# Kinetic Mechanisms of the Forward and Reverse pp60$^{c\text{-}src}$ Tyrosine Kinase Reactions

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ABSTRACT: The kinetic mechanism of the pp60$^{c\text{-}src}$ tyrosine kinase (*src* TK) reaction was investigated in the forward and reverse directions. In the forward direction, initial velocities obtained by varying ATP and the peptide $(FGE)_3Y(GEF)_2GD$ indicated a sequential addition of the two substrates. The peptide analog, $(FGE)_3F(GEF)_2GD$, was a competitive inhibitor versus the peptide substrate and a noncompetitive inhibitor versus MgATP. Interestingly, the tyrosine hydroxyl group imparts only a 6-fold increase in binding. AMP-PCP was a competitive inhibitor versus MgATP and a noncompetitive inhibitor versus the peptide substrate. These results prove that the addition of substrates is random. Furthermore, there appears to be little binding synergy as the $K_{iMgATP} \cong 2.4K_{mMgATP}$. The phosphorylated peptide $(FGE)_3$-pY-$(GEF)_2GD$ was a competitive inhibitor versus peptide and a noncompetitive inhibitor against MgATP, suggesting that a dead end complex can form between MgATP, the phosphorylated peptide product, and the enzyme. The reverse reaction was investigated by varying ADP and the phosphopeptide, $(FGE)_3$-pY-$(GEF)_2GD$. The initial velocity pattern was indicative of a sequential mechanism. There was even less binding synergy in the reverse direction as the $K_{iMgADP} \cong 1.4K_{mMgADP}$. AMP-CP was a competitive inhibitor versus MgADP and a noncompetitive inhibitor versus the phosphopeptide. $(FGE)_3F(GEF)_2GD$ was a competitive inhibitor versus the phosphopeptide and a noncompetitive inhibitor versus MgADP. These data prove that addition of the substrates in the reverse direction is random. $(FGE)_3Y(GEF)_2GD$ was a competitive inhibitor against peptide substrate and a noncompetitive inhibitor against MgADP; therefore a dead end complex can form between MgADP, $(FGE)_3Y(GEF)_2GD$, and the enzyme. These results indicate that the *src* TK reaction follows a sequential bi−bi rapid equilibrium random mechanism in both directions, with dead end complexes forming when either MgATP and $(FGE)_3$-pY-$(GEF)_2GD$ or MgADP and $(FGE)_3Y(GEF)_2GD$ bind to the enzyme. The kinetic constants determined from the forward and reverse reactions were used in the Haldane equation to determine a $K_{eq}$ constant for the forward reaction of 10.1, corresponding to a $\Delta G$ of −1.4 kcal/mol. This further confirms that the O−P bond of phosphotyrosine is similar in energy to that of the $\gamma$-phosphoryl of MgATP.

Tyrosine kinases have recently been shown to play critical roles in signal transduction pathways. Elevated tyrosine kinase activity has been correlated with various carcinomas, implicating tyrosine kinases as potential drug targets. Although understanding the mechanism of action of these target enzymes is critical for drug design, especially to correctly interpret specificity data, limited information exists regarding their kinetic mechanisms.

Cook et al. (1982) reported the first kinetic mechanism of a protein kinase. These workers concluded that the reaction catalyzed by PKA[1] proceeded by random addition of substrates and ordered release of products in the forward direction. However, Whitehouse et al. (1983) concluded that the mechanism of the PKA-catalyzed reaction was predominantly ordered, with ATP binding first. The mechanism of the human CSK TK reaction has been narrowed down to either a rapid equilibrium random or an ordered reaction with ATP binding first using poly-(Glu,Tyr) as a substrate (Cole et al., 1994). Posner et al. (1992) found that the kinetic mechanism of the EGFR TK reaction proceeds by a rapid equilibrium random mechanism when either poly-$Glu_6Ala_3$-$Tyr_1$ or [Val$^5$]-angiotensin II was used as a substrate. However, Erneux et al. (1983) concluded that the EGFR TK from A-431 cells followed an ordered mechanism where the synthetic peptide substrate bound first and ADP was the last product released. Walker et al. (1987) concluded that the human placental IR TK reaction proceeds by a rapid equilibrium random mechanism when a synthetic peptide was used as a substrate. Wong and Goldberg (1984) concluded that the v-*src* TK reaction proceeds by an ordered mechanism, with a productive reaction resulting only if ATP binds first because binding of [Val$^5$]-angiotensin II first excludes ATP binding.

