The Complete Pathway for Catalytic Activation of the Mitogen-activated Protein Kinase, ERK2*

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The mitogen-activated protein (MAP) kinase ERK2 is an essential signal transduction molecule that mediates extracellular signaling by all polypeptide growth factors. Full activation of ERK2 requires phosphorylation at both a threonine residue (Thr183) conserved in most protein kinases as well as a tyrosine residue (Tyr185) unique to members of the mitogen-activated protein kinase family. We have characterized the kinetic role of phosphorylation at each site with respect to the overall activation mechanism, providing a complete picture of the reaction steps involved. Phosphorylation at Tyr185 serves to configure the ATP binding site, while phosphorylation at both residues is required to stabilize binding of the protein substrate, myelin basic protein. Similar control mechanisms are employed to stabilize ATP and myelin basic protein in the phosphoryl group transfer reaction, accounting for the enormous increase in turnover rate. The mechanism of ERK2 activation is kinetically similar to that of the cell cycle control protein, cdk2/cyclinA. Phosphorylation of Tyr185 in ERK2 and association of cyclinA with cdk2 both serve to stabilize ATP binding. Subsequent phosphorylation of both enzymes on threonine serves to stabilize binding of the phosphoacceptor substrate.

Protein phosphorylation is the central mechanism for regulation of signal transduction. Consequently, the enzymes that catalyze these reactions, known as the protein kinases, are themselves necessarily subject to extensive regulatory control. To date, the established mechanisms for kinase regulation include protein-protein interactions (e.g. inhibition of PKA by the RI/RII regulatory subunits; activation of calmodulin-dependent kinases by Ca2+/calmodulin), and interaction of the catalytic core with autoinhibitory domains (e.g. smooth muscle myosin light chain kinase, PKC, pp60c-src, twitchin). In such cases, the relief of kinase inhibition is often achieved by interaction with second messengers (e.g. cAMP binding to RI/RII subunit of PKA, Ca2+ and diacylglycerol binding to PKC) or by protein phosphorylation (e.g. Ca2+/calmodulin-dependent kinase II; pp60c-src). The common theme in all cases is kinase activation by relief of competitive pseudo-substrate inhibition.

A separate class of regulatory mechanisms includes modifications that cause remodeling of the active site, resulting in altered turnover rates in addition to possible altered substrate binding affinity. The prevailing example is the activation of most protein kinases by phosphorylation at a single conserved residue in an “activation loop” structure located near the mouth of the active site (1). The most elaborate mechanism of this type is seen in the mitogen-activated protein (MAP) kinases, whose catalytic activation requires dual (tyrosine and threonine) phosphorylation (2), as opposed to phosphorylation at a single site.

The MAP kinase family comprises the ERK, JNK, and p38 subfamilies of kinases. All participate in three tier protein phosphorylation cascades that serve to mediate diverse cellular processes in response to extracellular signals. For example, the JNKs and p38 kinases serve primarily to mediate the effects of cellular stresses, inflammation, and apoptosis, while the ERKs are activated in response to proliferation and differentiation factors, mediated by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors, cytokine receptors and integrins. The prototypic member of the MAP kinase family is the extracellular-regulated kinase 2 (ERK2), whose activation requires phosphorylation at residues Tyr185 and Thr183 catalyzed by the upstream kinases MEK1 or MEK2. The direct targets of ERK2 classically include downstream protein kinases, nuclear transcription factors, cytoskeletal proteins, and other regulatory molecules. ERK2 is thus a crucial element for maintenance of cell homeostasis in response to changes in extracellular environment (for review see Refs. 3–5).

Because of their central role in signal transduction, the regulation and function of the MAP kinases has been the subject of intense interest since their discovery. As a result, abundant molecular biological, biochemical, and recently, structural information has accrued on ERK2 in particular. By comparison, little to no mechanistic information has been acquired on this enzyme, precluding correlation of its structure with its biological function. Thus, the detailed mechanisms by which changes in structure are linked to catalytic regulation are not known. Here, we provide the first description of the complete kinetic pathway for activation of the MAP kinase, ERK2. Phosphorylation at Tyr185 acts as a switch that independently controls ATP binding, while phosphorylations at both Tyr185 and Thr183 serve to stabilize the phosphoacceptor substrate. Our studies set the framework by which the structure/activity relationships of other MAP kinases, or protein kinases in general, may be defined or ultimately predicted.

