VOLUME 123, NUMBER 37
SEPTEMBER 19, 2001

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Protein Tyrosine Kinase Csk-Catalyzed Phosphorylation of Src Containing Unnatural Tyrosine Analogues

Dongxia Wang and Philip A. Cole*

Contribution from the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

Received February 28, 2001

Abstract: Using expressed protein ligation, five unnatural tyrosine analogues (amino-phenylalanine, homotyrosine, 2-methyl-tyrosine, (α*S*,*β*R)-*β*-methyl-tyrosine, and 2,6-difluoro-tyrosine) were incorporated into Src in place of the natural tail tyrosine residue. These semisynthetic substrates were evaluated as Csk substrates or allosteric activators. It appears that the tyrosine phenol hydroxyl is unlikely to be contributing significantly to Src's ground-state binding affinity for Csk. It has been observed that stabilizing tyrosine conformers can further optimize Src's already high substrate efficiency. These latter findings contrast similar studies with synthetic peptide substrates and highlight the value of investigation of protein kinase substrate selectivity with protein substrates.

Protein tyrosine kinases catalyze phosphoryl transfer of the γ-phosphoryl group of ATP to tyrosine residues of proteins. Determining the molecular basis of recognition of protein tyrosine kinase substrates is an active area of research in cell signal transduction.[1] Although much has been learned about peptide selectivity by protein kinases, relatively little is known about the molecular basis for selectivity of tyrosine within the context of protein substrates.[2] Indeed, preferences for individual amino acids surrounding a target tyrosine residue can vary significantly between peptide and protein substrates for some kinases as is the case for the protein tyrosine kinase Csk and its physiologic protein substrate Src.[3]

Csk-catalyzed Src phosphorylation is important in various cellular contexts including neuro-development, immune regulation, and oncogenesis.[4] Csk targets a specific tail tyrosine residue in Src and Src family members, triggering a conformational change that reduces Src's own catalytic activity.[4] While Csk can catalyze phosphorylation of synthetic peptides, it shows much greater phosphoryl transfer efficiency toward the folded Src proteins.[3,5]

Interestingly, a peptide library approach to map the sequence specificity shows little similarity to the residues found in the folded protein.[3] For example, the consensus sequence recognized within a peptide NH_2-Glu-Glu-Ile-Tyr-Phe-Phe-Phe-CO_2H looks little like the Src tail sequence NH_2-Glu-Pro-Gln-Tyr-Gln-Pro-Gly-Glu-Asn-Leu-CO_2H. Moreover, the consensus synthetic

* To whom correspondence should be addressed: (e-mail) pcole@jhmi.edu; (phone) 410-614-0540; (fax) 410-614-7717.

(1) Hunter, T. *Cell* **2000**, *100*, 113−127.

(2) (a) Shokat, K. M. *Chem. Biol.* **1995**, *2*, 509−514. (b) Songyang, Z.; Carraway, K. L.; Eck, M. J.; Harrison, S. C.; Feldman, R. A.; Mohammadi, M.; Schlessinger, J.; Hubbard, S. R.; Smith, D. P.; Eng, C.; Lorenzo, M. J.; Ponder, B. A. J.; Mayer, B. J.; Cantley, L. C. *Nature* **1995**, *373*, 536−539. (c) Till, J. H.; Annan, R. S.; Carr, S. A.; Miller, W. T. *J. Biol. Chem.* **1994**, *269*, 7423−7428. (d) Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M. *Nature Genet.* **2000**, *26*, 283−289. (e) Lee, T. R.; Niu, J.; Lawrence, D. S. *Biochemistry* **1994**, *33*, 4245−4250. (f) Lee, T. R.; Till, J. H.; Lawrence, D. S.; Miller, W. T. *J. Biol. Chem.* **1995**, *270*, 27022−27066.

