A Kinetic Approach for the Study of Protein Phosphatase-Catalyzed Regulation of Protein Kinase Activity†

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ABSTRACT: The activities of many protein kinases are regulated by phosphorylation. The phosphorylated protein kinases thus represent an important class of substrates for protein phosphatases. However, our ability to study the phosphatase-catalyzed substrate dephosphorylation has been limited in many cases by the difficulty in preparing sufficient amount of stoichiometrically phosphorylated kinases. We have applied the kinetic theory of substrate reaction during irreversible modification of enzyme activity to the study of phosphatase-catalyzed regulation of kinase activity. As an example, we measured the effect of the hematopoietic protein−tyrosine phosphatase (HePTP) on the reaction catalyzed by the fully activated, bisphosphorylated extracellular signal-regulated protein kinase 2 (ERK2/pTpY). Because only a catalytic amount of ERK2/pTpY is required, this method alleviates the need for large quantities of phospho-ERK2. Kinetic analysis of the ERK2/pTpY-catalyzed substrate reaction in the presence of HePTP leads to the determination of the rate constants for the HePTP-catalyzed dephosphorylation of free ERK2/pTpY and ERK2/pTpY•substrate(s) complexes. The data indicate that ERK2/pTpY is a highly efficient substrate for HePTP ($k_{cat}/K_m = 3.05 \times 10^6\ M^{-1}\ s^{-1}$). The data also show that binding of ATP to ERK2/pTpY has no effect on ERK2/pTpY dephosphorylation by HePTP. In contrast, binding of an Elk-1 peptide substrate to ERK2/pTpY completely blocks the HePTP action. This result indicates that phosphorylation of Tyr185 is important for ERK2 substrate recognition and that binding of the Elk-1 peptide substrate to ERK2/pTpY blocks the accessibility of pTyr185 to HePTP for dephosphorylation. Collectively, the results establish that the kinetic theory of irreversible enzyme modification can be applied to study the phosphatase catalyzed regulation of kinase activity.

Enzyme-catalyzed covalent modification of another enzyme is an important mechanism for the regulation of target enzyme activity. Protein phosphorylation is the most prevalent form of post-translational covalent modification that cells utilize to regulate enzyme activity and control various signal transduction pathways. Protein kinases, which catalyze protein phosphorylation reactions, have been extensively studied. Major insights for cellular signaling have been derived from studies of protein kinases, and it is common to view signaling pathways as cascades of protein kinase reactions. However, it is important to keep in mind that the level of cellular protein phosphorylation is controlled by the activities of both protein kinases and protein phosphatases. In addition, because many protein kinases are themselves regulated by phosphorylation (*1*), protein phosphatases can also directly affect the function of protein kinases by removing the phosphate(s) from the kinases.

To understand the role of protein phosphatases in cellular regulation, it is necessary to have a detailed understanding of how they catalyze the dephosphorylation of their physiological substrates. Unfortunately, direct measurement of the phosphatase-catalyzed dephosphorylation is more difficult to carry out, since the requirement for sufficient quantities of specifically and stoichiometrically phosphorylated proteins represents a significant technical hurdle. However, if the phosphatase substrates (i.e., phosphoproteins) possess an intrinsic enzyme activity that is regulated by this phosphorylation, then it should be possible to study the kinetic properties of the phosphatase by analyzing the effect of the phosphatase on the activity of the phosphorylated enzyme. This is essentially the same as the kinetic procedure by which the inactivation rate constant can be derived from irreversible modification of enzyme activity by small molecule modifiers in the presence of substrates (*2*).

In this study, we have shown that the kinetic theory of substrate reaction during modification of enzyme activity (*2*−*10*) can be applied to study the kinetic properties of a

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[1] Abbreviations: MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; MEK, MAP kinase/ERK kinase; HePTP, hematopoietic protein-tyrosine phosphatase; EDTA, ethylenediaminetetraacetic acid; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ERK2/pTpY, ERK2 phosphorylated on both Thr183 and Tyr185; ERK2/pT, ERK2 phosphorylated on Thr183.

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phosphatase-catalyzed kinase inactivation. As an example, we have analyzed the kinetics of the hematopoietic protein tyrosine phosphatase (HePTP) catalyzed inactivation of the bisphosphorylated extracellular signal-regulated protein kinase 2 (ERK2/pTpY). Biochemical and HePTP-/- mice studies suggest that HePTP may be a physiological negative regulator of ERK2 by selective dephosphorylation of pTyr185 in ERK2/pTpY (*11*, *12*). However, it is not known how efficiently can HePTP carry out the ERK2/pTpY dephosphorylation. From measurements of the effect of HePTP on the ERK2/pTpY-catalyzed phosphorylation of an ERK2 specific peptide substrate derived from the transcription factor Elk-1, we were able not only to obtain the apparent rate constants for irreversible inactivation of ERK2/pTpY activity from a single experiment, but also to ascertain the effect of ERK2 substrates on ERK2/pTpY dephosphorylation by HePTP. Because only a catalytic amount of ERK2/pTpY is required, this method alleviates the need for large quantities of phospho-ERK2. In addition, since the experimental conditions used resemble more closely the in vivo situation where the kinase substrate is constantly being turned over while the ERK2/pTpY enzyme is being modified, results from this method are more physiologically relevant. Collectively, the results show that this approach can be used to study any enzyme systems in which one enzyme acts to modulate the activity of another by covalent modification.

EXPERIMENTAL PROCEDURES

Materials. The substrate used for ERK2 kinase assay is a synthetic peptide derived from Elk-1 (residues 385−399, Ac−RRPRSPAKLSFQFPS−NH_2) which contains an ERK2 phosphorylation site Ser389 (*13*) and an ERK2 docking site sequence FQFP (*14*). The Elk-1 peptide was synthesized using standard protocol, purified by HPLC, and characterized by MALDI-TOF mass spectrometry by Alpha Diagnostic International. The purity of the peptide was determined to be close to 100%. Phospho(enol)pyruvate, NADH, lactate dehydrogenase, and pyruvate kinase were purchased from Sigma. 7-Methyl-6-thioguanosine was prepared according to the procedures described (*15*).

Protein Phosphatases. The N-terminal His_6-tagged HePTP was expressed in *Escherichia coli* BL21(DE3) and purified using standard procedures of nickel chelate affinity chromatography. The protein purity was over 90% judged by SDS−PAGE. The purified protein was made to 20% glycerol and stored at −80 °C.