We undertook a study of the *src* TK reaction to determine its kinetic mechanism utilizing a synthetic peptide as the substrate. In addition, we conducted similar mechanistic studies on the reverse of the *src* TK reaction. These two sets of kinetic data were used to determine the equilibrium

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[1] Abbreviations: AMP-CP, α,β-methyleneadenosine 5′-diphosphate; AMP-PCP, adenylylmethylenediphosphonate; CSK TK, C-terminal pp60$^{c\text{-}src}$ tyrosine kinase; EGFR TK, epidermal growth factor receptor tyrosine kinase kinase; G-6PDH, glucose 6-phosphate dehydrogenase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HK, hexokinase; IR TK, insulin receptor tyrosine kinase; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PKA, cAMP dependent protein kinase; PKI(5−24), TTYADFIASGRTGRRNAIHD *src* TK, pp60$^{c\text{-}src}$ tyrosine kinase; v-*src* TK, viral *src* tyrosine kinase.

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constant for the *src* TK reaction.

## MATERIALS AND METHODS

*Materials.* Nucleotides, PEP, PK, LDH, BSA, and pyruvate were purchased from Sigma Chemical Company or Boehringer Mannheim. $(FGE)_3Y(GEF)_2GD$ and $(FGE)_3$-pY-$(GEF)_2GD$[2] were purchased from Zeneca (Wilmington, DE). Nucleotides, PEP, and phosphorylated peptides were dissolved in 50 mM Hepes, pH 7.5. PK and LDH were dissolved in water.

*General Methods.* A Cary 5 spectrophotometer (Varian Instruments) or Molecular Devices (Menlo Park, CA) THERMOmax plate reader was used for UV−visible spectrophotometry.

*Protein Expression, Purification, and Activation. Src* TK was expressed in baculovirus as the N-85 protein deletion mutant[3] and purified according to Ellis et al. (1994). *Src* TK was activated by preincubating 10−20 $\mu$M enzyme with 20 mM $MgCl_2$ and 1 mM ATP at 4 °C for 30 min to allow for enzyme autophosphorylation (Barker et al., 1995).

*Spectrophotometric Assays.* Tyrosine kinase assays were performed as in Edison et al. (1995) using the peptide substrate, $(FGE)_3Y(GEF)_2GD$. The phosphorylation of peptide and production of ADP was coupled to the oxidation of NADH using PEP, PK, and LDH. The oxidation of NADH was monitored by following the decrease in absorbance at 340 nm. Reaction mixtures contained 1 mM PEP, 240 $\mu$M NADH, 45−65 units of LDH/mL, 15−30 units of PK/mL, 100 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 100 $\mu$M DTT, 20 $\mu$g of BSA/mL, and varying concentrations of ATP and peptide. Assays were initiated with 4 nM activated *src* TK.

The reverse of the kinase reaction was performed as described in Boerner et al. (1995) using the phosphorylated peptide, $(FGE)_3$-pY-$(GEF)_2GD$. The dephosphorylation of peptide and production of ATP was coupled to the reduction of NADP using glucose, glucose 6-phosphate dehydrogenase, and hexokinase. The reduction of NADP was monitored by following the increase in absorbance at 340 nm. Reaction mixtures contained 1 mM glucose, 240 $\mu$M $NADP^+$, 3.8 units of G-6PDH/mL, 2.5 units of HK/mL, 100 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 100 $\mu$M DTT, 20 $\mu$g of BSA/mL, and varying concentrations of ADP and phosphopeptide. Assays were initiated with 23.5 nM activated *src* TK.

