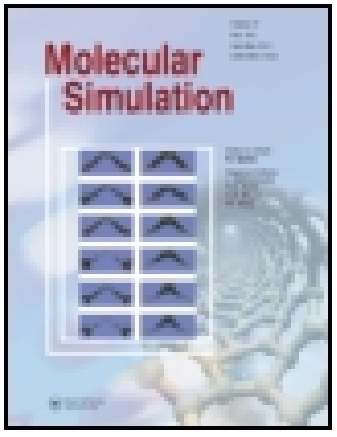

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On the accuracy of metadynamics and its variations in a protein folding process

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On the accuracy of metadynamics and its variations in a protein folding process

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Metadynamics and its variations are powerful tools for exploring the free energy landscape of physical, chemical and biophysical systems. However, previous tests of their accuracy were based on either low-dimensional systems or complicated systems without exact answers. Therefore their accuracy, particularly when used for high-dimensional biophysical systems, has not been rigorously tested. In this work we performed a series of simulations with metadynamics and its variations for a typical biophysical process – the folding of protein chymotrypsin inhibitor 2 (CI2) based on a coarse-grained structure-based model. The results revealed the power as well as limitations of the algorithms and provided some guidelines for using metadynamics and its variations in high-dimensional biological systems.

Keywords: metadynamics; accuracy; protein folding

1. Introduction

Computer simulations are powerful approaches in providing high-resolution information for processes of complex systems in solid state physics, biophysics and chemistry. However, the energy landscapes of these systems are often characterised by numerous local minima, which can easily trap conventional simulations and prevent them from sampling sufficient size of phase space comparable to experiments. This is usually referred as to the 'energy barrier problem'. Another difficulty faced by computer simulations is the 'entropic barrier problem', which is due to the extremely large size of the phase space related to the specific physical problem, for example the folding problem of proteins.[1] The huge size of the phase space itself poses a problem even if the free energy landscape (FEL) is relatively smooth.

People have developed various approaches to overcome the above difficulties. These approaches either directly modify the potentials of the physical systems or change the sampling process or alter their dynamics. The first category includes but not limited to the stochastic tunnelling method,[2,3] the energy landscape paving method,[4] the potential smoothing method,[5] etc. People have also developed a number of powerful methods belonging to the second category. For example, the popular replica exchange method,[6,7] also known as parallel tempering, couples the dynamics of several copies of the system at different temperatures or Hamiltonians and exchanges their conformations and velocities periodically. In this way it realises an automatic simulated annealing process and guarantees detailed balance and a canonical ensemble. The replica exchange method is one of the generalised ensemble approach, which also includes for example the entropic sampling method, the distributed algorithm employed in the folding@home project, the Wang–Landau algorithm,[8] a recent single-copy tempering method,[9] etc. The algorithms belonging to the third category usually change the dynamics of the system, for example the widely used SHAKE algorithm constrains the fast motions of the physical system thus making it possible to increase the time step in molecular dynamics (MD) simulations. The reversible reference system propagator algorithms (RESPA) method accelerates simulations with a separation of time scales or with long-range forces.[10] Various versions of coarse-grained (CG) methods also fall into this category.[11–15]

Most of the above-mentioned methods are designed to overcome the energy barrier problem. In contrast, there is relatively less attention on the entropic difficulties. For the systems such as proteins, the extremely large size of the phase spaces itself poses a problem, even the associated energy landscape is not very rugged. One interesting solution is the resolution-exchange simulation,[16] which couples the replicas at the all-atom level with those running at CG levels, with the assumption that the CG replicas explore the phase space much faster and thus are able to accelerate the sampling at the all-atom level. Another strategy is running a large number of short simulations starting from metastable states detected with non-equilibrium generalised ensemble algorithms.[17] Due to the large volume of the related works, a comprehensive review is not possible. We refer people to several excellent reviews for further information.[18,19]

Recently, an advanced method called metadynamics, also known as the hill method, has attracted more and

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more interests and has achieved successes in several research areas.[20–23] The method is closely related to the local elevation method, to the adaptive force bias method, and also to the Wang–Landau approach.[8,24–27] It employs two strategies to accelerate the sampling of phase space and calculation of free energy difference. One is to enforce escaping from local minima by periodically modifying the effective energy with small repulsive Gaussian potentials, thus overcoming the 'energy barrier problem'. The second is to concentrate sampling on the collective variables (CVs) corresponding to the slow motions of the system, therefore, effectively reducing the degrees of the freedom and alleviating the 'entropic barrier problem'. The combination of these two strategies makes metadynamics a very powerful algorithm for detecting metastable states and calculating free energy differences.

Since its invention, many variations of metadynamics were developed to improve either its efficiency or accuracy. For example, to guarantee a theoretical convergence, well-tempered metadynamics was developed by rescaling the Gaussian weight factor as a function of time.[28] As a further development of this method, reweighting techniques were invented to recover the unbiased probabilities on one CV from the biased data on another CV.[29,30] Umbrella sampling was also introduced into metadynamics to 'correct' the free energy surfaces (FESs).[24,31] To improve the efficiency of metadynamics, people developed the so called multi-walker algorithm, which simulated multiple copies (walkers) at the same time and allowed each copy feel the hill potentials added by the others.[32,33] Parallel tempering was also combined with metadynamics to take advantage of the power of both, mainly through two ways. One was running several replicas of metadynamics at the same CV(s) but at different temperatures and periodically exchanging their temperatures. The other was running multiple replicas at the same temperature but biasing them on different CVs [34]; the exchanges were between different CVs instead of temperatures. The latter algorithm was named the bias exchange metadynamics (BEMD).[35,36]

In recent years, metadynamics and its variations have been applied in many fields, particularly in biophysical systems such as the β-hairpin,[37] trp-cage,[38] HIV-1 protease,[39] insulin,[40] cyclophilin-A,[41] amyloid-related peptides,[42,43] human AChE gorge,[44] etc. However, we feel that the convergence and accuracy of the methods have not been rigorously examined particularly for complex systems, since previous computational experiments either used low-dimensional systems or biological systems with no exact answers for the tests.[21,45,46] The lack of clear statements for their accuracy and limitations in high-dimensional systems makes their usage a dangerous task and may even lead to wrong results. Their rigorous tests in biological systems become even more imperative with the increasing interests in using metadynamics in such fields nowadays.

