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Purification of Recombinant pp60$^{v\text{-}src}$ Protein Tyrosine Kinase and Phosphorylation of Peptides with Different Secondary Structure Preference†

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ABSTRACT: The expression of the transforming gene product of Rous sarcoma virus (pp60$^{v\text{-}src}$) in *Saccharomyces cerevisiae* has recently been reported (Kornbluth et al., 1987; Brugge et al., 1987). To carry out biochemical and structural studies of this enzyme, a facile purification was developed. The purification was accomplished in four chromatographic steps: Q–Sepharose, Affi-Gel Blue, phosphoagarose, and hydroxylapatite chromatography. The tyrosine kinase was isolated in milligram quantities as two highly active proteolytic fragments (52 and 54 kDa). Three model tyrosine kinase substrates with propensities to adopt helical or Ω-loop conformations were synthesized and characterized. The peptides were based on the sites of phosphorylation of pp60$^{v\text{-}src}$, lipocortin I, and lipocortin II. Circular dichroism spectroscopy was used to study the conformation of the helix-forming peptides in 50 mM Tris and in 50% trifluoroethanol/Tris. Peptide 1, which was designed to form an amphiphilic α-helix, displayed 24.2% helicity in buffer and 40.2% helicity in 50% TFE/buffer. Similar experiments for peptide 3, the other helix former, showed a lower helicity (8.1% helical and 26.0% helical in buffer and in 50% TFE/buffer, respectively). All three peptides were shown to be substrates for the recombinant tyrosine kinase. Kinetic measurements using high-voltage paper electrophoresis indicated that the helix-forming peptides exhibited low K_M values (~450 μM) for the purified *src* gene product, consistent with the notion that elements of secondary structure may be important in substrate recognition by tyrosine kinases.

The finding that protein tyrosine kinase activity is associated with the transforming gene product of the Rous sarcoma virus (Hunter & Sefton, 1986) was a major step in our understanding of neoplastic transformation at the molecular level. The oncogene-encoded protein tyrosine kinases have been the subject of intense research efforts focused predominantly on the elucidation of the intracellular modes of action of tyrosine kinases that lead to cell transformation (Jove & Hanafusa, 1987). Despite numerous efforts to identify crucial substrates for these enzymes, no single cellular phosphoprotein has surfaced as the mediator of transformation. Though protein phosphorylation plays a prominent role in the regulation of cellular metabolism, the details of the enzymatic mechanisms by which protein kinases catalyze phosphate transfer are not well understood. In the case of the tyrosine-specific protein kinases, essentially no data exist regarding the identity and the spatial arrangement of residues comprising the active site of these enzymes.

The transforming gene product of the Rous sarcoma virus pp60$^{v\text{-}src}$ was the first recognized tyrosine-specific protein kinase. Though it is the most thoroughly characterized member of this family of enzymes, analysis of its tertiary structure has been hindered by the lack of adequate quantities of purified pp60$^{v\text{-}src}$. Such studies usually require milligram quantities of active enzyme. Available lines of transformed cells produce only very limited amounts of pp60$^{v\text{-}src}$. To create an abundant source of v-*src* kinase, we inserted 5′ truncated *src* cDNA into a bacterial secretion vector. Unfortunately, our efforts resulted in the production of a polypeptide that lacked kinase activity. Brugge et al. (1987) and Kornbluth et al. (1987) recently reported the expression of enzymatically active Rous sarcoma virus gene product in *Saccharomyces cerevisiae*. The recombinant kinase produced in yeast was myristylated at its amino terminus and phosphorylated at tyrosine-416. It was found to have approximately the same in vitro activity as its wild-type counterpart expressed in Rous sarcoma virus transformed cells (Kornbluth et al., 1987).

Because of the facility with which yeast can be grown in

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Deceased. This paper is dedicated to the memory of Dr. Emil T. Kaiser for his friendship, leadership, and inspiration as a scientist and teacher.

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culture, we decided to determine the feasibility of obtaining pp60$^{v\text{-}src}$ in quantities sufficient for our studies. This paper describes the purification of pp60$^{v\text{-}src}$ from yeast and efforts to develop useful small peptide substrates for the enzyme.