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§ The abbreviations used are: PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; cdk, cyclin-dependent kinase; unP, unphosphorylated.
Mechanism of ERK2 Activation

MATERIALS AND METHODS

Materials

All chemicals were purchased from Fisher, except for ATP (Sigma), and [γ-32P]ATP (ICN).

Protein Expression and Purification

Myelin Basic Protein (MBP) Preparation—MBP was purified from a bovine brain acetone powder (Sigma B5058) by acid extraction followed by cation exchange chromatography as described previously (6).

MEK1 Expression and Purification—Recombinant human MEK1 (G7B; N4/S218D/M219D/N221D/S222D) was expressed in Escherichia coli and purified as previously described (6).

Mono-phosphorylated ERK2 Proteins—The plasmid encoding ERK2(T183A) was provided by Dr. Natalie Ahn. ERK2(Y185F) was generated by substitution of Tyr185 in wild-type ERK2 to phenylalanine using the QuikChange™ site-directed mutagenesis kit (Stratagene). ERK2 mutants were expressed in E. coli strain BL21 DE3 using the expression vector NpT7-5 with an incorporated N-terminal hexahistidine tag. Conditions for expression and purification were as previously described for wild-type ERK2(6). Briefly, mutants of ERK2 were purified by Ni2+-NTA affinity chromatography followed by FPLC Uno Q Bio-Rad then subject to kinetic analysis. Alternatively, the Ni2+-purified material was subject to in vitro phosphorylation by recombinant MEK1 for 2–3 h and then purified by chromatography over FPLC Uno Q Bio-Rad. The purified mono-phosphorylated proteins were subject to analysis by electrospray ionization mass spectrometry.

The mass analysis data are presented in Table 1.

The concentration of all ERK2 enzymes were determined spectrophotometrically based on an extinction coefficient (ε = 44,825 cm−1 M−1) calculated from its primary amino acid sequence and verified by SDS-PAGE densitometry using bovine serum albumin as a standard.

Kinase Assays and Data Analysis

Kinase activity was monitored by a radioisotope assay using MBP as substrate (6). 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, containing 0.1 μM [γ-32P]ATP, or 0.7 μM [γ-32P]ATP) for ERK2 or 0.13 μM [γ-32P]ATP for MEK1. Reactions were initiated by the addition of [γ-32P]ATP (500–1000 cpm/pmol) and allowed to proceed at 23°C for 30–60 min, after which time reactions were terminated with 25% acetic acid. The [γ-32P]MBP product was resolved from unincorporated [γ-32P]ATP by ascending chromatography on P81 phosphocellulose paper (Whatman), as described previously (6). Radioactivity in MBP was quantified by ascending chromatography on P81 phosphocellulose paper (Whatman) and verified by SDS-PAGE densitometry using bovine serum albumin as a standard.

| Enzyme | –MEK1 | +MEK1 | Mass difference |
|--------|--------|--------|----------------|
| ERK2(T183A) | 42144.0 ± 5.0 | 42214.0 ± 5.0 | 80 |
| ERK2(Y185F) | 42155.0 ± 5.0 | 42224.0 ± 5.0 | 79 |

*Mass units are in daltons.

ATPase Assays

ATPase activity of [Thr(P)183]ERK2 was determined using a radioisotope assay as described previously (6). Reactions were performed in phosphorylation buffer for a total volume of 10 μl at 23°C, typically, containing 3 μM ERK2 were initiated by the addition of [γ-32P]ATP (500 cpm/pmol) and then allowed to proceed for 2 h at 23°C, typically and then terminated at 1 ml of 0.1 N HCl.

ATPase activity of [Thr(P)183]ERK2 was determined using a coupled spectrophotometric assay, as described previously (6). The concentrations of the coupling reagents were as follows: 15 units/ml lactate dehydrogenase, 7.5 units/ml pyruvate kinase, 1 mM P-enolpyruvate, and 130 μM NADH. All reactions were performed in phosphorylation buffer with coupling reagents in a total volume of 75 μl at 23°C. Reactions were initiated by addition of 0.5 μM [Thr(P)183]ERK2 to the reaction mix containing ATP at various concentrations. Steady-state kinetic parameters for both [Thr(P)183]ERK2 and [Thr(P)183]ERK2 were determined from non-linear regression analysis of initial velocities as a function of ATP concentration using the Michaelis-Menten equation.

