Rate-Determining Steps for Tyrosine Phosphorylation by the Kinase Domain of v-fps†

Chengqian Wang,‡ Tae Ryong Lee,§ David S. Lawrence,§ and Joseph A. Adams*,‡

Department of Chemistry, San Diego State University, San Diego, California 92182-1030, and Departments of Chemistry and Medicinal Chemistry, Natural Sciences and Mathematics Complex, State University of New York, Buffalo, New York 14260-3000

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ABSTRACT: The rate-determining steps in the phosphorylation of four tyrosine-containing peptides by the kinase domain of the nonreceptor tyrosine protein kinase v-fps were measured using viscosometric methods. The peptides were phosphorylated by a fusion protein of glutathione-*S*-transferase and the kinase domain of v-fps (GST-kin) and the initial velocities were determined by a coupled enzyme assay. Peptides I (EEEIYEEIE), II (EAEIYEAIE), and III (DADIYDAID) were phosphorylated by GST-kin with similar kinetic constants. The viscosogens, glycerol and sucrose, were found to have intermediate effects on k_{cat} and no effect on $k_{cat}/K_{peptide}$ for the phosphorylation of these three peptides. The data are interpreted according to the Stokes−Einstein equation and a simple three-step mechanism involving substrate binding, phosphoryl group transfer, and net product release. Two competitive inhibitors (EAEIFEAIE and DADIFDAID) exhibited K_I values that are 6−10-fold higher than the $K_{peptide}$ values for their analogous peptide substrates. The data imply that peptides I−III are in rapid equilibrium with the enzyme and that k_{cat} is partially limited by both phosphoryl group transfer (40−100 s^{-1}) and product release (17−22 s^{-1}). GST-kin phosphorylates peptide IV (R_5AENLEYamide) with a low K_m (100 μM) and a k_{cat} that is 40-fold lower than that for peptide I. No effect of solvent viscosity was observed for the phosphorylation of this peptide on either k_{cat} or $k_{cat}/K_{peptide}$. This suggests that highly viscous solutions do not perturb structure and that the rate-determining step for this poor substrate is phosphoryl group transfer. The data indicate that the kinase domain of v-fps phosphorylates its best substrate with a chemical rate constant that is at least 5-fold lower than that for the serine-specific cAMP-dependent protein kinase and its best substrate LRRASLG (Adams & Taylor, 1992). Interestingly, both enzymes exhibit a similar affinity for their substrates and both enzymes release their products at a similar rate. This implies that the differences in catalytic efficiency between serine- and tyrosine-specific protein kinases lie exclusively in the rate constants for phosphoryl group transfer and not in substrate absorption or product desorption.

Protein phosphorylation is at the heart of most signaling processes within the cell. The addition of a single phosphoryl group from ATP to serine, threonine, or tyrosine side chains in a protein by protein kinases can have large effects on the activity and structure of the target. While protein kinases modulate an extensive list of physiological effects in the normal cell, their keen relevance for homeostasis is underscored by the effects of aberrant or oncogenic protein kinases. For example, genetically altered tyrosine protein kinases (TPKs)[1] have been implicated in a wide variety of human diseases including breast cancer (Slamon et al., 1987, 1989) and atherosclerosis (Ross, 1989). The TPK family is divided into two classes: receptor and nonreceptor TPKs. In general, the receptor TPKs are activated by the binding of a hormone, such as a growth factor, to an extracellular domain.

The available data on nonreceptor TPKs suggest that their activity is controlled through intrasteric phenomena [see review by Liu and Pawson (1994)]. The activity of the proto-oncogene, c-src, is repressed by interaction of its tyrosine phosphorylated C-terminus with its SH2 domain. Removal of this tyrosine in the oncogenic form, v-src, leads to a constitutively active enzyme that readily transforms cells (Cartwright et al., 1987; Kmiecik & Shalloway, 1987; Piwnica-Worms et al., 1987). The role of mutated nonreceptor TPKs in several disease states has been postulated. For example, chromosomal rearrangement of the nonreceptor TPK c-abl produces an oncogenic form, bcr-abl, that has been implicated in chronic myleogenous leukemia (Daley & Ben-Neriah, 1991). Although the catalytic efficiency of TPKs is closely linked to proper cellular function as well as disease, little is known about the mechanism of catalysis in receptor or nonreceptor TPKs. Some data suggest that TPKs are sluggish enzymes compared to their serine-specific counterparts possibly due to the weak nucleophilic character of the phenolic group of tyrosine compared to the alkyl hydroxy group of serine (Martin et al., 1990). However, the nonreceptor TPK, $p56^{lck}$, expressed in baculovirus shows high catalytic activity toward a peptide substrate with a k_{cat} value

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* Author to whom correspondence should be addressed.

‡ San Diego State University.

§ State University of New York.

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[1] Abbreviations: cAPK, cAMP-dependent protein kinase; GST, glutathione-*S*-transferase; GST-kin, glutathione-*S*-transferase linked to the N-terminus of the kinase domain of v-fps; Kemptide, peptide sequence L-R-R-A-S-L-G; Mops, 3-(*N*-morpholino)propanesulfonic acid; SPK, serine-specific protein kinase; TPK, tyrosine-specific protein kinase; Tris, tris(hydroxymethyl)aminomethane; v-fps, nonreceptor TPK of the Fujinami sarcoma virus.

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of approximately 6 s^{-1} (Ramer et al., 1991). This is only 3-fold less than that exhibited for cAPK (Adams & Taylor, 1992, 1993).

