Serine/threonine kinase of human Monkeypox virus: computational modeling and structural analysis

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Article

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Abstract

Kinases catalyze phosphoryl transfer from a nucleoside triphosphate (usually ATP) to an amino acid residue on a protein (for activation purposes). These enzymes are well-appreciated drug targets against different viruses and cancers. However, some poxviruses are human and animal pathogens that lack effective therapeutic agents. In poxvirus, the production of infectious particles in the infected cells depends on F10 protein kinase that activates numerous proteins involved in the assembly of new virions. The ongoing outbreak of the human monkeypox virus (hMPXV) sparked the need for efficient antiviral drugs to control such outbreaks and lower their burden. In this work, we employed state-of-the-art computational resources to elucidate the structure of the major kinase in hMPXV using AlphaFold2. The predicted structure shows the atypical nature of this kinase; nonetheless, the overall structural fold is roughly conserved. Calculations of binding free energy determined the hotspot residues contributing to phosphate source (ATP) via Molecular Mechanics with Generalized Born and Surface Area solvation (MM/GBSA). The structural analysis in this work provides the basis for setting up a thorough experimental investigation to understand the enzymatic mechanism and development of small-molecule inhibitors against such a critical target.

Introduction

Human monkeypox virus (hMPXV) (genus: Orthopoxvirus, family Poxviridae) is a large, brick-shaped, double-stranded DNA virus encoding ~ 200 proteins, many of which are required for the viral replication in the cytoplasm, rather than in the nucleus as most DNA viruses\(^1,2\). It is a zoonotic agent without a known reservoir; however, it is found in monkeys, squirrels, and anteaters in Central and West African countries\(^3\). It has about 200 genes, half of which are well conserved among members of the Orthopoxvirus genus whilst the remaining genes are special for viral infectivity and host-virus interactions\(^4\). In addition to the Monkeypox virus, Orthopoxvirus includes other human pathogenic species such as the Cowpox virus, Buffalopox virus, Vaccinia virus, and the eradicated smallpox agent, Variola virus\(^1,2\). Structurally, the virions are exceptionally complex, where the envelop membrane contains tubular lipoprotein subunits that arrange randomly. The core contains lateral bodies and a dumbbell-shaped structure harboring the genome and numerous viral proteins\(^2\).

The ongoing outbreak of hMPXV is the first widespread transmission outside the endemic region in Africa. Until the 10th of November 2022, 79,231 confirmed cases and 49 deaths had been reported from 110 countries/territories\(^5\). The phylogenetic analysis of hMPXV revealed two clades with different virulent rates: West Africa and Congo Basin. It is believed that hMPXV has reached Europe and North America through infected travelers from African passengers\(^6\). The infection in humans can be asymptomatic or usually with symptoms similar to those of smallpox virus infections: vesiculo-pustular rash, fever, swollen glands, and toxemia. The incubation period ranges from 5–20 days, with a case fatality rate approaching 10% if untreated\(^3,7\). The epidemiology of hMPXV infections has changed over the past two decades, most likely influenced by the stop of routine smallpox vaccinations that culminated...
in the waning of herd immunity\textsuperscript{7,8}. Reported complications and secondary infections include pneumonia, sepsis, encephalitis, and severe eye damage\textsuperscript{7}.

Protein phosphorylation, catalyzed by kinases, is a universal paradigm of activating proteins and enzymes that play crucial roles in diverse biological processes, including signal transduction and cell cycle\textsuperscript{9}. The vast majority of known kinases are Serine/Threonine kinases (STKs) which were historically considered to be present in eukaryotic organisms only. However, studies in the last three decades have identified STKs in bacteria and viruses\textsuperscript{10–13}. In Orthopoxvirus, \textit{B1R} and \textit{F10L} genes encode 303 and 439 amino-acid kinases\textsuperscript{12}, and temperature-sensitive mutants with non-functional F10 or B1 are defective in the maturation or production of infectious virions\textsuperscript{14}. The maturation of poxviruses is a complex process that is heavily dependent on structural changes of different proteins that are still poorly understood. B1 (\textasciitilde 34 kDa) was first annotated as serine/threonine kinase (STK) in 1989\textsuperscript{15}. However, F10 kinase was later identified in 1994 as the major contributor to viral protein phosphorylation reactions\textsuperscript{16}. F10 is also an STK in all poxviruses\textsuperscript{12}. In the vaccinia virus, F10 had been characterized biochemically as a dual specificity kinase (DSK) that phosphorylates serine, threonine, and tyrosine\textsuperscript{17} and it is regulated by autophosphorylation\textsuperscript{18}. It is \textasciitilde 50 kDa essential protein for viral morphogenesis and production of infectious particles\textsuperscript{18}. Interestingly, these kinases share low sequence similarity with other STKs from other domains or even within the same domain of life\textsuperscript{12}. Until now, no crystal structure for a pox viral kinase is publicly available; therefore, this study was performed to elucidate and analyze the structure of the STK of hMPXV using a computational approach.

