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Acidity of exogenous metal ion in the activation of calcineurin

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Abstract

The pH dependent activation of calcineurin by exogenous metal ion was studied over the pH range from 6.5 to 9.0 in increments of 0.5 pH units. Calcineurin activated by Co^{2+}, Ni^{2+}, or Mg^{2+} was characterized and compared to the pH dependency of the Mn^{2+}-activated enzyme (Martin, B.L., and Graves, D.J. (1986) *J. Biol. Chem.* **261**, 14545–14550). The pH dependency of the kinetic parameters varied with metal ion and subsequent analysis yielded estimates for the pK_a values for the enzyme-metal ion and the enzyme-metal ion-substrate complexes with each of the exogenous metal ions characterized. The evaluated pK_as for enzyme-metal ion (EM) complexes showed an inverse relationship with the pK_as of the M^{2+}-H_2O complex. In contrast, variation of the pK_as for the enzyme-metal ion-substrate (EMS) complexes showed no trend. These data support the hypothesis that exogenous metal ion functions to facilitate a proton transfer before the turnover of substrate with the acidity of the exogenous metal ion as a primary determinant of its participation.

Introduction

Critical to the regulation of cellular processes, protein serine and threonine phosphatases generally fall into one of four classes of cytosolic phosphatases with the designations types-1, -2A, -2B, and -2C phosphatases (Ingebritsen & Cohen,1983). Types-1, -2A, and -2B belong to the same gene family (Cohen *et al.* 1990) with all family members likely having intrinsic metal ions as identified in the resolved crystal structures of types-1 and -2B (Egloff *et al.* 1995; Griffith *et al.* 1995; Goldberg *et al.* 1995; Kissinger *et al.* 1995). All members of the gene family have a form, native or recombinant, activated by metal ions (King & Huang, 1983; Cai, *et al.* 1995; Chu *et al.* 1996). Although distinct from the protein phosphatases, some alkaline phosphatases (Coleman, 1982; Kim & Wyckoff, 1991) and the purple acid phosphatases (Mueller *et al.* 1993; Sträter *et al.* 1995) also are metalloenzymes and share characteristics involving the participation of metal ions in the hydrolysis of phosphate ester bonds.

Calcineurin contains iron and zinc (King & Huang, 1983; King & Huang, 1984) as intrinsically bound metal ions, but also has the requirement of exogenous metal for effective substrate turnover (King & Huang,, 1983; King & Huang, 1984; Li, 1984; Li & Chan, 1984; Pallen & Wang, 1984; Gupta *et al.* 1984; Wolff & Sved, 1985). The resolved crystal structure (Griffith *et al.*, 1995; Kissinger *et al.* 1995) of the enzyme shows iron and zinc at the putative active site, identified from the binding of inorganic phosphate from the buffer. Although a low concentration of Mn^{2+} was present in the protein solution during crystallization, no information about the presence or location of this metal ion in the structure of calcineurin was reported (Griffith *et al.* 1995; Kissinger *et al.* 1995). No inference on the function of exogenous metal ion can be made from these crystal structures. Previous studies of the activation of calcineurin by metal ions yielded data consistent with a model having hydrated exogenous metal ion bound at the active site and participating dir-

ectly in catalysis by transferring a proton to the leaving group of the substrate.

Hydrolysis of the phosphate ester bond is then catalyzed by the intrinsic iron by activation of water. These assigned roles are also consistent with NMR relaxation enhancement studies of Mn^{2+} binding (Haddy *et al.* 1996). No evidence is available showing the substitution of intrinsic metal ions by exogenous metal ions in the activation of calcineurin (Martin, 1997). Additionally, activation is not correlated with the insertion of exogenous metal ion into unfilled binding sites (Martin, 1997) of enzyme lacking stoichiometric levels of intrinsic iron and zinc.

Modulation of the redox potential has been suggested as a mechanism of regulating calcineurin with reports describing the inactivation of calcineurin by oxidizing agents (Wang *et al.* 1996; Yu *et al.* 1997; Carballo *et al.* 1999; Ferri *et al.* 1999; Furuke *et al.* 1999; Reiter *et al.* 1999; Bogumil *et al.* 2000; Sommer *et al.* 2000) and with some investigators assigning the effect to an alteration of the oxidation state of intrinsic iron found in the enzyme (Yu *et al.* 1997; Reiter *et al.* 1999; Sommer *et al.* 2000). Bogumil *et al.* 2000 provided evidence that chemical redox agents did not target the active site metal ions and did not affect the oxidation state of iron. Although showing activation of calcineurin by reducing agents, Sommer *et al.* 2000 also showed that the enzyme could be activated by non-reducing agents that did have the capability of chelating metal ions. This last observation was consistent with earlier work showing the apparent activation of calcineurin by dipicolinic acid (Martin, 1997). Specified roles for the various metal ions remain unclear.

