Reviving B-Factors: Activating ALK Mutations Increase Protein Dynamics of the Unphosphorylated Kinase

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Supporting Information

ABSTRACT: Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that can become oncogenic by activating mutations or overexpression. Full kinetic characterization of both phosphorylated and nonphosphorylated wildtype and mutant ALK kinase domain was done. Our structure-based drug design programs directed at ALK allowed us to interrogate whether X-ray crystallography data could be used to support the hypothesis that activation of ALK by mutation occurs due to increased protein dynamics. Crystallographic B-factors were converted to normalized B-factors, which allowed analysis of wildtype ALK, ALK-C1156Y, and ALK-L1196M. This data suggests that mobility of the P-loop, α C-helix, and activation loop (A-loop) may be important in catalytic activity increases, with or without phosphorylation. Both molecular dynamics simulations and hydrogen–deuterium exchange experimental data corroborated the normalized B-factors data.

KEYWORDS: B-factor, normalized, anaplastic lymphoma kinase, ALK, kinase, protein, dynamics, mutation, activation, crystallography, X-ray

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase and clinical target in oncology.¹ ALK mutation or overexpression has been implicated in a variety of tumor types. For example, ALK-fusion proteins that arise from gene translocations define a distinct molecular subtype of nonsmall-cell lung cancer (NSCLC) and lead to constitutive activation of the kinase and tumor formation.^{2,3}

Primary single amino acid missense mutations cause ligand-independent activation of the receptor tyrosine kinase ALK and are a major cause of neuroblastoma,⁴ the most common cancer in children under 12 months of age. Frequent mutations include F1174L, R1275Q, K1062M, Y1238S, L1196M, and T1151M.⁵ Gain of function mutations in the intact ALK gene have also been reported in anaplastic thyroid cancer.⁶

Since introduction of the first approved ALK inhibitor, crizotinib,⁷ reports of secondary missense mutations have been reported and are a major cause of acquired resistance in NSCLC.⁸ Reported crizotinib acquired resistance mutations include L1196M, G1269A, E1210K, S1206Y/C, G1202R, I1171T/N/S, C1156Y, and I151Tins.^{9–11} Following treatment with second-generation inhibitors (ceritinib, alectinib, and brigatinib) reported mutations include G1202R, F1174L/C, L1196M, I1171T/N/S, C1156Y, E1210K, D1203N, G1202del, and V1180L.¹² Of the second generation mutations, G1202R is by far the most frequent. In addition, some primary missense mutants in neuroblastoma are not sensitive to crizotinib treatment due to its modest ALK potency. Mechanisms involving ALK function can alter activation rates, catalytic activity, and

binding of ATP, inhibitor, or protein substrate.¹³ Treating acquired resistance and brain metastases have been a focus of many groups, leading to second^{10,14–16} and third generation¹⁷ inhibitors of ALK. To better understand ALK mechanisms of resistance with experimental data, we investigated the protein dynamics with multiple techniques and correlated those changes with catalytic activity of ALK oncogenic mutants.

The nonphosphorylated ALK kinase domain (KD) represents the basal kinase state. Overall, neuroblastoma-derived ALK mutations and many ALK-fusion secondary point mutations have their greatest effects on the activity of unphosphorylated ALK KD, promoting its constitutive autophosphorylation and, thus, ligand-independent signaling by the intact receptor.¹³ As a baseline, autophosphorylated, activated states of ALK increase k_{cat} for WT by 143-fold relative to unphosphorylated enzyme (Table 1). The likely mechanism for the ligand, dimer, or oligomer-induced activation of ALK involves the transphosphorylation of an activation loop (A-loop) tyrosine (Y1278) by the partner ALK protein kinase followed by transphosphorylation of the other two A-loop residues (Y1282 and Y1283).¹⁸

Several kinetic and binding parameters were calculated for the nonactivated and activated constructs of ALK kinase domain. The turnover rate, or k_{cat} (s^{-1}), and Michaelis constants for both

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density, indicating high dynamics, include residues 1124–1130 (in the P-loop), residues 1136–1143 (loop connecting $\beta 2$ and $\beta 3$), residues 1280–1285 (in the A-loop), and residues 1401–1411 at the C-terminus of the protein construct. Regions of the modeled protein structure that have high normalized B-factors are the N- and C-termini, the P-loop, the A-loop, and the C-terminal domain loop (1212–1222) connecting αD and αE .

Figure 2 shows the distribution of B' values for ALK, ALK-C1156Y, and ALK-L1196M. By definition, the average trends

Figure 2. Distribution of B'-factors for ALK (green), ALK-C1156Y (gray), and L1196M (orange) for all heavy atoms with bin size of 0.1 units.

toward 0.0 and values above 0.0 show increased motion, while those below 0.0 exhibit decreased motion. While ALK (green) shows a more central tendency toward 0.0, both mutants extend the distribution in both negative and positive directions. The relative B-factor values above 1.75 suggest more dynamic motion for the mutants. Interestingly, the mutants also show regions that exhibit less motion than WT. Although this spreading of the distribution could represent a more active signature, we were eager to examine the more nuanced nature of the B'-factor differences.

