Divalent Ion Effects and Insights into the Catalytic Mechanism of Protein Tyrosine Kinase Csk†

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ABSTRACT: Csk (C-terminal Src kinase) is a protein tyrosine kinase which catalyzes the transfer of the γ-phosphoryl group of ATP to the tyrosine hydroxyl of proteins in the presence of a divalent ion. Previous work with poly(Glu,Tyr) as the tyrosine-containing substrate and Mn as the divalent ion defined a ternary complex mechanism with ADP product release partially rate-determining [Cole, P. A., *et al.* (1994) *J. Biol. Chem. 269*, 30880-30887]. In this current study, ionic strength and divalent ion effects were probed. Increasing ionic strength led to a dramatic rise in the poly(Glu,Tyr) [4:1 poly(glutamate:tyrosine)] K_m and had little effect on the ATP K_m or k_{cat} in Csk-mediated phosphoryl transfer. This finding allowed the dead-end peptide inhibitor EDNEFTA to be characterized as a linear competitive inhibitor of poly(Glu,Tyr) and a linear noncompetitive inhibitor of ATP. Taken together with previous data, the overall kinetic mechanism could now be assigned as random substrate binding, ternary complex. Compared to Mn, Mg was shown to sustain phosphoryl transfer with a 2.5-fold higher k_{cat} but K_m's for ATP and poly(Glu,Tyr) that were some 15−20-fold higher. An elevated ADP K_i and microviscosity effects were most suggestive of a kinetic mechanism with fast ADP release, and the chemical step fully rate-determining in the Mg-dependent reaction. Steady-state kinetic analyses of Csk reactions with Co and Ni in addition to Mg and Mn on wild-type and D314E Csk with ATP and ATPγS [adenosine 5′-*O*-(3-thiotriphosphate)] as substrates were performed. The k_{cat} thio effects [k_{cat}(ATP)/k_{cat}(ATPγS)] were inversely correlated with metal thiophilicity in both wild-type and D314E mutant Csk reactions, although the relationship was less pronounced in the latter. These results appear to underscore the role of γ-phosphoryl hydrogen bonding/salt bridging in the wild-type Csk reaction transition state, which is somewhat perturbed in the D314E Csk reaction. In the case of the Ni reaction, the k_{cat} thio effect was reduced to about 2 in the wild-type and D314E mutant Csk reactions. Relevance with regard to the degree of nucleophilic attack in the transition state, i.e., associative vs dissociative character of phosphoryl transfer, is discussed.

C-Terminal Src kinase (Csk)[1] is a nonreceptor protein tyrosine kinase which is responsible for down-regulation of members of the Src family by catalyzing γ-phosphoryl transfer from ATP to specific C-terminal tyrosine residues (Okada *et al.*, 1991; Boerner *et al.*, 1996). Composed of SH3, SH2, and catalytic domains, Csk is a 50 kDa protein that belongs to a PTK family with at least one other member (Nada *et al.*, 1991; Klages *et al.*, 1994). Gene deletion of *csk* from mice leads to neural tube defects (Imamoto & Soriano, 1993). That overactive Src family members are at least partially responsible for this phenotype has been convincingly demonstrated (Nada *et al.*, 1993; Thomas *et al.*, 1995). Unlike many protein tyrosine kinases, Csk does not autophosphorylate at a significant rate (Amrein *et al.*, 1995; Cole *et al.*, 1994). It is readily overproduced and purified from *Escherichia coli* using chaperone-assisted protein expression (Amrein *et al.*, 1995; Cole, 1996). Using the random copolymer poly(Glu,Tyr) as the tyrosine substrate and Mn as the divalent cation, it has been shown that the Csk-catalyzed kinase reaction follows an intersecting line pattern consistent with a ternary complex mechanism (Figure 1) (Cole *et al.*, 1994). Under these circumstances, ADP product release was found to be partially rate-determining, with a first-order rate constant deduced to be comparable to that of the chemical step (both about 1 s^{-1}) (Cole *et al.*, 1994). A similar scheme was subsequently reported with the protein tyrosine kinase Fps (Wang *et al.*, 1996). There is significant interest in developing specific protein tyrosine kinase inhibitors as biological research tools and therapeutic

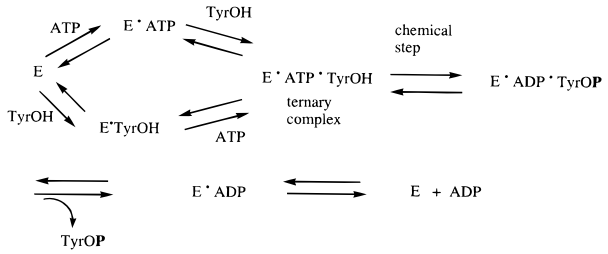

FIGURE 1: Ternary complex mechanism for Csk.

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[1] Abbreviations: Csk, C-terminal Src kinase; PTK, protein tyrosine kinase; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; poly(Glu,Tyr), random copolymer 4:1 poly(glutamate:tyrosine) molecular mass range 20−50 kDa from Sigma.

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agents (Levitzki & Gazit, 1995). It is anticipated that providing a detailed description of the catalytic mechanism of a protein tyrosine kinase should aid inhibitor development.

Our initial kinetic studies were carried out with Mn as the divalent cation, despite the expectation that Mg would play this role in vivo. Mn was employed because it had been reported to be 5−10-fold better in facilitating Csk-mediated phosphoryl transfer to both poly(Glu,Tyr) and casein (Okada & Nakagawa, 1988; Amrein *et al.*, 1995). The mechanistic basis for this difference was unclear and unexplored. In the serine-threonine kinase protein kinase A, Mn has been found to afford a slower turnover than Mg (Armstrong *et al.*, 1979; Grant & Adams, 1996).

In order to better understand Csk catalysis, a more thorough investigation of the role of the divalent ion appeared necessary. A systematic examination of catalytic effects of divalent ions has not been reported with a protein tyrosine kinase. In this study, it is shown that changing the divalent ion can alter the rate-determining step, probably by affecting the affinity of ADP for Csk. Furthermore, it is shown that though increasing ionic strength can weaken binding of poly-(Glu,Tyr) to Csk, it has minimal effect on k_{cat} or ATP affinity. An exploration of a range of divalent ions with Csk revealed that the magnitude of the k_{cat} thio effect was inversely correlated with metal thiophilicity. This supports the importance of γ-phosphoryl hydrogen bonding/salt bridging in the phosphoryl transfer transition state.