Materials and Methods

Proteins used as molecular weight markers were purchased from Pharmacia. ATP, raffinose, and galactose were obtained from the Sigma Chemical Co. Protease inhibitors were purchased from Boehringer Mannheim Biochemicals. *p*-Methylbenzhydrylamine and benzhydrylamine resins were Pierce products. All protected amino acids were purchased from Peninsula Laboratories Inc. [γ-^{32}P]ATP (3000 Ci/mmol) was supplied by NEN Research Products. ^{125}I-Labeled goat anti-mouse antibodies were from NEN. Radioactive decay was measured by using an LKB 1219 Rackbeta liquid scintillation counter. Fission fragment ionization mass spectrometric analysis was carried out at the Rockefeller University Biotechnology Mass Spectrometry Research Resource. Amino acid analysis of the peptides was performed by using a Dionex amino acid analyzer according to the method of Moore and Stein (1963) after hydrolysis of the peptides in 6 N boiling HCl at 110 °C for 24 h. High-performance liquid chromatography was carried out on a Perkin-Elmer Series 410 system, and effluent was monitored by means of a Perkin-Elmer variable-wavelength LC75 detector. Circular dichroism spectra were recorded on a Cary 60 spectropolarimeter calibrated with *d*-10-camphorsulfonic acid.

Enzyme Assay. Tyrosine kinase activity was assayed by using [Val^5]-angiotensin II octapeptide (AspArgValTyrValHisProPhe) as a phosphate acceptor. Reaction mixtures contained 3 mg/mL peptide, 400 μM ATP (specific activity, 50 μC/mmol), 20 mM $MgCl_2$, 14 mM sodium *p*-nitrophenyl phosphate, 100 μM sodium orthovanadate, 0.8 mg/mL BSA, 80 mM Tris-HCl (pH 7.5), and 5 μL of enzyme in a total volume of 25 μL. Incubation was carried out at 30 °C for 10 min, and the reaction was terminated by the addition of 60 μL of 20% trichloroacetic acid followed by centrifugation for 2 min in an Eppendorf microcentrifuge. A 50-μL portion of the supernatant was spotted on a 2 × 2 cm phosphocellulose paper which was then washed (3 × 4 min) in 0.5% phosphoric acid containing 0.25% sodium pyrophosphate. The extent of ^{32}P incorporation was measured by scintillation counting of the phosphocellulose paper.

Polyacrylamide Gel Electrophoresis. Proteins were separated and their molecular weights determined by electrophoresis through 1.5 mm SDS–polyacrylamide slab gels. The stacking gel contained 4% polyacrylamide, and the separating gel contained 12.5% polyacrylamide. The following standards (Pharmacia) were used to calibrate migration in the gel: phosphorylase *b* (94 kDa); bovine serum albumin (66 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20.1 kDa); and α-lactoglobulin (14.4 kDa).

Immunostaining. Following electrophoresis of proteins on 11 × 8 cm 10% polyacrylamide SDS–PAGE, gels were blotted onto nitrocellulose at 250 mA for 2.5 h. The nitrocellulose membrane was stained for 5 min with 0.1% Ponceau S dissolved in 5% trichloroacetic acid. Protein standards and other protein bands were marked with an indelible pen, and the membrane was destained with water. Nonfat dry milk (5% solution in PBS) was used for blocking nonspecific binding sites. Monoclonal antibody 327 (Lipsich et al., 1983) was then incubated with the membrane at a final concentration of 50 μg/mL. Immune complexes were detected by using radioiodinated goat anti-mouse second antibody (NEN) followed by autoradiography to visualize the bands.

Cell Growth. Yeast strain W303-1A bearing pp60$^{v\text{-}src}$ expressing plasmid was kindly provided by Dr. Sally Kornbluth of the Laboratory of Viral Oncology, The Rockefeller University. The plasmid was derived from the YEp51 vector and included the Leu$^+$ selectable marker, galactose-inducible promoter, and the complete v-*src* gene. A yeast inoculum was grown overnight to stationary phase in a synthetic medium containing 2% raffinose, 0.67% yeast nitrogen base, and all natural amino acids, except leucine, in a total volume of 2 L. Cells were transferred to 16 L of the same medium and grown in a 20-L autoclavable Nalgene carboy, with mechanical stirring and with rapid aeration of the medium at 30 °C. Growth was monitored periodically by measuring OD_{600}. When the cell density reached an OD_{600} of 0.9 (approximately 2.1×10^7 cells/mL) the synthesis of pp60$^{v\text{-}src}$ was induced by the addition of 40% galactose solution (950 mL), resulting in a final concentration of 2% galactose, and growth was continued for another 3 h. Yeast cells were subsequently harvested by use of a Millipore Pelican cassette system and were stored at −70 °C as a suspension in growth medium supplemented with 10% glycerol. Under these growth conditions the yield was 2.5 g of wet cell paste/L of culture.