Using WT ALK as a baseline, $\Delta B'$ values were generated by subtracting the WT B' value from each mutant B' value. $\Delta B'$ values greater than +0.5 are considered to be significant "warming" of the mutant, or increased disorder, relative to WT. $\Delta B'$ values less than -0.5 are considered to show considerable "cooling", or less disorder than WT protein. These differential B' values are depicted in Figure 3 for C1156Y (gray) and L1196M (orange)

Figure 3. Plot of $\Delta B'$ values for C1156Y (gray) and L1196M (orange) ALK KD by residue C α atom. Gaps represent unresolved protein regions.

(orange). While there are many regions that show $\Delta B'$ values greater than +0.5, including the P-loop, αC -helix, αD - αE -loop, $\beta 3$ - αC -loop, and A-loop, there are essentially no regions with $\Delta B'$ values less than -0.5. This data suggests that there are only significant increases in B', or motion, for the mutants relative to WT protein. Meaningfully "warmed" regions occur in the αAL -

helix at the base of the A-loop, consisting of residues 1272–1279. Other regions with $\Delta B'$ values greater than +0.5 include P-loop residues (1121–1129), αC -helix and downstream residues (1151–1165), and αD - αE loop residues (1212–1222).

Figure 4 shows the superposition of WT and mutant structures with regions colored red ($\Delta B'$ values greater than +0.5) and light

Figure 4. Normalized B-factor differences of ALK mutants relative to wildtype. Residues with $\Delta B'$ values greater than +0.5 units (C α atoms) are colored red for "warming" and $\Delta B'$ values less than -0.5 units (C α atoms) are colored light blue for "cooling" relative to WT (green). Top panel: C1156Y (gray) apo structure. Bottom panel: L1196M (orange) apo structure.

blue ($\Delta B'$ values less than -0.5). The top panel shows WT and ALK-C1156Y. The C1156Y mutation is located on the $\beta 3$ - αC -loop. The P-loop defines the upper border of the ATP-binding pocket and the tip extends to the A-loop and αC -helix, approaching the C1156Y side chain. At the base, the A-loop sits between the tip of the P-loop and the αC -helix and then extends out toward solvent. The αC -helix completes the triad, with residues having close contacts with C1156Y, the tip of the P-loop, and the A-loop. Overall, there is very little difference in backbone position of ALK and ALK-C1156Y. The most

for ALK, ALK-C1156Y, and ALK-L1196M. To simplify and align with the normalized B-factor analysis, only α atoms were extracted for investigation in the simulation. Figure S2 highlights the RMSF values by residue. Four key regions of the protein (activation segment, β 3- α C-loop, P-loop, and α D- α E-loop) show the least motion for wildtype protein relative to mutants, consistent with the normalized B-factor analysis.

To provide an additional comparison of C1156Y and L1196M structural dynamics relative to wild-type ALK, protein–deuterium exchange experiments were performed. Hydrogen–deuterium exchange (HDX) is a reaction that replaces deuterium atoms with hydrogen atoms, or vice versa. Proteins in solution are dynamic and allow exchange reactions to occur on solvent (D_2O) exposed regions. Based on the rate of deuterium incorporation, correlations with protein and nucleic acid dynamics have been proposed.³⁹ For both mutants relative to wildtype ALK (Figure S3), HDX results showed enhanced deuterium incorporation in representative P-loop (1119–1137, 1122–1139), α C-helix (1149–1164, 1149–1162), and activation loop (1276–1282, 1277–1282) containing peptides, indicating higher dynamics in these regions for the mutants. Three different peptides were also used as negative controls and showed no differential incorporation of deuterium (Figure S4). In a similar analysis, Hoofnagle and co-workers studied hydrogen/deuterium exchange/mass spectrometry on ERK and pERK showing enhanced incorporation of deuterium in regions of the protein that were both consistent and inconsistent with X-ray coordinate data.⁴⁰ B-factor data were not discussed in their analysis of the X-ray data.

Normalized B-factors are an under-utilized parameter in crystallography but can be extremely useful since most crystal structures provide atomic level resolution. To compare B-factors across crystallographic platforms, a normalized B-factor metric was used. We demonstrated the utility of this method to analyze protein dynamics by selecting simple systems with publicly available, high-resolution X-ray crystal data. Normalized B-factor evaluation for ALK-WT, ALK-C1156Y, and ALK-L1196M proteins were used to show flexibility differences and provide mechanistic insight into the activation of quiescent ALK. Differences in mobility were most pronounced in the P-loops, α C-helices, β 3- α C-loops, and A-loops. Mutations that disrupt the F1164 or F1271 hydrophobic cores or the stability of the P-loop, α C-helix, β 3- α C-loop, or A-loop may decrease stability. ALK kinetics data and three methods were used to show that selected clinical mutations are activating by increasing protein dynamics relative to wildtype ALK as baseline. Normalized B-factors add another dimension to protein crystal structures that can help distinguish two proteins that look similar by atomic position, provide protein dynamics, and inform structural and mechanistic insight into protein function.

■ ASSOCIATED CONTENT

Supporting Information

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Experimental and computational methods and structural data PDB information (PDF)

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Author Contributions

All authors contributed to this manuscript.

Notes

The authors declare no competing financial interest.

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