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Unbinding pathway energy of glyphosate from the EPSPs enzyme binding site characterized by Steered Molecular Dynamics and Potential of Mean Force

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ABSTRACT

The quantification of herbicides in the environment, like glyphosate, is extremely important to prevent contamination. Nanobiosensors stands out in the quantization process, because of the high selectivity, sensitivity and short response time of the method. In order to emulate the detection of glyphosate using a specific nanobiossensor through an Atomic Force Microscope (AFM), this work carried out Steered Molecular Dynamics simulations (SMD) in which the herbicide was unbinded from the active site of the enzyme 5- enolpyruvylshikimate 3 phosphate synthase (EPSPS) along three different directions.After the simulations, Potential of Mean Force calculations were carried, from a cumulant expansion of Jarzynski's equation to obtain the profile of free energy of interaction between the herbicide and the active site of the enzyme in the presence of shikimate-3 substrate phosphate (S3P). The set of values for external work, had a Gaussian distribution. The PMF values ranged according to the directions of the unbindong pahway of each simulation, displaying energy values of 10.7, 14.7 and 19.5 KJ mol^{-1}. The results provide a theoretical support in order to assist the construction of a specific nanobiossensor to quantify the glyphosate herbicide.

1. Introduction

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), which is present in algae, higher plants, bacteria, and fungi, catalyzes the reaction that involves the transfer of carboxyvinyl group of phosphoenolpyruvate (PEP) to the shikimate-3-phosphate (S3P), forming the 5-enolpiruvil shikimate-3-phosphate (EPSP). The herbicide glyphosate (GPJ) inhibits specific enzymes such as EPSPs, suspending the synthesis of aromatic amino acids. It preferably forms a stable ternary complex with the EPSPs and S3P substrate [1,2] (Fig. 1).

The detection and quantification of the glyphosate herbicide can be carried out by various analytical techniques [3–6]; however, the nanobiosensors arise as a quantification tool of high sensitivity, selectivity and short response time. It is noteworthy that this kind of detection comes from the deposition of a receiving layer (protein) in microcantilevers using an atomic force microscope (AFM) [7–9]. When the microcantilever is used as a force sensor for AFM, the distance curves can be used to find the forces that contribute to the deflection of the cantilever and a variety of biological and chemical noncovalent interactions can be mapped directly on the distance tip-sample, which is based on force measurement capability of the AFM [10].

In order to prevent time consuming experiments, Molecular Dynamics simulations can be used to emulate atomic force microscopy experiments, as previously mentioned, to provide a quantitative analysis of the effects of specific molecular interactions between the active site of the enzyme EPSPs, the shikimate-3-phosphate (S3P), and the herbicide glyphosate (GPJ). The probability that a ligand will escape from the active site through an exit channel is dependent on the free energy profile for passage of the ligand along the channel [12].

The determination of the absolute binding free energy for the protein-ligand systems can be performed by calculations of the potential of mean force (PMF). This approach is well based on statistical mechanics of liquids since the beginning of molecular mechanics [13,14].

In this work, we performed Steered Molecular Dynamics (SMD) simulations, and potential of mean force calculations, in order to provide a sufficient theoretical basis, at the atomic level, for possible

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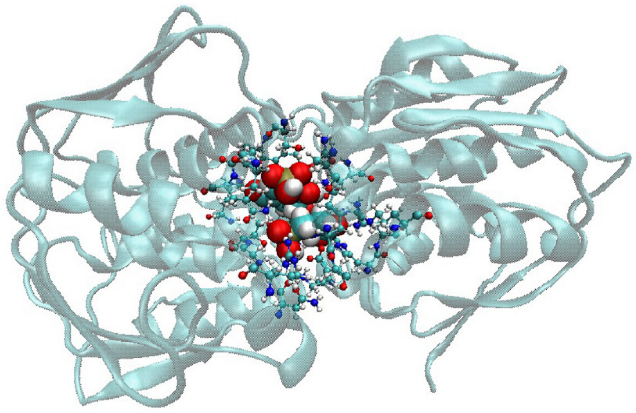

Fig. 1. Closed structure (new cartoon representation) of the enzyme EPSPs, the herbicide GPJ, the substrate S3P (van der Waals representation) and the active site residues (shown in ball and stick). Figure made by VMD software [11].

application in the development of a nanobiosensor for the detection of the herbicide glyphosate, using an atomic force microscope.