Purification of v-src Gene Product. The stored cell suspension was thawed and centrifuged to collect a 100-g pellet of induced yeast cells. All purification steps were carried out at 4 °C. The pellet was suspended in 75 mL of 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Thesit, and 0.5% sodium deoxycholate (buffer A) containing 1 mM DTT, 1 mM PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 2 μg/mL pepstatin.

Step 1: Cell Disruption and Centrifugation. Glass beads (200 mL, 450–500 μm) were added to the mixture, and the cells were homogenized on ice with an OmniMixer for 15 min. Supernatant was recovered, and the glass beads were washed with 125 mL of 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.1% Thesit, and 1 mM DTT (buffer B) supplemented with protease inhibitors. All buffers used in steps 1–3 were similarly supplemented with protease inhibitors. Combined filtrates were centrifuged at 10000 rpm in a Sorvall GS-3 rotor for 20 min. The pellet was discarded, and a volume of 25% solution of streptomycin sulfate was added to the supernatant to yield a final concentration of 1.25%. The mixture was incubated on ice for 10 min followed by centrifugation at 10000 rpm for 45 min in a Sorvall GS-3 rotor.

Step 2: Q–Sepharose Batch Adsorption. The clarified homogenate, supplemented with 5% betaine, was incubated for 15 min with Q–Sepharose (300 mL, Pharmacia), vacuum-filtered, and then washed with buffer B (1000 mL) containing protease inhibitors. The enzyme was subsequently batch-eluted with 0.3 M NaCl in buffer B (4 × 150 mL).

Step 3: Affi-Gel Blue. The combined eluate was diluted with 200 mL of buffer B and incubated with Affi-Gel Blue (Bio-Rad, 120 mL) for 1 h. The gel was vacuum-filtered and washed with 600 mL of buffer B. The enzyme was batch-eluted with 1 M NaCl in buffer B (3 × 70 mL). The eluate was applied (6–7 mL/min) to an Amicon desalting gel GH 25 column (5 × 50 cm), and the protein was eluted with buffer B containing protease inhibitors. Peaks corresponding to protein were identified by optical density readings at 280 nm.

Step 4: Phospho-Spectra/Gel A6R. The desalted sample was injected onto a Phospho-Spectra/Gel A6R (Spectrum Medical Industries Inc.) column (3.3 cm, 180 mL gel volume). The column was washed with 100 mL of buffer B, and enzyme was subsequently eluted overnight with a 0–500 mM NaCl gradient (875-mL total) at a flow rate of 1 mL/min. Fractions

Table I: Summary of the Purification of the v-*src* Gene Product

purification step[a]	total protein (mg)	total act. (nmol/min)	sp act. (nmol mg^{-1} min^{-1})	recovery (%)	purifn (x-fold)
(1) cell homogenate[b]	3290	1100	0.34	100	1
(2) Q–Sepharose	745	826	1.12	75	3.3
(3) Affi-Gel Blue	140.8	400	2.82	36	8.4
(4) phosphoagarose	13.8	175	12.6	16	38.0
(5) hydroxylapatite	1.5	93	58.1	8.4	174

[a] Details are given in the text. [b] Beginning with approximately 100 g of wet cell paste.

containing kinase activity were pooled and concentrated by using a 20-kDa cutoff cellulose acetate ultrafiltration membrane (Spectrum Medical). Buffer was exchanged to 10 mM sodium phosphate (pH 7.0), 0.01 mM $CaCl_2$, 0.1% Thesit, 10% glycerol, and 1 mM DTT (buffer C) by using the same ultrafiltration membrane.