*Data Analysis.* Initial rates were determined by a linear least-squares regression analysis of the change in absorbance as a function of time. The initial velocities as a function of both substrate concentrations were fitted to the equations for equilibrium ordered (1), sequential (2), and ping-pong (3) mechanisms using the equations from Cleland (1979). Inhibition data were fitted to the equations for competitive (4), noncompetitive (5), and uncompetitive (6) inhibition using the equations from Cleland (1979). The Haldane equation (7) was used to determine an equilibrium constant for the *src* TK reaction (Haldane, 1930).

$$v = VAB/(K_bA + AB + K_{ia}K_b) \quad (1)$$

$$v = VAB/(K_aB + K_bA + AB + K_{ia}K_b) \quad (2)$$

$$v = VAB/(K_bA + K_aB + AB) \quad (3)$$

$$v = VA/[K_m(1 + I/K_{is}) + A] \quad (4)$$

$$v = VA/[K_m(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

$$v = VA/[K_m + (A(1 + I/K_{ii}))] \quad (6)$$

$$K_{eq} = (V_f/V_r)(K_pK_q)/(K_aK_b) \quad (7)$$

In eq 1−6, $v$ is the measured velocity; $V$ is the maximum velocity; $A$ and $B$ are the substrate concentrations; $K_a$, $K_b$, and $K_m$ are the Michaelis constants for $A$, $B$, or the varied substrate, respectively; $K_{ia}$ is the dissociation constant for $A$; $K_{ib}$ is the dissociation constant for $B$; $K_{is}$ and $K_{ii}$ are slope and intercept inhibition constants, respectively. In eq 7, $V_f$ is the $k_{cat}$ for the forward reaction, $V_r$ is the $k_{cat}$ for the reverse reaction, $K_p$ and $K_q$ are the $K_m$'s for MgADP and phosphopeptide, and $K_a$ and $K_b$ are the $K_m$'s for MgATP and dephosphorylated peptide.

## RESULTS

Initial velocity studies were performed on the *src* TK reaction using MgATP, the peptide substrate $(FGE)_3Y(GEF)_2GD$, and preactivated enzyme. The reciprocal velocity was plotted as a function of the reciprocal peptide concentration at five different ATP concentrations (data not shown). The data clearly rule out a ping-pong mechanism (eq 3) and fit best to the equation for sequential addition (eq 2). The kinetic constants determined from these fits are shown in Table 1. The $K_m$ for $(FGE)_3Y(GEF)_2GD$ and the $K_m$ for MgATP are in good agreement with the previously determined values of 73 and 160 $\mu$M, respectively (Edison et al., 1995; Barker et al., 1995). The dissociation constants for peptide or MgATP are 2.4-fold greater than the $K_m$ values, indicating that binding of one substrate does not significantly affect binding of the other substrate.

To distinguish between a random or ordered addition of substrates, inhibitor studies using the ATP analog, AMP-PCP; the peptide substrate analog, $(FGE)_3F(GEF)_2GD$; and the phosphopeptide product of the forward reaction, $(FGE)_3$-pY-$(GEF)_2GD$, were performed. The inhibition constants determined from these studies are shown in Table 1. AMP-PCP was a competitive inhibitor versus MgATP and displayed noncompetitive inhibition versus $(FGE)_3Y(GEF)_2GD$ (Figure 1A). These results demonstrate that MgATP is not required to bind first. $(FGE)_3F(GEF)_2GD$ was noncompetitive versus MgATP (Figure 1B) and competitive versus $(FGE)_3Y(GEF)_2GD$, indicating that peptide substrate is not required to bind first. The phosphopeptide product, $(FGE)_3$-pY-$(GEF)_2GD$, was competitive versus $(FGE)_3Y(GEF)_2GD$ at nonsaturating MgATP concentrations and noncompetitive versus MgATP at nonsaturating $(FGE)_3Y(GEF)_2GD$ concentrations. These data indicate that the order of substrate addition is random in the forward direction. These results also indicate that a dead end complex can form between MgATP, $(FGE)_3$-pY-$(GEF)_2GD$, and the enzyme. The $K_{is}$ value for $(FGE)_3F(GEF)_2GD$ is about six times greater than

[2] Peptides are denoted by their one-letter amino acid code. Phosphorylated tyrosine is denoted by pY.