2. Computational details

2.1. Molecular dynamics simulation

The structure enzyme-substrate-ligand complex (PDB code: 1G6S [1]) stability between EPSPs and glyphosate was evaluated using the Molecular Dynamics protocol: Initially, the modeled system was solvated by filling the appropriated simulation box with SPC (simple point charges) [15] water model molecules. Sodium ions were used to achieve the ionic strength of 100 mM for the system, which was minimized using 10000 steps with the steepest descent method [16]. After minimization, the solvent was equilibrated by performing 100 ps molecular dynamics simulation at 50, 150 and 298 K, with non-hydrogen atoms positionally restrained (force constant 1.0×10^3 kJ mol^{-1} nm^{-2}). Following the solvent equilibration step, for each temperature a total of 10 ns molecular dynamics simulations was performed in an isothermal–isobaric (NPT) ensemble using velocity rescaling with a stochastic term for temperature coupling [17] and 1 bar pressure using Berendsen barostat [18] using the leapfrog algorithm [19] with a 2 fs time step. The configurations were recorded every 1 ps for analysis. These simulations were performed using the OPLS-AA force field [20] and the GROMACS 5.0.7 program [21].

The bonded force field parameters of glyphosate was obtained performing Hartree-Fock ab-initio calculations using the 6-31G* basis sets, and the program Orca 2.8 [22]. The atomic charges (non-bonded parameters) were calculated for the optimized molecule using a Restrained Electrostatic Potential method (RESP) [23], and using the NWCHEM 5.1 program [24].

2.2. Steered molecular dynamics

In order to simulate the AFM experiments, we performed the Steered Molecular Dynamics simulation (SMD), which was initially introduced by Izrailev in 1997 [25]. This method provides models to study mechanical properties of biomolecules, dissociation process by applying external forces or constraint distances on time scales covered by molecular dynamics simulations. The SMD has been widely used in the investigation of protein mechanical functions, such as the interaction process in protein-ligand complexes [8,26,27] and the diseases related to the protein structure stability [28].

One way of applying external forces in a protein-ligand complex is to restrict the binder at a point in space (contention period) by an external potential, for example, the harmonic. The point of contention is then displaced in a chosen direction forcing the binder to move from its initial position in the protein allowing it to explore new contacts along the unbinding path. Assuming a single reaction coordinate x, and an external potential $V = k(x\text{-}x_o\text{-}vt)^2/2$, where k is the stiffness of the containment point, and x_o is the initial position of the restraint point moving with a constant velocity v, the external force on the system can be expressed by the equation 1 [29],

$$F = k(x_0 + vt - x) \quad (1)$$

F corresponds to the force in which the molecule is being pulled by a harmonic spring with rigidity k and a constant velocity v.

In this work, different simulations were performed using the force field OPLS-AA and the GROMACS 5.0.7 program to determine a possible unbinding pathway of glyphosate from the active site of the enzyme EPSP. Thus, the steered molecular dynamics was carried out so that the glyphosate molecule (group zero) was removed from the enzyme on the presence of S3P molecule (group 1) from its initial position in the active site, along three possible unbinding pathways. These directions were chosen due to less steric repulsion. The three direction vectors are as follow: Direction A is determined by the vector from the nitrogen atom of the residue serine 197 (SER197:N) to the nitrogen atom of the ligand glyphosate (GPJ701:N1); Direction B is the vector from residue ASN336:C to GPJ701:N1; Direction C is from residue SER23:N to GPJ701:N1 (Fig. 2).

To mimic the AFM experiment, the explicit solvent of the minimized system was withdrawn and the SMD was performed using the constraint pulling method, in which the distance between the mass centers of the two groups (zero and 1) was restrained with a force constant, k, of 367 kJ mol^{-1} nm^{-1}, and in all dynamics the unbinding velocity, v, was 0.001 nm ps^{-1}. The SMD was done in the NVT ensemble (constant particle number, volume and temperature) using velocity rescaling with a stochastic term for temperature coupling with a heat bath temperature of T = 298 K for 4 ns with time step of 1 fs. All bond lengths were constrained using the LINCS algorithm [30]. A total of 80 SMD simulations were carried out for each of the three directions.

