UC Riverside
UCR Honors Capstones 2016-2017

Title
Changes in the Activation Loop: A Molecular Dynamics Study on the Effects of Phosphorylation on Monomeric CDK2

Permalink
https://escholarship.org/uc/item/01v3q3p4

Author
Madrigal, Jennifer Asenath

Publication Date
2017-12-08

eScholarship.org Powered by the California Digital Library
University of California

CHANGES IN THE ACTIVATION LOOP: A MOLECULAR DYNAMICS STUDY ON THE EFFECTS OF PHOSPHORYLATION ON FREE MONOMERIC CDK2

By

Jennifer Asenath Madrigal

A capstone project submitted for
Graduation with University Honors

May 18, 2017

University Honors
University of California, Riverside

APPROVED

____
Dr. Chia-en A. Chang
Department of Chemistry

____
Dr. Richard Cardullo, Howard H Hays Jr. Chair and Faculty Director, University Honors
Interim Vice Provost, Undergraduate Education

Abstract

Dysregulation of Cyclin Dependent Kinase 2 has been implicated in several forms of cancer. Of particular medical importance in molecular dynamics studies of CDK2 is elucidating the ways in which drug ligands can bind and inhibit the molecule most effectively. This study uses MD simulations to analyze differences in dynamics between phosphorylated and unphosphorylated monomeric CDK2, with the aim of providing additional information for improvement in CDK2 inhibitory drug design.

Among factors involved in CDK2 regulation include activation via phosphorylation of residue T160. This results in a more disordered activation loop, which loop includes the kinase-conserved DFG-motif. The DFG-in conformation allows ATP to bind and activate the kinase, while the DFG-out conformation is inactive. Inhibitors which bind in the inactive DFG-out conformation may be associated with slower off-rates than active-conformation binders, making dynamics of the DFG-loop an important factor in drug discovery.

Previous MD simulations involving CDK2 have used only the unphosphorylated monomeric form, assuming a linear activation process wherein cyclin binds CDK2 before T160 is phosphorylated. However, phosphorylation of T160 in monomeric CDK2 has been confirmed, meaning that a significant chemical species of the molecule has yet to be analyzed *in silico*, and phosphorylated T160 in the CDK2 monomer may result in a significant difference in protein dynamics and affinities for the free and bound states of various drug ligands. Here differences in protein dynamics are analyzed between phosphorylation states of T160 in monomeric CDK2, with an emphasis on the effects on the activation loop and DFG conformations.

Acknowledgements

Thanks are necessary to my PI for being an understanding and patient mentor, always willing to make time for me, answer my questions, and give input regarding my concerns. I must also thank all of my lab members for always being available to explain a process or theory, figure out correct parameterizations, troubleshoot my script-writing attempts, or tease out the flaws in my methodology.

Thanks to my lab members for always being available to explain a process or theory or to help parameterize correctly, troubleshoot a script, or tease out the flaws in my methodology. My time in lab has been a learning experience, and they were always patient as I came to know which resources to use for the field, how to better organize myself to be the most productive, et cetera.

Thanks to the weekly speakers in our joint group meeting laboratories for allowing me to assimilate the plethora of information and jargon needed to articulate and understand the field of computational chemistry. Over the course of two years these meetings have given me a wide appreciation for different aspects of computational chemistry and its applications. These examples of the scientific process have exposed me to a wide range of computational and analytical methods and ways of understanding that will much better prepare me as I learn to think like a scientist in graduate school.

The STEM Pathway (AKA STEM Connections) Program at UCR has been an invaluable support to me as a low-income transfer student-parent. Without the program and its administrators Marissa Carranza and Nhi Tran it is likely that I would not have succeeded as an undergraduate, much less in a laboratory, in Honors, or in my desire to

go to graduate school for a PhD. I have gone through many difficult times, and when I had no one else, I had my STEM family.

También doy gracias a mi esposo por estar a mi lado y para animarme durante este largo tiempo en el que gastamos tanto esfuerzo, dinero y trabajo para que yo pueda seguir mi pasión de estudiar las ciencias. Sin tu ayuda y sacrificios no habría sido posible hacer todo el trabajo ni la investigación científica ni desarrollo personal que he recibido. Aunque fue laborioso y a veces nos equivocamos en algunos aspectos, hemos llegado a la meta y estamos listos para seguir adelante con la familia rumbo a nuevas aventuras.

