

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 (Mn^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} , and 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, Ca^{2+} , and 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, pNPP² (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 $\mu\text{g/ml}$ calmodulin; and 2–10 $\mu\text{g/ml}$ calcineurin in 800 μl . 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

² Abbreviations used: EGTA, ethylene glycol bis(β -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 Zn^{2+} was a notable difference from other studies, although there was an early report of the activation of calcineurin by Zn^{2+} (9). Transition metal ions all activated calcineurin more effectively than did 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 Å) of the cation; and (5) minimal average distance (Å) 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^a

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

^aThe 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 M^{2+} radii and for M-L Distance (Å) are from Glusker (1993) *Adv. Protein Chem.* **42**, 9–19. The exception was 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.

^bThe values of minimum M-L distances for Sr^{2+} and Cd^{2+} were estimated from the linear relationship of radius (Å) and minimum M-L distances (Å) for metal ions with known values.

^cThe value for $\log K_1$ of pNPP with Fe^{2+} was estimated from the value measured for $\log K_1$ for P_i with Fe^{2+} based on 1:1 correlation of these parameters.

^dThe value for $\log K_1$ of pNPP with Tb^{3+} was estimated from the value measured for $\log K_1$ for EDTA with 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, Ca^{2+} compounds typically show a minimum metal to ligand distance of 2.23 Å and failed to support significant activity. Indeed, activity with 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 ($\text{M-H}_2\text{O}$); the exchange rate for water coordinated to the metal ion ($\log k_{\text{ex}}$ for H_2O); 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 $\text{p}K_a$ ($\text{M-H}_2\text{O}$), 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 Mg^{2+} and Ca^{2+} would be expected. Activation by Ca^{2+} was not observed. Similarly, the correlation of $\log k_{\text{cat}}$ to $\log K_1$ (pNPP) did not explain the difference between Mg^{2+} and Ca^{2+} . A single parameter was not sufficient to explain the differences between the various metal ions.

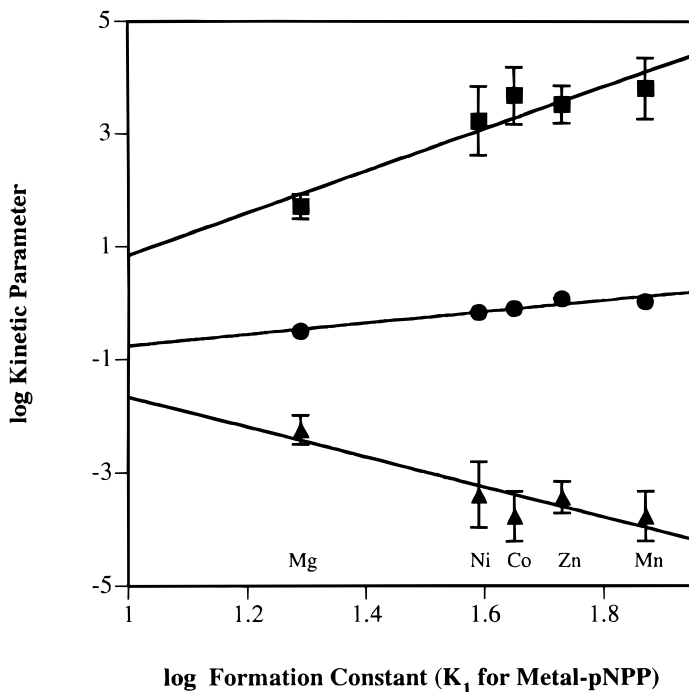


FIG. 1. Single regression relationships of metal ion activation kinetic parameters. The values for $\log k_{cat}$ (circles); $\log K_{act}$ (triangles); and $\log k_{cat}/K_{act}$ (squares) are plotted against $\log K_1$ (pNPP). The kinetic parameters are from Refs. 31 and 33. Error bars were omitted 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-H₂O). 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-H₂O) 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]