MD and EPR studies of the dynamics of the MTSL spin-label in the activation loop of Aurora-A kinase

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Abstract

Classical molecular dynamics (MD) simulations, within the AMBER program package that runs entirely on a CUDA-enabled NVIDIA graphic processing unit (GPU), were employed to study with low computational cost the dynamics of the methane-thiosulfonate spin label (MTSL) attached to the activation loop of Aurora-A kinase. MD provided information about the conformational space of MTSL in the protein environment; an isotropic and uniform distribution of orientations of the spin label in space was found due to the large exposure of the activation loop to the solvent water. A hydrodynamic approach was employed to determine the rotational protein tumbling, while the internal motion of the spin probe was determined from 94 GHz measurements that reflect the fast motions. The theoretical 9 GHz EPR spectra were calculated using configurations representing interactions between MTSL and water and the tyrosine residue 208 in the C-lobe and the comparison with experimental EPR spectra produced fits that successfully reproduced the experimental spectra in agreement with the average structures obtained from the MD trajectories showing the MTSL exposed to the solvent and probing the C-lobe of the protein.

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1. Introduction

Site-directed spin labelling (SDSL) combined with electron paramagnetic resonance (EPR) spectroscopy is a very powerful technique used widely for studying the structural properties and dynamical processes of biological systems.\(^1\)\(^2\) This has provided valuable information on many proteins such as T4 Lysozyme,\(^3\) lipoxygenase L-1,\(^4\) α-synuclein,\(^5\) bacteriorhodopsin,\(^6\) SNARE\(^7\) and NavMs.\(^8\) Studies on singly labelled proteins can reveal a wealth of information on the tumbling and diffusion properties of the target by analysis of the continuous-wave (CW) EPR lineshape.\(^9\)\(^10\)

In order to provide a realistic interpretation of the CW EPR spectrum of spin labelled systems, a description of the overall dynamics of the MTSL in the protein environment has to be found. Several different computational approaches to determine the conformational space and dynamics of spin label and subsequently simulate the EPR spectrum have been suggested by several groups previously. Some approaches are based on Brownian dynamics (BD), introduced by Robinson et al.\(^11\) and Steinhoff et al.\(^12\); and molecular dynamics (MD) methods introduced by Budil et al.\(^13\) and Oganesyan\(^14\)\(^-\)\(^15\) to generate stochastic dynamical trajectories of the spin labels and/or to derive diffusion parameters such as the rotational diffusion tensor, diffusion tilt angles and expansion coefficients of the orienting potentials that are then incorporated in the stochastic Liouville equation (SLE) for the calculation of the EPR spectrum. Other methods for the calculation of the EPR spectra include the stochastic Markov models\(^16\)\(^-\)\(^17\) and temperature scaling or replica exchange\(^18\)\(^-\)\(^19\) methods, including simulated tempering (ST) and parallel tempering (PT) that have been developed to improve sampling and kinetic information. There are also integrated computational approaches (ICA) that link a quantum mechanical (QM) method rooted on density functional theory (DFT) to the stochastic Liouville equation (SLE) equation in the Fokker-Planck (FP) form. A similar approach was used to calculate the CW EPR spectra of free radicals in their environments\(^20\)\(^-\)\(^22\) and to study the structure and dynamics of the MTSL side chain in protein systems, such as T4 lysozyme.\(^23\)\(^-\)\(^24\) The models produced were in good agreement with experimental data with a reasonable computational cost\(^25\)\(^-\)\(^27\) and consistent with data obtained by MD simulations.\(^28\) In these approaches QM methods were used to determine EPR parameters (g- and A-tensors), while coarse grained methods were used to produce a hydrodynamic model of the diffusion tensor\(^29\) that are the input of the SLE equation for the calculation of the CW EPR spectra.\(^30\) There are also computationally inexpensive approaches that can be used to predict the distribution of the conformations of the spin labels and distances between spin-labelled sites, such as MMM,\(^31\) PRONOX\(^32\) and MtsslWizard\(^33\).

