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A continuous spectrophotometric assay for mitogen-activated protein kinase kinases

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ABSTRACT

We describe a convenient and simple continuous spectrophotometric method for the determination of mitogen-activated protein kinase (MAPK) kinase activity with its protein substrate. The assay relies on the measurement of phosphoprotein product generated in the first step of the MAPK kinase reaction. Dephosphorylation of the phosphoprotein is coupled to a MAPK phosphatase to generate phosphate, which is then used as the substrate of purine nucleoside phosphorylase to catalyze the N-glycosidic cleavage of 2-amino 6-mercapto 7-methyl purine ribonucleoside. Of the reaction products ribose 1-phosphate and 2-amino 6-mercapto 7-methylpurine, the latter has a high absorbance at 360 nm relative to the nucleoside and, hence, provides a spectrophotometric signal that can be continuously followed. In the presence of excess phosphatase, the phosphorylated protein substrate molecules undergo dephosphorylation almost immediately after their formation; the steady-state use of the resultant inorganic phosphate is a reflection of the constant initial velocity of the exchange reaction. The validity of this method has been confirmed by using it to measure the activities of MEK1 (MAPK/ERK kinase 1) and MKK6 (MAPK kinase 6) toward their physiological substrates. Our findings of the MAPK kinases in the current study provide evidence that the substrate binding affinities of this subfamily of protein kinases are at the submicromolar concentration.

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Mitogen-activated protein kinases (MAPKs)[1] regulate diverse cellular programs, including embryogenesis, proliferation, differentiation, and apoptosis, based on cues derived from the cell surface and the metabolic state and environment of the cell. The three best characterized MAPK pathways are the extracellular signal-regulated kinase (ERK) pathway, which responds to stimuli that induce proliferation and differentiation, the c-Jun amino-terminal kinase (JNK) pathway, and the p38 kinase pathway, the latter two of which are activated in response to environmental stresses. MAPKs are activated by MAPK kinases (MKKs), which are dual specificity kinases that phosphorylate the conserved Thr and Tyr located within the TXY motif in the activation loop of MAPKs [1–6]. Seven members of this family of MAPK kinases have been isolated in mammalian cells, and specific MAPK kinases have been identified for each MAPK subgroup that allow for their selective activation [7–9]. The prototypic MAPK pathway, ERK1/ERK2, is selectively phosphorylated and activated by MKK1 and MKK2 (MAPK/ERK kinase 1 [MEK1] and MEK2), whereas the p38 pathway is activated by MKK3, MKK4, and MKK6. In contrast, the optimal activation of the JNK pathway requires the concerted action of two different MKKs: MKK4 and MKK7 [10,11]. Once activated, MAPKs phosphorylate a distinct spectrum of substrates, including key regulatory enzymes, cytoskeletal proteins, regulators of apoptosis, nuclear receptors, and many transcription factors. This range of substrates indicates a pivotal role for MAPKs in cellular signal transduction.

Because of the critical importance of MAPK in cellular signaling, the activity of the MAPK is tightly regulated by control of their activation through dual phosphorylation in the activation loop and

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[1] *Abbreviations used:* MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; MKK, MAPK kinase; MEK, MAPK/ERK kinase; MKP, MAPK phosphatase; PTPase, protein-tyrosine phosphatase; PNPase, purine nucleoside phosphorylase; MESG, 2-amino 6-mercapto 7-methyl purine ribonucleoside; Mops, 3-(*N*-morpholino)propanesulfonic acid; K52R, catalytically inactive ERK2 in which Lys52 was substituted with arginine; NTA, nitrilotriacetic acid; K53M, catalytically inactive p38α in which lysine-53 was substituted with methionine; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; cDNA, complementary DNA; EDTA, ethylenediaminetetraacetic acid; PVDF, polyvinylidene fluoride; P_i, inorganic phosphate; MTGua, 2-amino 6-mercapto 7-methylpurine.

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their inactivation by a family of MAPK phosphatase (MKP). The regulated dephosphorylation of MAPKs plays a key role in determining the magnitude and duration of kinase activation, thereby forming a negative feedback mechanism. The MKPs are dual specificity phosphatases that inactivate MAPKs through dephosphorylation of phosphothreonine and phosphotyrosine residues in the activation loop of MAPKs [12–15]. The MKPs belong to the protein-tyrosine phosphatase (PTPase) superfamily, which is defined by the PTPase signature motif $(H/V)C(X)_5R(S/T)$. In mammalian cells, at least 13 MKPs have been identified. These MKPs display distinct in vitro substrate preferences for the various MAPKs [16]. For example, MKP3 is predominantly localized in the cytoplasm and is highly specific in deactivation ERK1/2, whereas MKP5 selectively dephosphorylates p38 and JNK MAPKs.

In the current study, a continuous spectrophotometric assay for determination of the initial rate of the MAPK kinase-catalyzed reaction is presented. The assay incorporates two coupling enzyme systems: (i) a dual specificity MKP that dephosphorylates the phosphorylated MAPK product of the MAPK kinase reaction, and (ii) the system of Webb that uses purine nucleoside phosphorylase (PNPase) and its chromophoric substrate, 2-amino 6-mercapto 7-methyl purine ribonucleoside (MESG), for the quantitation of the resultant inorganic phosphate [17]. The usefulness of this method was demonstrated for the MEK1- and MKK6-catalyzed reactions, and the kinetic parameters of these two reactions with respect to wild-type ERK2 and p38α substrates were determined at pH 7.0 and 25 °C. The Michaelis–Menten constants for MKK-catalyzed reactions were determined to be at the submicromolar concentration, and the rate constants (k_{cat}) were 0.2 to 0.5 s^{-1}.

