Comparison of the Reaction Progress of Calcineurin with Mn^{2+} and Mg^{2+} †

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ABSTRACT: Activation of calcineurin by Mn^{2+} and Mg^{2+} was compared using a heavy atom isotope analogue of the substrate *p*-nitrophenyl phosphate (pNPP). Heavy atom isotope effects were measured for Mg^{2+} activation and compared to published results of the isotope effects with Mn^{2+} as the activating metal. Isotope effects were measured for the kinetic parameter V_{max}/K_m at the nonbridging oxygen atoms [$^{18}(V/K)_{nonbridge}$]; at the position of bond cleavage in the bridging oxygen atom [$^{18}(V/K)_{bridge}$]; and at the nitrogen atom in the nitrophenol leaving group [$^{15}(V/K)$]. The isotope effects increased in magnitude upon changing from an optimal pH to a nonoptimal pH; the $^{18}(V/K)_{bridge}$ effect increased from 1.0154 (±0.0007) to 1.0198 (±0.0002), and the $^{15}(V/K)$ effect increased from 1.0018 (±0.0002) to 1.0021 (±0.0003). The value for $^{18}(V/K)_{nonbridge}$ is 0.9910 (±0.0003) at pH 7.0. As with Mn^{2+}, the $^{18}(V/K)_{nonbridge}$ isotope effect indicated that the dianion was the substrate for catalysis, and that a dissociative transition state was operative for the phosphoryl transfer. Comparison to results for Mn^{2+} activation suggested that chemistry was more rate-limiting with Mg^{2+} than with Mn^{2+}. Changing the activating metal concentration showed opposite trends with increasing Mg^{2+} increasing the commitment factor and seemingly making the chemistry less rate-limiting. The influence of viscosity was evaluated as well to gauge the role of chemistry. The activation of calcineurin-catalyzed hydrolysis of pNPP[1] by Mg^{2+} or Mn^{2+} at pH 7.0 was compared in the presence of viscogens, glycerol and poly(ethylene glycol). Increasing glycerol caused different effects with the two activators. With Mn^{2+} as the activator, calcineurin activity showed a normal response with k_{cat} and k_{cat}/K_m decreasing with viscosity. There was an inverse response with Mg^{2+} as the activator as values of k_{cat}/K_m increased with viscosity. From values of the normalized k_{cat}/K_m with Mn^{2+}, the chemistry was found to be partially rate-limiting, consistent with previous heavy atom isotope studies (*22*). The effect observed for Mg^{2+} seems consistent with a change in the rate-limiting step for the two different metals at pH 7.0.

The regulation of many biological processes is accomplished by the formation and cleavage of phosphate esters, which is balanced by the interplay of protein kinases and phosphatases. Protein kinases and protein phosphatases are broadly classified by target specificity into serine and threonine specific, or tyrosine-directed enzymes. Protein serine/threonine phosphatases are seemingly distinguished by utilizing metal-ion catalysis in contrast to the exploitation of a nucleophilic cysteine by tyrosine phosphatases. There are four major protein serine/threonine phosphatase classes (*1*, *2*) designated types-1, -2A, -2B, and -2C and distinguished primarily by substrate specificities and susceptibility to specific phosphatase inhibitors. Phosphatases-1, -2A, and -2B share extensive sequence identity and are considered to belong to a single gene family (*3*). Recently crystal structures of both PP-1 (*3*, *4*) and calcineurin (PP-2B; *5*, *6*) have been obtained. These enzymes have very similar active sites, and these resolved crystal structures have identified the presence of two metal ions in the putative active sites.

As a member of this gene family, calcineurin is of considerable interest because of its target as a receptor of the cyclosporin-A/cyclophilin complex in T cells. Calcineurin has distinctive features making it a useful model for examining phosphatase catalysis. As isolated from bovine brain, calcineurin has intrinsically bound iron and zinc (*6*−*8*), but requires exogenous metal for activity (*8*−*14*). Without exogenous metal, the activity is virtually indistinguishable from buffer alone. Calcineurin also hydrolyzes low molecular weight phosphate esters (*15*−*17*), providing chemical tools to investigate catalysis (*17*−*21*). Kinetic studies of the activation by metals have suggested a catalytic role for exogenous metal (*18*, *20*, *21*) and supported the idea that the metal ion may provide determinants for the enzymatic reaction. Differences were observed between transition metals and Mg^{2+}, the lone nontransition metal characterized.

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[1] Abbreviations: CaN, calcineurin; CaM, calmodulin; pNP, *p*-nitrophenol; pNPP, *p*-nitrophenyl phosphate; PTPase, protein-tyrosine phosphatase; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 4-morpholinepropanesulfonic acid.

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Activation by Mn^{2+} and Mg^{2+} was compared by introducing perturbations into the calcineurin reaction conditions, including the inclusion of viscogen in the reaction mixture and the introduction of substrate labeled with heavy atom isotopes. These data obtained provide a background to interpret the activation of calcineurin by divalent metals.

