

## Evidence for a Structure–Function Relationship in the Activation of Calcineurin by Metal Ions

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Kinetic parameters for the metal ion activation of calcineurin at 30°C were found to be correlated to the formation constants ( $\log K_f$ ) of the activating metal ions ( $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Mg}^{2+}$ ) with the substrate *para*-nitrophenyl phosphate (pNPP). Metal ion activation measured by  $k_{\text{cat}}/K_{\text{act}}$  increased with increasing formation constant, although this correlation failed to explain the lack of activation by other metal ions having similar formation constants with pNPP,  $\text{Ca}^{2+}$ , and  $\text{Sr}^{2+}$ , for example. An empirical model describing metal ion activation was developed by inclusion of another constant for the metal ions, the hydrolysis constant ( $\text{p}K_a$ ,  $\text{M-H}_2\text{O}$ ) of water coordinated to the metal ion with an increase in the  $\text{p}K_a$  related to decreasing activation. The resulting two parameter equation improved the relationship between metal ion properties and enzyme activation. Nonactivating metal ions have the highest values of  $\text{p}K_a$  providing a possible explanation for their failure to cause activation. Derivation of the two-parameter equation inferred that the two properties of metal ions may be important determinants of the role(s) of exogenous metal ion in calcineurin catalysis. © 2000 Academic Press

### INTRODUCTION

Calcineurin, also designated protein phosphatase-2B, is a calmodulin activated phosphatase that is isolated with intrinsically bound iron and zinc (1,2), and uses exogenous metal ion for optimal activity (2–10) with effectively no catalytic activity in the absence of exogenous metal ion. The crystal structure of calcineurin has been resolved and showed both intrinsic metal ions coordinated to inorganic phosphate in the putative active site (11,12) with some features in common with the dinuclear purple acid phosphatases (13,14) and protein phosphatase-1 (15,16). The mammalian purple acid phosphatase is a di-iron containing enzyme that catalyzes the direct transfer from phosphate ester to water based on stereochemical studies; no phosphoryl-enzyme is formed (17). The related enzyme from kidney bean is a zinc-iron enzyme with no evidence yet collected to show that this isozyme generates a phosphoryl-enzyme intermediate. The crystal structure (13,14) of the kidney bean enzyme showed the metal ion centers located at the putative active site of the enzyme with the iron atom likely involved in catalysis and

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the zinc atom likely to participate in the binding of the phosphate group. A likely common mechanism for these purple acid phosphatases (13,14,17) was used as a model for the mechanism of calcineurin (18). There must be differences between these enzyme systems, however, because of the requirement for exogenous metal ion in calcineurin catalysis, but not with the purple acid phosphatases.

Various exogenous metal ions can function to activate calcineurin (2–10). Although often considered to require calcium, initial descriptions of the purification of calcineurin by the Klee and Cohen laboratories reported equivalent activation with  $Mn^{2+}$  or  $Ca^{2+}$  with the loss of sensitivity to  $Ca^{2+}$  only after affinity chromatography on immobilized calmodulin (19,20). Others have reproduced this observation (3–10,21,22) with  $Mn^{2+}$  shown to be targeted to the catalytic subunit (23,24). From mechanistic studies (25–33), divalent metal ion was proposed to have a direct role in the catalytic mechanism of the enzyme and not function simply as conformational metal. Metal ion activation of calcineurin has been characterized (28–33) and a data set of kinetic activation parameters has been accumulated. These data have now been compared to properties of the metal ions to develop insight about the determinants of metal ion activation of calcineurin.

## EXPERIMENTAL PROCEDURES

*Materials.* The substrate employed, pNPP<sup>2</sup> (Sigma 104 substrate), was purchased from Sigma, as were buffers, EGTA, DEAE-Sepharose, and phenyl-Sepharose. Bovine brain was from Pel-Freez and  $ZnCl_2$  was from Fisher Scientific. Chelex-100 was purchased from Bio-Rad Laboratories and all solutions used in the purification of protein were prepared from water depleted of trace metal ions with Chelex-100.

*Proteins.* Calcineurin was isolated from bovine brain by the method of Sharma *et al.* (34) except MOPS was substituted for Tris. Calmodulin was purified by the procedure of Sharma and Wang with a slight modification (35). Following DEAE-chromatography, the calmodulin fraction was loaded onto phenyl-Sepharose and eluted with EGTA (36). Protein concentrations were determined by the method of Bradford (37).