MATERIALS AND METHODS

General. Reagents were obtained from commercial sources (Cole *et al*., 1994) except where noted. Csk wild-type and D314E expression plasmids have been previously reported (Amrein *et al.*, 1995; Cole *et al.*, 1995). The GroESL expression plasmid was a gift from Dr. Paul Burn at Hoffmann-La Roche.

Peptide Synthesis. The peptide EDNEFTA was synthesized by Dr. Charles Dahl of Harvard Medical School by automated peptide synthesis. It was purified by C-18 reversed phase HPLC using acetonitrile/water/trifluoroacetic acid elution, and mass spectrometry showed the predicted molecular weight.

Protein Purification. Wild-type and D314E mutant Csk were prepared and purified as previously described (Amrein *et al.*, 1995) or using the modified method (Koegel *et al.*, 1994) described here. A culture containing 30 mL of *E. coli* strain M15 carrying the stably maintained plasmids pREP4groESL and pDS56csk (or pDS56/D314E csk) was grown in the presence of ampicillin (100 μg/mL) and kanamycin (25 μg/mL) in 2XYT media overnight at 37 °C in a shaker flask. This culture was used to inoculate 4 (3 L) Bellco flasks holding 500 mL of 2XYT, ampicillin (100 μg/mL), and kanamycin (25 μg/mL) (5 mL per flask) which were shaken at 37 °C. When the freshly inoculated cultures showed an OD_{595} of 0.8, they were induced with IPTG (final concentration 2 mM). After a further 4 h at 37 °C, the cells were harvested by centrifugation for 10 min at 3000*g* at 4 °C to afford 7 g of cell paste. The cell paste was frozen with liquid nitrogen and stored at −80 °C until use. It was thawed and resuspended to a final volume of 27 mL of lysis buffer (25 mM NaHEPES, pH 8.0, 150 mM NaCl, 5% glycerol, 5% ethylene glycol, 1 mM $MgSO_4$, 0.5 μg/mL DNase, 1 mM PMSF, and 10 μg/mL of each of the following: soybean trypsin inhibitor, antipain, pepstatin, leupeptin, and chymostatin). Cells were lysed by two passages through a French pressure cell (800 psi). The resultant suspension was treated with EDTA to a final concentration of 2 mM and then centrifuged for 30 min at 23000*g* at 4 °C. The supernatant was collected and then centrifuged at 100000*g* for 2 h. The supernatant (18 mL) from this spin was collected and was frozen with liquid N_2 and stored at −80 °C.

The thawed solution was dialyzed against 1 L of 25 mM HEPES, pH 7.7, for 2 h with one buffer exchange at 4 °C. The entire solution was then loaded onto a phosphotyrosine affinity column (15 × 2 cm) (Koegel *et al.*, 1994) which had been preequilibrated with 25 mM HEPES, pH 7.7. The flow rate was 1.5 mL/min, and 6 mL fractions were collected. After the column was washed with 100 mL of 25 mM HEPES, pH 7.7, a linear gradient was run up to 25 mM HEPES, pH 7.7−0.5 M NaCl over 300 mL. Both wild-type and D314E Csk eluted between approximately 150 and 300 mM NaCl. Fractions containing Csk were combined and dialyzed against 4 M $(NH_4)_2SO_4$ at 4 °C, and the flocculent white precipitate was collected by centrifugation for 45 min at 23000*g* at 4 °C. The pellet was resuspended in storage buffer (20 mM Tris−HOAc, pH 8.0) and dialyzed against storage buffer + 10% glycerol w/w and stored at −80 °C final concentration of 5−15 mg/L (49 mg total recovery). Protein concentration was determined using Bradford and UV assays. Using this purification scheme, both wild-type and D314E mutant Csk were homogeneous by SDS−PAGE and had a specific activity that was indistinguishable from material prepared using previously reported methods.

Kinetic Assays. Wild-type and mutant Csk assays were performed using the general methods described previously (Cole *et al.*, 1994). Standard conditions included 60 mM Tris-HCl, pH 7.4, 30 °C, 200 μg/mL bovine serum albumin (for Csk enzyme concentrations less than 500 nM), [γ-^{32}P]ATP or [^{35}S]ATPγS, 10 mM dithiothreitol, and optimal metal ion concentration. These were determined independently for each metal (Mg, Mn, Co, Ni), nucleotide (ATP, ATPγS), and enzyme type (wild type, D314E Csk) combination employing near-saturating substrate concentrations [poly-(Glu,Tyr), ATP greater than 3-fold K_m]. Optimal metal concentrations were 10−15 mM for $MgCl_2$, 2−6 mM for $MnCl_2$, 2−4 mM for $CoCl_2$, and 6−8 mM for $NiCl_2$ and were generally defined as the minimal metal concentration which would give at least 90% maximal rate. For Co and Ni, it was found necessary to use β-mercaptoethanol (2−4 mM) rather than dithiothreitol (10 mM) to avoid precipitation. It was shown that β-mercaptoethanol was about as effective (less than 10% difference in activity) as dithiothreitol for a Mn-containing reaction. Separation of phosphorylated poly(Glu,Tyr) was accomplished by using 10% SDS−PAGE as reported previously, and the radioactivity of gel slices was quantitated by liquid scintillation counting and corrected for counting efficiency using standards. Turnover numbers were measured in the linear range versus time, which was established for each metal−nucleotide combination independently under initial conditions (less than 10% turnover). All measurements were performed in duplicate.