**Computational Methods**

**Sequence retrieval and structure prediction**

From NCBI databases, the amino acid sequences of the F10 protein for the Monkeypox virus (NP_536469.1), Vaccinia virus (YP_232931.1), Cowpox virus (NP_619845.1), and smallpox virus (NP_042078.1) were retrieved. A position-iterative BLAST search against the Protein Data Bank (PDB) did not retrieve any homologous solved structure for the hMPXV sequence; hence, ab initio secondary structure prediction was made via three software that makes predictions via deep learning approach; AlphaFold2\textsuperscript{19,20}, RoseTTAfold\textsuperscript{21}, and trRosetta\textsuperscript{22}. In AlphaFold2 modeling, energy minimization using restrained gradient descent and Amber ff99SB force field\textsuperscript{23} was used to relax the predicted models, whereas RoseTTAfold and trRosetta are fully automated servers. The best model for each sequence was selected for quality assessment by the MolProbity server\textsuperscript{24}. Multiple sequence alignments of serine/threonine kinases from viruses, bacteria, fungi, and mammals were performed via Clustal\Omega\textsuperscript{25} to determine the catalytic domain and other conserved motifs.

**Biochemical properties**
Several biochemical characteristics of the STK enzyme were predicted using the ProtParam web interface\textsuperscript{26} and validated by the PepCalc server\textsuperscript{27}. The features include stability index, isoelectric point, Grand Average of Hydropathy (GRAVY), and AA composition, among others. The composition information of the secondary structure of STK was predicted via NetSurfP – 3.0\textsuperscript{28} and iStable 2\textsuperscript{29} webservers. Features were calculated, including the percentage of α-helix, β-sheet, and coils, in addition to surface accessibility and relative surface accessibility.

**Active site prediction**

Multiple tools were employed to identify the binding pocket residues of the enzyme, namely, CASTp 3\textsuperscript{30}, fPocket\textsuperscript{31}, DoGSiteScorer\textsuperscript{32}, and PrankWeb\textsuperscript{33}. Among them, PrankWeb was the best in comparison with the other tools as it revealed 11 mutual AA residues (Ile93, Ser94, Thr95, Gly96, Gly97, Tyr98, Gly99, Val101, Val108, Lys110, and Glu127) with those obtained from CB-Dock 2 server\textsuperscript{34} (utilize AutoDock Vina algorithm) upon docking of adenosine triphosphate (ATP).

**Molecular dynamics simulation**

The model of Monkeypox kinase was subjected to all-atom molecular dynamics simulation by GROMACS.2021.3 and the CHARMM36 forcefield\textsuperscript{35}. The model was solvated in a triclinic box with periodic boundary conditions, and box boundaries were at least 1 nm from the nearest protein atom. The TIP3P water model was used, and the system was neutralized by adding Na\textsuperscript{+} Cl\textsuperscript{−} ions at the physiological concentration (0.15 M). Energy minimization of the system was carried out using the steepest descent algorithm for a maximum of 15,000 steps so that no atom had a force >1000 kJ/mol/nm. The equilibrium was performed in two steps. First, the NVT canonical ensemble was adopted for two nanoseconds, where the Berendsen algorithm controlled the temperature at 310 K\textsuperscript{36}. The second step was applying the isothermal-isobaric NPT ensemble for another 2 ns. The temperature was controlled by a velocity-rescaling thermostat\textsuperscript{37} at the same degree (310 K), while the pressure was controlled via a Parrinello-Rahman isotropic barostat at 1 bar\textsuperscript{38}. Position restraints were applied to all bonds containing hydrogen atoms during the equilibration steps. For the production, position restraints were removed, and Leapfrog integrator was applied for 100 ns using the conditions of the last equilibrium step. Long-range electrostatic interactions were evaluated by the Particle Mesh Ewald method with a cutoff of 10 Å. The trajectory was analyzed using the built-in modules in GROMACS. Finally, the clustering of the frames was done with TTClust based on the Ca atoms using the Wald method\textsuperscript{39}. All cartoon representations were created by UCSF ChimeraX\textsuperscript{40}.

