Mechanism of Activation of ERK2 by Dual Phosphorylation*

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The mitogen-activated protein (MAP) kinases are characterized by their requirement for dual phosphorylation at a conserved threonine and tyrosine residue for catalytic activation. The structural consequences of dual-phosphorylation in the MAP kinase ERK2 (extracellular signal-regulated kinase 2) include active site closure, alignment of key catalytic residues that interact with ATP, and remodeling of the activation loop. In this study, we report the specific effects of dual phosphorylation on the individual catalytic reaction steps in ERK2. Dual phosphorylation leads to an increase in overall catalytic efficiency and turnover rate of approximately 600,000- and 50,000-fold, respectively. Solvent viscosometric studies reveal moderate decreases in the equilibrium dissociation constants ($K_d$) for both ATP and myelin basic protein. However, the majority of the overall rate enhancement is due to an increase in the rate of the phosphoryl group transfer step by approximately 60,000-fold. By comparison, the rate of the same step in the ATPase reaction is enhanced only 2000-fold. This suggests that optimizing the position of the invariant residues Lys$^{265}$ and Glu$^{269}$, which stabilize the phosphates of ATP, accounts for only part of the enhanced rate of phosphoryl group transfer in the kinase reaction. Thus, significant stabilization of the protein phosphoacceptor group must also occur. Our results demonstrate similarities between the activation mechanisms of ERK2 and the cell cycle control enzyme, Cdk2 (cyclin-dependent kinase 2). Rather than dual phosphorylation, however, activation of the latter is controlled by cyclin binding followed by phosphorylation at Thr$^{160}$.

Members of the family of protein kinases referred to as the MAP$^1$ kinases are critical components of the biochemical processes that define the essence of life, the ability of cells to sense their external environment and respond. The prototype member of the MAP kinase family that mediates signaling by all polypeptide mitogens is the extracellular signal-regulated kinase, ERK2. Like all MAP kinases, ERK2 participates in a three-tier protein phosphorylation cascade that, in response to growth factor receptor signaling, is activated by dual phosphorylation catalyzed by an upstream activating kinase, MEK1/MEK2. Among the targets of ERK2 are downstream kinases involved in cellular growth control as well as nuclear transcription factors. Thus, ERK2 provides an essential link in transducing the diverse signals from transmembrane growth factor receptors into gene regulatory events (for review see Refs. 1 and 2).

The x-ray crystallographic structure of ERK2 (357 amino acids) reveals a conserved catalytic core (residues 22–311) flanked by N- and C-terminal extensions that lie on the surface of the molecule. Like all protein kinases, the catalytic core is globular, consisting of an N- and C-terminal lobe, whose interface defines the active site cleft. Within the active site, the adenine ring of ATP is deeply buried, with the $\gamma$-phosphate pointing toward the mouth of the active site where protein substrates bind and where phosphorylation occurs (3).

All protein kinases display near their active sites an “activation loop,” the conformation of which must be optimized for high catalytic activity (4). For most, but not all, protein kinases, this activity is dependent upon phosphorylation at a single conserved residue located at the C-terminal end of the activation loop (4). In many cases, phosphorylation at this site is catalyzed by a heterologous kinase and constitutes a physiological mechanism for kinase regulation. For example, phosphorylation of the cyclin-dependent kinases at the conserved activation loop threonine (Thr$^{160}$ in Cdk2) by a cdk activating kinase (CAK) is critical for regulation of cell cycle progression (5). Similarly, the MAP kinases are regulated by phosphorylation by their upstream activators, the MAPK/ERK kinases (MEKs), in response to extracellular signaling (2).

The hallmark of the MAP kinases is their unique requirement for dual phosphorylation at a conserved threonine and tyrosine residue belonging to the consensus sequence TXY for catalytic activation (6). In ERK2, these sites are Thr$^{183}$ and Tyr$^{185}$. The phosphorylation of both of these residues is catalyzed by the dual-specific upstream kinases MEK1 or MEK2 (2). The structural role of both phosphorylations in catalytic activation has been revealed by comparison of the crystallographic structures of (non-p)ERK2 (3) and (pp)ERK2 (7). The major alteration upon dual phosphorylation is a closure of the active site cleft, which results in optimal alignment of the key catalytic residues that contact the phosphate groups of ATP. In addition, there is significant remodeling of the activation loop as well as the P$_{\alpha}$ surface pocket, the latter of which is necessary to accept the P$_{\alpha}$ proline residue essential for the recognition of all ERK substrates.

