Ca^{2+} Binding Site 2 in Calcineurin-B Modulates Calmodulin-Dependent Calcineurin Phosphatase Activity†

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ABSTRACT: Calcineurin is the Ca^{2+}- and calmodulin-dependent Ser/Thr phosphatase. Human calcineurin-Aα and wild-type or mutated calcineurin-Bs were coexpressed in *Escherichia coli* and purified by calmodulin−Sepharose affinity chromatography. Four calcineurin-B mutants were studied. Each had a single conserved Glu in the 12th position of one EF-hand Ca^{2+} binding site replaced by a Lys, resulting in the loss of Ca^{2+} binding to that site. Phosphatase activities of the enzymes toward a ^{32}P-labeled phosphopeptide substrate were measured. Inactivating Ca^{2+} binding sites 1, 2, or 3 in calcineurin-B reduced Ca^{2+}-dependent phosphatase activity of the enzymes in the absence of calmodulin with the site 2 mutation being most effective. Inactivating Ca^{2+} binding site 4 did not change enzyme activity or sensitivity to Ca^{2+} in either the absence or presence of calmodulin. The calmodulin-dependent phosphatase activity of the enzymes containing site 1, 2, or 3 mutations in calcineurin-B was also decreased compared to enzyme with wild-type calcineurin-B. Of these enzymes, the one with the site 2 mutation was most profoundly affected as determined by the magnitude of the shift in Ca^{2+} concentration dependence. Binding of a fluorescein-labeled calmodulin to the wild-type and the site 2 mutant enzymes was examined using fluorescence polarization measurements. The decrease in Ca^{2+} sensitivity for the enzyme with calcineurin-B site 2 inactivated is apparently due to a decrease in the affinity of that enzyme for calmodulin at low Ca^{2+} concentrations. These data support a role for Ca^{2+} binding site 3 in the carboxyl half of calcineurin-B in transmitting the Ca^{2+} signal to calcineurin-A and indicate that site 2 in the amino half of calcineurin-B is critical for enzyme activation.

Calcineurin-B (CN-B)[1] is an essential component of the heterodimeric Ca^{2+}- and calmodulin-dependent phosphatase, calcineurin. Although it is generally referred to as the regulatory subunit of the phosphatase, it also is important in establishing the structure of the enzyme catalytic subunit (*1*). The structural and activating functionalities of calcineurin-B have been assigned to the carboxyl and amino halves of the protein, respectively. These assignments are based on differences in the kinetics and affinity of Ca^{2+} binding to the two EF-hand Ca^{2+} binding sites in each half of the molecule and the reduction in enzyme activity when either of the Ca^{2+} binding sites in the amino half of the protein is inactivated by point mutation (*1*). For this study, calcineurin enzymes containing point mutations in each of the four calcineurin-B Ca^{2+} binding sites were used to examine the role of calcineurin-B in regulating the phosphatase.

Ca^{2+}-dependent activation of calcineurin is mediated by both calcineurin-B and calmodulin. The essential role of calcineurin-B in supporting calcineurin phosphatase activity (*2*, *3*) and the requirement of calcineurin-B for Ca^{2+} are both well established (*4*−*6*). Calcineurin-B has four EF-hand Ca^{2+} binding sites which are important for the structural integrity of the enzyme (*1*), for phosphatase activation (*6*, *7*), and for recognition of inhibitory complexes (*8*, *9*). The importance of Ca^{2+} in the interaction of calcineurin-A with calcineurin-B is suggested by data showing that when the subunits are expressed individually in an in vitro translation system, they require Ca^{2+} in order to form a complex (*10*). Without calcineurin-B the catalytic subunit of calcineurin has weak activity even in the presence of calmodulin (*2*, *3*). When the enzyme is reconstituted from isolated A and B subunits, the heterodimer has increased catalytic efficiency (increased K_{cat}/K_m) relative to isolated calcineurin-A (*7*). The essential role of Ca^{2+} binding to calcineurin-B for phosphatase activation was demonstrated using a calmodulin-independent fragment of calcineurin consisting of calcineurin-B and the catalytic domain of calcineurin-A plus a short extension of the regulatory domain to which calcineurin-B binds (*11*). This calcineurin fragment, which cannot bind calmodulin, remains completely dependent on Ca^{2+} for activity (*6*). Using this

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[1] Abbreviations: CN-B, calcineurin-B; CN-A, calcineurin-A; CaM, calmodulin; Fl-CaM, fluorescein-labeled CaM-T_{146}C; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; skMLCK, skeletal muscle myosin light chain kinase.

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calcineurin fragment, it was shown that calcineurin-B has a role in regulating calcineurin activity by modulating K_m of the enzyme (*6*). Binding of inhibitor complexes, such as the cyclosporin-A/cyclophilin complex, to calcineurin is also reported to be dependent on calcineurin-B but not calmodulin (*9*, *12*).

