CHAPTER 7 (b)

Effect of activation loop phosphorylation on Lemur Tyrosine Kinase 3 (LMTK3) activity: A Molecular Dynamics Simulation Study

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7b.1 Abstract

Eukaryotic protein kinases play a critical role in most of the human cancer. Protein kinases become catalytically active upon conversion from an inactive to an active conformation through phosphorylation of their activation loop. LMTK3 is an oncogenic kinase, found to be implicated in various types of cancer. Recent report says LMTK3 phosphorylation by CDK5 results in breast cancer tumorigenesis. We determined the probable activation loop in LMTK3 and carried out *in silico* phosphorylation at probable phosphorylation site (Thr189) in activation loop. We also studied the effect of phosphorylation on conformational dynamics of LMTK3. We also substituted Glu for phosphorylated Thr189 and noticed Glu does not mimic the effect of phosphorylation. Molecular dynamics analysis revealed that in phosphorylated LMTK3 domain there is a conformational change in the overall structure and the structure attain stability as compared to unphosphorylated and mutated LMTK3. We assumed, phosphorylation mediated conformational change may facilitate phosphoryl transfer reaction and it may activate LMTK3. These studies can be used to create hypotheses about the mechanisms of regulation by phosphorylation that can be tested further experimentally.

7b.2 Introduction

Eukaryotic protein kinases are the largest gene family that regulates several important cellular processes, such as cell growth and differentiation [114, 115]. Protein kinases share a conserved core consisting of two lobes, the N-terminal (small N-lobe) and C-terminal (large C-lobe). These two lobes form a deep pocket that accommodates an ATP [67]. The N-lobe consists of five β-strands and an α-helix (called αC-helix). The C-lobe contains α-helices and includes the activation segment which is 20-35 residues stretch located between a conserved DFG motif and APE motif that is flexible and its conformation can influence both substrate binding and catalytic efficiency. In addition the C-lobe serves as a docking site for substrate peptides/proteins [72,71].

Phosphorylation is the commonest posttranslational modification of proteins in eukaryotic cells [113]. Eukaryotic protein kinases (EPKs) are the family of enzymes that catalyse the phosphorylation reaction, and themselves regulated by phosphorylation [**11,71**]. The regulatory phosphorylation event in most EPKs occurs at activation loop of kinases, which is part of the activation segment 11,71. Phosphorylation of the activation loop is a major mechanism that induces the dynamic changes in activation process [12, 368,13, 14], that induces structural changes which stabilize the active conformation that catalyse the phosphoryl transfer reaction of γ-phosphate of an ATP molecule to the phospho acceptor site of substrate [11,368, 15]. Therefore activation-loop phosphorylation is crucial because it is required for the interconversion from an inactive to an active conformation of kinase. Generally the protein kinase domain is a structurally conserved protein domain containing the catalytic function of all protein kinases [369,291,370].

As we have already discussed LMTK3 and its implication in breast cancer [5, 427] and other cancers [87,367]. In addition to that, recently one of the *in vitro* studies has confirmed the ability of CDK5 to phosphorylate LMTK3 and results in breast cancer tumorigenesis [10]. CDK5 is a cytoplasmic proline-directed serine/threonine kinase which is expressed in many solid tumours [112]. But phosphorylation of LMTK3 in detail at molecular level has not been studied yet. In our computational study, we modelled 3D structure of LMTK3 using I-TASSER [99] as the crystallographic structure of LMTK3 is not available. Then we predicted the activation segment in LMTK3 by comparing the LMTK3 structure with well-known kinases (PKA, Pdb id- 1ATP; ERK2, Pdb id- 2ERK) and based on conserved motifs (DFG and APE) [72,71] activation segment was predicted. It has been reported that phosphorylation of these kinases in activation loop at threonine regulate the kinase activity [27-30]. So with this comparative study we predicted, threonine 189 (Thr-189) to be the phosphorylation site in LMTK3 which is present near to the APF motif in activation segment of LMTK3. To cross check our predicted phosphorylating site (Thr-189), we used NetPhos3.1 [428] server where it shows, the Thr-189 to be a probable phosphorylation site on LMTK3. Thereby in this study we carried out *in silico* phosphorylation of LMTK3 in activation

loop at Thr-189 and studied its effect on conformational dynamics upon phosphorylation and also studied ATP binding mechanism. MD simulation study demonstrated that, phosphorylation stabilize the structure of LMTK3 but facilitate significant conformational change in phosphorylated activation segment and also affect ATP binding site of LMTK3.