[3] The *src* TK protein used in these studies was produced in sf9 insect cells from a construct lacking the first 85 residues at the amino terminus (Ellis et al., 1994). This protein behaves similarly to protein expressed from a full-length construct that lacks the myristylation site. The amino acids are numbered according to the wild type human sequence.

Table 1: Kinetic Constants for the Forward and the Reverse *src* Tyrosine Kinase Reactions[a]

| Forward Reaction | | | | |
|---|---|---|---|---|
| substrate | $K_m$ ($\mu$M) | $k_{cat}$ (min$^{-1}$) | $K_{ia}$ ($\mu$M) | $K_{ib}$ ($\mu$M) |
| $(FGE)_3Y(GEF)_2GD$ | 82 ± 8 | 3100 ± 140 | 200 ± 30 | |
| MgATP | 80 ± 11 | | | 190 ± 20 |

| | | | inhib. constants | |
|---|---|---|---|---|
| inhibitor | varied substrate | inhib. pattern | $K_{is}$ ($\mu$M) | $K_{ii}$ ($\mu$M) |
| $(FGE)_3F(GEF)_2GD$ | $(FGE)_3Y(GEF)_2GD$ | C | 1180 ± 50 | |
| $(FGE)_3$-pY-$(GEF)_2GD$ | $(FGE)_3Y(GEF)_2GD$ | C | 1600 ± 200 | |
| AMP-PCP | $(FGE)_3Y(GEF)_2GD$ | NC | 790 ± 220 | 370 ± 40 |
| $(FGE)_3F(GEF)_2GD$ | ATP | NC | 1730 ± 580 | 1850 ± 490 |
| $(FGE)_3$-pY-$(GEF)_2GD$ | ATP | NC | 3200 ± 1100 | 2800 ± 800 |
| AMP-PCP | ATP | C | 230 ± 20 | |

| Reverse Reaction with Activated *src* TK | | | | |
|---|---|---|---|---|
| substrate | $K_m$ ($\mu$M) | $k_{cat}$ (min$^{-1}$) | $K_{ia}$ ($\mu$M) | $K_{ib}$ ($\mu$M) |
| $(FGE)_3$-pY-$(GEF)_2GD$ | 560 ± 30 | 360 ± 10 | 790 ± 80 | |
| MgADP | 13 ± 1 | | | 19 ± 1 |

| | | | inhib. constants | |
|---|---|---|---|---|
| inhibitor | varied substrate | inhib. pattern | $K_{is}$ ($\mu$M) | $K_{ii}$($\mu$M) |
| $(FGE)_3F(GEF)_2GD$ | $(FGE)_3$-pY-$(GEF)_2GD$ | C | 610 ± 30 | |
| $(FGE)_3Y(GEF)_2GD$ | $(FGE)_3$-pY-$(GEF)_2GD$ | C | 53 ± 1 | |
| AMP-CP | $(FGE)_3$-pY-$(GEF)_2GD$ | NC | 91 ± 5 | 107 ± 5 |
| $(FGE)_3F(GEF)_2GD$ | ADP | NC | 1860 ± 630 | 740 ± 140 |
| $(FGE)_3Y(GEF)_2GD$ | ADP | NC | 120 ± 10 | 57 ± 4 |
| AMP-CP | ADP | C | 54 ± 5 | |

[a] The kinetic parameters were calculated from the equation for sequential addition. The inhibition constants were calculated from the equations for either competitive or noncompetitive inhibition. All data are a representative set from experiments done in duplicate or triplicate. The errors shown are standard errors. To correct for the variable enzyme activity observed from day to day, a control reaction was run each day and used to adjust the $k_{cat}$ values relative to each other.

the $K_{ia}$ for $(FGE)_3Y(GEF)_2GD$, indicating that the tyrosine hydroxyl group increases binding by this factor (Table 1). The $K_{is}$ values for both $(FGE)_3F(GEF)_2GD$ and $(FGE)_3$-pY-$(GEF)_2GD$ are similar, which may indicate that the phosphate group by itself does not substantially reduce the affinity for the active site.