Much effort has been aimed toward characterizing the functional roles of metal ions in calcineurin catalysis (Martin & Graves, 1986; Martin & Graves, 1994; Hengge & Martin, 1997; Martin, 1997; Martin & Jurado, 1998; Martin & Rhode, 1999; Martin *et al.* 1999; Martin, 2000; Martin *et al.* 2000). Kinetic parameters for the activation of calcineurin by exogenous metal ions were found to be correlated to the formation constants (log K_1) of the activating metal ions (Martin, 2000) with the substrate *para*-nitrophenyl phosphate (pNPP[1]). The parameter, k_{cat}/K_{act}, increased with increasing formation constant. This correlation failed to account for the lack of activation by other metal ions with similar formation constants with pNPP. An empirical model describing metal ion activation was developed (Martin, 2000) by including another constant for the metal ions, the hydrolysis constant (pK_a M-H_2O) of water coordinated to the metal ion, with activity decreasing with increasing pK_a. The resulting two-parameter implied that the two properties (log K_1 pNPP and pK_a M-H_2O) of metal ions may be determinants of the role(s) of exogenous metal ion in catalysis. A one pH unit shift comparable to the difference in pK_a for water bound to Mn^{2+} ($pK_a = 10.6$) and Mg^{2+} ($pK_a = 11.4$) was observed in the apparent pH dependency was observed when Mg^{2+} was substituted for Mn^{2+} as the exogenous metal ion with pNPP as the substrate (Martin & Graves, 1986).

Experiments were performed to examine whether the acidity of the metal ion was a determinant of catalysis. If exogenous metal ion does function to facilitate a proton transfer to the substrate to enhance hydrolysis, then varying the exogenous metal ion would be expected to have a greater effect on the pH dependency of the k_{cat}/K_m parameter than the k_{cat} parameter because this parameter more directly reflects the interactions of the substrate with the enzyme-metal ion (EM) complex. This would be reflected in a higher correlation between the acidity of the metal ion and the pK_a for the EM complex (from the dependency of the k_{cat}/K_m parameter on pH) than between the acidity of the metal ion and the pK_a of the EMS complex (k_{cat} dependency on pH).

Experimental Procedures

Materials – Buffers, EGTA, and phenyl-Sepharose were purchased from Sigma Chemical. The substrate, pNPP (Sigma 104 substrate), was also purchased from Sigma. DE-52 cellulose was obtained from Whatman. Other chemicals (metal salts, etc.) were obtained from Fisher. All solutions were prepared from water that had been passed over a Chelex-100 column.

Proteins – Bovine brain calcineurin was isolated from bovine brain to apparent homogeneity using a modification of the method of Sharma *et al.* 1983 using MOPS buffer instead of Tris for the preparation of all solutions. Calmodulin was purified by the procedure of Sharma and Wang (Sharma & Wang, 1979) but including chromatography on phenyl-Sepharose with elution by EGTA (Gopolakrishna & Anderson, 1982). Protein concentrations were determined by the method of Bradford (Bradford, 1976).

Calcineurin assay and analysis of kinetic data – Kinetics were done at 30 °C with varied pNPP in 25 mM buffer, pH varied; 5–20 μg/ml calmodulin; and 5–20 μg/ml calcineurin with Mn^{2+} or Co^{2+}, added to

1.0 mM, with Ni^{2+} added to 2.0 mM, or with Mg^{2+} added to 20.0 mM, respectively. These concentrations represented 5 X K_{act} for the metal ions (Martin, 2000; Martin & Jurado, 1998). Calmodulin was added to the same concentration as calcineurin in units μg/ml; the resulting molar concentration was approximately 5-fold the molar concentration of calcineurin.

