Transient Protein-Protein Interactions and a Random-ordered Kinetic Mechanism for the Phosphorylation of a Transcription Factor by Extracellular-regulated Protein Kinase 2*

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No thorough mechanistic study of extracellular signal-regulated protein kinase 2 (ERK2) has appeared in the literature. A recombinant protein termed EtsΔ138, which comprises of residues 1–138 of the transcription factor Ets-1 is an excellent substrate of ERK2 (Waas W. F., and Dalby, K. N. (2001) Protein Exp. Purif. 23, 191–197). The kinetic mechanism of ERK2 was examined, with excess magnesium, by initial velocity measurements, both in the absence and presence of products at 27 °C, pH 7.5, and ionic strength 0.1 M (KCl). The velocity data are consistent with a steady-state random-ordered ternary complex mechanism, where both substrates have unhindered access to binding sites on the enzyme. The mechanism and magnitude of product inhibition by monophosphorylated EtsΔ138 is consistent with, but does not prove, the notion that ERK2 forms a discrete interaction with EtsΔ138 in the absence of active site interactions, and that this “docking complex” facilitates intramolecular phosphorylation of the substrate. The approximation of the steady-state data to a rapid equilibrium model strongly suggests that the formation of ERK2-EtsΔ138 complexes are transient in nature with dissociation constants of greater magnitude than the catalytic constant, of $k_{cat} = 17 \text{ s}^{-1}$. 

Extracellular regulated protein kinase 2 (ERK2)^1 is a prominent, ubiquitously expressed, 42-kDa cellular protein kinase, which is strongly activated by phorbol esters, growth factors, and serum (1). It is also activated, albeit more weakly, by a diverse range of general and cell type-specific stimuli, such as cell stresses, various cytokines, and insulin (1). ERK2 is some 85% identical in sequence (44 kDa) to ERK1 (2), which displays a similar profile of activation, activity, and expression. Both are associated with a myriad of biological processes, which include exit of cells from G0 into the G1 stage of the cell cycle, proliferation, mammalian synaptic plasticity and learning, glycogen metabolism, and many events that are often specific to certain tissues (3).

ERK2 is activated through a protein kinase cascade termed the mitogen-activated protein kinase (MAPK) pathway, usually by Ras, a small guanine nucleotide-binding protein that transduces extracellular signals to the cell nucleus. Mutations in the ras oncogene family are commonly found in both human and animal cancers and in particular tumors of the lung, thyroid, pancreas, colon, and some leukemias (4). When unregulated, Ras signaling can lead to the development of both primary tumors and metastases through the sustained action of downstream effectors such as ERK2. Events leading to the activation of the ERKs appear to be highly orchestrated, but while many proteins have been shown to interact with the major protagonists of the MAPK pathway the precise functions of protein-protein interactions within it are poorly understood (5).

Defining and understanding protein-protein interactions mediated by MAP kinases has far reaching consequences for both the design of inhibitors and for the understanding of their catalytic mechanism and regulation. While many protein-protein interactions cover large surface areas, making 10–40 contacts throughout the interface of a complex (6), protein recognition by signaling proteins is often modular in nature, where short strings (usually less than 10) of amino acids are recognized by specific domains. In this respect MAP kinases appear to be no different and in recent years several laboratories have identified MAP kinase-interacting sequences within cellular proteins that mediate their binding to cognate MAP kinases (7–13). Kornfeld’s recent classification places the known domains into two main categories termed the DEJL domain (docking site for ERK and JNK, LXL) (which has the consensus sequence; K/R-X-K/R-K/R-X$_{19-27}$-L/I-X-L/I or K/R-K/R-K/R-X$_{19-30}$-L/I-X-L/I) and the DEF domain (docking site for ERK, FXFP) (which has the consensus sequence; FXFP) (14).

Like all MAP kinases ERK2 catalyzes the transfer of the γ-phosphate of adenosine triphosphate to serine or threonine residues that precede proline (15–16). While this specificity is critical, further specificity is thought to be conferred through substrate docking domains, such as the DEJL domain, where interactions are made between ERK2 and the substrate at sites on the enzyme that can lie a considerable distance (>30 Å) from the active site (10). Although progress in identifying these sequences and demonstrating their involvement in mediating specificity (17) has advanced our knowledge considerably, pre-
cishly how they contribute to regulation and catalysis is still unclear.

