Investigation of DFG Motif Activation of Aurora A Kinase

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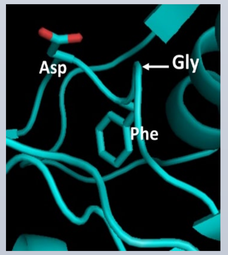
Abstract

Aurora A kinase (AurA) belongs to the serine/threonine protein kinase family and found in all eukaryotic organisms. To succeed at the job of activating G2 phase of the cell cycle for cell growth and preparing for mitosis, AurA is activated by autophosphorylation and TPX2 binding. TPX2 and ADP change the conformation to the kinase's active state. In particular, the DFG motif is of interest for research. The DFG motif is a catalytic triad that consists of aspartate-phenylalanine-glycine residues. The regulation of activation for AurA is based on interchanging conformations of DFG-in and DFG-out. When AurA is considered in DFG-in state, the aspartate reside faces in toward the active site which is favored when ADP binds. This allows for the interaction of the aspartate residue and magnesium ions associated with ATP. In the inactive state or DFG-out conformation, the aspartate rotates outward away from the magnesium ions. This study is focused on this major activation motif and how it functions when a residue in the activation loop is mutated to a different residue. In this case, the glycine residue was mutated to an alanine residue to investigate whether the DFG-motif will still be activation when ADP is present or if the added sterics inhibit AurA's activation. A wild-type AurA protein as expressed in *E.coli* and purified using a His tag NiNTA affinity column. An intrinsic fluorescence assay was used to measure ADP binding and was compared to results of a G276A mutation of AurA intrinsic fluorescence assay for measure of ADP binding. The AurA protein was characterized using gel electrophoresis and circular dichroism spectroscopy to test if the protein was properly folded.

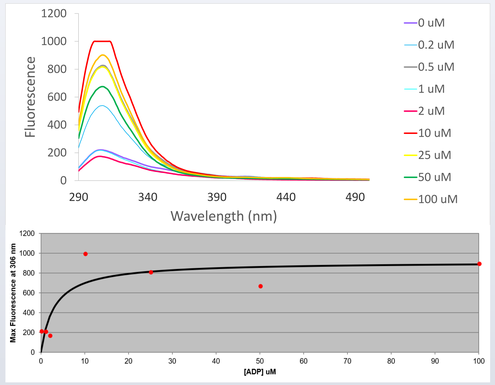
Background

Glycine is a small amino acid because it only has a hydrogen as a side chain. We mutated the gene for that glycine residue to alanine, which is slightly larger and has a CH_3 as a side chain. The idea of increasing the size of the residue that was originally a glycine has the possibility to change Aurora A's active conformation. The DFG motif is shown to the right. It is visible here that if the glycine was mutated, there could be steric hinderances caused between the alanine and the phenylalanine and causing it to inhibit activation. Experimentally, the goal was to successfully purify both a wild-type AurA and the mutation G276A for AurA. By characterizing both kinases, there should be results that show that the protein was folded and active. We then wanted to see if there were any differences between the wild-type and the mutant AurA characteristically and binding affinities (K_D) for ADP.



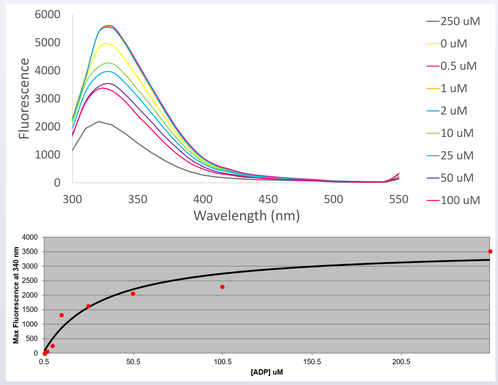
Results

Figure 1: Fluorescence Spectra and Binding Curve of AurA Cl2 C290A



The fluorescence characterization for AurA CL2 (C290A). A fluorescence spectrum with an excitation at 275 nm was used to create a binding curve of different concentrations of ADP. The wavelength that obtained the highest fluorescence emission was at 306 nm. The K_D for the binding curve was found to be 3.1±2.5 μM, indicating a high affinity for the binding of ADP.

Figure 2: Fluorescence Spectra and Binding Curve of AurA CL2 (C290A) G276A mutation



The results from the fluorescence characteristics using a microplate reader for the AurA Cl2 (C290A) G276A mutation with an excitation at 275 nm. The fluorescence data obtained was used to create the binding curve for ADP. The max wavelength of this assay was found at 340 nm. The binding curve made for this data had measured a K_D at 33 ± 2, indicating that the affinity for ADP ligand is much lower than the wild-type AurA CL2 (C290A).