EXPERIMENTAL PROCEDURES

Materials. Buffers, EGTA,[1] DEAE-Sepharose, and phenyl-Sepharose used in protein isolation were purchased from Sigma. The unlabeled substrate, pNPP (Sigma 104 substrate), was also purchased from Sigma. DE-52 cellulose used in the isolation of calcineurin was obtained from Whatman. The viscogens used, glycerol and poly(ethylene glycol), were from Aldrich Chemical. Other chemicals (metal salts etc.) were obtained from Fisher. Bovine brain was obtained from Pel-Freez. Solutions, including those used in the preparation of calcineurin, were prepared from water that had been passed over a Chelex-100 column. The MOPS buffer used in the assays was likewise passed over Chelex-100 after preparation of the solution. The pH was checked after passing over Chelex-100 and adjusted to 7.0 before use in assays. [^{14}N]-*p*-Nitrophenol, [^{14}N]-*p*-nitrophenyl phosphate, [^{15}N, bridge-^{18}O]-*p*-nitrophenyl phosphate, and [^{15}N, nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate were synthesized and purified as previously described (*23*). Diethyl ether used in extractions was distilled before use.

Proteins. Bovine brain calcineurin was isolated from bovine brain by the method of Sharma et al. (*24*) with MOPS buffers instead of Tris buffers. Calmodulin was purified by the procedure of Sharma and Wang (*25*) slightly modified to include a final chromatography step on phenyl-Sepharose with elution by EGTA (*26*). Protein concentrations were determined by the method of Bradford (*27*).

Calcineurin Assay. Calcineurin was assayed at 30 °C on a Cary Model 1E spectrophotometer with pNPP as the substrate by measuring the release of pNP at 410 nm. Standard reaction conditions were 25 mM MOPS, pH 7.0, 1.0 mM $MnCl_2$, 10 mM pNPP, 5−10 μg/mL calmodulin, and 5−10 μg/mL calcineurin in 800 μL reaction volume. Substrate kinetics were measured for a range of pNPP concentrations from 5.0 to 50.0 mM in the presence of 1.0 mM Mn^{2+} or 20.0 mM Mg^{2+}. These concentrations of metals represent 4 × K_{act}, respectively, for the metals. Kinetic parameters also were evaluated in the absence and the presence of added viscogen. The viscogens glycerol and poly(ethylene glycol) were added to final concentrations as indicated in the figures. Kinetic parameters, k_{cat} and K_m, were determined using EnzFitter (Elsevier BioSoft Inc.) or Enzyme Kinetics (Trinity Software) computer programs.

Heavy Atom Isotope Effect Measurements. The isotope effects[2] in this study were measured using an isotope ratio mass spectrometer by the competitive method with a doubly labeled substrate as described in an earlier investigation of calcineurin (*22*). The isotope effect experiments were performed at 37 °C in buffered solutions (0.1 M) of MOPS (pH 7.0) or TRICINE (pH 8.5) containing 20 mM or 100 mM $MgCl_2$. For measurement of each isotope effect, reactions were started with approximately 100 μmol of pNPP substrate. Reactions were performed and the isotopic ratio measured as previously described (*22*).

Isotope effects were calculated from the isotopic ratio of nitrogen in the pNP product at partial reaction (R_p), in the residual substrate (R_s), and in the starting material (R_o). Equation 1 was used to calculate the observed isotope effect from product and starting material at fraction of reaction *f*, and eq 2 was used to calculate the observed isotope effect from residual substrate and starting material. Thus, each experiment yields two independent measurements of the isotope effect:

$$\text{isotope effect} = \log (1 - f)/\log [(1 - f)(R_p/R_o)] \quad (1)$$

$$\text{isotope effect} = \log (1 - f)/\log [(1 - f)(R_s/R_o)] \quad (2)$$

At least four independent determinations were made of each isotope effect. The results calculated from R_p and R_o, and from R_s and R_o, were within experimental error in all cases, as were those measured at differing fractions of reaction. The observed ^{18}O isotope effects from these experiments were corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material (*29*). The isotopic ratio of nitrogen in the starting material, R_o, was determined both by direct combustion of samples of pNPP substrate and, as a control, by completely hydrolyzing samples of pNPP and analyzing the liberated nitrophenol following isolation by the same method used in the isotope effect experiments. The isotopic ratios obtained from both methods were the same within experimental error, showing that no isotopic fractionation occurs during the procedures used to recover pNP.

RESULTS AND DISCUSSION

The phosphatase activity of calcineurin can be activated by both transition and nontransition metals although distinct differences can be identified in the kinetic parameters, notably the estimation for K_{act} of the metal. Values for this parameter range from 0.2 to 0.4 mM for first-row transition metals to approximately 5 mM for Mg^{2+} (*30*). The underlying basis for the differences in metal activation is not clearly understood.