In this work, we investigated the relationship between the solution state CW EPR lineshape and the dynamics of the spin labelled activation loop of Aurora-A kinase using computational methods. Aurora-A kinase is a serine/threonine protein kinase that regulates mitotic entry, centrosome maturation and bipolar spindle assembly and is overexpressed in a number of cancers including breast, colorectal, ovarian, and glioma.\(^34\)\(^-\)\(^35\) Kinase activity is tightly regulated by conformational changes in a conserved region of the protein known as the activation loop upon phosphorylation of the threonine residues at positions 287 and 288 and binding of the activator protein Targeting Protein for Xklp2 (Figure 1).\(^36\)\(^-\)\(^37\)
Figure 1: X-ray crystal structure of the Aurora-A kinase protein based on the X-ray crystallography model with Protein Data Bank (PDB) identifier, 1OL7, showing the activation loop in green and the phosphorylated threonine residues at position 287 and 288.

The conformation of the activation loop may also be influenced by the binding of inhibitors to the active site of Aurora-A and, in the case of the potent and selective inhibitor MLN8054, the position of the activation loop main chain is moved by up to 19 Å. The understanding of kinase structure is mostly based on protein crystallography, which is limited by the requirement to trap the molecules within a crystal lattice. Therefore, studies of kinase activation, through characterisation of activation loop conformations in solution, are important to enhance the understanding of molecular processes related to diseases and to support the discovery of small molecule kinase inhibitors.

A common spin label employed to study structural and dynamic properties of biomolecules by EPR spectroscopy is MTSL (Figure 2) that is described in previous work and introduced into the activation loop of Aurora-A kinase at residue 288.

Figure 2: Structure of the MTSL side chain with the five dihedral angles indicated. The nitrogen and the oxygen atoms of the nitroxide group (NO) are represented in blue and red, respectively.

To allow this modification, the wild-type threonine residue was mutated to cysteine, while the threonine at position 287 was mutated to alanine in order to observe conformational changes in a more dynamic activation loop.

Here, we report a manageable workflow by using a variety of tools in order to determine all the different motional contributions to the overall dynamics of the MTSL and the environments mostly probed by the spin label in order to obtain the fundamental information necessary to simulate the EPR spectrum. MD simulations of opportune length were performed to determine how MTSL occupies space around the point of attachment in the loop. Subsequently, the average structures from MD were determined as representative of the ensemble and used for calculation of the relevant EPR parameters (g- and A-tensors) to
simulate the 9 GHz CW EPR spectrum of the spin-labelled Aurora-A kinase at 298 K in order to obtain information about the protein tumbling.

2. Methods

2.1 Force field parameterization of the MTSL side chain and MD simulation details

The latest AMBER force field\textsuperscript{43} (ff14SB), recommended for the study of protein dynamics was extended in order to perform MD simulations with AMBER 2014.\textsuperscript{44} The structure of the MTSL side chain was first optimized with the B3LYP hybrid functional using the 6-31G(d) basis set. This level of theory was used as it provided accurate experimental geometries of the MTSL spin label\textsuperscript{27} in previous work. Subsequently, in order to reproduce the electrostatic potential and hydrogen-bonding properties of the MTSL side chain, the atom-centred point charges were calculated with Hartree-Fock (HF) theory\textsuperscript{45-46} with the 6-31G(d)\textsuperscript{47} basis set, these DFT calculations were performed using Gaussian09 revision d.01.\textsuperscript{48} The electrostatic potential was calculated using the restrained electrostatic potential (RESP) procedure\textsuperscript{50}. The atom charges determined to extend the AMBER force field to the MTSL side chain were comparable with those reported in literature,\textsuperscript{25} to parametrize the NO bond, the bond angles, angle bending, regular torsions, non-bond interactions parameters were taken from reference 25.

