed very well with the IDP Nup153,²¹ is apparently unable to maintain the GB1 domain in its folded state; a very recent, unpublished update to the force field, which seeks to stabilize β -sheet structures, may rectify this problem (<http://kbff.chem.k-state.edu>). A similar destabilization of folded proteins has been reported by the Shaw group when using their TIP4P-D water model to simulate the proteins Ubiquitin and GB3: in both cases, it was noted that the water model may “slightly destabilize the folded states of proteins”, and further studies of the small proteins WW domain and Villin Headpiece suggested this destabilization was on the order of 2 kcal/mol.²⁰ Finally, while the Best & Mittal groups have shown good overall stability for four folded proteins simulated on a timescale of 200 ns, they also reported “larger amplitude dynamics in loop regions” for Lysozyme and Ubiquitin; this was especially apparent for Ubiquitin, for which the backbone order parameters in loop regions were much lower than in experiment.^{9, 26} It appears, therefore, that a challenge for the near future will be to attempt to find consistent pairwise force field parameters that work well for describing both IDPs and natively structured proteins.

One issue that we think is important to resolve at this stage is the extent to which protein force fields can correctly reproduce the interaction thermodynamics of individual types of amino acids in aqueous solution. As noted above, in developing the KBFF, the Smith group used KBIs to parameterize their force field to match the interaction thermodynamics of a number of analogs of the amino acids, representing side chains and/or backbone components, in various solvents.^{27–30} Here, in order to directly examine the interactions of a range of complete amino acids in water using the widely used AMBER force field,^{31–32} we: (a) report the use of MD simulations to measure the osmotic coefficients of 16 different amino acids and 4 different two-residue peptides, and (b), compare, where possible, the resulting osmotic coefficients with experimental data. The results point to clear dependences of the association thermodynamics on the choice of water model while also showing that for charged amino acids and two-residue peptides the results depend critically on the ion parameters and the partial charges of the termini, respectively. In addition, however, the results also indicate that the extent of agreement with experiment differs significantly for different types of amino acid, suggesting that a targeted reparameterization of the interactions of specific functional groups (e.g. hydroxyl) might be a profitable way to improve the accuracy of protein force fields.

Methods

Simulation Setup

All simulations were performed using GROMACS version 5.0, modified in-house to report forces exerted by a flat-bottomed potential function (FBP) at each timestep (see below).^{33–35} Amino acids were modeled using the AMBER ff99SB-ILDN force field (AMBER)^{31–32} in their zwitterionic state, with partial charges determined by Horn;³⁶ additional simulations were also run using the Kirkwood Buff Force Field (KBFF).^{16–18} The full list of amino acids

simulated in this study is given in Table 1. TIP3P,³⁷ TIP4P-Ew,³⁸ or TIP4P-D²⁰ water models were used with the AMBER protein force field, while the SPC/E³⁹ water model was used with the KBFF. Positively and negatively charged amino acids were neutralized with the addition of equal numbers of Cl⁻ and Na⁺ ions, respectively. Na⁺ ions were modeled using parameters developed either by Åqvist,⁴⁰ Joung & Cheatham,⁴¹ or Yoo & Aksimentiev.⁴² Cl⁻ ions were modeled using parameters reported by Dang⁴³ or by Joung & Cheatham.⁴¹ In cases where the Joung & Cheatham parameters were used, care was taken to ensure that the parameters appropriate to the chosen water model were selected. Histidine was modeled in its fully protonated state (Hip). Zwitterionic, two-residue peptides (Ala-Ala, Ala-Gly, Gly-Ala, Gly-Gly) were modeled initially using the appropriate N- and C-terminal residue types present in the AMBER force field, and, in a second set of simulations, using newly re-derived partial charge sets (see below).

Amino acids were randomly placed within a cube of side 4.8 nm, with the number of molecules necessary for a given molar concentration of 0.5, 1, or 2 M (33, 67, or 133 amino acid molecules); attempts were also made to compute osmotic coefficients at the much lower solute concentration of 0.1 M but were ultimately abandoned as it was difficult to obtain converged results (data not shown). Having added the solutes, the simulation box was extended by 2.4 nm in each direction along the z-axis to given final dimensions of 4.8 × 4.8 × 9.6 nm and then solvated with the chosen water model (see above). Energy minimization was completed using the steepest descent algorithm for 1000 steps, and each system was then incrementally heated to 298 K over the course of 350 ps, with a final 1 ns of equilibration MD being carried out prior to the beginning of production. Simulations were run in the NPT ensemble, with the temperature maintained at 298 K using the Nosé-Hoover thermostat and the pressure maintained at 1 bar using the Parrinello-Rahman barostat.^{44–46} Semi-isotropic pressure coupling was applied so that changes in the simulation box size in the z-direction could occur independently of changes in the x- and y- directions; following the simulation setup proposed by Luo and Roux,⁴⁷ the compressibility in the latter directions was set to 0 so that box size changes occurred only in the z-direction. A cutoff of 10 Å was applied to short-range, nonbonded interactions and the smooth particle mesh Ewald method was used to calculate long-range electrostatic interactions.⁴⁸ Bonds were constrained to their equilibrium lengths using the LINCS algorithm.⁴⁹ A 2.5 fs time step was used, and atomic coordinates of both the solute and the solvent molecules were saved every 1 ps. All production simulation were carried out for 100 ns; each such simulation took approximately 5.2 days to complete using TIP3P water or 7.6 days using TIP4P-Ew water on an 8-core server.