In this work, we evaluated the accuracy of metadynamics and its several variations with the folding process of chymotrypsin inhibitor-2 (CI2) and a structure-based CG model.[47] Although this protein model is simple, it is well appreciated by the community that it is able to characterise the key features of the protein folding process, such as the two-state or multi-states behaviours, the funnel shape of the energy landscape, the delicate enthalpy–entropy balance, the general structure of the transition states, etc.[1,48–54] Most importantly, the CG model makes it possible to obtain the 'exact answer' for the FEL, subjected to the accuracy of the model. Due to the limited capacity of modern computers, an exact answer is impossible for a more realistic model, such as the all-atom model. The 'exact answer' will be used as the standard for examining the accuracy of metadynamics and its variations.

2. Methods

2.1. Protein modelling and the conventional MD simulations

Protein CI2 (Figure 1), one of the best studied proteins, was used for all the tests. The protein was modelled by a structure-based Cα model, which was simple enough to allow the calculation of an exact answer. In fact, this model needed so little computational resources that a conventional MD simulation running on a desktop was

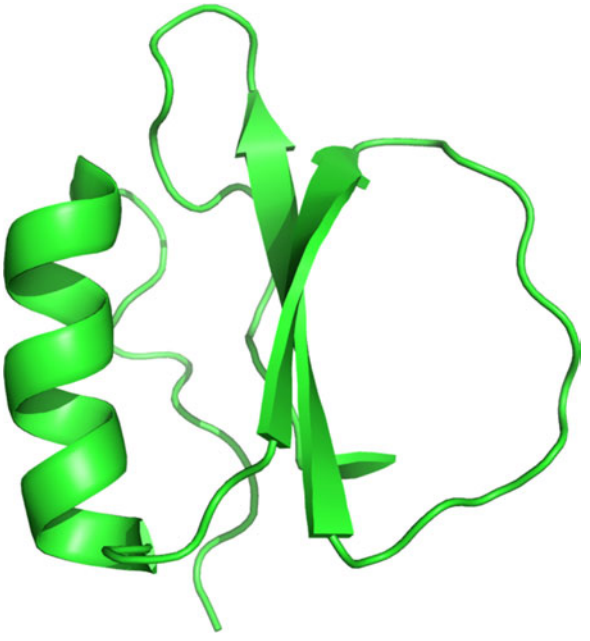

Figure 1. The native structure of protein CI2 (PDB code 1YPA), prepared using Pymol.

able to give converged results within hours; the convergence was not a problem. In contrast, a more realistic model, such as the all-atom model, will not allow such an answer, since conventional MD simulations based on the all-atom model cannot guarantee a thorough sampling of the relevant phase space while advanced sampling techniques used to accelerate sampling efficiency have their own weakness. It is also worth mentioning that the purpose of this work is not to give the realistic FEL of the protein, but to compare the outputs of different computational algorithms based on the same protein model; whether the CG model employed here is able to characterise the realistic folding process of protein CI2 is less relevant to this purpose, although it is able to reflect the major folding aspects as mentioned above.

The protein was modelled as a chain of beads, with each bead representing one amino acid. Conventional MD simulations were carried out on with Gromacs (version 4.5.3),[55] and the input files were prepared by the Smog web server.[56] The native contacts were determined based on the native structure of the protein (PDB code 1YPA, Figure 1) with a cut-off of 0.6 nm. The bond, angle and dihedral angle interactions were modelled as we did in our previous works.[52,53] The non-bonded native interaction was described by a 12-10 type Lennard-Jones potential and the corresponding cut-off was set to be 2.0 nm. The leap-frog stochastic dynamics integrator was used to evolve the equation of motion and to maintain the system at the desired temperature.[55] The friction constant in the stochastic dynamics was set to be 1.0 (in a reduced unit). The time step was chosen to be 0.0005 (in a reduced unit). More details of the model and parameters can be found at the Smog server.[56]

At first, 10 conventional MD simulations were performed at temperatures ranging from 70 to 300 K. The weighted histogram analysis method (WHAM) was used to post-process the data and calculate the heat capacity profile and the folding temperature T_f. After the folding temperature was determined, which is 130 K here, a long enough (800 ns, labelled as Sim-1) simulation under $0.98T_f$ was performed to get the FES, as shown in Figure 2(a). The results given by Sim-1 would be used as the standard to assess the accuracy of metadynamics and its variations. Note that in Sim-1 no advanced sampling techniques were employed to avoid possible defects associated with these techniques.

2.2. *Evaluation of the accuracy of the simulations*

All the protein structures generated by simulations were designated into either the folded state or unfolded state according to their Q values. The threshold was chosen to be $Q = 0.38$, where the free energy was a local maximum according to Figure 2(a). We defined $P_{F/U}$ as the ratio of the population (or probability) of the folded states over that of the unfolded state, and ΔF as the free energy barrier for the protein to escape from the native state to the transition state, as shown in Figure 2(a). We further defined two ratios, i.e.

$$R_P = \frac{P_{F/U}}{P^0_{F/U}} \tag{1}$$

and

$$R_F = \frac{\Delta F}{\Delta F^0}, \tag{2}$$

where the quantities with or without the superscript indicated that the values were calculated by Sim-1 (the standard) or the other simulations, respectively. The two ratios were used to assess how accurate the other simulations were able to reproduce the free energy difference and the free energy barrier, respectively. The latter quantity also reflected whether the folding pathway was correctly reproduced, since different pathways generally corresponded to different barriers. According to the definition, the ratios were closer to 1, the higher the accuracy was.

2.3. *Collective variables*

Three CVs were used throughout the tests, including the fraction of the native contact (Q), the radius of gyration (R_g) and the end-to-end distance (DIST). To ensure differentiability, the contact number between the ith Cα and jth Cα beads was calculated with

$$s_{ij} = \begin{cases} 1 & (\text{when} \quad r_{ij} \le 0) \\ \frac{1-(r_{ij}/r_0)^{10}}{1-(r_{ij}/r_0)^{12}} & (\text{when} \quad r_{ij} > 0), \end{cases} \tag{3}$$

where $r_{ij} = |r_i - r_j| - d_0$. Here, we set d_0 to 0.6 nm and r_0 to 0.4 nm, respectively. The radius of gyration was calculated with

$$R_g = \sqrt{\frac{\sum_i m_i |r_i - r_{com}|^2}{\sum_i m_i}}, \tag{4}$$

where m_i is the mass of the ith Cα bead and r_{com} is the centre of mass. The DIST was defined as the distance between the first and the last Cα beads.

