Substitution Studies of the Second Divalent Metal Cation Requirement of Protein Tyrosine Kinase CSK†

Gongqin Sun and Raymond J. A. Budde*

Department of Neuro-Oncology, Box 316, University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030

Received November 25, 1998; Revised Manuscript Received March 8, 1999

ABSTRACT: In addition to a magnesium ion needed to form the ATP−Mg complex, we have previously determined that at least one more free Mg^{2+} ion is essential for the activation of the protein tyrosine kinase, Csk [Sun, G., and Budde, R. J. A. (1997) *Biochemistry 36,* 2139−2146]. In this paper, we report that several divalent metal cations, such as Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} bind to the second Mg^{2+}-binding site of Csk with up to 13200-fold higher affinity than Mg^{2+}. This finding enabled us to substitute the free Mg^{2+} at this site with Mn^{2+}, Co^{2+}, Ni^{2+}, or Zn^{2+} while keeping ATP saturated with Mg^{2+} to study the role of the free metal cation in Csk catalysis. Substitution by these divalent metal cations resulted in varied levels of Csk activity, with Mn^{2+} even more effective than Mg^{2+}. Co^{2+} and Ni^{2+} supports reduced levels of Csk activity compared to Mg^{2+}. Zn^{2+} has the highest affinity for the second Mg^{2+}-binding site of Csk at 0.65 μM, but supports no kinase activity, acting as a dead-end inhibitor. The inhibition by Zn^{2+} is reversible and competitive against free Mg^{2+}, noncompetitive against ATP−Mg, and mixed against the phosphate accepting substrate, polyE_4Y, significantly increasing the affinity for this substrate. Substitution of the free Mg^{2+} with Mn^{2+}, Co^{2+}, or Ni^{2+} also results in lower K_m values for the peptide substrate. These results suggest that the divalent metal activator is an important element in determining the affinity between Csk and the phosphate-accepting substrate.

Protein tyrosine kinases (PTK)[1] are important enzymes in cellular signal tranduction that control cell growth and differentiation (*1*). Activation of these enzymes by disruption of their regulation, overexpression, or mutation often leads to transformation of the host cells. For this reason, many PTKs, such as growth factor receptors and members of the Src family, are key targets for drug discovery in proliferative diseases such as cancer (*2*, *3*). Csk is a soluble PTK structurally similar to members of the Src family. It regulates PTKs of the Src family by phosphorylation of a Tyr residue near their C-termini, which results in their inactivation (*4*, *5*).

Most enzymes that catalyze displacement at phosphoric esters (kinases and phosphatases) along with enzymes that carry out the synthesis and degradation of phosphodiester bonds (RNA and DNA polymerases and restriction endonucleases) have a requirement for two metal ions. Metal ions can act as essential cofactors through an indirect structural mode in which they facilitate the formation of the transition state, release of products, or through a mechanistic mode in which they or a water molecule bound to the metal play a direct role in catalysis. PTKs catalyze a bisubstrate phosphoryl-transfer reaction, with ATP−Mg complex as the phosphate donating substrate and a tyrosyl hydroxyl group in an appropriate protein structure as the phosphate acceptor. For maximal kinase activity, PTKs require Mg^{2+} at concentrations much higher than necessary to saturate ATP, suggesting an additional role for the divalent metal cation (DMC) in the catalytic activity of this class of enzymes (*6*, *7*). We recently demonstrated that, in addition to using Mg^{2+} to form ATP−Mg, PTKs also require an additional Mg^{2+} as an essential activator (*8*, *9*). Kinetic evidence suggests that the second Mg^{2+} appears to activate PTKs by one of two mechanisms: increasing the affinity for ATP−Mg or increasing the catalytic efficiency without affecting the binding of ATP−Mg. IRK and v-Fps utilize the first mechanism (*10*, *11*) while Src and Csk utilize the second (*8*). Both mechanisms are still poorly understood. Since the second Mg^{2+} appears to be a key component in their catalytic machinery, understanding its structural environment and how it activates PTKs will provide an important probe to the catalysis of this class of enzymes. Such information will also be useful for drug discovery efforts directed at the active site of PTKs. Furthermore, there is increasing evidence that the concentration of free Mg^{2+} in cells is tightly regulated (*12*, *13*) and that Mg^{2+} influx is an important step in the stimulatory action of growth factors (*14*). The sensitivity of PTK activity to Mg^{2+} fluctuation (*6*, *10*) may provide a mechanism for extracellular signals to modulate the activity of these enzymes (*8*). For these reasons, it is important that we further understand the interaction of free Mg^{2+} with PTKs and how that interaction accelerates the phosphorylation reaction. For the convenience of presentation in this paper, we designate the Mg^{2+} that binds to ATP as M1 and the free Mg^{2+} that serves as an essential activator as M2.

† This work was supported by NIH Grant CA53617.

* To whom correspondence should be addressed. Phone: (713) 792−3780. E-mail: rjabudde@mdanderson.org.

[1] Abbreviations: DMC, divalent metal cation; IRK, insulin receptor tyrosine kinase; PTK, protein tyrosine kinase.

10.1021/bi982793w CCC: $18.00
Published on Web 04/08/1999

The coordination and function of M1 in PTK-catalyzed phosphoryl transfer reactions are better understood than those of M2. The only direct structural information on M2 is from the recently published crystal structure of activated insulin receptor kinase (IRK), which has two Mg^{2+} ions coordinated within the active site (*15*). M1 is directly bound to the β- and γ-phosphate oxygens of ATP while M2 is bound to the β-phosphate oxygen of ATP and two amino acid residues, E1047 and D1150, either directly or indirectly through water molecules. This finding confirmed our earlier kinetic studies on the requirement of two Mg^{2+} by PTKs and the interaction of M2 with ATP is consistent with the evidence that the second Mg^{2+} activates IRK by increasing its affinity for ATP−Mg (*6*, *10*). But this structural information with regard to M2 is unlikely to extend to PTKs utilizing an apparently different mechanism of M2 activation, such as Csk and the Src family.