**Solvent Viscosimetric Studies**

Steady-state assays were carried out in buffer containing 0–40% sucrone, giving relative solvent viscosities of 1 to 4.2, respectively. Relative solvent viscosity was determined as previously described (6). A data set of initial velocities under conditions of saturating (or near saturating) ATP; varied MBP, and varied sucrose concentrations was obtained. Equation 2 relates the reaction velocity to the substrate concentration at any given relative solvent viscosity (see Refs. 8, 9).

\[
\eta = \frac{v}{v_{	ext{cat}}} = \frac{k_{\text{cat}}}{K_M + [S]} = \frac{k_{\text{cat}}}{K_M + \text{sol}}
\]

where \(v\) is the initial velocity, \(E\) is the enzyme concentration, \([S]\) is the MBP concentration, and \(\eta\) is the relative solvent viscosity. The kinetic constants \(k_{\text{cat}}, k_{\text{cat}}/K_M\), and \(k_{\text{cat}}/K_M\) are as defined in Scheme 1 and relate to the steady-state parameters as follows:

\[
k_{\text{cat}} = k_3 \times k_4 (k_3 + k_4)
\]

Optimal values for \(k_{\text{cat}}\), \(k_{\text{cat}}/K_M\), and \(k_{\text{cat}}/K_M\) were determined from the linear function of relative solvent viscosity (Figs. 2 and 4). The corresponding slope values provide the “viscosity effects” on \(k_{\text{cat}}\) (Eq. 5) or \(k_{\text{cat}}/K_M\) (Eq. 6).

Equation 2 was globally fit to the data, and a set of best-fit curves was obtained by non-linear regression analysis (not shown). The apparent and the fold decreases in \(k_{\text{cat}}\) (app) and \(k_{\text{cat}}/K_M\) (app) were plotted as a linear function of relative solvent viscosity (Figs. 2 and 4).

**RESULTS AND DISCUSSION**

We previously determined the net consequences of dual-phosphorylation on the kinetic mechanism of ERK2 activation (6, 7). In comparison to the unphosphorylated enzyme, [PPi]ERK2 displays a phosphoryl group transfer rate that is increased 60,000-fold and binding affinities for MBP and ATP that are increased by at least 100-fold and 12-fold, respectively. The net effect of these alterations is an approximate 50,000-fold increase in turnover rate \(k_{\text{cat}}\) with moderate decreases in the \(K_M\) values for both MBP (10-fold) and ATP (12-fold). A model of sequence of dual phosphorylation on the basis in the commitment of MBP to catalytic turnover. In the case of [PPi]ERK2, once bound to the active site, MBP is completely committed to phosphorylation. In contrast, the release rate of MBP from the active site significantly exceeds the rate of turnover in [unP]ERK2.

**Role of Phosphorylation at Tyr185**—To characterize the role of phosphorylation at Tyr185 separately from that at...
The initial rate of MBP phosphorylation was determined at varying concentrations of MBP and several fixed concentrations of ATP (18.8, 37.5, 75, 150, 300 μM; top to bottom). Data were analyzed as described under “Materials and Methods.” The concentration of enzyme was 0.1 μM.

Thr183, we constructed a mutant of ERK2 in which Thr183 was substituted with alanine. The steady-state kinetic parameters of the unphosphorylated form of this mutant proved to be identical to the unphosphorylated wild-type enzyme (Table II).

To generate the Tyr185 mono-phosphorylated form of ERK2 ([Tyr(P)185]ERK2), ERK2(T183A) was subjected to in vitro phosphorylation by recombinant MEK1, followed by purification of the phosphorylated enzyme by anion exchange chromatography. A single peak of protein eluted, and all fractions corresponding to the protein peak displayed identical specific enzyme activities toward MBP. Analysis of each column fraction by SDS-PAGE showed a single protein band that corresponded to ERK2 (42 kDa), and analysis by electrospray ionization mass spectrometry confirmed that the enzyme was phosphorylated to a stoichiometry of one mole of phosphate per mole of enzyme (see “Materials and Methods”).