Given the structural consequences of dual phosphorylation revealed by x-ray crystallography, it is not known how such changes in structure specifically affect the kinetics of individual reaction steps along the catalytic reaction pathway. Thus, a correlation between structure and mechanism, and therefore
regulation, cannot be made. In this study, the catalytic reaction pathway for nonphosphorylated ERK2 was determined and compared with that of the fully active, dual-phosphorylated enzyme. The results provide a quantitative understanding of the mechanistic basis for catalytic activation by dual phosphorylation. Finally, our studies reveal functional similarities between ERK2 and Cdk2 in terms of their mechanisms of activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals (KCl, EDTA, MgCl₂, MOPS, sucrose, dithiothreitol) were purchased from Fisher, except for ATP (Sigma), and [γ-³²P]ATP (ICN).

**MBP Preparation**—Myelin basic protein (MBP) was purified from a bovine brain acetone powder (Sigma B0508) by acid extraction followed by cation exchange chromatography as described previously (8).

**ERK2 Preparation**—Expression and purification of the recombinant rat ERK2 N-terminally fused to a hexahistidine tag was carried out as described previously (9). Briefly, ERK2 overexpressed in Esherichia coli was purified by Ni²⁺-NTA affinity chromatography followed by FPLC anion exchange chromatography on Uno Q (Bio-Rad). Select fractions from peak 1 (see "Results") were used for all experiments. (pp)ERK2 was generated as described previously (8). (non-p)ERK2 purified by Ni²⁺-NTA affinity chromatography was subject to phosphorylation by recombinant MEK1 in vitro, and the dual-phosphorylated material was further purified by FPLC anion exchange chromatography (Uno Q). ERK2 was confirmed to be dual-phosphorylated by electrospray mass spectrometry. Both (non-p)ERK2 and (pp)ERK2, purified as described above, were essentially homogeneous based on analysis by SDS-polyacrylamide gel electrophoresis. The concentration of ERK2 was determined spectrophotometrically based on an extinction coefficient (ε₂₈₀ = 44, 825 cm⁻¹ M⁻¹) calculated from its primary amino acid sequence (10).

**Kinase Assays and Data Analysis**—Kinase activity was monitored by a radioisotope assay in which the rate of incorporation of ³²P from [γ-³²P]ATP into MBP was directly measured. Reactions were carried out in phosphorylation buffer (20 mM MOPS, pH 7.4, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl₂, total containing 3 μM ERK2 and varied concentrations of MBP. Reactions were initiated by the addition of [γ-³²P]ATP (300–500 cpm/pmol) at varied concentrations and allowed to proceed at 23 °C for 45 min, after which time they were terminated with 25% acetic acid. The ³²P-labeled MBP product was resolved from unincorporated [γ-³²P]ATP by ascending chromatography on PS1 phosphocellulose paper (Whatman) as described previously (8). Radioactivity was quantified by Cerenkov counting.

Steady-state kinetic parameters were determined by nonlinear least squares analysis of initial velocity data obtained from several concentrations of MBP at several fixed concentrations of ATP. The following equation (Equation 1) for a two-substrate sequential reaction was globally fit to the data using the program Scientist (Micromath Inc.),

\[ v = V \cdot A \cdot B / (K_{A} \cdot K_{NH} + K_{NH} \cdot A + K_{NH} \cdot B + A \cdot B) \]  
(Eq. 1)

where \( v \) is the initial velocity, \( V \) is the maximal initial velocity, \( A \) and \( B \) are the fixed and varied substrates, respectively, \( K_{A} \) is the Michaelis constant, and \( K_{NH} \) is the dissociation constant for \( A \). \( k_{cat} \) was determined by dividing the maximal initial velocity by the enzyme concentration.

**Solvent Viscosimetric Studies**—Steady-state assays, as described above, were carried out in buffer containing sucrose ranging from 0 to 40%, to give relative solvent viscosities ranging between 1 and 4.2. Relative solvent viscosity was determined as described previously (8).