Preparation of Unphosphorylated and Bisphosphorylated ERK2. The N-terminal His_6-tagged ERK2 was expressed in *E. 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 anion exchange Mono Q column (Pharmacia) as described previously (*16*). The plasmid pET−His_6−ERK2−MEK1-(R4F) (a generous gift of Dr. Melanie Cobb) was used to coexpress a constitutively active MEK1 and an N-terminal His_6-tagged ERK2 in *E. coli* BL21(DE3). The expression and purification of ERK2/pTpY were carried out following the procedure described by Wilsbacher and Cobb (*17*). After the final FPLC step, about 3 mg of ERK2/pTpY was obtained from 6 L of culture. Electrospray ionization mass spectrometry analysis confirmed that the purified ERK2/pTpY was effectively homogeneous and was phosphorylated stoichiometrically to a ratio of 2 mol of phosphate per mole of ERK2.

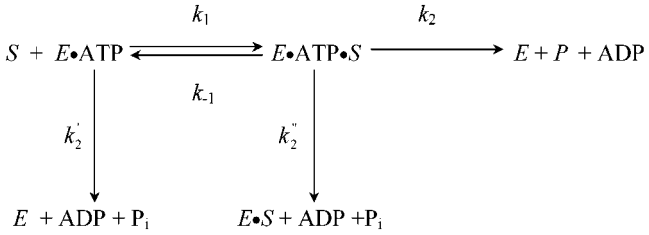
Enzyme Assays. Enzyme activity of the phosphorylated ERK2 was determined with the Elk-1 peptide as a substrate using a coupled spectrophotometric assay (*18*). The standard assay for ERK2, except where indicated, was carried out at 25 °C in 1.6 mL reaction mixtures containing 50 mM MOPS buffer, pH 7.0, 0.1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 1.0 mM phospho(enol)pyruvate, 200 μM NADH, 17.6 units/mL lactate dehydrogenase, 5.8 units/mL pyruvate kinase, and different concentrations of substrates. Reactions were initiated by the addition of ERK2 to the reaction mixture. Progress of the reaction was monitored by a continuous decrease in absorbance at 340 nm. Initial rates were determined from the linear slopes of progress curves obtained based on an extinction coefficient for NADH of 6220 $cm^{-1}M^{-1}$ at 340 nm. For ERK2/pTpY inactivation experiments, the time courses of ERK2/pTpY-catalyzed reaction in the presence of HePTP were monitored continuously by following the decrease of NADH at 340 nm, on a Perkin Elmer Lambda 14 spectrophotometer equipped with a magnetic stirrer in the cuvette holder, and the experimental data were analyzed using a nonlinear regression analysis program. The bisphosphorylated ERK2 was added last at a final concentration 58.8 nM to initiate the reaction. The concentrations of substrates and HePTP are given in the text or in the legends to the figures. Determination of the inactivation rate constants of ERK2/pTpY by monitoring the substrate reaction in the presence of HePTP was essentially carried out as previously described (*3*) except that the activity of ERK2/pTpY was monitored by a coupled enzyme assay system. In a theoretical study, it has been shown that the substrate reaction method using a coupled enzyme assay can be used for the study of enzyme inactivation kinetics (*19*). In the case of a two-substrate reaction (e.g., ATP and the Elk-1 substrate for the ERK2/pTpY reaction), it is assumed that one substrate for the enzyme reaction is present at a constant level and can be included in the kinetic constant without loss of generality (see Appendix). In control experiments, HePTP did not affect the activity of the coupling enzymes under identical conditions.

RESULTS

Characterization of the ERK2 Substrate Reaction. The goal of the present work was to develop a general kinetic approach for studying enzyme-catalyzed covalent modification of a target enzyme. As an example, we investigated the kinetics of ERK2/pTpY inactivation by HePTP during the ERK2/pTpY-catalyzed substrate turnover. To study the effect of HePTP in the ERK2/pTpY-catalyzed substrate reaction, we must fully characterize the ERK2/pTpY reaction. The kinase activity of ERK2/pTpY was measured with a synthetic peptide derived from Elk-1 (residues 385−399, Ac−RRPRSPAKLSFQFPS−NH_2) which contains an ERK2 phosphorylation site Ser389 (*13*) and an ERK2 docking site sequence FQFP (*14*). The Elk-1 peptide was chosen because it exhibits a K_m (∼30 μM) that is suitable for the kinetic analysis under initial rate conditions. In addition, to observe the effects of HePTP on free ERK2/pTpY and ERK2/pTpY-substrate complexes, the ERK2 substrate concentration should be around the K_m. Although myelin basic protein and the Elk-1 protein are more efficient substrates (*16*) than the

Elk-1 peptide, their low K_m values (in the low μM range) preclude their use in this method because the amount of products formed would not be sufficient to ensure accurate rate measurements under initial rate conditions.

Because ERK2/pTpY displays a significant amount of ATPase activity in the absence of any added protein/peptide substrate (*16*), the kinetic scheme of the ERK2/pTpY-catalyzed reaction at a saturating concentration of ATP can be written as



where S and P are the Elk-1 peptide substrate and the phosphorylated peptide product, respectively. E represents ERK2/pTpY or ERK2/pT. ADP is a common product of the kinase and ATPase reactions. Inorganic phosphate (P_i) and the phosphorylated Elk-1 peptide, on the other hand, are unique products of the ATPase and kinase reactions, respectively. The steady-state rate of formation of ADP (v_0), inorganic phosphate (v_0'), and phosphorylated peptide (v_0'') are given by

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

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

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

where K_m is the Michaelis constant for the Elk-1 peptide, k_2 is the rate constant for the Elk-1 peptide phosphorylation, k_2' is the ATPase activity for ERK2/pTpY, k_2'' is the ATPase activity for the ERK2/pTpY•Elk-1 peptide complex, and $\alpha = k_2''/k_2'$.