Calculating Osmotic Coefficients

For the purposes of measuring the osmotic pressure of the amino acid solutions, a flat-bottomed position restraint – acting along the z-direction – was applied to the heavy atoms of all solutes. The two ‘walls’ of this restraint function were placed such that solute molecules (but not water molecules) were restrained to the central 4.8 nm cube of the simulation box; the force constant for the position restraint was set to 1000 kJ·mol⁻¹·nm⁻². For the sake of converting between molar and molal concentrations, water molecules were counted by tabulating the number of water oxygen molecules found inside the region defined

by the flat-bottomed position restraints using VMD.⁵⁰ A view of an example system illustrating this set up is provided in Figure S1. The forces acting on the ‘walls’ were summed and written to disk at each time step. The forces were then converted to pressures by dividing by the combined surface area of the ‘walls’, i.e. the area of the x-y plane. The molal osmotic coefficient was then derived from the average osmotic pressure according to the equation:^{51–52}

$$\varphi = \frac{\pi \cdot V_w}{R \cdot T \cdot m \cdot v \cdot M_w}$$

where V_w is the partial molal volume of water (taken to be $0.018 \text{ L} \cdot \text{mol}^{-1}$), R is the gas constant, T is the temperature, m is the molality (determined, as noted above, by counting the number of water molecules in the central region of the system), M_w is the molecular weight of water ($0.018013 \text{ kg} \cdot \text{mol}^{-1}$), and v is the van’t Hoff factor (1 for neutral amino acids, 2 for charged amino acids, since they are accompanied by a counterion). For a very lucid derivation of the above relationship, see Janáček and Sigler.⁵² The computed osmotic coefficients were averaged over the 100 ns production period of the simulation, and errors were reported as standard deviations of the osmotic coefficients computed in five 20 ns blocks.

Control simulations

We performed a number of control simulations to ensure that the simulation setup was correct. First, we reproduced osmotic pressure simulations from the literature by simulating Gly with the KBFF⁵³ and by simulating NaCl with the CHARMM force field.^{47, 54–55} The osmotic coefficients resulting from our simulations of Gly were in line with those reported by the Smith group⁵³ (Figure S2A) and the values from our simulations of NaCl were in good agreement with those reported by the Roux group⁴⁷ (Figure S2B).

To ensure that our results were independent of simulation box size, we repeated simulations of both Thr and Gly with a box tripled in volume (dimensions of $6.92 \times 6.92 \times 13.84 \text{ nm}$). Figure S3A shows that the resulting osmotic coefficients were the same as those using a smaller box size, with values aligned to the 1:1 correlation line, and error bars on the same order of magnitude regardless of box size. To ensure that a 100 ns simulation length was sufficient to properly sample behavior, we repeated simulations for 2 M Gly with a tripled simulation time of 300 ns; the osmotic coefficients computed for each 100 ns block were consistent within the error (Figure S3B). To ensure that the osmotic pressure was independent of the external reference pressure applied (typically 1 bar),⁵³ we varied the external reference pressure from -150 to $+150 \text{ bar}$, for simulations of 2 M Thr in TIP3P water (Figure S4). Changing the external pressure did not have a significant effect on either the force exerted on the ‘walls’ (Figure S4A), or on the resulting osmotic coefficient (S4C); however, it did cause a monotonic but very small change ($\sim 1\%$) to the solute concentration by changing the number of water molecules present in the central cube of the box (S4B).

Finally, since all of our osmotic pressure simulations were carried out using the L-stereoisomers for each amino acid, while some of the experimental data used for comparison

(see below) were for systems of racemic mixtures of D- and L-enantiomers, it was of interest to determine whether there was any dependence of the computed osmotic coefficients on the relative populations of the two enantiomers. We therefore simulated systems of D- and L-alanine with a combined concentration of 2 M at each of the following mole fractions of L-enantiomers: 0, 0.25, 0.5, 0.75 and 1. As can be seen in Figure S3C, we did not find any appreciable dependence of the computed osmotic coefficients on the enantiomeric composition. This result, which is consistent with the experimental work by Robinson et al.⁵⁶ indicating no difference between the osmotic coefficients of D, L, and DL-alanine, suggests that the stereochemistry of the amino acids is unlikely to be a significant concern when comparing computed and experimental osmotic coefficients.

Sources of Experimental Data

Where possible, we compared the osmotic coefficients derived from MD simulation to those from experiment. For most of the uncharged amino acids (Gly, Ala, Val, Pro, Ser, Thr), we referenced the works of the Smith and Smith labs^{57–59} from the 1930s and 1940s. In the case of Ser, we cross-referenced the values from Smith & Smith⁵⁹ with those from Hutchens et al.,⁶⁰ who obtained coefficients at much higher concentrations and found excellent agreement between the two sets of data (see below). For the charged amino acids (Lys, Arg, Glu), we referenced the works of Bonner^{61–62} from the 1970s and 1980s. More recent measurements of osmotic coefficients by the Sadowski lab have yielded data for Leu, Met, and Cys,⁶³ while those by the Kunz lab have yielded data for Hip;⁶⁴ however, with the exception of Cys, the concentrations at which the osmotic coefficients were measured experimentally were too low for us to easily obtain statistically reliable estimates from simulations. Experimental osmotic coefficients for the four two-residue peptides Ala-Ala, Ala-Gly, Gly-Ala, and Gly-Gly were taken from the work of Smith and Smith.⁶⁵

Comparison with Experiment

In order to compare experimental and simulated osmotic coefficients at the same molal concentrations we chose to interpolate or extrapolate the osmotic coefficients from experimental data. Table 1 lists each of the amino acid systems simulated, the technique used for comparison with experiment, and the maximum solute concentration available from *in vitro* experiments; Figure S5 shows these maximum concentrations graphically. In some cases (Ala, Gly, Pro, Val, Thr), the osmotic coefficients as a function of solute concentration were described in the literature as a polynomial fit,^{57–58, 66} so the functions were interpolated to the concentrations that were simulated (or extrapolated in the case of Val from 0.7 m to 1.1 m). In other cases (Glu, Lys, Arg), the experimental data were collected at concentrations that exceeded those from our simulations, but had not been fit to a polynomial,^{61–62} so the osmotic coefficients were linearly interpolated from experimental data points to the concentrations that were simulated. In the case of Cys, the experimental data were collected at a concentration (1 m) that approached that used in our simulations (1.1 m);⁶³ these data were extrapolated using a simple, linear fit of the two highest concentrations measured experimentally (0.8 and 1 m).