K_m's were determined from rate measurements at a minimum of five substrate concentrations varied around K_m with the other substrate concentration fixed at near-saturation

Table 1: Steady-State Kinetic Parameters for Wild-Type and D314 E Csk Mutant Reactions as a Function of Divalent Ion with ATP (or ATPγS) and Poly(Glu,Tyr) as Substrates[a]

	ATP Reactions					
	wild-type Csk			D314E Csk		
metal	k_{cat} (min^{-1})	K_m(ATP)(μM)	K_m[poly(Glu,Tyr)](μg/mL)	k_{cat} (min^{-1})	K_m(ATP)(μM)	K_m[poly(Glu,Tyr)](μg/mL)
Mn	40 ± 5	12 ± 0.7	48 ± 2	0.0122 ± 0.001	34 ± 3	98 ± 8
Mg	108 ± 5	195 ± 19	654 ± 66	0.00527 ± 0.001	194 ± 10	1188 ± 92
Ni	15 ± 1	376 ± 55	218 ± 38	0.0011 ± 0.0001	−	−
Co	75 ± 5	216 ± 15	143 ± 11	0.005 ± 0.0005	378 ± 21	360 ± 42
	ATPγS Reactions					
	wild-type Csk			D314E Csk		
metal	k_{cat} (min^{-1})	K_m(ATPγS)(μM)	K_m[poly(Glu,Tyr)](μg/mL)	k_{cat} (min^{-1})	K_m(ATPγS)(μM)	K_m[poly(Glu,Tyr)](μg/mL)
Mn	2.1 ± 0.2	11 ± 1	51 ± 10	0.00262 ± 0.0003	<50	−
Mg	0.792 ± 0.01	88 ± 3	1690 ± 234	0.000313 ± 0.00004	−	−
Ni	6.8 ± 0.3	197 ± 69	<200	0.00072 ± 0.00006	−	−
Co	17 ± 1	159 ± 39	249 ± 39	0.0032 ± 0.001	−	−

[a] Assays were performed as described previously (Cole *et al.*, 1994) and under Materials and Methods. Data with wild-type Csk and Mn were taken from a previous report (Cole *et al.*, 1994). K_m data that display (−) in the D314E Csk reactions were unable to be measured because of signal-to-noise limitations, and the k_{cat}'s for these were approximated by using substrate concentrations which were fixed and saturating for the wild-type Csk reactions.

Table 2: Thio Effects on k_{cat} as a Function of Metal Thiophilicity for Wild-Type and D314E Csk[a]

metal	wild-type Csk thio effect	D314E Csk thio effect	preference for oxygen over sulfur
Mg	136	17	O>>>S
Mn	38	4.7	O>>S
Co	4.4	1.6	O>S
Ni	2.2	1.5	O≈S

[a] Thio effects are defined here as k_{cat}(ATP)/k_{cat}(ATPγS). Preferences for oxygen over sulfur based on analogous systems (Jaffe & Cohn, 1979; Pecoraro *et al.*, 1984). For related work on metal, amino acid interactions, see Martell and Smith (1974).

(>3K_m). Fits were performed using the nonlinear curve fit $V = V_mS/(K_m + S)$. The k_{cat}'s were obtained by measuring rates at saturation with both substrates and optimal metal concentrations. Measurements are displayed in Tables 1 and 2 and shown as ± standard error.

Inhibition experiments with peptide EDNEFTA were performed with the substrate and inhibitor concentrations displayed in Figure 3. Fits of competitive inhibition were carried out by fitting all of the data to the nonlinear curve fit of Kinetasyst according to the equation: $V = V_mS/[K_m(1 + I/K_{is}) + S]$. Fits of noncompetitive inhibition were performed by fitting all of the data to the nonlinear curve fit of Kinetasyst according to the equation: $V = V_mS/[K_m(1 + I/K_{is}) + S(1 + I/K_{ii})]$. Fits of uncompetitive inhibition were performed by fitting all of the data to the nonlinear curve fit of Kinetasyst according to the equation: $V = V_mS/[K_m + S(1 + I/K_{ii})]$. Comparisons of fits were carried out by visual inspection as well as by comparing the sum of squares of the residuals. 1/V vs 1/[ATP] with varying EDNEFTA concentrations (Figure 3b) gave the best fit to a noncompetitive model with the sum of squares of the residuals being 10-fold less than the uncompetitive model and 20-fold less than the competitive model. 1/V vs 1/[poly(Glu,Tyr)] with varying EDNEFTA concentrations (Figure 3a) fit about as well to a competitive model as a noncompetitive model (sum of the squares of the residuals residuals within 2-fold of each other). In the case of the noncompetitive fit, the EDNEFTA K_{ii} (41 ± 12 mM) was 6-fold larger than the EDNEFTA K_{is} (6.8 ± 1.0 mM). Therefore, the competitive model was invoked for Figure 3a since (a) the addition of a K_{ii} term did not lead to a significantly improved fit, (b) the K_{ii} in the noncompetitive model was implausibly large, and (c) there was good agreement between the K_m of EDNEYTA (Cole *et al.*, 1995) and the K_{is} of EDNEFTA in the competitive model. The kinetic constants ± standard error are shown in Figure 3.

Inhibition studies with ADP in the Mg reactions were carried out with independent plots of 1/V vs 1/[ATP] with varying ADP concentrations and 1/V vs 1/[poly(Glu,Tyr)] with varying ADP concentrations (data not shown). The ATP concentrations were varied over the range 25−750 μM, the poly(Glu,Tyr) concentrations were varied over the range 100−2400 μg/mL, and the ADP concentrations were varied over the range 0−250 μM. Each plot was also fit with competitive, noncompetitive, and uncompetitive models as described above. 1/V vs 1/[poly(Glu,Tyr)] with varying [ADP] was best fit with a noncompetitive model with the sum of squares of the residuals being 20-fold lower than in the competitive model and 40-fold lower than in the uncompetitive model. 1/V vs 1/[ATP] with varying ADP concentrations fit as well with a competitive model as with a noncompetitive model (sum of squares of the residuals within 2-fold of each other). In the case of the noncompetitive fit, the K_{ii} (724 ± 358 μM) was 20-fold larger than the K_{is} (35 ± 4 μM). Therefore, the competitive model was invoked for 1/V vs 1/[ATP] with varying [ADP] since (a) the addition of a K_{ii} term did not lead to a significantly improved fit, (b) the K_{ii} in the noncompetitive model was implausibly large, and (c) the pattern was similar to the Mn-dependent reactions (Cole *et al.*, 1994). The kinetic constants ± standard error are shown in the text.