The structural organization of the calcineurin heterodimer is the basis for all of the actions of calcineurin-B. It is, therefore, necessary to examine the calcineurin-A domain organization in order to understand enzyme regulation by the B subunit. Calcineurin-A consists of a catalytic domain and a regulatory region which has subdomains within it. The regulatory region of calcineurin-A encompasses the carboxyl half of that subunit. It extends from the catalytic domain as an elongated α-helix and folds back to cover the catalytic center (*13*). The regulatory region of calcineurin-A contains calcineurin-B and calmodulin binding domains as well as an autoinhibitory domain (*14*). Calmodulin binds reversibly in a Ca^{2+}-dependent manner to a domain between residues 400 and 425 (*15*, *16*). The primary autoinhibitory domain is located at the carboxyl terminus of calcineurin-A (*17*), but the region between the calmodulin binding and autoinhibitory domains may have an autoinhibitory function as it can modulate calmodulin-dependent activation (*7*, *14*). Calcineurin-B is noncovalently, but irreversibly, bound to calcineurin-A at the junction of the catalytic domain and the regulatory region (*13*, *18*). It has recently been shown that the calcineurin-B and calmodulin binding domains of calcineurin-A bind to each other and that Ca^{2+} binding to the activation sites in calcineurin-B results in displacement of the calmodulin binding domain from the calcineurin-B binding domain (*19*). The calcineurin-B-dependent displacement of the calmodulin binding domain is predicted to increase the affinity of calcineurin for calmodulin. The catalytic site has been localized to the central part of the molecule by strong sequence homology with the catalytic subunits of other Ser/Thr phosphatases (*20*, *21*) and by crystallographic localization (*13*, *18*). The amino terminus of calcineurin-A is important for calcineurin activity (*15*), and crystallographic data indicate that it participates in heterodimer formation by forming the outer part of a calcineurin-B binding pocket (*13*, *18*). This binding pocket forms around the carboxyl half of calcineurin-B and may contribute to stability of the heterodimer or to enzyme activation. The location of calcineurin-B at the junction of the catalytic and regulatory domains of calcineurin-A and the contacts with the amino terminus of calcineurin-A make calcineurin-B a determinant for the structure of the catalytic domain and a mediator of enzyme activation. In this paper we present evidence that the Ca^{2+} binding site 3 in the carboxyl half of calcineurin-B is important for enzyme activation and that the amino half of calcineurin-B stimulates phosphatase activity by increasing calmodulin affinity for the enzyme.

MATERIALS AND METHODS

Materials. The catalytic subunit of cAMP-dependent protein kinase A was from Boehringer Mannheim GmbH, Germany. The peptide (ARKEVIRNKIRAIGKMARVFSVLR) representing the calmodulin binding domain from calcineurin was synthesized at the UNMC Protein Structure core facility. All reagents were the highest grade available.

Site-Directed Mutagenesis. All mutations were introduced using the QuikChange (Stratagene) site-directed mutagenesis kit as described in the original report concerning these enzymes (*1*). In each of the four calcineurin-B mutants the Glu at the 12 position of one EF-hand Ca^{2+} binding loop was replaced with a Lys.

Expression of Calcineurin in Escherichia coli. The plasmids pETCN-α (*22*) encoding calcineurin-A and calcineurin-B in a dicistronic construct and pBB279 (*23*) containing *Saccharomyces cerevisiae* myristoyl-coenzyme A:protein *N*-myristoyltransferase were kindly provided by Drs. Liu and Gordon, respectively. Calcineurin enzymes were expressed in BL21 codon plus *E. coli* (Stratagene) and purified by affinity chromatography on calmodulin−Sepharose. Calcineurin enzymes were isolated without superoxide dismutase (SOD) and catalase or in the presence of 10 units/mL SOD and 0.2 μg/mL catalase to prevent oxidative inhibition of calcineurin (*24*). Purification on calmodulin−Sepharose and inclusion of SOD and catalase resulted in isolation of enzymes with specific activity that approached that measured for tissue homogenates (*24*), whereas enzyme isolated without SOD/catalase has 10−30-fold lower specific activity. Enzyme isolation conditions for individual experiments are given in the figure legends. The calcineurin-A subunit expressed in this system has an N-terminal polyhistidine tag and the calcineurin-B is myristoylated. Mutations at individual Ca^{2+} binding sites were introduced into calcineurin-B in the pETCN-α construct as described previously (*1*). The enzyme containing wild-type calcineurin-B is designated CN-WT, and the four heterodimers containing calcineurin-B with the single mutations $E_{42}K$, $E_{74}K$, $E_{101}K$, and $E_{152}K$ are designated M-1, M-2, M-3, and M-4, respectively.