MD simulations were carried out on MTSL spin labelled Aurora-A kinase, using the X-ray crystal structure of the Aurora-A kinase domain (residues 122-403 C290A C393A; PDB 4CEG\textsuperscript{50} with a resolution of 2.10 Å and R-value of 0.202). The pdb file was edited in the website WHATIF\textsuperscript{51} to mutate the native Threonine-288 to cysteine. Alanine substitutions were made at residues T287, C290 and C393 in order to produce a structural model similar to the experimentally studied protein. The missing crystallographic hydrogens and MTSL were added using the LEaP module AMBER. The protein was solvated using the Extended Simple Point Charge (SPC/E) water model (11896 water molecules) in a truncated octahedral box with a buffer of 12 Å between the protein atoms and the edge of the box. This water model was used as it predicts viscosities and diffusion constants closer to the experimentally observed data\textsuperscript{52} and reproduces crystallographic water positions more accurately than the commonly used Transferable Intermolecular Potential 3 Point (TIP3P) water model.\textsuperscript{53-54}

In order to obtain a good statistical sampling quality, multiple MD trajectories were performed\textsuperscript{55-56} using multiple initial starting structures of the spin-labelled system in which the MTSL was set in different configurations with varying values of the $\chi_1$, $\chi_2$ and $\chi_3$ dihedral angles (Table 1). These values were determined at the minima of the torsional profiles calculated in a previous work\textsuperscript{57}.

Table 1: Initial values of the $\chi_1$, $\chi_2$ and $\chi_3$ for trajectories from T1 to T6.

| Trajectory number (n°) | $\chi_1$ (°) | $\chi_2$ (°) | $\chi_3$ (°) |
|------------------------|--------------|--------------|--------------|
| T1                     | -160         | +80          | +90          |
| T2                     | +60          | -120         | +90          |
| T3                     | -60          | -160         | +90          |
| T4                     | -160         | +80          | -90          |
| T5                     | +60          | -120         | -90          |
| T6                     | -60          | -160         | -90          |
MD simulations for each trajectory were performed in three main steps: minimization, equilibration and production. Firstly, a short energy minimization was performed in two steps in order to clean the structure and to remove bad contacts using the Simulated Annealing with NMR-derived Energy Restraints (SANDER) module of AMBER. Three chloride ions were used to neutralize the net charge. In the first stage, the water molecules and counter ions were relaxed with 200 cycles of minimization. In the second step, the entire system as a whole was relaxed with 1000 cycles of minimization. Subsequently, the system was heated at constant volume for 20 ps from 10 K to 300 K with 10 kcal/mol weak restraints on the protein. This process was followed by two equilibration steps, the first was performed at constant pressure (1 atm) and temperature (300 K) for 200 ps with no restraints and the second was performed in a microcanonical (NVE) ensemble for 1 ns. The production step was performed in the NVE ensemble for at least 140 ns since transitions between rotamers occur on a time scale of ns and to ensure the full convergence of the root mean square deviation (RMSD) of the protein. All the calculations were performed using 1 fs integration time step and coordinates were collected every 2 ps, a total of 70000 frames were collected in each trajectory. The NVE ensemble was used since the interest of this work is on the dynamics of a system that would be perturbed by the use of a thermostat. This system is large enough (with 14000 atoms in total) that microcanonical and canonical ensemble are almost the same. The dynamical long-range electrostatic interactions were treated using a particle mesh Ewald (PME) algorithm with default parameters and a 10 Å cut-off Lennard-Jones. All the MD simulation were performed using one NVidia GTX 980 GPU and with AMBER software optimized to run entirely on a CUDA enabled NVIDIA GPU using a mixed-precision (single precision, double precision) model that is comparable with the double precision model on a central processing unit (CPU). We would also like to highlight the advantages of running MD over QM methods and on a computer with graphic processing unit (GPU). In a previous work, ab initio relaxed scans around the five dihedral angles of the MTSL were performed, five torsional profiles were computed in ~74 hours (3.1 days) on a common PC desktop (Intel CORE i7-4770 3.40 GHz CPU), considering systems composed of 23 to 41 atoms representing a short unit peptide on which the MTSL was gradually built. In contrast, one single MD trajectory of 140 ns of fully solvated spin-labelled protein system composed of 16273 atoms (4377 atoms of the protein and 11896 atoms of water) was calculated in only 81 hours (~41.05 ns/day equal to 3.4 days) using a dual Intel processor and one single NVidia GTX 980 GPU accelerator. This also helped to avoid issues related to queue limits and memory available to users in computer clusters.