Kinetic parameters, k_{cat} and K_m, were evaluated by varying pNPP concentrations at varying pHs ranging from pH 6.5 to pH 9.0 in increments of 0.5 pH units. The pNPP concentrations used were 5.0, 7.0, 10.0, 16.0, and 50.0 mM. Below pH 6.5 and above pH 9.0, enzymatic activity was low and the characterization of kinetics was difficult. MOPS and Bicine buffers were used; MOPS at pHs 6.5, 7.0, and 7.5, and Bicine at pHs 8.0, 8.5, and 9.0. No evidence for any buffer effect was observed. The extinction coefficient for pNP was measured at each pH studied to correct for the ionization of pNP. The $pK_{\underline{a}}$ values for the enzyme-metal ion complex and enzyme-metal ion-substrate complex were evaluated by fitting the data to either equation 1 or equation 2 (see p. 352, Fersht, 1999) as appropriate, where $(Y)_H$ is the value of the designated kinetic parameter (k_{cat} or k_{cat}/K_m) determined at each pH and (Y) is the pH-independent value; and K_{EM} and K_{EMS} are the ionization constants for the different enzyme forms. These are the enzyme-metal ion complex and the enzyme-metal ion-substrate complex. Earlier studies have supported the binding of metal prior to the binding of the substrate, pNPP (Martin & Rhode, 1999).

$$(k_{cat})_H = \frac{k_{cat}[H^+]}{K_{EMS} + [H^+]} \quad (1)$$

$$(k_{cat}/K_{m,pNPP})_H = \frac{(k_{cat}/K_{m,pNPP})[H^+]}{K_{EM} + [H^+]} \quad (2)$$

Data fitting and numerical estimates were done using the programs Enzyme Kinetics (Trinity Software), Deltagraph (SPSS, Inc.) and Prism 4 (GraphPad).

Results and Discussion

The hydrolysis of pNPP catalyzed by calcineurin in the presence of Ni^{2+}, Co^{2+}, or Mg^{2+} was characterized at pHs 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. Studies were limited to pNPP as substrate to obviate any interference from metal ion binding sites in a protein substrate. Using a peptide or protein substrate would necessarily introduce possible metal ion sites and complicate the analysis.

As in earlier studies of the role of exogenous metal ion in calcineurin catalysis, these studies were done without added Ca^{2+}. Numerous studies (Stewart *et al.* 1982; King & Huang, 1983; Stewart *et al.*, 1983; Gupta *et al.* 1984; Li, 1984; Pallen & Wang, 1984; Winkler *et al.*, 1984; Klee *et al.* 1985; Wolff & Sved, 1985; Seki, *et al.* 1995; Ladner *et al.* 1996) have demonstrated the activation of calcineurin by Mn^{2+} in the absence of Ca^{2+}. Among these studies are the reports of the initial isolation and characterization of calcineurin by the Klee and Cohen laboratories (Stewart *et al.* 1982; Stewart *et al.* 1983) which showed the loss of Ca^{2+} sensitivity after affinity chromatography on immobilized calmodulin. Recovered enzyme was then only activated by Mn^{2+}.

At each pH and with each metal ion examined, the dependency of calcineurin activity on pNPP concentration yielded a smooth hyperbolic plot indicative of normal Michaelis–Menten kinetics (not shown). Double reciprocal plots also showed a straight line (not shown) with the simplest interpretation consistent with the involvement of a single site for metal ion or a single class of sites with the same apparent affinity. Estimates for k_{cat} and K_m were evaluated and the resulting values plotted against pH with Figure 1 showing the profile for k_{cat} values. For all of the metal ions, activity was higher in the acidic portion of the pH range. In earlier studies (Martin & Graves, 1986) with Mn^{2+}, values of k_{cat} increased from 6.0 to 7.0 and then effectively remained constant through pH 8.8. For both Ni^{2+} and Co^{2+}, the profiles showed significant differences from Mn^{2+}. For Co^{2+}, the value of k_{cat} decreased with increasing pH, with the greatest change between 6.5 and 7.5. The profile with Ni^{2+} was remarkable, being essentially constant between pH 6.5 and pH 7.5 followed by a dramatic decrease from pH 7.5 to 8.0 and then remaining quite low and essentially constant for the rest of the range characterized. The profile found for Mg^{2+} was similar to that found with Co^{2+}. These k_{cat} profiles were different from the profile reported for Mn^{2+} (Martin & Graves, 1986) which showed an increasing k_{cat} value as the pH was raised from 5.8 to 7.0 followed by a plateau region from pH 7.0 to pH 8.8. With Mn^{2+}, the profile was consistent with a deprotonation step associated with higher activity. With the other metal ions tested, the profile was consistent with a deprotonation step being associated with the loss of activity as pH was increased. Mn^{2+} has the highest formation constant for association with pNPP and it is likely that this property has some role in the observed differences. Plots of log