The kinetic parameters for the ATPase activity of ERK2/pTpY in the absence of the peptide substrate were measured using a continuous spectrophotometric assay that couples production of ADP to oxidation of NADH, measured as a decrease in absorbance at 340 nm (*18*, see Experimental Procedures). Figure 1A shows the variation of initial rates of the ERK2/pTpY-catalyzed ATP hydrolysis with the concentrations of ATP. The experimental data fit the Michaelis−Menten equation with a $K_{m(ATP)}$ of 104.2 ± 6.1 μM, and a k_2' of 0.114 ±0.003 s^{-1}, at pH 7.0, 25 °C. We then used the same assay to determine the kinetic parameters of ERK2/pTpY kinase activity. Figure 1B shows the dependence of the initial rates of the ERK2/pTpY-catalyzed reaction on Elk-1 peptide substrate concentration. Again, there is measurable ATPase activity in the absence of any added peptide. As the peptide substrate concentration increased, the net rate of ADP release (ATPase plus kinase activities) increased. The experimental data fit well with eq 1 yielding $K_m = 29.3 \pm 5.8$ μM, $k_2' = 0.169 \pm 0.020$ s^{-1}, and $(k_2 + k_2'') = 0.622 \pm 0.017$ s^{-1}.

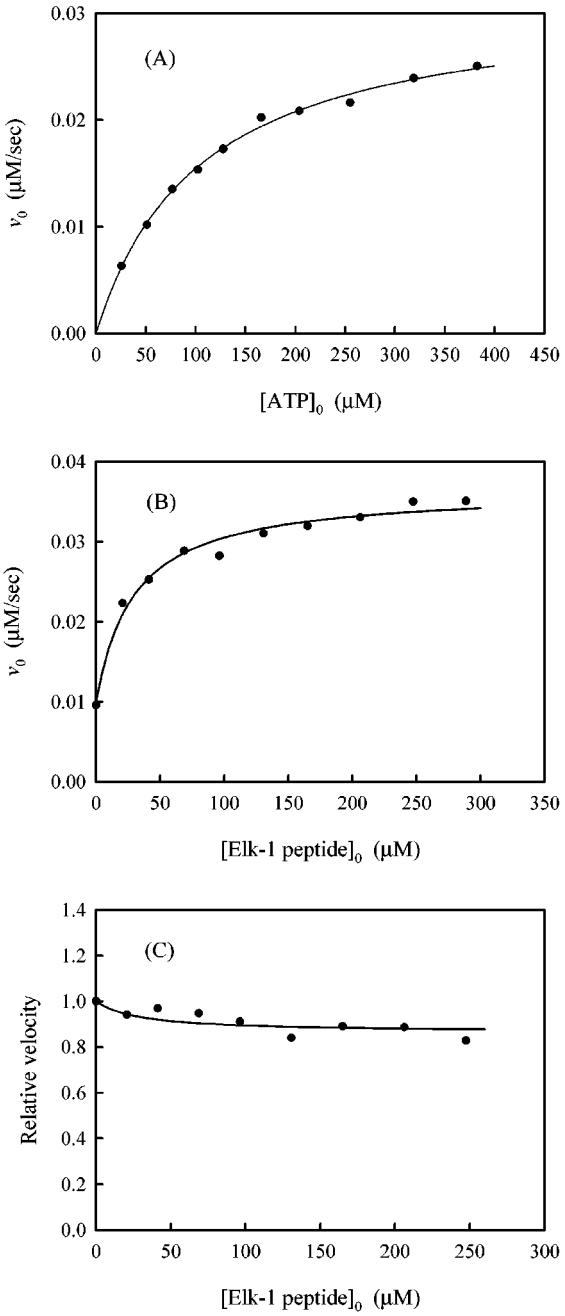


FIGURE 1: (A) Plot of initial rate of ERK2/pTpY-catalyzed ATP hydrolysis vs the ATP concentration. The reactions were followed by the production of ADP. Reaction mixtures contained standard assay buffer, 276.5 nM ERK2/pTpY and indicated concentrations of ATP, at pH 7.0 and 25 °C. (B) Plot of the initial rate of ERK2/pTpY-catalyzed reaction (ATPase plus kinase activities) vs the Elk-1 peptide substrate concentration. The reactions were followed by the production of ADP. Reaction mixtures contained standard assay buffer, 1 mM ATP, 58.8 nM ERK2/pTpY, and indicated concentrations of the peptide substrate, at pH 7.0 and 25 °C. (C) Plot of ATPase activity of ERK2/pTpY vs the peptide substrate concentration. Here, the reactions were followed by the production of inorganic phosphate. Reaction mixtures contains 50 mM MOPS, 0.1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP, 99 μM 7-methyl-6-thioguanosine, 0.125 mg/mL of purine nucleoside phosphorylase, 58.8 nM ERK2/pTpY, and indicated concentrations of the peptide substrate, at pH 7.0 and 25 °C.

Table 1: Kinetic Parameters of the Different Phosphorylated Forms of ERK2-Catalyzed Reactions

	k_2' (sec^{-1})	k_2'' (sec^{-1})	k_2 (sec^{-1})	$K_{m(Elk-1)}$ (μM)
ERK2/pTpY	0.169 ± 0.020	0.145 ± 0.017	0.477 ± 0.020	29.3 ± 5.8
ERK2/pT	0.014 ± 0.004	0.014 ± 0.004	0.129 ± 0.011	80.6 ± 16.7
ERK2	–	–	0.00484 ± 0.00051	2180 ± 320

To characterize the ATPase activity of ERK2/pTpY in the presence of the peptide substrate, we directly measured the inorganic phosphate released from the ERK2/pTpY-catalyzed hydrolysis of ATP by another enzyme coupled procedure, involving purine nucleoside phosphorylase and its chromophoric substrate, 7-methyl-6-thioguanosine (*15*). As shown in Figure 1C, progressive addition of peptide substrate resulted in a slight decline of the rate of phosphate production. As the peptide substrate concentration approached saturation, the ERK2/pTpY-catalyzed rate of phosphate release (ATPase activity) reached a constant value, suggesting that the EKR2/pTpY•peptide binary complex has a considerable ATPase activity. As the K_m for the Elk-1 peptide is known, the ratio of k_2'' to k_2', α, was determined to be 0.86 by fitting the experimental data to eq 2. Therefore, the values of k_2'' and k_2 can be calculated as $0.169 \times 0.86 = 0.145\ s^{-1}$ and $0.622 - 0.145 = 0.477\ s^{-1}$, respectively. All the kinetic parameters of the ERK2/pTpY-catalyzed reaction are summarized in Table 1.