Table of Contents

Abstract	ii
Acknowledgements	iii
Introduction	1
Methodology	2
Analytical Discussion	4
Conclusion	6
Citations	7

Introduction

CDK2 is a well-studied kinase[9], requiring a fully-active state conformation to allow ATP binding and phosphorylation of target peptide sequences[2]. Fully-active conformational changes are dependent on both CDK2 binding to cyclins (A, E) and

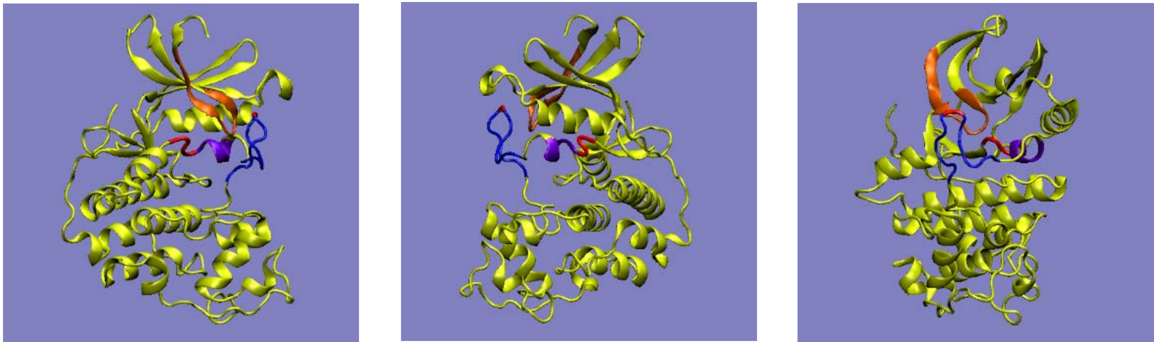

Figure 1:CDK2 Monomer shown rotated on the z-axis

phosphorylation of residue T160 by the kinase CAK[10]. Changes between active and inactive states require large conformational changes in several different areas, including the T-loop, DFG-loop, αC helix, and a glycine-rich loop[3,10].

Phosphorylation of residue T160 results in a more disordered activation loop, which includes the almost universally kinase-conserved 3-residue DFG-motif[5,7,8]. The added entropy of the phosphorylated T160 in the activation loop may provide a large enough change in free energy to flip the DFG-loop from DFG-in to DFG-out[4]. While the DFG-out conformation is inactive, the DFG-in conformation allows ATP to bind and activate the kinase. Type II inhibitors (which bind in the inactive DFG-out conformation) may be associated with slower off-rates than active-conformation binders[1], wherein a slower off-rate means a more effective inhibitory drug. Therefore, studies of dynamics

and conformations of the DFG-loop region are important in the discovery of new Type II inhibitors[11].

Previous MD simulations involving monomeric CDK2 have used only the unphosphorylated form, assuming a linear activation process wherein cyclin binds CDK2 before T160 is phosphorylated[3,6,12,13]. However, phosphorylation of T160 in *monomeric* CDK2 has been confirmed in vitro[4], meaning that a relevant chemical species of the molecule has yet to be analyzed in silico, and phosphorylated T160 in the CDK2 monomer may result in a significant difference in protein dynamics and affinities for the free and bound states of various drug ligands. Here differences in protein dynamics are to be analyzed between phosphorylation states of T160 in monomeric CDK2, with an emphasis on the effects on the activation loop and DFG conformation.

Methodology

MD simulations were performed using Amber14 with the Amber 1499SB force field for the protein and a one-off T2P force field for the phosphorylated threonine 160 (using a 2^- charge model). Protonation states for amino acid residues were checked using the MCCE program, and were assigned based on a pH of 7.4. Energy minimization was performed for hydrogen atoms, protein side chains, and the whole system for 500, 2,500, and 2,500 steps, respectively. Periodic boundary conditions were assumed and the particle mesh Ewald method was used to consider long-range electrostatic interactions.