2.4 Calculation of EPR parameters (g- and A-tensors)

The EPR parameters were calculated using the Gauge-Independent Atomic Orbital (GIAO) method, the B3LYP hybrid functional and the latest N07D basis set that has been used with success for accurate calculation of the magnetic tensors (Δgii = ±0.0005, ΔAii = ±1 G) in gas phase and in solution of nitroxide radicals at a reasonable computational cost and can be downloaded from the DREAMSLAB website. The Polarizable Continuum Model (PCM) was used to describe solvation in water since the experimental EPR spectrum was measured in water. All the EPR spectra were simulated using the open source Spinach software library, version 1.7.2996. The slow-motion regime EPR spectra were simulated using the gridfree context that uses Fokker-Planck formalism that generates spectra in good agreement with the spectra produced by the simulation software easySpin 5.0.20 (using the “chili” function) and with the microscopic-order-macroscopic-disorder (MOMD) approach developed by J. H. Freed and co-workers that represents an extension of the stochastic Liouville equation (SLE) developed by Kubo in the 1960’s and then adapted for EPR simulations by Freed and co-workers for the calculation of slow motion and rigid limit spectra. The Fokker-Planck formalism in Spinach generates spatial dynamics using lab space rotation generators for the three-dimensional rotational diffusion, \( \frac{1}{\tau_p} \left( \hat{L}_x + \hat{L}_y + \hat{L}_z \right) \), where \( \tau_p \) is the rotational correlation time.
and $\hat{L}_i$ represents the angular momentum (not spin) operators acting in the lab space to generate spatial rotations of the spin system from the starting Cartesian coordinates obtained from selected MD frames. The fast-motion regime EPR spectrum (7 ns for the fit of the 9 GHz spectrum) were simulated using the Bloch-Redfield-Wangsness relaxation theory.\textsuperscript{81-82}

2.5 Experimental section

Spin-labelled Aurora-A 122-403 T287A, T288C, C290A, C393A was produced as stated in earlier work\textsuperscript{57}. 50 μM MTSL-Aurora-A 122-403 T287A, T288C, C290A, C393A was used for CW EPR studies. 9 GHz measurements were performed using a Bruker Micro EMX spectrometer at 298 K, the modulation frequency was set at 100 KHz and the microwave power at 2.0 mW. The spin labelling efficiency was equal to 86% as measured following a published procedure.\textsuperscript{83} 93.778 GHz CW measurements was performed at 298 K on a Bruker E560 spectrometer. The magnetic field was calibrated using a Mn\textsuperscript{2+} power standard (0.02% MgO) and the procedure described by O. Burghaus \textit{et al}.\textsuperscript{84} Dual-scan measurements were made in order to avoid hysteresis effects and a modulation frequency of 100 KHz and low microwave power (0.0048 mW) were used to avoid distortion of the lineshape.

3. Results and discussion

3.2 Dynamics of the MTSL spin label in the activation loop of the Aurora-A kinase protein

All the six trajectories showed the MTSL fully probing the space around the point of attachment and exploring all the regions surrounding the loop. Appropriate sampling was obtained, simulation results converged to stable RMSDs (Figure 1 ESI) after 1-2 ns (for T1, T2 and T3) or after 20 ns (for T4, T5 and T6). The most populated values of $\chi_1$, $\chi_2$, $\chi_3$, $\chi_4$ and $\chi_5$ observed in the six trajectories were comparable with minima values of the torsional energy profiles obtained by QM in a previous work\textsuperscript{57}. Three minima were found in the torsional profile of $\chi_1$ corresponding to -160°, -60° and +60° and the same three minima were found in the plot of the occurrences (Figure 2 ESI) from which also the minima of $\chi_2$ were confirmed and so on for $\chi_3$, $\chi_4$ and $\chi_5$. The plot of the transitions of the five dihedral angles (\textit{e.g.} transitions in T1, Figure 3) over the time of the MD simulation showed slower transitions of $\chi_1$ due to the higher energy barriers of about 6 kcal mol\textsuperscript{-1} separating the conformational minima, while $\chi_2$, $\chi_4$ and $\chi_5$ undergo faster transitions due to the lower energy barriers of 1-4 kcal mol\textsuperscript{-1}. 
Figure 3: Plots showing the transitions of the $\chi_1$, $\chi_2$, $\chi_3$, $\chi_4$ and $\chi_5$ dihedral angles over 80 ns using the starting structure, T1.