Inactivation Kinetics of ERK2/pTpY by HePTP. HePTP has been implicated to negatively regulate the T cell antigen receptor signaling by dephosphorylating the pTyr residue in activated ERK2 (*11*, *12*). Phosphoamino acid analysis indicates that ERK2/pTpY treatment by HePTP results in rapid loss of phosphate from pTyr without hydrolyzing pThr (*11*, *20*). However, the kinetic parameters for the HePTP-catalyzed dephosphorylation of ERK2/pTpY have not been measured. We applied the kinetic theory of the substrate reaction during modification of enzyme activity (*2*, *3*) to the study of the dephosphorylation of bisphosphorylated ERK2 by HePTP. The method allows extraction of kinetic parameters for the HePTP-catalyzed ERK2/pTpY dephosphorylation from its effect on the ERK2/pTpY-catalyzed peptide phosphorylation reaction.

According to the substrate reaction theory, the product (ADP) formation of the ERK2/pTpY and ERK2/pT-catalyzed reaction with time, in the presence of HePTP, is given by

$$[P] = v_0^* t + \frac{v_0 - v_0^*}{k_{obs}}(1 - e^{-k_{obs}t}) \quad (4)$$

where v_0 and v_0^* are the initial rates of the bis- and monothreonine phosphorylated ERK2-catalyzed reaction, respectively, and k_{obs} is the apparent inactivation rate constant for the HePTP-catalyzed ERK2/pTpY dephosphorylation. When the total concentrations of both HePTP and ERK2/pTpY are much less than the corresponding dissociation constant for the ERK2/pTpY•HePTP complex, the expression of k_{obs} can be written as (for detailed derivation, see Appendix)

$$k_{obs} = \frac{\frac{k_c}{K_S}K_m + \frac{k_c'}{K_S'}[S]_0}{K_m + [S]_0}[M]_0 \quad (5)$$

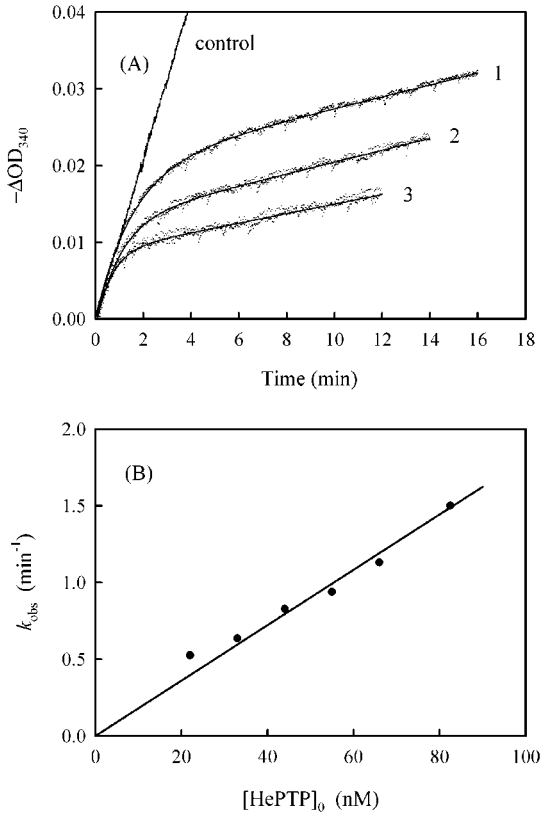


FIGURE 2: (A) Time course of the substrate reaction in the presence of different concentrations of HePTP. The reactions were followed by ADP formation. Final concentrations were 1 mM ATP, 103.1 μM Elk-1 peptide substrate, and 58.8 nM ERK2/pTpY in the standard assay buffer at pH 7.0, 25 °C. Concentrations of HePTP were 0 nM (control), 33 nM (curve 1), 55 nM (curve 2), and 82.5 nM (curve 3), respectively. The reaction was started by the addition of ERK2/pTpY to the reaction mixture. The data were fitted to eq 4 to determine the kenetic parameters, v_0, v_0^* and k_{obs}. The solid lines correspond to the best fit of the data to eq 4 by a nonlinear least-squares regression algorithm. (B) Plot of k_{obs} against the HePTP concentration for the inactivation of ERK2/pTpY by HePTP.

where k_c/K_S and k_c'/K_S' are the apparent second-order rate constants for dephosphorylation of free ERK2/pTpY and the ERK2/pTpY•substrate complex, respectively. $[M]_0$ is the total concentration of HePTP in the reaction system. If the ERK2 substrate has no effect on ERK2/pTpY inactivation by HePTP, then $k_c/K_S = k_c'/K_S'$, and the apparent inactivation rate constant will be independent of the concentration of substrate, i.e., $k_{obs} = k_c/K_S[M]_0$.

Figure 2A shows the time courses of the ERK2/pTpY-catalyzed Elk-1 peptide phosphorylation in the presence of different concentrations of HePTP. The progress curves start off linear (the initial-rate phase) but fall off with increasing time due to the action of HePTP. When t approaches infinity, $[P]$ approaches an asymptote with a positive slope. The fact that an increase of HePTP concentration does not affect the final level of residual ERK2/pT kinase activity suggests that the dephosphorylation of ERK2/pTpY is an irreversible

process and the dephosphorylation of pTyr in ERK2/pTpY is complete (*2*). In addition, the results also indicate that ERK2/pT still possesses about 10% of residual activity, which is represented by a constant slope of the straight line portions of the curves at different concentrations of HePTP (Figure 2A). We have determined in control experiments that the Elk-1 peptide (either phosphorylated or not) does not inhibit the HePTP-catalyzed hydrolysis of *p*-nitrophenyl phosphate and that HePTP does not dephosphorylate the Ser phosphorylated Elk-1 peptide (data not shown). Under the conditions employed in the present study, the time-dependent behavior of ERK2/pTpY inactivation can be well described by eq 4. The best-fit curves in Figure 2A were obtained by nonlinear regression analysis from which the kenetic parameters, v_0, v_0^*, and k_{obs} can be determined. As expected, v_0 and v_0^* are independent of HePTP concentration (data not shown) at fixed Elk-1 peptide concentration. Figure 2B shows the plots of the apparent inactivation rate constant, k_{obs}, against the concentration of HePTP. It can be seen from this figure that k_{obs} is proportional to $[HePTP]_0$, indicating that the reaction obeys the noncomplexion mechanism (i.e., case 2 in Appendix). This result is in agreement with the experimental conditions used in the present study since the Michaelis constant for the HePTP-catalyzed ERK2/pTpY dephosphorylation is in the micromolar range (Zhou et al., unpublished observation).