*Calcineurin assay and kinetics.* Calcineurin was assayed by measuring the release of pNP spectrophotometrically at 410 nm in a Cary model 1E spectrophotometer. The standard reaction conditions were 25 mM Mops, pH 7.0; 1.0 mM  $MnCl_2$ ; 10 mM pNPP; 2–10  $\mu g/ml$  calmodulin; and 2–10  $\mu g/ml$  calcineurin in 800  $\mu l$ . All activity measurements were corrected for basal activity in the absence of exogenous metal ion (<1%). The kinetic activation parameters for  $ZnCl_2$  were determined by varying the concentration of the metal ion: 1.00, 0.33, 0.20, 0.14, and 0.11 mM concentrations were used. Substrate concentrations used were 50.0, 16.0, 10.0, 7.0, and 5.0 mM. Initial rates were measured for a 10 min reaction. The kinetic parameters,  $k_{cat}$ ,  $K_m$ , and  $K_{act}$ , were estimated where  $K_{act}$  is the concentration resulting in 50% activation. Activation by  $FeSO_4$  was examined using solutions freshly prepared in Chelex-treated water.

## RESULTS AND DISCUSSION

With the exception of  $Zn^{2+}$ , the kinetic parameters for the activation of calcineurin at pH 7.0 by exogenous metal ions have been measured in other studies (31,33).  $Zn^{2+}$  was

<sup>2</sup> Abbreviations used: EGTA, ethylene glycol bis( $\beta$ -amino ethyl ether) $N,N'$ -tetraacetate; Mops, 3-( $N$ -morpholino)propanesulfonic acid; pNPP, *para*-nitrophenyl phosphate; and pNP, *para*-nitrophenol.

found to activate calcineurin with the following parameters:  $k_{\text{cat}} = 1.21 \pm 0.06 \text{ sec}^{-1}$ ;  $K_{\text{m}} = 3.6 \pm 0.5 \text{ mM}$ , and  $K_{\text{act}} = 0.37 \pm 0.03 \text{ mM}$ . The activation by  $\text{Zn}^{2+}$  was a notable difference from other studies, although there was an early report of the activation of calcineurin by  $\text{Zn}^{2+}$  (9). Transition metal ions all activated calcineurin more effectively than did  $\text{Mg}^{2+}$ , but differences between the various transition metal ions were also observed (33). Seeking an explanation for the differences between metal ion activators, the kinetic parameters were compared to known solution properties of the metal ions.

### *Enzyme Activity and the Properties of Exogenous Metal Ions*

Collected in Table 1 are some properties of relevant metal ion complexes that may be informative about the distinction between the metal ions. Values are included for the following properties: (1) rate constant ( $\log k_{\text{ex}}$ ) for the exchange reaction of coordinated water; (2) acid dissociation constants of coordinated water ( $\text{p}K_{\text{a}} \text{ M-H}_2\text{O}$ ); (3) formation constant ( $\log K_1$ ) of metal ions with pNPP; (4) size (radius in  $\text{\AA}$ ) of the cation; and (5) minimal average distance ( $\text{\AA}$ ) between the metal ion and an oxygen ligand (carboxylate). The last characteristic, minimal distance to an oxygen ligand, is used as a model for the interaction with the phosphate group oxygens. The properties for 6-coordinate zinc complexes are given because model studies (38) indicate a tendency toward the octahedral complexes for zinc with oxygen ligands.

TABLE 1  
Properties of Metal Ion Complexes<sup>a</sup>

$\text{M}^{\text{n}+}$	$\text{p}K_{\text{a}} (\text{H}_2\text{O})$	$\log k (\text{H}_2\text{O})$	$\log K_1 (\text{pNPP})$	$\text{M}^{\text{n}+}$ radius ( $\text{\AA}$ )	M-L Distance ( $\text{\AA}$ )
$\text{Mg}^{2+}$	11.4	5.2	1.29	0.65	1.95
$\text{Ca}^{2+}$	12.6	8.5	1.26	0.99	2.23
$\text{Sr}^{2+}$	13.2	8.7	1.12	1.13	2.41 <sup>b</sup>
$\text{Ba}^{2+}$	13.4	9.0	1.06	1.35	2.61
$\text{Mn}^{2+}$	10.6	7.5	1.87	0.80	2.08
$\text{Fe}^{2+}$	6.7	6.5	2.39 <sup>c</sup>	0.74	1.87
$\text{Co}^{2+}$	8.9	6.3	1.65	0.70	1.96
$\text{Ni}^{2+}$	9.9	4.3	1.59	0.66	1.93
$\text{Cu}^{2+}$	8.0	8.3	2.33	0.69	1.82
$\text{Zn}^{2+}$	9.0	7.7	1.73	0.71	1.97
$\text{Cd}^{2+}$	9.9	9.7	2.05	0.91	2.15 <sup>b</sup>
$\text{Tb}^{3+}$	8.16	8.7	2.03 <sup>d</sup>		

