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Detection of Ligand-induced Conformational Changes in the Activation Loop of Aurora-A Kinase by PELDOR Spectroscopy

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The structure of protein kinases has been extensively studied by protein crystallography. Conformational movement of the kinase activation loop is thought to be crucial for regulation of activity; however, in many cases the position of the activation loop in solution is unknown. Protein kinases are an important class of therapeutic target and kinase inhibitors are classified by their effect on the activation loop. Here, we report the use of pulsed electron double resonance (PELDOR) and site-directed spin labeling to monitor conformational changes through the insertion of MTSL [S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1 H-pyrrol-3-yl)methyl methanesulfonothioate] on the dynamic activation loop and a stable site on the outer surface of the enzyme. The action of different ligands such as microtubule-associated protein (TPX2) and inhibitors could be discriminated as well as their ability to lock the activation loop in a fixed conformation. This study provides evidence for structural adaptations that could be used for drug design and a methodological approach that has potential to characterize inhibitors in development.

Protein kinases are signaling enzymes that are regulated by molecular-switch mechanisms.[1,2] Different protein kinases have very similar structures when active, but can adopt a variety of divergent conformations when inactive. One common mechanism of kinase activation is phosphorylation of the activation loop, a dynamic region that spans a consensus sequence from Asp–Phe–Gly (DFG) to Ala–Pro–Glu (APE).[3] Active kinases adopt a DFG-in conformation needed for the correct placement of a catalytic Asp residue and for the central region of the activation loop to form part of the binding site for protein and peptide substrates. Despite intensive studies of kinases by X-ray crystallography,[4,5] there are few studies of conformational dynamics of the activation loops in solution.

Protein kinases are frequently dysregulated in human disease and are a common target for the development of new therapeutics. Most protein kinase inhibitors[6] compete for the ATP binding site and are further classified as either Type I, which bind to the kinase in its active state, or Type II, which bind to an inactive DFG-out conformation of the kinase and occupy an additional hydrophobic pocket within the active site. However, validation of the effect of inhibitors requires determination of the kinase-inhibitor structure by X-ray crystallography, and some compounds that would be classified as Type I based on their chemical structures induce DFG-flipped conformations of the activation loop that resemble Type II[7–9] inhibitors. Methods for analyzing the activation loop conformation in solution would be invaluable in the development and characterization of kinase inhibitors, enabling classification in the absence of crystal structures.[10–12]

Aurora kinases constitute a family of serine–threonine protein kinases whose localization and activities are precisely choreographed as a cell progresses through mitosis.[13–15] They play a major role in cell cycle progression and map to a chromosome region that is frequently amplified in tumors.[16] Aurora-A is activated by phosphorylation on Thr-288 and by the microtubule-associated protein, TPX2.[17] Crystal structures suggest that this involves a lever-arm-like movement of the Aurora-A activation loop from a relatively mobile conformation to a conformation that is stabilized by being hooked onto a short helical region in TPX2 (Figures 1 A and 1 B). However, this model has yet to be probed in solution.

Here, we investigate the application of site-directed spin labeling (SDSL) and pulsed electron–electron double resonance (PELDOR or DEER) spectroscopy[18–19] to measure distances between pairs of spin labels attached to Aurora-A in the presence or absence of ligands and the TPX2 protein. PELDOR separates dipole–dipole coupling between spins, which is inversely proportional to the cube of their distance. It can measure distances between spin labels on the nanometer scale (1.5–10 nm).[20] Dipolar spectroscopy has been successfully employed to study kinases.[21–23] However, to the best of our knowledge, this approach has not been applied to a kinase activation loop.

We analyzed the structure of Aurora-A kinase to identify suitable pairs of sites for site-specific labeling. Glu170 on the αB-helix of the kinase N-lobe was identified as a stable position that does not respond to inhibitors, and Ser284 and