We have taken a viscosometric approach toward obtaining detailed mechanistic information on nonreceptor TPKs. As a model system we chose the transforming agent of the Fujinami sarcoma virus, v-fps. V-fps is the proto-type for a specific subclass of nonreceptor TPKs that also contains the mammalian fer TPK and the Drosophila fps homologue (Alcalay et al., 1990; Hao et al., 1989; Katzen et al., 1991; Letwin et al., 1988; Roebroek et al., 1985). V-fps is a modular protein composed of an SH2 and kinase domain and a short, polybasic C-terminal tail. We have previously overexpressed and purified large amounts of the kinase domain of v-fps fused at its N-terminus with glutathione-S-transferase (Gish et al., 1995). This fusion protein, GST-kin, is an ideal model system for the kinetic analysis of nonreceptor TPK catalysis for several reasons. First, GST-kin is autophosphorylated in *Escherichia coli* and shows remarkable catalytic power toward small peptide substrates. Second, GST-kin is not inhibited by phosphorylation either in its kinase domain or in its C-terminal tail, so a fully active species can be studied. Third, the catalytic properties of the kinase domain of GST-kin can be analyzed without intrasteric influences from other domain structures.

The steady-state kinetic parameters for the phosphorylation of four peptides by GST-kin were measured as a function of added viscosogens. The peptides were selected based on their varying abilities to be phosphorylated by GST-kin. The results indicate that v-fps-catalyzed phosphorylation of tyrosine-containing peptides occurs by a catalytic scheme that is reminiscent of cAPK with one primary departure, namely, the rate of the chemical step in the v-fps reaction is at least, 5-fold slower. The binding of the peptide substrate and the net release rate of the products are close in value to those for cAPK (Adams & Taylor, 1992). cAPK phosphorylates Kemptide with an apparent second-order rate constant, $k_{cat}/K_{peptide}$, that is 30-fold higher than that for v-fps (Adams & Taylor, 1993; J. Lew and S. S. Taylor, unpublished results). We now report that the increased catalytic efficiency for this SPK does not stem from an improved k_{cat} or tighter binding of the substrate but rather from an improved chemical transfer rate relative to that of v-fps. The comparison of the SPK and TPK in this study illustrates the advantages of stabilizing the chemical transition state beyond that for product release to achieve efficient protein phosphorylation.

MATERIALS AND METHODS

Materials. Adenosine 5′-triphosphate (ATP), phospho(enol)pyruvate, magnesium chloride, sucrose, glycerol, nicotinamide adenine dinucleotide, reduced (NADH), 3-(*N*-morpholino)propanesulfonic acid (Mops), pyruvate kinase, type II, from rabbit muscle, and lactate dehydrogenase, type II, from bovine heart were purchased from Sigma. Ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) were purchased from Fisher.

Peptides. Peptides I and II (Table 1) were synthesized by the Microchemical Core Facility at San Diego State University using Fmoc chemistry on an Applied Biosystems 430A Peptide Synthesizer and purified by C-18 reverse phase chromatography on a Hewlett Packard HP1050 HPLC. Peptide III, EAEIFEAIE, and DADIFDAID were synthesized and purified by similar methods by the USC Microchemical Core Facility. Peptide IV (Table 1) was synthesized on the rink amide resin using Fmoc amino acids. A standard Fmoc peptide synthesis protocol was employed using a Biosearch 9600 automated peptide synthesizer. The peptide was simultaneously deprotected and cleaved from the resin by treatment with 90% trifluoroacetic acid, 10% thioanisole. The crude peptide was purified by cation-exchange chromatography on CM-Sephadex C-25, followed by HPLC using a C-8 preparative HPLC column. The purified peptide gave a satisfactory fast atom bombardment mass spectral analysis. The concentrations of peptides I−III were determined by complete turnover experiments. Typically, small quantities of the peptide (approximately 50 μM) were added to the cuvette containing high concentrations of ATP (3 mM). Small amounts of GST-kin were added, and the total absorbance change was recorded. The peptide concentration was measured using the extinction coefficient of 6.2 mM^{-1} for NADH.

Table 1: Steady-State Kinetic Parameters for the Phosphorylation of Peptide Substrates by GST-kin[a]

peptide	sequence[b]	k_{cat} (s^{-1})	$K_{peptide}$ (μM)	$k_{cat}/K_{peptide}$ ($mM^{-1}s^{-1}$)
I	EEEIYEEIE	13 ± 0.40	400 ± 50	32 ± 4.1
II	E**A**EIYE**A**IE	14 ± 0.82	400 ± 80	35 ± 7.3
III	**DAD**IY**DA**ID	14 ± 0.40	500 ± 80	28 ± 4.6
IV	**R_5**A**N**E**L**Yamide	0.35 ± 0.01	100 ± 17	3.4 ± 0.56

[a] All reactions were measured using 3 mM ATP, 13 mM $MgCl_2$, and 100−3000 μM peptides I−IV in 100 mM Mops (pH 7), 24 °C. [b] Residue changes relative to peptide I are indicated in bold type for peptides I−IV.

Protein. The fusion protein, GST-kin, was purified from *E. coli* according to previously published procedures (Gish et al., 1995). The concentration of the protein was determined by a Bradford assay. Significant improvements in the stability of the fusion protein were obtained by low-temperature storage. The enzyme was stored at −70 °C in a buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 10% glycerol. The frozen kinase buffer solution was thawed on ice (4 °C) and used immediately for each kinetic study. The $t_{1/2}$ for enzymatic activity is approximately 7 days at 4 °C and over two months at −70 °C.