Phosphatase Assay. The phosphatase activity assay was carried out as described (*6*). The substrate used was a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (DLDVPIPGRFDRRVSVAAE) (*25*), which was phosphorylated with ^{32}P-labeled ATP. The assay buffer consisted of 40 mM Tris-HCl, pH 7.5, 0.1 M KCl, 6 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 0.5 mM DTT, 10 units/mL SOD, and 0.2 μg/mL catalase. EGTA, $CaCl_2$, and calmodulin were present as described for individual experiments, and calcineurin was at a final concentration of 5 or 10 nM. The concentrations of all calcineurin enzymes were estimated using the molar extinction coefficient $\epsilon_{279} = 60\,935$. The reaction was started by addition of substrate to a final concentration of 1 μM. The concentration of substrate was based on the specific activity of [^{32}P]ATP used for the phosphorylation reaction. The incubations were carried out at 30 °C for 5 or 15 min before the reaction was stopped by addition of the ice-cold stopping solution (5% trichloroacetic acid, 0.1 M H_2KPO_4). Free phosphate was separated from peptide by passing the entire sample over a 0.5 mL column of Dowex AG 50W-X8 (200−800 mesh; Bio-Rad). Free ^{32}P passing through the column was mixed with scintillation cocktail and counted in a liquid scintillation counter. The concentration of free Ca^{2+} in the EGTA-buffered solutions was calculated as previously described (*6*, *26*).

Fluorescence Anisotropy. Fluorescence anisotropy, or polarized light fluorescence, measures changes in rotational freedom of a fluorescent probe (*27*−*29*). This assay was used

to estimate the affinity of calmodulin for the wild-type and M-2 enzymes. Calmodulin which had been engineered to contain a cysteine three residues away from the carboxyl terminus (CaM-T_{146}C) was generously supplied by Dr. Anthony Persechini (*30*). The CaM-T_{146}C was labeled with fluorescein at a 1:1 ratio to create Fl-CaM by incubating the CaM-T_{146}C with a 5-fold excess of fluorescein-5-maleimide. The reaction was quenched with DTT, and unincorporated reagents were separated from the Fl-CaM by gel filtration on Sephadex G-25. The Fl-CaM concentration was estimated using a fluorescein extinction coefficient of $\epsilon_{495} = 78\,000$. Polarization did not vary with Fl-CaM concentration between 0.1 and 2 nM whereas fluorescence intensity increased linearly in that range. All assays were run with 0.1 nM Fl-CaM in the binding mixture. Wild-type calcineurin and the M-2 enzyme were present at concentrations between 0.02 and 100 nM. Assay buffer contained 0.01 mg/mL BSA, 1 mM $MgSO_4$, 100 mM KCl, and 10 mM HEPES (pH 7.5), 1 mM EGTA, and $CaCl_2$ to produce the indicated concentration of free Ca^{2+}. Separate samples were prepared for each concentration of enzyme. Changes in fluorescence intensity were less than 10%, and polarization values were not corrected for effects of intensity change.

Data Analysis. All data were analyzed using the equations built into the Prism software package. Ca^{2+}-dependent activation of calcineurin phosphatase activity and binding as detected by fluorescence polarization measurement were analyzed according to the equation:

$$Y = Y_{min} + [(Y_{max} - Y_{min})/(1 + 10^{(\log EC_{50} - \log X)N_H})]$$

where Y is the measured value of activity or polarization, Y_{min} is the background value, Y_{max} is the maximum activity or polarization under the conditions of that run, X is the concentration of Ca^{2+}, and N_H is the Hill coefficient. Binding of calcineurin to Fl-CaM was analyzed according to the equation:

$$Y = Y_{max}X/(K_d + X)$$

where Y is the measured change in polarization, Y_{max} is the maximum change in polarization under the conditions examined, X is the concentration of calcineurin, and K_d is the dissociation constant.

RESULTS

Ca^{2+}-Dependent Activation of Calcineurin. The roles of individual Ca^{2+} binding sites in calcineurin-B were examined using enzymes containing point mutations in calcineurin-B that eliminated Ca^{2+} binding to one of the four Ca^{2+} binding sites (*1*). The Ca^{2+}-dependent, calmodulin-independent activation was examined first because that activation can be directly attributed to calcineurin-B. Eliminating Ca^{2+} binding at sites 1, 2, or 3 in calcineurin-B produced a significant decrease in Ca^{2+}-dependent activation of phosphatase activity, whereas mutation of site 4 had no effect on activity (Figure 1). The three enzymes that exhibited decreased activity also have decreased sensitivity to Ca^{2+} with the site 2 mutant showing by far the largest shift in Ca^{2+} concentration dependence (Figure 2, Table 1). The site 4 mutant and wild-type enzymes have similar maximal activity and Ca^{2+} sensitivity. The only significant difference between those two