Recently, following work from Karin’s group (9) on JNK (Jun N-terminal protein kinases), Yang et al. (18) proposed a “targeting” model for the phosphorylation of the ets domain transcription factor Elk-1 by ERK2, where ERK2 is proposed to target Elk-1 by binding to the DEJL domain, to form a “docking” complex (labeled as D in scheme 1A), which is an intermediate in the catalytic mechanism. The essential points of this model are: that interactions between ERK2 and Elk-1, within a docking complex, are mediated exclusively by interactions that lie outside of the active site of the MAPK. The docking complex (D) rearranges to bring a phosphoacceptor motif (Ser-Pro or Thr-Pro) into the active site to form the catalytic complex (C). k_c. Phosphorylation then occurs, k_p, followed by the exchange of ATP for ADP and rearrangement to form a new docking complex (D’), k_d. Phosphorylation of a second site then results through a number of repeated steps as shown before the complex finally dissociates, k_d. B, two-step distributive mechanism for p38 MAPK (19). In the distributive mechanism intermediary phosphoproteins are released from p38 MAPKs after each phosphorylation. For further phosphorylation to occur the phosphorylated protein must bind to p38 MAPKs again. For both mechanisms the binding of ATP to the enzyme is shown to occur before the binding of the protein substrate, although random-ordered binding does occur.

To critically investigate the targeting model and the role of a docking domain in mediating protein-protein interactions with ERK2 during substrate turnover, we developed a kinetic assay for ERK2 using EtsΔ138 a protein substrate that contains several putative DEJL domains such as the sequence, ^15KTEKVDLEL^23 near its N terminus. In the presence of excess magnesium at pH 7.5, ERK2 catalyzes the reaction shown in Scheme 2. The mechanism of this ERK2 reaction was examined by initial velocity measurements in the absence and presence of products. This study defines, for the first time, the basic steady-state kinetic mechanism for ERK2 using a model protein substrate that contains a putative ERK2 docking domain. The velocity data are consistent with a steady-state random-ordered ternary complex mechanism, where both substrates have unhindered access to binding sites on the enzyme and support a transient docking mechanism shown in Scheme 3 (upper pathway). To our knowledge, this is the first report of such a mechanism for a mitogen-activated protein kinase.

The recognition of a modular docking sequence represents an interesting, but not unique solution to the problem of multiprotein substrate recognition by enzymes. A similar strategy is thought to be used by 3-phosphoinositide-dependent protein kinase-1 (20), and the yeast prolly isomerase Ess 1 (21), while several other signaling enzymes including cyclin-dependent protein kinases (22) and various protein phosphatases (23) employ targeting subunits to recognize substrates. The present work lays the foundation for future structure/function studies, which will elaborate on the mechanism of ERK2 and provide an
interesting comparison with other enzymes that catalyze post-translational modifications of proteins.

**EXPERIMENTAL PROCEDURES**

Buffers and Reagents—Trizma (Tris) base was purchased from EM Industries (Gibbstown, NJ). All other buffer components and chemicals were obtained from Sigma. Qiagen Inc. (Santa Clarita, CA) supplied Ni-NTA-agarose. Protein kinase assays were conducted with Roche Molecular Biochemicals (Indianapolis, IN) special quality sodium adenosine triphosphate (Na$_2$ATP) and [$\gamma$-32P]ATP from ICN (Costa Mesa, CA).

The following buffers were employed. The assay buffer (A1) was 25 mM HEPES, pH 7.5, 2 mM DTT, 50 mM MgCl$_2$, and 10% (v/v) glycerol. Lysis buffer (L1) was 40 mM Tris-HCl, pH 7.0, 0.1% (v/v) β-mercaptoethanol, 0.03% (by mass) Triton X-100, 0.75 mM NaCl, 5 mM imidazole, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM TPCK. The activation buffer (P1) was 40 mM Tris-HCl, pH 8.0, 0.1% (v/v) β-mercaptoethanol, 0.02% (by mass) Brij-30, 200 mM imidazole. The HPLC buffer (H1) was 20 mM Tris-HCl, pH 8.0, 0.1% (v/v) β-mercaptoethanol, 0.02% (by mass) Brij-30, 0.1 mM EDTA, 0.1 mM EGTA. The activation buffer (P1) was 25 mM HEPES, pH 7.65, 2 mM DTT, 40 mM ATP, 20 mM MgCl$_2$, 0.5 mM EGTA.