Materials and methods

Materials

ATP, bacterial PNPase, and the ingredients to generate the PNPase substrate, MESG, were purchased from Sigma. MESG was synthesized as described previously [18]. 3-(*N*-morpholino)propanesulfonic acid (Mops) was purchased from Amresco. Other reagents were products of analytical grade without further purification. Double-deionized water was used throughout.

Preparation of unphosphorylated and bisphosphorylated ERK2 and p38

The pET15b plasmid containing rat ERK2 was a generous gift from Chao-Feng Zheng. The mutant ERK2/K52R was generated by polymerase chain reactions according to the standard procedure of the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the pET15b-His6-ERK2 plasmid as a template. The N-terminal His6-tagged wild-type ERK2 and ERK2/K52R mutant protein were expressed in *Escherichia coli* BL21(DE3) and purified using standard procedures of Ni^{2+}-nitrilotriacetic acid (NTA) metal affinity chromatography (Qiagen). The protein was further purified by FPLC (fast protein liquid chromatography) anion exchange Mono Q column (Pharmacia) as described before [19]. The plasmid pET-His6-ERK2-MEK1(R4F), a generous gift from Melanie Cobb, was used to coexpress a constitutively active MEK1 and an N-terminal His6-tagged ERK2 in *E. coli* BL21(DE3). The expression and purification of bisphosphorylated ERK2 (ERK2/pTpY) were carried out following the procedure described by Wilsbacher and Cobb [20]. The DNA sequence encoding mouse p38α was subcloned into the pET15b (Novagen). The mutant p38α/K53M was generated by polymerase chain reactions according to the standard procedure of the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the pET15b-His6-p38 plasmid as a template. Its identity was confirmed by DNA sequencing. The wild-type p38α was activated in vivo by coexpressing p38α and MKK6EE, a constitutively active MKK6. Both p38α and MKK6EE were subcloned into the same plasmid pETDuet-1 (Novagen). The N-terminally His6-tagged wild-type p38α, K53M mutant, and bisphosphorylated p38α/pTpY protein were expressed in *E. coli* BL21(DE3) and purified by Ni-NTA, followed by an anion exchange Source 15Q column [21]. The protein purity was greater than 95%, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Electrospray ionization mass spectrometry analysis confirmed that the purified ERK2/pTpY and p38α/pTpY were effectively homogeneous and were phosphorylated stoichiometrically to a ratio of 2 mol of phosphate per mole of MAPK. The concentrations of ERK2 and p38α were determined spectrophotometrically based on a theoretical molar extinction coefficient at 280 nm [22]. The purified proteins were made to 20% glycerol and stored at −80 °C.

MKPs and MAPK kinases

The human MKP5 complementary DNA (cDNA) was a generous gift from Eisuke Nishida and subcloned into pET15b vector (Novagen). The N-terminal His6-tagged MKP3 and MKP5 were expressed in *E. coli* BL21(DE3) and purified using standard procedures of Ni-NTA, followed by an anion exchange Source 15Q column. The cDNA for the constitutively active MAPK kinase MEK1 mutant G7B (ΔN4/S218D/M219D/N221D/S222D) in pRSETa was kindly provided by Natalie Ahn. The cDNAs for human wild-type MKK6 and MKK6EE, the constitutively active MKK6 mutant (S207E/T211E) in pET15b (Novagen), were kindly provided by Zhenguo Wu. Recombinant human MEK1/G7B and MKK6EE were expressed as an N-terminal His6-tagged protein in *E. coli* BL21(DE3) and purified using standard procedures of Ni-NTA, followed by an anion exchange Source 15Q column. All proteins prepared were subjected to SDS–PAGE analysis, and the contents of each were judged to be at least 95% pure. The purified protein were made to 20% glycerol and stored at −80 °C. Protein concentration was determined spectrophotometrically based on a theoretical molar extinction coefficient at 280 nm [22].

Enzyme assays for MKPs

Kinetic parameters for the dephosphorylation of the phosphorylated ERK2 and p38α were determined using a continuous spectrophotometric assay [19,21]. All experiments were carried out at 25 °C in a 1.8-ml reaction mixture containing 50 mM Mops (pH 7.0), 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $MgCl_2$, 100 μM MESG, and 0.1 mg/ml PNPase. The reactions were initiated by the addition of phosphatase unless indicated otherwise. The time courses of absorbance change at 360 nm resulting were recorded on a Lambda 45 PerkinElmer spectrophotometer equipped with a magnetic stirrer in the cuvette holder. Initial rates were determined from the linear slope of progress curves obtained, and the experimental data were analyzed using a nonlinear regression analysis program. Quantitation of phosphate release was determined using an extinction coefficient of 11,200 M^{-1} cm^{-1} for the phosphate-dependent reaction at 360 nm at pH 7.0 [23]. The concentration of MESG was determined at 331 nm using a molar extinction coefficient of 32,000 M^{-1} cm^{-1}.

Western blotting

The phosphorylation states of p38α and ERK2 were assessed by Western blot analysis using specific antibodies. The same amounts of p38α or ERK2 in various phosphorylation states were resolved by SDS–PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). After incubation of

the membranes with anti-phospho-Thr (Cell Signaling Technology, cat. no. 9381) and anti-phospho-Tyr (Cell Signaling Technology, cat. no. 9411) antibodies, specific immunocomplexes were detected by chemiluminescence using ECL (enhanced chemiluminescence) reagents. The membrane was finally exposed to X-ray film (Kodak).