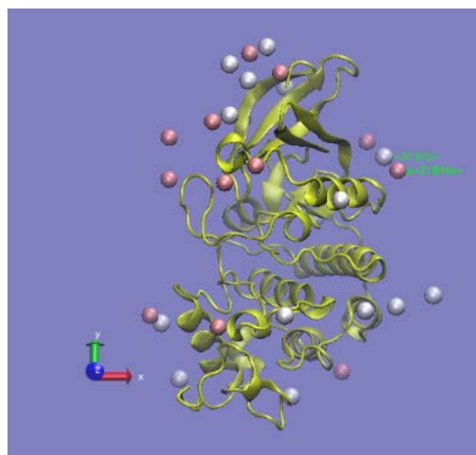

Figure 2: Apart from the usage of standard counter ions, additional salt was added to bring up the concentration to 40 mM.

The system was then solvated in a rectangular box of 10 Å with an explicit TIP3P water model by the tleap program in the Amber14 package for a total box size of 77 Å x 94 Å x 86.5 Å (626,000 Å^3 total for the pT160 system before equilibration). Grid placement of Na^+ and Cl^- was done to give an ionic concentration of 40mmol/L. Two additional counter ions of Cl^- were then added based on the Coulombic potential to keep the whole system neutral (reduced to charge 0.000001).

Before equilibration, energy minimization was run for the solvent waters and ions, keeping the protein and crystal waters restrained. Then a standard water minimization was done, first keeping the protein backbone restrained for 1,000 steps and then allowing a full-system minimization for 5,000 steps.

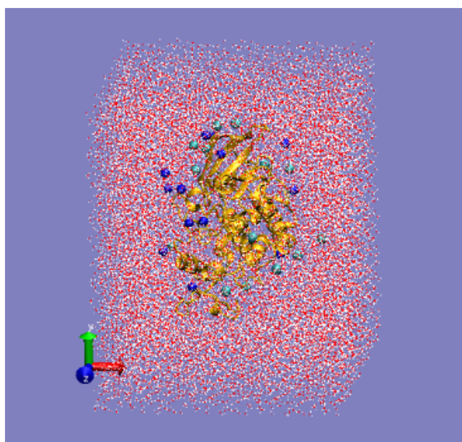

Figure 3: Energy minimization was run for solvent waters and ions with restrained protein and crystal waters. A full water minimization followed, along with a full-system minimization of 5,000 steps before the production run.

A short NVT (isothermic-isochoric) equilibration was performed for 10 ps with the protein fixed to stabilize the system before subsequent NPT (isothermic−isobaric) equilibration for 100 ps. The system was then gradually heated at NPT from 200-250K, 250-275K, and 275-300K for 20 ps per step. The temperature was then raised to 310K and allowed to equilibrate an additional 190 ps (a total of 360 ps for equilibration).

Production runs have been performed for 100 ns at 310 K for both the phosphorylated and unphosphorylated models. Resulting trajectories were collected every 1 ps with a time step of 2 fs at NPT. Langevin dynamics were used with a collision frequency of 2 ps^{-1} to maintain a temperature of 310K, and the SHAKE procedure was

used to constrain hydrogen atoms during MD simulations in order to optimize computational time.

Analytical Discussion

A thorough check was done on the equilibration and on the first 50 ns of production of both the T160 and pT160 systems to ensure no gross human or computational error. The 100 bar RMSF for pressure on the system was initially alarming (pT160 110.4 bar, T160 120.2 bar), but upon further inspection this fluctuation falls within the normal range for the NPT ensemble, and is not equivalent to a 100-bar fluctuation that would indeed be alarming in a large-scale macroscopic system. The difference in average pressure during equilibration was a factor higher for the phosphorylated system, at 27.3 bar vs. 2.8 bar for the non-phosphorylated system. This could have been concerning, however, during production the average smoothed out to -0.4 bar for the pT160 system and +0.4 for the T160 protein. All four average volumes remained very constant throughout the equilibration and production runs at 500,000 Å^3 with an RMSF of 505 Å^3. Interestingly, there was a difference in AMBER's calculated total energy between the two systems with an additional 1,000 kcal of energy calculated for the non-phosphorylated system.

The fitted regions were examined to confirm that the missing crystal-structure residues were fitted correctly. Preliminary analysis of the first 10 ns of the pT160 system indicate that there may be some strain in the region between residues 27-39 (large carbon RMSD) and residues 35-44 (elevated backbone RMSD). This is the location where the crystal structure coordinates were fitted with PDB 4FKV, a ligand-bound PDB (residues 37 to 40 were fitted from 4FKV and they have an RMSD of 55.157Å). However, after

analysis of the T160 system there are indications that this may not be due to an artifact of the residue fitting and could be due to the phosphorylation of the threonine 160 residue, as the T160 37-40 segment has less than half the RMSD at 22.141 Å. This large RMSD for both systems would be alarming were it not for the knowledge that this activation loop is known to be more disordered when it is not bound to a cyclin.