Expression and Purification of Ets Proteins

Ets138—A DNA sequence encoding Ets-1(1-138), which was PCR amplified from the full-length mature ets-1 cDNA cloned into PET28 (Invitrogen) was used to express Ets138 as an N-terminal, hexahistidine fusion protein, containing the sequence, GISHHHHHHSSLVRPSGH, immediately N-terminal to the initiating methionine, in Escherichia coli BL21(DE3 pLysS) as described previously (24).

Ets138-P—To generate Ets138 specifically phosphorylated on Thr138 for product inhibition studies, 50 μM Ets138 (13.3 mg, 750 nmol) was incubated with 5 nM ERK2 (3.2 μg, 0.075 nmol) in assay buffer A1 (15 ml) containing a large excess of ATP (2 mM). After 40 min at 27 °C the reaction was quenched on ice by the careful addition of 0.5M EDTA, 2 mM SDS-PAGE (not shown).

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His$_6$-tagged ERK2—Rat ERK2 was overexpressed under the control of the T7 promoter in E. coli. BL21 (DE3 pLysS) using the NpT7–His$_6$ rERK2 expression plasmid as previously described (25). The resulting protein, which contains the sequence MAHHHHHHH immediately N-terminal to the initiating methionine (Met) was expressed as the inactive enzyme. To be active ERK2 must be phosphorylated on Thr 183 and Tyr185. Cells expressing inactive ERK2 were resuspended in lysis buffer L1 and sonicated to lyse the cells. The lysate was cleared by centrifugation (16,000 × g, 20 min), before the supernatant was incubated for 50 min with gentle rocking at 4 °C with nickel-nitritotriacetic acid (Ni-NTA)-agarose beads (Qiagen), that had been pre-equilibrated in lysis buffer L1 (1 ml of beads per 10 g of wet cells). The beads were then transferred to a 25-cm$^3$ column (Bio-Rad) and washed with 20 column volumes of buffer W1. His$_6$-ERK2 was eluted from the column with 5 ml of elution buffer E1 into a tube containing EDTA and EGTA at final concentrations of 0.1 mM. Following dialysis into buffer S1 the protein was concentrated to 2 mg ml$^{-1}$ using a Centricon-10 spin column, before snap freezing in liquid nitrogen and storing at −80 °C in buffer S1.

To specifically phosphorylate ERK2 on Thr138 and Tyr185, a mutant of MKK1 (MKK1G75B), its cellular activator was employed. This mutant was expressed in E. coli as a hexahistidine fusion protein and purified essentially as described previously (26). Buffer H1 containing 0.05 mM NaCl, was pre-equilibrated in a 27 °C water bath. Frozen stocks of 2 mg ml$^{-1}$ ERK2 (10 mg, 245 nmol) and MKK1G75B (1 mg, 10.5 nmol) were thawed rapidly at 30 °C, centrifuged briefly (2 min, 13,000 rpm), and added to activation buffer P1 (35 ml) to final concentrations of 7 and 0.3 μM, respectively. After 3 h, the mixture was applied to a 1-ml column of DEAE-Sephrose FF (Amersham Biosciences) equilibrated in buffer H1 containing 0.05 mM NaCl. Elution of ERK2 resulted after applying 10-ml aliquots of incremental concentrations of NaCl (50 mM increments) in buffer H1 to the column. Fractions (1 ml) were analyzed for protein using the method of Bradford (27). A major protein peak (8-8.5 mg protein) eluted at 200 mM NaCl, which corresponds to ERK2. The collected fractions were diluted 5-fold and loaded onto a Mono-Q HR 10/10 column equilibrated in buffer H1 containing 0.05 mM NaCl. A linear gradient of 4.2 mM min$^{-1}$ NaCl at a flow rate of 1.5 ml min$^{-1}$ resolved ERK2. Fractions associated with a single peak (A$_{280}$) at 0.22-
mixture was incubated for 5 min before the reaction was initiated by the addition of ATP. Aliquots (5–10 μl) were taken at set time points and applied to 2 × 2-cm² P81 cellulose paper. The papers were washed for 3 × 10 min in 50 mM phosphoric acid (H₃PO₄), then in acetone and dried and the amount of labeled protein determined by counting the associated counts/min on a Packard 1500 scintillation counter at a linear range of 2. The Aₒρm, 1 mg mL⁻¹ of all proteins was determined by denaturing them in 6 M guanidine chloride following the method of Gill and von Hippel (29).