**ATPase Assays**—ATPase activity of (non-p)ERK2 was determined in a radioisotope assay in which the rate of ³²P decay was monitored. Reactions were performed in phosphorylation buffer in a total volume of 20 μl at 23 °C. Typically, reactions containing 3 μM (non-p)ERK2 were initiated by the addition of [γ-³²P]ATP (500 cpm/μmol) at various concentrations, allowed to proceed for 2 h at 23 °C, then terminated in 1 ml of 0.1 N HCl. To determine the amount of phosphate produced, the stopped reactions were incubated with 200 μl of charcoal solution (10% activated charcoal (Sigma C-6289), 10% acetic acid, 2.5 ml KH₂PO₄) for 1 h, centrifuged at maximum speed in a microcentrifuge for 30 min. Radioactivity in the supernatant was quantified by counting 500 μl of the supernatant using the Cerenkov method.

ATPase activity of (pp)ERK2 was determined using a coupled spectrophotometric assay (11). The coupling reagents and their concentrations were as follows: 15 units/ml lactate dehydrogenase, 7.5 units/ml pyruvate kinase, 1 mM P-enolpyruvate, and 130 μM NADH. All reac-

![FIG. 1. Anion exchange chromatography of (non-p)ERK2. ERK2 was purified by Ni²⁺ chelate chromatography and then further purified by FPLC over UnoQ (Bio-Rad). The Uno-Q trace is shown. Solid circles (●) correspond to A₂₈₀. Bars correspond to kinase activity toward MBP, determined as follows: 5 μl of each fraction was incubated with 100 μM MBP and 1 mM [γ-³²P]ATP (300 cpm/μmol) in phosphorylation buffer (15 μl total volume) for 1 h, and total ³²P-labeled MBP was produced was determined as described under "Experimental Procedures." Fractions 42 or 43 were used for all assays. The dashed line (— — —) indicates the salt gradient, which runs from 100 mM (fraction 38) to 250 mM (fraction 52) NaCl.](image-url)

**RESULTS AND DISCUSSION**

**Purification of (non-p)ERK2**—(non-p)ERK2 was expressed and purified from E. coli. Purification using anion exchange chromatography revealed two peaks of protein corresponding to ERK2, both of which displayed identical activities and activation properties. These observations are consistent with those reported previously during the crystallization of ERK2, in which only peak 1 formed crystals (9). Accordingly, we have used this fraction of ERK2 for all of our studies.

The activity of each fraction from peak 1 was determined. When assayed for kinase activity, it was found that the latter fractions of the peak exhibited substantially higher kinase activity than those eluting earlier (Fig. 1). The high specific activity associated with the later fractions may be attributable to enzyme that had undergone autophosphorylation during induction. In all our studies, however, fractions free of the high kinase activity (fractions 42–43, Fig. 1) were used. In these fractions, the profile of kinase activity corresponded to the ERK2 protein concentration exactly, and when subjected to phosphorylation by MEK1, the enzyme could be activated to a form that displayed high catalytic activity (k₅₀/Kₐ₅ = 1 μM⁻¹ s⁻¹ and k₅₀ = 10 s⁻¹) (8).

**Steady-state Kinetic Analysis**—The steady-state kinetic parameters for the phosphorylation of myelin basic protein by (non-p)ERK2 were determined. MBP phosphorylation was linear for at least 2 h, indicating that autoactivation of (non-p)ERK2 did not occur within this time frame. A data set of
initial reaction velocities obtained under conditions of varied MBP concentrations at several fixed concentrations of ATP was analyzed. Fig. 2 shows the experimental data and the best-fit regression curves in double reciprocal form. The regression analysis yielded values of $k_{cat} = 2 \times 10^{-4}$ s$^{-1}$, $K_{m\text{MBP}} = 50 \mu M$ and $K_{m\text{(ATP)}} = 700 \mu M$. The $k_{cat}$ value is down 50,000-fold from that of (pp)ERK2, whereas the $K_m$ values for MBP and ATP are each up by only 10-fold. The changes in the steady-state parameters correspond to an overall catalytic efficiency toward MBP ($k_{cat}/K_{m\text{MBP}}$), which is attenuated 600,000-fold.