In this communication, we probed the M2-binding site of Csk and found that several DMCs interact with this site with much higher affinity than Mg^{2+}. Since they have equal affinity for ATP, this finding enabled us to substitute M2 with different DMCs and keep Mg^{2+} bound to ATP as M1. Such studies provided further insight into the mechanism of PTK activation by M2 and potentially important clues for rational drug design targeting the active sites of these enzymes.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents. Csk was expressed as a fusion protein with glutathione *S*-transferase (*16*) and streptag (*17*) in *Escherichia coli* using a dual tag expression system (*18*). The two-step affinity purification and removal of GST was carried out as described (*18*). Csk used in this study was tagged with streptag, a 10 amino acid peptide, and purified to apparent homogeneity. Divalent metal cations in the form of chloride or acetate salts were purchased from Sigma-Aldrich Chemical Co. All other chemicals were purchased from Sigma Chemical Co.

Kinase Assays. For determination of PTK activity, we measured the phosphorylation of $polyE_4Y$ using the acid precipitation onto filter paper assay. The standard phosphorylation reaction contained 0.2 mM [γ-^{32}P]ATP (600 dpm $pmol^{-1}$), 1 mg mL^{-1} $polyE_4Y$, 6 mM $MgCl_2$, 75 mM EPPS-NaOH (pH 8.0), 5% glycerol, 0.002% β-mercaptoethanol, and appropriate amount of enzyme. The appropriate amount of enzyme transferred approximately 200 pmol of phosphate in 30 min under these conditions. When the K_m and V_{max} values were determined with regard to one substrate, the concentration of that substrate in the assays was varied between 20 and 200 μM (in the case of ATP) or mg mL^{-1} (in the case of $polyE_4Y$) while the concentration of the other reaction components remained constant.

Reversibility of Inhibition of PTKs by Divalent Metal Cations. To determine if the DMC inactivation or inhibition of PTK activity is reversible, the conditions that reverse the inactivation/inhibition were determined. For these experiments, phosphorylation reactions that are in the presence or absence of a DMC were started, and the time course of phosphorylation was monitored. After a linear reaction rate was established, EDTA was added to the reactions. The reaction rate after the addition of EDTA was determined. A comparison of the reaction rates indicates if EDTA reversed the inactivation or inhibition by the DMCs.

Table 1: Kinetic Values for Divalent Metal Cations that Support Csk Catalytic Activity

metal cation	% Csk activity[a]	$K_{0.5}$[b] (mM)	IC_{50}[c] (μM)	log $K_{d(ATP)}$[d]	$K_{m\ (ATP-DMC)}$ (μM)[e]
Mg^{2+}	100	2.3		4.86	66
Mn^{2+}	190	0.5	25	5.00	27
Co^{2+}	14	0.4	8	4.62	33
Ni^{2+}	7	0.5	13	4.54	82

[a] Csk activity was determined in the presence of 1 mM DMC and 0.2 mM ATP. The turn-over numbers in the presence of 1 mM Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} are 0.5, 0.95, 0.07, and 0.035 min^{-1}, respectively. [b] $K_{0.5}$ values were defined as the concentration of a given divalent metal cation that produced 50% of the optimal Csk activity for that divalent metal cation. [c] IC_{50} values are the concentrations of the divalent metal cations that resulted in 50% of the maximal inhibition of Mg^{2+}-supported Csk activity that was observed. For Mn^{2+}, the value is the concentration that activated Mg^{2+}-supported Csk activity 50% of the maximal activation observed. These inhibitory or stimulatory effects on Csk catalytic were determined in the presence of 6 mM $MgCl_2$ and 0.2 mM ATP. [d] Taken from ref *21*. [e] Determined in the presence of 1 mM DMC.

RESULTS

Mn^{2+}, Co^{2+}, and Ni^{2+} Support Csk Catalytic Activity. In addition to magnesium, Mn^{2+}, Co^{2+}, and Ni^{2+} can also support Csk catalytic activity (*19*). Our survey of 15 commercially available DMCs confirmed these results and did not reveal any other metals that are able to support Csk activity. Unlike Mg^{2+} which has a $K_{0.5}$ value for Csk of 2.3 mM, Mn^{2+}, Co^{2+}, and Ni^{2+} had much lower $K_{0.5}$ values of 0.4−0.5 mM (Table 1). The optimal concentration of Mg^{2+} is 5−10 mM, while optimal Csk activity was achieved at 1−2 mM Mn^{2+}, Co^{2+}, or Ni^{2+}, with higher concentrations resulting in reduced activity. At 1 mM DMC, the highest Csk activity is obtained with Mn^{2+}, while Ni^{2+} gives the lowest, in the following order: Mn^{2+} (190%) > Mg^{2+} (100%) > Co^{2+} (14%) > Ni^{2+} (7%). The higher Csk activity supported by Mn^{2+} is consistent with a previous report (*20*), yet the optimal concentrations of Co^{2+} and Ni^{2+} are lower than previously reported values of 2.5 and 6 mM (*19*), respectively. These higher values are probably the result of using higher concentration of reducing agent, which chelates divalent metal cations and thus lowers their effective concentrations in solution.