Kinetic Assay. The enzymatic activity of GST-kin was measured spectrophotometrically using a coupled enzyme assay. This assay couples the production of ADP with the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. In general, varying amounts of peptide I, II, or III were mixed manually with 3 mM ATP, 13 mM $MgCl_2$, and 0.15−1.3 μM GST-kin in a 50 μL (minimum volume) quartz cuvette containing 0.85 mM phospho(enol)-pyruvate, 0.25 mM NADH, 2 units of lactate dehydrogenase, and 0.5 units of pyruvate kinase. The total, free concentration of Mg^{2+} was calculated to be 9.4 mM on the basis of the dissociation constants of 0.0143 mM for Mg−ATP, 5 mM for Mg−PEP, and 19.5 mM for Mg−NADH (Martell & Smith, 1977). All reactions were measured in a Beckman DU640 spectrophotometer equipped with a microcuvette holder. All reactions were performed in a buffer containing 100 mM Mops, pH 7.0, in a final volume of 60 μL either in the presence or absence of glycerol or sucrose at 24 °C. Absorbance changes at 340 nm were collected over a time

range of 100−300 s. Less than 10% of the substrate peptides were consumed in each initial velocity measurement.

Viscosometric Measurements. The relative solution viscosities (η^{rel}) of buffers containing either glycerol or sucrose were measured relative to a standard buffer of 100 mM Mops, pH 7.0, at 24 °C, using an Ostwald viscometer (Shoemaker & Garland, 1962). Each viscosity measurement was carried out using 5.0 mL of buffer containing varying amounts of viscosogen. The amount of time required for each buffer to move through the markings on the viscometer was recorded. The relative viscosity of each buffer was calculated using eq 1:

$$\eta^{rel} = \frac{t\,(\%)}{t} \times \frac{\rho\,(\%)}{\rho} \quad (1)$$

where η^{rel} is the relative solvent viscosity, t (%) and t are the transit times for a given viscous buffer and the standard buffer, respectively, and ρ (%) and ρ are the densities of the viscous and standard buffers, respectively. The following relative solvent viscosities were obtained for the buffers (% viscosogen, η^{rel}). 20% glycerol, 1.8; 27% glycerol, 2.4; 33% glycerol, 2.8; 20% sucrose, 1.7; 25% sucrose, 2.1; 31% sucrose, 2.5. All solvent viscosity measurements were performed in triplicate and did not deviate by more than 2% in value.

Data Analysis. The steady-state kinetic parameters, V_{max} and $K_{peptide}$, were obtained by plotting the initial reaction velocity versus the total substrate concentration according to the following equation:

$$v = \frac{V_{max}[S]}{K_{peptide} + [S]} \quad (2)$$

where v is the initial reaction velocity, [S] is the total peptide concentration, V_{max} is the maximal reaction velocity, and $K_{peptide}$ is the Michaelis constant. The maximal reaction velocity was converted to k_{cat} by dividing V_{max} by the total enzyme concentration. The K_I values for the competitive inhibitor peptides were measured using Dixon plots (Dixon, 1953).

RESULTS

Steady-State Kinetic Parameters. The steady-state kinetic parameters for the phosphorylation of peptides I−IV are listed in Table 1. These data were obtained under fixed concentrations of ATP (3 mM) and $MgCl_2$ (13 mM) and varied substrate. Previous studies have shown that the K_m for ATP is 250 μM under conditions of 10 mM free Mg^{2+} (Gish et al., 1995) so that no free enzyme is present in these experiments. GST-kin was preequilibrated with ATP for approximately 3 min in the cuvette before the reaction was initiated with substrate. Enzyme samples that were preequilibrated with Mg·ATP overnight at 4 °C did not give higher initial velocities compared to the control with no ATP preincubation (data not shown). This implies that GST-kin is mostly autophosphorylated upon purification. The initial velocities of the fastest enzyme reactions ($v \approx 80$ μM/min) were not limited by the coupling enzymes since a linear dependence of v versus enzyme concentration was obtained up to initial velocities of 100 μM/min. At 950 μM of peptide II, initial velocities of 27, 37, 66, and 100 μM/min were obtained using 0.038, 0.062, 0.14, and 0.21 μM of GST-kin, respectively. Increasing the ATP concentration from 3 to 4 mM under conditions of 9.4 mM free Mg^{2+} had no effect on the initial velocity of the reaction when 0.6 or 1.6 mM of peptide II was used (data not shown).

Effects of Viscous Solutions on the Coupling Agents. Since the rate of substrate phosphorylation is monitored indirectly in a coupled enzyme assay, the ability of pyruvate kinase and lactate dehydrogenase to monitor the production of ADP in viscous media was tested. The initial velocities for the phosphorylation of peptide II were measured in highly viscous conditions as a function of the enzyme concentration to ensure that any rate decreases are not due to perturbation of the coupling agents. At 33% glycerol ($\eta^{rel} = 2.8$), 0.13, 0.26, and 0.51 μM GST-kin produced 24, 56, and 100 μM/min of ADP, respectively, using 0.63 mM of peptide II. Since linear rate behavior versus enzyme concentration was obtained at the highest tested relative viscosity, the coupling enzyme concentrations are sufficiently high so that their net velocity does not limit the observed initial velocity of GST-kin. The enzyme concentrations in all studies with viscosogens were adjusted so that initial velocities less than 100 μM/min were measured.