Transitions of the dihedral angle $\chi_3$, representing the disulphide bond of the side chain, were seen to occur very rarely, probably due to the high energy barrier of about 10-20 kcal mol$^{-1}$, they did not occur in the starting structures T2, occurred once in T3, twice in the T1, three times in T4 and T5, four times in T6. Another observed feature was the coupling between transitions of dihedral angles close to each other (Figure 3), e.g. the simultaneous transitions of all the five dihedral angles at 16.7 ns and the transitions of $\chi_1$ and $\chi_2$ between 23 and 55 ns.
3.3 Analysis of the main motional contributions in the protein systems

The 9 GHz EPR spectra are affected by both the slow rotational protein tumbling and by the fast internal motion of the spin label.\textsuperscript{29, 85-86} The rotational correlation time of the tumbling of the protein was determined using the Stokes-Einstein-Debye equation

\[
\tau_R = \frac{1}{4} \frac{\pi \eta R_h^3}{k_B T} = \frac{1}{6 \sigma_r},
\]

where \(k_B\) is the Boltzmann constant, \(T\) is the temperature, \(\eta\) is the viscosity of the water at 298 K, \(R_h\) is the hydrodynamic radius of the protein and \(D_r\) is the rotational diffusion coefficient. \(D_r\) was calculated for the PDB:4CEG structure of Aurora-A kinase using the program HYDROPRO\textsuperscript{86} and was equal to \(9.560 \times 10^6\) s\(^{-1}\), the corresponding \(\tau_R\) was equal to 17 ns.

In order to characterize the internal motion of the spin label, several considerations were made, there are many X-ray crystallographic and spectroscopic studies of the rotameric states of the MTSL indicating that its internal dynamics arise predominantly from transitions of \(\chi_4\)\textsuperscript{29, 87} since transitions of \(\chi_1\), \(\chi_2\) and \(\chi_3\) are hindered by the formation of hydrogen bonds between the sulphur atoms of the spin label and the hydrogen of the C\(\alpha\) in the protein backbone. In addition, our previous analysis of the potential torsional profiles of \(\chi_1\), \(\chi_2\) and \(\chi_3\) revealed well-defined minima and higher energy barriers separating them, corresponding to structures stabilized by hydrogen bonding.\textsuperscript{57} Transitions of \(\chi_5\) were seen to occur on a time scale of ps due to the small energy barriers (1 kcal mol\(^{-1}\)) and simulations using a \(\tau_R\) equal to ps were not seen to reproduce features in our experimental 9 GHz and 94 GHz EPR spectra. Hence, our attention was focused on the transitions of \(\chi_4\) and their speed was seen occurring on the ns time scale (Figure 3). In order to determine the correlation time of the internal motion of the spin label, a comparison between simulated and experimental 94 GHz spectra of the spin-labelled Aurora-A kinase, measured at 298 K was made (Figure 4). This frequency was chosen since fast motional processes are detected and slow motions are frozen out.\textsuperscript{85-86}
Figure 4: Comparison between experimental (h) and simulated (a, b, c, d, e, f and g) 94 GHz EPR spectra of Aurora-A kinase using values of $\tau_R$ equal to 1 ns (a), 3 ns (b), 5 ns (c), 7 ns (d), 9 ns (e), 11 ns (f) and 17 ns (g). The simulated EPR spectra were calculated keeping the magnetic parameters ($g$- and $A$-tensor) constant and no artificial line broadening parameters were included in the simulations.

The best fit was obtained using $\tau_R$ equal 7 ns, while simulations with $\tau_R$ from 1 ns to 5 ns provided too sharp resonances and simulations with $\tau_R$ from 9 ns to 17 ns produced further features in the spectra and revealed a shift to the right of the resonance at 3349 mT. Hence, it was possible to conclude that the magnitude of the internal motion of the MTSL can be approximated to 7 ns.
3.3 Analysis of the conformational states adopted by the Aurora-A kinase activation loop

In all the six MD trajectories it was observed that the activation loop oscillated between the two extreme conformational states, separated by the average distance of 7.2 Å (Figure 5) and explored many other conformational states in between characterized by a similar shape and dynamics due to the absence of specific interactions within the loop and/or between the loop and the surrounding side chains. All these structures were seen to be very exposed to the solvent water and consistent with published X-ray crystal structures. 