Data Analysis²

Reciprocals of 1/v against 1/s were checked for linearity, before the data were fitted to Equation 1 using a non-linear least squares approach, assuming equal variance for velocities, using the program Kaleidagraph 3.5 (Synergy software). The intercepts kₗapp and slopes Kᵢaghetti/Kₗapp obtained from these fits were then plotted against either the inhibitor concentration (i) for inhibition experiments) or the reciprocal of the non-varied substrate concentration (1/s) for initial velocity experiments. These plots were used to determine the appearance of the overall kinetic equation. Values for kinetic constants were then obtained using the program Scientist (Micromath) by fitting the kinetic data to the relevant overall equation. Data conforming to a sequential initial velocity pattern were fitted to Equation 2, data conforming to linear competitive inhibition were fitted to Equation 3, and data conforming to linear mixed inhibition were fitted to Equation 4.

The polypeptide Ets138 used in this study is an excellent model substrate for studies designed to critically examine the catalytic mechanism of ERK2. From a practical perspective it offers an excellent opportunity, because it can be expressed easily, purified to homogeneity, and assayed with high accuracy. From the biological perspective it is highly relevant, because it is a good substrate, which is a minimal model of the full-length transcription factor, Ets-1, which is a member of the ETS domain transcription factor family, several members of which are substrates of ERK2 in vivo (30). Ets138, like several other transcription factors, contains at least one sequence that conforms to the DEJL domain consensus sequence, suggesting that aside from its practical advantages it provides an opportunity to critically examine the structure and function of protein-protein docking interactions within the context of the ERK2 catalytic mechanism.

Preparation of Activated His₆-ERK2—Rat ERK2 was expressed as the inactive polypeptide in E. coli with an N-terminal hexahistidine tag. It must be phosphorylated on both Thr³⁸ and Tyr¹⁸⁵ to be fully active, which is achieved by incubating the inactive enzyme with the appropriate concentration of MgATP²⁻ and an active form of the physiological activator of ERK2, MKK1 in activation buffer P1. Separation of excess MgATP²⁻ and other salts on a DEAE column followed by a single high-resolution Mono-Q chromatography step is

²The parameters used in deriving equations are defined as follows; v, observed velocity; k_cat, catalytic constant; K_{iB}, apparent catalytic constant; s, concentration of substrate S; K_{mB}, apparent Michaelis constant for substrate S; a, concentration of substrate A; b, concentration of substrate B; i, concentration of inhibitor I; K_{iA}, inhibition constant for substrate A; K_{iB}, inhibition constant for substrate B; K_{mB}, Michaelis constant for substrate A; K_{mB}, Michaelis constant for substrate B; K_{iB}, apparent specific inhibition constant for inhibitor I; K_{app}, apparent competitive inhibition constant for inhibitor I.
sufficient to separate the activated ERK2 from the MKK1 and several minor ERK2 phosphoforms (Fig. 2A). As expected, the activated enzyme ran as a single 41.4-kDa band on a 10% SDS-PAGE gel, shifted by 60 Da from the inactive form that ran at 40.8 kDa (Fig. 2B). To confirm the stoichiometry and regioselectivity of phosphorylation, the activated enzyme was analyzed by LC-MS and tryptic analysis of a $^{32}$P-labeled preparation of ERK2, respectively. This analysis confirmed that the ERK2 used throughout these experiments was purified to homogeneity as the bisphosphorylated Thr$^{183}$, Thr$^{185}$ phosphoisoform. This preparation displayed a comparable activity to a previously reported preparation isolated from cells (31).