In the case of Ser, experimental data were reported by the Smith & Smith labs only up to a concentration of ~0.5 m;⁵⁹ however, additional data in the form of water activity coefficients

(γ) were reported up to a concentration of 3.7 m by Hutchens and coworkers.⁶⁰ We therefore converted these γ values to their molal osmotic coefficients by plotting molality versus $\ln(\gamma)$, as suggested by Hamer,⁶⁷ and used the trapezoidal rule to approximate the integral given by Hamer. The resulting values were checked against the data reported by the Smith & Smith labs, who reported both osmotic and activity coefficients for Ser,⁵⁹ and were found to be in excellent agreement. The osmotic coefficients were then linearly interpolated to the concentrations that were simulated as above. Finally, for three other systems that were simulated (Ile, Gln, Phe), no osmotic coefficient data at pH 7 appear to be available. Thus, for these systems, as well as for systems for which only very dilute data were available (see above), no quantitative comparison was drawn; these are specified as “too dilute” or N/A in Table 1.

Derivation of new partial charges for two-residue peptides

The AMBER force field contains three explicit residue types for each of the twenty common amino acids for describing: (a) N-terminal, (b) C-terminal, and (c) internal residue positions. The partial charges for these residue types were originally published in 1995 using methylammonium as a model for the N-terminal ammonium group and using acetate as a model for the C-terminal carboxylate group of each amino acid.⁶⁸ The AMBER force field's partial charges for the Ala-Gly two-residue peptide, therefore, are obtained by simply concatenating those of the N-terminal Ala residue type together with those of the C-terminal Gly residue type. For reasons outlined in Results, however, we chose to re-derive partial charges for each of the four two-residue peptides ‘from scratch’ in order to determine whether this would lead to improved agreement with experiment.

At the time that this work was carried out, the extremely useful R.E.D. server⁶⁹— which is commonly used for deriving partial charges on small molecules — was temporarily unavailable; however, we were able to find the R.E.D. utilities⁷⁰ compiled in an internet archive. We utilized programs from R.E.D. that employ the quantum chemistry program GAMESS^{71–72} for geometry optimization and electrostatic potential calculations and that employ AmberTools 11⁷³ for carrying out the restrained electrostatic potential fits involved in deriving the partial charges. We first verified that we could reproduce the partial charges for zwitterionic Ala that were recently reported by Horn³⁶ who had used the R.E.D. server. After finding good agreement, we derived partial charges for each of the entire two-residue peptides (Ala-Ala, Gly-Gly, Ala-Gly, and Gly-Ala) as follows. For initial geometry optimization, we began with an extended structure for Ala-Ala, built in PyMOL.⁷⁴ Hydrogen atoms were added with the GROMACS utility pdb2gmx. The initial geometry optimization in GAMESS – which was performed at the Hartree-Fock level of theory with the 6–31G* basis set – required an initial bond restraint in order to prevent the loss of an amine hydrogen to the carboxylate group (which would have destroyed the zwitterionic state). Using the geometry-optimized structure of Ala-Ala, we generated the other two-residue peptides Ala-Gly, Gly-Ala, and Gly-Gly by replacing side chain methyl group(s) with hydrogen. Each of these four structures underwent an additional round of geometry optimization in GAMESS, this time with no constraints. All structures were visually inspected to confirm maintenance of the zwitterionic state.

After this, the electrostatic potential map was computed using GAMESS, again at the Hartree Fock level of theory with the 6–31G* basis set. A Perl script downloaded from the R.E.D. server was used to generate intermediate files and to call AmberTools for two-stage RESP charge fitting using the default parameters. The GAMESS and RESP input files can be found in the Supporting Information. The new partial charges were incorporated into an in-house installation of the AMBER force field as new N- and C-terminal residue types, each being specific to its two-residue peptide; these residue types can also be found in the Supporting Information. Simulations of these two-residue peptides were carried out as described above, at 1 M and in TIP3P, TIP4P-Ew, and TIP4P-D water, as well as using KBFF in SPC/E water, and were then compared to experimental data reported by the Smith & Smith labs.⁶⁵

Results

All osmotic pressure measurements in MD simulations were performed using the approach developed by Luo and Roux,⁴⁷ following earlier work by Murad⁷⁵ (see Methods). In such simulations a semi-permeable ‘membrane’ is implemented in the form of a simple flat-bottomed potential function acting along the long axis of the simulation box, with the force exerted by solutes on this ‘membrane’ providing a measure of the osmotic pressure. A view of a typical simulation system is provided in Figure S1. Comparison of the osmotic pressure from a simulation with that expected from a corresponding ideal solution yields the osmotic coefficient (ϕ), which in turn provides a reasonably direct measure of the solute-solute interaction thermodynamics:⁵¹ if the interaction thermodynamics in the simulations are excessively favorable – as might be expected given some of the results recently reported for protein systems (see Introduction) – then the MD-computed osmotic coefficients will be too low relative to the experimental values. A full list of the systems that were simulated, using both the TIP3P and TIP4P-Ew water models, is provided in Table 1. As outlined in detail in Methods, all systems were simulated for 100 ns at solute concentrations of 0.5, 1 and 2 M with concentrations being converted from molar to molal for comparison with experiment; more precise measurements consistently resulted from the more concentrated simulations (see standard deviations with respect to concentration in Figure S6).