Effect of ERK2 Substrates on the HePTP-Catalyzed ERK2/pTpY Inactivation. Figure 3A shows the time courses of ERK2/pTpY inactivation by HePTP in the presence of different concentrations of ATP. Using procedures described above, the values of k_{obs} can be obtained by fitting the progress curves with eq 4. A plot of $k_{obs}/[HePTP]_0$ against the concentration of ATP is shown in Figure 3B. It can be seen from this figure that the $k_{obs}/[HePTP]_0$ value is independent of the concentration of ATP. This result indicates that ATP has no effect on the inactivation of ERK2/pTpY under the experimental conditions used, and therefore, the value of k_c/K_S in eq 5 is identical to that of k_c'/K_S'. Thus, the apparent second-order rate constants for the HePTP-catalyzed dephosphorylation of free ERK2/pTpY and the ERK2/pTpY•ATP binary complex are identical and also equal to $k_{obs}/[HePTP]_0$, which is $(3.05 \pm 0.11) \times 10^6$ M^{-1} s^{-1}, as determined from Figure 3B (Table 2).

Figure 4 shows the time courses of ERK2/pTpY inactivation by HePTP in the presence of different concentrations of the Elk-1 peptide substrate and at fixed saturating concentration of ATP (1 mM), which is within the physiological range. We found that as the peptide substrate concentration increased, the inactivation rate of ERK2/pTpY decreased dramatically, indicating the peptide substrate protects ERK2/pTpY against dephosphorylation by HePTP. Therefore, a high level of HePTP was used to increase the reaction rate of ERK2/pTpY dephosphorylation at higher concentrations of the peptide substrate (Figure 4B). As expected, the observed second-order rate constants ($k_{obs}/[HePTP]_0$) at two HePTP concentrations (curves 5 and 6 in Figure 4) were identical. A plot of $k_{obs}/[HePTP]_0$ versus the concentration of the peptide substrate is shown in Figure 5. It should be noted that in this case, k_c/K_S and k_c'/K_S' as described in eq 5 are the apparent second-order rate constants

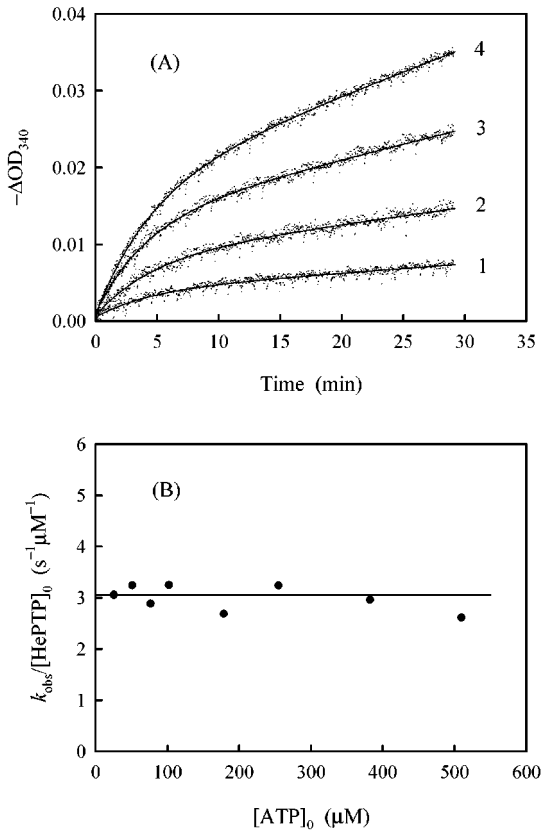


FIGURE 3: (A) Time course of the ERK2/pTpY substrate reaction at different concentrations of ATP in the presence of HePTP. The reactions were monitored by ADP formation. Final concentrations were 1.25 nM HePTP and 58.8 nM ERK2/pTpY in the standard assay buffer (pH 7.0) at 25 °C. Concentrations of the substrate (ATP) were 25.5 μM (curve 1), 76.45 μM (curve 2), 254.8 μM (curve 3), and 382.3 μM (curve 4), respectively. The solid lines correspond to the best fit of the data to eq 4 by a nonlinear least-squares regression algorithm. (B) Plot of $k_{obs}/[HePTP]_0$ against $[ATP]_0$ for the inactivation of ERK2 by HePTP. The values of the apparent inactivation rate constant were calculated from the time courses of substrate reaction shown in Figure 4A.

Table 2: Kinetic Parameters of the HePTP-Catalyzed Dephosphorylation of ERK2/PTpY

HePTP substrate	k_c/K_S (M^{-1} sec^{-1})
ERK2/pTpY[a]	$(3.05 \pm 0.11) \times 10^6$
ERK2/pTpY•ATP[a]	$(3.05 \pm 0.11) \times 10^6$
ERK2/pTpY·ATP[b]	$(3.18 \pm 0.16) \times 10^6$
ERK2/pTpY·ATP·peptide[b]	0

[a] Determined from the effect of ATP on the inactivation of ERK2/pTpY by HePTP. [b] Determined from the effect of the Elk-1 peptide on the inactivation of ERK2/pTpY by HePTP.

for the dephosphorylation of the ERK2/pTpY•ATP binary and ERK2/pTpY•ATP•peptide tertiary complexes, respectively. When the concentration of the peptide substrate was increased, $k_{obs}/[HePTP]_0$ decreased and approached zero. By fitting eq 5 to the experimental data, we determined the parameters to be $K_m = 31.3 \pm 7.4$ μM, $k_c/K_S = (3.15 \pm 0.14) \times 10^6$ M^{-1} s^{-1}, and $k_c'/K_S' = -(4.13 \pm 2.53) \times 10^5$ M^{-1} s^{-1}, respectively. Because the Elk-1 peptide protected ERK2/pTpY•ATP from inactivation by HePTP and because k_c'/K_S' derived from the curve-fitting of the data to eq 5 had a negative value with a very large standard deviation, we

