Characterization of pp60$^{c\text{-}src}$ Tyrosine Kinase Activities Using a Continuous Assay: Autoactivation of the Enzyme Is an Intermolecular Autophosphorylation Process†

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ABSTRACT: A continuous assay for pp60$^{c\text{-}src}$ tyrosine kinase (*src*TK) was developed. A lag in phosphorylation of the peptide RRLIEDAEYAARG was observed that could be eliminated by preincubation with MgATP. The induction time for this lag was dependent upon MgATP and *src*TK concentrations. When autophosphorylation was monitored by ^{32}P incorporation from [γ-^{32}P]ATP, a lag in the time course was also observed. These results demonstrate that autoactivation is an intermolecular process. The electrospray ionization mass spectrum of the enzyme before and after activation demonstrated an increase in the phosphorylation state of the enzyme after incubation with MgATP. The Δ85-N-terminal mutant protein and a full-length G2A pp60$^{c\text{-}src}$ mutant, which removes the myristylation site, used in these studies were partially phosphorylated on Y338 and Y530 as isolated. This is the first report of phosphorylation on Y338, but the significance of this site of phosphorylation is unknown. These phosphorylations were insufficient to activate the enzyme for transfer of the γ-phosphoryl of MgATP to the peptides. The unphosphorylated enzyme initially present was converted to a monophosphorylated species upon treatment with MgATP. Y-419 phosphorylation was evident only after treatment with MgATP. These data are consistent with autophosphorylation on Y-419 as predicted. Intermolecular autophosphorylation is consistent with the ability of *src*TK to dimerize, which is analogous to activation of receptor tyrosine kinases such as the EGF receptor kinase in response to growth factors. These results indicate that dimerization leading to activation does not require binding to the membrane or a hydrophobic N-terminus in the case of *src*TK.

The balance of protein phosphorylation and dephosphorylation is known to control many processes. This equilibrium, when applied to protein tyrosine residues, regulates cell differentiation and proliferation. The roles that these equilibria play in signal transduction pathways are under intense study. The tyrosine kinase (TK)[1] pp60$^{c\text{-}src}$ [2] is derived from the proto-oncogene which is the cellular equivalent of the Rous sarcoma virus transforming gene which encodes for pp60$^{v\text{-}src}$ (Bruggio & Erikson, 1977). The latter is required for transformation in animals infected with the virus (Hanafusa, 1977; Vogt, 1977). Activated pp60$^{c\text{-}src}$ has been linked to the pathogenesis of a number of carcinomas including breast (Hennipman et al., 1989; Ottenhoff-Kalff et al., 1992) and colon cancers (Cartwright et al., 1986). Therefore, this kinase and other members of the signal transduction pathway(s) in which pp60$^{c\text{-}src}$ is involved are targets for therapeutic intervention in these devastating diseases.

pp60$^{c\text{-}src}$ contains five sequence domains or regions with specific functions [for a review of the structure of the *src*TK family, see Bolen et al. (1992)]. The N-terminal protein sequence is hydrophobic and contains sites of S/T phosphorylation and myristylation. The SH2[3] and SH3 domains lack catalytic activity and are involved in protein−protein interactions. These domains as well as the catalytic TK domain (also known as SH1) are involved in cellular signal transduction [for a review of oncogenes and signal transduction, see Cantley et al. (1991); for a review of *src* family and hemopoietic signal transduction, see Bolen et al. (1992)]. The kinase domain is followed by a C-terminal noncatalytic region, which contains a regulatory site of phosphorylation, Y530[2,4] (Cooper & Howell, 1993; Pawson & Gish, 1992).

† A preliminary report of portions of this work was presented by Knight et al.

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[1] Abbreviations: AcCN, acetonitrile; AMPPCP, adenylyl methylenediphosphate; AMPPNP, adenylyl imidodiphosphate; ATP(γ)S, adenosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ESI-MS, electrospray ionization mass spectrometry; FFQ, Fast Flow Q Sepharose column; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; IEF, isoelectric focusing; LDH, lactate dehydrogenase; LSC, liquid scintillation counting; MAB, monoclonal antibody; MWCO, molecular weight cutoff; NADH, reduced nicotinamide adenine dinucleotide; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; PK, pyruvate kinase; RR-SRC, RRLIEDAEYAARG; Sf9, *Spodoptera frgiperda*; SH2, *src*-homology domain 2; SH3, *src*-homology domain 3; *src*, sarcoma; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; TBS, TRIS-buffered saline; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TK, tyrosine kinase; TRIS, tris-(hydroxymethyl)aminomethane.

[2] pp60$^{c\text{-}src}$ TK refers to the human protein and is generally abbreviated *src*TK unless specific mutants are identified. The two mutant proteins used in this work, N-85-*src*TK and myr(-)-*src*TK, refer to the 85 residue N-terminal deletion mutant and G2A pp60$^{c\text{-}src}$, respectively. Both mutant proteins displayed similar catalytic parameters toward substrates. pp60$^{v\text{-}src}$ refers to the gene product of the Rous sarcoma virus. In this work, *src*SH2 corresponds to the human pp60$^{c\text{-}src}$-homology 2 domain. Throughout this work, the residues are numbered according to the wild-type human sequence.

[3] Recent reports by Knight et al. (1994) and Boerner et al. (1994) suggest that the *src*SH2 domain may possess a low phosphatase activity, but the significance of this observation has not been established.

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TKs are often regulated by phosphorylation. Iba et al. (1985) proposed that there was a correlation between the level of phosphorylation and the transforming ability of pp60$^{c\text{-}src}$. Cartwright et al. (1986) have reported that the phosphorylation state of pp60$^{c\text{-}src}$ changes when complexed to the polyomavirus middle-T antigen, suggesting that complexation via domains such as SH2 or SH3 induces autophosphorylation. Phosphorylation of Y530 of pp60$^{c\text{-}src}$ appears to down-regulate the kinase activity [for a review, see Hunter (1987)]. In the current model, the *src*SH2 domain binds pY530 in the pp60$^{c\text{-}src}$ carboxyl terminus, leading to repression of kinase activity [for a review of pp60$^{c\text{-}src}$ regulation, see Cooper and Howell (1993)]. This residue is underphosphorylated or absent in transforming mutants of pp60$^{c\text{-}src}$. A specific kinase, Csk, is thought to catalyze the phosphorylation of Y530 (Okada & Nakagawa, 1989; Nada et al., 1991), although Cooper and MacAuley (1988) generated data that suggested that pp60$^{c\text{-}src}$ could also catalyze this event. Marin et al. (1991) have suggested that TPK-IIB, a TK isolated from spleen which does not require autophosphorylation for activity, may be involved in activation of members of the *src* family by catalyzing the phosphorylation of Y419.