Two substrate reaction kinetics with Mg, poly(Glu,Tyr), and ATP were performed with the substrate concentrations displayed in Figure 4. Fit to a sequential (ternary complex) mechanism was carried out by fitting all of the data to the nonlinear curve fit of Kinetasyst according to the equation: $V = V_mAB/[K_{ia}K_b + K_aB + K_bA + AB]$. Fit to a ping-pong mechanism (parallel line pattern) was carried out by fitting all of the data to the nonlinear curve fit of Kinetasyst according to the equation: $V = V_mAB/[K_aB + K_bA + AB]$. The sequential fit was superior based on a 13-fold lower sum of squares of the residuals and by visual inspection.

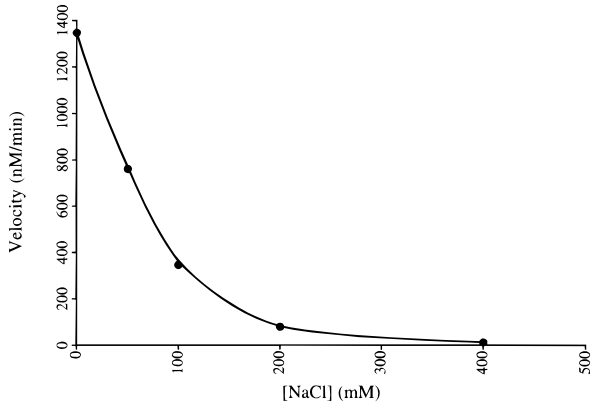

FIGURE 2: Rate of Csk-mediated poly(Glu,Tyr) phosphorylation vs NaCl concentration. Conditions: [$MnCl_2$], 2 mM; [poly(Glu,Tyr)], 200 μg/mL; [ATP], 100 μM; [Csk], 56 nM.

Kinetic constants ± standard error for the sequential mechanism are shown in Figure 4.

Sucrose microviscosity experiments were performed analogous to previously described methods (Cole *et al.*, 1994). Viscosity measurements were performed with a Canon−Fenske viscometer at 30 °C and referenced to buffer without added viscogen (sucrose w/v, relative viscosity; 16%, 1.55; 23%, 1.99; 30%, 2.5; 35%, 3.1). Data were fit to a linear curve fit, and slopes ± standard error are shown in Figure 5.

RESULTS

Ionic Strength. In order to be able to assess the effects of different divalent ion type on catalysis, an examination of the impact of ionic strength on Csk-mediated poly(Glu,Tyr) phosphorylation was needed. Thus, with fixed concentrations of ATP and poly(Glu,Tyr) and Mn, the effect of increasing salt concentration was determined. As can be seen (Figure 2), there was a profound rate loss with increasing NaCl concentration (from 0 to 400 mM). Similar effects were observed with KCl (data not shown). To better understand the effect of ionic strength on the reaction, measurements of K_m for ATP/poly(Glu,Tyr) and k_{cat} were undertaken at 50 mM NaCl. The K_m for poly(Glu,Tyr) was found to be 349 ± 38 μg/mL, the K_m for ATP 12.6 ± 0.5 μM, and the k_{cat} 35.3 ± 1.5 min^{-1}. In contrast, the 0 mM NaCl reaction showed a K_m for poly(Glu,Tyr) of 48 ± 2 μg/mL, a K_m for ATP of 12 ± 0.7 μM, and a k_{cat} of 40 ± 5 min^{-1} (Cole *et al.*, 1994). Thus increasing salt concentration has a dramatic effect on poly(Glu,Tyr) binding to Csk with minimal effects on nucleotide binding or catalysis. Given the polyionic nature of poly(Glu,Tyr), the altered K_m suggests either a salt-induced poly(Glu,Tyr) conformational effect or a weakening of a salt bridge interaction between poly(Glu,Tyr) and Csk. The lack of an ATP K_m effect and a k_{cat} effect argued against a global Csk active site structural change.

Having established an ionic strength-related poly(Glu,Tyr) K_m effect, we decided to reinvestigate the inhibition of Csk by a nonphosphorylatable peptide analog. Previously, short peptides were found to be relatively poor Csk inhibitors, and displayed nonlinear inhibition according to a Dixon plot (Cole *et al.*, 1994). Kinetic analysis in terms of discriminating random versus ordered substrate binding to Csk was hampered by the lack of a well-behaved dead-end peptide

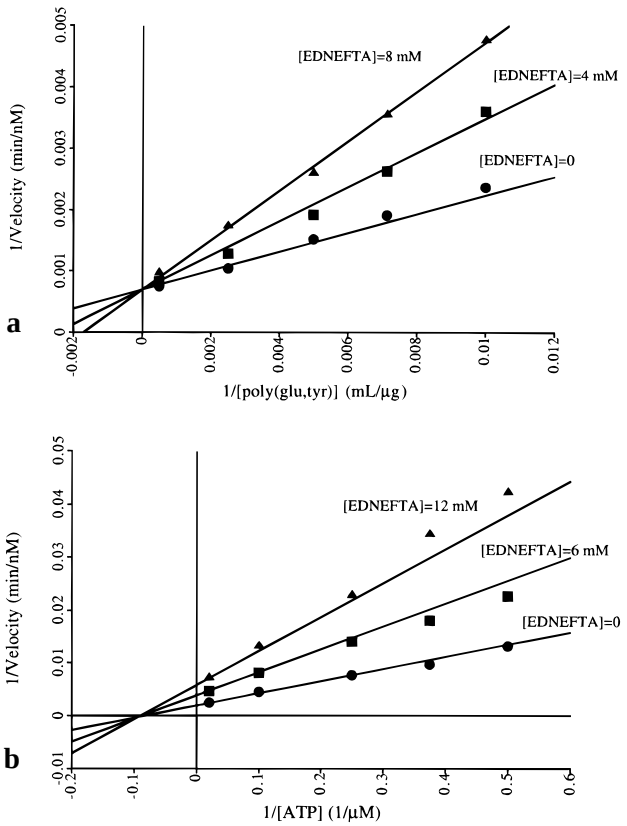

FIGURE 3: EDNEFTA as an inhibitor of Csk-catalyzed poly(Glu,Tyr) phosphorylation in the presence of 50 mM NaCl. (a) 1/velocity vs 1/[poly(Glu,Tyr)] with varying EDNEFTA concentrations (conditions: [$MnCl_2$], 2 mM; [ATP], 50 μM; [Csk], 80 nM); K_m[poly(Glu,Tyr)] = 221 ± 20 μg/mL, and K_{is} = 4.9 ± 0.7 mM. (b) 1/*V* vs 1/[ATP] with varying EDNEFTA concentrations (conditions: [$MnCl_2$], 2 mM; [poly(Glu,Tyr)], 100 μg/mL; [Csk], 80 nM); K_m(ATP) = 12 ± 1 μM, K_{is} = 6.7 ± 1.0 mM, and K_{ii} = 6.1 ± 0.5 mM.