Effect of Viscosity on Mn and Mg Activation of Calcineurin. These differences suggest differences in the catalytic process depending on the presence of Mg^{2+} or Mn^{2+}. One approach to identify the relative contributions of various steps to catalysis is the evaluation of the effects of viscosity on the kinetic parameters of the enzyme.

Experiments were performed with glycerol as viscogen. Glycerol is a microviscogen affecting both the viscosity of the solution and the rate of diffusion of small molecules. Poly(ethylene glycol) and other agents are macroviscogens, and influence only the bulk viscosity of the solution. Kinetic parameters are evaluated with varied concentrations of viscogen and plotted in the form $(k_{cat})_{ctrl}/(k_{cat})_\eta$ as a function of relative viscosity. These terms represent the kinetic parameters in the absence of viscogen ($k_{cat,ctrl}$) and in the presence of the viscogen ($k_{cat,\eta}$). For k_{cat}, a slope of 1.0 signifies that the chemistry is fast compared to the dissociation of product(s) and dissociation is likely rate-limiting. A

[2] The notation used to express isotope effects is that of Northrop (*28*) where a leading superscript of the heavier isotope is used to indicate the isotope effect on the following kinetic quantity; for example, $^{15}k_3$ denotes $^{14}k_3/^{15}k_3$, the nitrogen-15 isotope effect on the rate constant k_3.

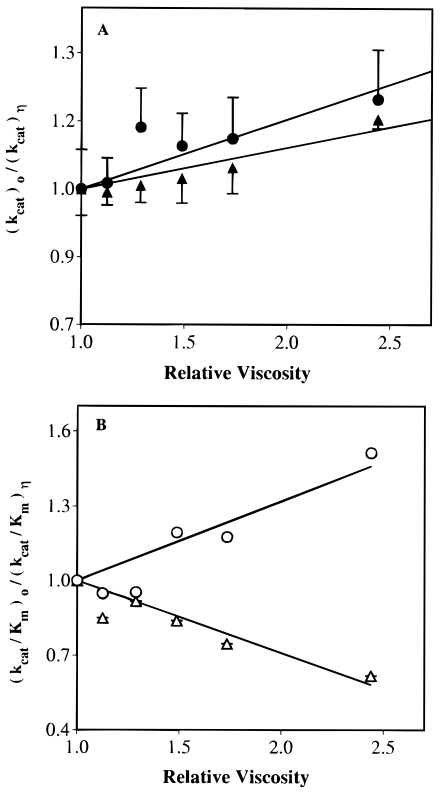

FIGURE 1: Viscosity dependence of calcineurin catalysis. Substrate kinetics were done in the presence of glycerol as indicated with Mn^{2+} (circles) or Mg^{2+} (triangles). The parameters k_{cat} and K_m were evaluated for the hydrolysis of pNPP by calcineurin in the presence of increasing glycerol. The parameters were plotted as normalized values in the form $(k_{cat})_{ctrl}/(k_{cat})_\eta$ or $(k_{cat}/K_m)_{ctrl}/(k_{cat}/K_m)_\eta$. Panel A shows the plot for k_{cat}, and panel B shows the plot for k_{cat}/K_m. Error bars are present in both panels, but are smaller than the symbol in panel B.

value <1.0 has decreased contribution of product dissociation with a low value (0.1−0.2) of $(k_{cat})_{ctrl}/(k_{cat})_\eta$ consistent with slow chemistry compared to product dissociation (*31*, *32*).

Ranging from 0 to 40% glycerol, the parameters k_{cat} and K_m were measured in the presence of either Mn^{2+} or Mg^{2+}. The parameter k_{cat} decreased with increasing viscosity with either metal. Shown in Figure 1A are the normalized plots for k_{cat} in the absence and presence of varied viscogen. Slopes were estimated as 0.15 for Mn^{2+} and 0.09 for Mg^{2+}. These slope terms are denoted as $(k_{cat})_\eta$ for each metal−enzyme pair. The dependency of the k_{cat}/K_m term showed significant differences and is diagrammed in Figure 1B. Surprisingly, the dependency on relative viscosity with Mg^{2+} showed an inverse viscosity effect compared to Mn^{2+}, with slopes measured as 0.32 and −0.29 for Mn^{2+} and Mg^{2+}, respectively, and denoted as $(k_{cat}/K_m)_\eta$. This was interpreted as caused by a different rate-limiting step for the Mg^{2+}-activated enzyme, consistent with the altered commitment to catalysis found with this metal.