2.4. *The base-metadynamics*

The 'base-metadynamics' is used to refer to the original version of metadynamics without introducing the well-tempered strategy or parallel tempering or the other techniques that were designed for further increasing the efficiency or accuracy. Four base-metadynamics

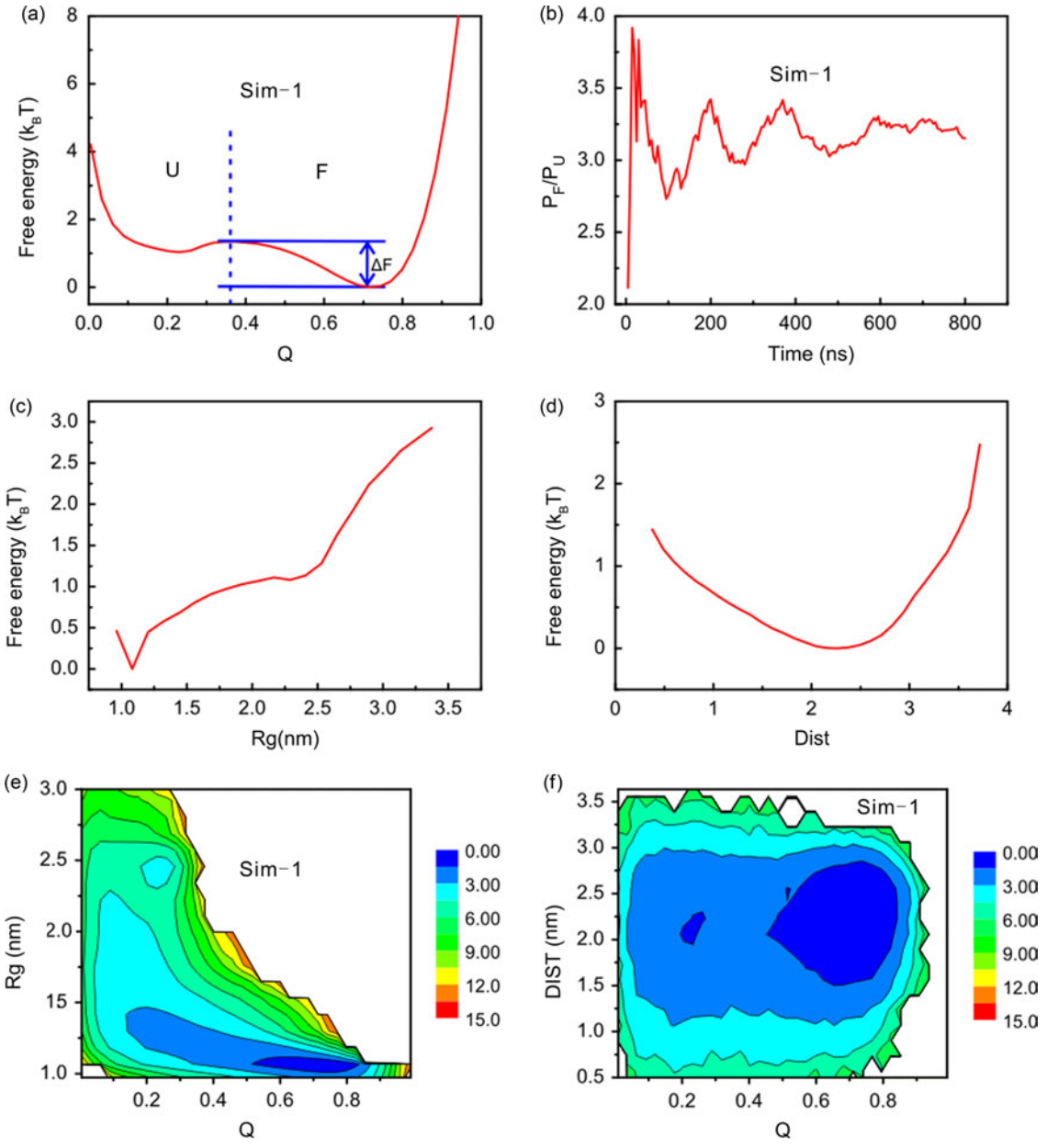

Figure 2. (a) Free energy as a function of Q calculated at the end of Sim-1 (800 ns). The temperature was $0.98T_f$. The label ΔF indicates the free energy barrier for the protein to escape from the native basin of attraction. (b) The convergence of Sim-1 measured by the population ratio between two states. (c, d) the one-dimensional projections of the FEL. (e, f) The two-dimensional projections of the FEL.

simulations were performed: three of them were biased on the CV Q with different Gaussian heights (Sim-2) and one was biased on the CV DIST (Sim-3). The Gaussian heights and widths are listed in Table I. In these simulations, the Gaussian potentials were added every 500 MD steps. The temperature was fixed at $0.98T_f$, and all the simulations in this work were also performed at this temperature unless indicated otherwise. The simulations were started from the native structure. We also performed simulations starting from the unfolded state and obtained similar results, due to the rapid structure transitions between the folded and unfolded states in the CG model. All the simulations for metadynamics were done by using the PLUMED plugin (version 1.3) for Gromacs (version 4.5).[57]

2.5. *Well-tempered metadynamics*

Six well-tempered metadynamics simulations were performed,[28] where the height of the Gaussian potential was decreased gradually to suppress the final fluctuation of the overall potentials to improve the theoretical convergence. Three CVs, including Q, R_g and DIST, were used to bias

Table 1. Parameters and results of the simulations.

