

Variation of the Energy Landscape of a Small Peptide under a Change from the ECEPP/2 Force Field to ECEPP/3

Frank Eisenmenger[†] and Ulrich H. E. Hansmann^{*,‡}

Institute for Biochemistry, Medical Faculty (Charité) of the Humboldt University Berlin, 10115 Berlin, Germany, and Swiss Center for Scientific Computing (SCSC), Federal Institute of Technology (ETH) Zürich, 8092 Zürich, Switzerland

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The *multicanonical ansatz* is used to study variations in the energy landscape of a small peptide, Met-enkephalin, under a change from the ECEPP/2 force field to ECEPP/3. Local minima with energies up to 5 kcal/mol higher than the global minima are sampled and classified according to H-bridges and backbone angles. The distribution and relative weight for various temperatures of the minima are calculated and compared for the two force fields. We demonstrate that while there are small differences in the energy landscape our results at relevant temperatures are robust under changes between ECEPP/2 to ECEPP/3.

Introduction

While there is considerable progress in numerical simulation of peptides and proteins (for a recent review see, for instance, ref 1), prediction of their low temperature conformations solely from first principles remains a formidable task. First, the interactions between the atoms in the molecules are described by force fields which depend in part on empirically determined parameters. It is still an open question which of the many force fields (AMBER,² CHARMM,³ ECEPP,^{4–7} ...) is the optimal choice (or if they are sufficiently accurate at all). The other mayor problem is common to many important physical systems. At low temperatures the energy landscape of proteins is characterized by a multitude of local minima separated by high energy barriers. In simulations based on classical molecular dynamics or Monte Carlo technique⁸ these barriers can be seldom crossed. Hence, only small parts of phase space are sampled (in a finite number of Monte Carlo sweeps) and physical quantities cannot be calculated accurately. For this reason, the “native” conformation of a protein (which should correspond to the global minimum in *free* energy) is often identified with the lowest *potential* energy configuration. In this approximation entropic contributions are ignored, but it allows application of optimization techniques like simulated annealing,¹⁰ genetic algorithms,⁹ or Monte Carlo with minimization¹¹ to tackle the protein folding problem. However, this approximation may be to crude. Especially for smaller peptides, entropic contributions are expected to be important.

It is not always clear whether the limited quality of numerical results is due to insufficient simulation algorithms or inadequate force fields. Recent progress in the development of Monte Carlo techniques may change this situation. Simulations in *generalized ensembles* promise a much better sampling of the phase space. A numerical comparison of some of these new algorithms can be found in ref 12. One of its more prominent exponents is the so-called *multicanonical* approach of Berg and co-workers¹³ who propose a weighting scheme which yields a flat probability distribution in energy. Hence, all energies have equal weight and an one-dimensional random walk in energy

space is carried out (when simulated with local updates) which ensures that the simulation will not get trapped in any local energy minimum. The Boltzmann distributions may be obtained for a given range of temperatures from one multicanonical simulation by reweighting the states.¹⁴ The method was exploited to model first-order phase transitions^{13,15} and spin–glass systems.^{16–18} The prediction of peptide and protein three-dimensional structures with multicanonical algorithms was first addressed in ref 19 for Monte Carlo methods and in ref 20 for molecular dynamics. Subsequent works include the study of coil–globular transitions of a model protein,²¹ helix–coil transitions of amino acid homo–oligomers,²² and the conformational sampling of a constrained peptide.²³

In the current article we use the multicanonical technique to compare distribution and relative weight of the local minima at room temperature and below for the closely related ECEPP/2 and ECEPP/3 force field in the case of the small pentapeptide Met-enkephalin. ECEPP/3 deviates from the previous ECEPP/2 by slightly different parameters due to improvements in the experimental measurements. We want to study the influence of such small changes on the energy landscape and our observed quantities. This should show us how much numerical simulations are affected by experimental uncertainties in the parameters of the force fields.

Methods

Multicanonical Algorithm. Although the multicanonical approach is explained in detail elsewhere (see, for instance refs 16 and 24), we briefly summarize the idea and implementation of the method for completeness.

Simulations in the canonical ensemble weight each configuration with $w_B(E, \beta) = e^{-\beta E}$ and yield the usual Boltzmann probability density distribution of energies:

$$P_B(E, T) \propto n(E)e^{-\beta E} \quad (1)$$

where $n(E)$ is the density of states with energy E and $\beta = 1/(k_B T)$, with temperature T and Boltzmann constant k_B .