inhibitor. It was postulated that the curvilinear inhibition could have been due to an ionic strength effect at high peptide concentration. We therefore repeated inhibition studies with EDNEFTA in the presence of 50 mM NaCl. It was anticipated that the ionic strength changes due to 50 mM NaCl would render inconsequential ionic strength effects due to peptide.

In the experiment with added NaCl, EDNEFTA was found to be a linear competitive inhibitor with respect to poly(Glu,Tyr) (K_{is} = 4.9 ± 0.7 mM) and a linear noncompetitive inhibitor with respect to ATP [K_{is} = 6.7 ± 1 mM, K_{ii} = 6.1 ± 0.5 mM, at 100 μg/mL poly(Glu,Tyr)] (Figure 3). These K_i data agree nicely with K_m (6.2 mM) data for the Csk substrate EDNEYTA (Cole *et al.*, 1995). Taken together with the previously obtained inhibition data, they now establish a random substrate binding, ternary complex mechanism for Csk.

WT Csk Kinetics with Mg. Reported studies with Csk demonstrated that Mg was significantly less efficient at catalyzing phosphoryl transfer compared to Mn. Confirming these findings, the rate of Csk phosphorylation with 200 μg/mL poly(Glu,Tyr) and 100 μM ATP was about 7 min^{-1} at optimal Mg concentration (10 mM), about 5-fold lower than the Mn-catalyzed reaction. A more detailed analysis revealed that the rate-reduction was not due to a k_{cat} effect. Indeed, the k_{cat} with Mg was determined to be about 108 ± 5 min^{-1}, 2.5-fold greater than the Mn turnover number. In contrast,

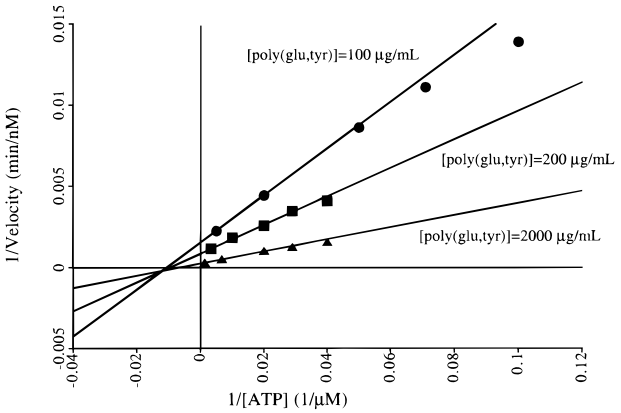

FIGURE 4: 1/velocity vs 1/[ATP] at varying poly(Glu,Tyr) concentrations with Mg (conditions: [$MgCl_2$], 10 mM; [Csk], 84 nM); K_m(ATP) = 186 ± 13 μM; K_m(poly(Glu,Tyr)] = 800 ± 89 μg/mL, and K_i(ATP) = 83 ± 16 μM.

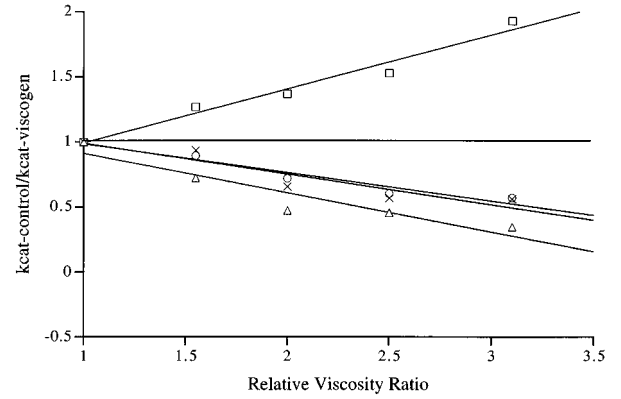

FIGURE 5: Sucrose microviscosity effect on k_{cat}: k_{cat}(control)/k_{cat}(viscogen) vs relative viscosity ratio for wild-type Csk using ATP, ATPγS, Mn, and Mg. Mn data taken from previously reported work (Cole *et al.*, 1994). General conditions: optimal metal concentration, fixed and saturating (>3-fold K_m) poly(Glu,Tyr) and nucleotide concentrations. (□) Mn/ATP, slope = +0.42 ± 0.04; (×) Mg/ATP, slope = −0.24 ± 0.06; (○) Mn/ATPγS, slope = −0.22 ± 0.03; (△) Mg/ATPγS, slope = −0.30 ± 0.06. Horizontal line at k_{cat}(control)/k_{cat}(viscogen) = 1 is theoretical and corresponds to the absence of viscogenic effects.