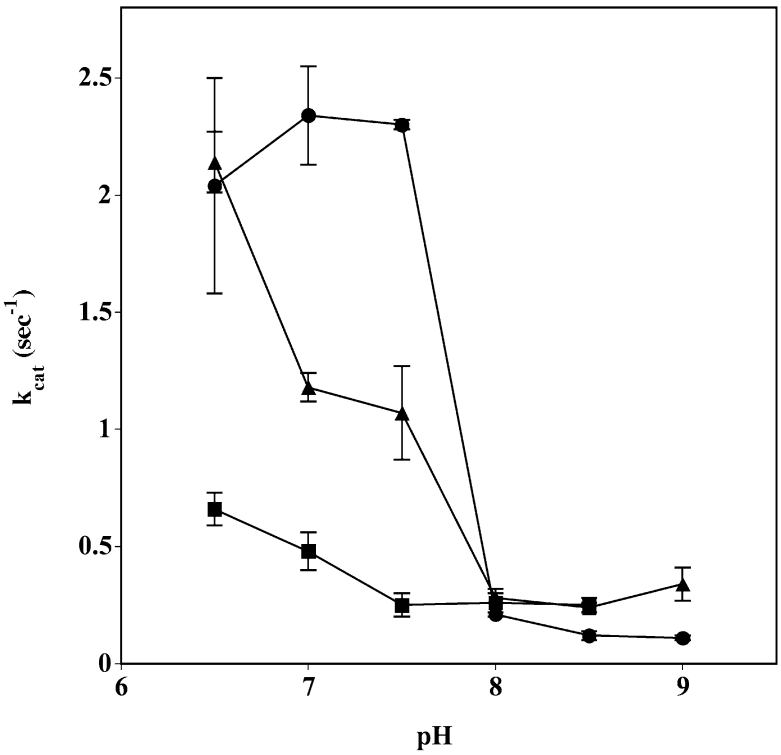

Fig 1. Profile of pH Dependency of Metal Ion Activation. The activation of calcineurin by Ni^{2+} (circles), Co^{2+} (triangles), and Mg^{2+} (squares) was characterized in the pH range from 6.5 to 9.0. Kinetic parameters were evaluated and the dependence of k_{cat} on pH is shown. Each initial velocity was measured in three to four replicates at each pNPP concentration and averaged. Other details are in the text.

k_{cat}/K_m showed less dramatic changes over the range of pH studied and were similar to plots observed with Mn^{2+} (Martin & Graves, 1986). Generally, the value of log k_{cat}/K_m decreased with pH (not shown). Ni^{2+} showed an anomalous spike at pH 7.5 followed by a dramatic decrease.

As described, equations 1 and 2 were used as appropriate to analyze the pH dependencies of the estimated kinetic parameters and yielded an estimate for the pK_a of the enzyme forms. Values were estimated for the pK_as for enzyme-metal ion complexes with each metal ion and for enzymemetal ion-substrate complex with each metal ion. The data were fit to equation 1 or 2 as appropriate (see p. 352, Fersht, 1999). Figure 2 demonstrates a representative fit of these equations for the kinetic parameters k_{cat} and k_{cat}/K_m with Mg^{2+} as the exogenous metal ion. The plots with the other metal ions were similar. The calculated values of pK_as for the EM (k_{cat}/K_m) and EMS (k_{cat}) complexes are collected in Table 1 along with values determined in the presence of Mn^{2+} (Martin & Graves, 1986).

Table 1. pK_a values of enzyme complexes vary with metal ion

Metal ion	H_2O	Enzyme-metal ion	Enzyme-metal ion-substrate
Co^{2+}	8.9	8.3 ± 0.4	7.2 ± 0.3
Ni^{2+}	9.9	8.1 ± 0.1	7.5 ± 0.4
Mn^{2+}	10.6	8.0	6.5
Mg^{2+}	11.4	7.6 ± 0.2	7.7 ± 0.5

Values for pK_a for M^{2+}-$(H_2O)_6$ complexes were taken from Perrin, 1974. The values for the Mn^{2+} complexes are from Martin & Graves, 1986.

Determinants of enzyme dissociation constants

Enzymatic pK_as were compared to the pK_a of the M^{2+}-$(H_2O)_6$ complex based on the hypothesis that this characteristic of the metal ion was a determinant of its participation in catalysis.