On the other hand, in the multicanonical approach configurations with energy E are updated with a weight

$$w_{mu}(E) \propto n^{-1}(E) = e^{-S(E)} \quad (2)$$

* Author to whom all correspondence should be addressed. New address: Institute for Molecular Science (IMS), Okazaki 444, Japan.

[†] Medical faculty of the Humboldt University Berlin.

[‡] Federal Institute of Technology Zürich.

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where $S(E)$ is the microcanonical entropy. A uniform distribution of the energy

$$P_{mu}(E) = n(E)w(E) = \text{const} \quad (3)$$

is obtained from the simulation with these weight factors. In this way information is collected over the whole energy range and from a single simulation one can obtain the canonical distribution for a wide range of temperatures by the reweighting techniques:¹⁴

$$P_B(T,E) \propto P_{mu}(E)w_{mu}^{-1}e^{-\beta E} \quad (4)$$

This allows one to calculate any thermodynamic quantity \mathcal{O} by

$$\langle \mathcal{O}_T \rangle = \frac{\int dE \mathcal{O}(E)P_B(T,E)}{\int dE P_B(T,E)} \quad (5)$$

However, unlike in a canonical simulation the weights $w_{mu}(E)$ are not *a priori* known (in fact, knowledge of the exact weights is equivalent with solving the system), and one needs its estimators for a numerical simulation. One way to obtain these estimators is the following iterative procedure. Starting with an initial guess $w_{mu}^0(E) = e^{-\beta_0 E}$, i.e., performing a canonical simulation at sufficient high temperature T_0 , improved estimators of the multicanonical weights are calculated from histograms $P_{mu}^{i-1}(E)$ of preceding simulations by

$$w_{mu}^i(E) = \frac{w_{mu}^{i-1}(E)}{P^{i-1}(E)} \quad (6)$$

For details, see refs 22 and 24. This method for calculating multicanonical weights is by no means unique, and while it is quite general, it has the disadvantage that it requires a certain number of extra iterations which is not *a priori* known.

Force Fields. In ECEPP⁴⁻⁷ the potential energy function E_{tot} is given by the sum of the electrostatic term E_C , 12-6 Lennard-Jones term E_{LJ} , and hydrogen-bond term E_{HB} for all pairs of atoms in the peptide together with the torsion term E_{tor} for all torsion angles:

$$E_{tot} = E_C + E_{LJ} + E_{HB} + E_{tor} \quad (7)$$

$$E_C = \sum_{(i,j)} \frac{332q_i q_j}{\epsilon r_{ij}} \quad (8)$$

$$E_{LJ} = \sum_{(i,j)} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} \right) \quad (9)$$

$$E_{HB} = \sum_{(ij)} \left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}} \right) \quad (10)$$

$$E_{tor} = \sum_l U_l (1 \pm \cos(n_l \chi_l)) \quad (11)$$

Here, r_{ij} is the distance between the atoms i and j , and χ_l is the torsion angle for the chemical bond l . In ECEPP bond lengths and bond angles (which are hard degrees of freedom) are fixed at experimental values and no out-of-plane deformation of peptide bonds is allowed, leaving the dihedral angles ϕ , ψ , ω , and χ as independent variables. The various parameters (q_i , A_{ij} , B_{ij} , C_{ij} , D_{ij} , U_l , and n_l) for the energy function were

TABLE 1: Point Charges for Terminal Groups of Met-Enkephalin

atom	ECEPP/2	ECEPP/3
N	-0.356	-0.332
H1, H2	0.176	0.076
C	0.450	0.517
O	-0.384	-0.351
O	-0.380	-0.334
H	0.204	0.235

determined by a combination of *a priori* calculations and minimization of the potential energies of the crystal lattices of single amino acids. In the light of more recent experimental findings the standard geometry and some energy parameters for prolyl and hydroxyprolyl have been updated. Together with a recalculation of partial atomic charges of main-chain atoms, this led to a revision of the ECEPP/2 parameter set to ECEPP/3. In the original formulation of the ECEPP/2 parameter set, the net charges of both the N- and the C-terminal residues, added up to the desired net charge of the molecule. Now each terminal amino acid residue carries a total charge of 0/+1/-1 at the N- or C-terminus, respectively. The correction avoids possible artifacts for systems with terminal groups of different types (e.g., one terminus charged, the other uncharged).