(3) (a) Cole, P. A.; Sondhi, D.; Kim, K. *Pharmacol. Ther.* **1999**, *82*, 219−229. (b) Sondhi, D.; Xu, W.; Songyang, Z.; Eck, M. J.; Cole, P. A. *Biochemistry* **1998**, *37*, 165−172. (c) Ruzzene, M.; Songyang, Z.; Marin, O.; Donella-Deana, A.; Brunati, A. M.; Guerra, B.; Agostinis, P.; Cantley, L. C.; Pinna, L. A. *Eur. J. Biochem.* **1997**, *246*, 433−439.

(4) (a) Brown, M. T.; Cooper, J. A. *Biochim. Biophys. Acta* **1996**, *1287*, 121−149. (b) Xu, W.; Harrison, S. C.; Eck, M. J. *Nature* **1997**, *385*, 595−602. (c) Sicheri, F.; Moarefi, I.; Kuriyan, J. *Nature* **1997**, *385*, 602−609.

(5) Wang, D.; Huang, X.-Y.; Cole, P. A. *Biochemistry* **2001**, *40*, 2004−2010.

10.1021/ja010540b CCC: $20.00
Published on Web 08/22/2001

Table 1. Relative Rates for Csk-Catalyzed Phosphorylation of Tyrosine Analogs

tyrosine analog	kinase rate of analogue in eSrc (V/E) (s^{-1})[a]	kinase rate of analogue in peptide k_{cat}/K_m (M^{-1} s^{-1})[b]	relative preference protein:peptide[c]
L-Tyr	0.11 ± 0.004	35	1
HomoTyr	0.0008 ± 0.0002	9.1	0.028
2-MeTyr	0.33 ± 0.04	32	3.3
β-MeTyr	0.66 ± 0.04	35	6.0
2,6F_2-Tyr	0.26 ± 0.005	105	0.8
NH_2Phe	*d*	*d*	

[a] The rates were measured at eSrc = 2 μM as described in the Experimental Section and shown ± standard error. [b] These values were taken from ref 6 and employed the peptide sequence EDNEXTA; the standard errors for these rates = ±20%. It should be noted that a similar length peptide derived from the tail of a Src family member is at least 20-fold less efficiently phosphorylated compared to the EDNEYTA sequence. [c] Relative preference of tyrosine analogue in eSrc protein versus peptide substrate referenced to L-Tyr. [d] Undetectable.

peptide derived from this library approach was processed >1000-fold more efficiently as a Csk substrate than a related peptide from the Src family, which is barely detectably a substrate.[3] The intact SH3-SH2-kinase construct, catalytically impaired Src protein substrate (dSrc, residues 83-533 K295M) is approximately 100-fold more efficiently phosphorylated by Csk than the consensus synthetic peptide.[5] Alanine scanning mutagenesis of the dSrc protein tail showed that only two amino acid residues, Y-3 (Glu) and Y-1 (Gln), are strongly required for Csk phosphorylation, with the other residues showing less than 2-fold effects on phosphorylation.[5]

Another unusual element of Csk recognition of dSrc is that dimerization or higher order oligomerization of dSrc may be important for Csk-mediated dSrc phosphorylation.[5] This model is supported by a combination of studies including the following: S-shaped saturation kinetic behavior in plots of velocity versus dSrc concentration; anti-Src antibodies which can enhance Csk phosphorylation of dSrc; and paradoxical stimulation of Csk phosphorylation of dSrc by the nonphosphorylatable dSrc mutant (Y527F). The use of phenylalanine as a mutant residue in Src can potentially complicate the interpretation of these stimulation studies because of its side chains inability to form a hydrogen bond at the Csk active site. In fact, the contribution of such a hydrogen bond in dSrc binding to Csk is unknown.

Csk shows a fairly relaxed selectivity for the natural tyrosine structure within the context of a synthetic peptide (Table 1).[6] This flexibility allowed the nature of the Csk-catalyzed phosphoryl transfer transition state to be probed with a series of fluorotyrosine-containing substrates.[7] In this manner, it was shown that the phopshoryl transfer rate was largely independent of the nucleophile pK_a, and the neutral phenol rather than the chemically more reactive phenoxide anion was required for enzymatic reaction, both consistent with a dissociative transition state.[7] Methyl-substituted tyrosine analogues as well as homotyrosine were also evaluated in the context of peptides and showed little to modest affects on k_{cat}/K_m (see Table 1).[6] Given the sequence selectivity differences between Csk phosphorylation of peptide versus protein substrates,[3,5] it was of interest to examine Csk's tolerance of unnatural tyrosine analogues within a natural protein substrate. In this study, we report on the effects of substitution of Src's tail with tyrosine analogues on interactions with Csk.