Initial velocity studies were also performed on the reverse of the *src* tyrosine kinase reaction using MgADP, the phosphorylated peptide substrate, $(FGE)_3$-pY-$(GEF)_2GD$, and preactivated *src* TK. The reciprocal velocity was plotted as a function of reciprocal phosphopeptide concentration at seven different ADP concentrations. The data fitted best to the equation for sequential addition. The kinetic constants determined from these fits are shown in Table 1. As with the forward reaction, binding of one substrate does not significantly affect binding of the other substrate.

To examine whether the order of addition for the reverse of the kinase reaction is random or ordered, inhibition studies using the ADP analog (AMP-CP), the peptide substrate analog [$(FGE)_3F(GEF)_2GD$], and the peptide product of the reverse reaction, [$(FGE)_3Y(GEF)_2GD$] were performed. AMP-CP was competitive versus MgADP and noncompetitive versus $(FGE)_3$-pY-$(GEF)_2GD$ (Figure 2A), indicating that nucleotide is not required to bind first. The substrate analog $(FGE)_3F(GEF)_2GD$ was competitive versus $(FGE)_3$-pY-$(GEF)_2GD$ and noncompetitive versus MgADP (Figure 2B), indicating that phosphopeptide is also not required to bind first. The peptide product of the reverse reaction, $(FGE)_3Y(GEF)_2GD$, was noncompetitive versus MgADP at nonsaturating $(FGE)_3$-pY-$(GEF)_2GD$ concentrations and competitive versus $(FGE)_3$-pY-$(GEF)_2GD$ at nonsaturating MgADP concentrations. These results are consistent with a random mechanism in which either MgADP or phosphopeptide can bind to the free enzyme. These results also demonstrate that a dead end complex can form between MgADP, $(FGE)_3Y$-$(GEF)_2GD$, and the enzyme.

The kinetic parameters generated from the kinetic studies of both the forward and reverse reactions were used in the Haldane relationship to calculate an equilibrium constant for the kinase reaction. The results yielded a $K_{eq}$ value of 10.1, corresponding to a $\Delta G$ of $-1.4$ kcal/mol. This value is in good agreement with the free energy (1.1 kcal/mol) previously estimated from analysis of the concentrations of reactants and products in the reverse of the *src* TK reaction (Boerner et al., 1995).

## DISCUSSION

Barker et al. (1995) and Boerner et al. (1995) reported and characterized the MgATPase activity of *src* TK, respectively. This activity demonstrated that a binary E·MgATP complex could form. These observations suggested that the kinetic mechanism for the *src* TK reaction was either ordered with MgATP binding first or random. Therefore, it was no surprise when the mechanism of the *src* TK reaction was found to be random. Examination of both the forward and the reverse of the *src* TK reactions demonstrates that the mechanism is rapid equilibrium random in both directions, with dead end complexes forming when either MgATP and $(FGE)_3$-pY-$(GEF)_2GD$ or MgADP and $(FGE)_3Y(GEF)_2GD$ bind to the enzyme. These data demonstrate that both of the substrate binding sites are accessible in the free enzyme. This mechanism is consistent with the previously determined mechanisms for the tyrosine kinases CSK TK (Cole et al., 1994), IR TK (Walker et al., 1987), and EGFR TK (Posner et al., 1992), but not with the studies of EGFR TK by Erneux et al. (1983). The random mechanism of the *src* TK reaction contrasts with the reported ordered addition with MgATP binding first in the v-*src* TK reaction, suggesting