Solvent Viscosimetric Analysis—The Michaelis-Menten parameters described above are a composite of microscopic rate constants combined in a manner dependent upon the order of substrate addition. The steady-state data for (non-p)ERK2 are consistent with both randomly and compulsorily ordered mechanisms. However, if the kinetic mechanism of this enzyme is ordered, it is necessarily ordered with ATP binding first. This is true because the crystal structure of (non-p)ERK2 has been obtained with bound ATP alone (3). In addition, (non-p)ERK2 displays measurable ATPase activity in the absence of MBP (see below).

Under saturating conditions of ATP, the catalytic mechanism of (non-p)ERK2 can therefore be described by Scheme 1. In this scheme, catalytic efficiency is given by $k_{cat}/K_{m\text{MBP}} = k_2k_3/k_3[k_2 + k_3]$, whereas the turnover rate is given by $[k_{cat} = k_3k_4/k_2 + k_3]$ (12). To solve for the microscopic constants, $k_2$, $k_3$, $k_4$, and $k_5$, we employed steady-state solvent viscosimetric techniques, which allow separation of the diffusive ($k_3$, $k_2$, $k_3$) from the nondiffusive ($k_5$) reaction steps (13–15). Initial velocity data were obtained as a function of MBP concentration at several fixed concentrations of sucrose, and the effect of solvent viscosity on $k_{cat}$ or $k_{cat}/K_{m\text{MBP}}$ was determined. Since it was not possible to carry out these experiments at a single, saturating concentration of ATP, because of the high $K_m\text{(ATP)}$ value, viscosity data were collected at several subsaturating ATP concentrations. The viscosity effect on $^{49}k_{cat}$ (Fig. 3A) or $^{49}k_{cat}/K_{m\text{MBP}}$ (Fig. 3B) was determined at each ATP concentration by plotting the fold decrease in the respective rate parameter as a function of the relative solvent viscosity. The viscosity effect is given by the slopes of the best-fit lines in Fig. 3, A and B. The true viscosity effect on $k_{cat}$ and $k_{cat}/K_{m\text{MBP}}$ was determined by extrapolation to infinite ATP concentration. We saw no significant viscosity effect on either rate parameter at any ATP concentration tested, and we therefore conclude that both $k_{cat}$ and $k_{cat}/K_{m\text{MBP}}$ are insensitive to the relative solvent viscosity.

The viscosity effect (designated by superscripted $\eta$) on $k_{cat}/K_{m\text{MBP}}$ is given by $[k_{cat}/K_{m\text{MBP}}]_{\eta} = k_3/k_2 + k_3 + k_4$), whereas the viscosity effect on $k_{cat}$ is given by $[k_{cat}]_{\eta} = k_3/k_2 + k_3 + k_4$ (16). The kinetic constants derived from the viscosity studies are shown in Table I. The lack of viscosity effect on $k_{cat}$ implies that the overall rate of substrate turnover is limited by phosphoryl group transfer ($k_3$) and that the diffusion of either product from the active site ($k_4$) is not rate-limiting. The rate of phosphoryl group transfer can therefore be assigned a value of 0.012 min$^{-1}$, which is 60,000-fold lower than that in (pp)ERK2.

The viscosity effect on $k_{cat}/K_{m}$ for ATP was also determined.

Initial velocities were determined at 1 mM MBP, 175 $\mu M$ ATP, and varied sucrose. Identical conditions, except in 300 $\mu M$ ATP, gave proportionally higher rates, indicative of true $k_{cat}/K_{m}$ conditions.
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### TABLE I
Kinetic and thermodynamic parameters for (non-p)ERK2 versus (pp)ERK2