It is interesting to note that while there is a greater disorder in the pT160 residue 37-40 segment over the same T160 segment, the pT160 overall backbone RMSD is 1.46 Å while the T160 RMSD is larger at 2.48 Å. This corresponds well with the unphosphorylated protein's additional 1,000 kcal of energy. Future analysis may delve into differences in individual residues or segments to determine if the overall protein is more disordered or if this additional disorder is due to differences in specific segments of the protein. MMPBSA energy calculations will also be useful in divining whether or not the entire protein is higher in energy or if this energy jump is due to specific high energy regions with an unphosphorylated T160.

No hydrogen bonds were found associated with the pT160 residue over the first 50 ns (with frames pulled every 500 steps), nor with the D145 residue in the DFG loop. However, the L12 helix adjacent to the T-loop (in the ATP-binding site) had an equilibrium between 0 and 1 unique H-bonds, with 4 instances of 2 H-bonds forming within the first 400 frames. Also in the PSTAIRE/alpha-C helix (residues 45-51) there was an equilibrium between 0 and 1 unique H-bonds, with several instances of 2 H-bonds forming and 4 instances of 3 H-bonds forming. The exact residues participating in the hydrogen bonding have not yet been analyzed.

Conclusion

There is much more analysis to be done on the production runs. This should include a thorough visual check to determine which residues and motifs to do analysis on (in addition to analysis of the G-rich loop, PSTAIRE helix/alpha-C helix, and the 4-residue hydrophobic spine, which are all implicated in movement from an inactive to active form of the enzyme). Further H-bonding analysis will be done to look for changes in the hydrogen bonding of amino acid side chains or backbones between the pT160 and T160 systems, and additional analysis will be done on RMSD/RMSF for important residues/motifs between the two systems.

Principle Component Analysis must be performed to plot histograms looking at protein side chains from residues interacting with the 160 residue in both runs. Plotting of backbone phi/psi angles is necessary, especially in the DFG region, to see if phosphorylation changes rigidity or preferred conformation of the protein backbone. MMPBSA energy calculations need to be completed to attempt to explain differences in dynamics via h-bonding, Van der Waals interactions, electrostatics, entropy differences, and dihedral energies.

After additional work into the analysis of the effects of phosphorylation, a clearer picture will emerge regarding how and where phosphorylation might affect the monomer. Additional investigation could then be done in the future such as running a greater time length of simulation or enhanced MD to look for DFG-flipping, or running simulations with inclusion of a magnesium ion, ATP ligand, or drug ligands.

Citations

1. Alexander, L. T.; Möbitz, H.; Drueckes, P.; Savitsky, P.; Fedorov, O.; Elkins, J. M.; Deane, C. M.; Cowan-Jacob, S. W.; Knapp, S., Type II Inhibitors Targeting CDK2. *ACS Chemical Biology* **2015**.

2. Andzelm, E. R.; Lew, J.; Taylor, S., Bound to activate: conformational consequences of cyclin binding to CDK2. *Structure* **1995**, *3* (11), 1135-1141.

3. Bártová, I.; Otyepka, M.; Kríz, Z.; Koca, J., Activation and inhibition of cyclin-dependent kinase-2 by phosphorylation; a molecular dynamics study reveals the functional importance of the glycine-rich loop. *Protein Sci* **2004**, *13* (6), 1449-57.

4. Besker, N.; Amadei, A.; D'Abramo, M., Molecular mechanisms of activation in CDK2. *Journal of Biomolecular Structure & Dynamics* **2014**, *32* (12), 1929-1935.

5. Brown, N. R.; Noble, M. E. M.; Lawrie, A. M.; Morris, M. C.; Tunnah, P.; Divita, G.; Johnson, L. N.; Endicott, J. A., Effects of Phosphorylation of Threonine 160 on Cyclin-dependent Kinase 2 Structure and Activity. *Journal of Biological Chemistry* **1999**, *274* (13), 8746-8756.

6. D'Abramo, M.; Besker, N.; Chillemi, G.; Grottesi, A., Modeling conformational transitions in kinases by molecular dynamics simulations: achievements, difficulties, and open challenges. *Front Genet* **2014**, *5*, 128.