Since Mg^{2+} plays two roles that are essential for the catalytic activity of Csk as demonstrated previously (*8*), the ability of Mn^{2+}, Co^{2+}, and Ni^{2+} to support Csk activity in the absence of Mg^{2+} indicates that they can substitute for Mg^{2+} at both M1 and M2. The different levels of catalytic activity supported by these DMCs are composite measures of their ability to serve as M1 and M2. Kinetic analyses indicate that the lower activity in the presence of Co^{2+} and Ni^{2+} is not the result of lower affinity for ATP−Co or ATP−Ni complexes compared to ATP−Mg (Table 1; K_m). Since Mn^{2+}, Co^{2+}, and Ni^{2+} have binding constants for ATP that are similar to Mg^{2+}-binding ATP (Table 1; K_d), the lower $K_{0.5}$ values indicate that they have higher affinity than Mg^{2+} for the M2-binding site of Csk. This possibility was further investigated by determining the effect of low concentrations (up to 0.2 mM) of these DMCs on Csk activity assayed with

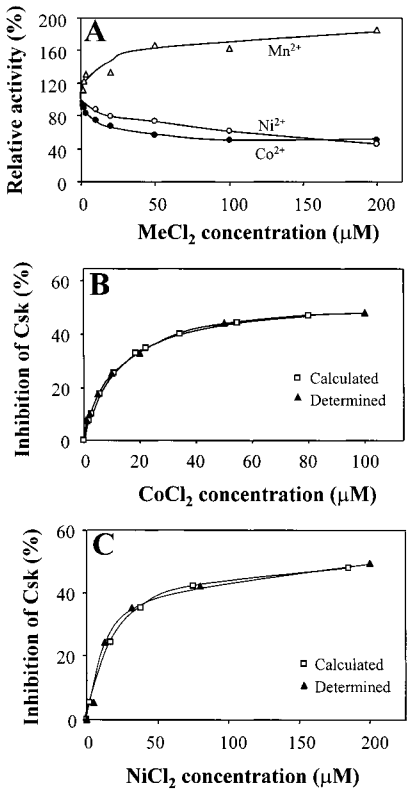


FIGURE 1: Effect of Mn^{2+}, Co^{2+}, and Ni^{2+} on Mg^{2+}-supported Csk kinase activity. (A) Concentration-dependent effect of Mn^{2+} (Δ), Co^{2+} (●), and Ni^{2+} (○) on Csk kinase activity. In the Csk activity assay, 6 mM Mg^{2+} and 0.2 mM ATP are present. Other conditions are the same as in the standard assay described in the Experimental Procedures. (B) Comparison between Co^{2+} inhibition of Csk with a calculated inhibition curve. The calculation was conducted using Microsoft Excel. Various curves were generated using assumed K_d values and maximal levels of inhibition. The calculated curve that best fits the determined curve as shown has a K_d of 12 μM and maximal level of inhibition of 54%. (C) Comparison between Ni^{2+} inhibition of Csk with a calculated inhibition curve. Curve fitting was conducted the same way as in panel B. A K_d value of 20 μM and maximal level of inhibition of 53% were derived.

6 mM Mg^{2+} and 0.2 mM ATP. The greater than 30-fold difference in concentrations between Mg^{2+} and other DMCs ensures that the majority of ATP is bound to Mg^{2+} and the effect of the other DMCs would be through interaction with presumably the M2-binding site. Under these conditions, Co^{2+} and Ni^{2+} were inhibitory, while Mn^{2+} stimulated Csk catalytic activity (Figure 1A). The inhibition by Co^{2+} and Ni^{2+} was partial, reaching plateaus of approximately 50% at 100 μM Co^{2+} and at 200 μM Ni^{2+}. The lack of complete inhibition by Co^{2+} and Ni^{2+} indicates that when this site is saturated with Co^{2+} or Ni^{2+}, Csk is less active compared to when this site is occupied by Mg^{2+}. The parabolic appearances of the inhibition curves suggest a saturatable single binding site for these metal cations. When the data were fitted to a parabolic equation assuming a single binding site (Figure 1, panels B and C), K_d values of 12 μM for Co^{2+} and 20 μM for Ni^{2+} were obtained. Best fittings of the data were obtained assuming that saturation by Co^{2+} inhibits Csk 52% and saturation by Ni^{2+} inhibits Csk 53%. Under these conditions, ATP is more than 95% saturated with Mg^{2+}, thus the inhibitory effects are exerted through binding to another site on Csk. Three observations suggest that this "inhibitory Co^{2+}- or Ni^{2+}-binding site" is the same as the M2-binding site: first, Co^{2+} and Ni^{2+} bind to this "inhibitory binding site" with affinity similar to their binding to the M2-binding site (see Figure 1 and Table 1); second, Csk is partially active when both the "inhibitory site" and the M2-binding site are saturated with Co^{2+} or Ni^{2+}; and third, the inhibition curves suggest a single binding site for Co^{2+} and Ni^{2+}. Mn^{2+} stimulated Csk activity up to 1.9-fold, with obvious stimulatory effect even at low micromolar concentrations, indicating a binding site that has a preference for Mn^{2+} over Mg^{2+}. Quantitative analysis of Csk activation by Mn^{2+} is complicated by its interaction with ATP at higher concentrations. Nevertheless, these results indicate that Mn^{2+} has higher affinity for the M2-binding site and is a more effective activator for Csk than Mg^{2+}. These results together indicate that Mn^{2+}, Co^{2+}, and Ni^{2+} can substitute for Mg^{2+} at M2 and all three bind to this site of Csk with higher affinity than Mg^{2+}. Csk is more active when Mg^{2+} at M2 is replaced with Mn^{2+}, and less active when Mg^{2+} at M2 is replaced with Co^{2+} or Ni^{2+}. Although the affinity of M2 for Csk is similar to M1 affinity for ATP, the $K_{0.5}$ is dependent on the concentration of both ATP and Csk, while IC_{50} is dependent only on the concentration of Csk. Since the concentration of ATP (0.2 mM) is much higher than that of Csk (0.06 μM) in the assay, the $K_{0.5}$ values for Co^{2+} and Ni^{2+} are much higher than the IC_{50} values.