Simulation and Technical Details

To investigate changes in the energy landscape under small variations of the force field, we have studied one of the simplest peptides, Met-enkephalin, which has the amino acid sequence Tyr-Gly-Gly-Phe-Met. This peptide is convenient for our purpose, since for the potential energy function ECEPP/2 the lowest energy conformation is known,^{11,25,26} and local minima with energies not much higher than the global minimum were sampled and classified for this molecule by Braun and co-workers.²⁷ We compare our results with theirs and calculate in addition the relative weight of the local minima at various temperatures, which is not possible with the method used by Braun *et al.*

For our simulations the backbone was terminated by a neutral NH₂ group at the N-terminus and a neutral COOH group at the C-terminus as in the previous works of Met-enkephalin. For Met-enkephalin the two versions of ECEPP differ in the point charges for terminal groups which are listed in Table 1. The peptide-bond dihedral angles ω were fixed at the value 180°, leaving 19 dihedral angles as independent variables. (We fixed these hard degrees of freedom to reduce the number of variables. It would be more rigorous to allow for bending of the peptide angles. However, in this article we are mostly interested in comparing two variants of a force field, not in reproducing experimentally found structures by numerical simulation, which allow us to choose a less rigorous approach.) Interactions of the molecule with solvent molecules were neglected and the dielectric constant ϵ was set equal to 2. We used the program SMC,²⁸ which was modified to accommodate the multicanonical ensemble.

The multicanonical weight factors were determined separately for both ECEPP/2 and ECEPP/3 by the iterative procedure described above. We needed 100 000 sweeps for their calculation. One MC sweep updates every dihedral angle of the molecule once. For each update of an angle a new randomly chosen value out of the interval $[-\pi, \pi]$ was proposed and the new configuration then accepted or rejected by the Metropolis criteria. All thermodynamic quantities were calculated for ECEPP/2 and ECEPP/3 separately from a production run of

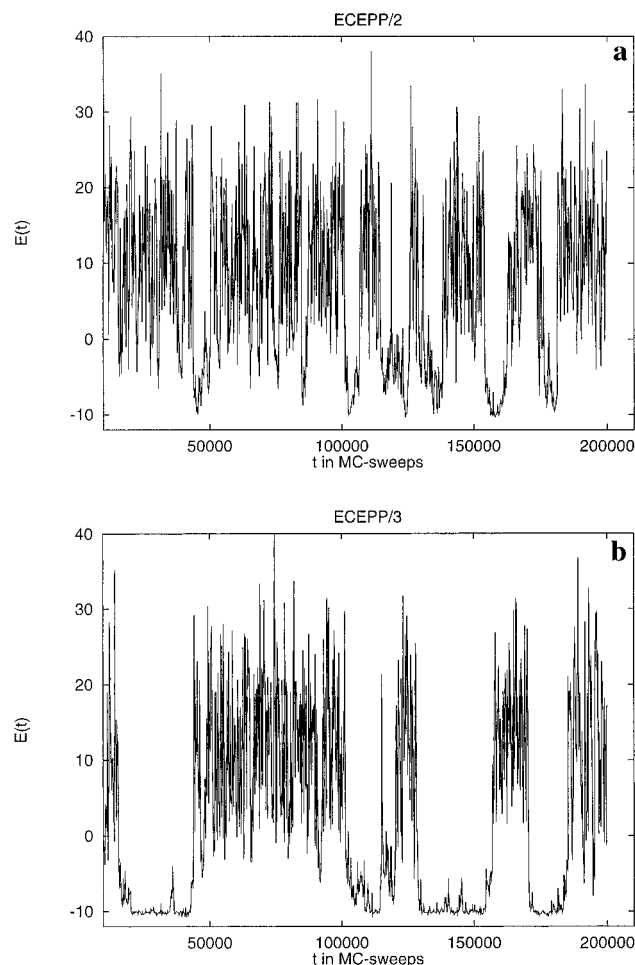


Figure 1. Time series of potential energy, obtained from a multicanonical simulation of 200 000 MC sweeps, using (a) the ECEPP/2 and (b) the ECEPP/3 force field.

200 000 MC sweeps following an additional 10 000 sweeps for equilibration each. The energy after each sweep was stored for further analysis. In all cases, each simulation started from a completely random initial conformation ("Hot Start"), but in the case of ECEPP/2 we also checked our results for shorter runs against those with an initial configuration of the known ground state ("Cold Start"), and we found that the results are in agreement with those from random initial conformations. This suggests that thermal equilibrium has been attained in our simulations. This conjecture is further supported by Figure 1, in which we show the time series of the energy for both production runs. In the course of the simulation, a 1d random walk in the energy between low energy states and high energy states is performed, as one would expect for local updates from the definition of the multicanonical ensemble. It is evident that low energy states which are separated in the time series by high energy states (which correspond to high temperature states) are uncorrelated. The number of such "tunnel" events is therefore a lower limit for the number of independent low energy states visited in the simulation.