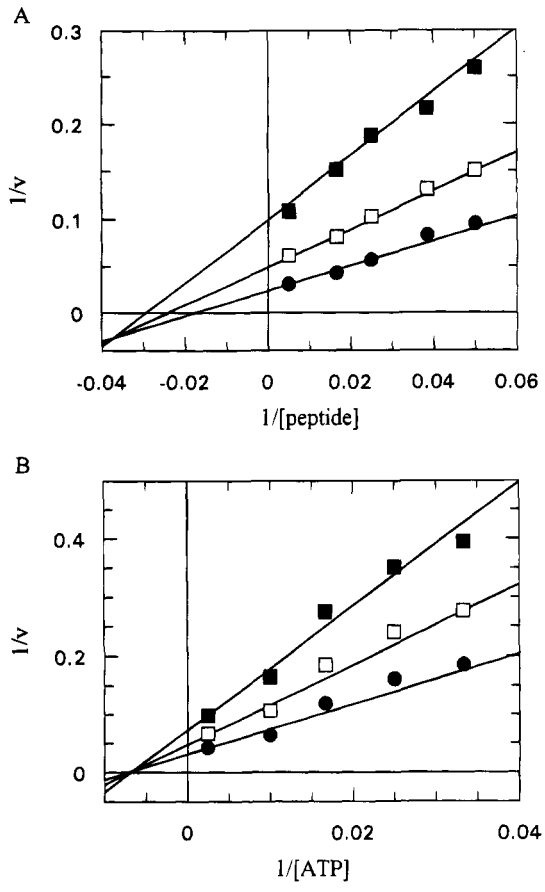


FIGURE 1: Inhibition of the *src* tyrosine kinase reaction. (A) Double-reciprocal plot of $1/v$ [1/(mM × $10^{-4}$ per min)] versus 1/[$(FGE)_3Y(GEF)_2GD$] (20, 26, 40, 60, and 200 $\mu$M) at 1.2 mM (■), 400 $\mu$M (□), and 0 $\mu$M (●) AMP-PCP. The concentrations of ATP and *src* TK were 100 $\mu$M and 4 nM, respectively. (B) Double-reciprocal plot of 1/v [1/(mM × $10^{-4}$ per min)] versus 1/[ATP] (30, 40, 60, 100, and 400 $\mu$M) at 2.5 mM (■), 1 mM (□), and 0 mM (●) $(FGE)_3F(GEF)_2GD$. The concentrations of $(FGE)_3Y$-$(GEF)_2GD$ and *src* TK were 60 $\mu$M and 4 nM, respectively. Curve fits were calculated from equations for noncompetitive inhibition for A and B.