Table 2: Inhibition of Csk Kinase Activity[a]

salt	% of inhibition of Csk
$BaCl_2$	0
$BeCl_2$	45
$CdCl_2$	**97**
$CaCl_2$	0
$CrCl_2$	ND[b]
$CuCl_2$	55
$EuCl_2$	62
$GaCl_2$	50
$FeCl_2$	11
$PbAc_2$	43
K_2PtCl_4	32
$SrAc_2$	3
$SnCl_2$	0
$ZnAc_2$	**100**

[a] The kinase activity was assayed in the presence of 6 mM $MgCl_2$ and 10 μM of the DMC to be tested. Other components of the reaction are as described for the standard assay reaction. [b] ND = not determined, since Cr^{2+} precipitates ATP on to the filter paper under our assay conditions, making it difficult to accurately determine its effect on the kinase activity.

Inhibition of Mg^{2+}-Supported Csk Catalytic Activity by Other DMCs. Although none of the other DMCs were able to support Csk activity in our assay, it is not clear which one of the two roles, M1 or M2, these other DMCs were not able to serve. It is possible that some of the DMCs could bind to the M2-binding site with high affinity even though they do not support Csk activity. We tested different DMCs at 10 μM for their ability to inhibit Csk catalytic activity in the presence of 6 mM Mg^{2+}. The concentration of 10 μM was chosen so that the large difference (600-fold) in the concentrations of Mg^{2+} and the DMCs minimizes any inhibitory effect exerted by complexing with ATP and this also avoided solubility problems of the metals. Among 14 additional DMCs tested (Table 2), Zn^{2+} and Cd^{2+} at 10 μM inhibited Csk activity more than 95% while several other DMCs showed moderate levels of inhibition. The IC_{50} values

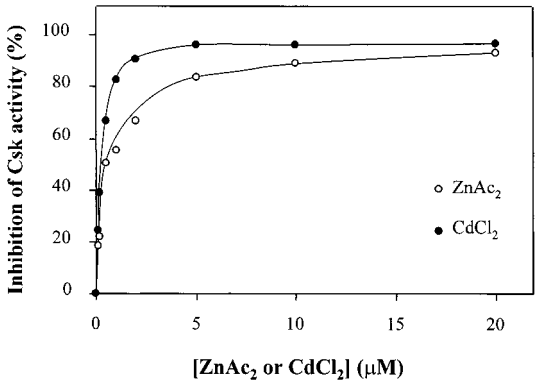


FIGURE 2: Inhibition profile of Mg^{2+}-supported Csk activity by $CdCl_2$ and $ZnAc_2$. The assay contained 6 mM $MgCl_2$ and 0.2 mM ATP.

of Cd^{2+} and Zn^{2+} inhibition of Csk under these conditions were 0.3 and 0.5 μM, respectively (Figure 2).

Zn^{2+} and Cd^{2+} Decrease Csk Catalytic Activity by Different Mechanisms. The presence of a DMC may cause a decrease in Csk activity by various mechanisms, such as binding to a specific site on the enzyme, nonspecific denaturation, oxidation of cysteine residues, binding to the substrate, etc. Interaction with $polyE_4Y$ as a reason is eliminated since the concentration of this substrate is far greater than the concentration of the inhibitory divalent metal cations used. If the inhibition is caused by binding to a specific site on the enzyme, the inhibition should be reversed by removal of the DMC from this reaction. We determined if the inhibition of Csk by Zn^{2+} and Cd^{2+} can be reversed by addition of Na_2EDTA. EDTA has much higher affinity for Zn^{2+} and Cd^{2+} than for Mg^{2+} (*21*) and thus preferentially chelates these DMCs even in the presence of much higher concentration of Mg^{2+}. Csk phosphorylation of $polyE_4Y$ with 0.2 mM ATP and 6 mM $MgCl_2$ was monitored in the presence or absence of 1 μM $ZnAc_2$ (Figure 3A). $ZnAc_2$ at 1 μM caused significant inhibition of Csk activity as expected (1.6 versus 8.2 pmol min^{-1}). At the indicated time after the start of the reaction, 0.2 mM Na_2EDTA was added. Removal of Zn^{2+} by EDTA from the reaction relieved its inhibition and allowed Csk activity to fully recover as indicated by the slope (9 pmol min^{-1}) that is similar to the positive control. This indicates that Zn^{2+} inhibits Csk activity by specific binding instead of other nonspecific mechanisms. In contrast, the inhibition/inactivation of Csk activity by Cd^{2+} is not reversed by addition of 0.2 mM Na_2EDTA (Figure 3B). The fact that it is not reversed by removal of Cd^{2+} with EDTA indicates that the decrease of Csk catalytic activity is not caused by Cd^{2+} binding to the M2-binding site but rather by modification of Csk. Cd^{2+} and some other transition metals are known to inactivate enzymes by oxidation of Cys residues that are important for their catalytic activity (*22*). When an additional reducing agent, such as 1 mM DTT, was added into the assay, Cd^{2+} does not cause a decrease of Csk catalytic activity (data not shown). In addition, inhibition by Cd^{2+} could be reversed by treatment with β-mercaptoethanol, confirming that the mechanism of Cd^{2+} inactivation of Csk is by oxidation. These results together indicated that Zn^{2+} inhibits Csk activity by binding to the enzyme in a reversible manner, while Cd^{2+} caused a decrease of Csk activity by modification, probably oxidation of cysteine residues. Fitting of the concentration-dependent inhibition