Proteins that require phosphorylation for its activation, there are many examples of constitutively active mutant in which the phosphorylatable residue is substituted with either aspartate or glutamate (negatively charged amino acid) [429-433]. We substituted glutamic acid (Glu) for phosphorylated Thr-189, and predicted that Glu does not mimic the effect of phosphorylation. We noticed mutated LMTK3 has no conformational change in the activation loop thus the mutated LMTK3 is more or less similar to that of unphosphorylated LMTK3. However in phosphorylated LMTK3 domain there is a conformational change in the overall structure that attain stability as compared to unphosphorylated and mutated LMTK3. We assumed phosphorylation mediated conformational change may facilitate phosphoryl transfer reaction and it may activate LMTK3. These studies can be used to create hypotheses about the mechanisms of regulation by phosphorylation that can be tested further experimentally. So the activation of LMTK3 through phosphorylation could leads to the breast cancer tumorigenesis. Our idea from this study can be used to create hypotheses about mechanisms of regulation by phosphorylation that can be tested further experimentally.

7b.3. Materials and Methods

7b.3.1. Prediction of activation segment and phosphorylation site in LMTK3 domain: A comparative study with other kinases (PKA and ERK2)

This protocol has been explained in Chapter 7a, under section 7a.3.1.

7b.3.2. *In silico* phosphorylation and mutation of LMTK3 domain at activation segment

Preparation of Phosphorylated LMTK3: Using xleap module of AMBER12 we added phosphate PO_4^{3-} group to the equilibrated structure of LMTK3 at Thr-189 of C-β atom by removing hydrogen (H) atom from hydroxyl group (OH).

Preparation of Mutated LMTK3: We substituted Glu for the Thr-189 using Chimera in order to check whether Glu mimic the effect of phosphorylation, as Glu is a negatively charged amino acid.

7b.3.3. Molecular Dynamics (MD) simulation of phosphorylated, unphosphorylated and mutated LMTK3 domain

LMTK3 structures were subjected to MD simulation. We used the Particle Mesh Ewald Molecular Dynamics (PMEMD) [305] module of AMBER12 software package [122]. AMBER ff99SB force field [306] protein parameters were used on the LMTK3 structures. Using implicit solvation method the corresponding topology and co-ordinate files were prepared for the modelled structures of phosphorylated, unphosphorylated and mutated LMTK3 domain. The resultant structures were then subjected to energy minimization by using 500 steps of steepest descent and another 500 steps of conjugate gradient. During energy minimization, we did not fix any restraint to hold the protein system. In the course of MD simulation, the systems were gradually heated from 0 to 300 K for slow relaxation of the built initial structure. In addition shake constraints [165] using a geometrical tolerance of 5×10^{-4} Å were imposed on all the covalent bonds involving hydrogen atoms. Subsequently MD was performed under constant pressure-temperature conditions (NPT) with temperature regulation achieved using Berendsen weak coupling method [166] (0.5 ps time constant for heat bath coupling and 0.2 ps pressure relaxation time). This was followed by another 200 ps of equilibration step. After equilibration of the system with constant temperature and pressure, the production MD runs were carried out for 30 ns in order to do the analysis of structure and properties.

7b.3.4. Trajectory analysis

Trajectory files obtained from MD simulations of phosphorylated, unphosphorylated and mutated LMTK3 domain were analyzed using cpptraj [358] module of AMBER12 package. Quality assurance such as Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF) and Radius of gyration (Rg), were performed. All the graphs were generated using xmgrace plotting tool. For inspecting the 3D structure of the molecule, we used UCSF Chimera [180], and VMD [179].