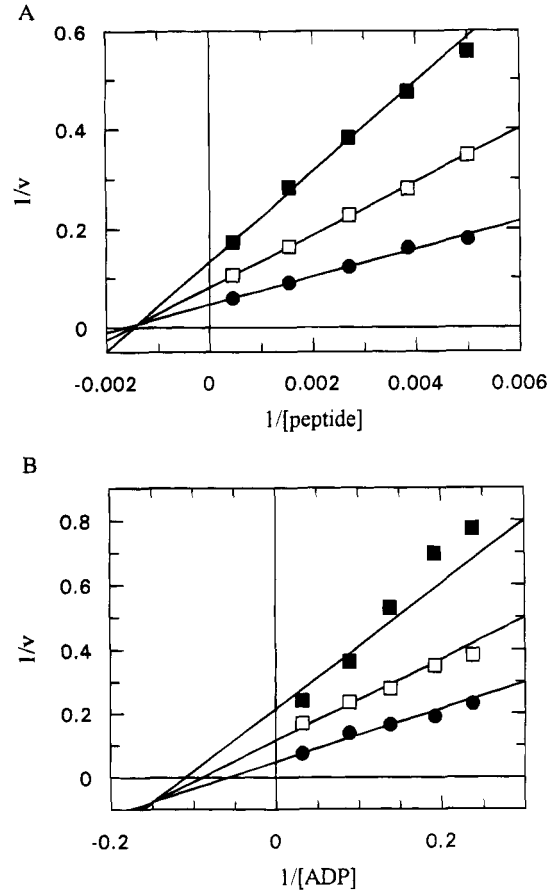


FIGURE 2: Inhibition of the reverse of the *src* tyrosine kinase reaction. (A) Double-reciprocal plot of $1/v$ [1/(mM × $10^{-4}$ per min)] versus 1/[$(FGE)_3$-pY-$(GEF)_2GD$] (200, 260, 370, 650, and 2200 $\mu$M) at 200 $\mu$M (■), 80 $\mu$M (□), and 0 $\mu$M (●) AMP-CP. The concentrations of ADP and *src* TK were 11.2 $\mu$M and 23.5 nM, respectively. (B) Double-reciprocal plot of $1/v$ [1/(mM × $10^{-4}$ per min)] versus 1/[ADP] (4.2, 5.2, 7.2, 11.2, and 31.2 $\mu$M) at 2.5 mM (■), 1 mM (□), and 0 mM (●) $(FGE)_3F(GEF)_2GD$. The concentrations of $(FGE)_3$-pY-$(GEF)_2GD$ and *src* TK were 370 $\mu$M and 23.5 nM, respectively. Curve fits were calculated from equations for noncompetitive inhibition for A and B.

that there are fundamental differences in these related enzymes. All of the reported kinetic mechanisms for protein kinases were determined using peptide substrates, which does not necessarily reflect what may be encountered with a true protein substrate. Future studies will concentrate on identifying a suitable protein substrate for *src* TK and comparing the kinetic mechanism determined with this substrate to that determined with a peptide substrate.

The ratio of $K_{iMgATP}/K_{mMgATP}$ is an estimate of the degree of binding synergy between the substrates in a two-substrate kinase reaction (Knight & Cleland, 1989).[4] The dissociation constant for MgATP from the binary complex can be obtained from either the $K_m$ determined from the ATPase activity (134 ± 19 $\mu$M ;Boerner et al., 1995) or from the $K_{iMgATP}$ determined in these studies. The two values are essentially the same within experimental error. These data suggest a synergy of approximately 2.4-fold. In the reverse direction, the ratio of $K_{iMgADP}/K_{mMgADP}$ yields a smaller degree of synergy of approximately 1.4.

[4] This is only strictly accurate when the binding of the Mg–nucleotide complex is at rapid equilibrium (Knight & Cleland, 1989; Viola et al., 1982). These conditions are usually achieved with a slow substrate, so it certainly holds here for the reverse reaction and likely for the forward reaction also.

The finding that the tyrosine hydroxyl only imparts a 6-fold increase in affinity is somewhat surprising. This likely reflects contribution of a number of interactions between the peptide amino acid residues and *src* TK. For comparison, the dissociation constant for the peptide substrate, LR-RASLG, with PKA is approximately 100-fold tighter than the dissociation constant for the inhibitor peptide, LR-RAALG. But Whitehouse et al. (1983) suggested that the inhibitor peptide does not interact with PKA in a similar fashion as the substrate. In addition, the peptide inhibitor, PKI(5–24), displays a dissociation constant >10 000-fold tighter than the ala peptide (Knighton et al., 1991). These results suggest that high-affinity binding is not simply the result of interactions with the hydroxyl group but is likely due to contribution of a number of interactions between the peptide amino acid residues and the enzyme.

*Relevance of the Kinetic Mechanism to Inhibitor Development.* The design of an inhibitor screen for a two substrate reaction requires knowledge of the enzyme kinetic parameters and mechanism. For example, assume that the kinetic mechanism of a kinase was ordered with MgATP binding first. The assay would require subsaturating concentrations of MgATP in order to detect inhibitors that are competitive versus the nucleotide. This requirement would limit the

sensitivity of simple binding assays for the detection of inhibitors that are competitive versus the phosphoacceptor site since these compounds would only bind to the E·MgATP complex. Therefore, a catalytic assay would be most appropriate. The finding that *src* TK proceeds via a random addition of substrates suggests that either binding assays or catalytic assays could be used to detect inhibitors that bind to either substrate site. Furthermore, the lack of significant binding synergy between the substrates in the *src* TK reaction simplifies the interpretation of the inhibitor data. The $IC_{50}$ of an inhibitor is increased by the factor $(1 + [S]/K_m)$ relative to the $K_i$ where S and $K_m$ refer to the substrate that competes with the inhibitor. The value of this factor calculated for one substrate will be essentially independent of the concentration of the other substrate used in *src* TK assays. In addition, the lack of substrate synergy suggests that the presence of one substrate in a binary complex would not affect the affinity of an inhibitor that bound to the other substrate site.

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