the K_m's were 654 ± 66 μg/mL for poly(Glu,Tyr) and 195 ± 19 μM for ATP, 15−20-fold higher than for the Mn case (see Table 1). Mechanistic studies revealed that a ternary complex mechanism was obeyed, with an intersecting line pattern observed (Figure 4). Furthermore, ADP was a noncompetitive inhibitor with respect to poly(Glu,Tyr) (K_{is} = 49 ± 4 μM, K_{ii} = 80 ± 4 μM, at a fixed ATP concentration of 150 μM) and competitive with respect to ATP (K_{is} = 33 ± 2 μM) (data not shown). This pattern was also similar to the Mn case (Cole *et al.*, 1994). The larger K_{is} in the Mg case [33 ± 2 μM vs 1.6 ± 0.2 μM in the Mn case (Cole *et al.*, 1994)] raised the possibility that ADP release would no longer be rate-determining in the Mg reaction. Indeed, a viscosity experiment with the microviscogen sucrose supported this expectation. Thus, the slope (−0.24 ± 0.06) of a plot of k_{cat}(control)/k_{cat}(viscogen) vs microviscosity was similar to previously determined nondiffusional rate-limited Csk-catalyzed reactions (WT Csk/Mn/ATPγS, slope = −0.22 ± 0.03; and D314E Csk/Mg/ATP, slope = −0.28 ± 0.06) (see Figure 5) (Cole *et al.*, 1994, 1995). Furthermore, ATPγS, which led to a significant reduction in k_{cat} (136-fold) in the Mg-dependent reaction (see Table 2), exhibited a k_{cat}(control)/k_{cat}(viscogen) vs relative microviscosity plot (WT Csk/Mg/ATPγS) with a very similar slope, slope = −0.30 ± 0.06 (see Figure 5). Note that the modestly negative slopes (−0.2 to −0.3) imply a mild rate-acceleration by sucrose in cases where product release is not rate-determining. This nonspecific viscogen effect with the Mg- and Mn-dependent reactions is general for Csk and has been discussed previously (Cole *et al.*, 1994). The negative slopes may be subtracted from the corresponding cases where slopes are positive to get a net effect (Blacklow *et al.*, 1988; Cole *et al.*, 1994). Taken together, these findings strongly argue in favor of the chemical step in both the MgATP and MnATPγS reactions being fully rate-determining, in contrast to the WT Csk/MnATP case, where ADP product release appeared to be partially rate-determining. The K_m's in the MgATPγS reaction for poly(Glu,Tyr) and nucleotide were within 3-fold of the corresponding K_m's of the MgATP reaction.

D314E Csk with Mg. Previously, it was shown that the Csk mutant D314E had greatly reduced catalytic efficiency with respect to the wild-type Csk reaction, down by some 10^3-10^4 fold (Cole *et al.*, 1995).[2] The k_{cat}, chemical step thio effect [k_{cat}(ATP)/k_{cat}(ATPγS)] in the D314E Csk mutant reaction was found to be smaller (4.7) compared to the wild-type (38) Csk reaction (Cole *et al.*, 1995). These results led to the proposal that the phosphoryl transfer transition state in the mutant reaction may have suffered from the lack of a strong hydrogen bond/salt bridge between one of the γ-phosphoryl oxygens and an active site H-bond donor (or metal). To attempt to probe this proposal, steady-state kinetics with D314E were investigated with Mg. As in the Mn reaction, there was a large k_{cat} reduction (20 000-fold) at optimal Mg concentration (15 mM) with minimal K_m effects (less than a factor of 2) for ATP and poly(Glu,Tyr) in the Mg reaction (see Table 1). The k_{cat} thio effect in the D314E Csk mutant reaction was determined to be 17 (see Table 2). Compared to the wild-type Csk thio effect with Mg (136), this represents about an 8-fold reduction (see Table 2). Such a thio effect reduction on going from wild-type to mutant Csk reactions is similar to the pattern observed in the Mn reactions.

Csk Catalysis with Co and Ni. The metals Mg and Mn have strong preferences for binding oxygen over sulfur in nucleotide complexes (Pecoraro *et al.*, 1984). In order to further evaluate the role of the divalent ion in the Csk reaction, metals which display a greater preference for sulfur were screened as potential substitutes in catalysis. Cadmium, which actually will bind sulfur ligands more tightly than oxygen (Pecoraro *et al.*, 1984), was tested and was not able to sustain Csk-mediated phosphoryl transfer (less than 1% of the rate of the Mn reaction). In contrast, Ni and Co (Jaffe & Cohn, 1979) were both able to facilitate Csk-mediated phosphoryl transfer at enzymatically significant rates. The k_{cat} for Csk kinase activity with Ni (optimal Ni concentration = 6 mM) was about 5-fold lower than with Mg, and Co

[2] The rate of the D314E mutant reported previously (Cole *et al.*, 1995) was 2−3-fold lower than reported here. This difference comes from the fact that the same Mn concentration (2 mM) was used in both wild-type and D314E mutant Csk assays in the previous work whereas the optimal Mn concentration (6 mM) for D314E Csk was used here. The K_m's of ATP and poly(Glu,Tyr) in the D41E Csk reaction with 6 mM Mn were unchanged. There was no significant change in the k_{cat} thio effect in the mutant reaction using 6 mM Mn, as both ATP and ATPγS reactions were similarly affected.

(optimal Co concentration = 2.5 mM) was within 2-fold of the Mg reaction. ATP K_m's in the Ni and Co reactions were within a factor of 2 compared to the Mg reaction, although poly(Glu,Tyr) K_m's were about 3.5-fold lower (see Table 1). Interestingly, the k_{cat} thio effects with wild-type Csk were more than an order of magnitude smaller with Co (4.4) and Ni (2.2) compared with Mg (136) and Mn (38) (see Table 2). Likewise, the thio effects with D314E Csk with these metals were also reduced (<2). It should be mentioned that the K_m's for the ATP vs the ATPγS reactions for Ni and Co were within 3-fold, arguing against major active site structural differences between the two nucleotide substrate reactions. That ADP release was not rate-limiting in the Ni and Co reactions is supported by (i) the relatively high K_i's of ADP in the Ni (72 ± 14 μM) and Co (55 ± 6 μM) reactions[3] and (ii) microviscosity studies [slopes of k_{cat}(control)/k_{cat}(viscogen) vs relative viscosity for Ni and Co were in the range −0.2 to +0.09 for both ATP and ATPγS wild-type Csk reactions; data not shown].

DISCUSSION

A detailed understanding of enzymatic phosphoryl transfer in protein tyrosine kinase reactions has been elusive. In part, this has stemmed from the inability to obtain milligram quantities of pure, well-behaved, nontruncated, nonchimeric enzymes. With many of these enzymes, autophosphorylation occurs at a significant rate and leads to altered enzyme behavior during turnover, making mechanistic studies complex. We have previously shown that protein tyrosine kinase Csk is suitable for such studies because of its straightforward overproduction and purification and low rate of background autophosphorylation. In this report, we describe an investigation of ionic strength, divalent ion specificity, and thio effects on wild-type and D314E mutant Csk.