The low slope value with the k_{cat} term for Mn^{2+} activation was consistent with the chemical step, phosphate ester cleavage, dominating the rate-limiting step of the reaction. A low value for the k_{cat}/K_m term indicated that substrate was not sticky, not surprising considering the high K_m of the substrate. The data were further analyzed assuming the model shown in Figure 2 as representative of calcineurin hydrolysis. Included in Figure 2 is a nonchemical step (k_3*) representing a hypothetical conformational change or other nonchemical step following binding of pNPP and preceding catalysis. The model is consistent with previous kinetic characterization of the reaction. The kinetic parameters in the absence and presence of viscogen can be described by eqs 3 through 6. The kinetic constants are as shown in Figure 2 with steps in the absence of viscogen represented by the terms $k_{-2}{}^0$ and $k_4{}^0$:

$$k_{cat} = \frac{(k_3^* + k_3)k_4}{(k_3^* + k_3) + k_4} \quad (3)$$

$$k_{cat}/K_m = \frac{k_2(k_3^* + k_3)}{k_{-2} + (k_3^* + k_3)} \quad (4)$$

$$(k_{cat})_\eta = \frac{(k_3^* + k_3)}{(k_3^* + k_3) + k_4{}^0} \quad (5)$$

$$(k_{cat}/K_m)_\eta = \frac{(k_3^* + k_3)}{k_{-2}{}^0 + (k_3^* + k_3)} \quad (6)$$

From these equations, the measured kinetic parameters, and the measured viscosity effects, estimates of rate constants for individual steps can be calculated. The calculations were straightforward for Mn^{2+} with a normal viscosity effect. The slopes with Mn^{2+} were estimated as 0.15 $(k_{cat})_\eta$ and 0.32 $(k_{cat}/K_m)_\eta$. Values for individual steps are collected in Table 1. The conformational (k_3*) and chemistry (k_3) steps were treated as a single process and represented with the term (k_3* + k_3). The value for (k_3* + k_3) was the rate-limiting rate constant evaluated and effectively matched the values of k_{cat} estimated for calcineurin hydrolysis. As expected from the low slope values, the constants for substrate association (k_2) and dissociation (k_{-2}) were significantly higher than for either the chemistry or the product dissociation steps.

The dependency of k_{cat} on Mg^{2+} showed a normal viscosity effect with a low-magnitude slope of 0.091. Using eqs 3 and 5, values for (k_3* + k_3) and k_4 were calculated for Mg^{2+} activation. Each of these individual steps was faster with Mn^{2+} than with Mg^{2+}, consistent with better activation typically found for Mn^{2+}. The k_{cat}/K_m term for Mg^{2+} showed an inverse viscosity effect with a slope less than zero (−0.29); k_{cat}/K_m increased with increasing relative viscosity. Equation 4 shows that k_{cat}/K_m will increase if either k_2 increases or k_{-2} decreases as viscosity increases. Equations 4 and 6 cannot be used to directly calculate values for k_2 and k_{-2}. Upper and lower bounds on these constants can be estimated if assumptions are made about the relative effect of the identity of the metal ion on k_2 and k_{-2}. One limiting situation is described when k_2 is independent of the metal, with the dissociation of substrate showing greater dependency. Then k_{-2} can be estimated from eq 4 as ≈0.83 s^{-1} with Mg^{2+} compared to 2.70 s^{-1} with Mn^{2+}. The other limiting situation is if k_{-2} is assumed to be independent of metal; then an estimate can likewise be made from eq 4 of 590 s^{-1} for k_2 compared to 232 s^{-1} for Mn^{2+}. Because k_2 and k_{-2} are the microscopic reverse of one another, then the metal ion must in reality affect both to the same degree. These assumptions, however, allowed estimations to be made

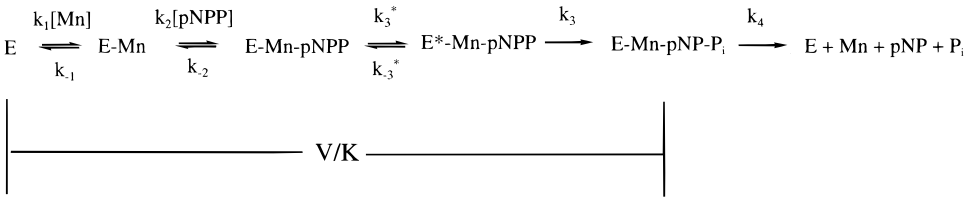

FIGURE 2: Reaction scheme for calcineurin-catalyzed hydrolysis. The kinetic reaction mechanism for calcineurin is shown with a hypothetical conformational step included (represented by k_3*). Steps after the association of the substrate pNPP are shown as irreversible as discussed in the text.