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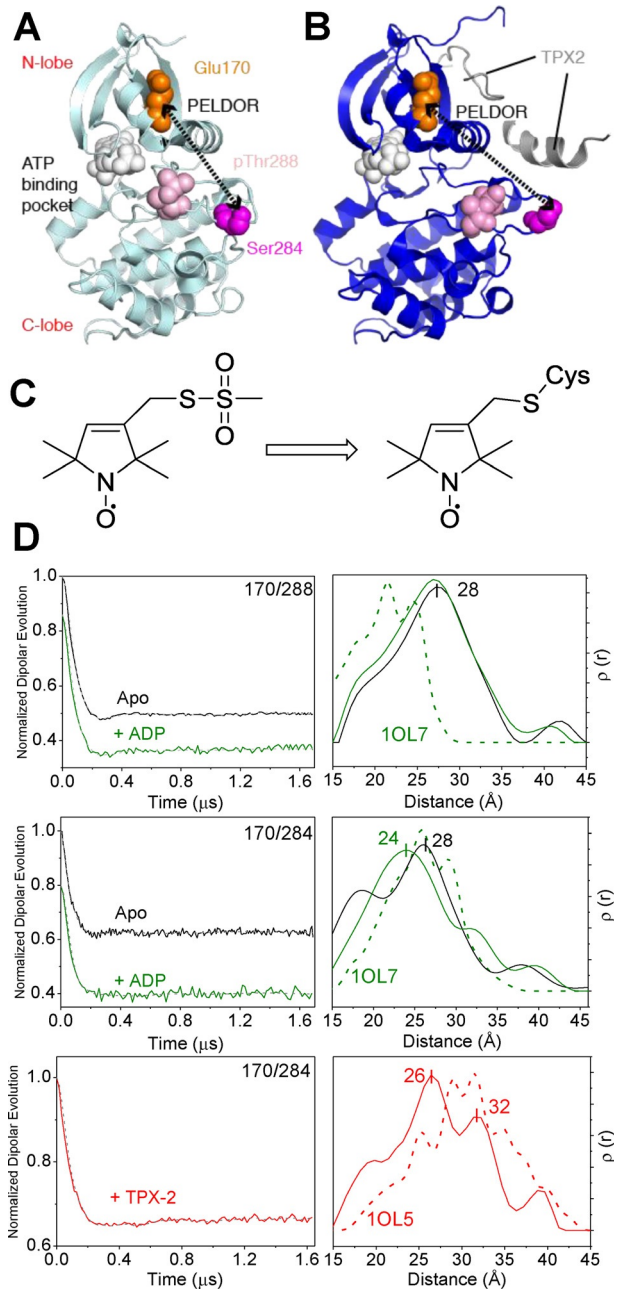

Figure 1. A) Cartoon of Aurora-A kinase (PDB:1OL7) showing the MTSL labeling sites (Glu170, Thr288 and Ser284). B) Cartoon of Aurora-A with TPX bound (PDB:1OL5) C) The chemical structure of the MTSL label and the product of its reaction with a cysteine residue on Aurora-A. D) Background-corrected PELDOR traces at 9 GHz for MTSL-labeled Aurora-A kinase variants and with a four-fold excess of ADP and TPX2 (left column). Form factor fits are given as a dashed line. Distance distributions derived using Tikhonov regularization ($\alpha = 100$) (right column). Rotamer library-derived distributions are given as dashed lines. All PELDOR traces before background correction are given in Figure S1.

Thr288 were chosen on the activation loop (Figure 1 A). These sites were mutated to cysteine in the background of an Aurora-A mutant (C290A, C393A) that lacks other surface cysteines. Recombinant proteins were labeled with MTSL [S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1 H-pyrrol-3-yl)methyl methanesulfonothioate] (Figure 1 C). We designed the system to be suitable for the detection of changes in the activation loop.

By using SDSL and PELDOR spectroscopy, distances were measured initially in the absence of a ligand. The PELDOR traces for MTSL-labeled Aurora-A variants E170C/T284C and E170C/T288C showed a shallow oscillation, indicative of a wide distribution of conformational states. The extracted distance distribution showed a dominant mean distance at 28 Å (Figure 1 D). Based on the acquisition window, the data are of sufficient quality to establish from the distribution a reliable mean and width with approximate shape. The wide distribution suggests spatial disorder of the Aurora-A kinase activation loop, consistent with crystal structures, as well as multiple internal rotamers of the MTSL.[24]

Comparison was made to the distribution created from a rotamer library[25] using PDB:1OL7. Although, a similar distribution width was found, the mean distance was offset in the case of the E170C/T288C variant, suggesting a difference in the conformation of the activation loop in solution. Continuous-wave electron paramagnetic resonance (EPR) spectra showed no significant dipolar broadening of the EPR line width, indicating that distances <15 Å are not significantly populated (Figure S2). Little difference was observed between the apo enzyme and with ADP (present in the crystal structures) for the E170C/T288C variant; however, the E170C/T284C variant showed a shift of the mean distance to 24 Å.