A plot of the pK_a for the enzyme-metal ion complex versus the pK_a of the M^{2+}-$(H_2O)_6$ complex did show a trend as shown in Figure 3. The estimated value of the pK_a decreased with increasing pK_a of the M^{2+}-$(H_2O)_6$ complex for relative metal ion. For all

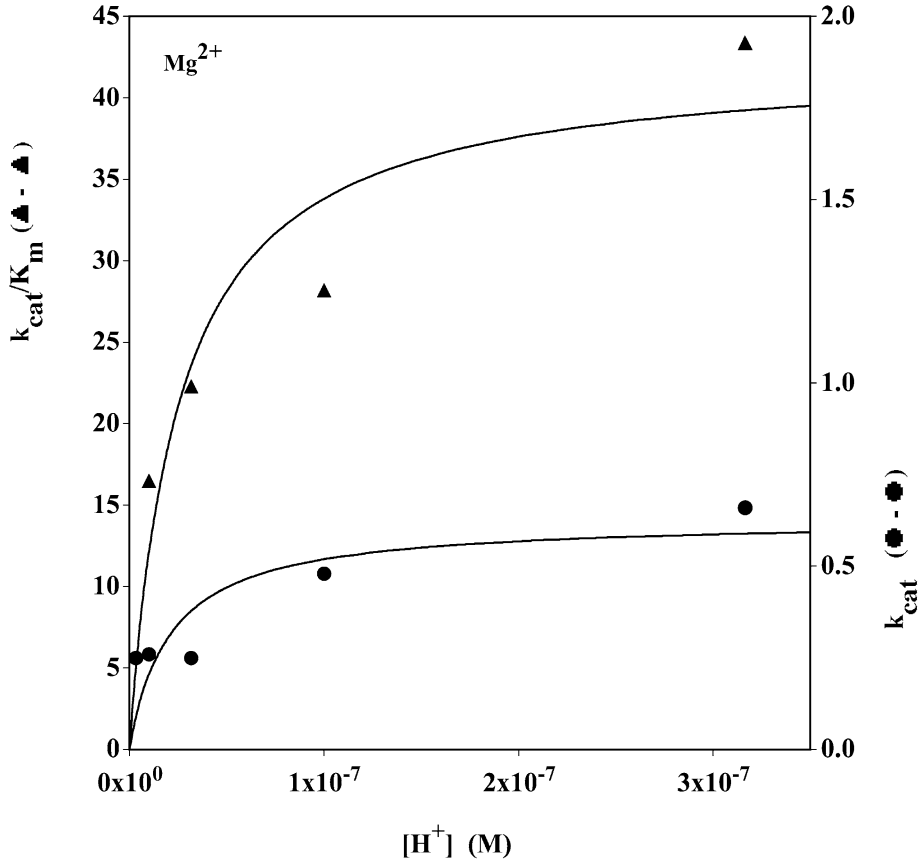

Fig 2. Analysis of pH dependency of Metal Ion Activation of Calcineurin. Shown are plots for Mg^{2+} as representative for each of the metal ions. The dependency of k_{cat} (circles) and k_{cat}/K_m (triangles) were fit as described in the text.

Table 2. Correlation of pK_a values to metal ion characteristics

Correlation coefficient to various independent variables			
Dependent variable	Cation radius	Marcus softness	Electronegativity
pK_a EM	0.08	0.94	0.91
pK_a EMS	−0.94	−0.49	−0.04

Similar values of correlation coefficients were found for the comparison of pK_a EM and pK_a EMS to charge:size ratio and electrode potential, respectively. These constants showed high correlation to cation radius and electronegativity, respectively, and thus were not listed in the table.

metal ions, the pK_a for the enzyme complex was lower than for the free metal ion in solution. The observed relationship was consistent with this property of the exogenous metal ion influencing the functional characteristic of the enzyme-metal ion complex. Further, this result was consistent with the predicted effect that variation in pH would have a greater influence on the pK_a of the EM complexes than on the pK_a for the EMS complexes.