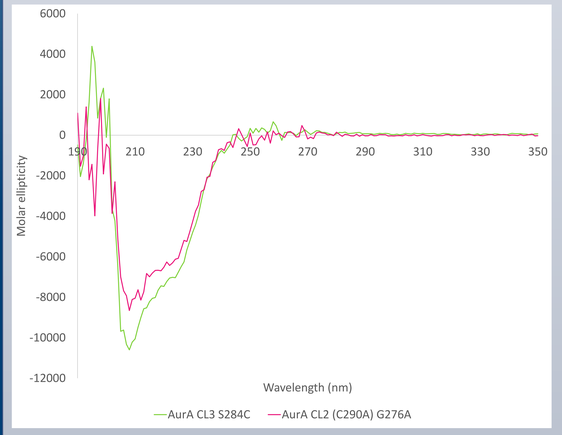


Figure 3: Circular dichroism molar ellipticity content of AurA CL3 S284C (WT) and AurA CL2 (C290A) G276A

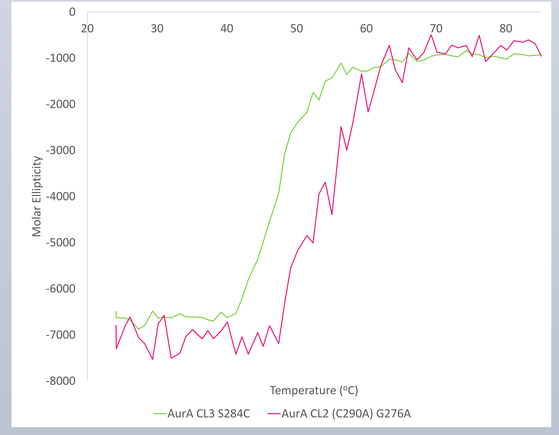


Figure 4: Thermal melt data from circular dichroism spectroscopy of AurA CL3 S284C (WT) and AurA CL2 (C290A) G276A

The circular dichroism molar ellipticity content (Figure 3) showed that both AurA CL3 S284C (WT) and AurA CL2 (C290A) G276A mutant has similar helical content. The thermal melt (Figure 4) of both AurA WT and G276A mutation resulted in about a 10 degree shift between the two. AurA CL2 (C290A) G276A experienced the higher melting point of the two. The two proteins can be said to similarly folded, but the G276A mutant is more thermally stable.

Methods

The Wild-Type AurA plasmid was a hexahistidine-tagged AurA. A Quikchange Lightning Mutagenesis kit using primers that encoded the G276A mutation was used to create the mutant G276A AurA plasmid.

Purification of the Proteins:

Day 1: 2 μL of plasmids of AurA Wild-Type and mutation G276A were transformed into BL21 DE3 RIL *E.coli* cells separately. Both were grown on agar plates with Kanamycin and Chloramphenicol antibiotics at 37˚C overnight.

Day 2: A colony was inoculated in 100 mL LB starter culture overnight at 37˚C and 250 RPM shaking.

Day3: Grew cells to OD 600=0.8 in 1 L TB broth at 37˚C and 250 RPM shaking. When the culture reached this point, the temperature was turn down to 18˚C for 30 minutes. 1 mL of 1M IPTG was added to the culture and was grown overnight at 18 ˚C at 250 RPM shaking.

Day 4: The 1 L culture was centrifuged at 5,700 RPM for 15 minutes at 4˚C. The supernatant was removed and the pellet was resuspended using lysis buffer in 1 mL increments. The lysate was then flash frozen in liquid nitrogen for 10 minutes.

Day 5: After thawing cells, a few grains of lyophilized DNase 1 was added to the cells. Cells were sonicated and the lysate was centrifuged at 20,000 RPM for 1 hour at 4˚C. The supernatant was removed and used for the purification by a Nickel Column. AurA was eluted in 5 1 mL fractions and fractions 2,3, and 4 were kept and used for dialysis for storage. The three fraction were put into a Snake Skin dialysis tubing in AurA storage buffer.

Assays:

A fluorescence assay was completed for both the AurA wild-type and G276A mutant of AurA. DTT and SDS buffer was used in the cuvette along with the different concentrations of ADP and 3 μL of AurA. The parameters for this assay included the excitation wavelength of 275 nm and slit width of 5 nm.

A Circular Dichroism spectroscopy experiment was also completed with 100 mM phosphate buffer pH 7.5 as the dilution buffer and background buffer.

Conclusions

We have successfully mutated a AurA CL (C290A) G276A plasmid by QuikChange Lightning Mutagenesis kit. Also we have utilized a repeatable protocol to purify both AurA CL2 (C290A) and the mutation of G276A. We have also characterized successfully the binding curve of both kinases using intrinsic fluorescence, melting temperature, and relative helical content. We are making efforts to advance our research in the following areas:

1. Successfully purifying a comparable AurA Cl2 (C290A) kinase.
2. Continuing to characterize different aspects of both the mutant and the wild-type.

Acknowledgements

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