2.3. Potential of mean force calculation

The Potential of Mean Force (PMF) was performed to determine the absolute free energy of interaction between EPSPs-S3P and GPJ in order to simulate the interaction between the AFM tip functionalized with EPSPs and glyphosate.

The PMF is a potential that is obtained by integrating the average strength of an ensemble of configurations, which displays an important role in the investigation of molecular processes in which the configurational space is described by a reaction coordinate.

The SMD is an effective method to explore mechanical and molecular processes accessible via AFM experiments. However, a directional dynamic simulation is a process of non-equilibrium, while the potential of mean force is an equilibrium property. Therefore, it becomes necessary a theory that connects the processes in equilibrium and non-equilibrium, which is through statistical mechanics of non-equilibrium, especially through the Jarzynski equality [31]. Thus, it is possible to extract equilibrium properties from non-equilibrium systems. The Jarzynski equality establishes a connection between the equilibrium free energy calculation and the work in non-equilibrium process, and therefore allows to calculate the PMF on non-equilibrium processes such as SMD simulations [27].

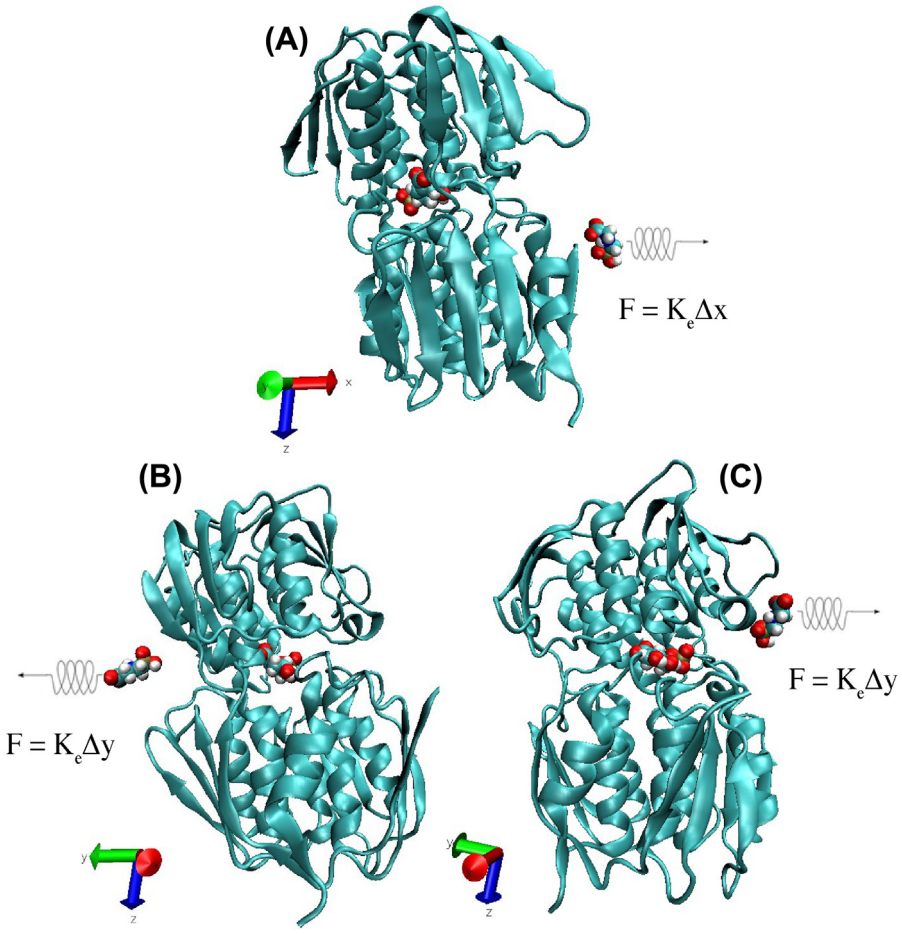

Fig. 2. Steered Molecular Dynamics in three possible unbinding pathways of the glyphosate herbicide, (A) Direction A; (B) Direction B and (C) Direction C. Figure made by VMD software [11].

The Jarzynski equality (Eq. (2)) is:

$$e^{-\beta\Delta F} = e^{-\beta W} \tag{2}$$

where ΔF represents the variation of the free energy between the initial and final states, W is the external work, $\beta=(K_BT)^{-1}$ and K_B and T are the Boltzmann constant and temperature, respectively. The most important property of this relationship is that it is not restricted only to equilibrium systems. The Equality is satisfied for any disturbance, since sufficient samples are performed [32].