Estimated values for the pK_as of the enzyme-metal ion-substrate complex did show some variation, but no similar relationship to the acidity of metal ions was found. There was no trend observed with these estimates when compared to the pK_a of the M^{2+}-$(H_2O)_6$ complex, as shown in the inset to Figure 3. An average value of 7.2 was calculated for all metal ions and is shown by the horizontal line in the figure. The estimated values for pK_as of the enzyme-metal ion-substrate complexes varied around this value, al-

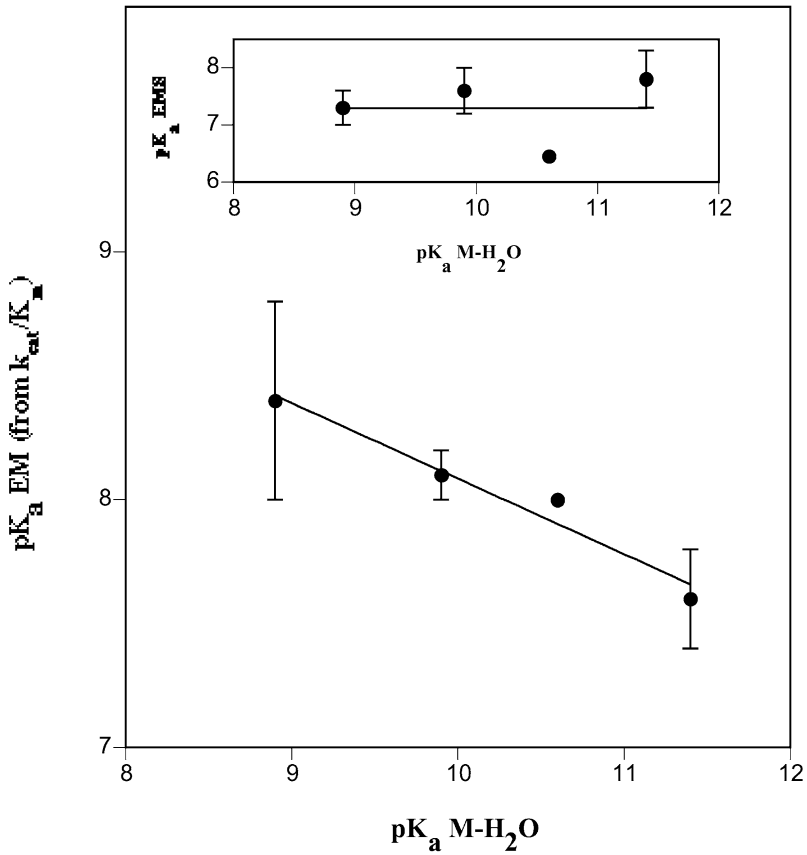

Fig 3. Relationship of pK_a values of the enzymes complexes to metal ion acidity. As described in the text, pK_as for the EM complexes with each metal ion were estimated from the dependency of log k_{cat}/K_m on pH. The line shown has the equation pK_a (EM) = 10.7 − 0.26 (± 0.06) $^*pK_a$ (M^{2+}-H_2O), with statistical parameters r = 0.95, F = 18.8, and p=0.05 as derived by linear regression analysis. The inset shows the data for pK_as of the EMS complexes calculated as described in the text. The line shown was drawn with slope zero and an intercept value equal to the average value of the calculated pK_as.

though the enzyme-metal ion complexes do not have the same pK_a. This result was consistent with there being no proton transfer during the cleavage step in the reaction catalyzed by calcineurin. This conclusion has been made in previous discussions about calcineurin catalysis from solvent isotope studies that showed no evidence for a proton transfer during the k_{cat} step (Martin & Graves, 1994) and from heavy atom isotope studies that were consistent with significant charge on the leaving group (Hengge & Martin, 1997).

There was a correlation between the pK_as for the EMS complexes and the formation constant of M^{2+}-pNPP complex (log K_1) as shown in Figure 4. The pK_a decreased with increasing stability of the M^{2+}-pNPP complex, indicating that a more acidic EMS complex was formed as the substrate complex was more stable. This relationship likely derived from the interaction of metal ion with the substrate being favored by increased partial negative charge in the enzyme complex and reflected some measure of selectivity for binding the substrate phosphate ester. The interaction of metal ion with pNPP must influence the reactive groups inasmuch as the pH profile is different for Mn^{2+} compared to Co^{2+}, Ni^{2+}, and Mg^{2+}. Of the metal ions characterized, Mn^{2+} has the highest formation constant with pNPP. These observations indicated that there is a relationship between the electronic nature of the different metal ions and their influence on the enzyme complex. Indeed, this was supported by the positive correlation between pK_as for the EMS complexes and the charge:size ratio for the metal ions (slope = +0.47 with r = 0.93; not shown). As shown in Table 2, pK_as for the EMS complexes were best correlated to properties related to size and the interaction with substrate. In contrast, pK_as for the EM complexes were best correlated to properties related to charge of the