In many cases, the activation of tyrosine kinases is autocatalytic. Purchio (1982) and Collett et al. (1983) demonstrated that the level of pp60$^{v\text{-}src}$ phosphorylation increased upon incubation of the protein with MgATP *in vitro*. This treatment also increases the specific activity of the kinase. Autophosphorylation can either be an inter- or an intramolecular event depending upon the kinase. In some cases, both routes have been proposed for the same enzyme. For example, cAMP-dependent protein kinase which phosphorylates S/T residues undergoes an intramolecular autophosphorylation (Todhunter et al., 1977; Rangel-Aldao, 1976). Swarup and Subrahmanyamm (1988) reported that autophosphorylation of a TK isolated from spleen (pp56^{lyn}) was an intramolecular event based upon a lack of dependence of autophosphorylation on the protein concentration, although the data were not presented. EGFR TK dimerizes (or aggregates) in response to EGF in cellular (Schlessinger et al., 1978) and *in vitro* assays (Yarden & Schlessinger, 1987). The mechanism of EGFR autophosphorylation *in vitro* in response to EGF is unclear. Some researchers have reported that the process is an intermolecular event (Yarden & Schlessinger, 1987; Rubin & Earp, 1983a,b), while Biswas et al. (19850 concluded that it was an intramolecular event. In the absence of ligand, the process appears to be intramolecular (Weber et al., 1984). Similarly, autophosphorylation of the insulin receptor tyrosine kinase (IRTK) has been reported to be an intermolecular (Cobb et al., 1989) and in another report an intramolecular event (Kwok et al., 1986).

Sugimoto et al. (1985) concluded that autophosphorylation of pp60$^{v\text{-}src}$ was an intramolecular event, but the report by Shoelson et al. (1989) that high concentrations of peptide substrates inhibited the autophosphorylation of this TK suggests that this is an intermolecular process. Feder and Bishop (1990) suggested that autophosphorylation of pp60$^{c\text{-}src}$ isolated from platelets was an intramolecular event, but Cooper and MacAuley (1988) demonstrated that intermolecular autophosphorylation was possible when a kinase-inactive pp60$^{c\text{-}src}$ mutant was coexpressed with active enzyme. In this work, a continuous assay is developed and used to determine the mechanism of autophosphorylation of pp60$^{c\text{-}src}$.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides, NADH, and PEP (Sigma Chemical Co. or Boehringer Mannheim) were dissolved in water, and stock solutions were titrated to pH 7.0 with dilute KOH. Adenosine [γ-^{32}P]triphosphate (3000 Ci/mmol) was purchased from Dupont NEN Research Products. Phosphopeptides were either obtained from the Glaxo collection, synthesized according to Gilmer et al. (1994), or purchased from Zeneca Bioproducts (Wilmington, DE). RRLIEDAEYAARG (RR-SRC) and $(FGE)_3Y(GEF)_2GD$ were purchased from Bachem California and Zeneca Bioproducts, respectively. Na_3VO_4 was purchased from LC Laboratories or Aldrich Chemical Co. and dissolved in 50 mM HEPES at pH 7.5 prior to use. Buffers (Sigma Chemical Co.) were titrated to the appropriate pH with KOH or HCl except where noted. PK, LDH (both from rabbit muscle, Sigma Chemical Co.), and BSA (Sigma Chemical Co.) were dissolved at 1−3 mg/mL in 50 mM HEPES at pH 7.5 prior to use. Perfusion HPLC columns (800 μm × 10 cm) were purchased from LC Packings (San Francisco, CA). All other reagents were reagent grade and used without further purification unless noted.

Methods

General Methods. Liquid scintillation counting was conducted on a Wallac 1214 counter using Scintiverse BD cocktail (Fisher Scientific). UV−visible spectrophotometry was conducted on either a Cary 5 spectrophotometer (Varian Instruments) or a Molecular Devices (Menlo Park, CA) THERMOmax plate reader. SDS−PAGE gels were conducted with either 8−16% or 12% Tris−glycine gels using a Novex minigel system and a Pharmacia−LKB Multidrive XL power source according to Laemmli (1970). Protein concentrations were determined according to the method of Bradford (1976). Perfusion capillary HPLC was conducted using a Hewlett Packard-1090 microbore pump system and an Applied Biosystems Instruments Model 788A UV−visible detector equipped with a capillary Z-flow cell (LC Packings). ESI-MS was conducted on a API III triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) equipped with an electrospray ion source. The scan range for protein samples was 900−1100 *m/z* with a 0.2 Da step size and a 3.0 s dwell time. ^{31}P NMR spectrometry was conducted on a Bruker AMX600 spectrometer using a 5 mm inverse probe at 25 °C.

Isolation of srcTKs. N-85-*src*TK and N-myr(-)-*src*TK were cloned and expressed in Sf9 cells using the AOX1 expression vector (Zhang et al., 1991). All subsequent purification steps were conducted at 4 °C. In a typical purification, Sf9 cells containing N-85-*src*TK from a 5 L fermentation were lysed with a polytron in lysis buffer (1 mM EDTA, 1 mM DTT, 0.5% sodium cholate, 0.1% NP40,

[4] The single-letter abbreviations are used for the amino acids. Peptides are named according to the one-letter designation for amino acid residues. Phosphorylated residues are abbreviated p*X*; for example, phosphotyrosine = pY. S/T indicates serine and/or threonine. Additional substitutions used: Ac, *N*-acetyl; #, amide.

0.1 mM Na_3VO_4, 1 mM EGTA, 10% glycerol, and 50 mM HEPES at pH 7.6) containing 250 mg/L each of aprotinin, leupeptin, bestatin, and pepstatin A. The lysate was centrifuged at 27K*g*, and the supernatant was removed. The pellet was resuspended in lysis buffer using a polytron and centrifuged at 27K*g*. The supernatant was removed, combined with the first supernatant (total protein 3 g), and loaded onto a 5 × 6 cm FFQ column equilibrated with Q buffer (1 mM EDTA, 1 mM DTT, 0.1 mM Na_3VO_4, 10% glycerol, and 25 mM HEPES at pH 7.6). The column was eluted with a linear gradient (1000 mL) from 0 to 400 mM NaCl in Q buffer. The material (0.6 g of protein) that eluted between 100 and 300 mM NaCl was pooled and loaded onto a 2.6 × 5 cm column containing MAB-327[5] cross-linked to protein A−Sepharose using dimethyl pimelimidate according to Harlow and Lane (1988). The antibody column was pre-equilibrated with buffer A (150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM Na_3VO_4, 10% glycerol, and 50 mM HEPES at pH 7.4). The column was washed in succession with buffer A, buffer A containing 1 M NaCl, and buffer B (1 M KSCN, 0.1 mM Na_3VO_4, 1 mM DTT, and 10% glycerol). N-85-*src*TK was eluted with 10 mM diethylamine containing 150 mM NaCl, 1 mM DTT, and 10% glycerol into 0.5 M Tricine at pH 8.0 (10:1 column eluant:Tricine buffer ratio). The eluant (20 mg of protein) was dialyzed against buffer C (50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 50 mM HEPES at pH 7.6). The stock enzyme was stored at 0.5−1 mg/mL in buffer C at −70 °C. N-myr(-)-*src*TK was obtained similarly except on a smaller scale (0.35 g of protein was applied to the FFQ column), and the 0.55 mg of final product was stored at 0.033 mg/mL in buffer C. The identity of the mutant proteins was confirmed by sequence analysis and purity assessed by SDS−PAGE (>95% purity). The N-terminal sequence analysis of N-85-*src*TK yielded two sequences, MVTTFVAL and VTTFVAL, at a ratio of 2.6:1. The former is consistent with the predicted sequence while the latter is consistent with loss of the N-terminal Met. In the case of N-myr(-)-*src*TK, the identity of the protein was confirmed by analysis of CNBr fragments, due to the inability to determine an N-terminal sequence for the intact protein. HPLC-ESI-MS of the protein demonstrated that the N-terminus was acetylated.