Step 5: Hydroxylapatite Chromatography. The resulting preparation was applied to a Bio-Gel HPHT high-performance hydroxylapatite column (Bio-Rad, 10 cm × 0.78 cm). The column was washed with 10 mL of buffer C, and enzyme was eluted with a 10–350 mM phosphate gradient (50-mL total) in buffer C. The fractions with tyrosine kinase activity were pooled and dialyzed against buffer B containing 50% glycerol and stored at −20 °C.[1]

Peptide Synthesis. Peptides were synthesized on a Beckman 990 peptide synthesizer using either *p*-methylbenzhydrylamine resin (peptide 1) or benzhydrylamine resin (peptides 2 and 3). The substitution level in all cases was 0.4 mmol/g. The coupling and deprotection procedures have been described previously (Yamashiro & Li, 1978). N-Protected amino acids (*t*-boc) were coupled as symmetric anhydrides by using DCC with the exceptions of asparagine and glutamine, which were coupled by hydroxybenzotriazole/DCC-mediated reactions. To acetylate N-terminal amino groups, completed peptides were treated with a 10-fold excess of acetic anhydride while still attached to the resin. After cleavage from the resins with anhydrous HF, peptides were purified by high-performance liquid chromatography on a Vydac C18 semipreparative column using a linear gradient of 0–50% acetonitrile in 0.1% aqueous TFA. The intramolecular disulfide bond between cysteines 1 and 11 in peptide 2 was formed by treating the HPLC-purified precursor with 20 mM potassium ferrocyanide as described (Moe & Kaiser, 1985). Peptide 2 was subsequently rechromatographed under the same experimental conditions. Peptides were characterized by amino acid analysis and by fission fragment ionization mass spectroscopy.

Kinetics of Peptide Phosphorylation. Phosphorylation reactions were carried out in a total volume of 25 μL for 25 min at 30 °C. Each reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 200 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol) and a peptide concentration range 50 μM–5 mM. Reactions were initiated by the addition of 3 μL of enzyme (70 μg/mL) and were terminated by incubation at 90 °C for 2 min. The reaction mixtures were subsequently microfuged for 2 min. The extent of phosphorylation for peptides 1 and 3 was determined by applying 5-μL aliquots of each reaction mixture to Whatman 3 MM paper and subjecting them to high-voltage electrophoresis at pH 1.9 (glacial acetic acid/formic acid/water, 78:25:897 v/v/v). Kinetic parameters of peptide 2 phosphorylation were determined by electrophoresis through a 0.75-mm 25% polyacrylamide SDS gel. In both types of assays phosphopeptides were located by autoradiography, corresponding regions were cut out, and the extent of ^{32}P incorporation was measured by scintillation counting.

[1] The enzyme stored in this manner lost activity gradually over several weeks. Typically, enzyme preparations were not saved beyond 10 weeks.

Results

Cell Growth and Enzyme Purification. Galactose induction of strain W303-1A has been shown to result in considerable attenuation of cell growth, presumably due to the toxicity of the expressed pp60$^{v\text{-}src}$ in the cells (Brugge et al., 1987; Kornbluth et al., 1987). Therefore, the exact timing for induction and harvest of cells was found to be critical. Consistent with earlier studies, we found that induction at a cell density of 2 × 10^7 cells/mL for a period of 3 h produced the best results. Typically, the cells were grown in 18-L quantities in a carboy (vide supra). The cell suspension was concentrated to approximately 0.5 L with a Millipore Pelican cassette system in 30 min. Cells were stored at −70 °C in the same medium supplemented with 10% glycerol. A combination of rapid steps was employed during the purification of the v-*src* gene product to prevent extensive proteolysis (Table I). The special features of the purification scheme include (1) batch adsorption and desorption of the enzyme to and from chromatographic resins, (2) the use of GH 25 cellulose (Amicon) gel filtration for rapid desalting of protein solutions, and (3) application of high-performance liquid chromatography. We were generally able to carry out the entire purification in less than 48 h. Use of the nonionic detergent Thesit, instead of Triton X-100 or Nonidet NP40, throughout the purification allowed the monitoring of column effluents at 280 nm. Inclusion of zwitterionic betaine in the first step improved enzyme recovery, most likely by breaking up protein aggregates present in the crude extract. The sodium chloride concentration required to elute enzyme from Q–Sepharose did not interfere with its subsequent binding to Affi-Gel Blue, thus allowing rapid transition between the two steps. We used Phospho-Spectra/Gel as the third step in purification instead of the more commonly used phosphocellulose, since this medium did not require pretreatment and was found to perform very consistently. High-performance hydroxylapatite chromatography was used as the final step. Two major bands were seen on a Coomasie blue stained SDS–polyacrylamide gel, corresponding to 52- and 54-kDa proteolytic fragments of v-*src* kinase (Figure 1). Immunostaining with monoclonal antibody 327 also revealed the presence of two pp60$^{v\text{-}src}$-related proteins with apparent molecular weights of 52 000 and 54 000 kDa (Figure 2). These two fragments were generated during the purification since immunoprecipitation from SDS cell lysate revealed the presence of an intact protein (Kornbluth et al., 1987). Unfortunately, proteolytic degradation of pp60$^{v\text{-}src}$ could not be prevented by inclusion of liberal amounts of protease inhibitors (PMSF, aprotinin, leupeptin, and pepstatin) in the buffers. However, this purification scheme yielded approximately 1.5 mg of the mixture of the two proteolyzed v-*src* gene products from 100 g of wet cell paste with an overall 174-fold purification. Its specific activity of 58.1 nmol min^{-1} mg^{-1} (3.3 μmol min^{-1} μmol^{-1}) is comparable with the reported turnover number of 2–3 μmol min^{-1} μmol^{-1} determined for pp60$^{v\text{-}src}$