Table 1: Rate Constants for Metal-Activated Catalysis[a]

rate constant	Mn^{2+}	Mg^{2+}
k_{cat} (s^{-1})	1.08 ± 0.12	0.35 ± 0.02
K_m (mM)	14.5 ± 1.9	4.8 ± 1.0
k_{cat}/K_m (M^{-1} s^{-1})	74.3	72.9
$(k_{cat})_\eta$	0.15	0.091
$(k_{cat}/K_m)_\eta$	0.32	−0.29
k_2 (M^{-1} s^{-1})	232	see text
k_{-2}^0 (s^{-1})	2.70	see text
$k_3^* + k_3$ (s^{-1})	1.28	0.38
k_4^0 (s^{-1})	7.20	3.85

[a] Kinetics were done at pH 7.0 as described in the text. The terms $(k_{cat})_\eta$ and $(k_{cat}/K_m)_\eta$ represent the slopes of plots of $(k_{cat})_{ctrl}/(k_{cat})_\eta$ or $(k_{cat}/K_m)_{ctrl}/(k_{cat}/K_m)_\eta$ against relative viscosity. The kinetic terms k_2, k_{-2}^0, $k_3^* + k_3$, and k_4^0 were calculated from these slopes using the measured kinetic parameters and eqs 3−6.

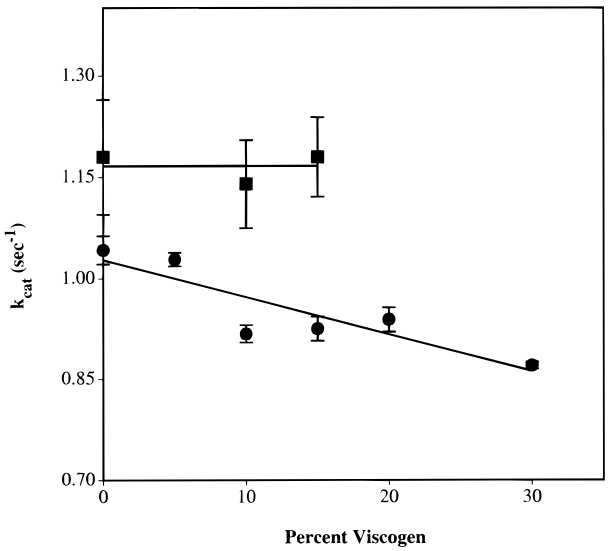

FIGURE 3: Effect of polyethylene glycol on calcineurin catalysis. Substrate kinetics were done in the presence of poly(ethylene glycol) with Mn^{2+} (circles) or Mg^{2+} (squares) as indicated. The parameters k_{cat} and K_m were evaluated for the hydrolysis of pNPP by calcineurin.

of the upper and lower bounds for these values and yielded estimates consistent with the observed kinetic parameters, particularly the lower K_m for pNPP in the presence of Mg^{2+}.

Similar kinetic experiments were done with poly(ethylene glycol) (average molecular weight 8000). Poly(ethylene glycol) is a macroviscogen and was expected to cause little change in the kinetics of the enzyme reaction. As shown in Figure 3, there was effectively no change in the k_{cat} parameter as PEG was increased, with a modest change in K_m for pNPP causing a concomitant change in k_{cat}/K_m. Because there was no effect on k_{cat}, it was concluded that bulk viscosity of the reaction solution had little kinetic effect.

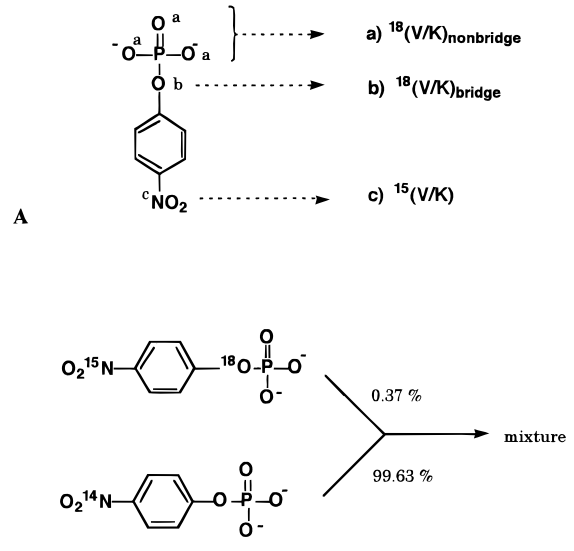

FIGURE 4: Structure of the heavy atom labeled pNPP substrate. The sites of heavy atom replacement are shown (panel A). Each molecule bearing an atom of O-18 in the position of interest also was labeled with N-15. Thus, the isotopic fractionation of nitrogen will act as a reporter for isotopic fractionation at the oxygen position. The two labeled versions of the substrate were mixed in the proportions shown (panel B), and the mixture was used as the substrate in the competitive method (*33*).