Results

Kinetic analysis of MKP3- and MKP5-catalyzed reactions

MKK6 phosphorylates p38 MAPK on Thr180 and Tyr182, the sites of phosphorylation that activate p38 MAPK. In a preliminary experiment, we examined the variously phosphorylated forms of p38α and ERK2 by performing Western blot analysis using anti-phosphothreonine and anti-phosphotyrosine antibodies. First, we phosphorylated p38α using active recombinant MKK6 and treated the resulting phospho-p38α with recombinant MKP5 at 25 °C (Fig. 1A). As shown in Fig. 1A, recombinant p38α is indeed effectively phosphorylated at both threonine and tyrosine residues in the activation loop by MKK6, and MKP5 caused a complete dephosphorylation of p38α /pTpY. Similarly, MEK1 is able to effectively phosphorylate Thr183 and Tyr185 in the activation loop of ERK2/pTpY in vitro, and a brief incubation at 25 °C of bisphosphorylated ERK2 with MKP3 resulted in a rapid loss of phosphate from both tyrosine and threonine (Fig. 1B).

The MESG/phosphorylase assay was developed to measure the kinetics of inorganic phosphate (P_i) release from GTPase and ATPase [17] and has been applied to the studies of the dephosphorylation of phosphoprotein by phosphatases [19,21,24]. The phosphatase catalyzes the dephosphorylation of phosphoprotein substrate, and the resulting P_i is then used by the PNPase to convert MESG to 2-amino 6-mercapto 7-methylpurine (MTGua) and ribo-1-phosphate. To validate the new assay system, the enzyme activities of MKP were examined first under the experimental conditions used for protein kinase assay (in the presence of Mg^{2+}). Previously, we reported the kinetic parameters for the dephosphorylation of p38α by MKP5 in the presence of 10 mM Mg^{2+}. In this study, using the continuous spectrophotometric assay, we determined the kinetic parameters for the MKP3-catalyzed ERK2 dephosphorylation. Fig. 2 shows typical progress curves of the MKP3-catalyzed reactions. The continuous assay for MKP3 was carried out in 1.8 ml of reaction mixture. After 5 min of equilibration at 25 °C, the reaction was initiated by adding a catalytic amount of MKP3 (52.5 nM) and absorbance at 360 nm was recorded. The initial rates of the dephosphorylation reaction were determined from the slopes of the initial parts of the progress curves. The inset of Fig. 2 shows the dependence of the initial rates of the MKP3-catalyzed reaction on ERK2/pTpY concentration. By fitting the Michaelis–Menten equation to the experimental data, the values of k_{cat} and K_m were determined to be 0.265 ± 0.006 s^{-1} and 0.199 ± 0.031 μM, respectively. For comparison, we also determined the kinetic parameters for the MKP3- and MKP5-catalyzed reactions in the absence of Mg^{2+} using the same procedure. All of the kinetic parameters of MKP-catalyzed reactions are summarized in Table 1. It can be seen from this table that when MKP activities were measured without Mg^{2+}, the k_{cat}/K_m values were 2- to 6-fold higher than those obtained with 10 mM Mg^{2+}.

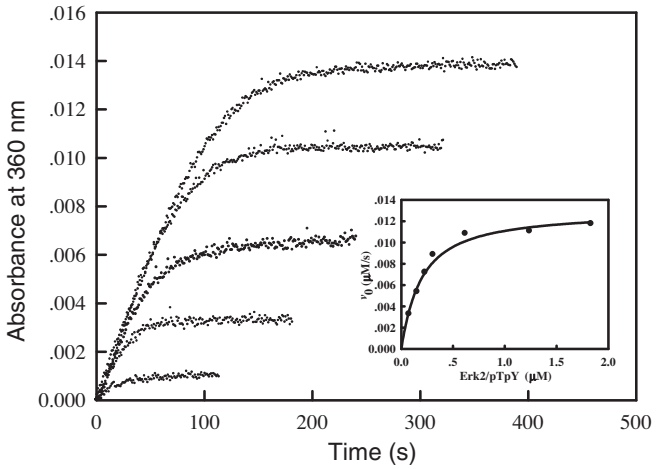


Fig.2. Time courses of MKP3-catalyzed ERK2/pTpY dephosphorylation reaction. The reaction mixture contained the standard buffer, 52.5 nM MKP3, and various concentrations of bisphosphorylated ERK2. The absorption at 360 nm was recorded following the addition of 52.5 nM MKP3 at pH 7.0 and 25 °C. Inset: Dependence of the initial rate of MKP3-catalyzed reaction on the concentration of bisphosphorylated ERK2. The solid line is the best fitting result according to Eq. (1), with k_{cat} = 0.265 ± 0.006 s^{-1} and K_m = 0.199 ± 0.031 μM.