Neutral amino acids

The osmotic coefficients computed for a variety of neutral amino acids in TIP4P-Ew water are plotted as a function of solute concentration in Figure 1A; a corresponding plot for TIP3P water is provided in Figure S7. At relatively low solute concentrations (i.e. 0.5 M), the computed osmotic coefficients of all tested amino acids are close to 1, but at higher concentrations they diverge, with the highest value (indicative of the most repulsive solute-solute interactions) being obtained with Pro and the lowest value being obtained with Phe (Figure 1A). The computed osmotic coefficients follow no clear trend with regard to the chemistry of the amino acids although, perhaps surprisingly, those of the polar amino acids Ser, Thr, and Gln tend to be lower than those of the aliphatic amino acids Leu and Ile. Visual inspection of the systems producing the lowest osmotic coefficients indicated that there was no obvious aggregation of the solutes. Figure 1B compares the computed osmotic coefficients of all tested uncharged amino acids at a 2 M concentration using both the TIP3P

(blue) and TIP4P-Ew (red) water models. From this it is apparent that simulations in TIP3P water always produce lower osmotic coefficients than the corresponding simulations in TIP4P-Ew water, which in turn indicates that solute-solute interactions are more attractive with the former water model. Interestingly, the statistical errors for the TIP3P simulations were also lower than for those of TIP4P-Ew water systems (Figure S6), consistent with improved sampling with the former water model due to its significantly lower viscosity (see, for example, ref. ⁶).

The real test of any force field, however, is to reproduce experimental data. For lower solute concentrations, issues with the precision of both the computed and the experimental osmotic coefficients make comparisons challenging. At the higher concentration of 2 M, however, comparisons can be made with more confidence for the five neutral amino acids that remain soluble at or very near these concentrations: Ala, Gly, Pro, Ser and Thr. Figure 2A compares the osmotic coefficients computed for these amino acids with the TIP3P water model with the corresponding experimental values; Figure 2B does the same for osmotic coefficients computed with the TIP4P-Ew water model. Interestingly, with both water models the relative ordering of the amino acids Ala, Gly, and Pro is very nicely reproduced, with the results for the TIP4P-Ew water model being in quantitative agreement with experiment (the black line in both Figures 2A and 2B indicates what would be expected if there was perfect agreement between simulation and experiment). With both water models, however, the osmotic coefficients computed for Ser and Thr are significantly lower than would be expected based on the values computed for other amino acids. Since both of these amino acids contain a side chain hydroxyl group, it seems reasonable to suspect that it is this group that contributes to excessively favorable solute-solute interactions in the simulation (see Discussion). Comparisons to experiment at lower solute concentrations are shown in Figure S8. Despite the increasing statistical uncertainties associated with these simulations, generally similar results are obtained; in particular, the correlations with experiment are considerably higher for the TIP4P-Ew water model at all solute concentrations tested. At a 1 M solute concentration, two additional amino acids can be added to the comparison with experiment (see Figures S8B and S8D). These are Val, which the simulations with TIP4P-Ew successfully identify as having a comparatively high osmotic coefficient, and Cys, to which the same force field combination assigns an unrealistically high osmotic coefficient. Relative to the results obtained at 2 M, the discrepancies with Ser and Thr at 1 M are less noticeable with the TIP4P-Ew water model (Figure S8D); with the better-sampled TIP3P water model, however, they remain quite obvious (Figure 8B).

Charged amino acids

Figure 3 shows osmotic coefficients obtained for the positively charged amino acids Hip (i.e. protonated His), Lys, and Arg; Figure 4 show results for the negatively charged amino acid, Glu. Since all of these simulations required the inclusion of counterions (Cl^- or Na^+) in order to neutralize the charge of the system, simulations were performed with a variety of ion parameters reported in the literature. Simulations of the positively charged amino acids indicated that solute-solute interactions became less attractive, and in some cases net-repulsive, with increasing solute concentrations (Figures 3A–C). As was the case with the neutral amino acids, use of the TIP3P water model resulted in consistently lower osmotic

coefficients than use of the TIP4P-Ew water model. In addition, use of the Dang Cl^- parameters resulted in consistently lower osmotic coefficients than use of the Joung & Cheatham Cl^- parameters. Figure 3D compares all of the computed osmotic coefficients at 2 M and, for Arg and Lys, shows the corresponding experimental values.⁶¹ In all cases, the computed osmotic coefficients were higher (i.e. more repulsive) than the experimental values, with the best results being obtained with a combination of the TIP3P water model and the Dang Cl^- parameters and the worst results being obtained with the TIP4P-Ew water model and the Joung & Cheatham Cl^- parameters.