Simulation	CVs	Height	Sigma	Bias factor	Length	R_P	R_F
1	–	–	–	–	800 ns	1.00	1.00
		0.1	2.5	–	100 ns	0.25	3.59
2	Q	0.01	2.5	–	100 ns	0.88	0.55
		0.005	2.5	–	100 ns	0.56	1.64
3	DIST	0.01	0.35	–	75ns	–	–
4	Q	1	2.5	15	50 ns	0.89	1.92
5	Q	0.1	2.5	5	50 ns	1.01	1.49
6	DIST	1	0.35	15	40 ns	1.46	1.68
7	DIST	0.1	0.35	5	50 ns	1.19	1.46
8	R_g	0.1	0.02	5	70 ns	0.48	0.10
9	R_g	0.1	0.02	2	80 ns	0.79	0.20
	Neutral	–	–	–		0.98	0.99
10	Q	0.005	2.5	–	90 ns/replica	–	–
	DIST	0.005	0.35	–		–	–
	R_g	0.005	0.02	–		–	–
	Neutral	–	–	–		0.99	1
11	Q	0.1	2.5	5	75 ns/replica	1.08	1.36
	DIST	0.1	0.35	5		0.99	1.29
	R_g	0.1	0.02	5		0.57	0.15

the sampling; one CV for each run. The Gaussian parameters and bias factors that control the decreasing rate of Gaussian potentials are given in Table 1. Note that the larger the bias factors are, the slower the decreasing rates are. The Gaussian potentials were added every 500 steps. All the simulations were performed at $0.98T_f$. Besides, a reweighting technique [29] was employed to post-process the data and recover the unbiased FES as a function Q from the biased data on the other CVs. The simulations were labelled from Sim-4 to Sim-9, respectively.

2.6. *Bias-exchange metadynamics*

Four replicas were used in the BEMD simulation (Sim-10). The first three were biased on Q, R_g and DIST, respectively. The fourth replica was a neutral one with no bias applied on any CVs. In the following text, the replica biased on Q is shorted as the Q-replica and the other replicas are similarly labelled. The height of the Gaussian potential was 0.005 kJ/mol and the potential was added every 500 steps. Replica exchanges were attempted every 15,000 steps. The time step was set to 0.5 fs and the temperature was $0.98T_f$.

2.7. *Well-tempered BEMD*

In this run the well-tempered algorithm was combined with BEMD to improve the final convergence.[28] All the set-up was the same as in Sim-10, except the Gaussian potentials were rescaled gradually with a bias factor of 5 from an initial height of 0.1 kJ/mol. The simulation was labelled as Sim-11.

3. Results and discussion

In Figure 2 we show the results of the long conventional MD simulations (Sim-1). It can be seen that the projection of FES on Q reproduces the two-state behaviour of the folding process of protein CI2. This behaviour is consistent with previous works [47,58] and is typical for many small globule proteins. The convergence of FES is tested in Figure 2(b), where the population ratio between two states is found to be saturated at about 600 ns, and the fluctuation after that time is very small, amounting to a free energy difference of $0.05k_BT$. In Figure 2(e),(f) we also show the projections of FES on two CVs, which are also consistent with previous works.[47,58] The FESs shown in Figure 2 are treated as the exact answer and used a standard for testing the other simulations.

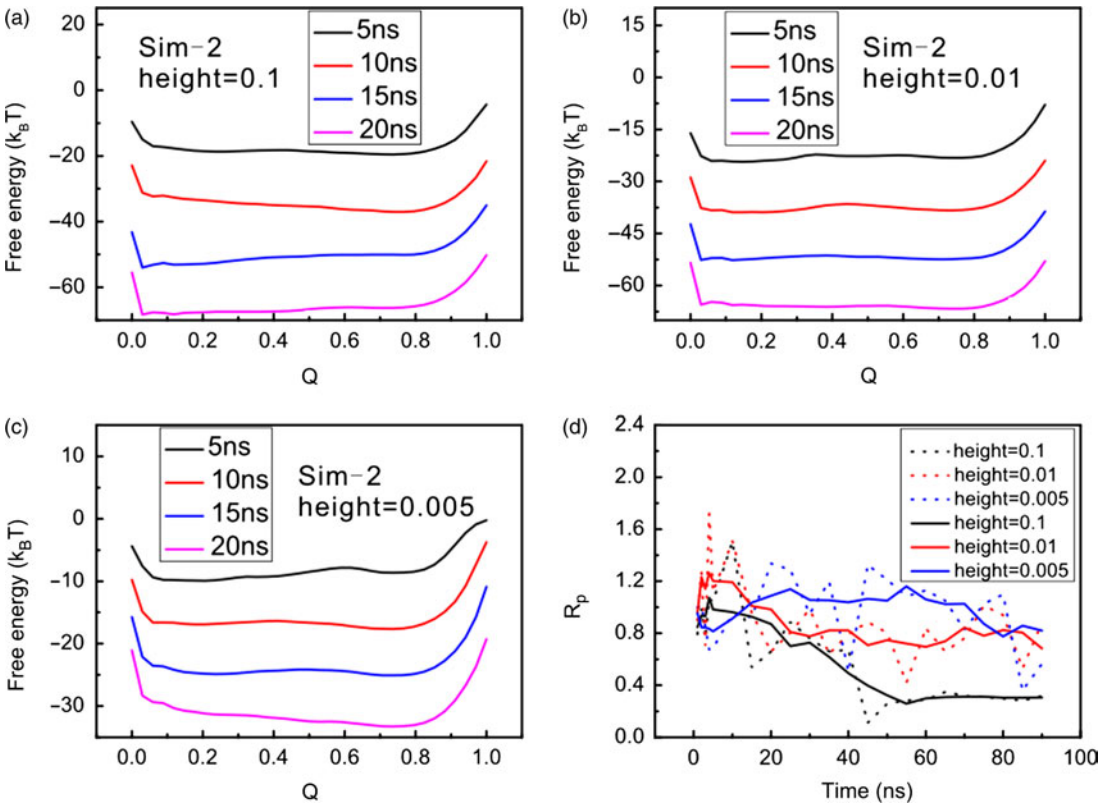

Figure 3. (ac) Free energy as a function of Q calculated at different simulation lengths and from metadynamics of different Gaussian heights. The Gaussian heights are labelled in the figures and their units are kJ/mol. (d) The convergence of R_P as a function of time; the dotted lines represent the raw data and the solid lines represent data obtained by averaging the raw FES over a sliding window of 20 ns.