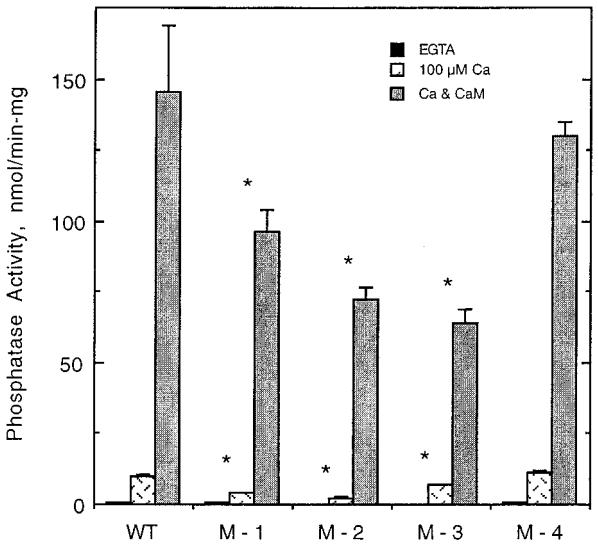

FIGURE 1: Calcineurin phosphatase activity of wild-type calcineurin and four calcineurin enzymes having a point mutation which disrupts Ca^{2+} binding to a single site in calcineurin-B. Black filled bars represent activity in the presence of 1 mM EGTA. Cross-hatched bars represent the activity in the presence of 30 μM Ca^{2+}. Gray filled bars represent activity in the presence of 30 μM Ca^{2+} plus 1 μM calmodulin. All enzymes were isolated in the presence of SOD/catalase; data were analyzed by ANOVA with individual comparisons by Dunnett's multiple comparisons test. An asterisk indicates a significant difference from control. Values presented are the mean ± SEM for $n = 6$.

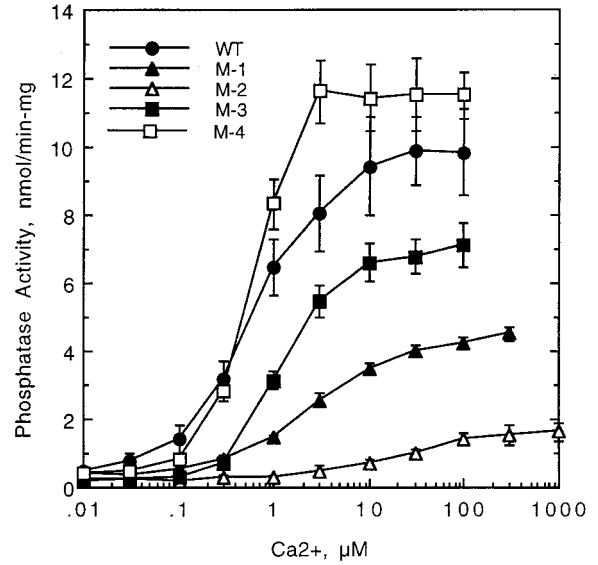

FIGURE 2: Ca^{2+} concentration dependence for activation of wild-type calcineurin and the four enzymes containing calcineurin-B with single point mutations. All enzymes were isolated in the presence of SOD/catalase. Values presented are the mean ± SEM for $n = 6$.

enzymes was an apparent increase in cooperativity in respect to Ca^{2+} for the site 4 mutant (Table 1).

Ca^{2+}- and Calmodulin-Dependent Activation of Calcineurin. Effects of calcineurin-B Ca^{2+} binding site mutations on the Ca^{2+}- and calmodulin-dependent activity of the four enzymes followed a pattern similar to that observed for the Ca^{2+}-dependent activation (Figure 1). Site 1, 2, and 3 mutants all had significant decreases in enzyme activity. The Ca^{2+} concentration dependence of calmodulin-mediated enzyme activation appears to be shifted slightly to the right for

Table 1: Ca^{2+} -Dependent Activation of Calcineurin Enzymes[a]

	calmodulin absent		calmodulin, 1.0 μM	
	K_{act} (μM)[b]	Hill coeff	K_{act} (μM)[b]	Hill coeff
wild type	0.67 (0.54−0.83)	1.20 (0.92−1.48)	0.78 (0.48−1.27)	1.65 (0.50−2.80)
M-1	2.67* (2.18−3.28)	0.96 (0.78−1.13)	1.21 (1.05−1.40)	1.96 (1.47−2.44)
M-2	56.4* (33.8−94.2)	0.79 (0.57−1.02)	3.10* (2.41−3.99)	2.31 (1.14−3.47)
M-3	1.23* (1.10−1.38)	1.50 (1.27−1.74)	1.28 (1.18−1.39)	2.10 (1.79−2.42)
M-4	0.62 (0.55−0.70)	1.88* (1.54−2.21)	0.83 (0.73−0.95)	2.30 (1.69−2.91)

[a] Phosphatase activity stimulated by Ca^{2+} or Ca^{2+} plus calmodulin. Activation constants and Hill coefficients for data presented in Figures 2 and 3. [b] Concentration of Ca^{2+} required for half-maximal activity. Values represent the mean of $n = 6$ with 95% confidence limits from analysis of grouped data in parentheses. An asterisk indicates a significant difference from WT enzyme as determined by nonoverlaping 95% confidence limits.