For the negatively charged amino acid, Glu, the computed osmotic coefficients appear to be even more strongly dependent upon the choice of ion parameters (Figure 4). Simulations performed with the Åqvist Na^+ parameters (dark blue and dark red symbols and bars in Figure 4) resulted in higher osmotic coefficients than those performed with the Joung & Cheatham Na^+ parameters (light blue and light red symbols and bars in Figure 4), which resulted in strikingly low osmotic coefficients. Upon visual inspection, the latter systems were found to contain large Na-Glu clusters; a similar aggregation phenomenon has been reported previously using the Joung & Cheatham parameters with TIP3P water for Na-Acetate by Yoo & Aksimentiev.⁴² To correct this aberrant aggregation, and to reproduce the osmotic coefficient of Na-acetate, Yoo & Aksimentiev modified Joung & Cheatham's Lennard-Jones parameters for interactions of Na^+ ions with carboxylate oxygens. Since it was of interest to see how this modification might affect the behavior of Na-Glu we conducted two additional sets of simulations in TIP3P water. In one, we applied the Yoo & Aksimentiev modification only to the Lennard-Jones parameters describing the interaction of Na^+ with the sidechain carboxylate group (Aksimentiev 1X, light green symbols and bar in Figure 4); in a second, we also modified the Lennard-Jones parameters for the interaction of Na^+ with the backbone carboxylate group (Aksimentiev 2X, dark green symbols and bar in Figure 4). The inclusion of these modifications led to a progressive increase in the computed osmotic coefficients and to a reduction in the amount of Na-Glu clustering. More importantly, as shown in Figure 4B, the modification suggested by Yoo & Aksimentiev dramatically improved the level of agreement with experiment relative to what was achieved with the original Joung & Cheatham parameterization.⁶² Interestingly, simulations of Glu with the Åqvist Na^+ parameters in TIP4P-Ew water also performed quite well despite the fact that these ion parameters were not originally derived for use with the TIP4P-Ew water model (Figure 4B).

The presence of clear solute aggregation in the Na-Glu system when using certain ion parameters is a significant concern and raises the issue of whether similar levels of aggregation might occur in systems containing mixtures of positively and negatively charged amino acids. A recent simulation study by the Chong group¹² using a large number of force field combinations has demonstrated (encouragingly) that salt-bridge interactions between charged organic solute molecules do not lead to the formation of long-lasting clusters. But to ensure that this is also true for complete amino acids, we simulated mixed Glu + Lys systems. The osmotic coefficients for the Glu + Lys system computed with both the TIP3P and TIP4P-Ew water models are shown in Figure S9. As seen with all other systems, the osmotic coefficients obtained with TIP4P-Ew are significantly higher than those obtained

with TIP3P. In neither case, however, were the computed osmotic coefficients so low as to indicate aggregation; the absence of any significant aggregation was confirmed visually.

Osmotic coefficients from force fields known to maintain IDP disorder

Since all of the results described above showed a clear dependence on the choice of water model, it was of interest to carry out additional simulations using the recently reported TIP4P-D water model developed by the Shaw group.²⁰ Simulations were performed of the following amino acids: Pro, Gly, Thr, Gln, and Phe, and compared to our earlier results with TIP3P and TIP4P-Ew water models. These amino acids were selected because of the low (Thr, Gln, Phe) or high (Pro) osmotic coefficients that were observed in simulation using the previous water models; Gly was simulated as it is the simplest amino acid and so forms a convenient reference point. With all five amino acids, use of the TIP4P-D water model led to an increase in the osmotic coefficients relative to the values obtained with the TIP3P or TIP4P-Ew models (green bars, shown at the 2 M concentration in Figure 5A, and at lower concentrations in Figure S10). This result is consistent with the previously reported tendency of TIP4P-D to increase the radii of gyration for IDPs and slightly destabilize folded proteins.²⁰ Interestingly, when we compared the magnitude of the *change* in the osmotic coefficient due to the use of TIP4P-D to the osmotic coefficient obtained from the corresponding simulation with TIP3P or TIP4P-Ew, we found a strong, negative correlation (Figure 5B): that is, low osmotic coefficients obtained with TIP3P or TIP4P-Ew water increased drastically when using TIP4P-D water, while high osmotic coefficients only mildly increased with this change in water model. Also of note is that in comparison with experiment, the resulting osmotic coefficients for Pro, Gly and Thr are all somewhat too high (Figure 5A), although in the case of Thr the value is in considerably better agreement with experiment than previously obtained with TIP3P or TIP4P-Ew.

As noted in the Introduction, the KBFF developed by the Smith group has also been shown to perform well in simulating IDPs,²¹ so it was of interest to simulate the same amino acids with this force field. With the exception of Gly, the osmotic coefficients computed with KBFF were higher than those obtained with the AMBER force fields regardless of which water model was used with the latter (yellow bars, shown at 2 M concentration in Figure 5A, and at lower concentrations in Figure S10); the exceptional result that we obtained for Gly is consistent with that reported previously by the Smith group.⁵³ For both Pro and Thr, the osmotic coefficient computed with KBFF was found to be considerably higher than the experimental value. While that is a somewhat disappointing result, it is notable that KBFF was the only force field combination tested that qualitatively reproduced the relative osmotic coefficients of Pro, Gly and Thr (Figure 5A). As was the case with TIP4P-D, the osmotic coefficients obtained for Gln and Phe with KBFF were much higher than those obtained with either the AMBER + TIP3P or AMBER + TIP4P-Ew force field combinations.

Two-Residue Peptides

Finally, to determine whether a force field combination that apparently works well for single amino acids might also work well for peptides, we simulated each of the following two-residue peptides: Ala-Ala, Ala-Gly, Gly-Ala and Gly-Gly. Experimental osmotic coefficients for each of these peptides have been determined at concentrations up to 1 m.⁶⁵

Encouragingly, all four force field combinations tested qualitatively reproduced the experimental data (Figure 6A). However, in contrast to the very good quantitative results obtained for both Ala and Gly when using the TIP4P-Ew water model (Figure 2B), simulations of these two-residue peptides resulted in osmotic coefficients that were well below those obtained from experiment (compare the red triangles in Figure 6A with the black line indicating perfect quantitative agreement). Relative to TIP4P-Ew, the results produced by the other force field combinations that we tested were qualitatively the same as seen with the single amino acids: simulations using the TIP3P water model (blue circles) reported osmotic coefficients that were consistently lower than those obtained with TIP4P-Ew, while simulations using the TIP4P-D water model (green squares) produced results that were higher than TIP4P-Ew. As was the case with single amino acids (Figure 5A), the KBFF produced the highest osmotic coefficients, even though our computed value for Gly-Gly ($\phi = 0.83$) is somewhat lower than that previously reported by the Smith group ($\phi = 0.88$).⁵³