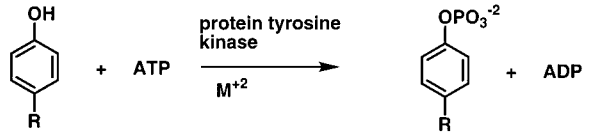

Figure 1. Protein tyrosine kinase catalyzed reaction. $M^{+2} = Mg^{2+}$ or Mn^{2+}; R = protein.

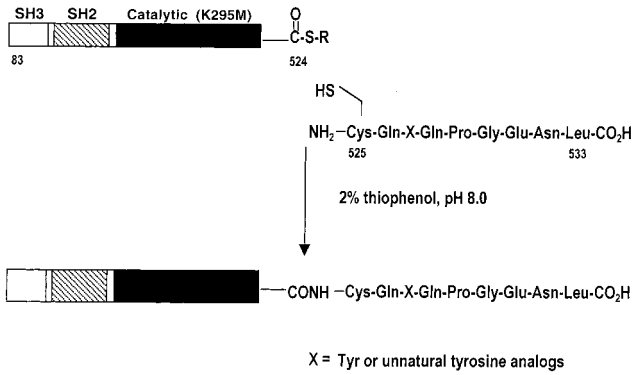

Figure 2. Incorporation of unnatural tyrosine analogues into eSrc using expressed protein ligation.

Results and Discussion

Of the possible strategies to incorporate unnatural amino acids into proteins of this size,[8,9] the expressed protein ligation approach was chosen because of its simplicity in engineering the C-terminii of recombinant proteins.[8] Given the recent success at expressing catalytically inactive Src in *E. coli*,[5] the residues 83-524 were subcloned into the commercially available pET vector pTYB2. This region was selected because it included the fully folded catalytic domain[10] and allowed for relatively short synthetic peptides to be attached (Figure 2). Ligation of synthetic peptides to the Glu-524 $^{\alpha}$thioester necessitated replacement of the wild-type Pro-525 residue in Src with a Cys (to generate eSrc); this residue change was predicted to be well-tolerated for recognition by Csk based on the previous site-directed mutagenesis studies described above.[5]

Six semi-synthetic eSrc's were produced, one containing a tyrosine at position 527 and the others containing unnatural amino acids at this position (Figure 3). While yields were modest (~0.25 mg/L of *E. coli* cell culture), high purity was verified by SDS-PAGE (Figure 4) and MALDI-MS, showing that the ligations proceeded essentially quantitatively. The semisynthetic proteins were further purified by a Zn column based on the affinity of an N-terminal His_6 tag and concentrated to about ~1 mg/mL.

These semisynthetic proteins were evaluated as Csk substrates using the previously described radioactive assay in which ^{32}P is transferred from labeled ATP to the Src protein and separated by SDSPAGE. Because of the complexity of the saturation curves of Csk with dSrc, the rates (see Table 1) of Csk-catalyzed

(6) (a) Kim, K.; Parang, K.; Lau, O. D.; Cole, P. A. *Bioorg. Med. Chem.* **2000**, *8*, 1263−1268. (b) Kim, K.; Cole, P. A. *J. Am. Chem. Soc.* **1997**, *119*, 11096−11097. (c) Kim, K.; Cole, P. A. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1205−1208.

(7) Kim, K.; Cole, P. A. *J. Am. Chem. Soc.* **1998**, *120*, 6851−6858.

(8) (a) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710. (b) Evans, T. C.; Benner, J.; Xu, M.-Q. *Protein Sci.* **1998**, *7*, 2256−2264.