Effects of Viscosogens on the Steady-State Kinetic Parameters. The initial velocities for the phosphorylation of peptides I−IV by GST-kin were measured under different viscosogen concentrations. The phosphorylation of peptide II in 0% and 20% glycerol is shown in the inset of Figure 1 as a plot of the observed initial rate constant, k_{obs} ($v/[E]_t$), versus peptide concentration. Typical Michaelis−Menten rate behavior was obtained in the absence and presence of the viscosogen. The dominant effect of this viscosogen is on k_{cat} with little or no effect on the apparent second-order rate constant, $k_{cat}/K_{peptide}$. The value of k_{cat} is lowered from 14 ± 0.30 to 8.2 ± 0.30 s^{-1} in 20% glycerol. Plots of $v/[E]_t$ versus [peptide II] were obtained at several relative viscosities using both glycerol and sucrose. For each viscosogen concentration, a standard plot was obtained in the absence of viscosogen and compared to the plot at that specific relative viscosity. This controls for any changes in the enzyme activity from one experiment to another. Figure 1 shows the ratio of the steady-state kinetic parameters in the absence and presence of added viscosogen as a function of the relative solvent viscosity (η^{rel}). In this plot, $k_{cat}°/k_{cat}$ and $(k_{cat}/K_{peptide})°/(k_{cat}/K_{peptide})$ are the ratios of those parameters in the absence ("°") and presence (no "°") of added viscosogen. Both parameters varied linearly with the relative solvent viscosity from 1 to 2.8. The slopes of these lines are designated as $(k_{cat})^{\eta}$ and $(k_{cat}/K_{peptide})^{\eta}$ and are listed in Table 2. This analysis was performed for peptides I and III (Table 1), and linear dependencies of the steady-state kinetic parameters were also obtained over a wide viscosity range. The slopes of these lines are also listed in Table 2.

The phosphorylation of peptide IV by GST-kin was monitored in a buffer containing 25% sucrose. Peptide IV was chosen as a control substrate for the application of viscosometric studies. Peptide IV binds well to the enzyme but shows a poor k_{cat} (Table 2). If a viscosity effect is measured for a good substrate [i.e., $(k_{cat})^{\eta}$ or $(k_{cat}/K_{peptide})^{\eta} > 0$], then a diminished effect should be measured for a poor substrate. This follows from the observation that a poor substrate is not converted to product under diffusion-limited kinetics since the chemical transformation is rate-limiting (Brouwer & Kirsch, 1982). No effect of solvent viscosity

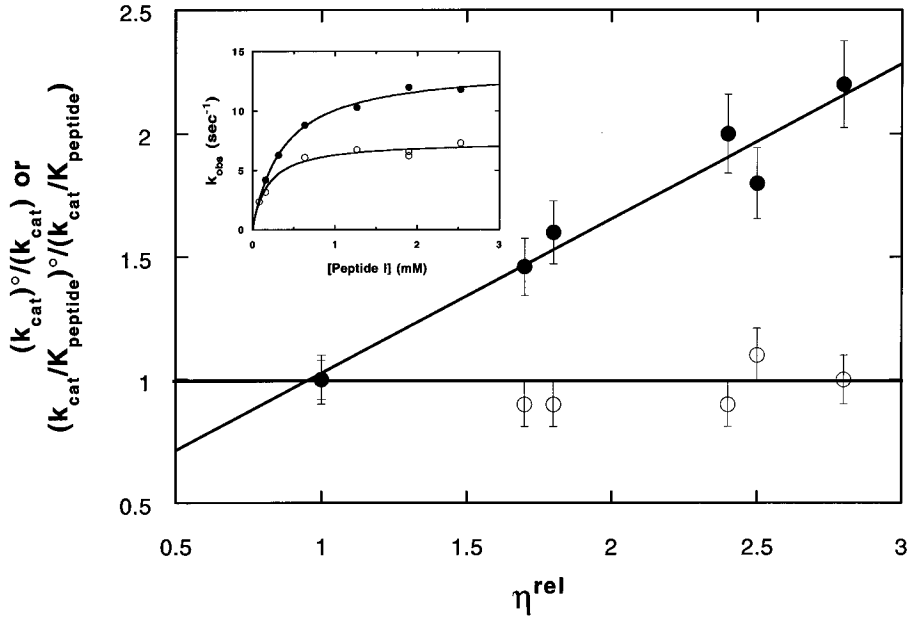

FIGURE 1: Effects of solvent viscosity on the steady-state kinetic rate parameters for the phosphorylation of peptide II by GST-kin. $(k_{cat})^\circ/k_{cat}$ (•) and $(k_{cat}/K_{peptide})^\circ/(k_{cat}/K_{peptide})$ (○) are the ratios of the observed steady-state kinetic parameters k_{cat} and $k_{cat}/K_{peptide}$ in the absence and presence of viscosogen, respectively, in 100 mM Mops (pH 7.0), 24 °C. The inset shows a representative plot of k_{obs} ($v/[E]_t$) as a function of the concentration of peptide II in 0% (●) and 20% (○) glycerol. The initial velocities were measured using 170 nM GST-kin, 3 mM ATP, and 13 mM $MgCl_2$ in 100 mM Mops (pH 7.0), 24 °C.