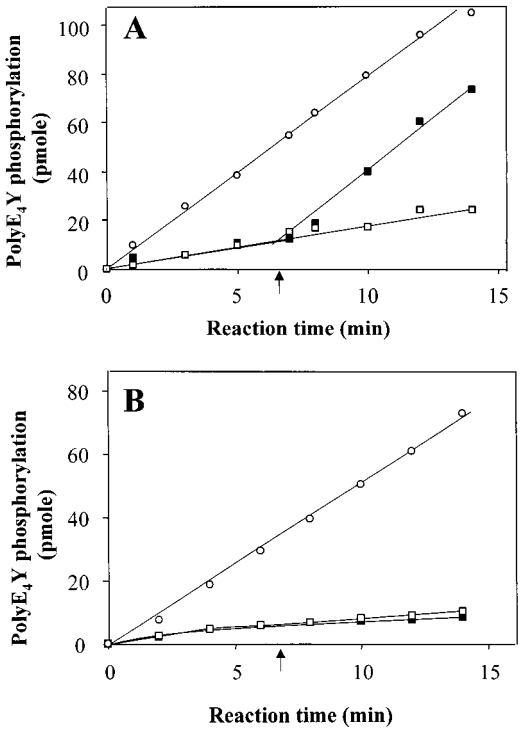


FIGURE 3: Reversibility of inhibition/inactivation of Csk activity by Zn^{2+} and Cd^{2+}. The reversibility of $ZnAc_2$ (A) and $CdCl_2$ (B) inhibition of Csk catalytic activity was determined. Assay conditions were the same as in the standard assay except the additions noted in the figure and below. The arrows indicate the time when 0.2 mM EDTA was added. (○) Control, no $ZnAc_2$ or $CdCl_2$ was present; (□) 1 μM $ZnAc_2$ (A) or $CdCl_2$ (B) is present; (■) 1 μM $ZnAc_2$ (A) or $CdCl_2$ (B) is present and 0.2 mM EDTA was added into the reaction at the time indicated by the arrows.

data by Zn^{2+} to a parabolic equation gave a K_d value of 0.65 μM.

The Inhibition of Csk Catalytic Activity by Zn^{2+} Is by Competing Against M2. We have previously shown (*8*) that the activation of Csk by free Mg^{2+} does not affect the affinity between Csk and ATP−Mg, but increases its V_{max}. Next, we determined if binding of Zn^{2+} interferes with the binding of ATP−Mg. Complexes between ATP and some DMCs, such as Cr^{2+}, have been reported to inhibit ATP utilizing enzymes as competitive inhibitors of ATP−Mg (*23*). Csk catalytic activity was evaluated at different concentrations of $ZnAc_2$ with ATP−Mg as the variable substrate in the presence of 6 mM Mg^{2+} (Figure 4A). Increases in the concentration of Zn^{2+} resulted in decreases in the V_{max} but did not affect the K_m for ATP−Mg. This result is not consistent with the possibility that the inhibition of Csk activity by Zn^{2+} is the result of ATP−Zn being a competitive inhibitor against ATP−Mg, thus leaving binding to the M2 site as a plausible explanation.

This possibility was directly examined by determining Csk activity in the presence of variable concentrations of free Mg^{2+} and Zn^{2+} (Figure 4B). Although M2 is not a substrate for the phosphorylation reaction, as an essential activator it can be treated as a substrate for the purpose of kinetic analyses (*24*). The concentration of free Mg^{2+} is calculated by subtracting the concentration of ATP from the total concentration of Mg^{2+}. We have previously shown that other components in our assay do not significantly chelate Mg^{2+} or reduce the concentration of free Mg^{2+} in the assay (*8*).

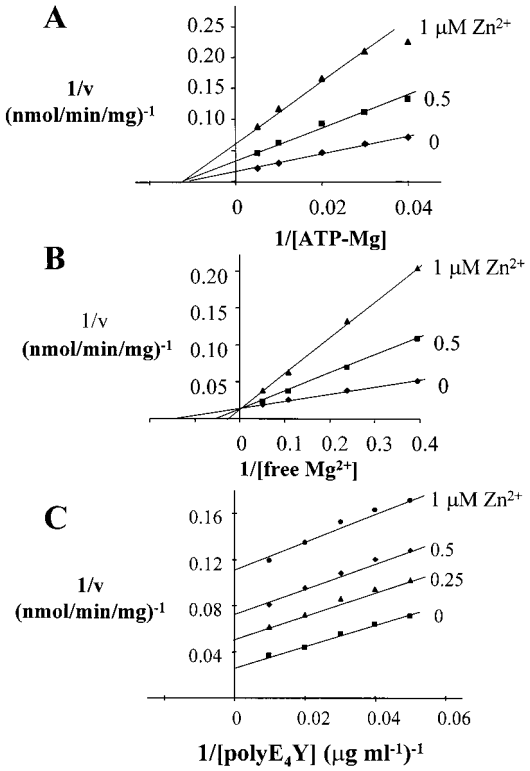


FIGURE 4: Effect of Zn^{2+} on the kinetic parameters of Csk activity with either ATP−Mg, free Mg^{2+}, or $polyE_4Y$ as the variable substrate. (A) ATP−Mg is the variable substrate and $MgCl_2$ concentration in the assays was 6 mM. (B) Mg^{2+} was the variable substrate and the ATP concentration in the assays was 0.2 mM. (C) Double reciprocal plots of Mg^{2+}-supported Csk activity versus variable concentrations of $polyE_4Y$ in the presence of increasing concentrations of $ZnCl_2$. Concentrations of $ZnAc_2$ in μM are noted for each plot.