<sup>a</sup>The values for each parameter included were taken from the following sources with exceptions noted: values for  $\text{p}K_{\text{a}} (\text{H}_2\text{O})$  from Perrin (1974) Buffers for pH and Metal Ion Control, p. 104, Halsted Press, New York. Values for  $\log k_{\text{ex}} (\text{H}_2\text{O})$  were taken from Hughes (1981) Inorganic Chemistry of Biological Processes, p. 45, Wiley, New York. Values for  $\log K_1 (\text{pNPP})$  from Massoud and Sigel (1988) *Inorg. Chem.* **27**, 1447–1453. Values for  $\text{M}^{2+}$  radii and for M-L Distance ( $\text{\AA}$ ) are from Glusker (1993) *Adv. Protein Chem.* **42**, 9–19. The exception was  $\text{Tb}^{3+}$ , for which the value of  $\text{p}K_{\text{a}} (\text{H}_2\text{O})$  was taken from Lange's Handbook of Chemistry (Dean, J. A., Ed.) (1995) p. 8.17, McGraw Hill, New York. and the value for  $\log k_{\text{ex}} (\text{H}_2\text{O})$  from Lincoln and Merbach (1995) *Adv. Inorg. Chem.* **42**, 2–88.

<sup>b</sup>The values of minimum M-L distances for  $\text{Sr}^{2+}$  and  $\text{Cd}^{2+}$  were estimated from the linear relationship of radius ( $\text{\AA}$ ) and minimum M-L distances ( $\text{\AA}$ ) for metal ions with known values.

<sup>c</sup>The value for  $\log K_1$  of pNPP with  $\text{Fe}^{2+}$  was estimated from the value measured for  $\log K_1$  for  $P_1$  with  $\text{Fe}^{2+}$  based on 1:1 correlation of these parameters.

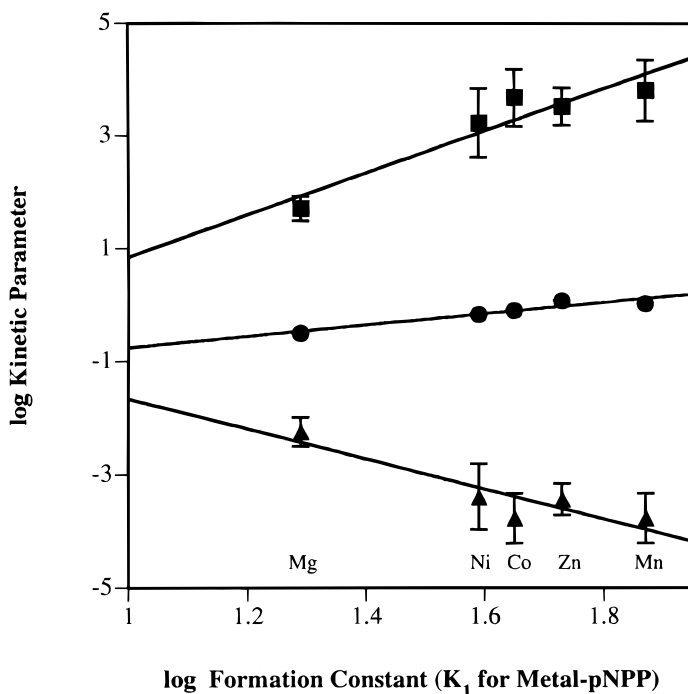
<sup>d</sup>The value for  $\log K_1$  of pNPP with  $\text{Tb}^{3+}$  was estimated from the value measured for  $\log K_1$  for EDTA with  $\text{Tb}^{3+}$  based from correlation analysis of the formation constants with these ligands.