7. Jeffrey, P. D.; Russo, A. A.; Polyak, K.; Gibbs, E.; Hurwitz, J.; Massague, J.; Pavletich, N. P., Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* **1995,** *376* (6538), 313-320.

8. Kornev, A. P.; Haste, N. M.; Taylor, S. S.; Eyck, L. F., Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proc Natl Acad Sci U S A* **2006**, *103* (47), 17783-8.

9. Maddika, S.; Ande, S. R.; Wiechec, E.; Hansen, L. L.; Wesselborg, S.; Los, M., Akt-mediated phosphorylation of CDK2 regulates its dual role in cell cycle progression and apoptosis. *J Cell Sci* **2008,** *121* (Pt 7), 979-88.

10. Morris, M. C.; Gondeau, C.; Tainer, J. A.; Divita, G., Kinetic mechanism of activation of the Cdk2/cyclin A complex. Key role of the C-lobe of the Cdk. *J Biol Chem* **2002**, *277* (26), 23847-53.

11. Sánchez-Martínez, C.; Gelbert, L. M.; Lallena, M. J.; de Dios, A., Cyclin dependent kinase (CDK) inhibitors as anticancer drugs. *Bioorg Med Chem Lett* **2015,** *25* (17), 3420-35.

12. Otyepka, M.; Kríz, Z.; Koca, J., Dynamics and binding modes of free cdk2 and its two complexes with inhibitors studied by computer simulations. *J Biomol Struct Dyn* **2002,** *20* (2), 141-54.

13. Zhang, B.; Tan, V. C.; Lim, K.; Tay, T., Molecular dynamics simulations on the inhibition of Cyclin-Dependent Kinases 2 and 5 in the presence of activators. *Journal of Computer-Aided Molecular Design* **2006,** *20* (6), 395-404.

EXTRAS/FOR DISCUSSION:

13. Shan, Y.; Seeliger, M. A.; Eastwood, M. P.; Frank, F.; Xu, H.; Jensen, M. Ø.; Dror, R. O.; Kuriyan, J.; Shaw, D. E., A conserved protonation-dependent switch controls drug binding in the Abl kinase. *Proceedings of the National Academy of Sciences* **2009,** *106* (1), 139-144.

6. Delk, N. A.; Hunt, K. K.; Keyomarsi, K., Altered subcellular localization of tumor-specific cyclin E isoforms affects cyclin-dependent kinase 2 complex formation and proteasomal regulation. *Cancer Res* **2009,** *69* (7), 2817-25.

8. Harper, J. W.; Elledge, S. J., Cdk inhibitors in development and cancer. *Curr Opin Genet Dev* **1996,** *6* (1), 56-64.

9. Hiromura, K.; Pippin, J. W.; Blonski, M. J.; Roberts, J. M.; Shankland, S. J., The subcellular localization of cyclin dependent kinase 2 determines the fate of mesangial cells: role in apoptosis and proliferation. *Oncogene* **2002,** *21* (11), 1750-8.

10. Jackman, M.; Kubota, Y.; den Elzen, N.; Hagting, A.; Pines, J., Cyclin A- and cyclin E-Cdk complexes shuttle between the nucleus and the cytoplasm. *Mol Biol Cell* **2002,** *13* (3), 1030-45.

18. Moore, J. D.; Yang, J.; Truant, R.; Kornbluth, S., Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and

Cdc2/cyclin B1. *J Cell Biol* **1999,** *144* (2), 213-24.

20. Orend, G.; Hunter, T.; Ruoslahti, E., Cytoplasmic displacement of cyclin E-cdk2 inhibitors p21Cip1 and p27Kip1 in anchorage-independent cells. *Oncogene* **1998,** *16* (20), 2575-83.

12. Jura, N.; Zhang, X.; Endres, N. F.; Seeliger, M. A.; Schindler, T.; Kuriyan, J., Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. *Mol Cell* **2011,** *42* (1), 9-22.

15. Lolli, G., Structural dissection of cyclin dependent kinases regulation and protein recognition properties. *Cell Cycle* **2010,** *9* (8), 1551-1561.

23. Seksek, O.; Bolard, J., Nuclear pH gradient in mammalian cells revealed by laser microspectrofluorimetry. *J Cell Sci* **1996,** *109 (Pt 1)*, 257-62.