The apparent K_a for free Mg^{2+} in the absence of $ZnAc_2$ was determined to be 6.6 mM. Presence of increasing concentration of Zn^{2+} up to 1 μM does not significantly change the V_{max} of the enzyme but increases the K_m for free Mg^{2+} to 40 mM. This kinetic pattern indicates that Zn^{2+} as a Csk inhibitor is competitive against free Mg^{2+}. The mutually exclusive binding by free Zn^{2+} and free Mg^{2+} to Csk clearly demonstrates that Zn^{2+} inhibits Csk catalytic activity by substituting for Mg^{2+} at the M2-binding site. A K_i for Zn^{2+} of 0.5 μM was obtained from these data, which is consistent with the K_d value determined above. This is a 13200-fold higher affinity than Mg^{2+} at the M2 binding site.

Involvement of M2 in the Interaction between Csk and the Peptide Substrate. We next examined if Zn^{2+} affects the binding of the peptide substrate (Figure 4C). Increasing concentrations of $ZnAc_2$ resulted in seemingly proportional decreases in both K_m and V_{max} when $polyE_4Y$ was the variable substrate. The kinetic pattern can be the result of either one of two inhibitory mechanisms, uncompetitive or mixed inhibition with similar effect on K_m and V_{max}. Two observations suggest that it is the latter. First, extension of the lines indicate that they intersect below the *x*-axis at a common spot. Second, similar plots with another substrate, RCM-lysozyme, resulted in lines that clearly intersect below the *x*-axis. These results indicate that the mechanism of inhibition with regard to the phosphate-accepting substrate is mixed and the effect on K_m and V_{max} is dependent on the substrate. This kinetic pattern reveals two insights into the mechanism of Zn^{2+} inhibition of Csk catalytic activity. First, we reported earlier (*8*) that increasing concentrations of free Mg^{2+} resulted in near proportional increases in the K_m and V_{max} of Csk when $polyE_4Y$ is the variable substrate. The fact that increasing concentrations of free Zn^{2+} has the opposite effect confirms that Zn^{2+} inhibits Csk catalytic activity by competing with the second Mg^{2+}. Second, the effect of Zn^{2+} on the affinity for the phosphate-accepting substrate indicates a potential interaction between the two. The involvement of M2 in peptide substrate binding was further studied. We determined how substitution of M2 by Mn^{2+}, Co^{2+}, or Ni^{2+} affects the interaction between Csk and the peptide substrate (Figure 5). Substitution of M2 with Mn^{2+}, Co^{2+}, or Ni^{2+} significantly affected both K_m and V_{max} values. The dependence of K_m values for the peptide substrate on the metal cation substituting for the second Mg^{2+} suggests that M2 is involved in the interaction between the enzyme and the peptide substrate. The V_{max} effect suggests that M2 does not only affect the interaction between the enzyme and the peptide substrate, it also significantly affects other aspects of the catalytic process.

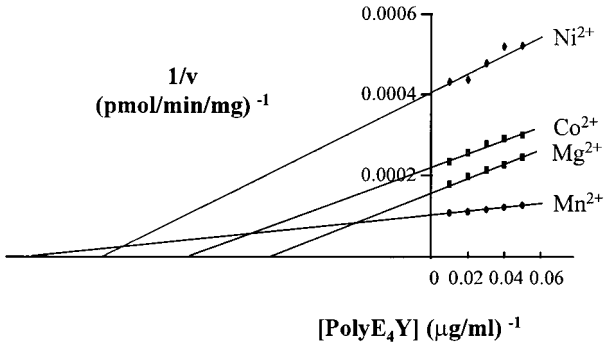


FIGURE 5: Effect of M2 substitution on the kinetics of Csk. Effect of 0.2 mM Mn^{2+} (○), Co^{2+} (□), or Ni^{2+} (◆) on the kinetic patterns of Mg^{2+}-supported Csk activity (■) with $polyE_4Y$ as the variable substrate was determined.

DISCUSSION

The effects of DMCs on PTK activity have been reported (*25*, *7*, *19*). Most studies examined the kinase activity of various PTKs in the presence of various DMCs. In a previous report (*8*), we found that Mg^{2+} plays two essential roles in the catalytic activity of PTKs. It binds to ATP to form the phosphate-donating substrate, ATP−Mg complex (M1), and it also acts as an essential activator (M2). The role and mechanism of M1 in PTKs are similar to most other ATP-utilizing enzymes and fairly well studied and understood (*26*, *27*), but there is little information concerning the role of M2 in PTK catalysis. The requirement of two DMCs make studies of the M2 site complicated, in that it is difficult to introduce a change to M2 without affecting M1. Here we report our findings that make such specific studies of the M2 site possible. We found that Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}, have much higher affinity for the M2-binding site of Csk than Mg^{2+} itself [more than 10000-fold difference in $K_{i(Zn^{2+})}$ versus $K_{a(Mg^{2+})}$]. This allows us to analyze the effect of DMC substitution of M2 without significantly affecting which DMC is bound to ATP as M1.