The geometry of the metal ion complexes may limit the ability of metal ions to activate calcineurin. All metal ions which support activity have a minimum metal to ligand distance in hexa-coordinate compounds within the range 1.91 to 2.10 Å; those metal ions which did not support activity have minimum metal to ligand distances outside this range (both greater and lesser). That activating metal ions all come within this range may be a hint that functional metal ion must be oriented suitably within a defined spatial region. A metal ion complex with a high metal to ligand distance may orient the metal ion too great a distance away from the substrate to effectively result in activation of the enzyme. For example,  $\text{Ca}^{2+}$  compounds typically show a minimum metal to ligand distance of 2.23 Å and failed to support significant activity. Indeed, activity with  $\text{Ca}^{2+}$  was not significantly different from activity in the absence of added exogenous metal ion. A substitution inert cobalt compound,  $\text{Co}(\text{NH}_3)_6^{3+}$ , has a similar metal to ligand distance as  $\text{Mg}(\text{H}_2\text{O})^{2+}$  and was found to activate calcineurin (33).

### *Dependence on Properties of Metal Ion Complexes*

These considerations do not provide any conclusive explanation for the differences between activating metal ions. Possible functions for metal ions in phosphate ester hydrolysis include: (1) activation of the nucleophilicity and/or basicity of coordinated water for attack on the phosphate ester; (2) coordination and activation of the phosphate ester substrate; and (3) stabilization of the developing leaving group. There are properties of metal ions which may influence the ability of a metal ion in these functions. The  $\text{p}K_a$  for water coordinated to metal ion is a critical determinant of the charge state and reactivity of coordinated water. Exchange of coordinated water with solvent will likely influence the activation of the water molecule with faster exchange likely be coupled with lowered reactivity. The association of metal ion with substrate as measured by the formation constant of the complex may directly measure the interaction of metal ion with the substrate or may be an indirect measure of a metal ion interaction with another ligand such as an amino acid in the enzyme. Thus, parameters to consider as determining metal ion activation include:  $\text{p}K_a$  for water coordinated to the metal ion (M-H<sub>2</sub>O); the exchange rate for water coordinated to the metal ion ( $\log k_{\text{ex}}$  for H<sub>2</sub>O); and the association of metal ion with the substrate ( $\log K_1$  with pNPP). Values for these parameters are provided in Table 1.

Correlation of the measured kinetic parameters to each these properties was attempted. There were no statistically significant correlations involving the  $K_m$  parameter. The parameter  $\log k_{\text{cat}}$  was moderately correlated ( $r = 0.76$ ) to  $\log k_{\text{ex}}$  and highly correlated ( $r = 0.95$ ) to  $\log K_1$  (pNPP).  $\log K_{\text{act}}$  and  $\log k_{\text{cat}}/K_{\text{act}}$  were moderately correlated ( $r = 0.69$  and  $0.71$ , respectively) to  $\text{p}K_a$  (M-H<sub>2</sub>O), but highly correlated ( $r = 0.91$  and  $0.94$ , respectively) to  $\log K_1$  (pNPP). The correlation plots with  $\log K_1$  (pNPP) are shown in Fig. 1. The relationships derived from these plots, however, did not explain the absence of activation by other metal ions. For example, the parameters  $\log K_{\text{act}}$  and  $\log k_{\text{cat}}/K_{\text{act}}$  increased and decreased, respectively, with  $\log K_1$  (pNPP), but from these relationships comparable activation by  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  would be expected. Activation by  $\text{Ca}^{2+}$  was not observed. Similarly, the correlation of  $\log k_{\text{cat}}$  to  $\log K_1$  (pNPP) did not explain the difference between  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . A single parameter was not sufficient to explain the differences between the various metal ions.



**FIG. 1.** Single regression relationships of metal ion activation kinetic parameters. The values for  $\log k_{cat}$  (circles);  $\log K_{act}$  (triangles); and  $\log k_{cat}/K_{act}$  (squares) are plotted against  $\log K_1$  (pNPP). The kinetic parameters are from Refs. 31 and 33. Error bars were omitted from the figure for clarity. In most cases, the error was less than the size of the symbol.