In order to investigate the energy landscape of our peptide, we not only stored the energy of the actual conformation after each MC sweep but also minimized a copy of this conformation if its energy was less than a certain limit, 0 kcal/mol. While this limit is somehow arbitrary, our experience shows that it allows a rough distribution between a "low temperature region" and a "high temperature region" (it is the expectation value of energy at $T = 300\text{K}$ where the specific heat has its maximum). From the minimization process we receive a local minimum

TABLE 2: Number of Conformers and Nonidentical Conformations Collected after Minimization for Both Choices of Force Fields

	ECEPP/2	ECEPP/3
minimized conformers $E < -6$ kcal/mol	62 142	109 863
nonidentical minimized conformers (≥ 1 dihedral differs by $> 1^\circ$)	1 677	1 574

conformation. Configurations which yield the same local minima belong to the same valley in the energy landscape and are therefore related to each other. Since we are mainly interested in local minima not too far away from the global minimum and to save disc space, we introduced a second limit. Only if the energy of the minimized configuration was below -6 kcal/mol (≈ 5 kcal/mol above the global minimum) it was stored for further analysis. In Table 2 we summarize the absolute number of local minima sampled in this way for both variants of the ECEPP force field.

While the set of configurations we received from our production runs are all local minima, due to limitations of the minimizer (a Newton–Raphson variant), they are not necessarily the lowest configurations associated with a certain valley in the energy landscape. Each valley is in itself rough and consists of subvalleys separated by energy barriers. Since we are not interested in the microscopic details of the energy landscape, we further clustered our local minima and identified each cluster by its member with the lowest energy. In this way, only a small number (less than five) of groups remains at the end of our clustering by which the overwhelming number of local minima is classified.

We describe now briefly our clustering technique. In a first step we sorted all minimized conformers according to their energy. In a second step, we looked for identical conformers. Two conformers were regarded as identical if they differ in no angle more than 1° (taking symmetries in account) and in their energies less than 0.0001 kcal/mol. For further clustering, we kept only the nonidentical conformations. Their number, which is also shown in Table 2, is our estimate for the total number of local minima with energy below -6 kcal/mol. It is interesting to observe that the number of nonidentical conformers sampled in the multicanonical runs is similar for each choice of force field, while the absolute number of sampled local minima varies much more (see Table 2). In a third step, using all backbone angles, the conformer (from our remaining set of nonidentical local minima conformations) with the lowest energy is compared to all other with higher energies, according to differences in their backbone dihedrals. The conformers having all their angles within 10° in comparison with the first one, with respect to symmetry, are put into the same cluster, which is represented by the first conformer. This procedure is repeated for the next conformer with higher energy, which was not counted in the first cluster by comparing its angles with the other remaining "free" conformers. In this way, we are left with 160 clusters for ECEPP/2 and 571 clusters for ECEPP/3. In the final step these clusters were classified into a few groups by using similarities in hydrogen bonding and the significant dihedrals, necessary to preserve the characteristic hydrogen bonds. The final groups which should represent the main valleys in the energy landscape of our peptide are characterized by their lowest energy conformer.

In Figure 2 we display how in the course of our simulations different valleys of the energy landscape (characterized by its corresponding group number) are visited. We show only that time when the simulation visits one of the four (three) most important clusters for ECEPP/2 (ECEPP/3), and no plot symbol