It is worth mentioning that within the framework of the structure-based model, it is well accepted that the fraction of native contacts Q is a good CV that corresponds to the slowest motion of the system and is suitable for describing the folding process.[47,59] Moreover, a very recent work pointed out that even for the folding simulations at the all-atom level, Q is still a good CV.[60] Since the proper usage of metadynamics depends strongly on the choice of good CVs, we evaluate the 'goodness' of the other CVs by checking their correlations with Q.

The results obtained with base-metadynamics (Sim-2) are shown in Figure 3. It can be seen that for all three runs with different Gaussian heights, the general shapes of the FESs are already roughly similar to the standard answer, even after a very short time of 5 ns. That is, there are no extremely large barriers and no hysteresis effect observed as a function of time, as will be discussed later. With further increasing the simulation length, the curves decrease uniformly, suggesting a good sampling quality in the relevant phase space. However, the population ratio

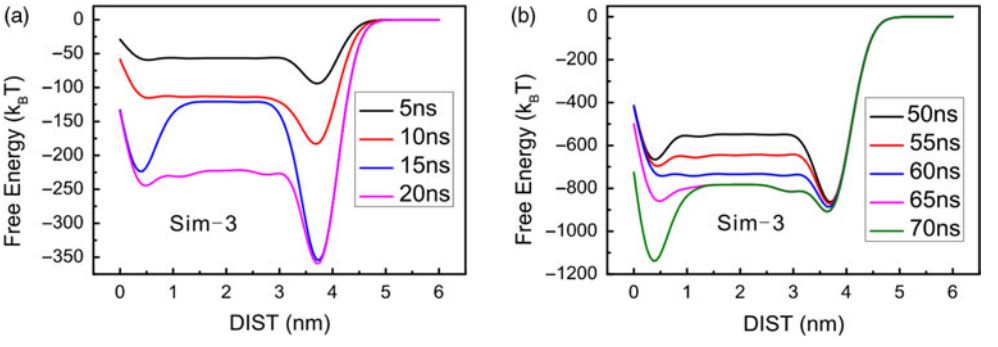

Figure 4. Free energy calculated from metadynamics biased on DIST, (a) is obtained at the early simulation stage and (b) at the later stage. The simulation lengths are indicated by the labels.

R_P between two states is under large fluctuations as a function of simulation time, as shown in Figure 3(d). This is a typical behaviour often observed in base-metadynamics. One advised strategy for suppressing such fluctuations is averaging the FES over some length. The results after averaging over a window of 20 ns are also given in Figure 3(d). The fluctuations are indeed suppressed and the curves become relatively smooth as a function of time. However, strictly speaking, the ratios R_P still deviate from unity even for simulations of length 100 ns, for all three simulations and particularly for the first one with a larger Gaussian height of 0.1 kJ/mol.

We also performed base-metadynamics biased on the CV DIST, which is not a good CV according to Figure 2 (f). The results are shown in Figure 4. It can be seen that the sampling goes back and forth several times between two meta-stable states and the free energy barrier separating them is extremely large, about $70k_BT$ at 10 ns and $400k_BT$ at the end of simulation. This is a typical hysteresis effect discussed by Laio and Gaervasio previously,[21] due to the neglecting of relevant degrees of freedom. This effect can be somehow alleviated by carefully controlling the way the Gaussian potentials are added, as will be discussed later.