Kinetics of MKK6-catalyzed dual phosphoryaltion reaction

In the current study, we first measured MKK6 activity using the inactive K53M mutant of p38α as a substrate to avoid possible complications from ATP hydrolysis by wild-type p38α. The MAPK kinase assay reported here is an extension of the well-characterized phosphate detection system originally described by Webb [17]. The main components of the coupled enzyme system in the continuous spectrophotometric assay of MAPK kinase are the MKP, PNPase, and MESG. The reaction scheme is summarized below:

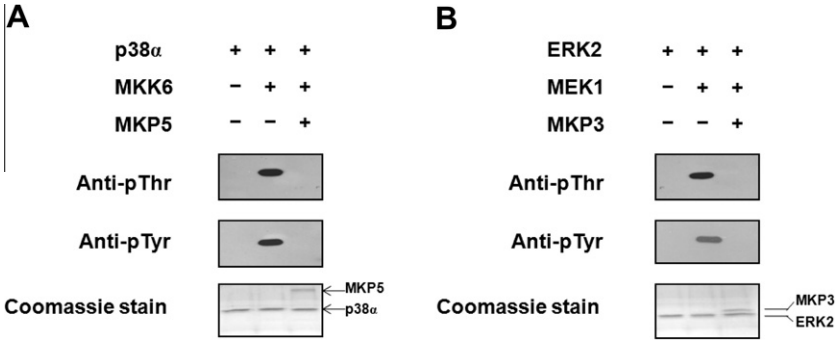


Fig.1. Western blot analysis of phosphorylation states of p38α (A) and ERK2 (B). Approximately 100 ng of various forms of p38α and ERK2 was transferred to PVDF membrane from 10% SDS–PAGE gel and probed with anti-Thr(P) and anti-Tyr(P) antibodies. The immunoactivity was detected by chemiluminescence. The lower panel of SDS–PAGE gel (Coomassie stained) shows the amounts of purified p38α and ERK2 and other proteins used in the experiment. MKK6 and MEK1 were not observed on the SDS–PAGE due to their low amounts.

Table 1
Kinetic parameters of MKP3 and MKP5 measured in the absence and presence of Mg^{2+}

Phosphatase	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
MKP3	ERK2/pTpY	0.301 ± 0.018	0.041 ± 0.012	(7.3 ± 2.5) × 10^6
MKP3 + Mg^{2+}	ERK2/pTpY	0.265 ± 0.006	0.199 ± 0.031	(1.33 ± 0.22) × 10^6
MKP5	p38α/pTpY	0.516 ± 0.013	0.0644 ± 0.01	(8 ± 1.3) × 10^6
MKP5 + Mg^{2+} [21]	p38α/pTpY	0.55 ± 0.01	0.14 ± 0.01	(4.01 ± 0.42) × 10^6

$$\text{MAPK} + \text{ATP} \xrightarrow{\text{MKK}} \text{Phospho-MAPK} + \text{ADP}$$

$$\text{Phospho-MAPK} + \text{H}_2\text{O} \xrightarrow{\text{MKP}} \text{MAPK} + \text{P}_i$$

$$\text{P}_i + \text{MESG} \xrightarrow{\text{PNPase}} \text{MTGua} + \text{Ribose-1-phosphate}.$$

With this technique, the formation of phosphoprotein in the protein kinase reaction is coupled to the phosphatase reaction to produce P_i, which in turn is coupled to the PNPase reaction with the concomitant conversion of MESG to MTGua.

To determine the optimal concentrations of MKP5, the effects of different levels of MKP5 are shown in Fig. 3. The continuous assay for MKK6 activity was carried out at 25 °C in 1.8-ml reaction mixtures. The reaction was initiated by the addition of a catalytic amount of MKK6 (55 nM), and the time course of P_i release was monitored at 360 nm. At low levels of MKP5, there was a marked initial lag phase before the linear phase was reached. As the levels of this coupling enzyme were increased, the time of the initial lag phase decreased and linear (steady-state) phases became essentially the same; therefore, the highest level of MKP5 was chosen for the standard assay. Fig. 4 shows the time-dependent P_i release (at 360 nm) for the MKK6-catalyzed reaction. The continuous assay was carried out at 25 °C in a 1.8-ml reaction mixture containing standard buffer (pH 7.0) and 200 nM MKP5. The reaction was initiated by the addition of a catalytic amount of MKK6 (55 nM), and a steady increase in aborbance at 360 nm was observed for at least 10 min. The fact that the P_i release at the steady state is linear with time indicates that all components of the reactions remain stable under the conditions of the assay. Thus, the slope of the linear absorbance trace was taken to be a measure of the initial rate of the MKK6-catalyzed reaction. The inset of Fig. 4 shows the effects of enzyme concentration on the rate of MKK6-catalyzed

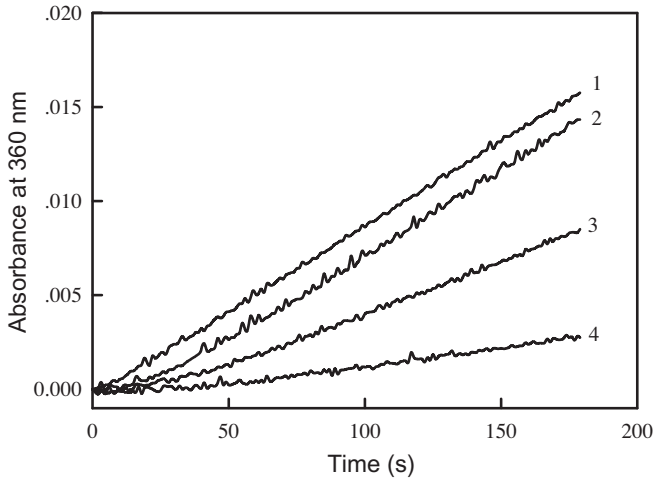


Fig.3. Effect of the levels of MKP5 on the coupled enzyme system. The reaction mixture contained the kinase assay buffer (50 mM Mops [pH 7.0], 100 mM NaCl, 0.1 mM EDTA, and 10 mM $MgCl_2$), 100 μM MESG, 0.25 mM ATP, and 0.1 mg/ml PNPase. The concentrations of MKP5 were 130, 52, 26, and 13 nM for curves 1, 2, 3, and 4, respectively. Reactions were initiated by the addition of 0.7 μM K53M, and the absorbance at 340 nm was monitored.