**Preparation and Characterization of Ets$^\Delta$138 and Ets$\Delta$138–P—**One of the most significant results of this work is the establishment of Ets$\Delta$138 as a substrate suitable for detailed kinetic studies of ERK2, because prior to this work no reliable substrate was available. For meaningful mechanistic investigations of protein kinases, homogeneous protein substrates that correspond to in vivo substrates of the enzymes are essential. This is particularly important in the case of the MAPKs, which bind substrates at sites outside the active site. While ERK2 does have many substrates in vivo none have been developed for mechanistic and biophysical studies for a number of reasons. Probably the major reason is that protein substrates are often difficult to isolate in a pure form in high yield, but they are also often difficult to assay and often they contain more than one phoshoacceptor site, which complicates an accurate kinetic analysis. For example, the best substrate previously available to assay ERK2 was myelin basic protein, which due to several post-translational modifications is purified with a high degree of heterogeneity (32, 33) and which is reported to be phosphorylated on both Thr$^{34}$ and Thr$^{37}$ under standard assay conditions (34). Ets$\Delta$138 is based on the N-terminal pointed domain (35) of the transcription factor Ets-1, a substrate of ERK2 in vivo (30). It was chosen because its expression and purification had been reported by Macintosh's laboratory (36), who had also solved the NMR structure of the polypeptide phosphorylated exclusively on Thr$^{38}$. The purification protocol described here utilizes an nickel affinity purification/enrichment step to separate Ets$\Delta$138 from the majority of the proteins present in E. coli, followed by ion exchange (Mono-Q) chromatography to separate it from proteolytically degraded forms of the protein. The procedure is reproducible, offering a highly pure preparation of homogeneous Ets$\Delta$138 (see Fig. 1A) in a yield of 60 mg liter$^{-1}$, which can be stored conveniently at 2 m$m$ in a solution of 2 m$M$ DTT (24).

For product inhibition studies we needed to prepare Ets$\Delta$138 stoichiometrically phosphorylated on Thr$^{38}$. Previous studies had established that Ets$\Delta$138 phosphorylation proceeds until about 1 mol/mol of phosphate is incorporated into the protein, however, Ets$\Delta$138 has one other site, Ser$^{29}$, that can also be phosphorylated by ERK2 if incubation proceeds for an extended length of time. The presence of the bisphosphorylated form can be detected routinely by SDS-PAGE, because it runs slightly slower than Ets$\Delta$138 and Ets$\Delta$138–P. It also elutes later than the Ets$\Delta$138 from a Mono-Q column and can also be detected easily by LC-MS. After incubating 13.3 mg of Ets$\Delta$138 with 3.2 mg of ERK2 for 40 min in the presence of a large excess of MgATP$^{2-}$, Ets$\Delta$138–P was identified. Contaminating phosphoisoforms of Ets$\Delta$138 were separated by Mono-Q chromatography (see "Experimental Procedures"). After purification, ~10.6 mg of Ets$\Delta$138–P was obtained with a yield of about 80%, whose purity was verified by LC-MS (Fig. 1B) and SDS-PAGE (not shown). Like Ets$\Delta$138 this could be frozen in 2 m$M$ DTT without loss of activity.

**Initial Rate Measurements in the Absence of Products—**To define the basic kinetic mechanism we examined the dependence of product formation on the concentrations of each substrate by the method of initial rates (28). Preliminary experiments established that the P81 binding assay that we employed detected 97 ± 3% of the phosphorylated protein product (Ets$\Delta$138–P) in the presence of up to 300 $\mu$m substrate protein, Ets$\Delta$138, and was therefore suitable for an extensive kinetic analysis (24). The appearance of product with time under initial rate conditions was linear under all experimental conditions and was highly reproducible, to within 10%. Figs. 3 and 4 show that double reciprocal plots of $1/v$ versus $1/[Ets^\Delta138]$ or $1/[MgATP^{2-}]$ are linear and display a pattern of intersecting lines below the abscissa at a common vertical coordinate. This is consistent with a sequential mechanism where both substrates react before either dissociates from the enzyme (37). The kinetic parameters were obtained by fitting all the initial rate data using Equation 2, for a ternary complex and are recorded in Table I. Alternative kinetic models failed to account for the substrate dependence satisfactorily.

**Initial Rate Measurements in the Presence of Products—**To analyze the kinetic mechanism further, product inhibition studies (38) were carried out and a mode of inhibition was assigned to each product/substrate pair (see Table II). Product inhibition studies are an extremely useful way to elucidate the order of binding of substrates, in part, because of the ease by which product inhibition characteristics can be predicted. Product inhibitors are classified according to whether they affect the apparent specificity constant, $k_{cat}/K_{m}^{p}$ (competitive inhibition), the apparent catalytic constant $k_{cat}^{p}$ (uncompetitive) or both (mixed). By plotting the data in reciprocal form as $1/v$ against $1/[substrate]$ at varied concentrations of inhibitor one can determine the mechanism by noting whether an inhibitor affects the slope or intercept of a plot. A competitive inhibitor affects only the slope, an uncompetitive inhibitor affects only the intercept, while a mixed inhibitor affects both the slope and intercept of such plots.