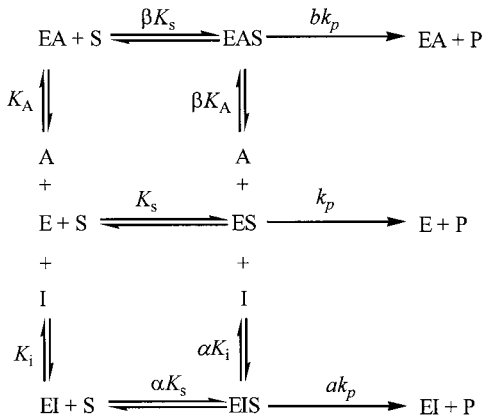
Several lines of evidence support our contention that the substitution DMCs are specifically interacting with the M2-binding site of Csk. First, the stability constant between ATP

and all the DMCs used for the study are similar, and therefore, the proportion of ATP complexed with each metal cation is determined by their relative concentrations. Since the concentration of Mg^{2+} is at least 30-fold higher than the substitution DMCs in our assay, more than 95% of ATP is complexed with Mg^{2+}. In the case of more detailed kinetic characterization carried out with Zn^{2+}, more than 99.9% of ATP is bound with Mg^{2+}. Second, increasing concentrations of Zn^{2+} did not affect the affinity for ATP−Mg, indicating that the metal substitution did not affect the function of M1. Third, Zn^{2+} inhibited Csk activity as a competitive inhibitor against free Mg^{2+}. The mutually exclusive binding to Csk provides the most direct kinetic evidence that the substitution cation is interacting at the M2-binding site. Finally, increasing concentrations of Zn^{2+} had the opposite effect on Csk kinetics as increasing Mg^{2+} concentration in the assay. Such antagonistic effects suggest that Zn^{2+} is competing with Mg^{2+} for the same M2-binding site.

M2 substitution with Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} indicates that their ability to support Csk catalytic activity is roughly correlated to their ability to serve the function of M2. The chemical bases for the observed biochemical differences are not clear, but other chemical properties of a DMC, such as coordination number, polarizability, cation size, metal−ligand bond distance, etc., are expected to contribute to their ability to perform a particular biochemical function (*28*, *29*). Detailed structural and kinetic studies may reveal the chemical basis for the biochemical observations. The high-affinity binding between the M2-binding site of Csk and Mn^{2+}, Co^{2+}, Ni^{2+}, and especially Zn^{2+} is not surprising chemically. Due to differences in their atomic structures, these metal cations bind to most ligands much tighter than Mg^{2+} (*21*).

The mode of inhibition of Csk activity by Zn^{2+} is competitive against M2, noncompetitive against ATP−Mg and mixed against the phosphate-accepting substrate. Since Zn^{2+} is competitive against the free Mg^{2+}, the binding equilibrium can be represented in Scheme 1 (*24*). In this scheme, E is Csk; S is the substrate being considered; A is Mg^{2+} as M2; I is Zn^{2+}; P is the reaction product. Since the reaction rate of Csk approaches zero when the concentration of free Mg^{2+} approaches zero, k_p is close to or equal to zero. Since at the lowest concentration of free Mg^{2+} we have used, 0.1 mM, Csk activity is approximately 1% of its maximal activity at optimal Mg^{2+} concentrations, factor *b* is far greater than 100. When ATP−Mg is the substrate in consideration, both α and β equal 1. When the substrate is the phosphate-accepting substrate, β ranges from slightly greater than 1 for RCM-lysozyme to approaching the value of *b* for $polyE_4Y$ (*8*). Determination of a value for α using steady-state kinetics is complicated by the fact that the apparent K_s or K_i is a function of not only E versus EI or E versus ES, they are also a function of EA, EAS, K_A, β, and *b*. Our results indicate that the apparent affinity of Csk for Zn^{2+} or $polyE_4Y$ are significantly enhanced by the binding of the other. Since the affinity for the peptide substrate is significantly affected by the metal cation at M2, it is possible that M2 interacts with the peptide substrate in the active site of the enzyme. Such a suggestion awaits to be confirmed by structural studies. Although a large number of kinases require a second Mg^{2+} for catalysis, the kinetic effect, structural coordination and the mechanism of its involvement in catalysis varies widely within a single enzyme family (*29*). For example, the cAMP-dependent protein kinase has two Mg^{2+} in the active site, but binding of the second Mg^{2+} reduces the V_{max} and the K_m for ATP−Mg (*30*, *31*), significantly different from the responses of any PTK that has been studied. Currently the only structural information on M2 of PTKs are from the crystal structure of IRK (*15*), in which two Mg^{2+} were found in the active site. M2 in this structure is bound to ATP and away from the peptide substrate. The close proximity and interaction between M2 and ATP confirmed the kinetic conclusion that the bindings of M2 and ATP−Mg to IRK are mutually inclusive. But this information is not applicable to Csk and the Src family, since kinetic studies established that they are activated by M2 by a different mechanism than IRK (*8*). While M2 activates IRK by increasing its affinity for ATP−Mg, it does not affect this kinetic parameter of Csk and Src. The decreasing K_m values for $polyE_4Y$ with increasing concentration of Zn^{2+} indicate that binding of Zn^{2+} to Csk-$polyE_4Y$ complex stabilizes (i.e., tighter binding) this complex compared to when Mg^{2+} is bound to the M2-binding site. This effect is the opposite of that when Mg^{2+} concentration is increased. Together these observations suggest that one role of M2 as an activator is to destabilize the interaction between Csk and tyrosyl substrate. Although this notion of an activator destabilizing enzyme substrate interaction contradicts the commonly held belief that "a higher affinity substrate is a better substrate", it has been proven both theoretically and experimentally (*32*).