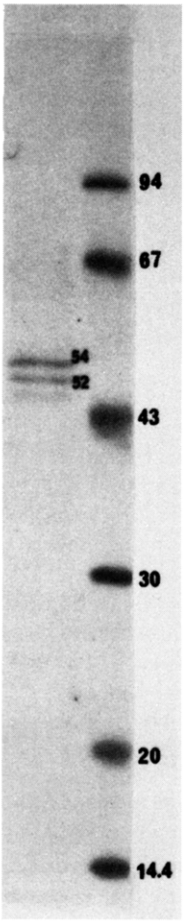

FIGURE 1: SDS–polyacrylamide electrophoresis of the purified v-*src* gene product.

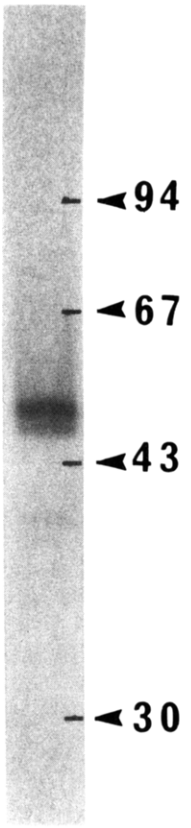

FIGURE 2: Western blot analysis of the purified v-*src* gene product.

isolated from transformed cells (Richert et al., 1982; Graziani et al., 1983). It is lower than the specific activity of 270 nmol min^{-1} mg^{-1} for the 59.8/57.2-kDa recombinant protein tyrosine kinase encoded by the Abelson murine leukemia virus (Foulkes et al., 1985) and the specific activity of 202 nmol min^{-1} mg^{-1} for the partially purified 50-kDa protein tyrosine kinase from bovine spleen (Kong & Wang, 1987).

Peptide Substrates for the v-src Gene Product. Though excellent peptide substrates with high-affinity constants are available for serine/threonine protein kinases, none that show similar affinity constants are available for protein tyrosine kinases. The catalytic subunit of cAMP-dependent protein kinase is able to phosphorylate a peptide substrate (kemptide) with kinetic parameters comparable to those of physiological protein substrates (Kemp et al., 1977). The conformation of this peptide when bound at the active site of the catalytic subunit has been determined to be a random coil by NMR studies (Rosevear et al., 1984). On the basis of these observations we elected to explore the possibility that protein tyrosine kinase substrate specificity may be governed to some extent by the three-dimensional structure of the substrate. Three peptides containing tyrosine residues were synthesized by solid-phase methodology. The peptides were designed for their ability to form helical and Ω-loop structures. Helical structure was represented by amphiphilic α-helix based on an altered sequence surrounding the Tyr-416 autophosphorylation site from v-*src* tyrosine kinase (peptide 1) and by a regular α-helix based on the sequence encompassing the phosphorylation site in lipocortin I (peptide 3) (Pepinski & Sinclair, 1986). The peptide substrate designed to form an Ω-loop (peptide 2) was based on the sequence encompassing the