srcTK Radioisotopic Assay. The enzyme was assayed during purification using a Gibco BRL protein tyrosine kinase assay kit according to the manufacturer's instructions with RRLIEDAEYAARG (RR-SRC) as the phosphoacceptor and [γ-^{32}P]ATP as the donor. Initially, purified *src*TK was assayed for its ability to incorporate ^{32}P from [γ-^{32}P]ATP into the peptide substrate RR-SRC (Casnellie et al., 1982). The reaction mixture typically contained buffer D (20 mM MgCl, 0.1 mM DTT, 20 μg/mL BSA, and 100 mM HEPES at pH 7.5), 1 mM ATP with [γ-^{32}P]ATP (25 000 cpm/nmol), 1 mM RR-SRC, and 0.2 μM *src*TK in an assay volume of 10 μL. The reaction was initiated by addition of *src*TK and incubated at 23 °C. After a designated time, the reaction was quenched with 40 μL of cold 10% TCA (w/v), and 20 μL aliquots in duplicate were applied to P81 phosphocellulose disks (Gibco BRL) and filtered to immobilize the phospho-RR-SRC peptide. Excess [γ^{32}P]ATP was removed by rinsing 3× with 0.5 mL of 1% acetic acid (v/v). The P81 disks were then counted using a liquid scintillation counter.

srcTK Spectrophotometric Assay. Kinetic studies were conducted by coupling the phosphorylation of RR-SRC and the other peptides by MgATP, to the oxidation of NADPH via PK, PEP, and LDH. The conditions were 1 mL (final volume) of buffer D containing 1 mM PEP, 0.25−0.3 mM NADH, 10−20 units of PK, and 20−30 units of LDH. Reactions were initiated with 5−40 μL of *src*TK (final concentrations ranged from 0.04 to 0.6 μM unless noted). In preliminary experiments, the amount of coupling enzymes was both increased and decreased to assure that the rates of the reaction were limited by *src*TK. Activated *src*TK was produced by preincubating 5−15 μM enzyme with 1 mM ATP and 20 mM $MgCl_2$ for 30 min at 4 °C. In preliminary experiments, it was determined that this was more than sufficient to yield complete activation. The activated enzyme was stable when stored at 4 °C over 8 h. The mechanism of inhibition of N-85-*src*TK by MgATP(γ)S was examined by varying [MgATP] at 0, 52, and 104 μM concentrations of the nucleotide analog.

Autophosphorylation. The rate of incorporation of ^{32}P from [γ-^{32}P]ATP into N-85-*src*TK was determined in 20 μL of buffer C containing 20 mM $MgCl_2$, 100 μM ATP with [γ-^{32}P]ATP (1 μCi/nmol), and 10 μM *src*TK. The reaction was initiated by addition of ATP and incubated at 4 °C. At designated times, 1 μL aliquots were removed in duplicate and applied to P81 phosphocellulose disks on a vacuum manifold to immobilize the *src*TK. Excess [γ^{32}P]ATP was removed by rinsing 3× with 0.5 mL of 1% TCA (v/v). The disks were subjected to liquid scintillation counting.

The dependence of the rate of incorporation of ^{32}P from [γ-^{32}P]ATP into N-85-*src*TK protein on the enzyme concentration was monitored by SDS−PAGE. Stock enzyme (0.7 mg/mL in buffer C) was serially diluted 7 times by a factor of 2 into buffer C. Autophosphorylation was initiated by addition of 10 μL aliquots of the 0.7, 0.35, 0.18, 0.088, 0.044, 0.022, 0.011, 0.0055 mg/mL solutions of enzyme to 10 μL of 60 mM HEPES at pH 7.4 containing 20 mM $MgCl_2$, 0.2 mM DTT, 40 μM EDTA, 50 μg/mL BSA, 0.3% NP40, 140 μM Na_3VO_4, and 120 μM [γ-^{32}P]ATP (1 μCi/10 μL). These solutions were incubated at 30 °C for 10 min, and then 15 μL aliquots were diluted with SDS−PAGE buffer to a final concentration of 1 ng/μL. Aliquots (10 μL) were then analyzed by SDS−PAGE using 12% Tris−glycine minigels. The gels were dried and exposed to autoradiograph film.

The autophosphorylation reaction was also examined by HPLC/ESI-MS. The 11−33 μL samples contained 20 mM $MgCl_2$ and 0.9 mM ATP and 90% buffer C. In separate experiments, 0.9 mM ATP(γ)S was substituted for ATP. The reaction solutions were incubated for 30 min at 4 °C, and 10 μL aliquots were injected onto the HPLC column. The amount of phosphorylation was determined by comparing the intensities produced by the various species in the deconvoluted mass spectra.

Determination of the Phosphorylation Sites of N-85-TK. Purified N-85-*src*TK was isolated by RP-HPLC before and after treatment with MgATP (1 mM in buffer C) using a capillary perfusion column (800 μm × 30 cm) and a gradient of 13.5−58.5% AcCN containing 0.05% TFA in 5 min.

[5] The monoclonal antibody MAB-327 was a kind gift of J. Brugge of Ariad Pharmaceuticals, Inc. (Cambridge, MA). MAB-327 was derived from original immunizations with pp60$^{v\text{-}src}$ TK and recognizes determinants in the SH3 domain (Lipsche et al., 1983).