Table 2: Effects of Solvent Viscosity on the Steady-State Kinetic Parameters[a]

peptide	$(k_{cat})^{\eta}$ [b]	$(k_{cat}/K_{peptide})^{\eta}$ [b]	K_d (μM)[c]	k_3 (s^{-1})[d]	k_4 (s^{-1})[d]
I	0.74 ± 0.080	~0	1500 ± 500	50 ± 8	18 ± 2
II	0.63 ± 0.072	~0	1100 ± 310	38 ± 5	22 ± 3
III	0.51 ± 0.090	~0	1200 ± 320	32 ± 7	25 ± 4
IV	~0	~0	100[e]	0.35	>3.5[f]

[a] All reactions were measured using 3 mM ATP and 13 mM $MgCl_2$ in 100 mM Mops (pH 7), 24 °C. [b] $(k_{cat})^{\eta}$ and $(k_{cat}/K_{peptide})^{\eta}$ are the slopes of the plots of k_{cat}°/k_{cat} and $(k_{cat}/K_{peptide})^\circ/(k_{cat}/K_{peptide})$ versus η^{rel}. [c] The thermodynamic dissociation constants for the peptides were determined from eq 5. [d] $k_3 = k_{cat}/[1 - (k_{cat})^{\eta}]$ and $k_4 = k_{cat}/(k_{cat})^{\eta}$. These rate constants are derived from the ratio of eq 3 and the expression for k_{cat} (Adams & Taylor, 1992). [e] When there is no effect of viscosity on k_{cat} and $k_{cat}/K_{peptide}$, $K_{peptide}$ and K_d are identical (see eq 5). [f] Since $(k_{cat})^{\eta}$ for peptide IV is zero, only a lower limit can be placed on k_4.

on $k_{cat}/K_{peptide}$ or k_{cat} was obtained for the phosphorylation of peptide IV (data not shown). At 25% sucrose ($\eta^{rel} = 2.1$), k_{cat} and $K_{peptide}$ values of 0.33 ± 0.02 s^{-1} and 90 ± 40 μM were obtained, which are equal to the values at 0% sucrose (Table 1). The lack of an effect on both k_{cat} and $k_{cat}/K_{peptide}$ indicates that the viscosogens are not acting as inhibitors of the enzyme reaction but rather are exerting their effects through alterations in the microviscosity of the buffer. Furthermore, these data indicate that the viscosogen is having no measurable influence on the structure of the enzyme and the orientation of active-site residues.

Inhibition Studies using In-2 and In-3. Two peptides, EAEIFEAIE (In-2) and DADIFDAID (In-3), based upon the sequences of two substrate peptides (peptides II and III, respectively) were designed that lack a tyrosine in the P position. These peptides were tested for their inhibitory efficacy with GST-kin. The initial velocity of the enzyme reaction was monitored at saturating ATP (3 mM), high $MgCl_2$ (13 mM), varying substrate (peptide II) and varied, fixed inhibitor concentrations. Double-reciprocal plots of $1/v$ versus 1/[S] yielded a series of straight lines with intersection points on the $1/v$-axis (data not shown) indicating that both In-2 and In-3 are competitive inhibitors of GST-kin. The initial velocities of the enzyme reactions were then measured at fixed peptide II concentrations and variable inhibitor concentrations to obtain K_I values for In-2 and In-3 using Dixon plots. Values of 4.6 ± 0.74 and 3.0 ± 0.28 mM were obtained for In-2 and In-3 using this analysis.

DISCUSSION

The increasing data base on protein kinase structure now permits a thorough analysis of catalytic function. Perhaps the best-studied protein kinase is the catalytic subunit of cAPK. Extensive mechanistic studies have shown that this enzyme will phosphorylate serine-containing peptides with a random kinetic mechanism (Kong & Cook, 1988). Viscosometric studies indicate that the maximal rate constant, k_{cat}, is diffusion limited, indicating that the release of one of the products controls maximum turnover (Adams & Taylor, 1992). The rate of phosphoryl group transfer is predicted to be at least 10-fold larger than k_{cat} (20 s^{-1}). Although the K_m for its best substrate, Kemptide, is low, the enzyme binds the peptide with low affinity. The K_m and K_d for the substrate is 20 and 2400 μM, respectively, at 10 mM free Mg^{2+} (Adams et al., 1995; Kong & Cook, 1988). Although the fast rate constant for phosphoryl group transfer cannot enhance k_{cat}, it has a profound influence on k_{cat}/K_m and accounts for the enzyme's high catalytic efficiency.

There are two major classes of protein kinases that are distinguished by their ability to phosphorylate serine versus tyrosine residues. To understand why these two classes of protein kinases phosphorylate different amino acid side chains, it is first important to determine complete kinetic mechanisms for both so that structure−function studies can be applied. We now report that the kinetic mechanisms for these two enzyme classes are surprisingly similar. The major difference lies in the more than 5-fold slower rate constant for phosphoryl group transfer from ATP to tyrosine. Both

the binding of the substrate and net release rate of the products are similar between v-fps and cAPK. The effects on phosphoryl group transfer do not greatly impact maximum substrate turnover but rather impart its primary influence on the apparent second-order rate constant, $k_{cat}/K_{peptide}$.

Interpretation of Viscosity Data. The effect of solvent viscosity on the rate parameters for enzyme-catalyzed reactions has been given a substantial amount of theoretical and experimental consideration [e.g., Brouwer & Kirsch (1982), Caldin (1964), Nakatani and Dunford (1979)]. For a simple bimolecular process in solution, the association and dissociation rate constants are inversely related to the intrinsic solvent viscosity at constant temperature (Caldin, 1964) so that the ratio of each rate constant in the absence and presence of viscosogens can be equated directly to the relative solvent viscosity (η^{rel}). Scheme 1 describes the