Heavy Atom Isotope Effects. Information distinguishing chemical and structural steps also can be obtained from enzymatic studies using substrate labeled with heavy atom isotopes. Using the competitive method yielding isotope effects on *V/K* (*33*), heavy atom isotope effects were measured for the hydrolysis of labeled pNPP (Figure 4) by calcineurin with Mg^{2+} as the activator. Previously, this approach was recently used with calcineurin to probe the effects of calmodulin and Mn^{2+} on the transition state (*22*). The isotope effects are sensitive only to steps from substrate binding up to and including the first irreversible step. The kinetic order has been studied[3] and showed that metal binding to calcineurin precedes pNPP binding as represented by the scheme in Figure 2. Any binding steps or conformational changes (k_3* step in Figure 2) that occur before binding of pNPP will have no effect on the isotope effects and are not considered in the isotope effects. The chemical step of phosphoryl transfer is considered essentially irreversible (*17, 18, 20*).

The isotope effects for the Mg^{2+}-activated CaN reaction with pNPP are listed in Table 2 with their standard errors. The isotope effects were measured at pH 7.0 and pH 8.5; pH 7.0 is the optimal pH for Mn^{2+} activation of calcineurin, but Mg^{2+} activation is lower than Mn^{2+} at this pH. Mg^{2+}

[3] Martin, B. L., and Rhode, D. J. (1998) submitted.

Table 2: Heavy Atom Isotope Effects for Calcineurin with Mn^{2+} and Mg^{2+} [a]

pH	metal	^{18}O-bridge	^{15}N	^{18}O-nonbridge
		Calcineurin with Mg^{2+} (Current Paper)		
7.0	20 mM Mg	1.0154 ± 0.0007	1.0018 ± 0.0002	0.9910 ± 0.0003
7.0	100 mM Mg	1.0130 ± 0.0006		
8.5	20 mM Mg	1.0198 ± 0.0002	1.0021 ± 0.0003	
		Calcineurin with Mn^{2+} (Reference *22*)		
7.0	1 mM Mn	1.0072 ± 0.0011	1.0006 ± 0.0005	
7.0	5 mM Mn	1.0103 ± 0.0014	1.0011 ± 0.0003	
8.5	1 mM Mn	1.0115 ± 0.0012	1.0014 ± 0.0001	0.9942 ± 0.0007
		Monoanion Hydrolysis (Reference *23*)		
		1.0106 ± 0.0003	1.0005 ± 0.0002	1.0224 ± 0.0005
		Dianion Hydrolysis (Reference *23*)		
		1.0230 ± 0.0005	1.0034 ± 0.0002	0.9993 ± 0.0007
		Alkaline Phosphatase (Reference *23*)		
6.0		1.0005 ± 0.0002	1.0003 ± 0.0003	0.9979 ± 0.0005
8.0		1.0003 ± 0.0004	1.0003 ± 0.0002	0.9982 ± 0.0001
		Protein Tyrosine Phosphatase (*Yersinia*) (Reference *39*)		
7.0		1.0142 ± 0.0004	0.9999 ± 0.0003	0.9981 ± 0.0015

[a] For measurements of heavy atom isotope effects for the calcineurin reaction, calmodulin was included at 5-fold molar excess over calcineurin.

activation is greater at pH 8.5 than at pH 7.0. These experiments were done with a 5-fold molar excess of calmodulin present and 20 mM Mg^{2+} (approximately 4-fold K_{act}) similar to the studies with Mn^{2+}. Based on the pK_a of 4.96 reported for pNPP (34), substrate was present essentially completely as the dianion at the pH values used in this study. Therefore, there will be no nonbridge oxygen isotopic fractionation affecting the population of the dianion protonation state of the substrate which would affect the observed values for $^{18}(V/K)_{nonbridge}$.

Whether the chemical step in an enzymatic reaction is rate-limiting and the isotope effects are the intrinsic effects for the chemical step can be determined by comparing isotope effects on enzymatic reactions with effects on uncatalyzed reactions because the possible suppression of isotope effects is not manifested in uncatalyzed reactions. The contribution of the chemical step can also be evaluated by determining the enzymatic isotope effects at optimal and nonoptimal pHs. At the nonoptimal pH, chemistry is slower and likely more rate-limiting. Full intrinsic isotope effects will be expressed at the optimal pH only if the commitment factor is negligible (*35*). A large commitment factor will completely suppress isotope effects and will yield isotope effects of unity at optimal and nonoptimal pH values and indicates that a nonchemical step after substrate binding is completely rate-limiting for V/K as found for alkaline phosphatase (*23*). A smaller commitment factor would suppress but not abolish isotope effects on the chemical step, and reactions at a nonoptimal pH will have reduced commitment and yield increased isotope effects closer to the intrinsic ones.