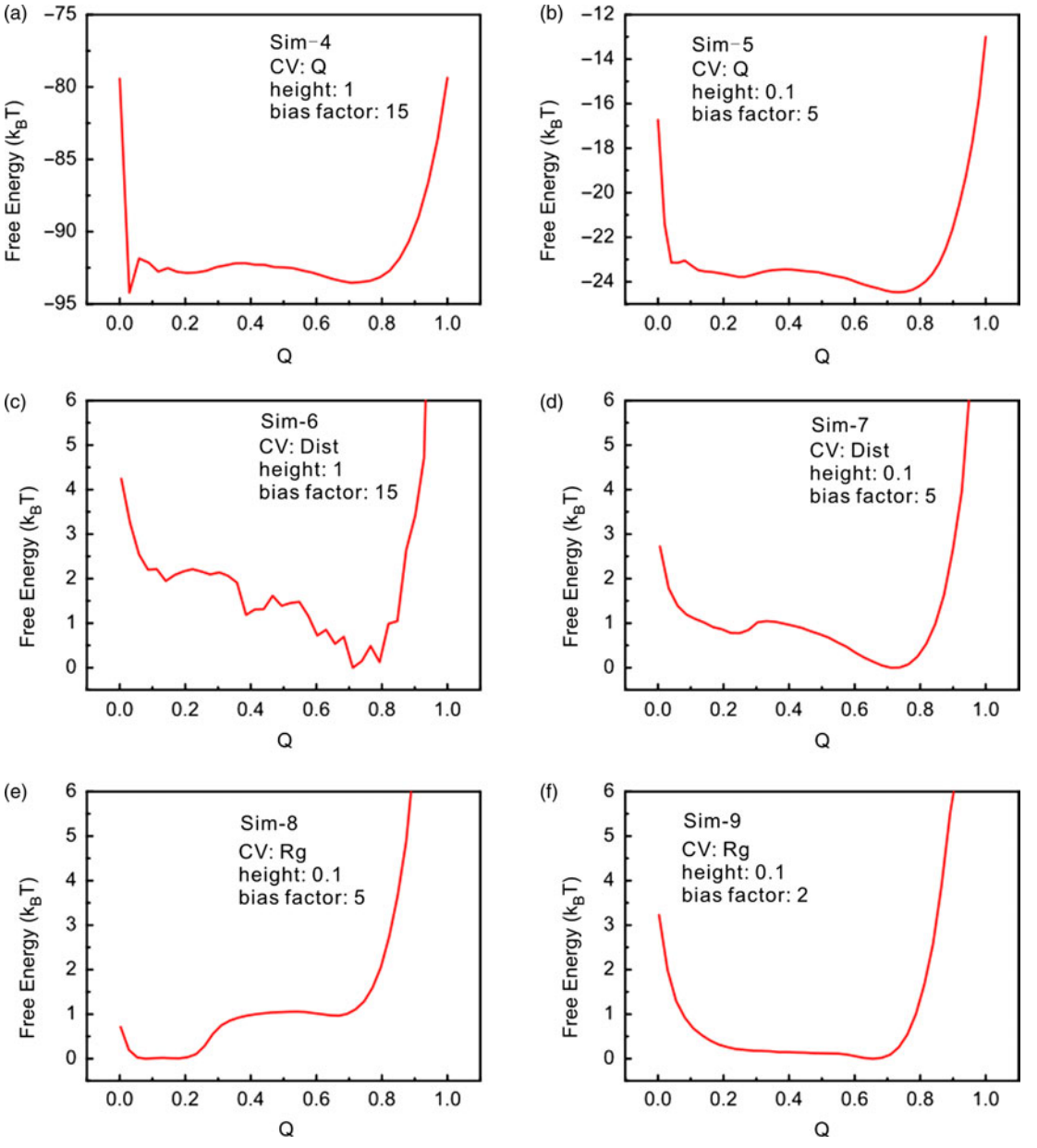

Figure 5. Free energy as a function of Q calculated from six well-tempered metadynamics with different CVs, Gaussian heights and bias factors. The unit for Gaussian heights is in kJ/mol. Note that the reweighting technique was used to obtain (c–f).

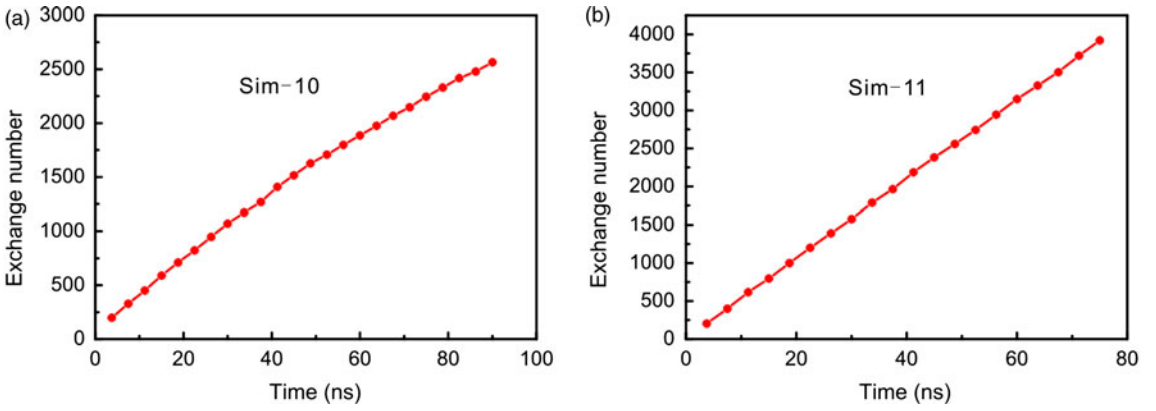

Figure 6. The number of successful exchanging events of the neutral replica as a function of time, calculated for BEMD (a) and well-tempered BEMD (b), respectively.

Well-tempered metadynamics is developed in order to give a better convergence by suppressing the final fluctuation of FES, which is of order of the Gaussian height. To test the accuracy of such an algorithm, we performed six well-tempered metadynamics with different CVs and bias parameters, labelled from Sim-4 to Sim-9, respectively. The obtained FESs as a function of Q are shown in Figure 5. Note that for the simulations with bias on the CVs other than Q, a reweighting technique was employed to recover the unbiased FES as a function of Q from the biased data on the other CVs.[29] According to Figure 5, most FESs are generally similar to the exact answer. However, strictly speaking, the accuracy is not satisfying, measured by the ratios R_P and R_F shown in Table 1. Two hints may be drawn from these simulations. First, in general, smaller Gaussian heights and faster decreasing rates lead to a better accuracy. For example, Sim-5 gives better results than Sim-4. Second, even if a less relevant CV such as DIST is used, Sim-6 and Sim-7 still give reasonable results. However, this may not be a

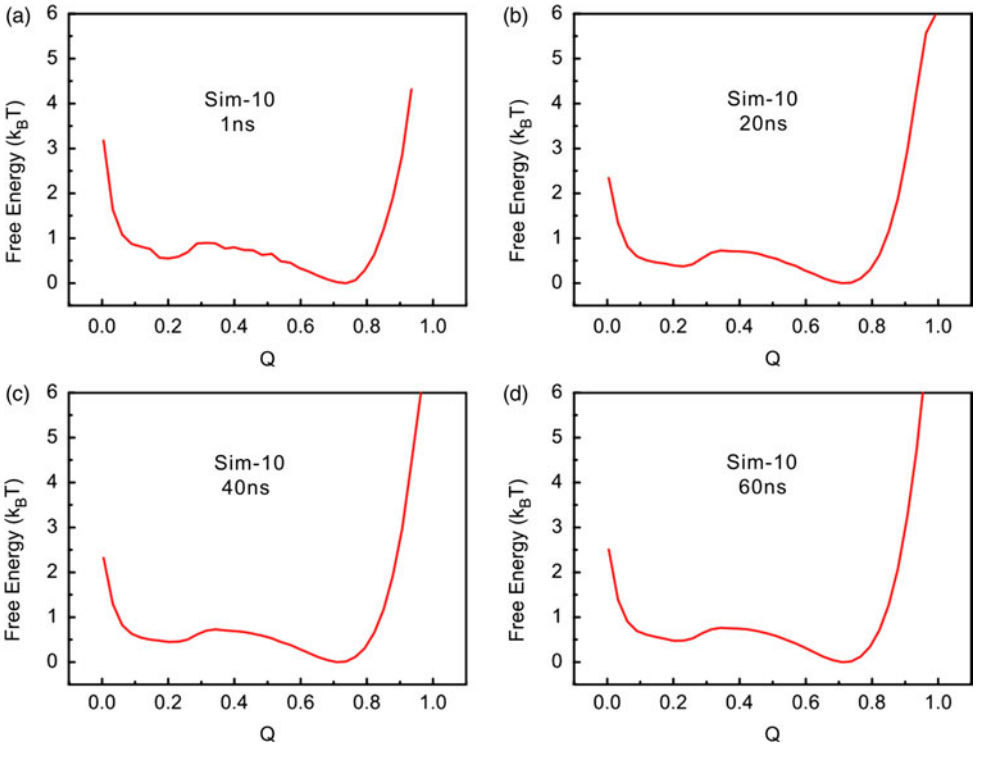

Figure 7. Free energy as a function of Q calculated solely from the neutral replica. Different figures correspond to different simulation lengths.

general phenomenon since the free energy barriers in our protein model are small and therefore can be crossed without the help of Gaussian potentials.