A cumulant expansion of the Jarzynski's equation can be used to circumvent the difficulty of estimating the exponential average of the right side of Eq. (2). The average logarithm of an exponential can be expanded in terms of cumulants. The Eq. (3) shows the first and second cumulants.

$$loge^{x} = x + \frac{1}{2}\left(x^2 - x^2\right) + \ldots \tag{3}$$

In this case, when all other cumulants terms are neglected, the variable x must be, imperatively, sampled by a Gaussian distribution. Using this expansion, the free energy can be obtained from Eq. (4)

$$F_{\lambda(\tau)} - F_{\lambda(0)} = W(\tau) + \frac{\beta}{2}\left(W(\tau)^2 - W(\tau)^2\right) + \ldots \tag{4}$$

where, $F_\lambda(\tau) - F_\lambda(0)$ is the free energy variation, $\beta = 1/k_BT$ and $W(\tau)$ is the work. When the work follows the Gaussian distribution, the formula until second order can be used since the third and higher cumulants are identically zero [33,34].

From the 80 SMD simulations, the work and the potential of mean force were obtained for each chosen direction, using the Octave 3.8.1 software [35]. The Shapiro-Wilk test (R-Statistics 3.0.2 software [36]) was performed to evaluate if the data set distribution, related to the work values obtained in each simulation, follows a normal distribution.

3. Results and discussion

The Molecular Dynamics simulation, in the presence of explicit solvent (spc water), displayed a very low structural fluctuation, 0.17 nm ± 0.011 nm, and an inexpressive mobility of glyphosate within the EPSPs active site.

For each SMD simulation, a graphic of strength versus time can be made as displayed in Fig. 3. By the analysis of three representative simulations, it is possible to observe (Fig. 3) that the paths along the directions A, B and C showed maximum forces around 2150, 2350 and 1530 kJ mol^{-1} nm^{-1} (3570, 3900 and 2540 pN), respectively. These values have the same order of magnitude of SMD simulations done in our previous work, which extraction forces ranged from 1100 pN to 2100 pN [8].

The herbicidal unbinding pathway requires a smaller force along the direction C, but showed the same two moments of maximum strength, 200 and 600 ps, which are related to the coordinates where the hydrogen interactions of glyphosate and some residues of EPSPs-S3P active site are more intense. At 200 ps, the GPJ herbicide interacted mainly with GLU341, HIS385, ARG386, LYS22, GLN171, ARG124, GLY196, ARG344 and ASP313 residues. At 600 ps

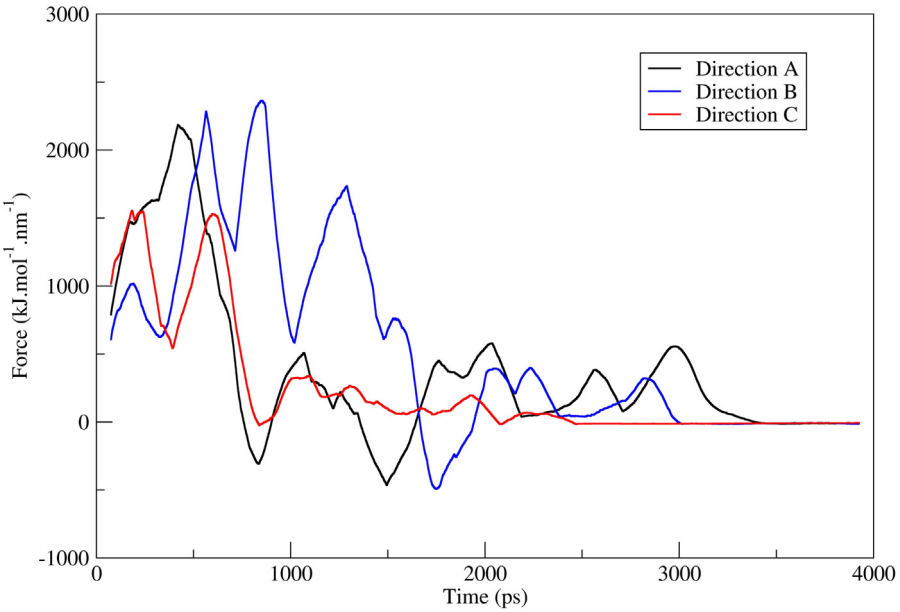

Fig. 3. One force profile vs time, applied along direction A (black), B (blue) and C (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the GPJ herbicide interacts only with ASN336, ARG124, LYS340, GLN171 and SER169.