HPLC-purified protein was then subjected to speed-vacuum lyophilization and reconstituted in 100 μL of digestion buffer (100 mM NH_4HCO_3, pH 8.5). Trypsin was added at an enzyme:N-85-*src*TK ratio of 1:25 and allowed to incubate at 37 °C for 12 h. The digestion was quenched by addition of 1 μL of TFA. Aliquots of the digests were then analyzed by HPLC/ESI-MS using a 320 μm × 30 cm Poros R2/H capillary perfusion column connected to the mass spectrometer. The spectrometer was scanned from 450 to 2050 Da in 3.2 s using a dwell time of 1 ms and a step size of 0.5 Da. Phosphorylated peptides were identified using a method analogous to that described by Ding et al. (1994). Phosphopeptides were either isolated and sequenced by gas phase Edman sequencing and/or were subjected to LC/MS/MS analysis.

Dependence of ADP Production on Glycerol Concentration. Glycerol concentration was varied from 0.1% to 10.1%, and the final steady-state rates were determined using the spectrophotometric assay in the presence of 1 mM ATP and in the absence of a peptide phospho-acceptor.

Preparation of NMR Samples. The glycerokinase and ATPase activities of N-85-*src*TK were also examined by ^{31}P NMR with 0.6 mL reactions containing 20 mM $MgCl_2$ and 100 mM HEPES at pH 7.5. The two enzyme reactions contained 0.234 μM N-85-*src*TK and either 5mM ATP and 10% glycerol or only 5 mM ATP. Two matching control reactions lacked only enzyme. Additional controls were prepared that contained 5 mM DL-glycerophosphate with and without N-85-*src*TK. All samples were incubated for 48 h at 25 °C and made 0.3 mM in EDTA prior to obtaining ^{31}P NMR spectra. Proton-decoupled ^{31}P NMR spectra (256 transients) were acquired with 4096 data points over 8193 Hz with a 45° observation pulse and an interacquisition delay of 1 s at 25 °C and referenced to glycerophosphate which was assigned a chemical shift of 4 ppm. The identity of the new products was confirmed by addition of authentic phosphate and glycerophosphate to the samples at pH 7.5. The production of glycerol-1-P was also confirmed using glycerol-1-P dehydrogenase and hydrazine according to the method of Lang (1981).

Data Analysis. Attempts to fit the nonlinear progress curves obtained for the phosphorylation of peptides by unactivated *src*TK to a model for a first-order approach to a steady-state rate were unsuccessful; therefore, induction or transient times were determined from the intersection of the asymptotes for the initial and final steady-state phases of the progress curves. The initial rates for linear progress curves and the final steady-state rates of nonlinear progress curves were determined by a linear least-squares regression analysis of the change in absorbance as a function of time (eq 1). The kinetic parameters for substrate hydrolysis were determined by fitting the initial velocities as a function of substrate concentration (0.2−3 or 4 × K_m) to eq 2 by nonlinear least-squares regression using GraFit (Leatherbarrow, 1992).[6] A molecular weight of 52 000 for N-85-*src*TK was used in all calculations. Initial velocity data were collected as a function of inhibitor concentration to determine the kinetic mechanism of inhibition by MgATP(γ)S. The data obtained for the inhibition of MgATP(γ)S versus

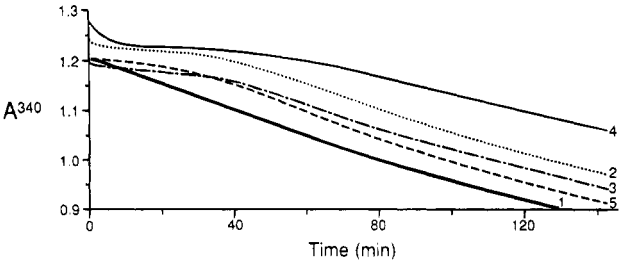

FIGURE 1: Progress curves of the phosphorylation of 0.24 mM RR-SRC in the presence of 1 mM ATP after preincubation of 90 nM N-85-*src*TK (final concentration in the 1 mL assay) in 0.84 mL of standard reaction solution with 0.119 mM (1) ATP, (2) AMPPNP, (3) AMPPCP, and (4) ATP(γ)S. In these cases, PK, LDH, RR-SRC, and 1 mM ATP (final volume) were added in 0.16 mL after 1 h at 25 °C to start the reaction. The final concentration of the nucleotide analogs in the reactions was 100 μM. Curve 5 is a standard reaction initiated with N-85-*src*TK (without preincubation). The induction times for curves 2−5 are 40.8, 36.8, 50.1, and 28.7 min, respectively. The relative steady-state velocities for curves 1−5 are 0.91, 0.91, 0.87, 0.59, and 1, respectively. The initial burst in the consumption of NADH evident in curve 4 and to a lesser extent curve 2 is likely due to the presence of contaminating ADP in the nucleotide preparations (see Figure 3).

MgATP (varied from 0.2 to 3 × K_m) were analyzed according to the models for competitive inhibition (eq 3), noncompetitive inhibition (eq 4), and uncompetitive inhibition (eq 5). The best fit of the data was obtained with the competitive model:

$$Y = v_1 X + B \quad (1)$$

$$v = V_m[S]/(K_m + [S]) \quad (2)$$

$$v = V_m[S]/\{K_m(1 = [I]/K_{is}) + [S]\} \quad (3)$$

$$v = V_m[S]/\{K_m(1 + [I]/I_{is}) + [S](1 + [I]/K_{ii})\} \quad (4)$$

$$v = V_m[S]/\{K_m[S](1 + [I]/K_{ii})\} \quad (5)$$

RESULTS

The spectrophotometric assay utilized in this work allows continuous monitoring of *src*TK activity. Preincubation of N-85-*src*TK with MgATP lead to linear progression curves (see Figure 1, curve 1). The K_m for MgATP in the presence of 0.24 mM RR-SRC was 0.16 ± 0.02 mM.[7] The kinetic parameters for N-myr-*src*TK were similar. In Table 1, the kinetic parameters for the phosphorylation of several peptides using this assay are reported. In the absence of a peptide phosphoacceptor, there was a background rate that was proportional to the glycerol concentration (see Table 2). This rate increased linearly with glycerol concentrations up to 1 M which suggests that the K_m for glycerol is greater than or equal to this concentration. The turnover number at 1 M glycerol was approximately 3.52 ± 0.66 min^{-1}, which compares to a turnover number for 0.24 mM RR-SRC under the same conditions of 5.92 ± 0.42 min^{-1}.[8] Richert et al. (1982) reported that *v-src*TK displayed a glycerokinase

[6] Direct comparison of the results obtained from this graphical data analysis program with the programs of Cleland (1979) yielded essentially identical results.

[7] The kinetic constants toward both RR-SRC and MgATP were essentially the same when the radioligand assay was utilized.

[8] In the RR-SRC experiments, the concentration of glycerol was 11 mM. The contribution of glycerol phosphorylation to the reaction rate should be minimal of this concentration especially since the presence of the peptide substrate should preclude glycerol binding productively.