**Results And Discussion**

**Biochemical properties**

The STK was predicted as neutral based on the isoelectric point and the almost equal number of positively and negatively charged residues (51 versus 52, respectively). The enzyme is predicted to be
stable since its high stability index, and the estimated half-life for proteolysis of such protein in *E. coli* was long (Table 1). The pI and molecular weight predictions are consistent with the biochemical analysis of vaccinia virus STK that shares > 95% sequence identity with hMPXV. The composition information of the secondary structure of STK was predicted via NetSurfP – 3.0 and iStable 2 webservers. Features, including the percentage of α-helix, β-sheet, and coils, in addition to surface accessibility as well as relative surface accessibility, were calculated. According to NetSurfP – 3.0 and iStable 2, 57% of the protein was predicted to be buried, while the remaining 43% was exposed (Figure S1). In addition, the relative surface accessibility diagram validates the previous results, as most of the peaks had values less than 1.

Concerning secondary structure composition, 44% of the residues are within coils, while α-helices harbor 42% of the amino acids. The remaining amino acids (14%) are expected to be in β-sheets. Although it represented the lowest percentage, β-strands were expected to be part of the active site since it lies between the two lobes. However, except for the glycine-rich loop (a.k.a P loop) (discussed below), no conserved motifs can be detected by multiple sequence alignment of known STKs orthologs along with the hMPXV STK (Figure S2). Indeed, the sequence identity drops to ~ 15% between hMPXV F10 and its orthologs in other genera within the *Poxviridae*.

Table 1

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (Da)</td>
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</tr>
<tr>
<td>pI</td>
<td>7.63</td>
</tr>
<tr>
<td>Positive residues</td>
<td>51</td>
</tr>
<tr>
<td>Negative residues</td>
<td>52</td>
</tr>
<tr>
<td>Estimated half-life</td>
<td>&gt; 10 hours</td>
</tr>
<tr>
<td>Instability index</td>
<td>32 (stable)</td>
</tr>
<tr>
<td>Aliphatic index</td>
<td>93.69</td>
</tr>
<tr>
<td>GRAVY&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.151</td>
</tr>
</tbody>
</table>

<sup>a</sup> isoelectric point, <sup>b</sup> GRAVY: grand average of hydropathy

**Model quality and overall fold**

The model obtained from AlphaFold2 has an excellent quality according to the Ramachandran plot (Figure S3) and physics-based evaluation tests implemented in MolProbity (Table S1) with an overall score of 1.39 (97th percentile). The MolProbity is an overall computational score equivalent to the x-ray resolution of experimentally solved models. Furthermore, according to ERRAT and VERIFY
assessments, the model has an overall quality factor of 96.1, and 94.34% of the residues have an averaged 3D-1D score ≥ 0.2.

Despite the low sequence identity with known kinases, the hMPXV STK was predicted to have the common structural features, namely, two lobes with a cleft in-between and the glycine-rich loop at the substrate pocket. The STK was predicted in the apparently “open” conformation as a typical kinase with two lobes. Unlike other known STKs from bacteria and eukaryotes, the modeled kinase of hMPXV has additional helices and β-sheets in both lobes. The N-lobe has a stretch of ~25 residues that extend onto the C-lobe to form two β-helices (5 and 6 in Fig. 1) that behave as a structural part of the C-lobe. The N-lobe has seven helices and six anti-parallel β-sheets, whereas the C-lobe consists of 5 helices and four β-sheets. The first 20 residues of the N-lobe comprise a disordered arm, which may be a regulatory sequence for intracellular localization.