The isotope effect having the largest magnitude and, therefore, the most sensitive to commitment factors is the $^{18}(V/K)_{bridge}$ effect. The increase in magnitude of this isotope effect from 1.0154 at pH 7.0 to 1.0198 at pH 8.5 indicates that chemistry is only partially rate-limiting at pH 7.0 and somewhat more so at pH 8.5. If only the phosphoryl transfer step in the scheme in Figure 2 is isotope-sensitive, the observed isotope effect will be given by eq 7 where $^{KIE}k_{intrinsic}$ represents the intrinsic isotope effect (*36*) with the commitment factor equal to $(k_3/k_{-3}^*)(1 + k_3^*/k_{-2})$.

$$^{KIE}(V/K)_{observed} = (^{KIE}k_{intrinsic} + c_f)/(1 + c_f) \qquad (7)$$

If one simplifies the mechanism by combining the hypothetical conformational change and the catalytic step into a single step, then the commitment is approximated by $(k_3^* + k_3)/k_{-2}$ (see Table 1). This simplification allows for estimation of the commitment factor of approximately 0.47 for Mn^{2+}, yielding calculated intrinsic values of 1.0105 for the $^{18}(V/K)_{bridge}$ effect and 1.0009 for $^{15}(V/K)$, both approximately the same as experimental values at pH 8.5.

With Mg^{2+}, k_{-2} could not be directly calculated from eqs 4 and 6 as done with Mn^{2+} because of the inverse viscosity effect observed. Values could be estimated from limiting situations as previously described. Using the estimated values for k_{-2} yielded commitment factors of 0.46 (considering k_2 constant) or 0.14 (considering k_{-2} constant) for Mg^{2+} in the two limiting examples. The value of 0.46 for the commitment factor was nearly identical to that found with Mn^{2+}. Applying this value to the observed isotope effects with Mg^{2+} gave intrinsic values for the $^{18}(V/K)_{bridge}$ effect of 1.0225 and for $^{15}(V/K)$ of 1.0026. Using the commitment factor of 0.14 yielded intrinsic values for $^{18}(V/K)_{bridge}$ of 1.0176 and for $^{15}(V/K)$ of 1.0021. The differences between the intrinsic values assuming the upper and lower commitment factors are sufficiently small as to not affect the interpretation (see next section). For both isotope effects, the values were not too different from the observed values at pH 8.5 with Mg^{2+}. These findings suggest that the isotope effects measured at pH 8.5 are close to the intrinsic ones and can be used to approximate the transition state.

Isotope effects evaluated in the uncatalyzed hydrolysis of pNPP serve as a basis for the comparison of enzymatic reactions. Hydrolyses of the phosphate dianion and monoanion forms have been well characterized and provide limitations on the interpretation of isotope effects. Hydrolysis of the dianion is characterized by departure of a fully negatively charged leaving group with a highly dissociative transition state. Observed isotope effects can then be attributed to almost complete cleavage of the P−O bond with the ^{15}k isotope effect reflecting delocalization of the negative charge in the aromatic ring of the leaving group (*37*). Near-

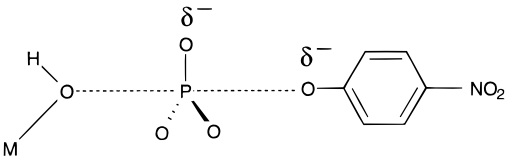

FIGURE 5: Transition state structure for calcineurin catalysis. The transition state structure for the calcineurin-catalyzed reaction of pNPP was inferred from the isotope effect data. The P−O bond to the leaving group is at least two-thirds broken with partial negative charge on the leaving group.

unity values for $^{18}k_{nonbridge}$ are consistent with studies suggesting that metaphosphate should be represented as a hybrid structure with no increase in P−O bond order in the metaphosphate-like transition state (*38*). For the monoanion reaction, the leaving group is protonated concurrently with bond cleavage and yields a decreased value for the $^{18}k_{bridge}$ isotope effect. There is almost no ^{15}k isotope effect measured because the leaving group is protonated and there can be no charge delocalization. The deprotonation of the monoanion phosphoryl group in the transition state causes the large $^{18}k_{nonbridge}$ observed for monoanion hydrolysis.

Significance of Heavy Atom Isotope Effects. Experiments with Mg^{2+} as an activator indicate that essentially full activation is achieved at 20 mM concentration of the metal ion (*11*, *30*). The measured isotope effects in the presence of Mg^{2+} were compared to those found in studies with Mn^{2+} (*22*). The $^{18}(V/K)_{nonbridge}$ effect at pH 8.5 with Mg^{2+} is 0.9910, which is slightly larger (more inverse) than nonbridge effects measured for the uncatalyzed reactions or for other enzymatic phosphate monoester reactions and larger than measured for calcineurin and Mn^{2+}. The inverse nonbridge isotope effects were consistent with a dissociative transition state similar to the uncatalyzed reaction of the dianion in solution. The same was concluded for studies with Mn^{2+} although the isotope effects were less (smaller inverse effect). A change to an associative transition state would be expected to yield normal, not inverse, isotope effects.