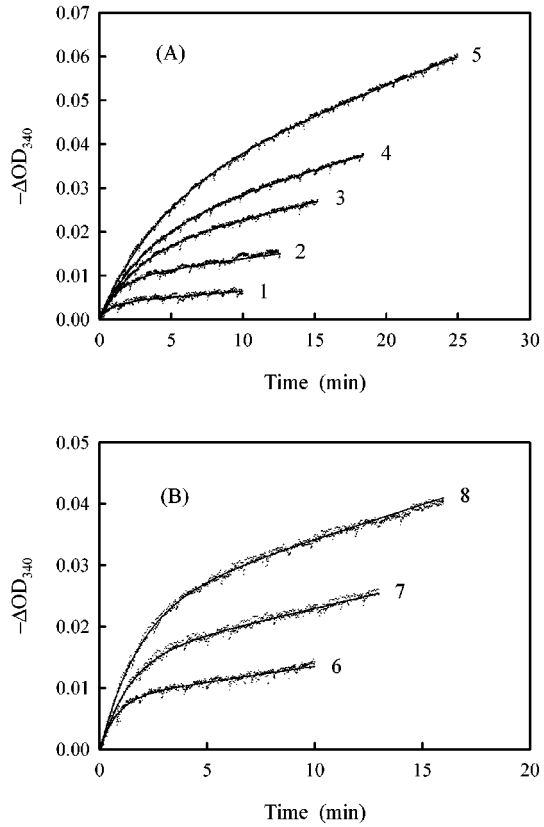


FIGURE 4: Time course of the ERK2/pTpY substrate reaction at different Elk-1 peptide substrate concentrations in the presence of 5.5 nM HePTP (A) or 27.5 nM HePTP (B). All reactions were followed by ADP formation. (A) Concentrations of the Elk-1 peptide substrate were 0 μM (curve 1), 17.19 μM (curve 2), 34.38 μM (curve 3), 51.56 μM (curve 4), and 68.75 μM (curve 5), respectively. (B) Concentrations of the Elk-1 substrate were 68.75 μM (curve 6), 103.1 μM (curve 7), and 137.5 μM (curve 8), respectively. The reaction was started by the addition of 58.8 nM ERK2/pTpY to the reaction mixture in the standard assay buffer (pH 7.0) at 25 °C. The solid lines correspond to the best fit of the data to eq 4 by a nonlinear least-squares regression algorithm.

suspected that k_c'/K_S' might be in fact zero. If this is the case, eq 5 can be simplified as

$$k_{obs}/[M]_0 = \frac{(k_c/K_S)K_m}{K_m + [S]_0} \quad (6)$$

When the experimental data of Figure 5 were fitted to eq 6, a remarkable correspondence was observed. The continuous line in Figure 5 represents the best fit of the experimental data to eq 6 yielding $k_c/K_S = (3.18 \pm 0.16) \times 10^6$ M^{-1} s^{-1} and $K_m = 22.3 \pm 0.3$ μM, respectively. The K_m so determined is comparable to that determined directly from the kinase assay (29.3 $\pm$ 5.8 μM, Table 1), and the apparent second-order rate constant, k_c/K_S, for the ERK2/pTpY•ATP binary complex is identical to the corresponding value for the same ERK2/pTpY•ATP binary complex obtained from assessing the effect of ATP on the HePTP-catalyzed ERK2/pTpY dephosphorylation (Table 2). As an additional test, a plot of $[HePTP]_0/k_{obs}$ against [Elk-1 peptide] (Figure 5, inset) gives a straight line, again indicating that the value of k_c'/K_S' in eq 5 is equal to zero. Taken together, our results indicate that binding of ATP to ERK2/pTpY does not affect the dephosphorylation of ERK2/pTpY by HePTP, whereas

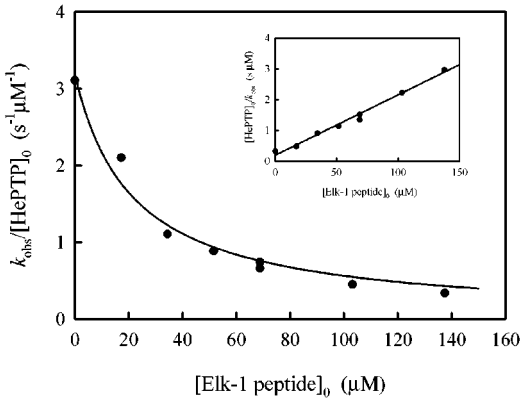


FIGURE 5: Plot of $k_{obs}/[HePTP]_0$ against $[Elk\text{-}1\ peptide]_0$ for the inactivation of ERK2/pTpY by HePTP. The values of the apparent inactivation rate constant were calculated from the time courses of substrate reaction shown in Figure 5. The solid line is the best fitting result according to eq 6 with $k_c/K_S = 3.18 \times 10^6$ M^{-1} s^{-1} and $K_m = 22.3$ μM. The inset shows a plot of $[HePTP]_0/k_{obs}$ against $[Elk\text{-}1\ peptide]_0$ for the same data.

binding of the Elk-1 peptide substrate to the ERK2/pTpY•ATP complex completely protects against HePTP mediated ERK2/pTpY inactivation.

Kinetic Properties of ERK2/pT and Unphosphorylated ERK2. As shown above, the Thr183 phosphorylated form of ERK2 (ERK2/pT) obtained in the present study displayed about 10% residual activity compared to the fully active bisphosphorylated ERK2 at 103 μM Elk-1 peptide concentration (Figure 2A). This is in contrast to some early reports suggesting that the monophosphorylated ERK2s have little kinase activity. Therefore, it is of interests to determine the biochemical properties of the different phosphorylated forms of ERK2.

To prepare ERK2/pT, we incubated bisphosphorylated ERK2 with HePTP until no further decrease in ERK2 kinase activity was observed to ensure complete tyrosine dephosphorylation of ERK2/pTpY. The kinetic parameters of ERK2/pT with the Elk-1 peptide substrate were determined using the method described for ERK2/pTpY (Table 1). In addition, we also measured the kinetic parameters of the unphosphorylated ERK2 using a radioisotope assay in which the rate of incorporation of ^{32}P from (γ-^{32}P) ATP into the Elk-1 peptide was directly measured (*16*). As shown in Table 1, ERK2/pT exhibited dramatically higher kinase activity than that of the unphosphorylated ERK2. Since the initial rate for the ERK2/pT catalyzed reaction, v_0^*, is constant in the presence of increasing concentrations of HePTP (Figure 2A), it is unlikely that the kinase activity in the ERK2/pT sample is due to contaminating ERK2/pTpY. In addition, the K_m values for ERK2/pT and ERK2/pTpY reactions are significantly different, further suggesting that the observed kinetic properties of ERK2/pT are intrinsic to the monophosphorylated ERK2, not from contaminating ERK2/pTpY.