The biochemical studies on vaccinia virus F10 kinase suggested a membrane association of STK with the internal vesicles, membranous fragments derived from the Endoplasmic reticulum (ER), and the ER-Golgi intermediate compartment (ERGIC) at the assembly location of poxviruses. The same study also reported that deletion of the first 21, 69, or 91 residues from the N-terminal abolished the biological function in vitro as well as the enzymatic activity of the STK. It is still unclear whether these deletions induced improper folding or blockage of the association with the lipid membranes.

The active site pocket

Multiple computational tools were employed to identify binding pocket residues of the enzyme, namely, CASTp, fPocket, DoGSiteScorer, and PrankWeb. Among them, PrankWeb was the best in comparison with the other tools as it revealed 11 mutual AA residues (Ile93, Ser94, Thr95, Gly96, Gly97, Tyr98, Gly99, Val101, Val108, Lys110, and Glu127) with those obtained from CB-Dock 2 server (utilize AutoDock Vina) upon docking of adenosine triphosphate (ATP). The pocket volume (solvent accessible) was calculated to be 1717.3 Å³ by CASTp.

From the initial visual analysis, the groove between lobes was expected to be the pocket for substrate binding. Molecular docking of ATP (by GNINA 1.0) has predicted a reasonable binding pose in which the purine ring has an H-bond with Asn342, while the pentose ring oxygen is interacting via another two hydrogen bonds with Ser94 and Ser259 (Fig. 2). Similarly, the terminal gamma phosphate has an H-bond with the amino group of Gly96 at the glycine-rich loop. However, these interactions were slightly modified after 100ns of MD simulation (discussed below). The glycine-rich-loop (a.k.a. P-loop) is a stretch of 7–10 residues interacting with the ATP to facilitate phosphoryl transfer to the other peptide substrate during catalysis. The consensus sequence for non-viral kinases is GX₁GX₂ΦGX₃V, where X is any residue and Φ is a hydrophobic residue (tyrosine or phenylalanine). The glycine-rich loop consensus sequence in poxvirus is STGGYGIV.
In nearly all known kinases, the active form is marked by a salt bridge between two conserved residues, lysine and glutamate, near the active site\textsuperscript{43–47}. The Lysine residue is located in a β-sheet near the glycine-rich loop, whereas the glutamate is part of the activation helix. In the model of hMPXV, the glutamate (E127) is projecting from a helix in the C-lobe (α13 in Fig. 1), while lysine (Lys110) is at a similar location as seen in eukaryotic and prokaryotic kinases, and the two residues were well oriented to form salt bridge even in the absence of the ATP.

The real function of this pair in hMPXV kinase can be deciphered by the construction of mutant STK. The catalytic loop (His305 - Asn312) comprises a short stretch of charged residues that interact with the phosphate moiety and Mg\textsuperscript{2+} ions. The orientation and folding of the catalytic loop are highly similar to the ATP-bound eukaryotic MEK1 structure\textsuperscript{48}. The Asp311 in the catalytic loop is expected to facilitate the nucleophilic attack of the oxygen on the ATP gamma phosphate group. The active conformation of MEK1/2 dual kinases is characterized by the formation of the salt bridge between the K-E conserved pair; however, the salt bridge in STK of hMPXV is formed and sustained irrespective of ATP binding to its pocket.

**MD simulation**

The apo model shows good stability over the simulation period (100 ns) without experiencing unfolding events. The presence of ATP in the active site enhanced STK model stability. The deviations of backbone atoms from the initial structure were limited, roughly within 1 Å in both systems (Fig. 3A). However, the backbone deviations of the ATP-bound system were milder and fastly equilibrated. The backbone-based clustering of the ATP-bound trajectory showed that all frames after 20ns are grouped into three clusters with an RMSD difference of 1.54 Å between their representatives (Figure S4).

The stability of the model was also inferred by the calculations of the radius of gyration, the solvent-accessible surface area (SASA), and the total number of H-bonds (Figs. 3B, 3C, and 3D). In the presence of the ATP, the average radius of gyration decreased slightly as a response to the transition from open conformation to the closed (or partially closed) state in which the ATP becomes surrounded by residues and loops originally positioned away from the active site in the apo conformation. The radius of gyration fluctuates within 2Å for the Apo and 1Å for ATP-bound owing to the restriction of N lobe movement by the presence of the ATP. Similarly, the SASA decreased in the ATP-bound system because the presence of ATP occupied an area accessible to solvent molecules in the apo system.