### *An Empirical Quantitative Model for Metal Ion Activation*

Although single parameter models could not explain the ability of metal ions to serve as activators, the trends identified in Fig. 1 did provide a starting point. For example,  $Ca^{2+}$  has a lower value for  $\log K_1$  (pNPP) and would be predicted toward a lower value of  $k_{cat}/K_{act}$  based on this property alone. Similarly, the value of  $k_{cat}/K_{act}$ , would be expected to be lower based on the trend with  $pK_a$  (M-H<sub>2</sub>O). Regression analysis was extended to include multiple properties. The kinetic parameters with  $Mn^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Zn^{2+}$ , and  $Mg^{2+}$  were fit to a multiparameter equation using a stepwise-regression algorithm contained in the Statview statistical package (Abacus Concepts) and yielded the following two parameter equations with the properties  $pK_a$  (M-H<sub>2</sub>O) and  $\log K_1$  (pNPP) of the evaluated metal ions as variables (Eqs. [1] and [2]):

$$\log k_{cat} = 0.86 (\pm 0.15) \log K_1 - 0.06 (\pm 0.03) pK_a + 0.92$$

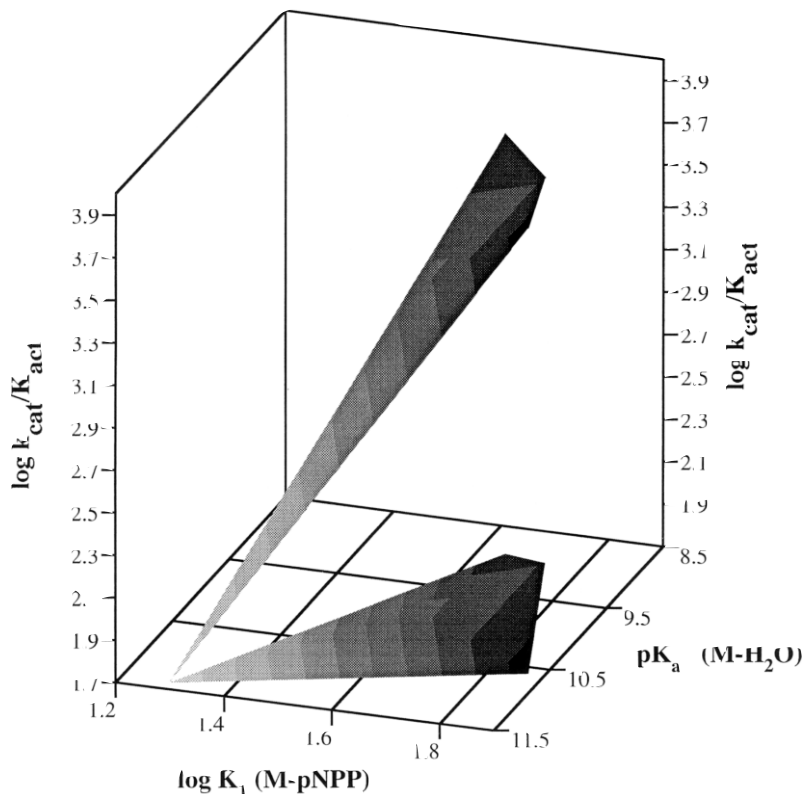
with statistical parameters:  $r = 0.98$ ;  $F = 31.6$  ( $P = 0.03$ ) [1]

$$\log k_{cat}/k_{act} = 3.1 (\pm 0.6) \log K_1 - 0.26 (\pm 0.12) pK_a + 0.66$$

with statistical parameters:  $r = 0.98$ ;  $F = 27.5$  ( $P = 0.035$ ) [2]

For both kinetic constants, inclusion of  $\log k_{\text{ex}}(\text{H}_2\text{O})$  as a third variable did not improve the data fit and caused a detrimental change in the statistical characteristics. Two parameter fits using  $\log k_{\text{ex}}(\text{H}_2\text{O})$  and  $\log K_1(\text{pNPP})$ , or  $\log k_{\text{ex}}(\text{H}_2\text{O})$  and  $\text{p}K_a(\text{M-H}_2\text{O})$  did not yield statistically significant equations. Moreover, the properties  $\log k_{\text{ex}}(\text{H}_2\text{O})$  and  $\log K_1(\text{pNPP})$  were identified as colinear variables for the available data set. The magnitude of the regression coefficients did seem to indicate that  $\log K_1(\text{pNPP})$  was more important than  $\text{p}K_a(\text{M-H}_2\text{O})$  in the activation process. Step-wise regressions of  $\log K_{\text{act}}$ ,  $\log K_m$ , or  $\log k_{\text{cat}}/K_m$  did not provide any statistically significant multivariate parameter fits.