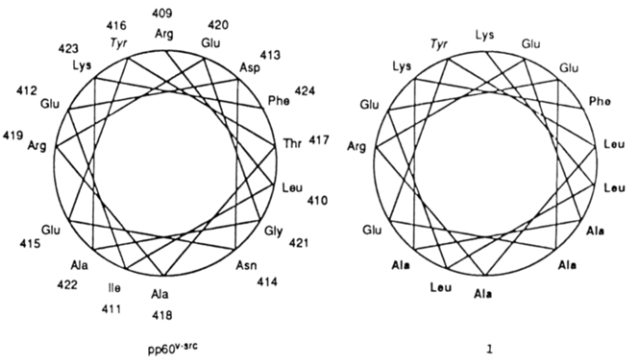

FIGURE 3: Sequences of peptide 1 and the autophosphorylation site of $pp60^{v\text{-}src}$ viewed by helical wheel representation. Amino acid residues in the hydrophobic face of the helix in peptide 1 are boldfaced; the phosphorylatable Tyr is italicized.

phosphorylation site from lipocortin II (Glenney & Tack, 1985). Peptide 1 was designed as an amphiphilic α-helical structure based on the $pp60^{v\text{-}src}$ autophosphorylation site. As shown by helical wheel diagrams in Figure 3, the amphiphilic segregation of the charged and hydrophobic residues surrounding the site of autophosphorylation of $pp60^{v\text{-}src}$ was exaggerated to give peptide 1. This approach has proven successful in modeling many amphiphilic peptide structures (Kaiser & Kezdy, 1984). To imitate more closely natural protein substrates for tyrosine kinases, the peptides were acetylated at N-terminal residues, and C-terminal carboxyl groups were replaced by amide groups, eliminating the existence of charged groups at these sites. On the basis of our earlier findings with substrates for the catalytic subunit of the cAMP-dependent protein kinase (Mobashery & Kaiser, 1988; Miller & Kaiser, 1988), we expected that C- and N-terminal protection would not be likely to impair the ability of our peptides to bind to the $pp60^{v\text{-}src}$ active site. Furthermore, the Ω-loop structure was mimicked by incorporating cysteine as

