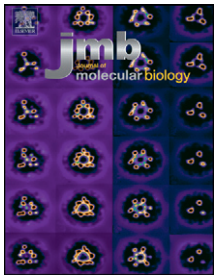

Extracellular-Regulated Kinase 2 Is Activated by the Enhancement of Hinge Flexibility

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http://dx.doi.org/10.1016/j.jmb.2014.02.011
Edited by I. Stagljar

Abstract

Protein motions underlie conformational and entropic contributions to enzyme catalysis; however, relatively little is known about the ways in which this occurs. Studies of the mitogen-activated protein kinase ERK2 (*e*xtracellular-*r*egulated protein *k*inase *2*) by hydrogen-exchange mass spectrometry suggest that activation enhances backbone flexibility at the linker between N- and C-terminal domains while altering nucleotide binding mode. Here, we address the hypothesis that enhanced backbone flexibility within the hinge region facilitates kinase activation. We show that hinge mutations enhancing flexibility promote changes in the nucleotide binding mode consistent with domain movement, without requiring phosphorylation. They also lead to the activation of monophosphorylated ERK2, a form that is normally inactive. The hinge mutations bypass the need for pTyr but not pThr, suggesting that Tyr phosphorylation controls hinge motions. In agreement, monophosphorylation of pTyr enhances both hinge flexibility and nucleotide binding mode, measured by hydrogen-exchange mass spectrometry. Our findings demonstrate that regulated protein motions underlie kinase activation. Our working model is that constraints to domain movement in ERK2 are overcome by phosphorylation at pTyr, which increases hinge dynamics to promote the active conformation of the catalytic site.

Introduction

The activation of MAP kinases is controlled by phosphorylation at Thr-Xxx-Tyr sequences within the activation loop, catalyzed by dual-specificity MAP kinase kinases (MKKs). Phosphorylation of both Thr and Tyr residues is required, and negligible activation is seen with phosphorylation of either residue alone or mutation of either or both residues to acidic amino acids. Solvent viscometric steady-state rate measurements have shown that the mechanism of activation by phosphorylation is dominated by rate enhancement of steps involving phosphoryl group transfer [1].

X-ray structures of ERK2 (*e*xtracellular-*r*egulated protein *k*inase *2*) in its inactive, unphosphorylated (0P) and active, dual phosphorylated (2P) forms provide important insights into the structural changes underlying ERK2 activation [2,3]. Dual phosphorylation rearranges the activation loop from an inactive conformation that precludes substrate binding to an active conformation that enables recognition of the Ser/Thr-Pro phosphorylation motif [3]. In addition, ion pair interactions between pThr183 in the activation loop and Arg65 and Arg68 in helix αC enable communication between N- and C-terminal domains. Finally, activation loop rearrangement opens a high-affinity binding site for a docking motif found in substrates and scaffold proteins [4,5].

Biophysical measurements suggest that ERK2 is also regulated at the level of protein dynamics. Hydrogen-exchange mass spectrometry (HX-MS) revealed changes in hydrogen–deuterium exchange (HX) rates within localized regions of the kinase upon activation by phosphorylation [6]. In particular, HX increases within residues $LMETD_{109}$, which form the hinge region between N- and C-terminal domains. Structural differences between 0P-ERK2 and 2P-ERK2 in this region are not obvious, suggesting that phosphorylation does not affect conformation but instead alters conformational

mobility. In accordance, site-directed spin label-electron paramagnetic resonance spectroscopy measurements of ERK2 showed changes in correlation rates at the hinge upon ERK2 phosphorylation, without changes in the local environment [7]. Together, these observations suggest that ERK2 activation modulates protein motions at the hinge.

Studies of protein kinases have shown the importance of domain movements for catalytic function. For example, in the catalytic (C) subunit of cAMP-dependent protein kinase (PKA), nucleotide and substrate binding elicits N- and C-terminal domain rotation to form a closed conformation (Fig. 1a) [8,9]. By contrast, X-ray structures of both 0P-ERK2 and 2P-ERK2 show open conformations, raising questions about how the necessary domain movements needed for closure could be achieved. One clue is that 0P-ERK2 and 2P-ERK2 bind with similar affinities to the nucleotide analog, AMP-PNP, yet differ in the extent to which AMP-PNP binding protects from hydrogen exchange with solvent, measured by HX-MS (Fig. 1b). In particular, 2P-ERK2 shows a greater extent of HX protection by AMP-PNP binding within the Mg^{2+} positioning loop (DFG motif), located at the interface between N- and C-terminal domains [10]. Thus, nucleotide has two binding modes that distinguish the 0P-ERK2 and 2P-ERK2 kinase activity states.