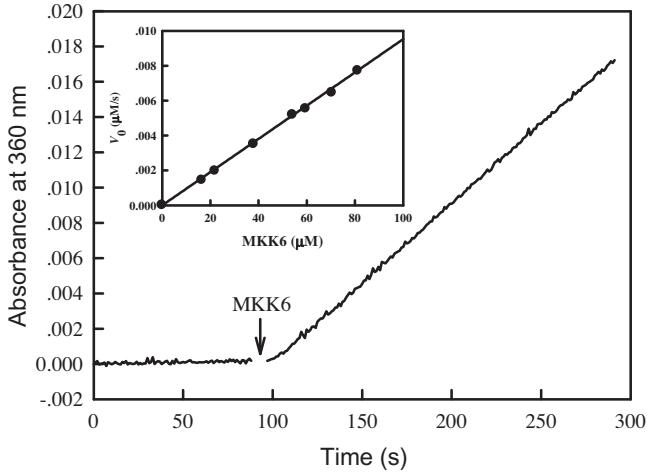


Fig.4. P_i release for the MKK6-catalyzed reaction, as measured by absorbance at 360 nm with the MESG assay system. The solutions at 25 °C contained standard assay buffer mixture, 250 mM ATP, 200 nM MKP5, and 0.7 μM K53M at pH 7.0. The reaction was initiated with 55 nM MKK6. Inset: Linearity of the protein kinase activity of MKK6 in the coupled enzyme assay. Initial rate is plotted against varying concentrations of MKK6 added to the standard assay.

reaction. The response is linear up to 80 nM MKK6 with the K53M mutant (0.7 μM) as a substrate. The linear relationship between the initial velocity and enzyme concentration further validates the coupled enzyme assay system for MKK6 activity.

The usual procedure to determine the kinetic parameters of an enzyme-catalyzed reaction is to carry out the reactions at a series of substrate concentrations and measure the reaction rates (slopes) during the initial stages of the reactions. In practice, it is not always easy to obtain accurate values of the slopes of the rate curves given that nonlinear initial rates are frequently observed, especially in the cases of lower substrate concentrations. However, in the cyclic system of kinase and phosphatase used in this study for interconverting the phosphorylated and unphosphorylated K53M, the substrate concentration always remains a constant during the MKK6-catalyzed reaction. Thus, the dependence of the initial velocity on the substrate concentration can be obtained by a modified titration experiment [25]. The procedure is to add increasing amounts of substrate to a relatively dilute solution of the enzyme and then calculate the reaction rate from the slope of linear absorbance trace between two substrate titrations. The data can then be transformed into substrate concentration versus velocity data and analyzed by a nonlinear least squares computer program. Fig. 5 depicts a typical titration curve obtained by adding incremental amounts of substrate K53M to a 1.8-ml assay solution. Because changes in effective volume of less than 2% during a substrate addition experiment may be neglected, there is no need for a dilution correction. A plot of the initial velocity versus K53M concentration is shown in the inset of Fig. 5. By fitting the Michaelis–Menten equation to the experimental data, the values of K_m and k_{cat} for the MKK6-catalyzed K53M phosphorylation were determined to be 0.152 ± 0.002 s^{-1} and 0.16 ± 0.009 μM, respectively. Thus, the use of this assay considerably simplified the performance of assay experiments.

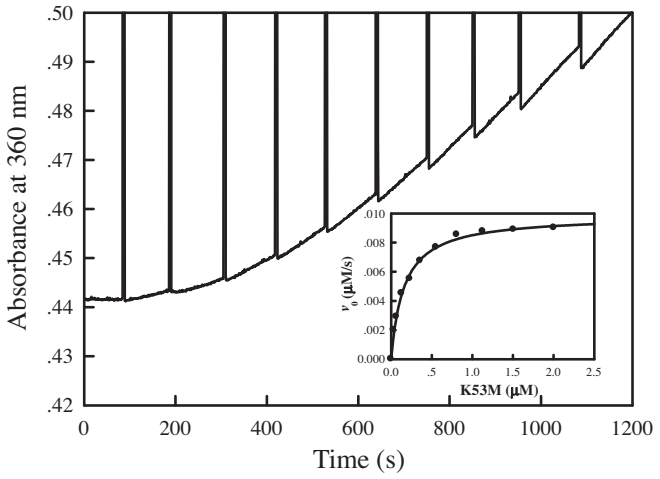


Fig.5. Change in absorbance at 360 nm for a titration of the MKK6-catalyzed reaction with substrate K53M in 50 mM Mops buffer (pH 7.0) and 25 °C. Aliquots (1–10 μl) of K53M solution (120 μM) were added into a 1.8-ml reaction mixture containing 250 mM ATP, 200 nM MKP5, and 55 nM MKK6. Stirring speed was set to 375 rpm. Inset: Plot of initial rate of the MKK6-catalyzed reaction versus K53M concentration. The diluting volume was not corrected. The solid line is the best fitting result according to the Michaelis–Menten equation, with k_{cat} = 0.152 ± 0.002 s^{-1} and K_m = 0.16 ± 0.009 μM.