7b.4. Results and Discussions

7b.4.1. Activation segment and phosphorylation site prediction

Activation segment and phosphorylation sites (**Figure 7a.1 and 7b.2)** have been predicted and demonstrated in **Chapter 7a**, section 7a.4.1. Thr-189 observed to be the probable phosphorylation site in LMTK3.

7b.4.2. Molecular Dynamics (MD) simulation analysis

To examine the conformational changes, the phosphorylated, unphosphorylated and mutated LMTK3 structures, were subjected to MD simulation upto 30 ns. The snapshots of LMTK3 structures are depicted in **Figure 7b.1.**

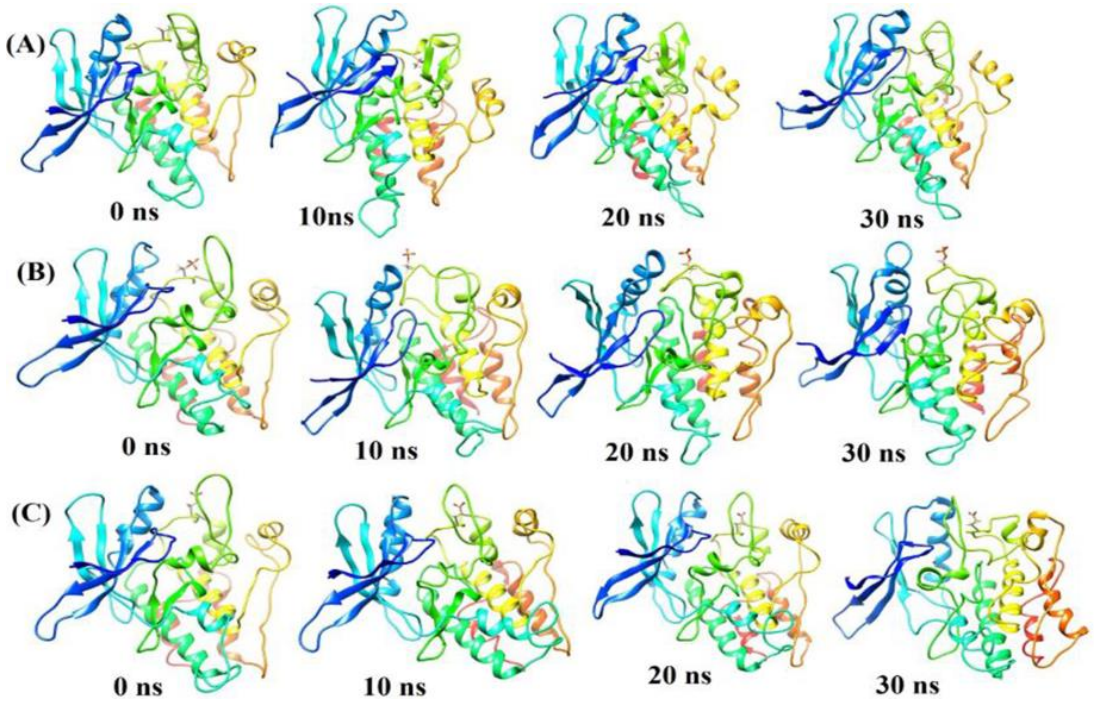

Figure 7b.1*. Snapshots of (A) unphosphorylated (B) phosphorylated (C) mutated LMTK3 domain*

7b.4.2.1. Stability and Flexibility analysis from RMSD and RMSF

Overall structural stability of phosphorylated LMTK3 was analysed from Root Mean Square Deviation (RMSD) and compared with unphosphorylated and mutated LMTK3. The RMSD analysis was done based on C-α atoms, depicted in **Figure 7b.2**, we noticed, the structures started converging from its reference structure after 5 ns in

LMTK3 and attained the stable conformation after 15 ns with RMSD near 2.5 Å (in phosphorylated LMTK3), 3.5 Å (in unphosphorylated LMTK3) and 4 Å (mutated LMTK3) during the course of MD run**.** The lack of gross structural changes throughout the MD simulation confirmed that all the respective structures were stable. However RMSD of loop region (Gly184 to Ser196) in phosphorylated, unphosphorylated and mutated region is observed to be, 2.8Å, 2.5 Å and 1.4 Å respectively shown in **Figure 7b.3**. Phosphorylated loop region shows high RMSD value (2.8 Å) than the unphosphorylated and mutated loop region.