(9) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188. (b) Arslan, T.; Mamaev, S. V.; Mamaeva, N. V.; Hecht, S. M. *J. Am. Chem. Soc.* **1997**, *119*, 10877−10887. (c) Xiao, G.; Parsons, J. F.; Tesh, K.; Armstrong, R. N.; Gilliland, G. L. *J. Mol. Biol.* **1998**, *281*, 323−339. (d) Thorson, J. S.; Chapman, E.; Murphy, E. C.; Schultz, P. G.; Judice, J. K. *J. Am. Chem. Soc.* **1995**, *117*, 1157−1158.

(10) Yamaguchi, H.; Hendrickson, W. A. *Nature* **1996**, *384*, 484−489.

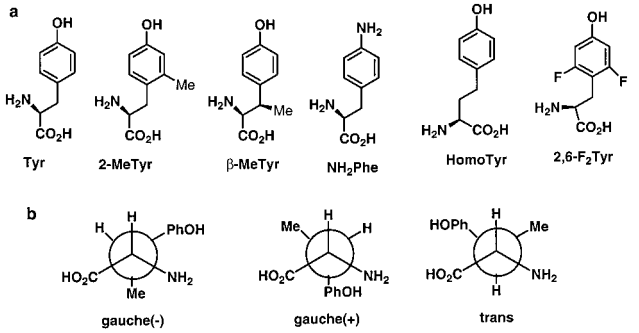

Figure 3. (a) Tyrosine analogues incorporated into eSrc at position 527. (b) Potential χ_1 rotamers for β-MeTyr.

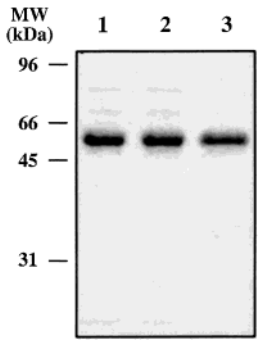

Figure 4. Coomassie-stained 10% SDSPAGE of selected semisynthetic eSrc proteins. Approximately 2 μg of purified Tyr-eSrc (lane 1), β-MeTyr-eSrc (lane 2), and NH_2Phe-eSrc (lane 3) are shown here.

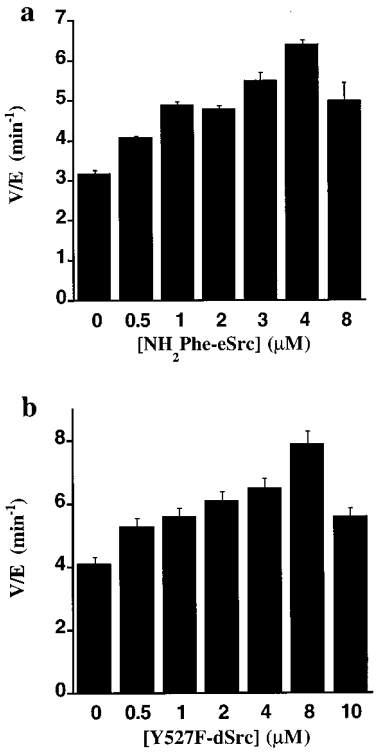

Figure 5. Plots of Csk kinase velocity versus [NH_2Phe-eSrc] and [Y527F-dSrc] in the presence of fixed dSrc substrate concentration (0.4 μM).

phosphorylation of these proteins were characterized at a fixed and relatively low Src protein concentration to approximate k_{cat}/K_m conditions. It was initially and reassuringly shown that the standard tyrosine containing form of semisynthetic eSrc (Tyr-eSrc) was only 2-fold lower than dSrc, a rate similar to that of Csk phosphorylation of fully recombinant P525A-dSrc reported previously.[5] This suggests that the conditions of expressed protein ligation/semisynthesis do not adversely affect the properties of Src as a Csk substrate, compared to Src generated as an intact recombinant protein.

Our next substrate investigated was NH_2Phe-eSrc. Csk-catalyzed phosphorylation was shown to be undetectable (<200-fold the rate of Tyr-eSrc). This was not surprising based on previous work with peptide substrates[11] as well as other kinases in which the substrate hydroxy nucleophile is replaced by an amino group.[12] Why this should be the case is not entirely clear for a reaction with a dissociative transition state, but perhaps the subtle electronic and geometric differences between nitrogen and oxygen as a nucleophile contribute to the inability of the anilino moiety to be processed in the enzyme-catalyzed reactions.