It has been shown that the addition of monovalent salts such as 50 mM NaCl had a significant effect on raising the K_m of poly(Glu,Tyr) without affecting the k_{cat} or the K_m of ATP. The value of this observation relates not only to characterizing the poly(Glu,Tyr)−Csk interaction. It also has allowed the analysis of a relatively weak dead-end peptide inhibitor which in the presence of high salt displays linear inhibition patterns. The dead-end peptide analog EDNEFTA was a competitive inhibitor of poly(Glu,Tyr) and a noncompetitive inhibitor of ATP. These results in conjunction with previously reported findings (Cole *et al.*, 1994) now support a random ternary complex mechanism. This pattern is similar to that recently reported for Src (Boerner *et al.*, 1995). The salt sensitivity of poly(Glu,Tyr)−Csk binding should also serve as a warning to other workers in the field who may be comparing different levels of activity under varying conditions with poly(Glu,Tyr) and protein tyrosine kinases.

The large K_m variations as a function of divalent ion type also serve as a caution to those engaged in biochemical signal transduction studies. Since the protein tyrosine kinase field lacks standardization in terms of divalent ion type and concentration employed, in vitro signal transduction studies of activation and inhibition may be difficult to compare. As noted in this work, the nature of the rate-limiting step (ADP release versus the chemical step) can be affected by altering the divalent ion (Mn vs Mg). Related effects have also been seen previously in the protein serine/threonine kinase protein kinase A (Adams & Taylor, 1993). As is clear from our current Csk work, the alterations in the kinetic parameters (K_m of ATP and k_{cat}) are not due primarily to ionic strength effects. The ionic strength changes caused by different divalent ions should not be as pronounced as those due to 50 mM NaCl, where ATP K_m and k_{cat} effects were not observed. Rather, MgADP most likely has lower Csk active site affinity compared to MnADP. Accelerated MgADP product release could then manifest itself in a fully rate-determining chemical step with this physiologically relevant cation.

An investigation of the enzyme activity of D314E Csk mutant as a function of divalent ion types was also undertaken. Based on sequence homology, Asp-314 has been proposed to function as a catalytic base (Taylor *et al.*, 1995). Recent studies have cast doubt on this assignment (Cole *et al.*, 1995). While the D314E Csk mutant was found to be significantly catalytically impaired, the mechanistic basis for this impairment was not felt to be due to an inability to deprotonate the substrate tyrosine hydroxyl. More recently, a trifluorotyrosine-containing peptide, which should already be deprotonated at neutral pH, was found to be approximately as poor a kinase substrate with D314E (>1000-fold reduced from wild type) as the tyrosine-containing peptide (unpublished data).

The catalytic activity in the mutant enzyme reactions was reduced by about 10 000-fold for all of the divalent ions examined. Moreover, with the exception of Mn, the optimal metal concentrations between D314E mutant and wild-type Csk were very similar for both. The lack of a significant rate difference among the various divalent ions in the mutant enzyme reactions is evidence that a similar defect is present in all of the metal-dependent D314E mutant reactions.

To better define the implications of the thio effect observed in our previous work (Cole *et al.*, 1995), and the importance of γ-phosphoryl hydrogen bonding in the transition state of this protein tyrosine kinase reaction, we explored the effect of other divalent cations in the wild-type and D314E mutant Csk reactions. A range of metals with varying thiophilicities was investigated. Thus, Mg has a greater preference for oxygen over sulfur compared to Mg whereas Co and Ni have increased relative affinities toward sulfur (Jaffe & Cohn, 1979; Pecoraro *et al.*, 1984). The k_{cat}'s with these different metals in the ATP reactions were within about a factor of 7 of each other, suggesting that the overall catalytic mechanism was not greatly affected by the choice of metal. Moreover, based on ADP K_i's and viscosity effects, the chemical step was likely fully rate-limiting in all cases except the Mn/wild-type Csk reaction.

Mg showed a rather pronounced thio effect (136) in the wild-type reaction, with only 2−3-fold K_m effects. In the D314E mutant reaction, the k_{cat} thio effect with Mg was significantly reduced (17), as in the Mn case. These results are consistent with the interpretation of a γ-phosphoryl hydrogen bond change between wild-type and D314E Csk. In contrast, the Co and Ni reactions showed 30−60-fold reduced k_{cat} thio effects (4.4 for Co, 2.2 for Ni) compared to Mg as well as small effects on K_m (Table 2). The D314E mutant thio effects were only slightly smaller than the wild-type reaction. In analyzing the basis for the thio effect

[3] These K_i's for ADP were obtained using a Dixon plot ($1/V$ vs I), assuming competitive inhibition vs ATP, at fixed substrate concentrations.

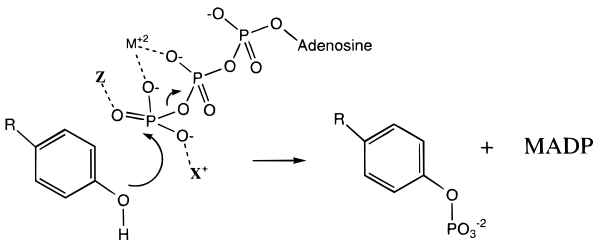

FIGURE 6: γ-Phosphoryl hydrogen bonding/ionic interaction in phosphoryl transfer catalyzed by Csk. X and Z are hypothetical, as yet unidentified hydrogen bonding or salt bridging amino acid side chains or active site metal ions. Evidence for their importance comes from thio effect experiments. They appear to be at least partially disrupted in the D314E Csk-catalyzed reaction.

dependence on metal type, it is helpful to consider that there is an equilibrium between two types of metal−ATPγS complexes: M−O (divalent ion bound to β- and γ-nonbridging phosphoryl oxygens of ATPγS) and M−S (divalent ion bound to β-oxygen and γ-sulfur). With Mg and Mn, the equilibrium between M−O and M−S lies heavily toward M−O. Mg−O/ATPγS appears to bind about as well to the Csk active site as MgATP but is 136-fold poorer a substrate. In contrast, Ni−S/ATPγS, expected to be in 1:1 equilibrium with Ni−O, is processed by Csk at a comparable rate to NiATP. It seems likely that Mg−S would be an efficient substrate but is minimally populated in the equilibrium, and therefore does not contribute significantly to enzyme turnover. Moreover, Ni−O/ATPγS is probably a poor substrate.