The effect of TPX2 on the activation loop of Aurora-A phosphorylated on T288 was investigated with a PELDOR experiment on MTSL-labeled E170C/T284C Aurora-A. This resulted in a pronounced change in the distance distribution compared to the apo enzyme, consistent with a different conformational state of the activation loop (Figure 1 D). There was a sharp peak in the distance distribution at 34 Å, which is in reasonable agreement with the conformational change observed in the crystal structure. Small-molecule inhibitors of Aurora kinases, such as compounds **1** and **5**, have been identified as potential cancer therapeutics,[26] and clinical trials are ongoing (Figure 2 A).[27] To validate the site-directed-labeling EPR approach, we tested imidazo[1,2-a]pyrazine-based (compounds **2** and **3**)[28] and imidazo[4,5-b]pyridine-based inhibitors (compounds **4** and **5**),[29] which dock into the ATP binding site but do not affect the activation loop, as observed by X-ray crystallography (Figures 2 B and S3).[28,29] The results showed little change in the mean and width of the distribution (Figure 2 C), indicating minimal perturbation of the activation loop, a distinct difference compared to TPX2.

A crystal structure of compound **1**[30] bound to Aurora-A (PDB:2WTV) showed that the drug induces an ordered and closed, inactive, conformation of the Aurora-A activation loop (Figure 2 D).[7] The conformational change in the activation loop moves the Cα of Val279 by approximately 19 Å compared with its position in the ADP-bound structure. However, other crystal structures (Figure 2 E) showed an alternative conformation of Aurora-A bound to compound **1** (PDB codes:2WTW and 2X81).[7,31] The specific conformation induced by MLN8237, an Aurora-A inhibitor closely related to compound **1**, might explain its activity in neuroblastoma cells.[32] The conformation of