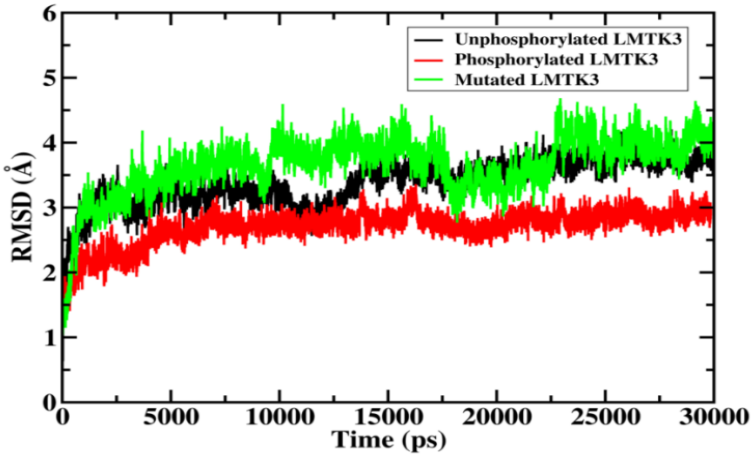

Figure 7b.2. *RMSD plot of Cα atom over time course of MD simulation for phosphorylated (red), unphosphorylated (black) and mutated (green) LMTK3 domain*

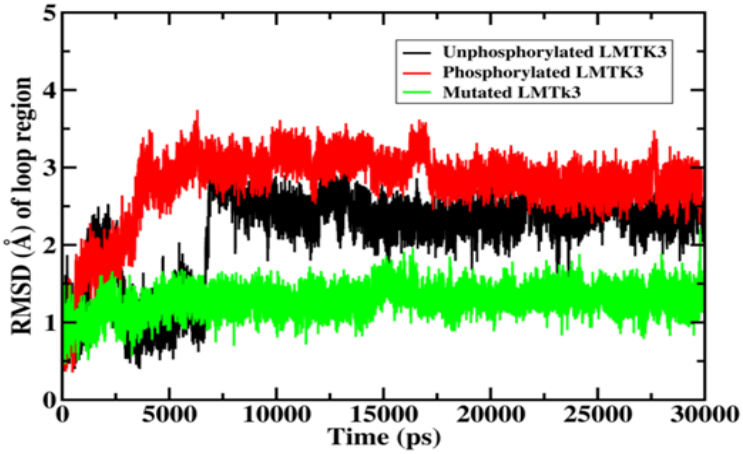

Figure 7b.3. *Loop region RMSD plot of Cα atom over time course of MD simulation for phosphorylated (red), unphosphorylated (black) and mutated (green) LMTK3 domain*

For the loop region, we also analysed the potential energy as a function of RMSD shown in **Figure 7b.4**. The Phosphorylated loop region initially change its conformation from its initial structure during the course of MD simulation from 1 Å to 3.5 Å with a potential energy of -7500 kcal/mol (**Figure 7b. 4 B**), however, the lowest energy conformers settles within RMSD value 1 to 1.5 Å with the potential energy of -10700 kcal/mol depicted in **Figure 7b. B**. Whereas unphosphorylated and mutated loop region does not show much conformational changes and shows the RMSD value ranges between 1 to 1.5 Å with a potential energy of -7500 kcal mol^{-1} (**Figure 7b. 4 A and C**) and the lowest energy conformers settles for both the structures around 1.5 Å with the same potential energy as that of phosphorylated loop region (-10700 kcal mol^{-1}). We see there is a less conformational change in unphosphorylated and mutated loop region from the initial structure throughout the MD simulation (**Figure 7b. A, C**).Whereas phosphorylated loop region undergoes much conformational changes from the initial structure throughout the MD simulation shown in **Figure 7b B**.