The dependency of  $\log k_{\text{cat}}/K_{\text{act}}$  on  $\text{p}K_a(\text{M-H}_2\text{O})$  and  $\log K_1(\text{pNPP})$  is shown in Fig. 2 as an x-y-z plot with the shape radiating into z-space from the data point for  $\text{Mg}^{2+}$ , the lowest value of  $\log k_{\text{cat}}/K_{\text{act}}$ . The coefficients for these two parameters were opposite in sign so greater effectiveness was favored by a higher  $\log K_1(\text{pNPP})$  and a lower  $\text{p}K_a(\text{M-H}_2\text{O})$ ; that is, tighter association and greater acidity tended to favor greater effectiveness of the metal ion as an activator of calcineurin. The figure also depicts the projection of the shape onto the bottom grid (x-y plane) to show limiting values of the metal ion prop-



**FIG. 2.** Plot of multi-property relationship of metal ion activation kinetic parameters. Equation 1 defined a funnel-like shape projecting upward into the  $\log k_{\text{cat}}/K_{\text{act}}$  plane. The bottom grid displays the downward projection of the funnel shape onto a two-dimensional surface and the shape of the projection indicates the limiting values of  $\text{p}K_a(\text{M-H}_2\text{O})$  and  $\log K_1(\text{pNPP})$  suitable for enzyme activation based on available information.

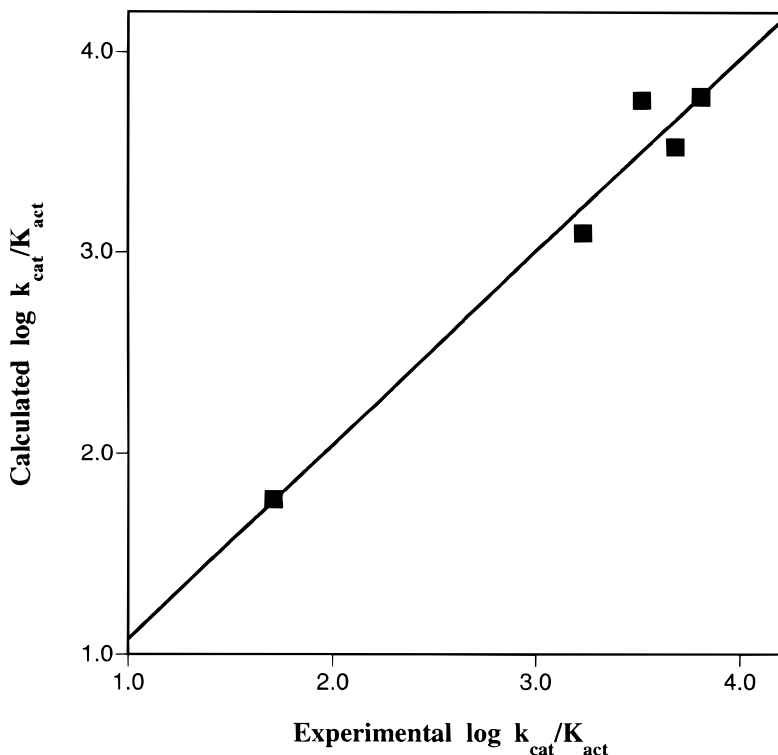
erties for enzyme activation. As a first approximation, only metal ions with values of  $pK_a$  ( $M-H_2O$ ) and  $\log K_1$  ( $pNPP$ ) within these limits would be expected to support significant calcineurin activity. A plot of Eq. [2] was similar (not shown).

Calculated (from Eq. [1]) values for  $\log k_{cat}/K_{act}$  are collected in Table 2 along with the experimentally determined values. There was high correlation (Fig. 3) between the calculated and experimental values, although the relative order of  $Co^{2+}$  and  $Zn^{2+}$  was interchanged. Similar results were found using Eq. [2] (not shown). The two parameter fits from both equations yielded predicted values which were better correlated to the experimental values than did the best single parameter fit ( $\log K_1$ ). Equation [1] was used to predict values of  $22.6 M^{-1} s^{-1}$  and  $6.0 M^{-1} s^{-1}$  for  $k_{cat}/K_{act}$  with  $Ca^{2+}$  and  $Sr^{2+}$ , respectively, as these metal ions have a low values for  $\log K_1$  with pNPP and are not very acidic. The predicted value for  $Ca^{2+}$  was only 0.4% the value measured for  $Mn^{2+}$  (Table 2) consistent with the relative failure of  $Ca^{2+}$  and  $Sr^{2+}$  to activate calcineurin. Similar calculations using Eq. [2] yielded estimates for  $k_{cat}$  and  $K_{act}$  with  $Ca^{2+}$  of  $0.25 s^{-1}$  and 11 mM, respectively. Using these predicted parameters as a guide, kinetics were done using  $Ca^{2+}$  from 5.6 to 50.0 mM (approximately 0.5 to 5.0 times predicted  $K_{act}$ ). Even with 50.0 mM pNPP (varied from 5.0 to 50.0 mM), measured activity was indistinguishable from activity measured in the absence of metal ion. No kinetic parameters were evaluated for  $Ca^{2+}$  and the predicted expectations could not be validated. The larger size of  $Ca^{2+}$  and the greater metal to ligand distance may preclude effective orientation of the metal ion in the activation site.