Scheme 1

$$E\cdot ATP + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot ATP\cdot S \xrightarrow{k_3} E\cdot ADP\cdot P \xrightarrow{k_4}$$

minimal kinetic mechanism for the phosphorylation of peptide substrates by GST-kin under saturating concentrations of ATP. In this mechanism, substrate binds the E·ATP binary complex by the association and dissociation rate constants, k_2 and k_{-2}, respectively. The catalytic step, k_3, describes the favorable, unimolecular rate constant for the transfer of the γ phosphoryl group of ATP to the hydroxyl of tyrosine. This step is presumed to be favorable since the reverse reaction for many protein kinases (i.e., phosphorylation of ADP) is very slow. The rate of the reverse reaction catalyzed by calmodulin-dependent protein kinase is approximately 3500-fold slower than for the forward rate (Kwiatkowski et al., 1990). The overall equilibrium constant for Kemptide phosphorylation catalyzed by cAPK was determined by ^{31}P NMR to be approximately 3000 at pH 7.2 (Qamar et al., 1992) suggesting that phosphoryl transfer step is favorable. The final step in Scheme 1 (k_4) describes the net bimolecular rate constant for the release of both products. Since the viscosity measurements cannot distinguish between the dissociation rate constants for the phosphorylated peptide and ADP, it is possible that k_4 may be limited by either product or partially limited by both.

By applying the steady-state assumption, a rate law describing Scheme 1 can be written and the relevant steady-state kinetic parameters, k_{cat} and $k_{cat}/K_{peptide}$, can be extracted. By inserting viscosity dependencies on k_2, k_{-2}, and k_4 into these kinetic parameters, linear functions of the ratios of k_{cat} and $k_{cat}/K_{peptide}$ in the absence and presence of viscosogen versus η^{rel} are obtained. Equations 3 and 4 represent the slopes of these equations for both steady-state kinetic parameters:

$$(k_{cat})^{\eta} = \frac{k_3}{k_3 + k_4^{\circ}} \tag{3}$$

$$(k_{cat}/K_{peptide})^{\eta} = \frac{k_3}{k_{-2}^{\circ} + k_3} \tag{4}$$

where $(k_{cat})^{\eta}$ and $(k_{cat}/K_{peptide})^{\eta}$ are the slopes of $(k_{cat})^{\circ}/k_{cat}$ and $(k_{cat}/K_{peptide})^{\circ}/(k_{cat}/K_{peptide})$ versus η^{rel}, respectively, and k_{-2}° and k_4° are the rate constants in the absence of viscosogen. These two relationships are identical in form to those solved for several enzymes including phosphotriesterase (Caldwell et al., 1991) and the C-subunit of cAPK (Adams & Taylor, 1992).

By combining eqs 3 and 4 and the steady-state kinetic expressions for k_{cat} and $k_{cat}/K_{peptide}$, the individual steps in Scheme 1 can be measured for the phosphorylation of the peptides by GST-kin. Since $(k_{cat}/K_{peptide})^{\eta}$ is zero (Table 2), all four peptides dissociate faster than they are phosphorylated by GST-kin (i.e., $k_{-2} \gg k_3$). This implies that the substrates are in rapid equilibrium with the enzyme, and no definitive value can be placed on their dissociation rate constants. Since intermediate values for $(k_{cat})^{\eta}$ are obtained for peptides I−III, the rate constant for phosphoryl group transfer must be partially rate-determining at high substrate concentration. Table 2 lists the rate constants for phosphoryl group transfer and net product release for peptides I−III. For these peptides, the rate constants of phosphoryl group transfer and product release are close in value indicating that GST-kin does not discriminate between these two substrates. Although the exact value of k_{-2} in Scheme 1 cannot be determined, the K_d for substrate binding to the binary complex, E·ATP, can be estimated. In Scheme 1, $K_{peptide}$ can be related to the individual rate constants when $k_{-2} \gg k_3$ by the following equation:

$$K_{peptide} = K_d \times \frac{k_4}{k_3 + k_4} \tag{5}$$

where $K_d = k_{-2}/k_2$. The thermodynamic dissociation constants for peptides I−IV can be estimated from the values of $K_{peptide}$, k_3, and k_4. These values are listed in Table 2. Peptides I−III bind with similar affinity while peptide IV binds 10-fold more tightly.

The sequence of peptide I is based on the optimum substrate for v-fps derived from a random peptide library (Songyang et al., 1995). As a control we prepared peptide II, which we expected to serve as a relatively ineffective substrate. Songyang et al. (1995) found, by employing a combinatorial library, that the glutamic acid residues at the P − 3 and P + 2 positions are important for substrate recognition. Consequently, we replaced each of these negatively charged residues with an alanine moiety. Much to our surprise, we found that the kinase domain of v-fps phosphorylates peptide II as efficiently as peptide I. To probe further the substrate specificity of GST-kin, all of the glutamate residues in peptide II were replaced by aspartate residues (peptide III). Again, GST-kin phosphorylates this peptide as effectively as peptides I and II. Thrombin cleavage studies have previously shown that removal of GST does not influence the steady-state kinetic parameters for the phosphorylation of peptide I (Gish et al., 1995) so that these similarities are not an artifact of the fusion system. However, the random peptide library was used with the entire v-fps oncoprotein while this viscosometric study was performed with the isolated kinase domain. It is possible that other domains in v-fps influence substrate specificity. Previous studies have shown that the SH2 domain of v-fps enhances the activity of the kinase domain 10-fold toward the substrate poly-glu-tyr (Koch et al., 1989). The SH2 domain may influence the values for $K_{peptide}$ as well as k_{cat}. Proof of this hypothesis awaits the kinetic elucidation of the protein

construct containing both the SH2 and kinase domains of v-fps.