The unbinding along the direction A showed only one significant maximum strength peak at 430 ps, which can be attributed to the rupture point of the main herbicide interactions with the active site composed by ARG124, LYS411, LYS22, HIS385, GLU341, GLY96, ARG386 and ARG384 residues.

Moreover, the path along the direction B had the highest interaction strength (850 ps) among the three paths, and displayed two more significant peaks with 2250 and 1730 kJ mol^{-1} nm^{-1} (3740 and 2870 pN), at 575 and 1300 ps, respectively. The most intense force peak, at 850 ps, interacts with SER23, LYS22, THR97, GLN171, ARG27 and TYR200 residues. The second most intense peak, at 575 ps, interacts only with THR97, SER23, LYS22 and ARG386 residues. And the last peak, at 1300 ps, interacts with ARG27, SER23, THR204 and MET178 residues.

All these force peaks mentioned previously are related to glyphosate interactions with the residues of the active site involving hydrogen bonds and electrostatic interactions. The negative force values correspond to the rupture of these intermolecular interactions. The lower force peaks are related to herbicide contacts with other regions of the enzyme outside of the active site.

In order to evaluate the normality of a variable quantitatively, the Quantile-Quantile Plot was used (Fig. 4). The results of the non-parametric Shapiro-Wilk gave more objective results of adherence to normal distribution, as shown in Table 1. In this test, four points along the reaction coordinate (simulation time) were selected, for each direction, to verify if the data distribution obtained for the accomplished work, in each trajectory, follows a Gaussian distribution. A significance level of 10% was chosen, which was considered the hypothesis test as: H_0: The distribution of the data set follows a normal distribution and H_1: The data set distribution does not follow a normal distribution.

The Q-Q plots for the three directions (Fig. 4) shows a distribution of points near the reference line, indicating a variable with normal distribution.

According to Table 1, it is observed that all the values obtained for the p-value are greater than 10%; thus, the null hypothesis is not rejected, and so there is statistical evidence, at the level of significance of 10%, that the distribution of the work values obtained on three directions, in these four points chosen along the reaction coordinate, follows a Gaussian distribution. Thus, the number of SMD simulations is sufficient to calculate the potential of mean force calculation according to the methodology of the cumulant expansion of Jarzynski equation.

Considering the expressive difference among the three unbinding pathway directions and the distance vs time is linearly proportional ($r > 0.979$, support information), the variable time was considered as the normalization factor to compare the PMF energies. Thus, Fig. 5 shows the PMF calculated for the three directions in function of the simulation time. The lowest free energy barrier occurs along the direction C, approximately 10.7 kJ.mol^{-1}, followed by the direction A and direction B with energy barriers around 14.7 and 19.5 kJ mol^{-1}, respectively. These values of energy barriers (unbinding process) can be compared with the free energy of binding ($\Delta G_{Binding}$) for the receptor-ligand (EPSP-Glyphosate) complex. Thus, in order to validate the PMF calculations, the $\Delta G_{Binding}$ was calculated using the experimental value of inhibition constant (K_i) which ranges from 12 mM to 0,15 μM [37], and the equation 5 [38],

$$\Delta G_{Binding} = RT \ln (K_i) \quad (5)$$

where, R is the universal constant of gas and the T is the absolute temperature in kelvin.

Therefore, the experimental value obtained of $\Delta G_{Binding}$ by the equation 5 ranged from −11.0 kJ mol^{-1} to −38.9 kJ mol^{-1}, suggesting that the PMF theoretical estimative to calculate the energy involving the bind/unbind process between receptor-ligand complex is appropriate. However, it worth nothing to observe that K_i experimental results were obtained in aqueous solution, in the presence of an appropriate ionic strength; and our simulations setup described the system in vacuum (NVT ensemble) to mimic the AFM experiments. Considering that these differences can affect significantly the binding energy value, SMD simulations in the presence of water molecules should be made to predict the specific binding energy between enzyme-ligand complexes.