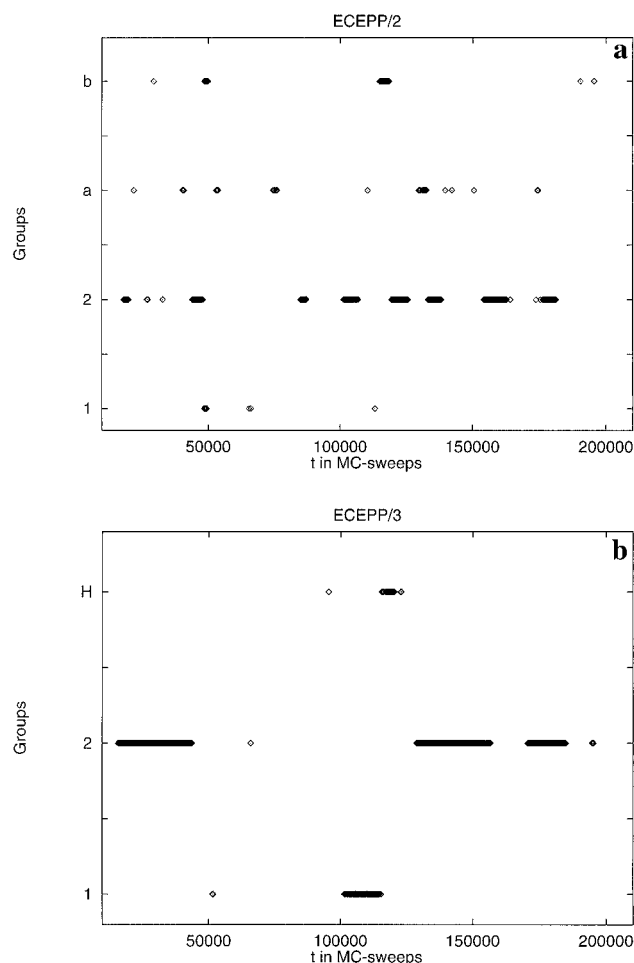


Figure 2. Time series of clusters, obtained from a multicanonical simulation of 200 000 MC sweeps, using (a) the ECEPP/2 and (b) the ECEPP/3 force field.

means that the simulation did not visit one of these valleys. As one can see there are different slices in which the multicanonical simulation moved only inside of a valley separated by jumps between different valleys. Again the number of these slices will give a lower limit on the number of independent low energy states visited in the simulation.

Results and Discussion

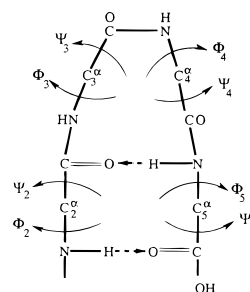
Using the techniques above we were able for each choice of the force field to classify the large number of local minimum conformations sampled in our multicanonical runs into a few groups which represent more than 80% of the sampled local minimum conformations and which correspond to the important valleys in the energy landscape of Met-enkephalin. In Figure 3 the dominant groups for both variants of the ECEPP force field are shown. In addition, we present in Table 3 the dihedral angles for the global minimum conformations obtained by our method. These structures are also shown in Figure 4.

The global minimum for ECEPP/3 has the characteristic hydrogen bond of the NH-group of Phe(4) with the carbonyl group of Try(1) resulting in a β -bend between the first and fourth residue. To preserve this hydrogen bond only the backbone angles of Gly(2) and Gly(3) have to be conserved and therefore one finds a large number of variants differing from the global minimum structure in the other backbone and side-chain dihedrals in this group (which we call group "1"). The second group ("2") is characterized by the local minimum configuration with the second lowest energy in the ECEPP/3 force field. This configuration has two possible hydrogen bonds between N—H

a ECEPP/2 with fixed Omega

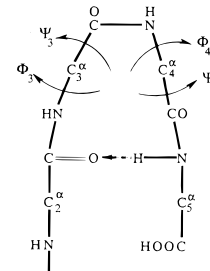
(2) F/F* D C* A F/C, A 65 %

$E_{\min} = -10.72$ kcal/mol



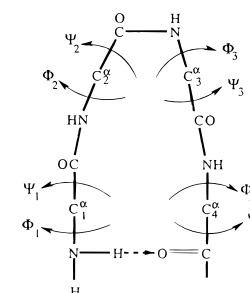
(a) F/F* E, D C* D E, F 9 %

$E_{\min} = -9.76$ kcal/mol



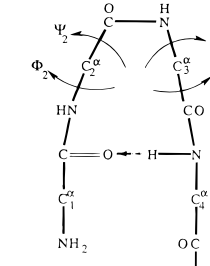
(b) F/F* C D* E x 12 %

$E_{\min} = -9.52$ kcal/mol



(1) x C* D E x 3 %

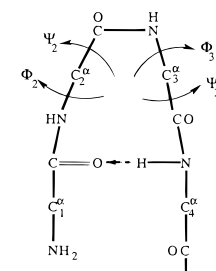
$E_{\min} = -9.46$ kcal/mol



b ECEPP/3 with fixed Omega

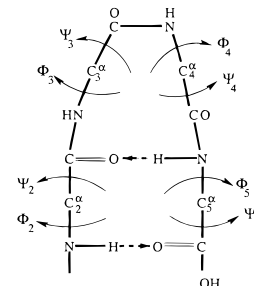
(1) x C* D E x 13 %

$E_{\min} = -10.85$ kcal/mol



(2) F/F* D C* A F/C, A 75 %

$E_{\min} = -10.81$ kcal/mol



(H) x A A A x 5 %

$E_{\min} = -10.56$ kcal/mol

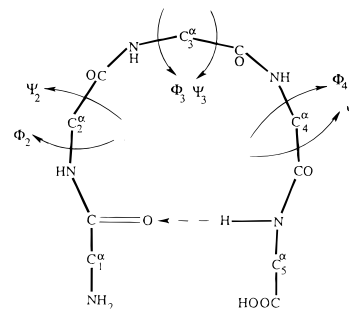


Figure 3. Most frequent low energy conformers for (a) ECEPP/2 and (b) ECEPP/3. Shown are also their Zimmermann codes, the frequency with which they appear in the multicanonical simulation, and the potential energy of their lowest energy exponent E_{\min} .