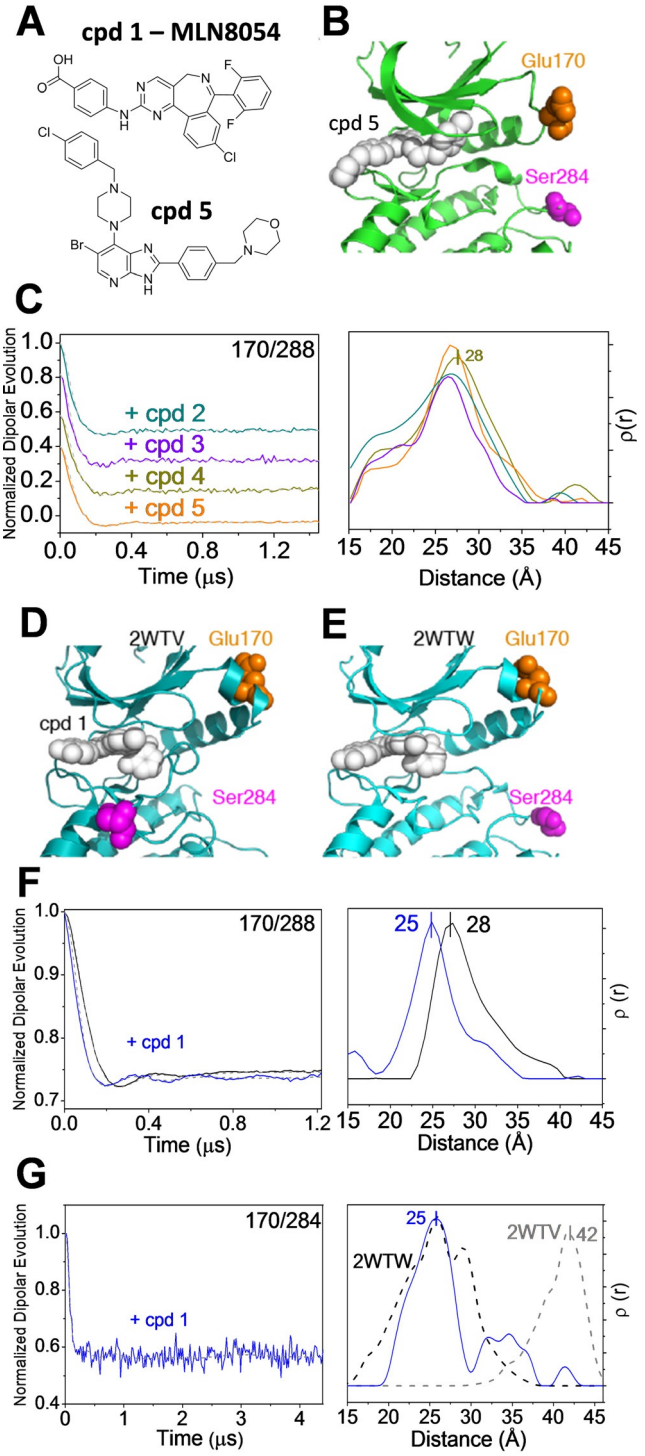

Figure 2. A) Chemical structures of compounds **1** and **5**. B) Cartoon of the Aurora-A binding site for compound **5** in PDB:2X6G. C) Background-corrected PELDOR traces at 9 GHz for MTSL-labeled Aurora-A E170C/T288C with a four-fold excess of inhibitors (left column). Form factor fits are given as a dashed line. Distance distributions derived using Tikhonov regularization ($\alpha = 100$) (right column). D) Cartoon of the Aurora-A binding site of compound **1** in PDB:2WTV. E) Cartoon of the Aurora-A binding site of compound **1** in PDB:2WTW. F) The same as (C), but at 34 GHz. G) The same as (C) for Aurora-A E170C/S284C. Rotamer library-derived distributions are given as dashed lines. All PELDOR traces before background correction are given in Figure S4.

the kinase in solution in the presence of the compound is an open and important question.

PELDOR experiments on MTSL-labeled E170C/T288C Aurora-A in the presence of compound **1** revealed a clear change in the oscillation, reflected by a change in the major peak of the distance distribution (Figure S5). The distance distribution appears to narrow with the presence of compound **1** (absent with compounds **2**–**5**), suggesting a greater order of the conformation of the activation loop. Further experiments at 34 GHz (Figure 2F) gave a well-defined oscillation and confirmed the result. The distribution is narrower and shifts to a shorter dominant distance of 25 Å, as compared to Aurora-A alone.

Key measurements were repeated to evaluate changes in MTSL-labeled Aurora-A E170C/S284C on ligand binding (Figure 2G). Upon addition of compound **1**, we observed a change in the mean distance and a narrowing of the distribution, which is absent in the case of compound **5** (Figure S6). Interestingly, the rotamer library produced with PDB:2WTV (Figure 2G, grey dash) showed a significant difference with a mean distance of 42 Å, suggesting the conformation of the activation loop is more akin to the DFG-in state adopted in PDB:2WTW, which differs from that induced by compounds **2**–**5**, because the N and C lobes of the kinase are twisted. There is perhaps only a minor contribution from the DFG-up state in PDB:2WTV. Subsequent addition of TPX2 caused a broad distance distribution profile, which is distinct from that of TPX2 and compound **1** alone, indicating a dynamic conformation that results from the opposing tendencies of TPX2 and compound **1** (Figure S7).

Recently, the structures of a number of mammalian kinases bound to ligands and substrates have been solved using X-ray crystallography. Although structures for open and closed forms are available for certain kinases, it is becoming clear that they occupy several conformational states, and that our knowledge of these states in solution is poor. In particular, the dynamics of the activation loop and how it responds to ligands is poorly characterized. PELDOR is complementary to other biophysical methods used to study kinase activation loops and holds many advantages, such as the smaller size of their labels compared to Förster resonance energy transfer (FRET) and no issues with limitations of molecular weight or difficulties with chemical exchange in NMR. However, one weakness of methods such as PELDOR and FRET is that the measurements are of the synthetic modifications of the protein, not the main chains and side chains of the amino acids. Indeed, the full extent of the movement of the activation loop will be understood through ongoing work by using molecular dynamics to implement the distance constraints provided by the PELDOR measurements to model the full conformational change of the activation loop. Inarguably, a combination of approaches will be required to solve this problem.

The studies here provide key information on adaptations to inhibitor binding. Our results confirm that PELDOR is a suitable approach to discriminate between different classes of ligand that do, or do not, influence the conformation of the kinase. Overall, the results suggest a correlation between the trends

found for inhibitors and TPX2 with PELDOR spectroscopy and the observed conformational changes in the activation loop as judged by X-ray crystallography. Although the differences in the mean of the PELDOR distributions for the inhibitors are small, they show a clear trend and are able to discriminate whether or not inhibitor binding to the ATP site influences the activation loop. This work suggests that PELDOR spectroscopy could be a potential method to classify inhibitors by their effects on the conformation of the kinase activation loop as well as the study of native substrates in solution. Moreover, the method has sufficient sensitivity to detect the influence of TPX2 on the activation loop of Aurora-A in the absence of an inhibitor, and shows that this is altered in the presence of compound **1**.

Our ultimate aim is to exploit the information gleaned from these studies to generate plausible models of the structure and dynamics of human kinases. This may enable the development of inhibitors that exploit specific conformational states that are not accessible for study by other methods.

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