Given that TIP4P-Ew nicely reproduced the osmotic coefficients of both Gly and Ala (Figure 2B), it was surprising to find such poor agreement with experiment for two-residue peptides containing the same residues. One key difference between the simulations of single amino acids and the simulations of two-residue peptides however, is that the partial charges for the former were derived from QM calculations performed recently on the complete, zwitterionic amino acids,³⁶ while the partial charges for the latter, taken directly from the AMBER force field, were originally derived from QM calculations performed 20 years ago on smaller fragments meant to represent the charged termini.⁶⁸ We were therefore interested in determining whether a re-derivation of partial charges, specific for each of the four two-residue peptides, might lead to altered predictions of the osmotic coefficients (see Methods). This was indeed the case (Figure 6B). With all three water models, the osmotic coefficients shifted to higher values when the newly reparameterized charges were used, and encouragingly, the values obtained with the TIP4P-Ew water model became much closer to the experimental values (red triangles in Figure 6B).

Discussion

Following earlier work by Murad,⁷⁵ a number of reports have described the use of osmotic pressure simulations as a means of validating and/or reparameterizing biomolecular simulation force fields. These include reports from the Roux group,⁴⁷ whose protocol we have followed closely here, the Aksimentiev^{42, 76}, Smith⁵³ and Mackerell⁷⁷ groups, as well as work on carbohydrates from our own group.⁷⁸ The principal results of this study are as follows: (a) the combination of AMBER + TIP4P-Ew produces excellent osmotic coefficients for the neutral amino acids Ala, Gly, Pro and Val (the latter at 1 M) but noticeably underestimates the osmotic coefficients of the hydroxyl-containing amino acids Ser and Thr, (b) the osmotic coefficients obtained with TIP3P are uniformly lower than those obtained with TIP4P-Ew, which, in turn, are much lower than those obtained with the newly developed TIP4P-D water model, (c) the osmotic coefficients of the charged amino acids Arg, Glu, His and Lys are sensitive to the parameters assigned to the neutralizing counterion, and the poor agreement obtained with the original Joungh & Cheatham parameters for Na-Glu is almost entirely corrected by the modification proposed by Yoo & Aksimentiev,⁴² (d) the osmotic coefficients of the four two-residue peptides tested here are

much too low when using the original AMBER charges with TIP4P-Ew water but are in good agreement with experiment when simulations are run with partial charges derived specifically for each peptide. In what follows, we consider each of the above points in turn.

The results shown in Figure 2B indicate that the osmotic coefficients of some amino acids are accurately reproduced by the AMBER force field while those of other amino acids are not. This is, in some sense, an encouraging result as it suggests the possibility that the previously reported failures to describe the behavior of IDPs discussed in the Introduction might be due more to inadequate parameterization of specific amino acids rather than to a general failing of the force fields. If true, this could call into question strategies that explicitly take general approaches in attempts to solve the problems.^{9, 20} The comparatively poor results obtained with Ser and Thr suggest that solute-solute interactions involving the sidechain hydroxyl group are excessively favorable when using the AMBER + TIP4P-Ew force field combination. In contrast, the poor results obtained for Cys at 1 M (Figure S8) suggest that interactions involving the sulfhydryl group might be insufficiently favorable. Interestingly, both of these results are consistent with the Nerenberg group's recent calculations¹³ of the enthalpies of vaporization (H_{vap}) for a wide range of small molecules: in their study, the H_{vap} values of four alcohols (methanol, ethanol, etc) were too favorable by 1.3 ± 0.6 kcal/mol, while the H_{vap} values of four simple thiols were too unfavorable by 0.9 ± 0.6 kcal/mol. One minor complication with interpreting the result for Cys is the possibility of partial ionization of the sidechain which might occur in the experiment but which is not modeled in the simulations: depending on the exact numbers assigned to the pKa values of the ionizable groups in Cys, we estimate that 5 – 10% of the sidechains will be ionized. However, since ionization would lead to the formation of two solute species from one, it would be expected to result in an increased osmotic coefficient; for this reason, we think that ionization is unlikely to explain the discrepancies with experiment for this amino acid. In the near future, it should be possible to follow the approaches outlined previously by the Roux⁴⁷ and Aksimentiev groups^{42, 76} – and by us recently in the context of carbohydrates⁷⁸ – and adjust the Lennard-Jones parameters for solute-solute interactions involving the hydroxyl and sulfhydryl groups in order to improve agreement with experiment.

It is quite possible that the interactions of other amino acids will also require adjustment, although a current paucity of experimental osmotic coefficient data may place limits on what can be definitively concluded at this stage. Obvious candidates for further examination are Phe and Gln, for which widely disparate osmotic coefficients were obtained depending on the force field combination used (see Figure 5). The very low osmotic coefficients obtained for these two amino acids with the TIP3P and TIP4P-Ew water models are potentially significant for at least two reasons. The first is that the amidic residues (Gln and Asn) and aromatic residues (Phe, Tyr, Trp) were found in abundance at the aggregation interface of the villin headpiece in the MD simulations reported by the Zagrovic group.¹⁹ The second is that the IDP Nup153, which was recently shown to undergo an unphysical collapse using multiple force fields in MD,²¹ contains 9 Gln and 7 Phe residues, which together account for 20% of its sequence. While both observations would be consistent with the self-interactions of amidic and aromatic residues being excessively favorable in the AMBER force field,

directly demonstrating this will require additional experimental data as the osmotic coefficients of these amino acids are, unfortunately, not currently available.