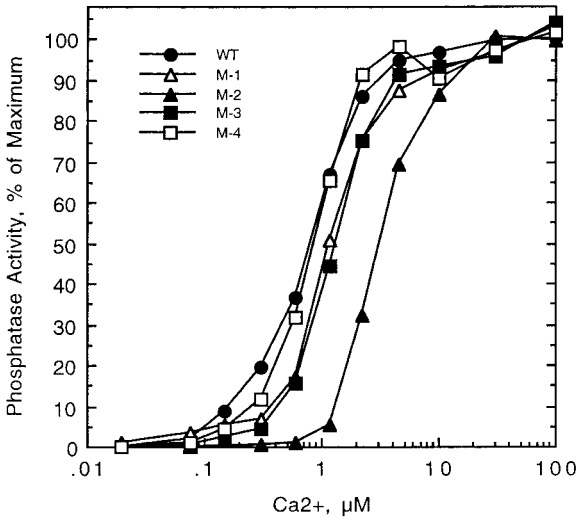

FIGURE 3: Ca^{2+} concentration dependence for activation of wild-type calcineurin and the four enzymes containing calcineurin-B with single point mutations in the presence of 1 μM calmodulin. Maximum activity for each enzyme was used as the 100% value for that enzyme. Activity in the presence of 1 mM EGTA was subtracted from all values as the Ca^{2+}- and calmodulin-independent activity. Enzymes were isolated without SOD/catalase present. Values presented are the mean for $n = 6$.

enzymes with mutations in sites 1 or 3 of calcineurin-B but was not changed for the site 4 mutant (Figure 3). The largest, and the only significant, shift in Ca^{2+} concentration dependence for calmodulin activation of the phosphatase occurred with the site 2 calcineurin-B mutant (Figure 3). In the presence of 1 μM calmodulin the M-2 enzyme was approximately 4-fold less sensitive to Ca^{2+} than the wild-type enzyme. The experiments represented in Figure 3 utilized enzymes isolated without SOD/catalase. The assays were also done using enzymes isolated in the presence of SOD/catalase, and a significant shift to the right in the Ca^{2+} dependence of calmodulin stimulation was found for the M-2 enzyme. The M-2 enzyme was also different from the other enzymes in the degree to which it is dependent on calmodulin for activation. In the absence of calmodulin the M-2 enzyme has almost no activity, but in the presence of calmodulin it has phosphatase activity comparable to the enzymes with

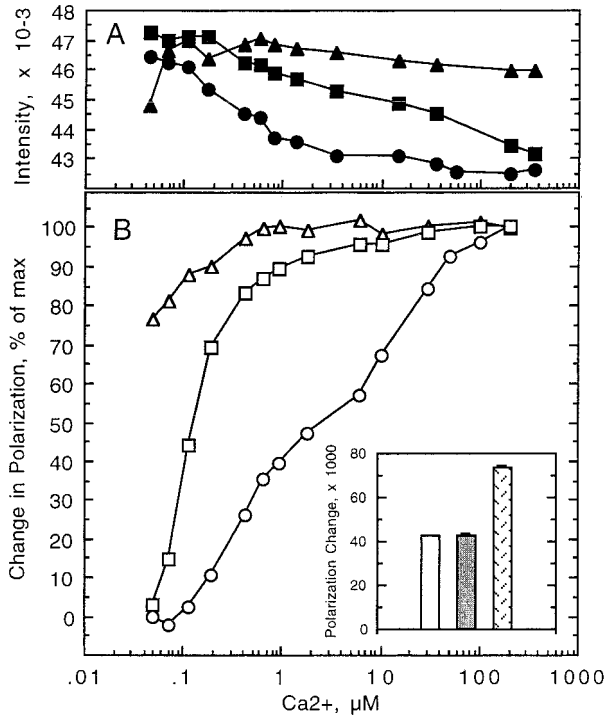

FIGURE 4: Fluorescence intensity (panel A) and polarization (panel B) of Fl-CaM itself (circles) and in the presence of 100 nM calcineurin calmodulin binding domain peptide (triangles) or 100 nM wild-type calcineurin (squares) as a function of Ca^{2+} concentration. The inset is the maximum change in polarization for Fl-CaM itself (open) or in the presence of the peptide (gray) or calcineurin (hatched). All enzymes were isolated in the presence of SOD/catalase. Values represent the mean ± SEM for $n = 4$.

site 1 or site 3 mutations (Figure 1). Data from the Ca^{2+} dependence and Ca^{2+} and calmodulin dependence for enzyme activation experiments are summarized in Table 1.