After we validated and standardized the coupled enzyme assay system, we set out to measure the kinase activity of MKK6 with the wild-type p38α as a substrate. In the presence of excess phosphatase, the phosphorylated protein substrate molecules undergo dephosphorylation almost immediately after their formation; therefore, all protein substrate molecules are in the unphosphorylated state when the reaction system reaches the steady state. Therefore, under this condition, the steady-state use of the resultant P_i reflects the constant initial velocity of the protein kinase-catalyzed reaction. Fig. 6 shows the dependence of the initial rate of the MKK6-catalyzed reaction on the p38α concentration. By fitting the Michaelis–Menten equation to the initial rates versus [p38α] data, the k_{cat} and K_m values were determined to be 0.243 ± 0.003 s^{-1} and 0.205 ± 0.012 μM, respectively. Thus, with wild-type p38α as substrates, the k_{cat}/K_m values determined at pH 7.0 and 25 °C for MKK6 reactions are greater than 10^6 M^{-1} s^{-1}.

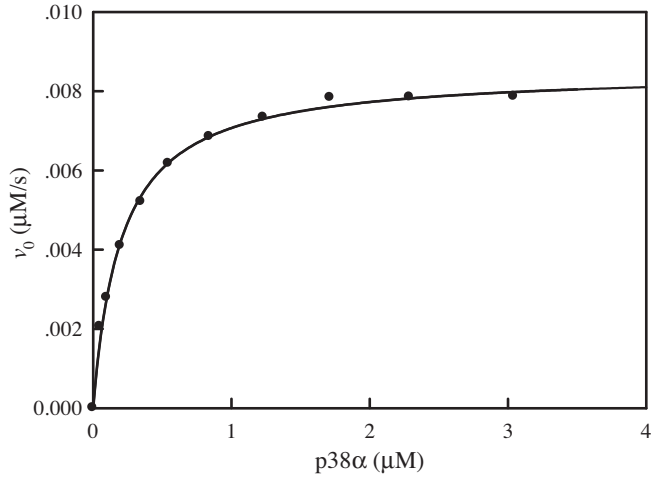
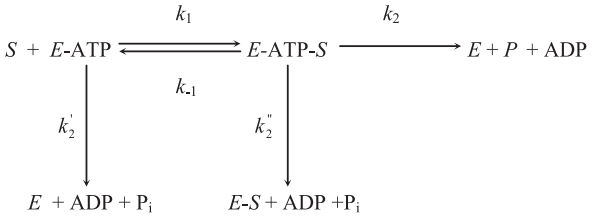


Fig.6. Plot of the initial rate of MKK6-catalyzed reaction versus the p38α concentration. Reaction mixtures contained standard assay buffer, 0.25 mM ATP, 200 nM MKP5, 35 nM MKK6, and the indicated concentrations of p38α at pH 7.0 and 25 °C. The solid line is the best fitting result, according to the Michaelis–Menten equation, with k_{cat} = 0.243 ± 0.003 s^{-1} and K_m = 0.205 ± 0.012 μM.

Kinetics of MEK1-catalyzed dual phosphorylation reaction

MEK1 is highly specific for two isoforms of the MAPK family: ERK1 and ERK2. As another example, we determined the kinetic parameters of the MEK1-catalyzed reaction using the current method. Unlike MKK6, MEK1 displays significant ATPase activity; therefore, the mechanism of MEK1-catalyzed reaction at a saturating concentration of ATP should be written a follows:



where *S* and *P* are the protein substrate and phosphoprotein product, respectively. ADP is a common product of kinase and ATPase reactions. P_i ad phosphoprotein, on the other hand, are unique products of ATPase and kinase reactions, respectively. The steady-state rates of formation of ADP, P_i, and phosphorylated protein are given by

$$v_0 = \frac{\{k_2' K_m + (k_2 + k_2'')[S]_0\}[E]_0}{K_m + [S]_0} \quad (1)$$

$$v_0' = \frac{(k_2' K_m + k_2''[S]_0)[E]_0}{K_m + [S]_0} \quad (2)$$

$$v_0'' = \frac{k_2[S]_0[E]_0}{K_m + [S]_0} \quad (3)$$

MKP3 is a dual specificity phosphatase that can dephosphorylate both P-Tyr185 and P-Thr183 in bisphosphorylated ERK2. In the presence of excess phosphatase, because the phosphoprotein product was dephosphorylated immediately, the P_i became a common product of kinase and ATPase reactions as well, In this case, the steady-state rate of phosphate release is also given by Eq. (1).