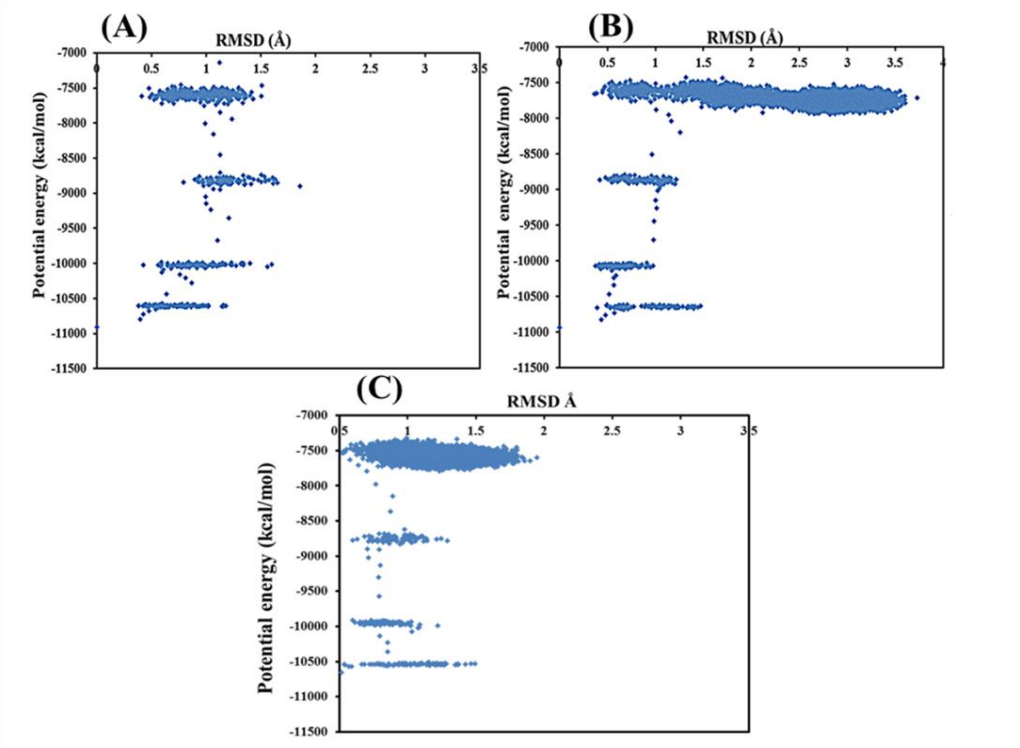

Figure 7b.4. *Potential energy as a function of RMSD for loop region of LMTK3 domain in (A) unphosphorylated, (B) phosphorylated (C) mutated LMTK3 domain*

In order to check the flexibility of phosphorylated-LMTK3 we analyse Root Mean Square Fluctuation (RMSF) for the C-α atoms and compared with unphosphorylated and mutated LMTK3. From the **Figure 7b.5**, we see there is a fluctuation near the phosphorylated residue (Thr-189) between residue indexes 150 to 200 with 3 high peaks (2.8Å, 3.5Å and 3.8Å), but there is less fluctuation is case of unphosphorylated LMTK3 region, and there is no fluctuation in the mutated LMTK3 near the mutated (Glu-189) region, but shows fluctuations far from the mutated region (200-250). From this observation we can say Glu does not mimic the effect of phosphorylation in terms of stability and fluctuation. The fluctuation for phosphorylated, unphosphorylated mutated residue for C-β atom observed to be 16 Å, 10 Å and 9 Å respectively throughout the MD simulation. Similarly we have also analysed the fluctuation in the loop region (Gly184 to Ser196) for C-β atom, the phosphorylated, unphosphorylated and mutated loop fluctuates from its initial position by 14.5 Å 11 Å and 10 Å respectively throughout the MD simulation. The fluctuations in the loop region depicted in **Figure 7b.5**.