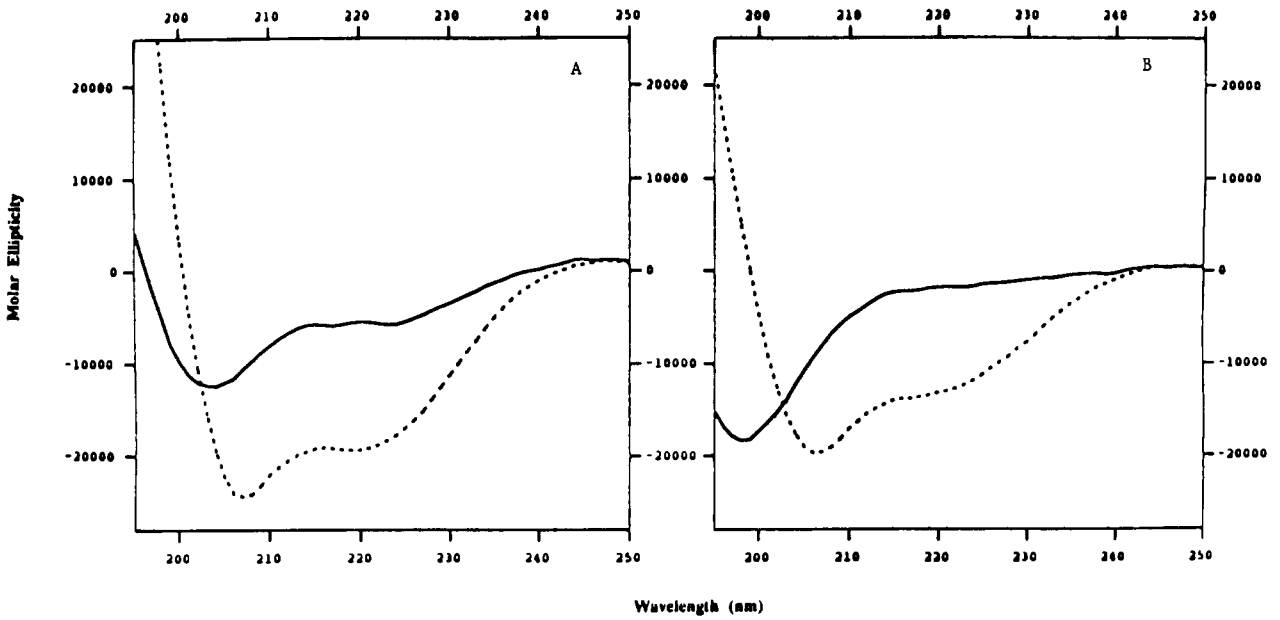

FIGURE 4: Circular dichroism spectra of peptides 1 (100 μM) (A) and 2 (120 μM) (B) in 50 mM Tris, pH 7.5 (solid lines), and in 50% TFE/50 mM Tris, pH 7.5 (broken lines).

Table II: Kinetic Parameters of Peptide Substrates for *v-src* Gene Product[a]

peptide	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m
[Val^5]-angiotensin II	0.94 ± 0.40	10.6 ± 4.5	11.4
1	0.48 ± 0.14	0.8 ± 0.2	1.7
2	1.18 ± 0.11	10.1 ± 0.7	8.6
3	0.44 ± 0.17	10.3 ± 0.8	23.4

[a] Details of assay conditions are given in the text.

both C- and N-terminal residues of the peptide, which were oxidized to form a disulfide bond.

Ac-Lys-Leu-Leu-Glu-Glu-Ala-Glu-Tyr-Leu-Ala-Arg-Glu-Ala-Ala-Lys-Phe-NH_2 1

Ac-Cys-Ser-Thr-Pro-Pro-Ser-Ala-Tyr-Gly-Ser-Cys-NH_2 2

S —— S

Ac-Ile-Glu-Asn-Glu-Glu-Gln-Glu-Tyr-Val-Gln-Thr-Val-Lys-NH_2 3

Circular dichroism spectroscopy was used to study the conformation of the helix-forming peptides in solution. Figure 4A shows that in 50 mM Tris peptide 1 displayed minima at 208 and 222 nm, characteristic of an α-helix. Upon addition of 50% trifluoroethanol, a structure-inducing solvent α-helicity was increased. Quantitative estimates of percent helicity for peptide 1 using the method of Hennessey and Johnson (1981) were 24.2% in buffer alone and 40.2% in 50% TFE/buffer, with the remainder of the molecule in a predominantly random coil conformation. Similar experiments for peptide 3 (Figure 4B) showed a lower tendency to form an α-helix. In 50 mM Tris this peptide was essentially all random coil. In the presence of 50% TFE/buffer the α-helicity increased from 8.1 to 26.0%.

As indicated in Table II, all three peptides served as substrates for the recombinant tyrosine kinase. Kinetic measurements were obtained by using high-voltage paper electrophoresis (Wong & Goldberg, 1983) and polyacrylamide electrophoresis. Experiments were performed at saturating concentrations of ATP (200 μM) and Mg^{2+} (10 mM) to arrive at K_m and V_{max} values for the peptides. Due to the rapid decrease of the recombinant enzyme activity, kinetic parameters could only be measured on a partially active preparation. Peptides 1 and 3—the helix formers—exhibited K_m values approximately half that of [Val^5]-angiotensin II, while peptide 2 showed a slightly higher K_m. Peptide 3 appears to be a better substrate than [Val^5]-angiotensin II by a factor of 2, according to the parameter V_{max}/K_m. It is interesting that the V_{max} numbers appear to approach a limiting value of approximately 10–11 nmol min^{-1} mg^{-1} with three of the four peptides listed in Table II.

Discussion

We have reported here a facile purification of the recombinant v-*src* gene product from *Saccharomyces cerevisiae*. The purification protocol yielded a protein that showed tyrosine-specific kinase activity and migrated on SDS–polyacrylamide gel as two closely spaced bands with apparent molecular weights of 52 000 and 54 000. Partially proteolyzed preparations of pp60$^{v\text{-}src}$ have been reported when transformed cells (Courtneidge et al., 1980) or tumor tissue (Richert et al., 1982) was used as the source of the protein. Similarly, when human platelets were used as the source of pp60$^{c\text{-}src}$, the enzyme preparation contained two proteolytic fragments with molecular weights of 52 000 and 54 000 (Presek et al., 1988). To purify intact recombinant kinase, it may be necessary either to clone in a protease-deficient yeast strain or to alter residues at the site of proteolysis by site-directed mutagenesis, thus rendering the protein less susceptible to proteases. To that end, we are presently attempting to identify N-terminal residues in both fragments to improve the stability of the recombinant enzyme by protein engineering. The ability to prepare milligram quantities of intact pp60$^{v\text{-}src}$ tyrosine kinase should facilitate biochemical studies aimed at characterization of this important enzyme.