The results displayed in Fig. 5 confirm the previous data force profile (Fig. 3), wherein the unbinding process of glyphosate from

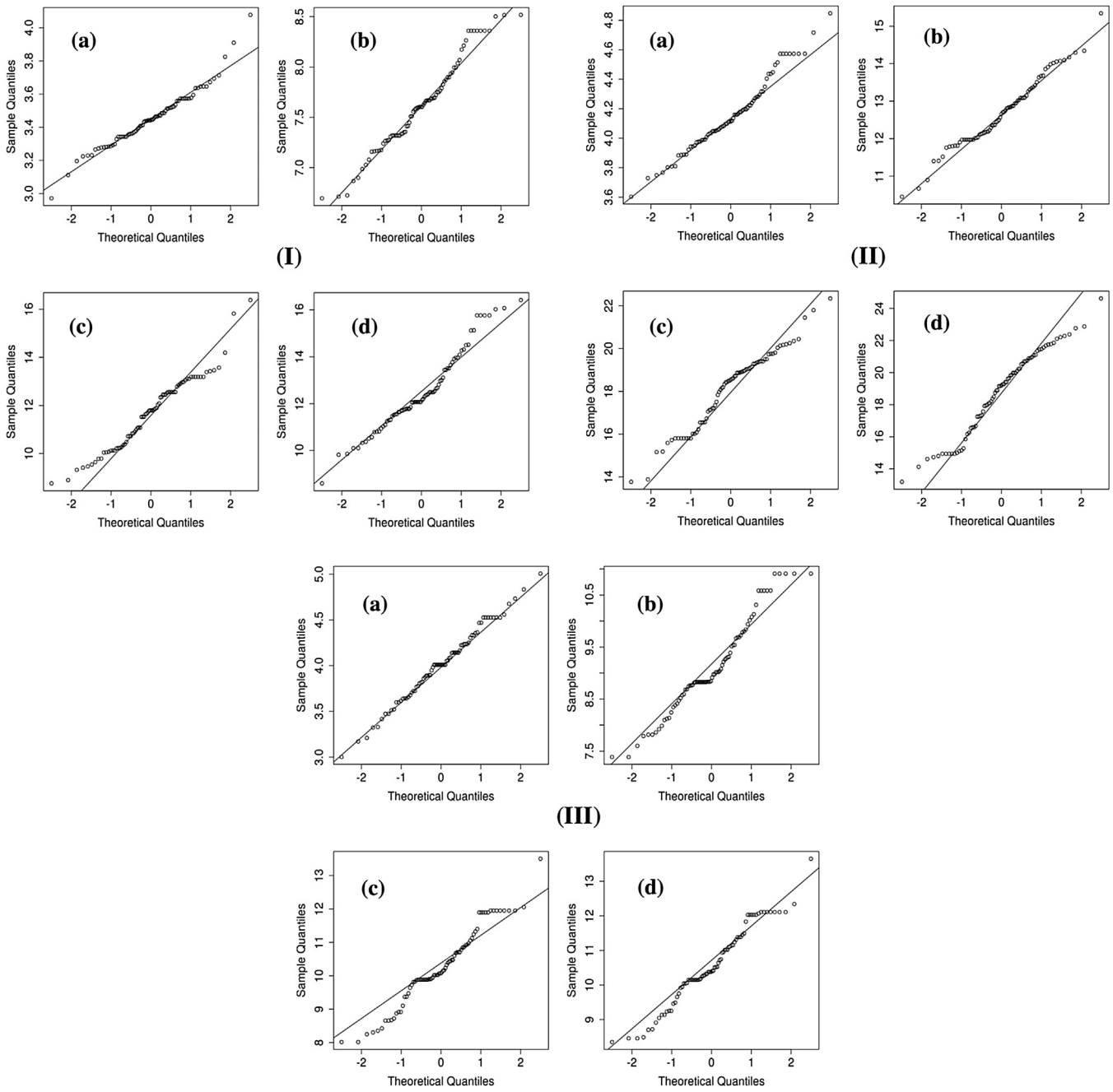

Fig. 4. Quantile-Quantile Plot. (I) – Direction A, (a) work in 300 ps, (b) work in 500 ps, (c) work in 1000 ps, (d) work in 1500 ps; (II) – Direction B, (a) work in 500 ps, (b) work in 1000 ps, (c) work in 1500 ps, (d) work in 2000 ps; (III) – Direction C, (a) work in 300 ps, (b) work in 1000 ps, (c) work in 1500 ps, (d) work in 1750 ps.