For ERK2/pT, the overall substrate turnover number k_{cat} for the Elk-1 peptide phosphorylation was 27-fold higher than that of the unphosphorylated ERK2 and only 3.7-fold lower than that of the fully active ERK2/pTpY. The overall catalytic efficiency, also known as substrate specificity constant k_{cat}/K_m, was 720-fold higher than that of the unphosphorylated ERK2 and only 10-fold lower than that of the fully active ERK2/pTpY. Thus, in comparison with

the bisphosphorylated ERK2, the monophosphorylated ERK2/pT shows significant residual enzyme activity. These results further support our conclusion that a single phosphorylation in the activation loop of ERK2 produces an intermediate activity state (*16*).

DISCUSSION

The goal of this study was to establish an efficient system for studying the dephosphorylation of physiological substrates by protein phosphatases. Protein phosphatases form a large family of enzymes that, together with protein kinases, control the cellular phosphorylation level. Defective or inappropriate regulation of protein phosphatase activity leads to aberrant phosphorylation, which contributes to the development of many human diseases (*21*). Up to now, most mechanistic studies on protein phosphatases have been conducted with artificial chromogenic substrates and phosphopeptides. Further understanding of the specific functional role of protein phosphatases in cellular signaling requires detailed investigation with physiological substrates, i.e., phosphoproteins. However, our ability to study the phosphatase-catalyzed substrate dephosphorylation has been limited in many cases by the difficulty in preparing sufficient amount of stoichiometrically phosphorylated proteins.

Because the activities of protein kinases are often themselves regulated by phosphorylation, protein kinases form a special class of substrates for protein phosphatases. Because protein kinases possess intrinsic enzyme activity, it should be possible to study the phosphatase-catalyzed dephosphorylation of the protein kinase by treating the phosphatase as an irreversible kinase modifier during the kinase catalyzed reaction. The main advantage of this approach is that only a catalytic amount of phosphorylated kinase is required, thus alleviating the need for preparing large quantities of phosphorylated enzyme. To establish the utility of this approach to study the phosphatase-catalyzed regulation of kinase activity, we have applied the kinetic theory of the substrate reaction during modification of enzyme activity to investigate ERK2 inactivation by HePTP. We were able to determine the kinetic constant for the HePTP-catalyzed dephosphorylation of ERK2/pTpY from the analysis of the effect of HePTP on the substrate reaction catalyzed by ERK2/pTpY. The obtained results are physiologically relevant because inside the cell, ERK2 is surrounded with mM ATP and various protein/peptide substrates.

Analyses of the time courses of ERK2/pTpY inactivation by HePTP in the presence of different concentrations of ATP suggest that ATP binding to ERK2/pTpY has no effect on the HePTP-catalyzed ERK2/pTpY dephosphorylation. Thus, the second-order rate constants for the inactivation of free ERK2/pTpY and ERK2/pTpY•ATP are identical and equal to $(3.05 \pm 0.11) \times 10^6$ M^{-1} s^{-1}. This value is similar to the k_{cat}/K_m determined directly by following the inorganic phosphate produced in the ERK2/pTpY dephosphorylation reaction by HePTP (2.2×10^6 M^{-1} s^{-1}, Zhou et al., unpublished observation), thus validating the utility of this assay for determining the kinetic constant of the phosphatase reaction. The high k_{cat}/K_m value also indicates that ERK2/pTpY is a highly efficient substrate for HePTP. As a comparison, the k_{cat}/K_m for the hydrlysis of *p*-nitrophenyl phosphate (a commonly used chromogenic substrate for protein phosphatases) by HePTP is 780 M^{-1} s^{-1}, which is more than 3 orders of magnitude lower than that for ERK2/pTpY.

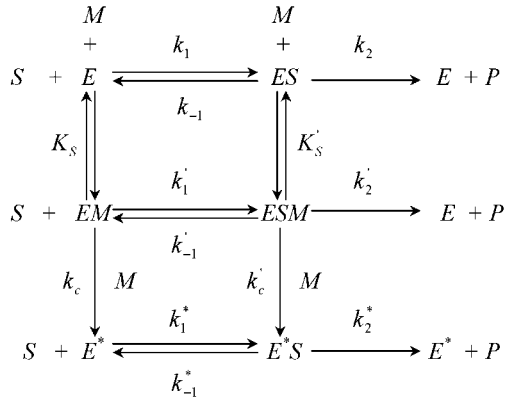
We also analyzed the inactivation kinetics of ERK2/pTpY by HePTP in the presence of the Elk-1 derived peptide substrate. The second-order rate constant for the inactivation of ERK2/pTpY•ATP determined from these sets of experiments $(3.18 \pm 0.16) \times 10^6$ M^{-1} s^{-1}) is essentially identical to that obtained from studying the effect of ATP (Table 2). Intrestingly, our results show that the phosphatase activity of HePTP toward ERK2/pTpY is competitively inhibited by the binding of the Elk-1 peptide to ERK2/pTpY, suggesting that the binding of the ERK2 substrate and the binding of HePTP to ERK2/pTpY are mutually exclusive. Since the Elk-1 peptide can effectively block HePTP's activity toward ERK2/pTpY and since most ERK2 protein substrates contain similar consensus recognition sequence as the Elk-1 peptide, the conclusions are applicable to other ERK2 protein substrate as well. We know from structural studies that Tyr185 is buried and inaccessible to the solvent in unphosphorylated ERK2 (*22*). Upon Tyr185 phosphorylation, the activation loop is refolded which enables the phosphate on Tyr185 to interact with Arg189 and Arg192 in ERK2, leading to the formation of the P+1 binding site for substrate recognition (*23*). Results from this work show that removal of the phosphate group from pTyr185 by HePTP led to a 10-fold decrease in k_{cat}/K_m for ERK2/pT (Table 1). Taken together, the structural and kinetic data suggest that pTyr185 is important for ERK2/pTpY substrate recognition and the binding of ERK2 substrate to ERK2/pTpY blocks the accessibility of pTyr185 to HePTP for dephosphorylation.

In summary, we have shown that the kinetic theory of substrate reaction during modification of enzyme activity can be applied to study kinase dephosphorylation by protein phosphatases. Using this approach, we have shown that HePTP is a highly efficient enzyme toward its physiological substrate, ERK2/pTpY. Further, we have shown that the dephosphorylation of Tyr185 in ERK2/pTpY is blocked by the Elk-1 peptide but not by ATP. Finally, we have shown that the monophosphorylated ERK2/pT has significant kinase activity. The main advantages of this approach include the facts that (1) only small amounts of the kinase and phosphatase are required and (2) the results are directly physiologically relevant. In addition, because the assays presented here require only small amount of protein and they are rapid in real time, they should facilitate further studies employing mutant phosphatases and/or kinases to determine the molecular basis for substrate recognition.