It was well-known that a large number of cellular proteins are phosphorylated on tyrosine in Rous sarcoma virus transformed cells, but the question of substrate specificity has only recently begun to be addressed in a more systematic fashion (Hunter & Cooper, 1985). In particular, it appears likely that some conformational factors may play a function in substrate specificity requirements.

Recently, Tinker et al. (1988) have reported that peptides with tendencies to form β-turns may be recognized as substrates by a protein tyrosine kinase from the leukemia virus transformed LSTRA cell line. Our own analysis of the sites of protein (auto)phosphorylation has also revealed that sec-

ondary structure—or higher ordered—structures may play a role in substrate recognition by protein tyrosine kinases. Unfortunately, inspection of the primary sequences surrounding the sites of (auto)phosphorylation in a number of proteins did not yield a consensus sequence for phosphorylation sites. In the case of a number of phosphorylated proteins including pp60$^{v\text{-}src}$, lactate dehydrogenase, EGF receptor, Neu receptor, insulin receptor, integrin, polyoma middle T, pp90$^{gag\text{-}yes}$, p130$^{gag\text{-}fps}$, and p87$^{gag\text{-}fes}$, the third residue to the N-terminal side of the phosphorylated tyrosine may be either Asp, Glu, or Asn. Otherwise, no common denominator is discernible from the primary sequence of these proteins. This observation is in sharp contrast with the case of the catalytic subunit of the cAMP-dependent protein kinase—the prototypic protein serine/threonine kinase—for which Arg-Arg-X-Ser is the primary recognition sequence. Should secondary structural parameters prove important for substrate recognition by protein tyrosine kinases, it will present a point of contrast with the catalytic subunit, since a substrate bound to the active site of the latter has been shown to assume a random coil conformation by NMR studies (Rosevear et al., 1984).

To investigate the importance of secondary structures for protein tyrosine kinase substrates, we studied the phosphorylation sites in pp60$^{v\text{-}src}$ and lipocortins I and II. According to the empirical method of the Chou–Fasman analysis (Chou & Fasman, 1978) the sequence surrounding the phosphorylation site in lipocortin II favors formation of an Ω-loop, while those of lipocortin I and pp60$^{v\text{-}src}$ have propensities to form helical structures. The phosphorylatable tyrosine residues in a number of proteins are often surrounded by amino acids having considerable likelihood of forming Ω-loops (Leszczynski & Rose, 1986). On the one hand, because Ω-loops are located on protein surfaces and therefore subject to posttranslational modifications, it seemed conceivable that this particular structure might be important for recognition by tyrosine kinases. On the other hand, helices are common secondary structures in most proteins. Indeed, in lactate dehydrogenase, the one in vitro substrate for pp60$^{v\text{-}src}$ for which a crystal structure is known (Rossman et al., 1971), the phosphorylated tyrosine residue falls in an α-helical region (Cooper et al., 1984).

The three synthetic peptides 1–3 served as substrates for the v-*src* gene product (Table II). The helix formers (peptides 1 and 3) showed the lowest K_m values for any peptidic substrate for pp60$^{v\text{-}src}$. However, we find it intriguing that the V_{max} numbers approach a limiting value of ~10–11 nmol min^{-1} mg^{-1} for three of four peptides listed in Table II. This is reminiscent of findings with serine proteases, where the involvement of a covalent acyl enzyme intermediate was suggested (Hess, 1971). Perhaps an active-site residue in pp60$^{v\text{-}src}$ may be phosphorylated prior to the transfer of phosphate to the substrate tyrosine residue or, alternatively, a crucial rate-limiting conformational change may be responsible. Insofar as catalysis by the catalytic subunit does not apparently involve a phosphoenzyme intermediate (Ho et al., 1988), this may give rise to yet another point of contrast between serine/threonine and tyrosine kinases. Current efforts are focused on the analysis of other peptidic substrates for the v-*src* gene product, which may extend and clarify these observations.

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