While the accuracy of the osmotic coefficients for some amino acids will remain uncertain until direct experimental data are forthcoming, there is no doubt that the water model used in the simulations plays a significant role in determining the values obtained. Our results are consistent with previous work examining small molecule associations in water: the Chong group showed that simulations of sidechain analogs of charged amino acids exhibited larger association constants in TIP3P water than in TIP4P-Ew water,¹² and we previously showed that for concentrated amino acids, the first peak in the radial distribution function for interactions of the NH_3^+ and COO^- groups was significantly larger with TIP3P water than with TIP4P-Ew water.⁶ Our results are also consistent with studies of the relative effects of the two water models in simulations of proteins. The Paschek and García groups examined the stability of the Trp-cage mini-protein and found it to be more unfolded in TIP4P-Ew water than in TIP3P water.⁷⁹ Similarly, the Shaw group found that for multiple proteins, R_g values were larger when simulated using TIP4P-Ew water than when using TIP3P water²⁰ and became larger still when using their TIP4P-D water model, again consistent with the trends that observed here for amino acids (Figure 5A) and two-residue peptides (Figure 6). Moving forward, therefore, it appears that the choice of water model is likely to play an important role in determining the strengths of inter-solute interactions.

The results shown in Figures 3 and 4 indicate that for interactions of charged amino acids, the choice of ion parameters also becomes an important consideration. The Dang Cl^- and Åqvist Na^+ parameters, both of which originate in the 1990s, have been shown to lead to crystallization artifacts at low concentrations.⁴¹ This problem was overcome with the parameters developed much more recently by Joung & Cheatham,⁴¹ so it is somewhat surprising that simulations using the earlier parameter sets resulted in better agreement with experiment for the osmotic coefficients of charged amino acids. In fact, as noted by the Aksimentiev group⁴² and reiterated here, use of the Joung & Cheatham Na^+ parameters with carboxylate-containing solutes can lead to aggregation. It is therefore encouraging to find that the inclusion of the modifications suggested by the Aksimentiev group for TIP3P water significantly improve agreement with experiment, and when applied to both carboxylate groups of Glu (i.e. sidechain and backbone) lead to near-quantitative agreement (Figure 4B). This result strongly suggests that the Aksimentiev group's TIP3P parameters are to be preferred for future simulations of systems in which Na^+ -carboxylate interactions are likely to play an important role. For TIP4P-Ew water, the best current choice – at least for reproducing the osmotic coefficient of Na-Glu – are the parameters derived by Åqvist, although since they were derived many years prior to the development of the TIP4P-Ew model, the good result should probably be considered fortuitous. Given that these parameters have other failings (namely, crystallization artifacts with Cl^- at low concentrations mentioned earlier), however, it would seem to be worth following the Aksimentiev group approach and seeking a modification to the Joung & Cheatham parameters for Na^+ -carboxylate interactions in TIP4P-Ew. A similar strategy would also seem to be worth applying in the near future to the Cl^- -Lys and Cl^- -Arg interactions in TIP4P-Ew since neither of the ion parameter sets tested here produced very good agreement with experiment for these amino acids.

Still another factor that we think affects the ability to reproduce experimental osmotic coefficients of peptides is the set of partial charges assigned to the terminal groups in the AMBER force field. The decision to recalculate partial charges for the two-residue peptides studied here was inspired in part by the large differences we noticed between the charges assigned to the NH_3^+ and the COO^- groups in the existing AMBER force field and those found in the charge sets recently reported by Horn³⁶ for single amino acids (which, in turn, were very similar to the values that we had derived earlier⁶). Relative to the original AMBER charge set, recalculation of the charges decreased the magnitude of the net charges on the terminal NH_3^+ and COO^- groups by ~ 0.2 e, changed the net charge on the peptide group from -0.07 to -0.24 e and changed the partial charge on the C-terminal Ca atoms from -0.05 to 0.20 . Use of these new partial charges in TIP4P-Ew water improved the computed osmotic coefficients for all four two-residue peptides tested, with the result for Gly-Gly changing by more than 0.3 units (Figure 6B). Using the same partial charges in TIP4P-D water resulted in osmotic coefficients that were too high compared with experiment although it is worth noting that using the original partial charges with this water model led to surprisingly good agreement with experiment (Figure 6A). This result should, however, probably be considered fortuitous given the consistent overestimation of the osmotic coefficients that we observed for systems of individual amino acids using this water model (Figure 5A). Of course, recalculating the partial charges on the terminal amino acids will almost certainly not solve the problems outlined in the Introduction of protein aggregation and IDP collapse seen with the AMBER force field as there are only two such groups in a given polypeptide chain. Nevertheless, for smaller systems – especially short peptides – the present results suggest that a need to recalculate partial charges for the termini might be a potential factor to consider if poor agreement with experimental observables is encountered.²