and C=O groups of the Gly(2) and Met(5) residue forming a Π' -type β -turn between the second and last residue. Since more backbone dihedrals have to be conserved to preserve the two

TABLE 3: Dihedral Angles of Our Estimates of the Global Minima for ECEPP/2 and ECEPP/3^a

		ECEPP/2	ECEPP/3
1, Tyr	ϕ	-86.3	-162.7
	ψ	153.7	-41.7
	ω	180.0	180.0
	χ_1	-179.8	-174.2
	χ_2	-111.4	-85.2
	χ_6	145.3	2.8
2, Gly	ϕ	-161.5	65.8
	ψ	71.1	-87.0
	ω	180.0	180.0
3, Gly	ϕ	64.1	-157.3
	ψ	-93.5	34.9
	ω	180.0	180.0
4, Phe	ϕ	-81.7	-158.8
	ψ	-29.2	159.5
	ω	180.0	180.0
	χ_1	179.8	52.4
	χ_2	-100.0	-96.0
	ϕ	-80.7	-82.4
5, Met	ψ	143.5	134.1
	ω	180.0	180.0
	χ_1	-65.1	-66.1
	χ_2	-179.2	-179.6
	χ_3	-179.3	-179.9
	χ_4	60.1	60.1
E_{tot} (ECEPP/2), kcal/mol		-10.72	-9.46
E_{tot} (ECEPP/3), kcal/mol		-10.81	-10.85

^a The peptide bond angles were fixed to $\omega = 180^\circ$ in the simulations.

hydrogen bonds, the configurations of this group vary much less than in the case of the previous group. Despite their differences (see Figures 3 and 4) the lowest energy exponent of both groups differs by only 0.04 kcal/mol in potential energy, so that one can regard the global minimum of Met-enkephalin in the ECEPP/3 force field as degenerated. However, the electrostatic energy for the ECEPP/3 minimum is raised substantially if ECEPP/2 charges are used for this structure. The total potential energy increases from $E_{tot} = -10.84$ kcal/mol (ECEPP/3) to $E_{tot} = -9.46$ kcal/mol (ECEPP/2), while the energy of the other configuration changes only from $E_{tot} = -10.81$ kcal/mol (ECEPP/3) to $E_{tot} = -10.72$ kcal/mol (ECEPP/2), which is the global minimum of Met-enkephalin in the ECEPP/2 force field.^{25,26} Configurations of both group 1 and 2 were also found in our ECEPP/2 simulation.

We remark that our peptide seems to have a much richer spectrum of low energy local minimum configurations in the ECEPP/3 force field than in the case of ECEPP/2 but show in Figure 3b only one more (the other contribute with less than 1% each) group. Conformers of this group ("H") are characterized by a hydrogen bond between the C=O group of the Tyr(1) and the N-H group of Met(5) residues. Together with the backbone dihedrals of Gly(2), Gly(3), and Phe(4), this hydrogen bonding resembles that of an α -helix. Its lowest energy exponent has a potential energy of -10.56 kcal/mol, which is only 0.25 kcal/mol higher than the ground state. No such conformation was found in the ECEPP/2 simulation. In the case of ECEPP/2, two more groups appeared with a frequency of more than 1% and are shown in Figure 3a. Both are II' type β -turns. Group "a" is characterized by single hydrogen bond between Gly(2) and Met(5). The lowest energy conformation in this group has a potential energy of -9.74 kcal/mol, which makes this group the one with the second lowest potential energy, separated by ≈ 1 kcal/mol from the ground state. Group "b" has its hydrogen bond between Tyr(1) and Phe(4); its lowest energy exponent has a potential energy of -9.52 kcal/mol. Both groups were also found in the ECEPP/3 simulation but with frequencies less than 1% of the nonidentical conformers.