Table 1: Kinetic Parameters of *src*TK toward Peptides and MgATP As Determined with the Continuous Assay

varied substrate[a]	TK form	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
RRLIEDAEYAARRG	N-85-*src*TK	2.8	208	74.5
DRVYIHPF	N-85-*src*TK	>8.5[b]	nd	4.13
DRVYVHPF	N-85-*src*TK	4.6	49.7	11
ATP[c]	N-85-*src*TK	0.16 ± 0.02	23.6 ± 4.2	128 ± 21
ATP[d]	N-85-*src*TK	0.15	16.9	112
ATP[d]	myr(-)-*src*TK	0.13	33.8	273

[a] When the peptide substrate was varied, the concentration of ATP was 1 mM. When ATP was varied, the RR-SRC concentration was 0.24 mM. The standard errors were less than or equal to ±10% of the reported values except where noted. [b] Due to the solubility of the peptide, the highest concentration tested was 8.5 mM, and this did not result in saturation. The k_{cat}/K_m was estimated from the rates at 1 and 2 mM peptide. [c] These values are the average of three determinations, and the errors reported are the standard deviations. [d] There was 0.005% NP in these experiments.

Table 2: Dependence of the Rate of MgADP Production on Glycerol Concentration[a]

[glycerol]		
%	M	rate (μM/min)
0.1	0.011	0.041
1.1	0.120	0.058
2.6	0.283	0.093
5.1	0.55	0.148
10.1	1.1	0.317

[a] The concentration of N-85-*src*TK was 90 nM. The values reported are the average of two determinations after subtraction of the background rate (0.021 μM/min) in the absence of N-85-*src*TK. The deviation from the mean was less than or equal to 10% in all cases.

activity. To confirm that the background rate was due to glycerol phosphorylation and not an enhanced ATPase activity, the reaction was also monitored by ^{31}P NMR. The reaction of N-85-*src*TK in the presence of 10% glycerol and MgATP (multiplets centered at −5.6, −10.6, and −19 ppm due to the α-, β-, and γ-phosphoryls of the nucleotide were observed) produced new ^{31}P NMR resonances due to glycerophosphate (4 ppm) and phosphate (2.2 ppm). Approximately 1.8 mM glycerol-1-P was produced after 48 h, and considerably less phosphate was observed. In the presence of only MgATP, 0.7 mM phosphate was observed after 48 h which indicates that the enzyme also possesses a slow ATPase activity. In both cases, the resonances due to the α- and β-phosphates of MgADP were evident (−5.8 and 9.8 ppm, respectively). Subsequent experiments were performed with minimal glycerol (≤0.1%) to minimize this activity. The ATPase activity could be minimized by storage of the preincubated protein at 4 °C after autoactivation. Furthermore, both of these activities should be inhibited by the presence of peptide substrates in the reactions.[8]

In the absence of preincubation with MgATP, coupling of the phosphorylation of RR-SRC and concomitant production of MgADP to the oxidation of NADH resulted in nonlinear progress curves indicative of a lag in the rate of RR-SRC phosphorylation (see Figure 1, curve 5). Similar results were obtained with myr(-)-*src*TK (data not shown). The induction times were estimated from the intersection of the asymptotes to the two phases of the curves. The lag was not due to inefficiency of the coupling system as increasing or decreasing the amount of coupling enzyme had no effect on the observed lag or the final steady-state rates.

For example, Knight and Cleland (1989) reported that the presence of even slow substrates of authentic glycerokinase inhibited the contribution of water, an alternate phosphoacceptor, to the rate of MgADP production from MgATP.

Table 3: Effect of the Concentration of MgATP on the Induction Times for Phosphorylation of RR-SRC

[MgATP] (mM)	induction time (min)	rel final steady-state velocity
1	29	1
0.1	34	0.51
0.05	48.6	0.3

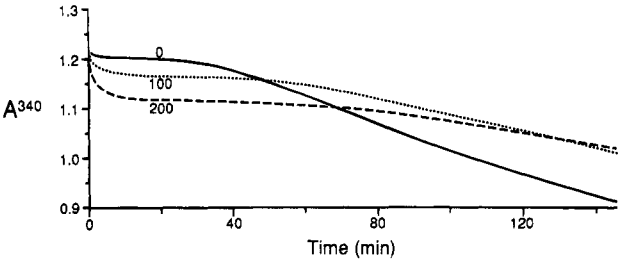

FIGURE 2: Progress curves of the phosphorylation of RR-SRC after a 3 h preincubation with 0, 119, and 238 μM ATP(γ)S (final concentrations of 0, 100, and 200 μM in the actual assay). The other conditions were as in Figure 1. The induction times were 36, 58, and 78 min, and the relative steady-state rates were 1, 0.57, and 0.43 for 0, 100, and 200, respectively. The initial burst in the consumption of NADH evident in the presence of ATP(γ)S is due to ≈3% contaminating ADP in the nucleotide analog preparation. (The concentration of contaminating ADP was measured using the coupling system in the absence of *src*TK.)

The induction time decreased as the concentration of ATP was increased (see Table 3). Preincubation of the enzyme with the components of the assay other than MgATP had no effect on the observed lag (data not shown). Analogs of ATP did not support autoactivation (curves 2−4 in Figure 1). MgATP(γ)S inhibited both the rate of autoactivation as well as the final steady-state rate (see Figure 2). The phosphorothioate analog was a competitive inhibitor versus MgATP (data not shown) with a K_i of 22 ± 2 μM, which compares to a K_m for ATP of 170 ± 17 μM in the same experiment. The lag in peptide phosphorylation was dependent upon the concentration of N-85-*src*TK (see Figure 3 and inset). A similar dependence on TK concentration was observed when the reaction was monitored by the transfer of ^{32}P to RR-SRC (see Figure 4 and inset). Similar results were obtained with myr(-)-*src*TK (data not shown). The apparent lag in substrate phosphorylation was increased in the presence of $(FGE)_3Y(GEF)_2GD$ (I), a peptide substrate that yields a substantially lower K_m than that displayed by RR-SRC.[9] The induction time increased from 24 to 34 min when the concentration of I was increased from 50 to 200 μM at 0.14 μM N-85-*src*TK. A similar trend was observed at 0.42 μM N-85-*src*TK where the values went from 10 to

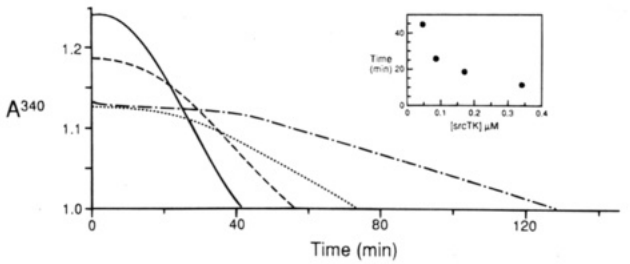

FIGURE 3: Progress curves of the phosphorylation of RR-SRC in the presence of 0.045, 0.09, 0.181, and 0.362 μM N-85-*src*TK. The inset is the dependence of the induction time determined from the progress curves on the concentration of N-85-*src*TK in the assay.