Recently, protein dynamics in ERK2 were analyzed by Carr–Purcell–Meiboom–Gill NMR relaxation dispersion experiments, measuring millisecond timescale exchange between conformational states in Ile, Val and Leu side-chain methyl groups [11]. In 0P-ERK2, relaxation dispersion measurements reported fast conformational exchange processes (e.g., A ⇌ B interconversion) in Ile/Leu/Val residues, with little or no evidence for coupling between these residues. However, in 2P-ERK2, residues throughout the kinase core could be fit globally, consistent with a single exchange process with rate constant $k_{ex} = k_{AB} + k_{BA} = 300\ s^{-1}$ and p_A and p_B populations of 20% and 80%, respectively. The residues appeared throughout the N- and C-terminal domains, except for the MAP kinase insert. Thus, changes in dynamics accompany phosphorylation and activation of ERK2, where internal motions become dominated by a slow-exchange process characterized by interconversion between two major conformational states. The fact that the global process involves residues throughout the N- and C-terminal domains suggests that the underlying motion involves domain movements. This is consistent with phosphorylation-induced changes in the interactions between Mg^{2+}-AMP-PNP and the DFG motif, although other structural models are possible. Taken together, the findings imply that, in solution, 0P-ERK2 is constrained from hinge motions, which in turn interferes with domain movement. Phosphorylation to form 2P-ERK2 bypasses these constraints, allowing optimized configuration of the catalytic site.

Here, we address the hypothesis that regulation of hinge motions in ERK2 is relevant to the mechanism of kinase activation by phosphorylation. We show that introducing hinge mutations that increase backbone flexibility allows adoption of the active-state nucleotide binding mode, without activation loop phosphorylation. Monophosphorylation of ERK2 at Tyr185 is sufficient to increase HX at the hinge and also reduces HX in the Mg^{2+} positioning DFG motif upon AMP-PNP binding, suggesting that pTyr phosphorylation enhances hinge flexibility and domain movements needed for active-state nucleotide binding. The hinge mutations promote activation of a normally inactive monophosphorylated form of ERK2, where Thr is phosphorylated and Tyr is mutated to Glu. Thus, mutations that enhance hinge flexibility bypass the need for Tyr phosphorylation, arguing that that hinge flexibility is coupled to activation. We propose that Tyr phosphorylation has the distinct role of controlling hinge dynamics in ERK2 in order to promote the switch in nucleotide binding and domain movement, events that are crucial for kinase activation.

Results

Hinge sequences in ERK2 and other protein kinases

In previous studies, AMP-PNP binding led to steric protection from HX within the N-terminal domain and hinge regions to levels that were comparable between 0P-ERK2 and 2P-ERK2 [10]. In contrast, greater protection from HX was observed in 2P-ERK2 compared to 0P-ERK2 within the conserved DFG motif, which is located within the C-terminal domain where it forms a coordination site for nucleotide-bound Mg^{2+}. A model to explain the HX patterns proposed domain movements leading to differing interdomain conformations for 0P-ERK2 and 2P-ERK2, respectively (Fig. 1b). Interconversion between open and closed conformations would be consistent with these movements, although other models are possible. We hypothesized that constraints at the hinge region prevent domain movement in 0P-ERK2, whereas activation loop phosphorylation releases these constraints and increases hinge backbone flexibility in 2P-ERK2.

X-ray structures of PKA C-subunit show the apo enzyme in an open conformation, where the N- and C-terminal lobes are too far apart to enable catalysis [8]. In the ternary complex with nucleotide and substrate, the structure undergoes domain closure to a closed conformation, involving hinge bending and sideways rotation about the lobes (9; Fig. 1a). Based on NMR and molecular dynamics, this has been attributed to underlying motions of the Gly loop and ATP binding pocket and requires integrity of