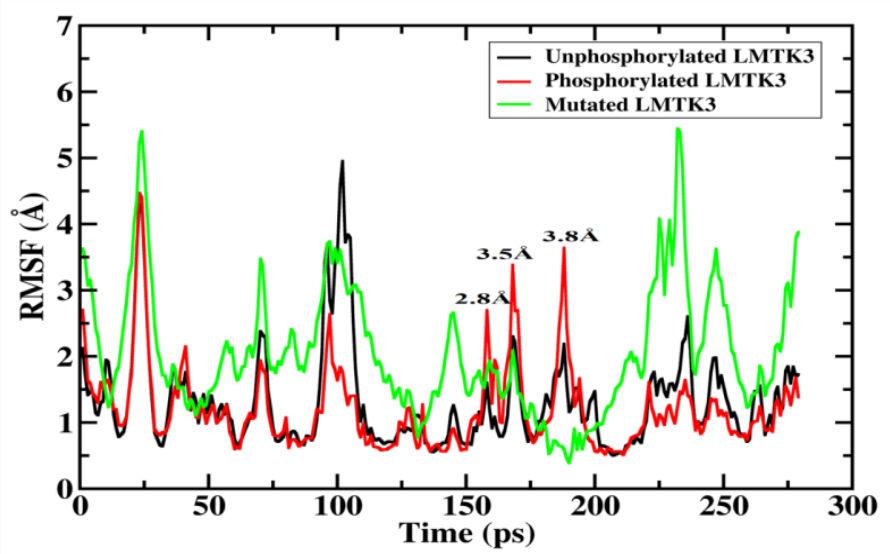

Figure 7b.5. *RMSF plot of Cα atom over time course of MD simulation for phosphorylated (red), unphosphorylated (black) LMTK3 and mutated (green) LMTK3 domain*

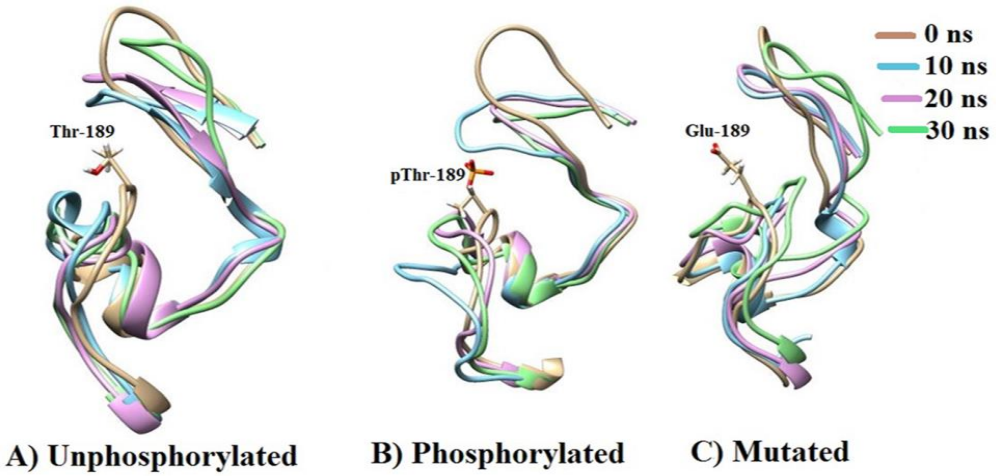

Figure 7b.6. *Flexibility and conformational change in activation segment of (A) unphosphorylated (B) phosphorylated and (C) mutated LMTK3 domain during the course of MD simulation.*

7b.4.2.2. Radius of gyration

In order to check the compactness of phosphorylated, unphosphorylated and mutated LMTK3 we performed radius of gyration (Rg) analysis. From **Figure 7b.7**, in all the three structures we see Rg value showed a drift between 19.7 Å and 18.7 Å and reached a stable conformation after 18.75 Å. We can infer proper folding of all the three protein structure. From the plot we can infer that there is not much degree of compactness in the phosphorylated LMTK3 during the course of MD simulation, thereby confirming the stability in phosphorylated LMTK3.