We then tested the performance of the BEMD (Sim-10), where three replicas were biased on Q, R_g and DIST, respectively, and one was not biased on any CV (the neutral replica). The number of successful exchanging events as a function of time is calculated for the neutral replica and is shown in Figure 6. The line is almost straight and the average exchanging probability is close to 19%, demonstrating that the exchanges happened at a reasonable rate. The FESs at different simulation lengths are given in Figure 7. Note that they were calculated only based on the data of the neutral replica, to avoid possible problems associated with the applied bias in the other replicas. It can be seen that a BEMD run for a length of 1ns already gives a roughly correct FES, and the other three FESs from simulations longer than 20 ns are almost indistinguishable from each other and agree very well with the exact answer. Correspondingly, the values of R_P and R_F are almost independent of the length of the simulation after 20 ns, and they are very close to unity at the end of the simulation (Table 1). Overall, BEMD was able to reproduce the exact answer with a high accuracy within 20 ns, which was two orders of magnitude faster than the time needed by the conventional MD simulation.

We also calculated the FESs as a function of time from three biased replicas, as shown in Figure 8. It is found that they decrease uniformly as the simulation length increases, suggesting a good sampling quality in the relevant phase space and free of the hysteresis problem observed in Figure 4. However, the accuracy of the FESs obtained in such a way is not as good as that calculated from the neutral replica. As exemplified in Figure 8(d), the ratio R_P calculated from the Q-replica displays large fluctuations during the whole simulation and is not converged even after 100 ns, in a sharp contrast to that from the neutral replica. It is also noted that a smoothing strategy is not helpful since the general trend of R_P is still increasing at the end of simulation. The convergence of the Q-replica is similar to that shown in Figure 3(d). Therefore, the hint for using BEMD simulations is only the data from the neutral replica are reliable for calculating the FES if the exact answer is paramount.

Finally, we tested the combination of the BEMD and the well-tempered technique (Sim-11). In this case the number of successful exchanging events is linear as a function of time and the average probability is about 38% (Figure 6(b)), significantly higher than that in Sim-10. The FES calculated from the neutral replica is almost the same as that from Sim-10 and with the exact answer (Figure 9 (a)). The result is also independent of the simulation length after the length exceeds a certain threshold (figure not

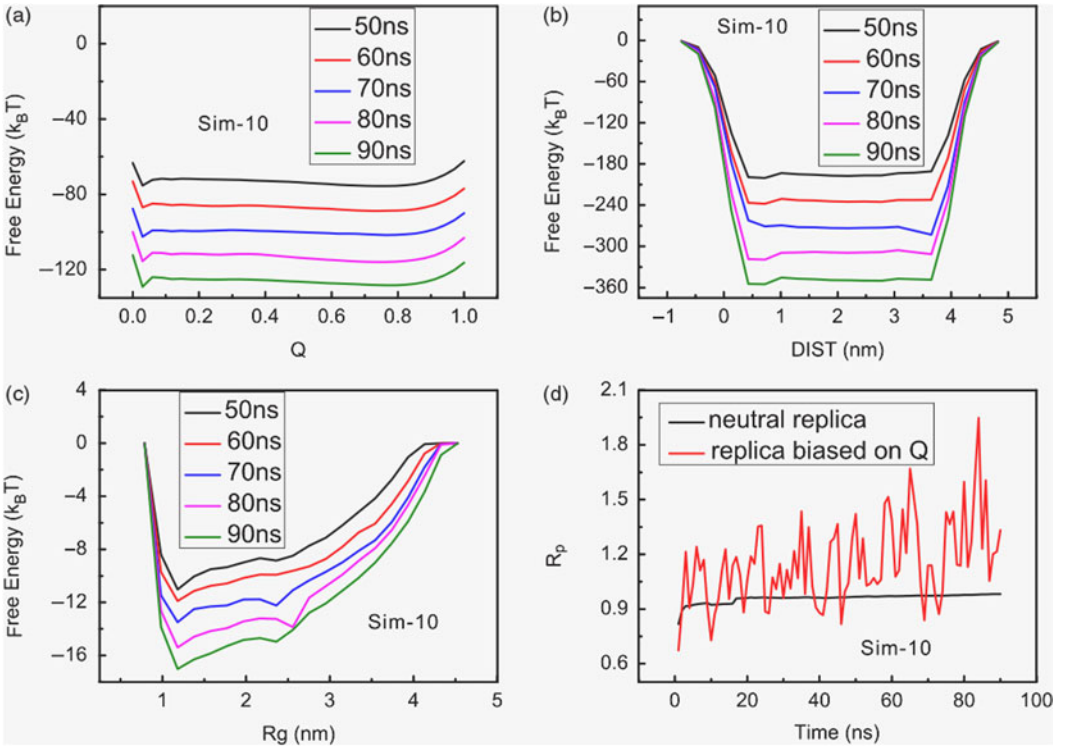

Figure 8. Free energy calculated at different simulation lengths and from different replicas of the BEMD simulation. (a–c) correspond to the Q-, DIST-, and R_g-replica, respectively. (d) The convergence of the neutral and the Q-replica during the simulation, measured by the population ratio R_P.