In conclusion, the present study serves as a continuation of previous studies performed by the Roux⁴⁷ and Aksimentiev groups,^{42, 76} and more recently our own group,⁷⁸ in using calculations of osmotic coefficients as a basis for testing and adjusting the thermodynamics of inter-solute interactions in MD simulations. In particular, this work highlights the potential use of osmotic coefficient data for identifying areas for improvement in protein force fields, something that, with the exception of the pioneering works of the Smith group,^{16–18} has thus far attracted little attention. The results obtained for Ser and Thr point to potential shortcomings in the AMBER force field's description of inter-solute interactions involving the hydroxyl group, and given how widespread the hydroxyl group is, it is possible that similar shortcomings will be found in other biomolecular systems; we have, for example, recently found that the strengths of sugar-sugar interactions are significantly exaggerated with the AMBER-compatible GLYCAM06 force field.⁷⁸ Work to establish and correct such errors more generally is currently underway in our laboratory. Notwithstanding the challenges associated with identifying experimental osmotic coefficient data in the literature, we think that it is clear that such data allow for valuable comparisons between the solute-solute interaction thermodynamics observed in MD simulations and those determined from experiment. Since such comparisons can reveal inaccuracies and suggest routes for reparameterizing force fields, they can, in turn, facilitate the construction of more fully

transferrable force fields applicable to modeling of biomolecular interactions in aqueous solution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Osmotic Coefficients of the Amino Acids

A. Plot of osmotic coefficients (ϕ) for 16 amino acids in TIP4P-Ew water with respect to solute concentration. Small aliphatic residues are shown in blue, large aliphatic residues and aromatics in green, and polar residues in red. Error bars are standard deviations obtained from dividing data into 5 contiguous blocks of 20 ns. **B.** Comparison of osmotic coefficients of the amino acids at 2 M in both TIP4P-Ew water and TIP3P water. Data are sorted in increasing order of osmotic coefficients in TIP4P-Ew water.

Figure 2. Comparison with Experimental Data

A. Comparison of osmotic coefficients for zwitterionic amino acids from simulation (y-axis) with those from experiment (x-axis) at 2 M in TIP3P water. The black line represents a perfect 1:1 correspondence between simulation and experiment. The colored line represents the linear regression line. Error bars are shown for simulation data only. **B.** Same as **A**, but showing results for TIP4P-Ew water.

Figure 3. Osmotic Coefficients for Positively Charged Amino Acids

A. Plot of osmotic coefficients for Arg-Cl versus solute concentration for various force field and parameter combinations. **B.** Same as A but showing results for Lys-Cl. **C.** Same as A but showing results for Hip-Cl. **D.** Comparison of osmotic coefficients for the same amino acids at 2 M with experiment (grey).

Figure 4. Osmotic Coefficients for Glutamate

A. Plot of osmotic coefficients for Na-Glu versus solute concentration for various force field and parameter combinations. **B.** Comparison of osmotic coefficients for Na-Glu at 2 M with experiment (grey).

Figure 5. Osmotic Coefficients Using Different Force Fields

A. Comparisons of osmotic coefficients from simulations of five amino acids (Pro, Gly, Thr, Gln, and Phe) at 2 M using AMBER99sb-ildn with TIP3P (blue), TIP4P-Ew (red), and TIP4P-D (green) water; using the KBFF with SPC/E water (yellow); and from experiment (grey). **B.** Scatterplot showing the change in osmotic coefficient that results when TIP4P-D is used in place of TIP3P (blue) and TIP4P-Ew (red) water plotted versus the osmotic coefficient obtained with TIP3P or TIP4P-Ew; all data points correspond to a 2 M solute concentration.

Figure 6. Osmotic Coefficients for Two-Residue Peptides

A. Comparison of osmotic coefficients for two-residue peptides Gly-Gly (GG), Gly-Ala (GA), Ala-Gly (AG), and Ala-Ala (AA) from simulation (y-axis) with those from experiment (x-axis) at 1 M using original AMBER charges. **B.** Same as A but showing results obtained using re-derived partial charges for each peptide. The data for KBFF are the same in both plots. The black line represents a perfect 1:1 correspondence between simulation and experiment.

Table 1

Simulation and Experimental Details

Amino Acid ^a	Method For Comparison with Experiment ^b	Max. Exp. Conc. (m) ^c	ϕ or γ (Exp.)? ^d	Simulated with TIP4P-D/KBFF? ^e
Ala ⁵⁸	Literature Polynomial	1.9	ϕ	
Cys ⁶³	Linear Extrapolation	1.0	ϕ	
Glu ⁶²	Interpolation	4.1	ϕ	
Phe	N/A	N/A	N/A	Yes
Gly ⁵⁷	Literature Polynomial	3.3	ϕ	Yes
Hip ⁶⁴	Too Dilute	0.6	ϕ	
Ile	N/A	N/A	N/A	
Lys ⁶¹	Interpolation	3.7	ϕ	
Leu ⁶³	Too Dilute	0.2	ϕ	
Met ⁶³	Too Dilute	0.2	ϕ	
Pro ⁵⁹	Interpolation	7.2	ϕ	Yes
Gln	N/A	N/A	N/A	Yes
Arg ⁶¹	Interpolation	5.4	ϕ	
Ser ⁶⁰	Interpolation	3.7	γ	
Thr ⁵⁹	Literature Polynomial	2.1	ϕ	Yes
Val ⁵⁸	Literature Polynomial	0.7	ϕ	
Glu+Lys	N/A	N/A	N/A	
Ala-Ala ⁶⁵	Interpolation	1.1	ϕ	Yes
Gly-Gly ⁶⁵	Interpolation	1.9	ϕ	Yes
Ala-Gly ⁶⁵	Interpolation	1.1	ϕ	Yes
Gly-Ala ⁶⁵	Interpolation	1.3	ϕ	Yes

^aList of amino acids simulated in this study with TIP3P and TIP4P-Ew water. Superscript numbers indicate the references for the sources of experimental data.

^bData were compared by using polynomials found in literature, by our own linear extrapolation, or by linearly interpolating between two data points.

^cThe maximum molal concentration for which experimental data were reported.

^dDenotes whether experimental data were reported as the osmotic coefficient (ϕ) or the activity coefficient (γ).

^eIdentifies amino acids that were also simulated using TIP4P-D water and using the KBFF with SPC/E water.