Scheme 1: Binding Equilibrium between the Enzyme, a Substrate, Divalent Metal Cation Activator and the Substituting Metal Inhibitor



The inhibition of Csk by Zn^{2+} in vitro which we have demonstrated may present an interesting biological problem. Zinc is the second most abundant trace element with the average person containing 2.3 g of zinc compared to 4 g of iron. More than 300 zinc-containing enzymes are known. The concentration of free Zn^{2+} in the cell is not known, but most is bound by metallothioneins as a storage pool (*33*). Changes in the redox status can release Zn^{2+} from metallothioneins (*34*). Reports on the concentration of free Mg^{2+} in the cell range from 0.3 to 2 mM (*35*, *36*). The IC_{50} for Zn^{2+}-dependent inhibition of Csk in the presence of 2 mM Mg^{2+} is 0.4 μM. Thus, a submicromolar concentration of free Zn^{2+} would be inhibitory to Csk. A recent report on the regulation of NMDA receptor and its interaction with the alternate splice version of Src has indicated that Zn^{2+} plays a role in controlling synaptic signaling within the nervous

system (*37*). However, the potential of Zn^{2+} to act as a potent PTK inhibitor was not noted.

Due to the large number of different diseases associated with aberrant PTK activity, most pharmaceutical companies are developing PTK inhibitors. In the screening of active-site-directed inhibitors, most researchers use either Mn^{2+} or a mixture of Mg^{2+} and Mn^{2+}, rather than Mg^{2+} in their in vitro assay (*38*). The result from this study would suggest that Mn^{2+} would occupy the M2-binding site and may very well give misleading results as it affects the affinity of the phosphate-accepting substrate and thus may affect the binding of inhibitors that are competitive to this substrate.

REFERENCES

1. Brown, M. T., and Cooper, J. A. (1996) *Biochim. Biophys. Acta 1287*, 121−149.
2. Levitzki, A., and Gazit, A. (1995) *Science 267*, 1782−1788.
3. Klohs, W. D., Fry, D. W., and Kraker, A. J. (1997) *Curr. Opin. Oncol. 9*, 562−568.
4. Cooper, J. A., and Howell, B. (1993) *Cell 73*, 1051−1054.
5. Sun, G., Sharma, A. K., and Budde, R. J. A. (1998) *Oncogene 17*, 1587−1595.
6. White, M. F., Haring, H.-U., Kasuga, M., and Kahn, C. R. (1984) *J. Biol. Chem. 259*, 255−264.
7. Budde, R. J. A., Ramdas, L., and Ke, S. (1993) *Prepr. Biochem. 23*, 493−515.
8. Sun, G., and Budde, R. J. A. (1997) *Biochemistry 36*, 2139−2146.
9. Sun, G., and Budde, R. J. A. (1997) *Arch. Biochem. Biophys. 345*, 135−142.
10. Vicario, P. P., Saperstein, R., and Bennum, A. (1988) *Arch. Biochem. Biophys. 261*, 336−345.
11. Saylor, P., Wang, C., Hirai, T. J., and Adams, J. A. (1998) *Biochemistry 37*, 12624−12630.
12. Roof, S. K., and Maguire, M. E. (1994) *J. Am. Coll. Nutr. 13*, 424−428.
13. Beeler, T., Bruce K., and Dunn, T. (1997) *Biochim. Biophys. Acta 1323*, 310−318.
14. Hwang, D. L., Yen, C. F., and Nadler, J. L. (1993) *J. Clin. Endocrinol. Metab. 76*, 549−553.
15. Hubbard, S. R. (1997) *EMBO J. 16*, 5572−5581.
16. Smith, D. B., and Johnson, K. S. (1988) *Gene 67*, 31−40.
17. Schmidt, T. G. M., and Skerra, A. (1993) *Protein Eng. 6*, 109−122.
18. Sun, G., and Budde, R. J. A. (1995) *Anal. Biochem. 231*, 458−460.
19. Grace, M. R., Walsh, C. T., and Cole, P. A. (1997) *Biochemistry 36*, 1874−1881.
20. Okada, M., and Nakagawa, H. (1988) *J. Biochem. (Tokyo) 104*, 297−305.
21. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*, 3rd ed., Clarendon Press, Oxford.
22. Yuan, C. J., Huang, C. Y., and Graves, D. J. (1994) *J. Biol. Chem. 268*, 17683−17686.
23. Janson, C. A., and Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2572−2574.
24. Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, New York.
25. Koland, J. G., and Cerione, R. A. (1990) *Biochim. Biophys. Acta 1052*, 489−498.
26. Mildvan, A. S. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 103−126.
27. Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877−919.
28. Glusker, J. P. (1991) *Adv. Protein Chem. 42*, 1−75.
29. Villafranca, J. J., and Nowak, T. (1992) *Enzymes 20*, 63−94.
30. Cook, P. F., Neville, M. E, Jr., Vrana, K. E., Hartl, F. T., and Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794−5799.
31. Mildvan, A. S., Rosevear, P. R., Fry, D. C., Bramson, H. N., and Kaiser, E. T. (1985) *Curr. Top. Cell. Regul. 27*, 133−144.
32. Fersht, A. (1984) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman & Co, New York.
33. Coleman, J. E. (1992) *Annu. Rev. Biochem. 61*, 897−946.
34. Maret, W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 237−241.
35. Maguire, M. E. (1990) *Metal Ions Biol. Syst. 26*, 135−153.
36. Altura, B. M., Durlach, J., and Seelig, M. S. (1987) in *Magnesium in Cellular Processes and Medicine* (Altura, B. M., Durlach, J., and Seelig, M. S., Eds.) pp 1−4.
37. Zheng, F., Gingrich, M. B., Traynelis, S. F., and Conn, P. J. (1998) *Nature Neurosci. 3*, 185−191.
38. Ramdas, L., and Budde, R. J. A. (1988) *Cancer Biochem. Biophys. 16*, 375−385.

BI982793W