The activation of calcineurin by terbium ( $Tb^{3+}$ ) was used as a test of Eqs. 1 and 2 for characterizing activation of calcineurin. Choosing  $Tb^{3+}$  also provided a chance to evaluate whether the model defined by the equations fit only main-table elements or more broadly within the periodic table. Calcineurin was previously found to be activated by  $Tb^{3+}$  and the measured kinetic parameters did result in the highest level of  $k_{cat}/K_{act}$  ( $9750 M^{-1} s^{-1}$ ) for any of the metal ions characterized (31).  $Tb^{3+}$  typically has a higher formation constant with ligands and is more acidic than  $Ca^{2+}$  and any of the metal ions characterized. The values of these properties for  $Tb^{3+}$  are similar to values for  $Cu^{2+}$  providing another perspective on calcineurin activation. With values for  $pK_a$  ( $Tb^{3+} - H_2O$ ) = 8.16 and  $\log K_1$  ( $Tb^{3+} - pNPP$ ) = 2.03, Eq. [1] predicted that  $Tb^{3+}$  would be a good activator of enzyme activity; a high value for  $k_{cat}/K_{act}$  would be mea-

TABLE 2  
Predicted Values for Metal Ion Activation

Activator	Experimental $\log k_{cat}/K_{act}$	Predicted $\log k_{cat}/K_{act}$	Percentage difference from experimental $\log k_{cat}/K_{act}$
$Mg^{2+}$	1.71	1.77	3.5 (increase)
$Mn^{2+}$	3.80	3.78	0.5 (decrease)
$Co^{2+}$	3.68	3.53	4.1 (decrease)
$Ni^{2+}$	3.23	3.10	4.0 (decrease)
$Zn^{2+}$	3.51	3.76	6.8 (increase)
$Tb^{3+}$	3.99	4.90	22.8 (increase)
$Ca^{2+}$	Not done	1.36	—
$Sr^{2+}$	Not done	0.79	—



**FIG. 3.** Comparison of experimental and calculated values. Shown is the plot of calculated and experimental values of  $\log k_{\text{cat}}/K_{\text{act}}$ . The equation of the line is Predicted  $\log k_{\text{cat}}/K_{\text{act}} = 0.96 * \text{Experimental } \log k_{\text{cat}}/K_{\text{act}} + 0.11$  ( $r = 0.98$ ).

sured with the predicted value of  $\log k_{\text{cat}}/K_{\text{act}} \approx 4.90$ ). Indeed,  $\text{Tb}^{3+}$  was predicted to be the most effective activator based on the  $k_{\text{cat}}/K_{\text{act}}$  parameter. The experimental (3.99) and predicted (4.90) values of  $\log k_{\text{cat}}/K_{\text{act}}$  were in good agreement, but the difference was greater than found for other metal ions (Table 2), indicating limitations of the current quantitative model. In a similar calculation, Eq. [2] yielded an overestimated predicted value of  $k_{\text{cat}}$  for  $\text{Tb}^{3+}$  activation with a predicted  $k_{\text{cat}}$  value =  $2.1 \text{ s}^{-1}$  compared to an experimental  $k_{\text{cat}}$  value  $\approx 0.78 \text{ s}^{-1}$ . The predicted value of  $K_{\text{act}}$  was calculated as  $0.026 \text{ mM}$ ; an underestimation of the experimental value of  $0.08 \text{ mM}$ . These differences between predicted and experimental values for  $\text{Tb}^{3+}$  likely result from the different properties of  $\text{Tb}^{3+}$  compared to the main-group transition metal ions.  $\text{Tb}^{3+}$  and the other lanthanides generally form nine-coordinate complexes, not six-coordinate complexes prevalent with the transition metal ions. These different characteristics may skew the fit characterized by Eq. [1] and [2] when nonmain table metal ions are used. Alternatively, the underestimation of  $k_{\text{cat}}/K_{\text{act}}$  by this approach may also signal that there is a limiting value for this term; a maximum effectiveness for activation of calcineurin by any metal ion. The interaction and appropriate orientation of metal ion may provide constraints on the association between calcineurin and metal ion and subsequently provide restrictions on substrate turnover.