Overall, the simplest interpretation is that M−S/ATPγS complexes faithfully mimic the reactivity of MATP complexes whereas M−O/ATPγS complexes do not.[4] M−O/ATPγS complexes are poor presumably because of an inability of the γ-phosphoryl sulfur to form a stable hydrogen bond/salt bridge with an active site partner. The M−S/ATPγS complexes have the standard free γ-phosphoryl oxygen atoms able to form hydrogen bonds analogous to MATP. It is deduced that hydrogen bonding/salt bridging to the γ-phosphoryl oxygens is an important feature of catalysis (see Figure 6).

One of the major unanswered questions about enzyme-catalyzed phosphoryl transfer is the degree of associative or dissociative character in the transition state (Knowles, 1980). In an associative transition state, a relatively tightly packed pentacoordinate phosphorus is envisaged, with a large proportion of nucleophile to phosphorus and leaving group to phosphorus bond formation (Figure 7). In contrast, a dissociative transition state, analogous to an S_N1 reaction in organic chemistry, involves formation of a metaphosphate-like species (Figure 7). Such a species features substantial leaving group departure prior to nucleophilic attack. In mechanistic analyses of nonenzymatic phosphate monoester, phosphoryl transfer reactions, dissociative transition states are strongly supported (Admiraal & Herschlag, 1995; Herschlag & Jencks, 1990; Jencks, 1969; Hengge *et al.*, 1994). Inverse nonbridging thio effects [k_{cat}(O)/k_{cat}(S)] also support dissociative mechanisms (Breslow & Katz, 1968). Sulfur, less electronegative than oxygen, should be better equipped

[4] Other factors such as metal electrophilicity (Gupta *et al.*, 1976), coordination geometry (Huheey, 1983), and changes in atomic radii (Huheey, 1983) could contribute to rate effects for the various divalent ion reactions. None of these other parameters displays a simple relationship with the k_{cat} thio effects reported here.

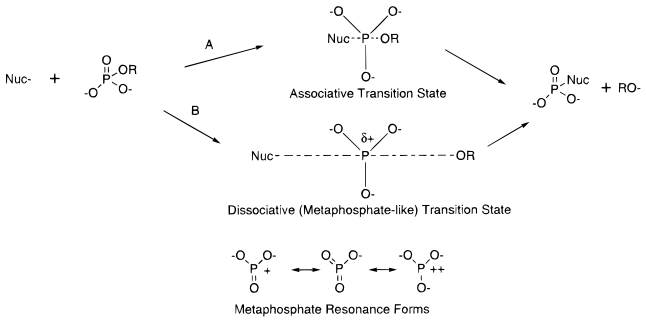

FIGURE 7: Transition states of phosphoryl transfer.

to stabilize an electropositive phosphorus as found in a metaphosphate. In comparison, phosphate diesters and triesters appear to show more associative character in nonenzymatic phosphoryl transfer reactions (Herschlag *et al.*, 1991; Herschlag, 1994). In these cases, thio effects are typically 4−11 and 10−200 for diesters and triesters, respectively, suggesting that the greater electronegativity of oxygen activates the phosphate toward attack by the nucleophile. The situation with enzymatic phosphate monoester reactions is less clear cut (Herschlag & Jencks, 1990), although the most rigorous studies have appeared to support dissociative transition states (Hollfelder & Herschlag, 1995; Hengge *et al.*, 1994, 1995; Weiss & Cleland, 1989; Jones *et al.*, 1991).

Hydrogen bonding of the γ-phosphoryl group, which has been shown to be important in Csk catalysis as described above, could be expected to be important in a dissociative mechanism for precise orientation of ATP in the ternary complex. Hydrogen bonding could also be expected to be important in an associative mechanism for orientation as well as activation of the phosphate toward attack by a nucleophile. Very strong γ-phosphoryl hydrogen bonding would certainly destabilize metaphosphate generation, but there is no evidence that γ-phosphoryl hydrogen bonding is very strong or that it occurs after the leaving group has departed. The lack of a large thio effect particularly in the Ni reaction (2-fold) argues against a highly associative transition state (expected thio effect greater than 10). As mentioned, sulfur is thought to destabilize an associative transition state because it is less electronegative than oxygen. The net electronegativity of the NiATPγS complex should be lower than the NiATP complex. Therefore, the thio effect results do not argue in favor of an associative transition state and if anything point to a more dissociative one (the lack of an inverse thio effect in the Csk reaction does not disprove a dissociative mechanism since even in the nickel reaction, active site geometry may be perturbed by sulfur). Moreover, the observation that a catalytic base most likely does not act early in creating a phenoxide (the absence of nucleophile activation) (Cole *et al.*, 1995, and above) and the lack of large solvent isotope effects (Cole *et al.*, 1994, 1995) are also consistent with a dissociative transition state. In summary, there is no significant evidence that Csk alters the usual dissociative course of nonenzymatic phosphate monoester transfer reactions.

Thio effect results with D314E Csk are also noteworthy. There is a decreased k_{cat} thio effect for all metals examined in the D314E Csk reaction compared to the wild-type Csk reaction. These findings suggest that a defect in the mutant enzyme is a decreased tendency to hydrogen bond the

γ-phosphoryl group in the transition state. The lack of this hydrogen bonding could cause misalignment of ATP in the active site, impairing its reaction with tyrosine.

Finally, it should also be mentioned that cell signal transduction studies have benefited from the use of thiophosphoryl tyrosine-containing proteins as "long-lived phosphotyrosine" analogs (Zhao, 1996; Tegge, 1994; Tonks *et al.*, 1988). The increased in vivo stability of thiophosphates is presumed to be related to a sluggish rate of phosphotyrosine phosphatase hydrolysis compared to the corresponding phosphates. The enzymatic preparation of tyrosine thiophosphates can be difficult. As such, the finding that Ni and Co can accelerate the rate of thiophosphoryl transfer (4−20-fold greater than Mn and Mg) catalyzed by a protein tyrosine kinase with ATPγS and a peptide substrate may have practical synthetic utility.

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