The conventional substrate for characterization of MAP kinase activity has traditionally been MBP. Under initial rate conditions, the phosphorylation of MBP by ERK2 occurs at a single site (Thr183) (10), which displays a specificity constant among the highest of all known ERK2 substrates (kcat/Km = 2.4 μM⁻¹ s⁻¹) (6). We determined the steady-state kinetic parameters of [Tyr(P)185]ERK2 with respect to the phosphorylation of MBP (Fig. 1). The overall rate of substrate processing (kcat) was near 1000-fold higher than that of [unP]ERK2 and 50-fold lower than that of the fully active [PP]ERK2. The Km values for ATP, which is 12-fold higher in [unP]ERK2 compared with [PP]ERK2, was completely restored by phosphorylation at Tyr185 alone. Phosphorylation at Tyr185 did not affect the Km value for MBP.

To determine the consequence of Tyr185 phosphorylation on the individual kinetic steps of the reaction pathway, solvent viscometric experiments were carried out. In such experiments, initial-rate measurements are performed in buffer containing increasing concentrations of a microviscosogen (8, 11). We have employed sucrose as the microviscosogenic agent and, under such conditions, all kinetic steps (Scheme 1) can be resolved (9). Scheme 1 depicts the addition of substrate MBP to the enzyme-ATP complex. As practiced by tradition, substrate binding (k2, k−2) can be separated from product release (k4) by varying the substrate concentration because the net reaction rate at low substrate concentration is given by [kcat/Km] = k−2k4/k3 + k4, while at high substrate levels it is given by [kcat/Km] = k−2k4/k3 + k4 (12). The steady-state rate parameters can be further resolved into their respective microscopic kinetic parameters by varying the relative solvent viscosity, allowing those reactions that are diffusive in nature (k2, k−2, and k4) to be separated from those that are not (k1).

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whereas the rate of substrate dissociation from the enzyme-substrate complex ($k_2$) is not.

Initial velocity data were collected under conditions of saturating ATP, varied MBP, and varied relative solvent viscosities ranging from 1 to 4.2 (0–40% sucrose). Viscosity effects on $k_{cat}$ for both substrates, as well as on $k_{cat}$ (Fig. 2, A–C), were determined, and the kinetic constants of all steps along the reaction pathway were calculated (see “Materials and Methods”). The derived rate constant values, as well as the true thermodynamic $K_p$ values ($k_2/k_3$) predicted for both ATP and MBP binding to [Tyr(P)185]ERK2, are shown in Table III. Phosphorylation at Tyr185 completely restores the affinity of ATP binding to that seen in the dual-phosphorylated enzyme, has no effect on the binding of MBP, and increases the rate of phosphoryl group transfer by ~1000-fold.

**Role of Phosphorylation at Threonine 183**—To characterize the role of phosphorylation at Thr183 separately from that at Tyr185, a mutant of ERK2 was generated in which Tyr185 was substituted with phenylalanine. The unphosphorylated mutant, ERK2(Y185F), displayed steady-state kinetic properties identical to that of unphosphorylated wild-type [unP]ERK2 (Table II). ERK2(Y185F) was subject to in vitro phosphorylation by MEK1 followed by purification by anion exchange chromatography. Analysis by SDS-PAGE and electrospray ionization mass spectrometry confirmed that the purified enzyme was effectively homogenous and was stoichiometrically phosphorylated to a ratio of one mole of phosphate per mole of enzyme (see “Materials and Methods”).

[Tyr(P)185]ERK2 was subject to the same routine of steady-state (Fig. 3) and solvent viscometric (Fig. 4, A–C) analyses as was performed on [Tyr(P)185]ERK2. The steady-state kinetic parameters derived for [Tyr(P)185]ERK2 are listed in Table II. Kinetic constants for the individual reaction steps are listed in Table III. Phosphorylation at Thr183 resulted in increases in the rate of phosphoryl transfer and the overall turnover rate, both of ~80-fold, and a partial (2.5-fold) restoration of the low $K_{cat}$ defined by [PP]ERK2. Notably, the value for $K_{cat}$ was essentially unchanged from that of [unP]ERK2. Since no viscosity effects were seen on $k_{cat}/K_m$ or $k_{cat}$, we conclude that phosphorylation at Thr183 does not affect ATP binding affinity.