Ca^{2+}-Dependent Calmodulin Binding to Calcineurin. Calmodulin binding to calcineurin was measured to determine if the shift in Ca^{2+} concentration dependence for calmodulin activation of the enzyme with a site 2 mutation was accompanied by a decrease in calmodulin affinity for that enzyme. For these experiments a fluorescence polarization binding assay which utilizes a fluorescein-labeled calmodulin (Fl-CaM) was used. Calmodulin was specifically fluoresceinated on a cysteine engineered three amino acids from the carboxyl terminus of the protein (*30*), and fluorescent polarization for solutions containing 0.1 nM Fl-CaM was measured. Changes in fluorescence polarization are dependent on the rotational velocity of the fluorescent species and can indicate conformational changes in the labeled protein or binding of the labeled protein to a larger molecule. In the absence of calcineurin the fluorescein-labeled calmodulin undergoes a Ca^{2+}-dependent increase in polarization with an EC_{50} of 2.54 ± 0.06 μM and a Hill coefficient of 0.57 ± 0.04 (Figure 4B). The observed Ca^{2+}-dependent increase in fluorescence polarization for Fl-CaM in the absence of calcineurin is consistent with the Fl-CaM undergoing a conformational change as it binds Ca^{2+}. In the presence of 100 nM peptide representing the CaM binding domain of calcineurin the Fl-CaM binding was 75% of maximum at 45 nM Ca^{2+}, the lowest concentration tested. The increased affinity of Fl-CaM for Ca^{2+} is consistent with the properties of this peptide to stimulate Ca^{2+} binding to

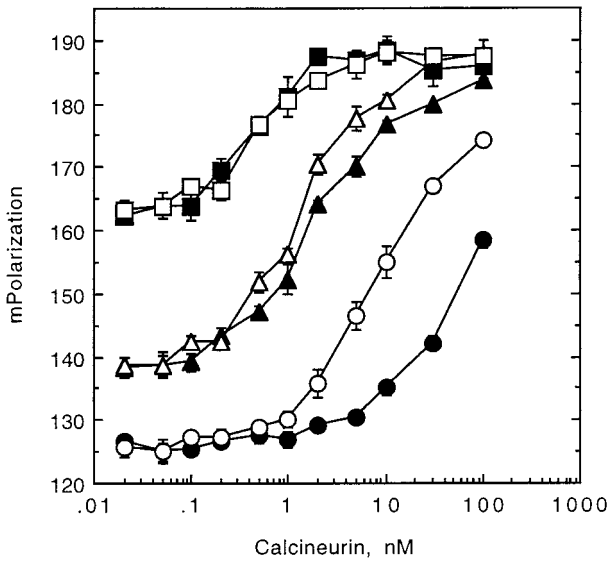

FIGURE 5: Fluorescence polarization of Fl-CaM in the presence of 200 nM (○, ●), 1.0 μM (△, ▲), and 200 μM (□, ■) Ca^{2+} as a function of wild-type (open symbols) and M-2 (filled symbols) enzyme concentration. Enzymes were isolated without SOD/catalase present. Values represent the mean ± SEM for $n = 6$.

calmodulin (*6*). The increase in polarization was the same in the presence and absence of peptide, a finding consistent with the peptide being small and not significantly changing the size of Fl-CaM. In the presence of 100 nM wild-type calcineurin the measured change in polarization is greater than for Fl-CaM alone (Figure 4, inset). In addition, the change in polarization occurs at a lower Ca^{2+} concentration, EC_{50} is 0.13 ± 0.01 μM, and exhibits cooperativity in respect to Ca^{2+} with a Hill coefficient of 2.27 ± 0.25 (Figure 4B). Ca^{2+}-dependent changes in fluorescence intensity were observed for Fl-CaM itself and for Fl-CaM plus calcineurin but not for Fl-CaM plus peptide (Figure 4A). The changes were less than 10% of initial values and did not correlate with Ca^{2+} dependence for changes in polarization; therefore, no corrections of polarization values for intensity changes were made. These data indicate that the degree of Fl-CaM saturation with Ca^{2+} can be determined by measuring fluorescence polarization in the absence of enzyme and that Fl-CaM binding to calcineurin can be measured at any degree of Fl-CaM Ca^{2+} saturation. Therefore, the affinity of the wild-type and M-2 enzymes for Fl-CaM was determined under various degrees of Ca^{2+} saturation by examining changes in fluorescence polarization with increasing enzyme concentrations.

Affinity of Fl-CaM for Calcineurin. The affinities of calmodulin for wild-type calcineurin and the M-2 enzyme were estimated by measuring changes in fluorescence polarization of solutions containing 0.1 nM Fl-CaM as a function of enzyme concentration. Ca^{2+} concentration was initially set at 200 nM to maximize the calcineurin-dependent signal of the system and was sequentially increased to 1.0 and 200 μM, concentrations which are believed to produce partial and complete saturation of Fl-CaM with Ca^{2+} (Figure 4B). The affinity of each enzyme for Fl-CaM increased as the Ca^{2+} concentration was increased from 200 nM to 200 μM (Figure 5) but did not change when Ca^{2+} was further increased to 300 μM (not shown). Under low Ca^{2+} conditions the affinity of Fl-CaM for the mutant enzyme was reduced substantially in comparison to the native enzyme (Figure 5). As the Ca^{2+} concentration was increased to partially saturate Fl-CaM with Ca^{2+}, the difference in affinity of the two enzymes for Fl-CaM decreased. With Ca^{2+} at 200 μM, a concentration that is expected to saturate all Ca^{2+} binding sites on Fl-CaM, there was no difference in the affinity of the wild-type and M-2 enzymes for Fl-CaM.