Having established NH_2Phe-eSrc is not a Csk substrate, we tested it as a potential inhibitor or allosteric activator for Csk phosphorylation of dSrc. As described above, previous work had shown that Y527F-dSrc was not a competitive inhibitor of Csk-mediated phosphorylation of dSrc, but in fact could activate such phosphorylation, presumably by promoting dimerization.[5] A nonphosphorylatable dSrc analogue, which still had a hydrogen-bonding functional group in place of the hydroxy moiety, might form a much tighter active site interaction with Csk than Y527F-dSrc does and serve as a competitive inhibitor rather than activator. However, NH_2Phe-eSrc was also found to stimulate the rate of phosphorylation of dSrc (Figure 5). The degree and concentration dependence of activation of dSrc phosphorylation by NH_2Phe-eSrc was quite comparable with that shown by Y527F-dSrc, both showing saturation of the effect in the range of 8−10 μM. This suggests that the hydrogen-bonding capability of Tyr and NH_2Phe at position 527 does not significantly strengthen the Csk−dSrc interaction. This is an important observation because hydrogen bonds to both Csk Arg-318 and Asp-314 are thought to be rendered by the substrate tyrosine phenol in the ground state. While these hydrogen bonds are no doubt important in the transition state,[13] our data suggest they are not critical in ground-state binding of Csk to protein substrate.

We next examined the Csk phosphorylation of methyl- and methylene-substituted tyrosine derivatives (see Figure 3) incorporated into eSrc at position 527. Recent work with a short synthetic peptide (NH_2-EDNEXTA-CO_2H; X = Tyr analog) showed that HomoTyr, 2-MeTyr, and β-MeTyr substitutions were all fairly well tolerated; 2-MeTyr and β-MeTyr substituted peptides showed similar substrate efficiency to Tyr-containing peptide and Homo-Tyr-containing peptide was about 4-fold less efficient (Table 1).[6] Results with the corresponding semisynthetic proteins differed considerably (Table 1). The rate of Csk-catalyzed phosphorylation of HomoTyr-eSrc was about 140-fold lower than Tyr-eSrc whereas 2-MeTyr-eSrc and β-MeTyr-eSrc were 3- and 6-fold more rapidly phosphorylated, respectively. The relative preferences for these analogues to be phosphorylated in a synthetic peptide versus Src are shown in Table 1. These results support the possibility that Csk shows enhanced sensitivity to conformational effects of the tyrosine undergoing phosphorylation in the context of its physiological protein substrate compared to a short peptide substrate.

A formal possibility accounting for the results of these various analogues is that the methyl-substituted analogues induce subtle

(11) Cole, P. A. Unpublished work.
(12) Knight, W. B.; Cleland, W. W. *Biochemistry* **1989**, *28*, 5728−34.
(13) Williams, D. N.; Wang, D.; Cole, P. A. *J. Biol. Chem.* **2000**, *275*, 38127−38130.

phenol pK_a changes, which alter interactions with the active site residues or otherwise affects catalysis. It should be pointed out, however, that *m*- and *p*-methyl substitution of phenol appears to change the phenol pK_a by less than 0.5 units.[14] To more intensively examine this issue, we incorporated the analogue 2,6F_2-Tyr (which has been measured to have a pK_a of 8.1, about 2 units lower than tyrosine[7]) into eSrc. Interestingly, 2,6F_2-Tyr when incorporated into a synthetic peptide was previously shown to be 3-fold more efficiently phosphorylated by Csk compared to Tyr.[6] In the context of the eSrc protein, there was a 2.4-fold rate enhancement over Tyr-eSrc. Thus, the large (2 unit) pK_a drop in 2,6F_2-Tyr compared to Tyr induces very similar rate effects in peptide and protein substrate, arguing that the transition states with peptide and protein are both likely to be dissociative. These results suggest that it is unlikely that the methyl- and methylene-substituted tyrosine analogue rate differences between peptide and protein substrates are due to subtle pK_a effects.