The following results were obtained and are discussed below. (i) With Ets$\Delta$138–P as product inhibitor a mixed inhibition pattern with respect to MgATP$^{2-}$ (Fig. 5) and a competitive

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1. M. Rainey, manuscript in preparation.
2. W. F. Wass and K. N. Dalby, unpublished observations.
the investigators showed that although initial velocity and product inhibition and indeed isotope exchange patterns were consistent with a rapid-equilibrium assumption, isotope exchange rates showed that the rapid-equilibrium assumption was not valid.

Rapid chemical-quench experiments from our laboratory suggest that product release may be partially rate-limiting for the phosphorylation of Ets138, which suggests that the rapid-equilibrium assumption is not valid for the phosphorylation of Ets138 by ERK2. Therefore, the most satisfactory explanation for the kinetic observations is a steady-state mechanism according to Scheme 4 where the binding of substrates is not at equilibrium, but close. While the ordered Theorell-Chance mechanism has the same form of initial velocity equation as a rapid-equilibrium random-ordered mechanism (which fits the data) it can be discounted for two reasons. First, ERK2 has an extremely high ATPase activity suggesting that MgATP$^{2-}$ binds ERK2 in an activated conformation (43). Second, ERK2 forms complexes with protein substrates in the absence of MgATP$^{2-}$ (18). These observations demonstrate that binding pockets on the free enzyme for both protein and nucleotide substrates are accessible, which is not compatible with a compulsory ordered mechanism. The data also supports the idea that despite the large surface area thought to constitute the interface between ERK2 and Ets138 the dissociation of these proteins is relatively rapid. For the rapid-equilibrium model to approximate the kinetic data the dissociation constants $k_{-2}$ and $k_{-3}$ in Scheme 4 must be of similar magnitude to the catalytic constant for the reaction of $k_{cat} = 17$ s$^{-1}$ (42).

**Random-ordered Substrate Binding**—A random-ordered mechanism of substrate binding requires that both substrates have unrestricted access to binding sites on the enzyme and that binding of the second substrate to its cognate binary complex occurs in a productive as opposed to a non-productive binding mode. While many laboratories have reported results supporting a random-ordered mechanism of substrate binding for various protein kinases, in most cases the phosphoacceptor used was a relatively small peptide (44). The substrate used in this study, Ets138, is a globular protein substrate (18 kDa) that can, in principle, hinder access of MgATP$^{2-}$ to the catalytic active site. The nucleotide binding pocket of ERK2 is deep in a cleft between its two lobes and could be inaccessible in the presence of a bound protein substrate. Thus, the random-ordered mechanism reported here is significant, because it demonstrates that each substrate can gain access to the binding sites despite the potential for steric hindrance.

Such access is not compatible with the structure of the ternary complex of cAPK, where the active site of cAPK closes around the substrates to properly align catalytic residues (45). While kinetic (46–49) and structural (50) data for cAPK support the concept that conformational transitions accompany substrate binding, it is not clear precisely how such conformations are linked to the catalytic mechanism. While it is likely that cAPK utilizes an induced fit type mechanism (51) to allow the unhindered access of both substrates to the active site this has not been critically addressed in the literature for any protein kinase. In this respect it is of considerable interest that ERK2 phosphorylation sites are found in flexible regions of proteins and that the flexibility of ERK2 in regions thought to be important for substrate recognition are affected by the activation state of the enzyme (52).

The kinetic mechanism described here is also interesting in light of an earlier report that the closely related protein kinase, p38 MAPKα, binds the substrate transcription factor, ATF2

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**Table I**

**Kinetic and thermodynamic parameters for the phosphorylation of Ets138 by ERK2**

| Kinetic constant (Value) |  
|-------------------------|
| $k_{cat}$               | $17.0 \pm 3.0$ s$^{-1}$ (42) |
| $K_{M}^{B}$             | $18.0 \pm 2.0$ $\mu$M (32) |
| $K_{M}^{A}$             | $14.0 \pm 5.0$ $\mu$M (32) |
| $K_{A}$                 | $9.0 \pm 1.0$ $\mu$M (32) |
| $K_{D}$                 | $65.0 \pm 5.0$ $\mu$M (32) |

* Initial velocities were measured using 2 nM ERK2, 20 mM MgCl$_2$, 2 mM DTT, MgATP$^{2-}$ (32.1–710 $\mu$M), and Ets138 (5.6–100 $\mu$M) at pH 7.5, 27 °C, ionic strength 0.1 M (KCl). The data were fitted to Equation 1 according to the parameters in Table I.