DISCUSSION

Calcineurin's regulatory subunit, calcineurin-B, is absolutely essential for phosphatase activity of the enzyme (*2*, *3*) and for some calcineurin−protein interactions (*10*, *12*) such as immunosuppressant binding. It acts to establish and maintain the structure of the holoenzyme as well as supporting activation in response to Ca^{2+} and calmodulin. The structural component of calcineurin-B is believed to reside in the carboxyl half of the protein as the two EF-hand Ca^{2+} binding sites in that half of the protein have very slow exchange of Ca^{2+} and are likely to remain Ca^{2+} saturated at all times (*1*). The regulatory function of calcineurin-B is presumed to reside in the amino half of the protein. This half of the protein also contains two EF-hand Ca^{2+} binding sites, but they have relatively low affinity for Ca^{2+} and are presumed to activate the phosphatase by reversibly binding Ca^{2+} during Ca^{2+} transients (*1*, *6*). The amino terminus of calcineurin-A forms part of a calcineurin-B binding cleft (*13*, *18*), and when that domain of calcineurin-A is proteolyticaly removed from calcineurin-A, the phosphatase loses activity (*15*). The intramolecular mechanism for transmission of the Ca^{2+} signal from calcineurin-B to the catalytic domain of calcineurin-A is not known. Because calcineurin phosphatase activity is dependent on an intact calcineurin-A amino terminus, it is likely that the amino-terminal domain contributes to effective transmission of the Ca^{2+} signal from calcineurin-B to the catalytic domain of calcineurin-A. It is presumed that a conformational change(s) occur(s) within calcineurin-A upon Ca^{2+} binding to calcineurin-B and that the conformational change activates the enzyme either by exposing the catalytic center or modifying the conformation of that center.

Data presented in Figure 1 support the assignment of the activating function to the amino half of calcineurin-B but extend the critical activating sites to include the third Ca^{2+} binding site. Mutation of Ca^{2+} binding site 3 in calcineurin-B to prevent Ca^{2+} binding reduces both the Ca^{2+}-dependent and Ca^{2+}/calmodulin-dependent phosphatase activity. In fact, the site 3 mutation had the greatest effect to decrease calmodulin-dependent activity of the enzyme. In addition, there is a slight shift in the Ca^{2+} dependence for calmodulin-independent enzyme activation to the right for M-1 or M-3 and a profound shift with M-2. When calmodulin is not present, this shift can represent a decrease in Ca^{2+} affinity at the remaining activation sites or it may result from activation that is dependent on Ca^{2+} binding to the mutated site. With the site 1 and 3 mutants it is most reasonable to conclude that the affinity for Ca^{2+} of one or both of the remaining activating sites is reduced. With the site 2 mutant the shift in Ca^{2+} dependence is approximately 100-fold, and the degree of activation is less than 20% of the wild-type enzyme. Because of the magnitude of the shift in Ca^{2+} dependence and the profound inhibition of activity, activation of M-2 may be dependent on some restoration of Ca^{2+}

binding to the mutated site. It is clear that mutation of Ca^{2+} binding site 2 in calcineurin-B has the greatest influence on enzyme activation.

In either the absence or presence of calmodulin the M-4 enzyme is indistinguishable from the wild-type enzyme except that M-4 has slightly more cooperativity in respect to Ca^{2+}-dependent activation. From previous studies it is known that one high-affinity, slowly exchanging Ca^{2+} binding site is lost from enzymes containing calcineurin-B with mutation of either site 3 or site 4 (*1*). Therefore, the lack of effect of the site 4 mutation on calcineurin activity cannot be due to restoration of Ca^{2+} binding to that site. These data indicate that Ca^{2+} binding site 4 in calcineurin-B is not involved in transmitting the Ca^{2+} signal from calcineurin-B to calcineurin-A and by extension show that this site is entirely structural. It is clear, however, that site 3 is important in transmitting the Ca^{2+} signal from calcineurin-B to calcineurin-A. Because site 3 is a slow exchanging site and is the only part of calcineurin-B with contacts to the amino terminus of calcineurin-A, it is still regarded as a structural Ca^{2+} binding site. The structural alterations resulting from a site 3 mutation interfere with both the Ca^{2+}-dependent and the Ca^{2+}/calmodulin-dependent transmission of signals to the catalytic domain. Because Ca^{2+} binding site 3 is the only part of calcineurin-B with significant contacts with the amino terminus of calcineurin-A, this is consistent with the activation signal being dependent upon a proper interaction of calcineurin-B with the amino terminus. Loss of site 2 renders the enzyme essentially unresponsive to Ca^{2+} in the absence of calmodulin, and loss of site 1 substantially reduces activity. These results are consistent with sites 1 and 2 being the activating sites and suggest that site 2 has the more important role in enzyme activation.