The effect of solvent viscosity on the phosphorylation of peptide IV provides a good control for the interpretation of viscosometric effects and offers new insight into the substrate specificity of the kinase domain of v-fps. Elevated solvent viscosity ($\eta^{rel} = 1-4$) is not expected to adversely affect the structure of protein kinases. Viscous solutions of 36% glycerol have no effect on the circular dichroism spectra or the stability of the catalytic subunit of cAPK indicating that the structure of this enzyme is intact in this viscosogen (Adams & Taylor, 1992). The lack of an effect on the steady-state kinetic parameters for peptide IV (Table 2) indicate that viscosogens do not influence greatly the structure of the kinase domain or act as inhibitors of the enzyme's activity. Either of these effects would be expected to influence the steady-state kinetic parameters. Since the rate-determining step in the phosphorylation of peptide IV is k_3 at high substrate concentrations (Table 2), the value of $K_{peptide}$ is equal to the K_d on the basis of eq 5. Peptide IV lacks four amino acids on the C-terminal side of the tyrosine. These residues have been determined by the degenerate library approach to be essential for substrate recognition (Songyang et al., 1995). In particular, a strong preference for glutamic acid in the P + 1 position was found. Nonetheless, peptide IV bound with a 10-fold lower K_d than peptides I−III.[2] This tighter binding must be due to the presence of additional arginines at the N-terminus, implying that the active site of the kinase domain of v-fps may be further extended. This may have significant relevance toward the design of potent active-site-directed inhibitors of v-fps. We are currently designing variants of the peptides in Table 1 to test the importance of this extended active-site pocket in catalysis and inhibition.

Competitive Inhibition Studies. The activity of GST-kin decreases upon the addition of two peptides, In-2 and In-3, whose sequences were based upon peptides II and III (Table 1) but lack phosphorylatable tyrosines. Since inhibition of the enzyme was overcome at high substrate concentrations (peptide II), In-2 and In-3 are behaving as competitive inhibitors that bind in the peptide substrate binding site. The K_I values for both inhibitors are 6−11-fold higher than the $K_{peptide}$ values for their analogous substrates. There are several explanations for these discrepancies. First, the $K_{peptide}$ is not a true measure of substrate affinity for the enzyme active site. Second, $K_{peptide}$ is less than K_d (as determined by viscosometric experiments) as a consequence of the relatively fast rate of phosphoryl group transfer and relatively slow rate of product release (eq 5). However, even taking this into account, the K_I values exhibited by In-2 and In-3 are still 2−4-fold greater than the experimentally-derived K_d values for the corresponding substrates. This difference may be due to the absence of the hydroxyl moiety in the inhibitor peptides. However, the salient point is that the thermodynamic dissociation constants measured by the viscosometric and inhibition methods are larger than the Michaelis constants of the substrates with a rapid equilibrium kinetic mechanism.

Comparison of v-fps and cAPK. The data presented herein permit a detailed comparison of the catalytic properties of SPKs and TPKs by evaluating the individual steps in the mechanism. In depth viscosity studies of the catalytic subunit of cAPK illustrate that the substrate Kemptide is in rapid equilibrium with the enzyme and that the rate of phosphoryl group transfer from ATP to serine is approximately 10-fold higher than the release of the products ($\geq$200 versus 20 s^{-1}) under conditions of 10 mM free Mg^{2+} (Adams & Taylor, 1992; J. Lew and S. S. Taylor, unpublished results). A comparison of this kinetic mechanism with that for the kinase domain of v-fps shows many similarities. Scheme 2 lists the rate constants for the phosphorylation of peptide II and Kemptide by the kinase domain of v-fps and the catalytic subunit of cAPK, respectively.[3]

Scheme 2

$$E\cdot ATP + S \overset{K_d}{\rightleftharpoons} E\cdot ATP\cdot S \xrightarrow{k_3} E\cdot ADP\cdot P \xrightarrow{k_4}$$

cAPK	2400 μM	$\geq$200 s^{-1}	20 s^{-1}
v-fps	1100 μM	38 s^{-1}	22 s^{-1}

Two similarities are apparent in Scheme 2. First, peptide II and Kemptide bind to the active sites of their respective enzymes with similar affinities although the peptides differ in size, charge, and amino acid composition. Second, the net rate constant for the release of the products is the same. The most significant difference between the two kinetic mechanisms lies in the 5-fold or more slower rate of phosphoryl group transfer in GST-kin compared to cAPK. This difference has only a small effect on the maximal rate constant, k_{cat} (20 s^{-1} in cAPK and 14 s^{-1} in GST-kin), but has a very large effect on $k_{cat}/K_{peptide}$. The $k_{cat}/K_{peptide}$ value for cAPK is 1000 mM^{-1} s^{-1} (J. Lew and S. S. Taylor, unpublished results) while that for GST-kin is 35 $mM^{-1}s^{-1}$ (Table 2). This 30-fold difference does not originate from tighter binding of the substrate. We consider the 2-fold difference in K_d values in Scheme 2 to be insignificant compared to the 30-fold difference in $k_{cat}/K_{peptide}$ for the two enzymes. Furthermore, this difference in $k_{cat}/K_{peptide}$ does not stem from a larger maximal rate constant, k_{cat}, but rather from a smaller $K_{peptide}$ for cAPK. The catalytic efficiency of cAPK as measured by the apparent second order rate constant, $k_{cat}/K_{peptide}$, is higher than GST-kin because it maintains a high rate of phosphoryl group transfer relative to the net release rate of the products ($k_3 \gg k_4$), thereby, lowering the steady-state dissociation constant, $K_{peptide}$ (see eq 5).