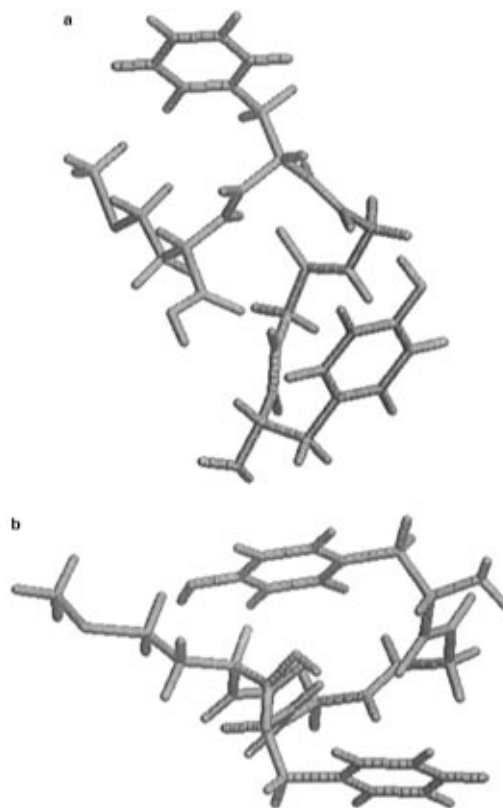


Figure 4. Ground state configurations of (a) ECEPP/2 and (b) ECEPP/3. The plots were drawn using RASMOL.

For ECEPP/2 our local minimum conformations resemble those found in previous work,²⁷ where unlike in the present work the peptide angles were released. Fixing these angles therefore seems to be a reasonable approximation. But while our classification of low energy local minima can also be obtained with other methods, the multicanonical approach allows in addition the estimation of the relative weight of these conformers as a function of temperature; i.e., entropic contributions can be considered. As an example we show in Figure 5 the relative weight of the dominant groups of conformation for both ECEPP/2 and ECEPP/3. Note that around 300 K the percentage of configurations which are in none of our groups (and hence do not belong to the important valleys) decreases dramatically for both force fields. At high temperatures coil structures dominate, which show large flexibility and therefore a broad energy spectrum. On the other hand, at low temperatures ordered structures are expected which are each confined to one of the valleys in the energy landscape. In the case of the ECEPP/2 simulation, the percentage of configurations of type 1 (the ECEPP/3 ground state) never exceeded 4%. Configurations of type 2 (the ground state for ECEPP/2) are found with much higher probability, but at room temperature they contribute to only about 30% of the conformers, which confirms previous work.¹⁹ For ECEPP/3 we find that the probability of configurations of type 2 is comparable to that of the ECEPP/2 simulation. At room temperature they contribute again to around 30% of the conformers. Conformations of type 1 appear with much higher probability than in the ECEPP/2 simulation, but that of type 2 are still dominant in the range of shown temperatures. At relevant temperatures the probability for finding the ECEPP/2 ground state (type 2) is twice as high as that of finding the ECEPP/3 ground state (type 1). Of course we expect that at $T = 0$ K we will find only the ECEPP/3 ground state (type 1); however, the energy difference is much too small (about 0.04 kcal/mol) to favor them at finite temperatures against type 2

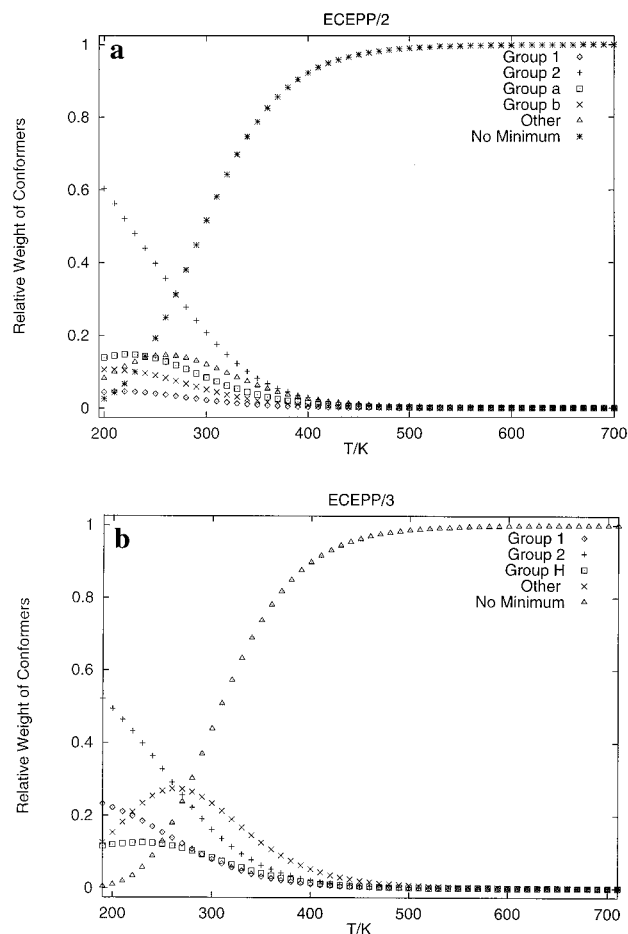


Figure 5. Relative weight of the important groups of configurations in the case of (a) ECEPP/2 and (b) ECEPP/3. We have summed up all local minima which do not belong to one of the major groups in "Other". Likewise "No Minimum" contains all configurations which could not be minimized to a local minimum with potential energy below -6 kcal/mol (see text).