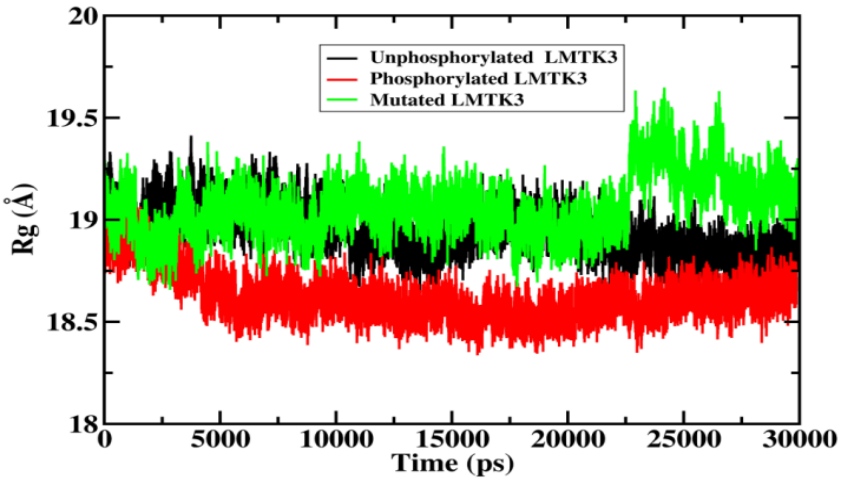

Figure 7b.7. *Radius of gyration as a function of time course of simulation for unphosphorylated, phosphorylated and mutated LMTK3 domain.*

7b.4.2.3. Energetics

From the potential energy plot (**Figure 7b.8**), we see the phosphorylated and unphosphorylated LMTK3 structure have a potential energy value of around -7900 kcal/mol and -7800 kcal/mol respectively while in case of mutated LMTK3, potential energy value is around -6600 kcal/mol during the course of MD simulation. We can say that the phosphorylated LMTK3 structure is more stable than the other structures.

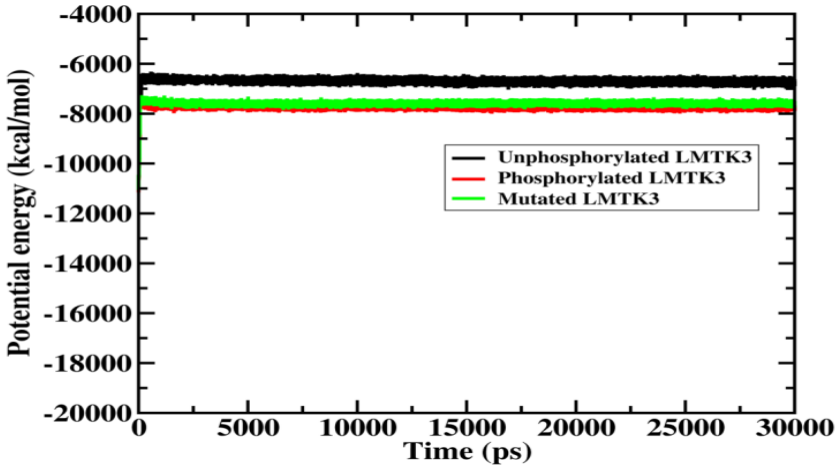

***Figure 7b.8**. Potential energy plot as a function of time for unphosphorylated (black), Phosphorylated (red) and mutated LMTK3 (green) domain*

For all the three systems, electrostatic interactions were analysed from the last trajectories as a function of time, electrostatic interactions for unphosphorylated LMTK3 found to oscillate around -18500 to -20500 kcal/mol whereas electrostatic interactions in phosphorylated and mutated LMTK3 were found to oscillate around -18400 to -20000 kcal/mol as a function of time **(Figure 7b.9**).