APPENDIX

In the presence of a substrate, the mechanism of kinase inactivation by a phosphatase can be described as follows (Scheme 1): where E and E* represent the phosphorylated and dephosphorylated forms of the kinase and S, P, and M are the substrate, product, and phosphatase, respectively. In the case of a two-substrate reaction, it is assumed that one substrate for the enzyme reaction is present at a constant level and can therefore be included in the kinetic constant without loss of generality. The equation for irreversible modification with complex formation before the chemical modification step have been given before (*2*, *8*). On the basis

Scheme 1



of Scheme 1, we have

$$[E_T] = [E] + [ES] + [EM] + [EMS] \quad (A1)$$

$$[E_T^*] = [E^*] + [E^*S] \quad (A2)$$

$$[E]_0 = [E_T] + [E_T^*] \quad (A3)$$

As before, it is assumed that the steady state of the substrate reaction is rapidly established, and the formation of the EM and EMS complexes are fast reactions relative to the chemical modification step. Therefore, the following relations hold at any time:

$$[E] = \frac{K_m[E_T]}{\left(1 + \frac{[M]}{K_S}\right)K_m + \left(1 + \frac{[M]}{K'_S}\right)[S]} \quad (A4)$$

$$[ES] = \frac{[S][E_T]}{\left(1 + \frac{[M]}{K_S}\right)K_m + \left(1 + \frac{[M]}{K'_S}\right)[S]} \quad (A5)$$

$$[EM] = \frac{K_m[E_T][M]/K_S}{\left(1 + \frac{[M]}{K_S}\right)K_m + \left(1 + \frac{[M]}{K'_S}\right)[S]} \quad (A6)$$

$$[EMS] = \frac{[S][E_T][M]/K'_S}{\left(1 + \frac{[M]}{K_S}\right)K_m + \left(1 + \frac{[M]}{K'_S}\right)[S]} \quad (A7)$$

$$[E^*] = \frac{K_m^*[E_T^*]}{K_m^* + [S]} \quad (A8)$$

$$[E*S] = \frac{[S][E_T^*]}{K_m^* + [S]} \quad (A9)$$

The rate of the kinase inactivation is given by

$$-\frac{d[E_T]}{dt} = k_c[EM] + k'_c[EMS]$$
$$= \frac{k_c\left(\frac{K_m}{K_S}\right) + k'_c\left(\frac{[S]}{K'_S}\right)}{\left(1 + \frac{[M]}{K_S}\right)K_m + \left(1 + \frac{[M]}{K'_S}\right)[S]}[M][E_T]$$
$$= k_{obs}[E_T] \quad (A10)$$

where

$$k_{obs} = \frac{k_c\left(\frac{K_m}{K_S}\right) + k'_c\left(\frac{[S]}{K'_S}\right)}{\left(1 + \frac{[M]}{K_S}\right)K_m + \left(1 + \frac{[M]}{K'_S}\right)[S]}[M]$$

To obtain the analytical expression of $[E_T]$ with time, we have to find the exact conditions under which k_{obs} can be regarded as a constant. When $[S]_0 \gg [E]_0$ and the comsumption of substrate can be neglected during the modification reaction, these conditions are found to be

(1) $[M]_0 \gg [E]_0$; in this case, $[M] = [M]_0$, and

$$k_{obs} = \frac{k_c\left(\frac{K_m}{K_S}\right) + k'_c\left(\frac{[S]_0}{K'_S}\right)}{\left(1 + \frac{[M]_0}{K_S}\right)K_m + \left(1 + \frac{[M]_0}{K'_S}\right)[S]_0}[M]_0 \quad (A11)$$

(2) $[M]_0, [E]_0 \ll K_S, K'_S$; in this case, [EM] and [EMS] can be neglected compared to $[M]_0$ and $[E]_0$, and

$$k_{obs} = \frac{\frac{k_c}{K_S}K_m + \frac{k'_c}{K'_S}[S]_0}{K_m + [S]_0}[M]_0 \quad (A12)$$

This result indicates that if the concentrations of the phosphatase and the kinase are much less than the corresponding dissociation constants, the apparent rate constants for complexing and noncomplexing types are identical in form and therefore no longer distinguishable, and in this case, only the values of k_c/K_S and k_c/K'_S can be determined experimentally. Thus, when k_{obs} is a constant during the modification reaction, integration of eq A10 with $t = 0$, $[E_T] = [E]_0$ yields

$$[E_T] = [E]_0 e^{-k_{obs}t} \quad (A13)$$

The rate of product formation while the kinase is being modified is

$$\frac{d[P]}{dt} = k_2[ES] + k_2^*[E^*S]$$

$$= \frac{k_2[S]_0[E_T]}{K_m + [S]_0} + \frac{k_2^*[S]_0[E_T^*]}{K_m^* + [S]_0}$$

$$= \frac{k_2[S]_0[E]_0}{K_m + [S]_0} e^{-k_{obs}t} + \frac{k_2^*[S]_0[E]_0}{K_m^* + [S]_0}(1 - e^{-k_{obs}t}) \quad \text{(A14)}$$

It can easily be shown that the equation for product concentration at any time t is

$$[P] = v_0^* t + \frac{v_0 - v_0^*}{k_{obs}}(1 - e^{-k_{obs}t}) \quad \text{(A15)}$$

where

$$v_0 = \frac{k_2[S]_0[E]_0}{K_m + [S]_0}$$

$$v_0^* = \frac{k_2^*[S]_0[E]_0}{K_m^* + [S]_0}$$

are the initial velocities of the phsphorylated and dephosphorylated kinase catalyzed reactions, respectively. Since ERK2/pTpY shows a significant ATPase activity, when its activity was measured by the coupled enzyme assay method, the net rate of ADP release measures both ATPase and kinase activities. Thus, the expressions of v_0 and v_0^* are given by

$$v_0 = \frac{\{k_2' K_m + (k_2 + k_2')[S]_0\}[E]_0}{K_m + [S]_0} \quad \text{(A16)}$$

$$v_0^* = \frac{\{k_2'' K_m^* + (k_2^* + k_2''^*)[S]_0\}\,[E]_0}{K_m^* + [S]_0} \quad \text{(A17)}$$

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