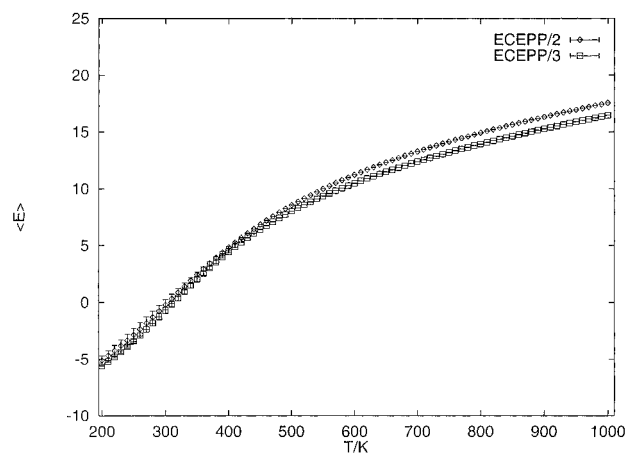


Figure 6. Average potential energy as a function of temperature for both choices of force fields.

structures. This proves that at least for small peptides entropic contributions cannot be neglected. Any pure optimization method (in the potential energy) may lead to configurations which are of only limited significance at relevant temperatures.

In the final plots we show how the variations in the force field affect two important thermodynamic quantities. Figure 6 displays the average energy $\langle E \rangle$ as a function of temperature. Again, we observe only little differences between the ECEPP/2 and ECEPP/3 force fields for the physically relevant temperature

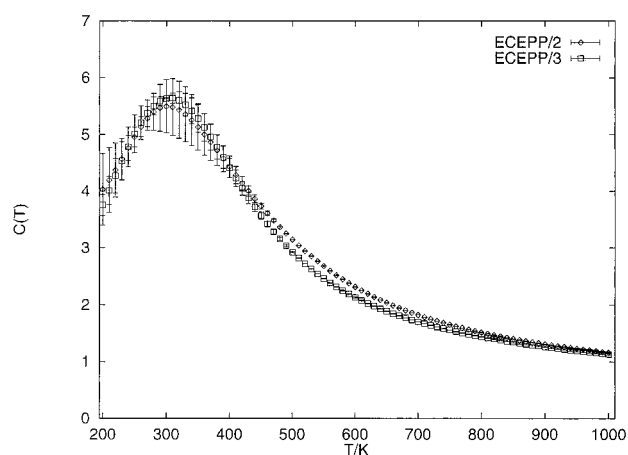


Figure 7. Average of specific heat as a function of temperature for both choices of force fields.

range. Below 400 K these differences are within the error bars, while for higher temperatures the ECEPP/3 energies are systematically lower by a small amount. Similar results were found for the specific heat $C(T)$ as function of temperature shown in Figure 7. Here we define

$$C(\beta) = \frac{\beta^2 \langle E^2 \rangle - \langle E \rangle^2}{5} \quad (12)$$

Neither the position of its maximum nor its shape or height varies much, indicating that the transition between ordered and disordered states is of the same kind for both variants of the force field. Note that the position of the peak in specific heat corresponds to the increase of structures which belong to one of the main groups and the decrease of configurations which do not belong to any of the significant energy valleys.

Conclusion

We used the multicanonical approach to study variations of the energy landscape of Met-enkephalin under a change from the ECEPP/2 force field to ECEPP/3. In both cases, the low energy local minima were sampled and classified into a small number of groups which correspond to essential valleys of the energy landscape. We studied their distribution and relative weight at various temperatures. While the energy landscape differs in detail and the global minimum is not the same, our physical results are not affected much by changes between the two force fields. Our analysis demonstrates that at least for small peptides it is not sufficient to search only for the global minimum in *potential* energy, since this conformation may be of limited significance at room temperature. Our work corroborates the well-known fact that small flexible molecules exist at room temperature in an ensemble of low energy conformations (see, for instance, ref 1 and work cited therein) whose relative weight has to be determined. Finally, our results show that numerical simulations of peptides are stable under small changes in the utilized force fields. Especially thermodynamic quantities are not changed much. This is an important observation since all force fields rely on experimentally determined parameters and are therefore known only within certain error bars.

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