The all-atoms residue motions during the simulation were calculated by the root-mean-square fluctuation (RMSF). The most fluctuating residues were those in disordered loops between α-helices or β-sheets. This is normal behavior as the residues are highly affected by the motion of the solvent. ATP binding was also stable, as evident by the little changes in its RMSD compared to its initial binding pose predicted by molecular docking.

The stabilized binding pose for ATP shows that the purine ring is buried into the hydrophobic region and stabilized by two hydrogen bonds linking the purine ring to the carboxyl amide group of Pro245 (Fig. 4).
In addition, the ribose ring has another H-bond contact with Ser94, whereas the triphosphate end established four H-bonds with Lys309, Lys110, and Asp343 (two bonds). The later interactions are expected to be stabilized for subsequent phosphoryl transfer to serine or threonine residue on the substrate peptide. Additional hydrogen bonds were also seen between the phosphate moiety and water molecules coordinating the Mg$^{2+}$ (Fig. 5). The phosphate moiety was stabilized via coordinating Mg$^{2+}$ ions with Asp311, Asn312, Asp343, and possibly Asp345. Hence, the DFD (343–345) in hMPXV is believed to be the equivalent to the conserved DFG motif in eukaryotic and bacterial kinases.

The segment (Ser347 to His365) after the DFD motif containing a short helix is expected to be the activation loop. In S. aureus PknB kinase (PDB: 4EQM), this loop occludes the formation of the conserved salt bridge between K39-E58 in the active form by displacing the activation helix away from the ATP-binding site; thus, it regulates the transition to the active state. In contrast, the corresponding loop in hMPXV kinase is away from the E127 residue; therefore, it is expected to modulate the peptide substrate for receiving the phosphate group. Similarly, another short loop near the ATP pocket (Ala115-Thr126) may play a crucial role in phosphate transfer to the protein substrate. Indeed, serine and threonine residues on each loop are close to each other and to the phosphate moiety of the ATP pocket. Both loops seem responsible for phosphate transfer reaction to the protein substrate.

MD simulation predicted octahedral coordination of Mg$^{2+}$ ions by D311, N312, D343, and water molecules (Fig. 5). In most kinases, the purine ring is stacked by a phenylalanine residue at the active site. However, during the simulation, the nearest phenylalanine residue (F253) sustained its orientation. Only the first aspartate residue of the DFD motif was involved in interactions with ATP or the divalent ion (Mg$^{2+}$). The perpendicular orientation of the H305 side chain traps the movement of the following phenylalanine (F344) sidechain ring. On the other hand, the D345 side chain is always in a salt bridge interaction (2.77 ± 0.2 Å) with the terminal amino group of the K356 side chain (Figure S5).

A decomposition scheme was followed to calculate the binding free energy via MM/GBSA for the last 50 ns of the simulation trajectory to evaluate the energy contributions of different residues at the active site. The most contributing residues are graphically summarized in Fig. 6. Residues involved in hydrogen bonding with the ATP have an average binding free energy of −3.36 kcal/mol. These results are helpful for the identification or de novo design of inhibitors against hMPXV.

**Conformational changes**

Proteins are dynamic macromolecules whose subtle conformational changes (native ensemble) can be sampled by unbiased atomistic molecular dynamics simulations. The native ensemble was explored in the two simulation runs (Apo & ATP-bound). The distance analyses between certain residues showed that both lobes of the Apo form structure had experienced large-scale motions (Fig. 7A-C). The motion of the N-lobe was reduced when complexed with ATP, whereas the C-lobe retained roughly the same translocation moment pattern in both systems (Apo versus ATP-bound). The motion of lobes is facilitated by three disordered loops (A115-S120, I146-T150, and L255-S295) (Fig. 7C).
Interestingly, the lateral motion of the C-lobe was sustained in both states (apo & ATP-bound). This motion is likely due to the availability of additional conformational space to be occupied when interacting with the peptide substrate(s). The most changes were seen in the residues corresponding to the activation segment (dashed circle in Fig. 7A) that translated towards the phosphate in the ATP-bound state (Fig. 7B). This observation supports the functional role of these residues as the activation segment (via autophosphorylation) or it may play roles in phosphoryl transfer reaction to other protein substrates, most likely via S347 residue following the DFD motif. Despite the larger changes experienced by the triphosphate moiety during the simulation, the glycine-rich loop showed limited conformational changes (Fig. 7D).