The kinetic mechanisms in Scheme 2 outline the individual steps for both tyrosine and serine phosphorylation catalyzed by v-fps and cAPK. The similarities and differences drawn from this scheme raise an important question. Do these two mechanisms represent general kinetic pathways for protein phosphorylation? The catalytic subunit of cAPK is the only SPK for which detailed kinetic information is available regarding individual steps (Adams & Taylor, 1992). Whether other SPKs phosphorylate substrates with similar rate

[2] The degenerate peptide library search does not necessarily select peptides with high affinity but rather selects peptides with high values of $k_{cat}/K_{peptide}$.

[3] The rate constants, k_3 and k_4, for the kinetic mechanism of cAPK in Scheme 2 were measured in 100 mM Mops, pH 7.0, under conditions of saturating ATP (1.0 mM), varied Kemptide, and 10 mM free Mg^{2+} (J. Lew and S. S. Taylor, unpublished results). The K_d for Kemptide and cAPK was estimated from the K_I value for a competitive inhibitor, LRRAALG, at 10 mM free Mg^{2+} in 100 mM Mops, pH 7.0 (Kong & Cook, 1988). The K_d for peptide II is based on the viscosometric analysis (Table 2).

constants awaits further kinetic studies. Recent viscosometric studies on the nonreceptor TPK, csk (C-terminal src kinase), indicate that k_{cat} is partially rate-limited by both phosphoryl group transfer and product release (Cole et al., 1994). This finding is consistent with the data in this manuscript and suggests that the kinetic mechanism for v-fps in Scheme 2 may be a general mechanism that can be applied to other nonreceptor TPKs.

REFERENCES

Adams, J. A., & Taylor, S. S. (1992) *Biochemistry 31*, 8516−8522.

Adams, J. A., & Taylor, S. S. (1993) *J. Biol. Chem. 268*, 7747−7752.

Adams, J. A., McGlone, M. L., Gibson, R., & Taylor, S. S. (1995) *Biochemistry 34*, 2447−2454.

Alcalay, M., Antolini, F., Van de Ven, W. J., Lanfrancone, L., Grigani, F., & Pelicci, P. G. (1990) *Oncogene 5*, 267−275.

Brouwer, A. C., & Kirsch, J. F. (1982) *Biochemistry 21*, 1302−1307.

Caldin, E. F. (1964) *Fast Reactions in Solution*, Wiley, New York.

Caldwell, S. R., Newcomb, J. R., Schlecht, K. A., & Raushel, F. M. (1991) *Biochemistry 30*, 7438−7444.

Cartwright, C. A., Eckhart, W., Simon, S., & Kaplan, P. L. (1987) *Cell 49*, 83−91.

Cole, P. A., Burn, P., Takacs, B., & Walsh, C. T. (1994) *J. Biol. Chem. 49*, 30880−30887.

Daley, G. Q., & Ben-Neriah, Y. (1991) *Adv. Cancer Res. 57*, 151−184.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Gish, G., McGlone, M. L., Pawson, T., & Adams, J. A. (1995) *Protein Eng. 8*, 609−614.

Hao, Q., Heisterkamp, N., & Groffen, J. (1989) *Mol. Cell Biol. 9*, 1587−1593.

Katzen, A. L., Montarras, D., Jackson, J., Paulson, R. F., Kornberg, T., & Bishop, J. M. (1991) *Mol. Cell Biol. 11*, 226−239.

Kmiecik, T. E., & Shalloway, D. (1987) *Cell. 49*, 65.

Koch, C. A., Moran, M., Sadowski, I., & Pawson, T. (1989) *Mol. Cell Biol. 9*, 4131−4140.

Kong, C.-T., & Cook, P. F. (1988) *Biochemistry 27*, 4795−4799.

Kwiatkowski, A. P., Huang, C. Y., & King, M. M. (1990) *Biochemistry 29*, 153−159.

Letwin, K., Yee, S., & Pawson, T. (1988) *Oncogene 3*, 621−627.

Liu, X., & Pawson, T. (1994) *Rec. Prog. Hormone Res. 49*, 149−160.

Martell, A. E., & Smith, R. M. (1977) *Critical Stability Constants*, Vol. 3, Plenum, New York.

Martin, B. L., Wu, D., Jakes, S., & Graves, D. J. (1990) *J. Biol. Chem. 265*, 7108−7111.

Nakatani, H., & Dunford, H. B. (1979) *J. Phys. Chem. 83*, 2662.

Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., & Cheng, S. H. (1987) *Cell 49*, 75−82.

Qamar, R., Yoon, M.-Y., & Cook, P. F. (1992) *Biochemistry 31*, 9986−9992.

Ramer, S. E., Winkler, D. G., Carrera, A., Roberts, T. M., & Walsh, C. T. (1991) *Proc. Natl. Acad. Sci. (U.S.A.) 88*, 6254−6258.

Roebroek, A. J. M., Schalken, J. A., Verbeek, J. S., Van den Ouweland, A. M. W., Onnekink, C., Bloemers, H. P. J., & Van de Ven, W. J. M. (1985) *EMBO J. 4*, 2897−2903.

Ross, R. (1989) *Lancet 1*, 1179−1182.

Shoemaker, D. P., & Garland, C. W. (1962) *Experiments in Physical Chemistry*, 2nd ed., McGraw-Hill, New York.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & McGuire, W. L. (1987) *Science 235*, 177−182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Stuart, S. G., Udove, J., Ullrich, A., & Press, M. F. (1989) *Science. 244*, 707−712.

Songyang, Z., Carraway, K. L., Eck, M. J., Feldman, R. A., Mohammadl, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., & Cantley, L. C. (1995) *Nature 373*, 536−539.

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