Phosphorylation of Thr183 resulted in an increase in the commitment of MBP to catalytic turnover. Catalytic commitment reflects the extent to which the net rate of substrate binding ($k_{cat}/K_m$) is limited by diffusion, and is given by the viscosity effect on $k_{cat}/K_m$ ($k_{cat}/K_m$). The value of $k_{cat}$ ($K_m$) (given by the slope of the line in Fig. 4C) corresponds to the propensity for MBP to undergo phosphoryl group transfer as opposed to dissociation from the active site. $K_m = k_f/(k_{-k} + k_f)$. The effect of phosphorylation on the catalytic commitment of MBP can be seen as a progressive increase in $k_{cat}/K_m$ as ERK2 is phosphorylated on Thr183 and then at Tyr185 (Fig. 4C). The values for $k_{cat}/K_m$ for [unP]ERK2, [Thr(P)183]ERK2, and [PP]ERK2 are ~0 (Ref. 7), 0.1 (Fig. 4C) and ~1 (Ref. 6). The increased catalytic commitment of MBP in response to phosphorylation at Thr183 is attributable mostly to the 80-fold increase in the rate of phosphoryl group transfer, as opposed to only minor changes in MBP binding affinity.

**Mechanism of Catalytic Activation**—Knowledge of the catalytic reaction pathway for all phosphorylated forms of ERK2 allows a detailed kinetic analysis of the mechanism by which dual phosphorylation results in catalytic activation. We previously characterized the net effects of dual phosphorylation on the kinetic parameters of ERK2 (Tables II and III) (6, 7). Here we describe the separate roles of phosphorylation at each site on the overall activation process.

The complete activation of ERK2 by dual phosphorylation involves substantial increases in binding affinities ($k_{cat}/K_m$) of both ATP and MBP (12- and >100-fold, respectively). Phosphorylation at Tyr185 alone is sufficient to induce maximal ATP binding affinity and, on its own, has no effect on the binding of MBP. Conversely, ATP binding is not affected by phosphorylation at Thr183. In addition, the fold change in ATP affinity induced by Tyr185 phosphorylation is not affected by bound

**Table III**

| Kinetic constants for MBP phosphorylation |
|------------------------------------------|
| [unP]ERK2 | [P-Tyr185]ERK2 | [P-Thr183]ERK2 | [PP]ERK2 |
| $k_{cat}$ | 0.003 ± 0.048 | 0.01 ± 0.02 | 0.029 ± 0.004 | 0.18 ± 0.05 |
| $k_{cat}/K_m$ | 0.04 ± 0.02 | −0.02 ± 0.05 | 0.10 ± 0.02 | 1.0 ± 0.1 |
| $k_{cat}/K_m$ | 0.01 ± 0.05 | 0.3 ± 0.05 | 0.03 ± 0.02 | −0.08 ± 0.04 |
| $k_{cat}/K_m$ | 0.01 ± 0.05 | 0.3 ± 0.05 | 0.03 ± 0.02 | −0.08 ± 0.04 |

$^a$ Data are taken from Ref. 7.

$^b$ Data are taken from Ref. 6.
that 1) enhance ground-state binding of ATP and 2) optimize the possible basis for the limited (1000-fold) activation of the transition-state complex according to classical theory. To explain may therefore be attributed to relative stabilization of the phosphoryl group transfer in response to dual phosphorylation state Michaelis complex (13). The 60,000-fold increased rate of the transition-state structure relative to the ground-classically accounted for by hypothesizing active site stabilization of the rate of the chemical transfer step (7). Enzymatic catalysis is affinities, dual phosphorylation causes a dramatic increase in with the other in a highly synergistic fashion to achieve greater MBP binding (Fig. 5). Thus, each phosphorylation site interacts and changes in ATP binding affinity (Fig. 5).

In contrast, the binding of MBP is dependent upon phosphorylation at both Tyr185 and Thr183. Moreover, phosphorylation at either site alone shows little to no effect on the energetics of MBP binding (Fig. 5). Thus, each phosphorylation site interacts with the other in a highly synergistic fashion to achieve greater than 100-fold overall enhancement in MBP binding affinity.