| Parameter | (non-p)ERK2 | (pp)ERK2 | Fold alteration (pp)ERK2/(non-p)ERK2 |
|-----------|-------------|----------|-------------------------------------|
| $k_{cat}$ | $0.012 \pm 0.009 \text{ min}^{-1}$ | $10 \pm 2 \text{ s}^{-1}$ | 50,000 |
| $k_{cat}/K_m^{(MBP)}$ | $4.0 \pm 1 \text{ M}^{-1} \text{s}^{-1}$ | $2.4 \times 10^{6} \text{ M}^{-1} \text{s}^{-1}$ | 600,000 |
| $k_{cat}/K_m^{(ATP)}$ | $0.3 \pm 0.1 \text{ M}^{-1} \text{s}^{-1}$ | $0.2 \pm 0.1 \times 10^{8} \text{ M}^{-1} \text{s}^{-1}$ | 700,000 |
| $k_m^{(MBP)}$ | $50 \pm 10 \mu\text{M}$ | $4.2 \pm 0.8 \mu\text{M}$ | 0.1 |
| $K_m^{(MBP)}$ | $700 \pm 150 \mu\text{M}$ | $47 \pm 8 \mu\text{M}$ | 0.07 |
| $K_m^{(ATP)}$ | $50 \pm 10 \mu\text{M}$ | $0.5 \pm 0.5 \mu\text{M}$ | $<0.01$ |
| $K_m^{(ATP)}$ | $700 \pm 150 \mu\text{M}$ | $57 \pm 8 \mu\text{M}$ | 0.07 |
| $V_{max}$ | $0.0026 \pm 0.0003 \text{ min}^{-1}$ | $0.08 \pm 0.05 \text{ s}^{-1}$ | 2000 |
| $k_1$ | $0.0026 \pm 0.0003 \text{ min}^{-1}$ | $0.08 \pm 0.05 \text{ s}^{-1}$ | 2000 |
| $K_m$ | $1600 \pm 500 \mu\text{M}$ | $180 \pm 20 \mu\text{M}$ | 0.1 |
| $K_d$ | $1600 \pm 500 \mu\text{M}$ | $180 \pm 20 \mu\text{M}$ | 0.1 |

a Values for the kinase reaction of (pp)ERK2 are from Prowse et al. (8).
b Substrate is MBP.

No viscosity effect on this parameter was observed. Thus, the lack of a viscosity effect on both $k_{cat}/K_m^{(MBP)}$ and $k_{cat}/K_m^{(ATP)}$, as well as on $k_{cat}$, supports a kinetic scheme in which both substrates exist in rapid equilibrium with the ternary Michaelis complex, whose breakdown to form products is entirely limited by phosphoryl group transfer. In such a model, the true affinities ($K_m$) of both substrates to form the ternary complex are given by their $K_m$ values. Thus, our steady-state data reveal that dual phosphorylation enhances the affinity of ATP binding by approximately 12-fold, whereas the affinity for MBP is enhanced $\approx 100$-fold.

**ATPase Activity**—An important consequence of dual-phosphorylation is the optimization of the alignment of the invariant residues, Lys$^{52}$, which coordinates to the $\alpha$- and $\beta$-phosphates of ATP, and Glu$^{69}$ which stabilizes Lys$^{52}$, in (pp)ERK2 (7). To determine the relative contribution of these interactions to the overall increase in the phosphoryl group transfer step of the kinase reaction, we compared the fold increase in catalytic parameters of the kinase reaction to those of the ATPase reaction (Table I). The steady-state parameters and individual rate constants for the ATPase reaction for both (non-p)ERK2 and (pp)ERK2 were determined by solvent viscometric analysis (see “Experimental Procedures”). Similar to the kinase reaction, the major effect of dual phosphorylation was a large (2000-fold) increase in the phosphotransfer rate, whereas the observed increase in ATP binding affinity was only 12-fold. However, the 2,000-fold increase in the rate of phosphotransfer is significantly less than the 60,000-fold rate enhancement of this step seen in the kinase reaction. This finding suggests that the net stabilization of the transition-state complex for phosphoryl group transfer to MBP occurs only in part by stabilization of the ATP moiety and that significant stabilization of the protein phosphoacceptor substrate moiety must also occur.

**Mechanism of Activation by Dual Phosphorylation**—We previously characterized the kinetic reaction pathway of the dual-phosphorylated form of ERK2 (8). In comparison to (pp)ERK2, (non-p)ERK2 displays $k_{cat}/K_m^{(MBP)}$ and $k_{cat}$ values that are decreased by approximately 600,000- and 50,000-fold, respectively (Table I). Yet, the $K_m$ values for MBP and ATP are each increased by only 10-fold. Therefore, the exceedingly low activity of (non-p)ERK2 is due to the dramatically decreased rates of substrate capture and turnover and not the inability to saturate the enzyme with substrate under steady-state conditions.