* $k_{cat}$ was found to vary between the values of 15–20 s$^{-1}$ for several different preparations of the enzyme.

* Determined from fitting of the substrate dependence data in Figs. 3 and 4 according to Equation 2.

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**DISCUSSION**

**Product Inhibition Patterns**—The kinetic dependence on substrate concentration (Figs. 3 and 4) and the product inhibition patterns (Figs. 5–8) are consistent with a rapid-equilibrium random-ordered ternary complex mechanism with two abortive complexes, $E_{B}G_{A}D_{P}$ and $E_{B}G_{A}D_{P}$ (Scheme 4). This model describes the kinetic data extremely well and describes a situation where the interconversion of ternary complexes is rate-limiting. The rapid-equilibrium model has been proposed for other protein kinases such as c-Src (39) and kinase insert domain receptor (40). However, product inhibition data are not definitive evidence for a rapid-equilibrium mechanism, because experimental error can hide subtle differences predicted for some non-equilibrium mechanisms. Therefore, a more sensitive test, such as an isotope exchange experiment is required (41).

This was clearly demonstrated by Gulbinsky and Cleland (42) a number of years ago for *E. coli* galactokinase. In this study the investigators showed that although initial velocity and product inhibition and indeed isotope exchange patterns were consistent with a rapid-equilibrium assumption, isotope exchange rates showed that the rapid-equilibrium assumption was not valid.

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$^5$ W. Waas, unpublished observations.
prior to MgATP$^{2-}$ (53). This compulsory ordered ternary complex mechanism was later questioned by Chen et al. (54) using a peptide, which unfortunately was a rather poor substrate ($K_m = 800 \mu M$). Chen et al. (54) argued for a compulsory ordered ternary complex mechanism where MgATP$^{2-}$ bound first. In light of our experiments it would seem plausible
that p38 MAPKα follows a random-ordered kinetic mechanism.

Docking Complexes—As mentioned earlier several laboratories have examined the binding interactions between MAPKs and other proteins (7–13). In an excellent study using a glutathione S-transferase pull-down approach, Yang et al. (18) showed that deletion mutants of the ets transcription factor Elk-1, which contains a DEJL domain, similar in sequence to that found in Ets-1 could form complexes with ERK2 even when the C terminus of the protein containing all the phosphoacceptor sites was deleted (18). Furthermore, they showed that point mutations in the DEJL domain abrogated the interaction between ERK2 and Elk-1. In another thorough study Zhou et al. (55) used isothermal calorimetry to quantify the interactions between ERK2 and several peptides derived from DEJL-like sequences and showed that the dissociation constants for these peptides reach as low as 80 μM. These and other studies (7–13) support the concept of a “docking complex,” which we define as a complex between a MAPK and substrate mediated exclusively by interactions that lie outside of the active site of the MAPK.

Two related pieces of evidence, presented here, support the notion that a discrete ERK2-EtsΔ138 docking complex also exists. First, the competitive inhibition data of EtsΔ138–P (Fig. 6) suggests that the phosphorylation of Thr38 does not significantly alter the stability of its interactions with ERK2 (Table II). This is consistent with the notion that Thr38 does not make significant stabilizing interactions with the active site, which are expected to be abrogated by Thr38 phosphorylation. Second, the strong mixed inhibition observed for EtsΔ138–P toward MgATP2− (Fig. 5) provides a compelling argument for the formation of the abortive complex, E−PωMgATP (EREleftΔ138−P) (Scheme 4). Steric arguments predict that Thr(P)38 cannot occupy the active site of ERK2 in this abortive complex, suggesting that the stability of the complex derives from interactions that lie outside of the active site. It should be noted however, that E−A−P abortive complexes have been reported for other kinases such as liver fructokinase (56) and yeast galactokinase (42). In these cases it would appear that an enzyme conformation exists that accommodates the binding of both product molecules while at the same time avoiding a steric clash. This remains a possibility for ERK2 also. However, in light of the evidence from several laboratories for the existence of stable docking complexes (in the absence of phosphoacceptor site interactions) (7–13, 55) the docking complex remains an attractive hypothesis. Such a mechanism is also consistent with a random-ordered mechanism of substrate binding, where the active site of the docking complex is accessible to MgATP2−.