In addition to the significant increases in substrate binding affinities, dual phosphorylation causes a dramatic increase in the rate of the chemical transfer step (7). Enzymatic catalysis is classically accounted for by hypothesizing active site stabilization of the transition-state structure relative to the ground-state Michaelis complex (13). The 60,000-fold increased rate of phosphoryl group transfer in response to dual phosphorylation may therefore be attributed to relative stabilization of the transition-state complex according to classical theory. To examine the possible basis for the limited (1000-fold) activation of this step in response to phosphorylation at Tyr185, we characterized the kinetics of the ATPase reaction. The rate of phosphoryl group transfer to water in the absence of MBP was isolated using solvent viscometric techniques. The phosphoryl transfer rate was maximally enhanced by phosphorylation at Tyr185 alone, as phosphorylation at Thr183 alone had no effect on ATPase kinetics (Table IV, Fig. 5). Furthermore, the fold increase in phosphoryltransfer rate in both the ATPase (2000-fold) and kinase (1000-fold) reactions was similar (Fig. 5), consistent with the hypothesis that activation is due to stabilization of similar chemical structures. These data suggest that phosphorylation at Tyr185 serves to induce structural changes that 1) enhance ground-state binding of ATP and 2) optimize alignment of ATP for phosphotransfer to different acceptors. In contrast, phosphorylation at Thr183 enhances only phenomena specifically linked to MBP, perhaps through optimizing alignment of this substrate in both the ground and transition states as well (Fig. 6).

Comparison to cdks/cyclinA—While the MAP kinases classically mediate early responses to mitogen signals, the family of cell cycle control proteins, known as the cyclin-dependent kinases (cdks), serves to directly control progression through the cell division cycle (14). In addition to the central role that both enzyme families play in cell cycle control, the MAP kinases and cdks share a number of similarities in their structures and catalytic functions. For example, they display significant sequence homology to each other, share similar substrate sequence specificities (15, 16), and employ two-step mechanisms for catalytic activation. Based on protein kinase structures solved to date, phosphorylation at the conserved threonine residue in the activation loop serves in general to configure this loop structure by coordinating to three positively charged side chains that arise from the small and large lobes of the catalytic core (1, 17, 18). Unique to the MAP kinases as well as the cdks, however, is an additional regulatory step in their pathways to catalytic activation. In the MAP kinases such a step corresponds to tyrosine phosphorylation, while in the cdks the equivalent step is the binding of cyclin. We have found that Tyr185 phosphorylation of ERK2 and cyclinA binding to cdk2—serve similar kinetic roles with respect to catalytic activation. Both modifications result in maximal increases in affinity for ATP and in the rate of phosphotransfer to water. In both enzymes, these kinetic parameters are unaffected by subsequent threonine phosphorylation, which instead serves to align the phosphoacceptor substrate. Thus, two enzymes employing biochemically distinct mechanisms for regulation achieve catalytic activation through kinetically similar means.

Crystallographic data on ERK2 offers some insights regarding possible kinetic consequences of the observed structural changes that occur upon dual phosphorylation (17, 19). Phosphorylation at Thr183 contributes to a large restructuring of the
Mechanism of ERK2 Activation

Table IV

| Kinetic constants for ATPase reaction |
|---------------------------------------|
| [unP]ERK2 | [P-Tyr<sup>185</sup>]ERK2 | [P-Thr<sup>183</sup>]ERK2 | [PP]ERK2<sup>a</sup> |
| <br>
| $k_{cat}$ (min<sup>-1</sup>) | 0.0026 ± 0.0003 | 7.7 ± 0.5 | 0.008 ± 0.0005 | 5.0 ± 3.0 |
| $k_{cat}/K_m$ | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.01 ± 0.00 | 0.04 ± 0.05 |
| $K_m$ (mM) | 0.0026 ± 0.0003 | 7.7 ± 0.5 | 0.008 ± 0.0005 | 5.0 ± 3.0 |
| $K_m$ (ATP, mM) | 1.6 ± 0.5 | 1.0 ± 0.5 | 1.0 ± 0.8 | 0.12 ± 0.02 |
| $K_m$ (ATP, mM) | 1.6 ± 0.5 | 1.0 ± 0.5 | 1.0 ± 0.8 | 0.12 ± 0.02 |

<sup>a</sup> Most of the data are taken from Ref. 6.