Table 1
Shapiro-Wilk test.

Directions	P-Value			
Direction A	**W in 300 ps**	**W in 500 ps**	**W in 1000 ps**	**W in 1500 ps**
	0.02167	0.0863	0.02589	0.01948
Direction B	**W in 500 ps**	**W in 1000 ps**	**W in 1500 ps**	**W in 2000 ps**
	0.09391	0.2919	0.0393	0.03267
Direction C	**W in 300 ps**	**W in 1000 ps**	**W in 1500 ps**	**W in 1750 ps**
	0.9526	0.01072	0.03206	0.05707

the active site of EPSPs is favorably along the direction C. However, the functionalization of enzymes on the tip of the cantilever of AFM can occur randomly, which may results different orientations between the enzyme active site and glyphosate. Thus, the specific interaction energy between glyphosate and EPSPS should vary between the energy obtained by the potential of mean force in three directions presented in this study.

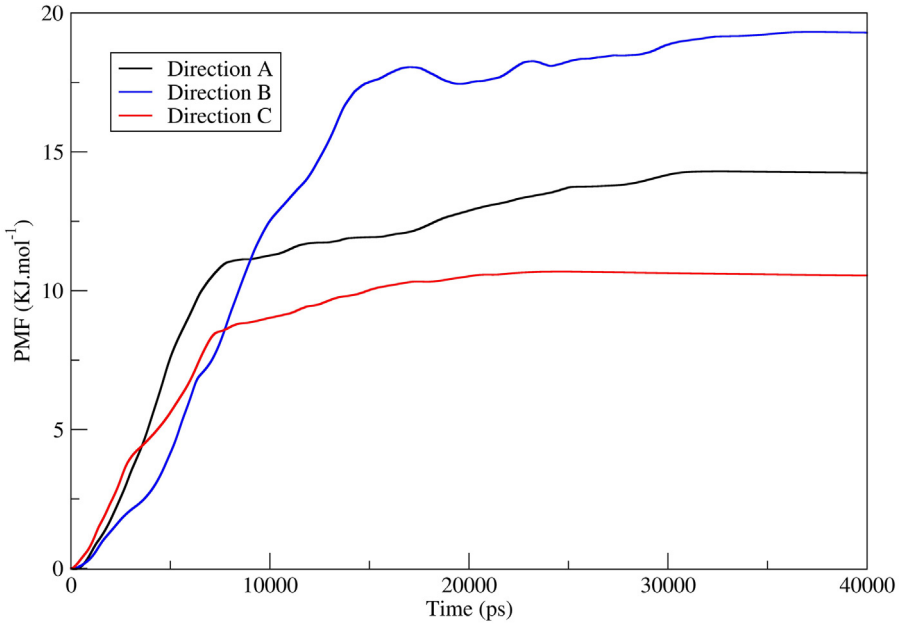

Fig. 5. Potential of Mean Force of the SMD simulations along three directions: Direction A (black); B (blue) and C (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

This work permitted to obtain the force and the energy of interaction between the herbicide glyphosate and the region of the active site of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPs), in the presence of shikimate-3-Phosphate substrate (S3P), using Steered Molecular Dynamics simulations and Potential of Mean Force through the cumulant expansion of the Jarzynskiís equation. Three directions of unbinding pathways were chosen due to steric repulsion, and the force profile, for the unbinded herbicide from the active site of the enzyme, showed that less force is required for removing the herbicide along the direction C compared to the direction A and B. The energetic values of the potential of mean force corroborate with previous data, since the PMF obtained was 10.7, 14.7 and 19.5 kJ mol^{-1}, for the directions C, A and B, respectively. The number of SMD simulations was satisfactory because the set of values obtained for the external work, for the simulation along the three directions, presented a Gaussian distribution. The emulation of the AFM experiments through directional molecular dynamics simulations promoted a quantitative view of the free energy profile involved in the interaction between the herbicide glyphosate and the active site of the enzyme EPSPs in the presence of S3P substrate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmgm.2016.11.010.

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