The two mechanistic extremes with respect to proton transfer to the leaving group are (a) leaving group departure as the fully protonated form as in aqueous monoanion hydrolysis and in PTPase reactions; and (b) leaving group departure as the anion as in the aqueous dianion hydrolysis and in reactions of PTPase mutants in which the general acid has been mutated (*39*). The first type, with a departing group bearing no negative charge, has very small or unity values for the $^{15}(V/K)$ isotope effect and values of about 1.01 for $^{18}(V/K)_{bridge}$ isotope effects. Leaving group departure as the pNP anion results in isotope effects of from 1.002 to 1.003 for $^{15}(V/K)$ and values of 1.02−1.03 for $^{18}(V/K)_{bridge.}$. For the calcineurin reaction with Mg^{2+} as the activator, using either the upper or the lower estimate for the commitment factor yielded intrinsic isotope effects in the leaving group which indicated that it bears significant negative charge in the transition state and, therefore, bond cleavage must be significant. The magnitudes of $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ are about two-thirds of the way from the values exhibited when charge neutralization on the leaving group is complete and those seen when a full negative charge resides on the leaving group. If the numbers at pH 8.5 are taken as approximating the full intrinsic ones representing the transition state, then the P−O bond is about two-thirds broken and about two-thirds of a charge resides on the leaving group. The leaving group isotope effects potentially can be diminished either by metal ion coordination or by protonation of the leaving group, either of which will neutralize charge in the transition state. Thus, it is possible that the reaction is even more dissociative with charge on the leaving group partially neutralized.

It is also possible the values at pH 8.5 are still slightly suppressed from their intrinsic values; the simplification applied earlier to allow estimation of the commitment factor ignores the internal commitment k_3/k_{-3}*. Such a commitment, if present at all, must be small since the intrinsic values for $^{15}(V/K)$ and for $^{18}(V/K)_{bridge}$ with Mg^{2+} calculated from the estimated commitment factor, as well as these experimental isotope effects measured at pH 8.5, approach their maximum values.

Figure 5 shows a diagram of the transition state inferred by the isotope effect data. The isotope effect data require that P−O bond cleavage is about two-thirds or more complete in the transition state. The inverse values observed for $^{18}(V/K)_{nonbridge}$ imply that the transition state remains dissociative with little nucleophilic participation, as in the solution reactions of phosphate monoesters.

Protonation of phosphate esters results in inverse ^{18}O isotope effects of about 0.984 (*40*). Because the substrate was present in solution essentially completely (>99%) as the dianion, if protonation occurred either during binding or during catalysis the full equilibrium inverse isotope effect would be manifested in the observed $^{18}(V/K)_{nonbridge}$ effect. Because this isotope effect of 0.9910 is only slightly more inverse than that measured with other phosphatases requiring the dianion as the substrate, protonation of the phosphoryl group either upon binding or during catalysis is excluded in the mechanism of calcineurin.

It is interesting to compare these results with those observed with Mn^{2+} activation. The commitment factors calculated from the kinetic data in Table 1 yield predicted intrinsic values of 1.011 ± 0.001 for $^{18}(V/K)_{bridge}$ and 1.0009 ± 0.0005 for ^{15}k. These are close to the experimental values obtained when chemistry was made more rate-limiting by raising pH or omitting calmodulin. These values are also smaller than the values with Mg^{2+} as the activator. If indeed they are intrinsic values, the data indicate that charge neutralization of the leaving group in the transition state is more complete with Mn^{2+} than with Mg^{2+}. This could either be because of stronger electrostatic interactions with Mn^{2+} than with Mg^{2+}, or, if charge neutralization is accomplished via protonation by a metal-bound water molecule, be due to the greater acidity of Mn^{2+}-bound water. Alternatively, the transition state with Mn^{2+} as the activator could be earlier with bond cleavage to the leaving group less advanced. Another significant difference between Mn^{2+}- and Mg^{2+}-supported catalysis was the effect of each metal on the commitment factor of the reaction. Increasing Mn^{2+} was found to reduce commitment factors and make pNPP a less sticky substrate. The opposite was found with Mg^{2+}. Increasing the concentration of Mg^{2+} from 20 to 100 mM at pH 7.0 caused a small change in the observed value for $^{18}(V/K)_{bridge}$ from 1.0154 to 1.0130, suggesting that the commitment factor was increased by the increased Mg^{2+} concentrations.

SUMMARY

These data suggested that cleavage of the phosphate ester linkage was the most significant factor contributing to the rate-limiting step of calcineurin catalysis. The choice of metal activator influenced individual steps in the reaction. Specifically, the choice of metal influenced the interaction of substrate with the enzyme at the k_2 and k_{-2} steps. Although quantitative assessment of the effect was not possible, boundary situations were considered. The overall catalytic process was impacted by the effect of metal on the steps represented by $k_3^* + k_3$. The nature of the transition state was similar regardless of the metal ion used and was dissociative as with other phosphatases (*23*, *39*, *41*−*44*), but, in contrast, the leaving group in the calcineurin reaction had a partial negative charge with both Mn^{2+} and Mg^{2+}.

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