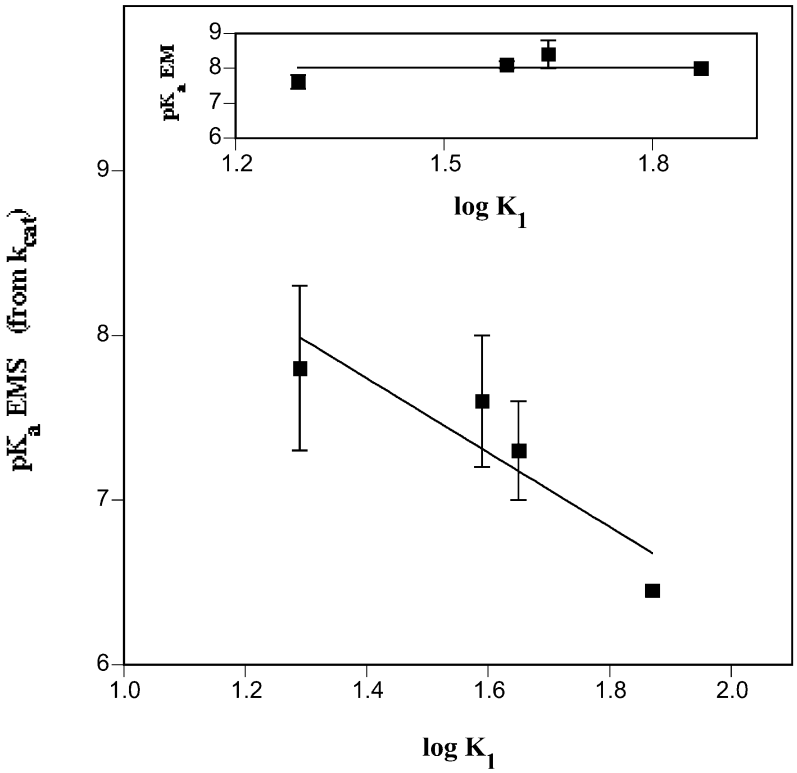

Fig 4. Relationship of pK_a values of the enzymes complexes to the formation Constant of Metal Ion with pNPP. As described in the text, pK_as for the EMS complexes with each metal ion were estimated from the dependency of log k_{cat} on pH. The line shown has the equation pK_a (EMS) = 10.5 − 2.05 (± 0.67) *log K1 (M^{2+}-pNPP), with statistical parameters r = 0.91, F = 9.3, and p = 0.09 as derived by linear regression analysis. The inset shows the data for pK_as of the EM complexes calculated as described in the text. The line shown was drawn with slope zero and an intercept value equal to the average value of the calculated pK_as.

metal ion. Additional study is required to evaluate any significance of this observation.

Functional significance of metal ion acidity

The data indicated that the acidity of exogenous metal ion was a determinant of its participation in calcineurin catalysis and provided additional insight into the possible role of exogenous metal ion. First, M-H_2O, not M-OH, was indicated as the preferred active species inasmuch as enzyme function decreased as the pH increased for Co^{2+}, Ni^{2+}, and Mg^{2+}. The metal ion complex likely did not act as a catalytic base in the reaction. Second, the pK_a of the EM complex decreased inversely with the pK_a of M-H_2O providing additional evidence that exogenous metal ion participates directly in the reaction, likely by stabilizing a developing charge. The M-H_2O may transfer a proton or participate in stabilization through hydrogen bonding. Mutagenesis of histidine-151 to glutamine has provided evidence for involvement of this residue in catalysis and Mertz *et al.* 1997 have discussed the participation of His151 in a charge relay with a hydrated metal ion functioning to activate a catalytic nucleophile. The data reported here are consistent with such a charge relay and support the involvement of exogenous metal ion within this charge relay. Participation is manifested before the hydrolytic step.

Acknowledgements

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Footnotes

[1]Abbreviations used: EM, enzyme-metal ion complex; EMS, enzyme-metal ion-substrate complex; EGTA, ethylene glycol *bis*(β-amino ethyl ether)N,N′-

tetraacetate; pNP, *para*-nitrophenol; pNPP, *para*-nitrophenyl phosphate.

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