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Our data support a simple model by which dual phosphorylation of ERK2 achieves catalytic activation. The complete pathway involves phosphorylation at Tyr<sup>185</sup>, which serves to control ATP binding, while phosphorylation at both sites is required for binding of MBP. A similar mechanism is likely used to stabilize the transition-state complex for phosphoryl group transfer. This hypothesis is based on three lines of evidence. 1) Kinetic isolation of the phosphotransfer reaction in the ATPase pathway demonstrates that maximal stabilization of the transition-state is achieved by phosphorylation at Tyr<sup>185</sup> alone. This is likely to be true in the kinase pathway as well, since 2) MBP does not influence the effect of Tyr<sup>185</sup> phosphorylation on ATP binding in the ground-state, and 3) the fold enhancement of the phosphotransfer rate by Tyr<sup>185</sup> phosphorylation is similar in both the ATPase and kinase pathways, consistent with similar chemical structures being stabilized in both reactions. In all, the evidence suggests that stabilization of nucleotide binding and orientation are achieved exclusively by phosphorylation at Tyr<sup>185</sup>, while phosphorylation at Thr<sup>183</sup> serves solely to align the protein phosphoacceptor species. Our data demonstrate functional conservation in the catalytic mechanisms for regulation of ERK2 and cdk2/cyclinA that is revealed only at the kinetic level.

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Activation loop (17), whose conformation may be predicted to be critical for binding MBP and other protein substrates. A link between phosphorylation, restructuring of the activation loop, and substrate binding affinity has been observed in cdk2/cyclinA, in which significant changes in activation loop structure induced by phosphorylation at Thr<sup>210</sup> (equivalent to Thr<sup>182</sup> in ERK2) (18, 20) correlates with a 50-fold increase in affinity for a peptide substrate (21) (also see Ref. 22). In addition, the phosphoryl group oxygens on phospho-Tyr<sup>185</sup> in [PP]ERK2 coordinate to the side chains of two arginine residues optimizing the conformation of a pocket that binds the substrate Pro<sup>1</sup> proline residue (17). Together, these structural changes in response to dual phosphorylation likely account for much of the enhanced (≥100-fold) binding affinity with respect to MBP.

In all protein kinases, an invariant active site lysine residue (Lys<sup>52</sup> in ERK2) plays a critical role in stabilizing the transition-state by coordination to the α- and β-phosphoryl group oxygens of ATP (see Ref. 23). Substitution of this residue to alanine in ERK2 results in a dramatic reduction in turnover rate, with little effect on ground state ATP binding (24). Similarly, in [unP]ERK2, Lys<sup>52</sup> does not make contact with ATP (19), and this protein displays dramatically reduced phosphotransfer kinetics with only moderate decreases in ATP affinity (7). Although the crystal structure of [PP]ERK2 does not contain ATP, it is presumed that dual phosphorylation acts in part to optimize the alignment of Lys<sup>52</sup>.

Both crystallographic (23) and biochemical studies (25) on PKA show that ATP binding affinity arises largely from hydrogen bonding and hydrophobic interactions with the ATP adenine ring. The equivalent interactions between ATP and ERK2, as well as those involving Lys<sup>52</sup>, are some 20 Å away from the phosphoryl group on Tyr<sup>185</sup>. Thus, phospho-Tyr<sup>185</sup> must communicate with the ATP binding site through a long-range atomic network in the protein. Similar long-range communication with ATP has been reported in PKA (26). A definitive correlation between Tyr<sup>185</sup> phosphorylation, changes in enzyme structure, and ATP binding must necessarily await a crystal structure of [P-Tyr<sup>185</sup>]ERK2.

Fig. 6. Role of phosphorylation at Tyr<sup>185</sup> and Thr<sup>183</sup> on the stabilization of ground-state and transition-state structures along the kinase reaction pathway. Phospho-Tyr<sup>185</sup> serves to stabilize ATP in the ground-state Michaelis complex, while phospho-Thr<sup>183</sup> and phospho-Thr<sup>185</sup> are required for stabilization of MBP. Similar roles for each phospho-amino acid are predicted in stabilizing the analogous transition-state structures for phosphoryl group transfer. However, while phospho-Thr<sup>183</sup> is required to stabilize MBP in the transition-state, the relative contribution from phospho-Thr<sup>185</sup> cannot be determined since the E-MBP transition-state complex cannot be isolated. Phospho-Tyr<sup>185</sup>, Tyr<sup>(P)</sup>Thr<sup>183</sup>, pThr<sup>183</sup>, Thr<sup>(P)</sup>Thr<sup>183</sup>.
Mechanism of ERK2 Activation

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