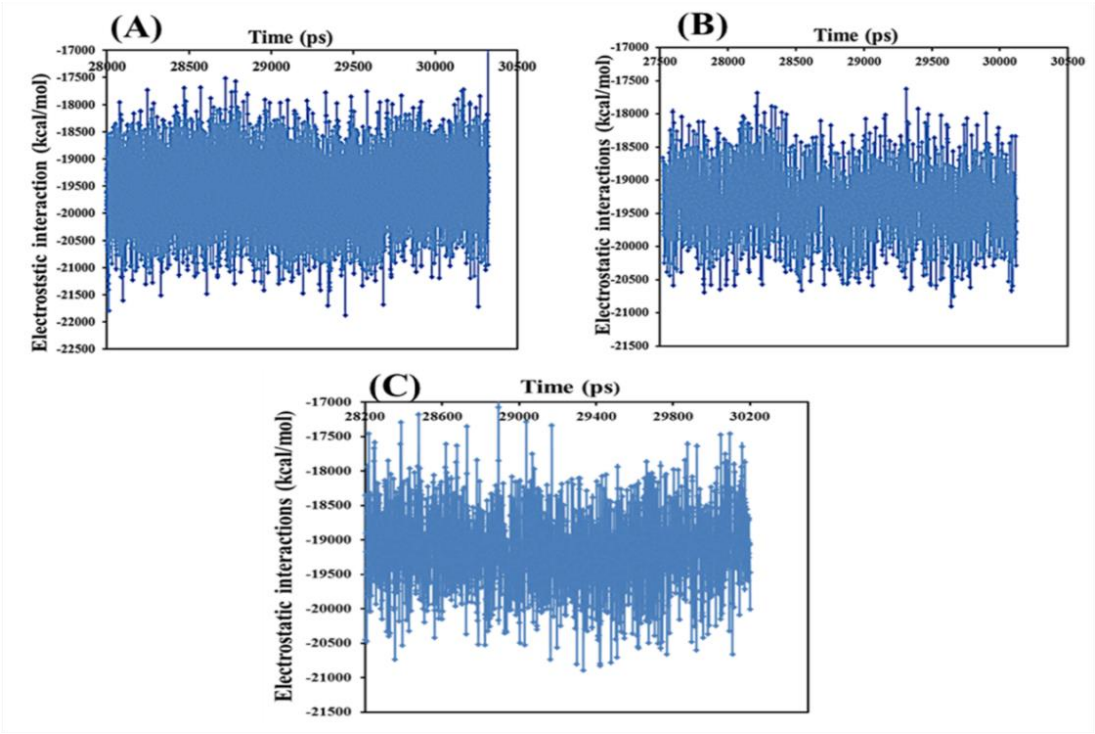

Figure 7b.9. *Electrostatic interaction for (A) unphosphorylated (B) phosphorylated and (C) mutated LMTK3 domain as a function of time.*

7b.5. Conclusion

In this computational study, we determined the probable activation segment in LMTK3. Then we studied the dynamics of LMTK3 upon phosphorylation and compared its dynamics with unphosphorylated and mutated LMTK3 structures. RMSD and potential energy analysis reveal that phosphorylated, unphosphorylated and mutated LMTK3 structures are stable during the MD simulation. Despite the stability of the structures, we see, mainly the phosphorylated activation segment undergoes much conformational changes as compared to unphosphorylated and mutated one. We also inferred that Glu(189)Thr mutation in phosphorylated LMTK3 does not mimic the effect of phosphorylation. The conformational change in the phosphorylated activation segment of LMTK3 was found to have significant impact on the ATP binding site. As a result the binding mode of ATP was found to be different in phosphorylated LMTK3 as compared to unphosphorylated LMTK3. The conformational change in ATP binding site may facilitate the catalysis of phosphoryl transfer reaction in LMTK3 to its

substrates and may lead to the breast cancer tumourogenesis. In this work, we have restricted our efforts in predicting relatively modest, localized conformational changes in phosphorylated LMTK3, we have determined the LMTK3 undergoes significant conformational change mainly those portions closest to the phosphorylated threonine-189. This modelling technology may thus be used to create hypotheses about mechanisms of regulation by phosphorylation in LMTK3 that can be further studied experimentally.