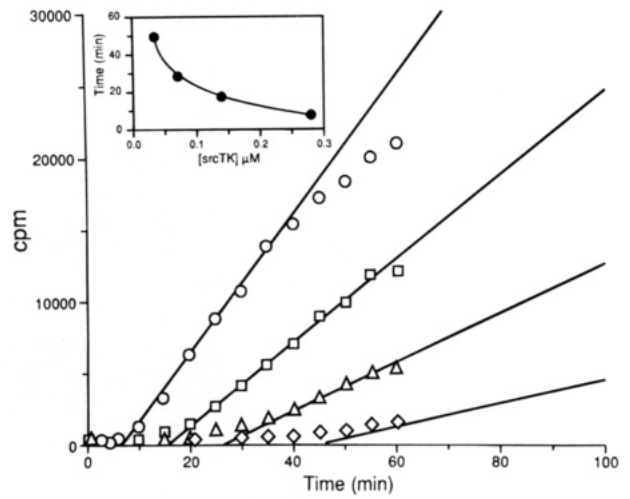

FIGURE 4: Progress curves of the incorporation of ^{32}P into RR-SRC at 0.27 (○), 0.135 (□), 0.067 (△), and 0.034 (◇) μM N-85-*src*TK. The concentration and specific activity of ATP were 1 mM and 25 000 cpm/nmol, respectively. The inset is a plot of the induction time as a function of N-85-*src*TK concentration. Similar results were obtained in two additional experiments.

15 min. Finally, the rate of autophosphorylation of N-85-*src*TK also displayed a similar lag (Figure 5). The maximal amount of ^{32}P incorporation corresponded to greater than 0.5 equiv based upon the protein concentration and a molecular weight of 52 000. SDS−PAGE analysis of autophosphorylation of N-85-*src*TK also demonstrated a dependence on the amount of ^{32}P incorporation on the enzyme concentration (see Figure 6).

The presence of a phosphopeptide, TSTEPQpYQPGENL, which binds to the *src*SH2 domain had only a small effect on the rate of autoactivation and a similar effect on the final steady-state rate (see Figure 7). The unphosphorylated peptide, TSTEPQYQPGENL (100 μM), had no effect on either rate (data not shown). In additional experiments, similar results were obtained with Ac-pYEEIE, which has higher affinity for this SH2 domain (Gilmer et al., 1994). Once again, the unphosphorylated peptide AcYEEIE had no effect on the kinetics.

In Figure 8, the mass spectra of N-85-*src*TK before and after preincubation with MgATP are shown. There are multiple species present (see Table 4).[10] Initially, species were resolved with masses corresponding to the protein ±

[9] R. J. A. Budde (M. D. Anderson, University of Texas, Houston, TX) reported that $(FGE)_3Y(GEF)_2GD$ was a "good" substrate for pp60$^{c\text{-}src}$ (personal communication). Edison et al. (1995) reported that this peptide yielded a K_m of 73 μM as well as a 7-fold higher V_m and a 250-fold higher k_{cat}/K_m than the values obtained with RR-SRC.

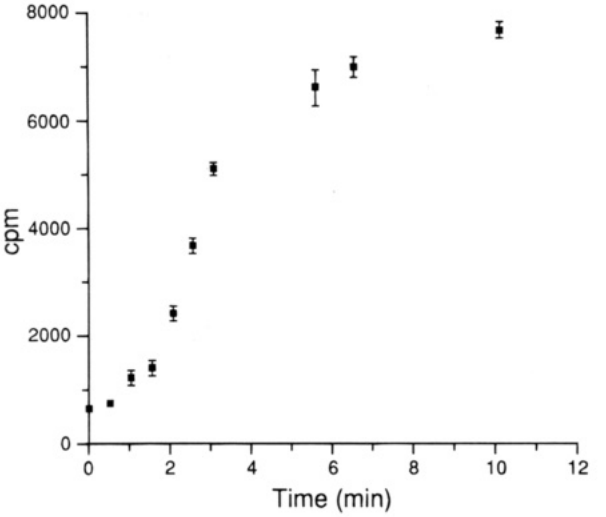

FIGURE 5: Autophosphorylation of 10 μM N-85-*src*TK in the presence of 100 μM MgATP (specific activity 1 μCi/nmol) at 4 °C. The points represent the average of two determinations, and the error bars are standard deviations. Similar results were obtained in three other experiments.

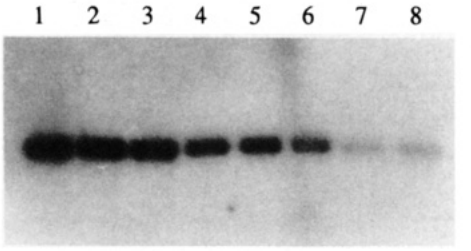

FIGURE 6: SDS−PAGE analysis of the dependence of the amount of ^{32}P incorporation into N-85-*src*TK on the enzyme concentration. The concentrations of N-85-*src*TK in the original reaction solutions were (1) 6.75, (2) 3.38, (3) 1.69, (4) 0.845, (5) 0.422, (6) 0.211, (7) 0.105, and (8) 0.053 μM. The amount of protein applied to each lane was 10 ng.

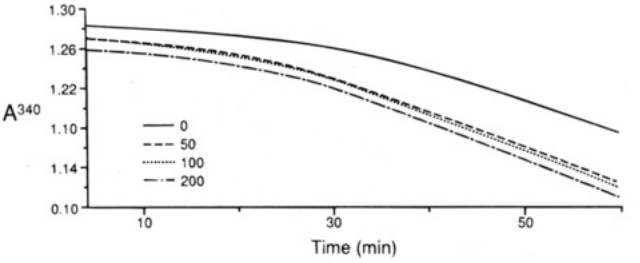

FIGURE 7: Progress curves of the phosphorylation of 0.24 mM RR-SRC by 0.09 μM N-85-*src*TK in the presence of 0, 50, 100, and 200 μM TSTEPQpYQPGENL. The induction times and relative steady-state rates were 29.7, 19.7, 22, and 22 min and 1, 1.18, 1.16, and 1.14, respectively.

the N-terminal methionine as well as mono- and diphosphorylated forms of the protein containing the Met. It is likely that small amounts of the mono- and diphosphorylated form that lack the N-terminal methionine are present, but these were not resolved from the other components in the mass spectra. Mass spectral analysis of the peptides obtained from tryptic digestion of the protein indicated that Y338 and Y530 were partially phosphorylated in the protein as isolated, but the peptide containing Y419 was not phosphorylated.