The kinetic parameters for the ATP hydrolyzing activity of MEK1 can be determined by the MESG/phosphorylase system of Webb [17]. Fig. 7A shows the variation of initial rates of the MEK1-catalyzed ATP hydrolysis with the concentrations of ATP. The experimental data fit the Michaelis–Menten equation with a $K_{m(ATP)}$ of 53.27 ± 6.64 μM and a k_2' of 0.085 ± 0.004 s^{-1} at pH 7.0 and 25 °C. To measure the kinase activity of MEK1 in the MKP3/phosphorylase-coupled assay system, we used an inactive mutant of ERK2, K52R, as a substrate. Fig. 7B shows the dependence of the initial rate of the MEK1-catalyzed reaction on the K52R concentration. The nonzero intercept is due to the ATPase activity of MEK in the absence of any added ERK2. It can be seen from this figure that K52R shows a strong substrate inhibition. Therefore, the expression of the initial rates of P_i release in the presence of MKP3 (ATPase plus kinase activities) should be written as

$$V_0 = \frac{\{k_2' K_m K_i + (k_2 + k_2'')K_i[S]_0\}[E]_0}{K_m K_i + K_i[S]_0 + [S]_0^2} \quad (4)$$

By fitting Eq. (4) to the experimental data shown in Fig. 7B, the kinetic parameters for the MEK1-catalyzed reaction with respect to K52R were determined to be K_m = 0.129 ± 0.021 μM, K_i = 0.671 ± 0.117 μM, k_2' = 0.0744 ± 0.004 s^{-1}, and $(k_2 + k_2'')$ = 0.408 ± 0.030 s^{-1}.

To further characterize the effects of protein substrate on the ATPase activity of MEK1, we monitored the ATPase activity of MEK1 in the presence of various concentrations of K52R by the MESG/phosphorylase method. Progressive addition of protein substrate (K52R) resulted in the decline of the rate of phosphate

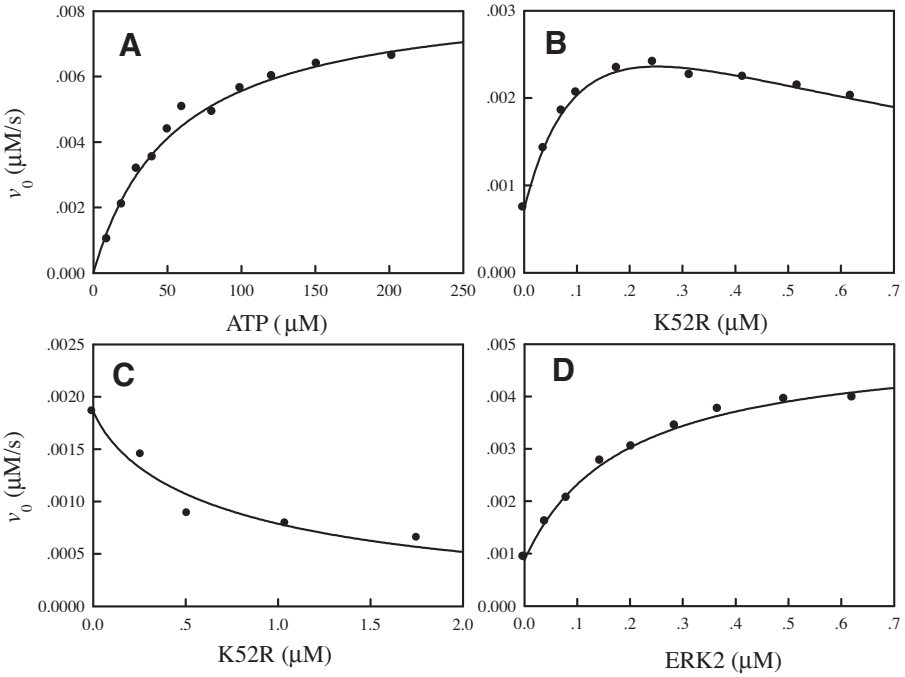


Fig.7. (A) Plot of the initial rate of MEK1-catalyzed ATP hydrolysis versus the ATP concentration. Reaction mixtures contained standard assay buffer, 17.6 nM MEK1, and indicated concentrations of ATP at pH 7.0 and 25 °C. The solid line is the best fitting result, according to the Michaelis–Menten equation, with $k_2' = 0.0853 \pm 0.004\ s^{-1}$ and $K_{m(ATP)} = 53.27 \pm 6.64$ μM. (B) Plot of the initial rate of MEK1-catalyzed reaction (ATPase plus kinase activities) versus the K52R concentration. Reaction mixtures contained standard assay buffer, 0.5 mM ATP, 100 nM MKP3, 9.9 nM MEK1, and the indicated concentrations of K52R at pH 7.0 and 25 °C. The solid line is the best fitting result, according to Eq. (4), with $k_2' = 0.0744 \pm 0.004\ s^{-1}$, $k_2'' + k_2 = 0.408 \pm 0.030\ s^{-1}$, $K_i = 0.671 \pm 0.117$ μM, and $K_m = 0.129 \pm 0.021$ μM. (C) Plot of ATPase activity of MEK1 versus the K52R concentration. Reaction mixtures contained the standard buffer, 17.6 nM MEK1, and indicated concentrations of K52R at pH 7.0 and 25 °C. The solid line is the best fitting result, according to Eq. (6), with $\alpha = k_2'/k_2''$ 0.899 ± 0.038. (D) Plot of the initial rate of MEK1-catalyzed reaction (ATPase plus kinase activities) versus the ERK2 concentration. Reaction mixtures contained standard assay buffer, 0.5 mM ATP, 100 nM MKP3, 9.9 nM MEK1, and the indicated concentrations of ERK2 at pH 7.0 and 25 °C. The solid line is the best fitting result, according to Eq. (1), with $k_2' = 0.088 \pm 0.008\ s^{-1}$, $k_2'' + k_2 = 0.503 \pm 0.017\ s^{-1}$, and $K_m = 0.184 \pm 0.024$ μM.