### *Mechanistic Role of Exogenous Metal Ion*

Activation of calcineurin by various metal ions was found to be distinct as evidenced by the varied  $k_{\text{cat}}/K_{\text{act}}$  values. Properties of the metal ion complexes seemingly important for effective enzyme activation were identified. The identified properties were the formation constant of substrate-metal ion interaction ( $\log K_1$  with pNPP) and the  $\text{p}K_{\text{a}}$  of water coordinated to metal ion ( $\text{M}-\text{H}_2\text{O}$ ). Equations were empirically developed for the relationship of  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{act}}$  to these characteristics. Identification of these properties was consistent with the catalytic role previously assigned (28) to one of the metal ions. The function of this metal ion was proposed as interacting with substrate and stabilizing the developing leaving group; this function can now be assigned to the exogenous metal ion required for optimal calcineurin activity. The remaining function, activation of water for the cleavage reaction (28), is assigned to one of the intrinsic metal ions (11,12) as found in catalysis by the purple acid phosphatases (13,14,39). Such a role for intrinsic metal ion(s) would be consistent with the reported inactivation of calcineurin by oxidizing reagents and protection by superoxide dismutase (40). Using substitution inert cobalt compounds (33), exogenous metal ion participation in calcineurin activity was concluded to function through an outer sphere coordination interaction. Indeed, calcineurin catalysis may be partially limited by this interaction based on comparisons to other phosphatases (33).

Similar mechanisms may be operational with other metal ion-activated enzymes. For example, consider alkaline phosphatase which is also a metallo-phosphatase (see reference 41 for a mini-review). Unlike calcineurin and the purple acid phosphatases, the reaction mechanism of alkaline phosphatase generates a phosphoryl-enzyme intermediate with a nucleophilic serine attacking the phosphate ester. There are similarities, however, in the chemistry of these enzymes. The hydroxyl group of the active site serine is activated by intrinsic metal ion (zinc) as the water is activated by metal ions in calcineurin and the purple acid phosphatases. Similar to calcineurin, some forms (typically from mammalian sources) of alkaline phosphatase also require exogenous metal ion for optimal catalysis by stabilizing the active site particularly the appropriate orientation of the substrate. In these forms of alkaline phosphatase, intrinsic metal ions are not sufficient to achieve effective catalysis. Murphy and Kantrowitz (41) suggest that mammalian alkaline phosphatases may require exogenous metal ( $\text{Mg}^{2+}$ ) to induce the appropriate structure of the active site for catalysis. Exogenous metal ion likely functions the same in calcineurin catalysis. The recognition of exogenous metal ion by calcineurin may be critical for appropriate regulation of its activity.

### *Summary*

Calcineurin activation by metal ions was characterized by an empirical model relating activation to properties of the activating metal ions. Activation (compared using  $\log k_{\text{cat}}$  or  $\log k_{\text{cat}}/K_{\text{m}}$  values) was seemingly connected to two variables, with greater activation correlated to higher formation constants between metal ion and substrate pNPP and to lower  $\text{p}K_{\text{a}}$  values for water coordinated to the activating metal ion. Based on the developed model, a specific function was tentatively assigned to exogenous metal ion in the mechanism of calcineurin. These data were also suggestive that the same approach could be applied to other catalyzed reactions, involving phosphate ester chemistries and other metal ion activated reactions. An example of the hairpin

class of catalytic RNA recently was shown to be activated by  $\text{Co}(\text{NH}_3)_6^{3+}$  (42) with the authors concluding that exogenous metal ion served to orient the active site and stabilize the reaction intermediate. Using outer sphere coordination, as does calcineurin, the action of exogenous metal ion in this reaction is likely directed by the interaction with substrate. It is feasible that examination of other metal ions in the hairpin RNA reaction would provide a similar fit as found for calcineurin.

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