It is especially noteworthy that 2-MeTyr and β-MeTyr show enhanced efficiency over naturally occurring Tyr in the context of the protein, rather uncommon for highly evolved, native molecular interactions. Both 2-MeTyr and β-MeTyr show increased barriers to side chain bond rotation as well as shifts in equilibrium among various conformers.[15,16] β-Methyl-tyrosine and β-methyl-phenylalanine analogues have been studied intensively in peptide systems and these analyses indicate that the χ_1-trans rotamer is preferentially stabilized severalfold in β-MeTyr (Figure 3b).[16,17] Although we cannot rule out the possibility that increased hydrophobic contacts made by 2-MeTyr and β-MeTyr contribute to increased phosphorylation efficiency, given the relatively high efficiency of β-MeTyr-eSrc as a Csk substrate it is reasonable to speculate that this tyrosine conformation is especially favored by Csk in the context of the Src protein substrate. In contrast, the longer linker between backbone and phenyl ring in Homo-Tyr relative to Tyr has increased flexibility. Moreover, the increased distance and change in orientation between the protein backbone and the phenol hydroxy group in HomoTyr versus Tyr could also contribute to HomoTyr-eSrc's decreased substrate efficiency.

Conclusions

In summary, we have investigated two features of Src's efficiency as a Csk substrate using unnatural tyrosine analogues. First, it appears that the tyrosine phenol hydroxyl is unlikely to contribute greatly to Src's ground state affinity for Csk binding. Second, it has been observed that stabilizing tyrosine conformers can further optimize Src's already high substrate efficiency. That tyrosine rotamer preferences may be especially important within the context of proteins versus peptides highlights the value of investigation of protein kinase substrate selectivity with protein substrates. In future work, it will be interesting to explore the significance of substrate tyrosine conformational preferences in other protein kinase−protein substrate interactions and how these preferences may relate to overall sequence selectivity and regulation.

Experimental Section

Unnatural Amino Acids. Reagent grade Fmoc-HomoTyr and Fmoc-NH_2Phe were obtained commercially. 2-MeTyr, β-MeTyr, and 2,6F_2-Tyr were prepared using tyrosine phenol-lyase and converted to their Fmoc derivatives as reported previously.[6,7]

Peptide Synthesis. All synthetic peptides were prepared using the Fmoc strategy on a 0.1 mmol scale.[7] Peptides were cleaved and deblocked using reagent K and purified to greater than 95% homogeneity by reversed-phase HPLC using a water:acetonitrile:0.05% trifluoroacetic acid gradient. Correct peptide structures were confirmed by electrospray ionization mass spectrometry.

Expressed Protein Ligation. DNA corresponding to residues 83-524 of Src K295M and carrying a His_6 N-terminal tag was subcloned into pTYB2 (NEB). After confirming the correctness of the open reading frame by DNA sequencing, the src-pTYB2 plasmid was transformed into *E. coli* (BL21 DE3 strain), and the cells (2−6 L) were grown in shaker flasks at 37 °C in LB media containing ampicillin (100 μg/mL), followed by induction with IPTG (0.5 mM) at A_{600}-0.6 at 16 °C for 20 h. Cells were lysed in lysis buffer (25 mM Na-Hepes, pH 8.0/150 mM NaCl/1 mM $MgSO_4$/5% ethylene glycol/5% glycerol) in the absence of added thiols and loaded on to 2−5 mL of chitin resin as described.[5,7a] The column was washed with 10 column volumes of equilibration buffer (25 mM Na-Hepes, pH 7.0/250 mM NaCl/1 mM Na-EDTA/0.1% Triton X-100) and then 10 column volumes of ligation buffer (25 mM Na-Hepes, pH 8.0/250 mM NaCl/1 mM Na-EDTA). Ligations were carried out by addition of 1 mM peptide, 2% thiophenol in ligation buffer at room temperature for 24 h as described previously.[7a] The reaction mixtures were dialyzed against 20 mM Na-Hepes (pH 7.5), 200 mM NaCl, and 2 mM DTT at 4 °C. The semisynthetic proteins were purified over 2 mL of Zn chelate column.[5] The dialyzed protein solution was loaded onto the column at a flow rate of 0.5 mL/min. This was followed by washing with 50 mM and 100 mM imidazole-containing buffers (20 mM Na-Hepes, pH 7.5, 200 mM NaCl, 1 mM DTT, 2 mL each). The protein was eluted from the column with 200 mM imidazole-containing buffer and fractions containing the desired protein were combined and concentrated to 0.5−1 mg/mL by ultrafiltration. Proteins appeared nearly homogeneous by SDS-PAGE and showed the correct MW by MALDI-TOF MS (±150 daltons) with little if any unligated materials present.