Transient Protein Interactions—The possible existence of a docking complex raises a number of questions concerning its stability and structure and the potential role it plays in facilitating the catalytic mechanism of ERK2. According to the model of Yang et al. (18) (Scheme 1A) by maintaining contact at a DEJL domain and simply “sliding” the active site of ERK2 from one phosphorylation site to the next, kφ−1, ERK2 could phosphorylate several residues on a substrate such as Elk-1 without dissociating fully from it. While simple in concept, as mentioned in the Introduction, this model is not consistent with kinetic observations that we have previously reported for the phosphorylation of the transcription factor ATF2 by the related enzyme p38 MAPKα. In this case the data supports the distributive mechanism of Scheme 1B (19). In the present work the close approximation of the kinetic data in Figs. 3–8 to the rapid-equilibrium model strongly suggests that like p38 MAPKα-ATF2 complexes, complexes of ERK2 and EtsΔ138 are not long-lived on the time scale of the reaction. Rather, they are relatively transient and dissociate with rate constants of similar magnitude to the catalytic constant of kcat = 17 s−1 (42). Thus, a processive mechanism mediated by interactions between a MAPK and a substrate docking domain (Scheme 1A) is not supported by kinetic evidence from two different studies for two different MAPKs.

Transient Docking—However, the existing data does support a transient docking model for the phosphorylation of a substrate, shown in the upper pathway of Scheme 3. Characterizing this mechanism, which is essentially the same mechanism as step 1 in Scheme 1B, is the dissociation of the MAPK-protein complex after each phosphorylation event. Thus, according to this model a docking complex forms with rate constant, k1, mediated in part by a docking domain, which in some cases binds to a region on the reverse side of ERK2, that lies more than 30 Å from the active site (10). The docking complex (D) contains phosphorylation sites that potentially lie in the “intramolecular sampling space” of the active site. Conformational transitions bring an opportunistic Thr-Pro or Ser-Pro motif to within the immediate vicinity of the active site, where it binds, k2. The active site then wraps around it lining up the catalytic residues and this alignment leads to the phosphorylation of the substrate, k3. Dissociation of both MgADP− and the monophosphorylated protein from the enzyme, k−3, then follows. Alternatively, a Thr-Pro or Ser-Pro motif could bind within the active site of the enzyme first, k′1, followed by interaction of ERK2 with the DEJL domain to stabilize the protein-protein interaction, k′2. While peptides corresponding to sequences surrounding known phosphorylation sites in proteins are phosphorylated by ERK2 with Kr values in the millimolar range (15) the possible charge complementarity between a DEJL domain and the C terminus of ERK2 suggests that strong long-range electrostatic interactions could favor initial docking via a DEJL domain (10). Further studies are required to fully address the contributions made by individual amino acids to both complex formation and the kinetic mechanism.

Conclusion—The kinetic data are consistent with the idea that significant conformational flexibility accompanies substrate binding to facilitate unhindered access of both EtsΔ138

6 The magnitude of the apparent competitive inhibition constant toward EtsΔ138 of Kp38 = 26 μM, when the concentration of MgATP is 267 μM is of similar magnitude to the Michaelis constant for EtsΔ138 of Km = 18 μM.

7 Because the rapid-equilibrium model cannot be assumed the kinetic data does not furnish the dissociation constant for the abortive complex. However, the apparent uncompetitive inhibition constant is only 5-fold greater than the Michaelis constant for EtsΔ138, suggesting that EtsΔ138–P and EtsΔ138 bind to E-ATP with a similar affinity.

8 Basic residues in DEJL-like domains are thought to form ionic interactions with acidic residues on ERK2, such as Asp136 and Asp139 (10).
and MgATP$^2–$ to binding sites on the enzyme. The mechanism and magnitude of product inhibition by EtsA138–P is consistent with, but does not prove, the notion that ERK2 forms a discrete interaction with EtsA138 in the absence of active site interactions, and that this docking complex facilitates intramolecular phosphorylation of the substrate. The approximation of the steady-state data to a rapid-equilibrium model strongly suggests that the formation of ERK2-EtsA138 complexes are transient in nature with dissociation constants of greater magnitude than the catalytic constant, of $k_{cat} = 17 \text{ s}^{-1}$.

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