production (Fig. 7C). In the absence of MKP3 (ATPase activity only), the expression of the initial rates of P_i release is given by

$$v_0' = \frac{(k_2' K_m K_i + k_2'' K_i [S]_0)[E]_0}{K_m K_i + K_i [S]_0 + [S]_0^2} \tag{5}$$

and the relative enzyme activity can be written as

$$\alpha = \frac{v_0'}{k_2'[E]_0} = \frac{(K_m K_i + \alpha K_i [S]_0)}{K_m K_i + K_i [S]_0 + [S]_0^2}, \tag{6}$$

where $\alpha = k_2'/k_2''$. Based on the experimentally measured values of K_m and K_i, the ratio of k_2'' to k_2' was determined to be 0.899 ± 0.038 by fitting Eq. (6) to the experimental data shown in Fig. 7C. These results indicate that ATPase activity of MEK1 is not in competition with its protein kinase activity, and binding of protein substrate to MEK1 only slightly inhibits its ATPase activity. Finally, using the wild-type ERK2 substrate, we also conducted a kinase activity assay of MEK1 by following the P_i formation in the MKP3/phosphorylase-coupled system. In contrast, at 0.5 mM ATP, the enzyme activity shows a hyperbolic concentration dependence on ERK2 (Fig. 7D). The experimental data fit Eq. (1) with $K_m = 0.184 \pm 0.024$ μM, $k_2' = 0.088 \pm 0.008\ s^{-1}$, and $(k_2' + k_2'') = 0.503 \pm 0.017\ s^{-1}$. Assuming the same α value for the wild-type ERK2, the values of k_2'' and k_2 for MEK1-catalyzed wild-type ERK2 phosphorylation can be calculated as $0.088 \times 0.899 = 0.079\ s^{-1}$ and $0.503 - 0.079 = 0.424\ s^{-1}$, respectively. All of the kinetic parameters of MKK-catalyzed reaction toward wild-type substrate are summarized in Table 2. These values are in reasonable agreement with the values obtained from different assay techniques [26].

Table 2
Kinetic parameters of MKK6 and MEK1 for wild-type MAPK substrates

MAPK kinase	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
MEK1 (G7B)	ERK2	0.503 ± 0.017	0.184 ± 0.024	$(2.73 \pm 0.35) \times 10^6$
MKK6EE	p38α	0.243 ± 0.003	0.205 ± 0.012	$(1.2 \pm 0.08) \times 10^6$

Discussion

Protein phosphorylation is one of the most important processes for cellular regulation and signal transduction in eukaryotic cells. The enzymes responsible for catalyzing this reaction are protein kinases. Two methods are commonly used to assay protein kinase-catalyzed reaction in the literature. The first method is the radioactive assay, in which kinase activity is determined by quantifying ^{32}P incorporation from $[\gamma - {}^{32}P]$ATP into protein substrate. Although this method is effective in obtaining kinetic constants, it suffers from major disadvantages of being discontinuous and, consequently, not particularly sensitive to deviations from linearity in kinetic measurements. In the second method, kinase activity is determined by a continuous spectrophotometric assay that uses pyruvate kinase, lactate dehydrogenase, and NADH to measure ADP formation by kinase reaction [27]. This assay is more widely applicable but suffers from the fact that it needs to follow small changes against an already high initial absorbance. It is difficult to measure a change of less than 0.5 μM/min (which translates to 0.003 A/min at 340 nm) because the molar extinction coefficient of NADH oxidation is only 6220 $M^{-1}\ cm^{-1}$. Therefore, the pyruvate kinase/lactate dehydrogenase-coupled system cannot be applied to determine the kinetic parameters of protein kinase reaction with a submicromolar K_m value. In addition, the activated MAPK displays

significant ATPase activity and will rapidly exhaust the available ATP in the reaction system. Thus, for most MAPK kinase kinetic studies, an inactive mutant of MAPK was commonly used as substrate to avoid possible complications from the hydrolysis of ATP by the activated wild-type MAPK [26,28]. As shown in this study, however, the point mutation in MAPK may interfere with the MKK-catalyzed reaction and, therefore, yield different kinetic parameters.

In the current study, we have developed a fast spectrophotometric method for MAPK kinase activity assay that incorporates the continuous assay system of Webb for P_i [17]. The problems in the pyruvate kinase/lactate dehydrogenase-coupled system are circumvented in the coupled system described here because the linear increase in absorbance is a reflection of the constant initial velocity of protein kinase reaction due to the continual regeneration of its substrate. The cyclic nature of the coupled enzyme system allows for the ready determination of the initial velocity of the protein kinase reactions with lower substrate concentrations. The data presented also show that kinetic parameters for enzymes can be estimated by the described titration method using substrate addition–slope tangent analysis [25]. Thus, compared with many other laborious kinase assay procedures, the method outlined offers the advantages of rapidity, accuracy, and simplicity given that isolation of reaction products is not required. The coupled enzyme system was applied to measure the activity of two MAPK kinases, MEK1 and MKK6, and should be applicable to other protein kinases. However, this will depend on availability of the appropriate protein phosphatase. A drawback of this system is the possible effect at non-neutral pH conditions on the assay components. The spectrophotometric signal at 360 nm is limited to a pH range of approximately 6.5 to 8.8, and the stability of MESG is pH dependent. Thus, the correction for the instability of MESG at alkaline pH values must be considered. Despite this drawback to the system, the coupled enzyme system should make a valuable addition to the current assay systems for the kinetic analysis of MAPK kinases at neutral pH.

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