Csk Assays. Kinase assays were carried out as described previously.[5] In brief, reactions were performed at 30 °C and pH 7.4 with 1.5 nM Csk, 1−2 μM of semisynthetic eSrc protein, 2 mM $MnCl_2$, 50 μM ATP, 0.2 μCi of [γ-^{32}P] ATP, 60 mM Tris-HCl, 10 mM DTT, 200 μg/mL BSA in 15 μL reaction volume for 2 min. Reactions were initiated with Csk and quenched with 7.5 μL of aqueous Na-EDTA (100 mM, pH 8). A 20 μL aliquot of the quenched reaction mixtures was loaded on to a 10% SDS-PAGE. The gels were stained with Coomassie, which revealed the protein band corresponding to phosphorylated eSrc, which was subsequently excised with a blade. The radioactivity of gel slices was quantitated by liquid scintillation counting. In all cases, reaction of the limiting substrate did not exceed 10%. As discussed previously, Csk phosphorylation of dSrc does not follow typical Michaelis−Menten kinetics[5] and thus relative rates are compared at fixed eSrc concentrations and shown in Table 1. All rate measurements were performed in duplicate on at least two separate occasions with good reproducibility.

Acknowledgment. We are grateful to the NIH and Burroughs Wellcome Fund for financial support and to members of the Cole lab for helpful discussions and advice.

JA010540B

(14) Dean, J. A. *Lang's Handbook of Chemistry*, 14th ed.; McGraw-Hill: New York, 1992.

(15) (a) Anastasakos, L.; Wildman, T. A. *J. Chem. Phys.* **1993**, *99*, 9453−9459. (b) Seeman, J. I.; Secor, H. V.; Breen, P. J.; Grassian, V. H.; Bernstein, E. R. *J. Am. Chem. Soc.* **1989**, *111*, 3140−3150. (c) Bello, J. F.; Llama, E. F.; Del Campo, C.; Cabezas, M. J.; Sinisterra, J. V.; Arias, M. S. *J. Mol. Catal.* **1993**, *78*, 91−112.

(16) (a) Hruby, V. J.; Toth, G.; Gehrig, C. A.; Kao, L.-F.; Knapp, R.; Lui, G. K.; Yamamura, H. I.; Kramer, T. H.; Davis, P.; Burks, T. F. *J. Med. Chem.* **1991**, *34*, 1823−1830. (b) Hruby, V. J.; Li, G.; Haskell-Luevano, C.; Shenderovich, M. *Biopolymers* **1997**, *43*, 219−266. (c) Casimir, J. R.; Iterbeke, K.; Van Den Nest, W.; Trescol-Biemont, M.-C.; Dumortier, H.; Muller, S.; Gerlier, D.; Rabourdin-Combe; Tourwe, D.; Paris, J. *J. Pept. Res.* **2000**, *56*, 398−408. (d) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N.-N.; Marsden, B. J.; Wilkes, B. C. *J. Med. Chem.* **1991**, *34*, 3125−3132. Huang, Z.; He, Y.-B.; Raynor, K.; Tallent, M.; Reisine, T.; Goodman, M. *J. Am. Chem. Soc.* **1992**, *114*, 9390−9401.

(17) The preference for the χ_1-trans rotamer is true only for the α-S, β-R isomer of β-methyl-tyrosine; the α-S, β-S isomer not studied here would show preference for the χ_1-gauche(−) rotamer.