The distance/length of the salt bridge between K110 and E127 in the apo and the ATP-bound is comparable and seems not to be affected by ATP absence (Fig. 7E). In the ATP-bound model, the abrupt increase in the distance at 30-35ns was due to the temporary deviation of the K110 side chain towards the phosphates of ATP to establish temporary hydrogen bonds. However, in the apo system, a transient hydrogen bond was formed between K110 and D343 in the DFD motif.

**Conclusion**

Our structural study elucidates the secondary structure of hMPXV STK and provides a basis for understanding the enzymatic mechanism and the design of potential antiviral drugs against hMPXV. Future work will aim to screen potential small-molecule inhibitors that establish stable interactions with the identified hotspot residues at the ATP-binding pocket. In addition, the structural details presented in this study may serve as a basis for further investigations of the molecular mechanism and kinetics of such atypical kinases.

**Declarations**

**Data availability statement**

The results of modeling via AlphaFold2, RosetTAfold, and trRosetta are available at https://github.com/Jameel9/STK_hMPXV_paper. Other data are available upon request.

**Acknowledgments**

We thank Bibliotheca Alexandrina for providing access to their High-Performance Computing facility.

**Conflict of interest**

There are no conflicts of interest regarding this paper or its publication.

**Author contribution**
JMA conceptualized and designed the study, performed modeling and calculations, analyzed the data, and wrote the manuscript. HAA contributed to the first draft’s design, biochemical modeling, and writing. AAE & MMA supervised the study and contributed to the proofreading of the final manuscript.

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Data availability

Data are available on the following link: https://doi.org/10.5281/zenodo.7380089

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**Figures**

![Figure 1](image)

**Figure 1**

*Structural architecture of the STK of hMPXV.* The left panel shows lobes of STK in different colors, along with the secondary structure’s succession. A dashed-black line shows the removed disordered 20 residues at the N-terminal. The residues shown in the ball-stick representation in the right panel are the conserved lysine-glutamate pair next to the active site in nearly all known kinases.
Figure 2

**Predicted binding pose and interactions of ATP in the active site.** Green spheres are Mg$^{2+}$ ions. The predicted interactions of ATP (yellow sticks) are presented in different colors: Red, hydrogen bonds, gray; hydrophobic interactions, orange; salt bridges. The glycine-rich-loop is shown in magenta.

Figure 3
Stability analysis after 100ns of MD simulation for apo (blue) and ATP-bound (red) systems. A) RMSD of backbone atoms compared to the initial structure. B) Radius of gyration of the model. C) Solvent accessible surface area (nm²) over the simulation duration. D) the total number of the formed Hydrogen bonds in the systems.

Figure 4

Residues and ATP fluctuation analysis over the simulation period. A) Cartoon representation showing the most flexible regions that showed elevated RMSF seen in B. C) RMSD of ATP in comparison to the predicted binding pose by GNINA. D) The number of hydrogen bonds established between ATP and the protein during the simulation time.
**Figure 5**

*ATP binding pose after 100 ns of MD simulation.* Red spheres are water molecules participating in hydrogen bonds (red dash lines) and/or coordination of Mg$^{2+}$ (green spheres). Important loops are colored differently; the DFD motif is in blue, the catalytic loop in magenta, and the glycine-rich loop in yellow.

**Figure 6**

*MM/GBSA results of ATP binding to the kinase.* **A)** The contributions of important residues (with ΔG < −2 kcal/mol) at the ATP binding site. **B)** Surface rendering of the kinase showing the spatial distribution of hotspot residues that established contacts with ATP (yellow sticks) over the simulation period.
Figure 7

**Conformational analysis.** **A)** and **B)** Conformational space visited by the apo and ATP-bound forms of the kinase, respectively. **C)** and **D)** Distance analysis between selected residues in both conformations. **E)** Distance between K110-E127 (salt bridge) over the simulation period.

**Supplementary Files**

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- Supplementarymaterial.docx