Figure 5: Conformational states of the activation loop separated by 7.2 Å revealed by MD

The distances, in the average structures obtained from the six MD trajectories, between N- and C-lobes and the activation loop were seen to be comparable to those observed in the starting X-ray crystal structure (Figure 3 ESI) and equal to 12.8 Å and 11.3 Å, respectively. However, MD revealed that the distances between the C-lobe and the activation loop are ~1-2 Å less than the distance observed in the 4CEG X-ray crystal structure (~13 Å).

3.4 Interpretation of the 9 GHz EPR spectrum of the Aurora-A kinase protein

In order to elucidate the EPR spectrum of the Aurora-A kinase, the average structures obtained from the six trajectories were considered; in four of these structures the MTSL was exposed to the solvent and pointed to different positions in space and faraway from the N- and C-lobes, while in the remaining two, the spin label was interacted with tyrosine 208 in the α-helix of the C-lobe (Figure 6).
**Figure 6:** Representative average structures obtained from two MD trajectories showing the MTSL fully exposed to the solvent (red MTSL) and probing the C-lobe (blue MTSL). The blue MTSL represents trajectories T3 and T5, while the red MTSL represents trajectories T1, T2, T4 and T6. The remaining average structures showed similar configurations but were omitted to improve the clarity.

These average structures were calculated to the first frame after the equilibration process in order to remove the rotational and translational contributions, in this case they are a good representation of the ensemble since the activation loop did not change significantly during the MD simulation and the spin label was seen prevalently diffusing in water in all the trajectories. MD revealed that the activation loop and spin label were very much exposed to the solvent, the latter was seen to diffusing along circular trajectories. The effect of protein tumbling provided an isotropic and uniform distribution of conformations that remained constant in space and time (Figure 7).

**Figure 7:** Distribution in the space of the oxygen atom (red dots) of the NO group of the MTSL.

In this case the spin system was seen evolving similarly in each point of the space and the effect of different orientations of the A- and g-tensors with respect to specific laboratory frame (aligned to the magnetic field) can be considered lost.

In previous work, it was found that the single occupied molecular orbital (SOMO) is very localized in the NO moiety and therefore no significant variation in the values of the A- and g-tensors were observed for geometric changes of the side chain of the spin label (changes can be observed only upon variations in the geometry of the pyrrole ring of the MTSL) and consequently no significant changes in the EPR spectra were observed.⁵⁷-⁸⁹

After these considerations, we calculated the 9GHz EPR spectra using the g- and A-tensors calculated using structures shown in Figure 8, at the B3LYP/N07D level of theory using the PCM water model, representing the configurations revealed from the average structures (Figure 6).
The experimental 9 GHz EPR spectrum of the Aurora-A kinase protein, measured at 298 K, shows a typical multicomponent lineshape and was fitted as in previous work using two isotropic spectral components (Figure 9).\textsuperscript{74-90}

**Figure 8:** (A) MTSL interacting with an explicit molecule of water, (B) MTSL interacting with the tyrosine 208

Both the configurations in Figure 8 provided reasonable fits to the 9 GHz EPR experimental spectrum that was successfully simulated summing in a 1:1 ratio of two spectra obtained using isotropic rotational correlation times of 7 ns and 17 ns, representing the internal motion of the spin label and the slow protein tumbling determined above, respectively. Spectrum s3 in Figure 9 contains also a low amount of free spin label equal to the 14%. The spectra were summed making the assumption that the slow exchange between multiple protein conformations is independent from the fast exchanges between the multiple conformations of the spin label.\textsuperscript{74} During the course of the MD simulations, the spin label was seen to interact with also other residues of the protein but poor fits were obtained using the EPR parameters calculated from these configurations and a few examples are shown in Figure 4 ESI. This can be explained considering that the system is very exposed to the water solvent so