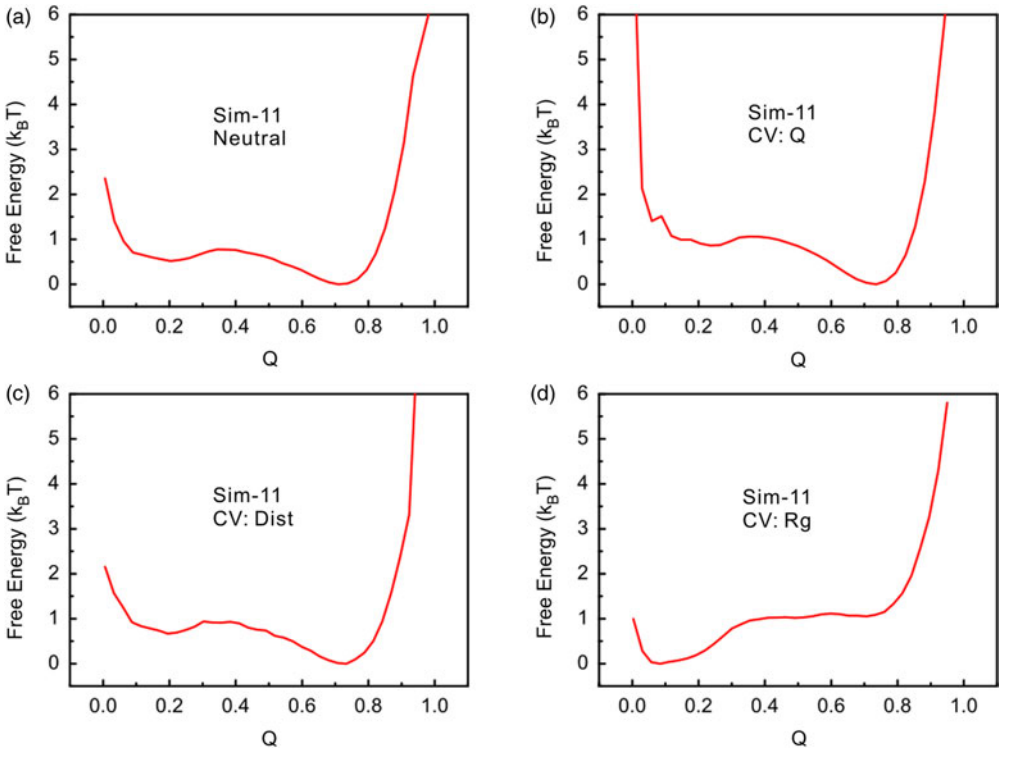

Figure 9. Free energy as a function of Q calculated from different replicas of the well-tempered BEMD simulation. Note that the reweighting technique was used to obtain the last two figures.

shown), as can be expected since the Gaussian height was gradually decreased and became minimal in the late stage of the simulation.

Since the well-tempered method employed in Sim-11 allowed the usage of the reweighting technique to recover unbiased distribution on one CV from biased data on the other CVs, we calculated the FES as a function of Q by reweighting the data from the DIST-replica and Rg-replica, respectively. The results are given in Figure 9, also shown is the FES calculated directly from the Q-replica. The ratios that characterise their accuracy are given in Table 1. It can be seen that the ratios R_P obtained from the Q-replica and DIST-replica are close to unity, while ratios R_F are 30% over-estimated. Notably, the result reweighted from the R_g-replica is poor, with the unfolded state over-populated and the free energy barrier significantly under-estimated. It is worth pointing out that this problem can be more or less solved by using a bias factor that decreases the Gaussian height faster (data not shown), suggesting again the importance of carefully choosing parameters in metadynamics.

4. Conclusion

In recent years, metadynamics and its variations become popular tools for investigating the FEL of physical, chemical and particularly biomolecular systems. They are extremely powerful and have achieved many successes in a variety of fields. However, the proper usage of such tools is not a simple task since they require prior knowledge of the underlying dynamics (CVs), and the choice of algorithms and parameters is not trivial either. To make clear of their power as well as limitations in particular in biological systems, we tested several metadynamics variations with a typical biological process – the folding of protein CI2. This testing system is simple enough to allow the calculation of the exact answer to be compared with, and also complex enough for detecting possible defects of the algorithms that may not appear in low-dimensional systems.

Based on the testing data, it was found that base-metadynamics was able to reproduce the rough shape of the FES, and the smaller the Gaussian height, the better the accuracy. The base-metadynamics was also very efficient, the general shape of the FES could be obtained from a 10 ns run, which was two orders of magnitude faster than the conventional MD simulation. However, it was also found that the result from base-metadynamics was not very satisfying – the population ratio could be four times underestimated for the simulations with larger Gaussian height.

The well-tempered method is designed to suppress the final fluctuation of the FES and improve the accuracy. According to our tests, the employment of the well-tempered method indeed improved the results. Plus, it

allowed us to recover the FES as a function of one CV from the data of other CVs. Although of the improvement, the population ratio R_P and barrier ratio R_F usually ranged from 0.5 to 1.6, suggesting a reasonable but not very accurate result. In addition, it was not easy to determine the bias factor that gauged the decreasing rate, since it needed a prior estimation of the free energy barrier and the volume of the basins of attraction.

BEMD is an important development since it allows the usage of a number of CVs simultaneously and thus alleviates the dilemma of choosing CVs. Our tests showed that the FES calculated from the neutral replica agreed very well with the exact answer. However, the replicas other than the neutral replica could only give a rough shape for FES, similar to the base-metadynamics and well-tempered metadynamics. Therefore, the suggestion is that using the neutral replica only for the FES calculation in the case the accuracy is paramount.

BEMD can also be combined with the well-tempered algorithm to further improve the performance, if the free energy barrier and the volume of the basins can be estimated *a priori*. In addition, the well-tempered algorithm allows one to use the reweighting technique to recover unbiased probability on one CV from biased data on other CVs. This is particularly useful in computational studies of biological systems. Our tests showed that the calculated FESs from replicas other than the neutral replica gave roughly correct results.

In a short summary, for biological processes such as protein folding, the base-metadynamics is very powerful for a quick sweep of the FEL if the most relevant CVs are known *a priori*; its well-tempered variation gives a more accurate FES subject to the prerequisite that the Gaussian parameters are correctly given; BEMD is a good choice for lazy users who do not want to be bothered by choosing CVs and Gaussian parameters; if an accurate FES is paramount for the study, our suggestion is using BEMD and using the neutral replica only for calculating the FES. We believe that people who are interested in the computational study of physical, chemical and biological systems will find this work helpful.

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