[10] IEF gels of the protein preparation also demonstrated the presence of four to six species. The isoelectric points of these species were centered around pH 6.0. All of these species reacted with MAB-327.

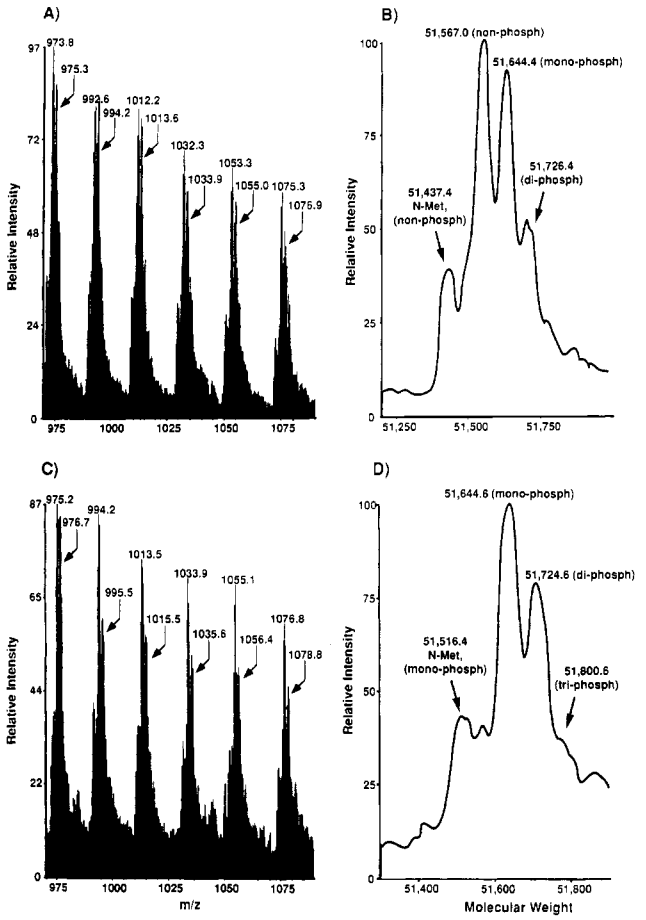

FIGURE 8: ESI-MS of N-85-*src*TK before (A) and after (C) incubation with 1 mM MgATP. The +53 to +48 ions are shown. The spectra reconstructed using Hypermass (PE-Sciex) are shown in (B) and (D) for the stock enzyme and the enzyme after treatment with MgATP, respectively. The identity of the species are summarized in Table 4.

Table 4: Phosphorylation Sites and States of N-85-*src*TK

mass	identity	phosphorylated sites
Before Treatment with MgATP		Y338,[a]Y530[a]
51 437.4	N-85-*src*TK − Met	
51 567	N-85-*src*TK	
51 645.4	N-85-*src*TK + phosphate	
51 726.4	N-85-*src*TK + 2 phosphates	
After Treatment with MgATP		Y338,[a] Y530,[a] Y419[b]
51 516.4	N-85-*src*TK − Met + phosphate	
51 644.6	N-85-*src*TK + phosphate	
51 724.6	N-85-*src*TK + 2 phosphates	
51 800.6	N-85-*src*TK + 3 phosphates	

[a] The tryptic peptides containing these tyrosines were only partially phosphorylated. [b] Greater than 70% of the peptide fragment containing Y419 was phosphorylated after treatment with MgATP.

Upon reaction with MgATP, the two unphosphorylated forms were converted to monophosphorylated forms, and the amount of the diphosphorylated form present was also increased. A small amount of triphosphorylated species is also evident after reaction with MgATP. The increase in phosphorylation is primarily due to Y419 phosphorylation as the peptide obtained from the tryptic digest containing this residue was 70% phosphorylated after treatment with MgATP. There was no change in the phosphorylation state of the protein upon incubation with 1 mM MgATP(γ)S.

DISCUSSION

The kinetic parameters obtained using the continuous spectrophotometric assay for *src*TK activity were essentially identical to those obtained using the fixed-time ^{32}P assay with RR-SRC as a substrate. The lack of uniformity in assay conditions precludes a definitive comparison of the results obtained using the spectrophotometric assay for *src*TK activity to those reported in the literature. The k_{cat}/K_m for angiotensin II (DRVYIHPF) calculated from the data reported by Lydon et al. (1992) for recombinant myr(-)-*src*TK expressed in Sf9 cells was 8.3 mM^{-1} min^{-1}, which compares favorably to the value reported in Table 1 for the same peptide, although the K_m reported for the peptide was at least 4-fold lower than that determined in this work. The K_m for DRVYVHPF (^{5}V-angiotensin II) reported by Budde et al. (1993) was 3-fold lower than that observed in this work, but the unknown concentration of recombinant enzyme and enzyme immunoprecipitated from HT29 cells used in those experiments precludes a comparison of k_{cat} and k_{cat}/K_m. The data reported in this work for the angiotensin peptide analogs suggest a 2-fold specificity of *src*TK for smaller hydrophobic residues at the Y+1 position.[11] This is in contrast to the results reported for pp60$^{v\text{-}src}$ where these two peptides yield similar specificity constants (Wong & Goldberg, 1983). The continuous assay does not require a specific net charge on peptide substrates; therefore, it can be used to further delineate the sequence specificity of *src*TK.

pp60$^{c\text{-}src}$ possesses an inherent glycerokinase activity. The observation that the rate of ADP production was linear with glycerol concentration up to 1 M suggests that the activity is not due to a contaminating glycerokinase. The latter should yield a lower K_m, typically in the micromolar range (Knight & Cleland, 1989). Richert et al. (1992) reported that pp60$^{v\text{-}src}$ also displayed glycerokinase activity with a linear increase in rate up to 3 M glycerol. Richert (1983) reported that the catalytic subunit of cAMP-dependent protein kinase also possessed a slow glycerokinase activity. The low catalytic efficiency of these activities suggests that it is unlikely that this activity is physiologically relevant, although the possibility of additional physiologically relevant phosphoacceptor substrates that do not contain tyrosine cannot be ruled out.[12]

When the activity of the *src*TK mutants was assessed without preincubation with MgATP, there was a lag in the phosphorylation of substrate and production of MgADP. This in itself was not surprising as pp60$^{c\text{-}src}$ is known to require activation by phosphorylation on Y-419. The enzyme as isolated from baculovirus was not phosphorylated at this site, but treatment with MgATP results in phosphorylation of this position. There was no evidence of transfer of the thiophosphoryl group from MgATP(γ)S to the enzyme. Intramolecular autophosphorylation of the enzyme should have yielded first-order progress curves for substrate phosphorylation. In that case, the induction time would be independent of the enzyme concentration, but this was not observed. In

[11] The peptides are numbered according to the following system. The residues C-terminal to the tyrosine are given positive values beginning with Y equal to 0. The residues N-terminal to the tyrosine are given negative values beginning with Y equal to 0.