**Figure 9:** Comparison between theoretical and experimental 9 GHz EPR spectra of MTSL spin-labelled Aurora-A kinase, (A) EPR spectra simulated using A- and g-tensors from structure A in Figure 8, (B) EPR spectra simulated using A- and g-tensors from structure B in Figure 8. (s1) EPR spectra simulated with $\tau_R$ equal to 7 ns, (s2) EPR spectra simulated with $\tau_R$ equal to 17 ns, (s3) EPR spectra obtained summing in the a 1:1 ratio of spectra a and b, (s4) experimental 9 GHz EPR spectrum.
the effect of the interactions between the MTSL and the residues of the protein can be considered too small to affect the EPR spectrum.

4. Conclusion and future work

The work described here demonstrated the advantages of running MD simulations on a GPU that provided a reliable and realistic description of the conformational space of the MTSL in the full protein environment in a short time frame. Conformational states of the MTSL obtained from MD simulations were in good agreement with those obtained from QM calculations performed previously with the advantage that the dynamics of the full system were considered. MD revealed that the activation loop oscillated between two conformational states separated by about 7 Å and the distances between activation loop and the N- and C- lobes were seen to be consistent with those observed in the X-ray crystal structures. The experimental 9 GHz EPR spectrum of the Aurora-A kinase measured at 298 K was easily simulated with two rotational correlation times equal to 7 ns and 17 ns, respectively, indicating isotropic rotational protein tumbling and isotropic rotational diffusion of the spin label. The agreement between simulated and experimental 9 GHz EPR spectra was consistent with that observed in the average structures obtained from the MD trajectories that showed the MTSL exposed to the solvent and interacting with the tyrosine 208 in the C-lobe. A realistic picture of the MTSL in the Aurora-A kinase was provided in a relatively simple way using publically available software without over parameterizations. This detailed protocol can be easily applied to other spin and/or biological systems to test a broad applicability. Now that we determined the rotational protein tumbling of the Aurora-A kinase, the effect on the 9 GHz CW EPR spectrum of inhibitors and binding partners can be tested.

Author contribution

R.B., A.J.F. and M.G.C designed the research. M.G.C. designed and organized the work described in this paper, performed the spectroscopic experiments, the MD and DFT calculations, calculated the theoretical EPR spectra and analysed the data. S.G. Burgess made the MTSL-spin labelled Aurora-A used for EPR spectroscopy. M.G.C. wrote the paper with contributions from R.B., S.G.B. and A.J.F.

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Electronic supporting information (ESI):

**Figure 1:** Plots of the root mean square deviation (RMSD) of the protein backbone of T1, T2, T3, T4, T5 and T6, considering the C$_\alpha$, C, N, O atoms.

**Figure 2:** Plots of the occurrences of the $\chi_{i-1}$-$\chi_i$ dihedral angles for T1, (A) $\chi_1$-$\chi_2$, (B) $\chi_2$-$\chi_3$, (C) $\chi_3$-$\chi_4$ and (D) $\chi_4$-$\chi_5$. The most populated regions are white and the least populated regions are black.
Figure 3: Comparison of the distances between the N- and C-lobes and the activation loop observed in the average structures obtained from the MD trajectories T1, T2, T3, T4, T5, T6 and the starting X-ray crystal structure (PDB 4CEG). The Cα of the alanine 46 in the N-lobe and Cα of the MTSL (residue 288) in the activation loop, and the tyrosine 208 in the C-lobe and Cα of the MTSL were considered for the measurements.
Figure 4: Comparison between theoretical (c and d) and experimental (e) 9 GHz EPR spectra measured at 298 K. The theoretical spectra c and d were calculated summing in the same amount two isotropic components obtained one with $\tau_R$ equal to 7 ns and the other with $\tau_R$ equal to 17 ns. The EPR magnetic parameters were calculated using the structures c and d in the insert, respectively. The configuration c represents an interaction between the MTSL and the alanine 46 and tryptophan 151 located in the N-lobe of the protein, while the configuration d represents an interaction with the glutamine 209 located in the C-lobe of the protein.