The extreme rate enhancements caused by dual phosphorylation are the most dramatic of any protein kinase for which the kinetic basis for activation by phosphorylation has been investigated. The large increases in catalytic efficiency and turnover rate are attributable to an approximate 60,000-fold increase in the rate of phosphotransfer, 12-fold higher binding affinity for ATP, and a minimum 100-fold higher affinity for MBP. The ability to bind MBP more tightly correlates to a remodeling of the $\text{P}_{\perp}$ surface pocket that functions to bind the essential proline residue found in all ERK2 substrates; this is achieved in part by hydrogen bonding between the phosphoryl group oxygens of PO$_{\gamma}$-Tyr$^{185}$ and the side chains of Arg$_{180}$ and Arg$_{182}$ (7). Although our data provides only a lower limit value on the absolute enhancement in substrate binding affinity, it should be noted that tighter binding of the substrate to (pp)ERK2 will not result in higher catalytic efficiency, because the rate of MBP binding to (pp)ERK2 already occurs at the diffusion-controlled limit (8). Thus, the catalytic efficiency of the dual-phosphorylated enzyme depends only on the rate of substrate encounter.

Dual phosphorylation results in a rotation of the N- and C-terminal lobes, which closes the active site cleft and optimizes the alignment of the essential catalytic residues, Lys$^{52}$ and Glu$^{69}$ (7). This results in a 12-fold tighter binding of ATP to the enzyme, and a 2000-fold increase in the rate of phosphoryl group transfer with respect to the ATPase reaction. Nonetheless, this increase in phosphotransfer rate is approximately 30-fold down from that seen in the kinase reaction, demonstrating that stabilization of the phosphate moieties of ATP accounts for only a portion of the overall enhanced rate of chemistry with respect to the phosphorylation of protein substrates.

Correlation of the ATPase and kinase activities of ERK2 reveals similarities in its mechanism of activation to that of Cdk2/cyclin A. For example, activation of both enzymes involves two steps. In Cdk2/cyclin A, these steps are cyclin binding followed by phosphorylation at Thr$^{160}$ (5), whereas in ERK2, phosphorylation at Tyr$^{185}$ followed by phosphorylation at Thr$^{183}$ is required (6, 17). In Cdk2/cyclin A, the rate of phosphotransfer in the ATPase reaction is unchanged by phosphorylation at Thr$^{183}$ (18). This is consistent with crystallographic information which shows that the role of phosphorylation at Thr$^{183}$ is not to align the conserved lysine (Lys$^{185}$) and glutamate (Glu$^{181}$) side chains, thus stabilizing ATP. Instead, alignment of these residues in Cdk2 is achieved by the binding of cyclin. However, the rate of the same step in the kinase reaction of Cdk2/cyclin A is achieved nearly 3000-fold (18). Thus, although cyclin binding serves to stabilize the ATP portion of the transition state for phosphoryl group transfer, phosphorylation at Thr$^{160}$ serves to stabilize the protein phosphoac-
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ceptor group. Thr\textsuperscript{160} in Cdk2 and Thr\textsuperscript{183} in ERK2 occupy structurally analogous roles (4); both coordinate to a positively charged triad of arginine side chains that bridge the N- and C-terminal lobes of the catalytic cores. Thus, it is possible that phosphorylation at Thr\textsuperscript{183} in ERK2 may function analogously to that at Thr\textsuperscript{160} in Cdk2/cyclin A, whereas phosphorylation at Tyr\textsuperscript{185} in ERK2 may play a role analogous to cyclin binding.

In summary, we have demonstrated that dual phosphorylation of ERK2 results in 10–100-fold greater rate enhancements compared with other protein kinases in which activation is dependent on phosphorylation at only a single site. Most of the increase in catalytic power in ERK2 is attributable to an increased rate of phosphotransfer, which is coordinately accomplished by two independent mechanisms: 1) stabilization of the phosphate moieties of ATP via alignment of Lys\textsuperscript{52} and Glu\textsuperscript{69} and 2) stabilization of the protein phosphoacceptor group. In Cdk2, we propose that these may be separately achieved by cyclin binding and phosphorylation at Thr\textsuperscript{160}, respectively (18). We hypothesize that a similar mechanism of activation may be functionally conserved in ERK2, except that stabilization of the ATP and protein phosphoacceptor groups may instead be controlled separately by phosphorylation at Tyr\textsuperscript{185} and Thr\textsuperscript{183}.

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