[12] Lee et al. (1995) have recently reported that pp60$^{c\text{-}src}$ will catalyze the phosphorylation of peptides containing a number of different aromatic and acyclic alcohols.

addition, incorporation of ^{32}P into *src*TK displayed a lag, rather than the first-order dependence on time predicted for a unimolecular reaction. Therefore, we conclude that autophosphorylation of *src*TK at the concentrations used in these studies is primarily an intermolecular event. The observation of intermolecular autophosphorylation does not appear to be the result of the phosphorylation state of the initial enzyme preparation since preliminary experiments with a double mutant (Y338F, Y530F) also yielded a dependence of the induction time on the enzyme concentration (Barker and Knight, unpublished results).[13] The induction time for autophosphorylation of this mutant still displays a dependence on the enzyme concentration (Barker and Knight, unpublished results). Furthermore, close examination of the dependence of the induction time on *src*TK concentration suggests that the enzyme may possess reasonable affinity for itself (K_m less than 1 μM).

The results reported in this work are consistent with those of Cooper and MacAuley (1988), who concluded that intermolecular autophosphorylation was possible. These authors observed transphosphorylation of a kinase-inactive mutant, pp60$^{c\text{-}src}$, by the active enzyme. On the other hand, Feder and Bishop (1990) reported that pp60$^{c\text{-}src}$ isolated from platelets underwent intramolecular autophosphorylation, but the highest concentration used in those studies was 4 nM. It is possible that there is a low rate of intramolecular autophosphorylation which is exceeded by the rate of intermolecular reaction at higher concentrations of *src*TK. In fact, close examination of the data reported by Feder and Bishop (1990) suggests that the rate of autophosphorylation may not have been increasing linearly with the enzyme concentration at higher concentrations. Sugimoto et al. (1985) concluded that autophosphorylation of pp60$^{v\text{-}src}$ was an intramolecular event. This could be the result of a difference in the mechanism between the viral and human enzymes, or it could be due to the different conditions used in these studies. Furthermore, these authors use ATP concentrations of 0.4 and 20 μM, and their enzyme was stored in 20% glycerol; therefore, the glycerol concentration varied in their reactions. The glycerokinase activity of pp60$^{v\text{-}src}$ (Richert et al., 1992) could have resulted in consumption of ATP at higher protein concentrations and resulted in the apparent lack of dependence of autophosphorylation on protein concentration in these studies. Barker and Knight (unpublished results) have made similar observations at high glycerol concentrations and low ATP concentrations. Furthermore, it is likely that the viral enzyme displays an ATPase activity as reported for *src*TK in this work.

The X-ray structure of the kinase domain of the insulin receptor tyrosine kinase (IRTK) provided an explanation for a trans-autophosphorylation event for this kinase (Hubbard et al., 1994). In this structure, Y1162, a site of IRTK autophosphorylation, is situated in the kinase active site and precludes binding of MgATP. Movement of the loop containing this tyrosine is required to yield an active enzyme. Phosphorylation of this site by the other β-subunit of the insulin $\alpha_2\beta_2$ tetramer prevents the loop from occupying this site and results in active kinase. This transphosphorylation is analogous to an intermolecular autophosphorylation by two monomers. While it would be attractive to propose a similar role for Y419 in pp60$^{c\text{-}src}$, the data presented in the accompanying work (Boerner et al., 1995) demonstrate that MgADP can bind to the unactivated form of the enzyme. It is still possible that with the additional phosphoryl, presumably coordinated to the metal ion, that MgATP is prevented from binding to the unactivated form of the enzyme and prevents intramolecular autophosphorylation.

The significance of an intermolecular autophosphorylation of pp60$^{c\text{-}src}$ versus the intramolecular event is that activation of one molecule of the enzyme possibly by an intramolecular event or interaction with some other activator could then lead to a chain reaction involving intermolecular events. pp60$^{c\text{-}src}$ is associated with cellular membranes, and therefore the effective concentrations could be such that intermolecular autophosphorylation would be greatly favored. The X-ray crystallographic structure of the SH3SH2 domain of pp56^{lck} indicated that dimerization was possible via interactions between the SH3 domain of one monomer with the SH2 domain of the other monomer for this member of the *src*TK family (Eck et al., 1994). In addition, data recently generated in these labs are not inconsistent with the hypothesis that the presence of ligands for the other domains of pp60$^{c\text{-}src}$ induces self-association of the full-length and N-85 enzymes (Willard et al., unpublished results). Self-association of the enzyme may induce transphosphorylation analogous to IRTK. In contrast to the conclusions reported by Liu et al. (1993), the presence of phosphopeptides that are recognized by the *src*SH2 domain does not appear to dramatically enhance the rate of autophosphorylation. These data suggest that simply binding to a phosphoprotein is not sufficient to increase the rate of autophosphorylation. These data suggest that one role of signaling components may be to induce *src*TK self-association such that intermolecular autophosphorylation can occur. For example, self-association could occur through the binding of two *src*TK molecules through a third protein. This could occur through interactions with the SH2 or SH3 domains or possibly a combination of the two.

The ESI-MS spectra of the pp60$^{c\text{-}src}$ mutants expressed in baculovirus demonstrate that the enzyme as isolated is partially phosphorylated on Y338 and Y530 and partial loss of the N-terminal Met has occurred. The latter is further supported by the N-terminal sequence analysis. A number of groups have produced pp60$^{c\text{-}src}$ by a similar route (Zhang et al., 1991; Budde et al., 1993; Saya et al., 1993) without characterizing the phosphorylation state of the protein. Y530 is a known site of intracellular phosphorylation of pp60$^{c\text{-}src}$, but phosphorylation of Y338 has not been reported. The significance of this phosphorylation site is unknown but under investigation. Based upon the lack of initial activity, we can state that monophosphorylation of either Y530 or Y338 is not sufficient to activate the enzyme as a catalyst in the direction of peptide phosphorylation. Furthermore, the diphosphorylated species (Y530 and Y338 phosphorylated) initially present is essentially inactive in the forward catalytic reaction, since the protein as isolated has only very low kinase activity. The results in the accompanying paper demonstrate that at least one or more of the species initially present is active in the direction of phosphoryl transfer from the phosphopeptide to MgADP (Boerner et al., 1995). We do not know whether the diphosphorylated and triphosphorylated forms of the pp60$^{c\text{-}src}$ produced upon phosphorylation of Y419 are active. Future experiments will

[13] This mutant is isolated in a homogeneous unphosphorylated form (Ellis et al., unpublished results).

address the molecular identity(s) responsible for the catalytic activity in the reverse direction when Y419 is not phosphorylated as well as the catalytic activity of hyperphosphorylated forms of *src*TK containing phosphorylated Y419.

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