Calcineurin is highly susceptible to inactivation during isolation (*24*, *31*). Therefore, an alternative explanation for the decrease in activity of enzymes containing mutations is that they are inherently more unstable than the wild-type enzyme. This could result from greater susceptibility to reversible oxidative inactivation (*24*, *32*) or an irreversible denaturation of the enzyme. Although both of these mechanisms may contribute to the lower activity observed, they would be expected to have a similar relative effect for enzymes stimulated by Ca^{2+} alone or by Ca^{2+} plus calmodulin. A reduction of the concentration of active enzyme would also not be expected to shift the Ca^{2+} dependence of the remaining active enzyme. These two features are clearly not observed for any of the three enzymes with reduced activity; therefore, the mutations in calcineurin-B which decrease enzyme activity and shift the Ca^{2+} dependence of that activity must prevent calcineurin-B from inducing conformational changes in calcineurin-A that result in enzyme activation.

The restoration of activity to M-2 in the presence of calmodulin may indicate that calmodulin binding allows a conformational change in calcineurin-B that is blocked in the calmodulin free state. In this regard, the recent finding that the calmodulin binding domain of calcineurin-A interacts with the region of the calcineurin-B binding domain that binds to the amino half of calcineurin-B (*19*) provides a reasonable explanation for these findings. If Ca^{2+} binding to site 2, but not site 1, in calcineurin-B is sufficient to displace the calmodulin binding domain from the calcineurin-B binding domain, then the M-1 enzyme could be activated by Ca^{2+} alone but the M-2 enzyme could not. Calmodulin binding to calcineurin-A would remove the calmodulin binding domain as a constraint on calcineurin-B and, therefore, would restore activity to the M-2 enzyme by allowing site 1 mediated Ca^{2+}-dependent interactions of the amino half of calcineurin-B with the primary calcineurin-B binding domain of calcineurin-A.

The shift to the right in the Ca^{2+} dependence of calmodulin-stimulated activation of M-2 indicates that the site 2 deficit can be overcome by calmodulin binding at high Ca^{2+} concentrations. There are at least two different mechanisms that would explain these results. Either calcineurin-B modulates the affinity of calcineurin for calmodulin so the activation curves shown in Figure 3 are representative of calmodulin binding to the enzymes or calmodulin may bind to all of the enzymes with the same affinity and Ca^{2+} binding to the inactive site 2 in the M-2 enzyme is restored, but with decreased affinity, when calmodulin is bound to calcineurin-A. In the second scenario the M-2/calmodulin complex would remain inactive until Ca^{2+} bound to calcineurin-B site 2. Target-induced restoration of Ca^{2+} binding to mutated EF-hand Ca^{2+} binding sites in calmodulin has been reported (*33*) so it is not unreasonable to predict that the Ca^{2+} binding deficit observed for the isolated calcineurin-B (*1*) could be reversed to some degree in the heterodimer. To determine if the M-2 enzyme has reduced affinity for calmodulin, a direct binding assay was used.

The Ca^{2+}-dependent increase in fluorescence polarization for Fl-CaM in the absence of target shown on Figure 5 is consistent with Ca^{2+} binding to the Fl-CaM and inducing a conformational change that reduces the rotational freedom of the fluorescein. The increased sensitivity to Ca^{2+}, the positive cooperativity, and the greater change in fluorescence polarization seen when calcineurin is present are all consistent with Fl-CaM binding to a larger protein with a standard basic amphipathic α-helical calmodulin binding domain. When Fl-CaM binding was examined at 200 nM free Ca, there was a 9-fold difference in affinity between the native enzyme and the M-2 enzyme. At 1 μM Ca^{2+} the Fl-CaM is partially saturated by Ca^{2+}, and the affinity for each of the enzymes is increased so that there is no longer a significant difference in Fl-CaM affinity between them. Further increasing the Ca^{2+} concentration to 200 μM saturates all Ca^{2+} binding sites on Fl-CaM, resulting in an additional increase in the affinity of each of the enzymes for calmodulin, and renders them indistinguishable. These results indicate that under Ca^{2+} limited conditions calcineurin-B modulates affinity of calcineurin for calmodulin. It is not known if the mutated site 2 in calcineurin-B remains inactive or if the Ca^{2+} binding properties of that site are restored when the mutant calcineurin-B is incorporated into the heterodimer or when M-2 binds calmodulin.

The shift in the Ca^{2+} concentration dependence for calmodulin stimulation of the M-2 enzyme as compared to the wild-type enzyme strongly supports the hypothesis that calmodulin-dependent activation of calcineurin can be modulated by structural changes at sites apart from the calmodulin binding and autoinhibitory domains. Because calmodulin-dependent activation of calcineurin is believed to be dependent on displacement of the autoinhibitory domain, manipulations of calcineurin-B were not expected to affect

the Ca^{2+} dependence of calmodulin-dependent stimulation. The shift in the Ca^{2+} concentration dependence for calmodulin-dependent activation of M-2 demonstrates conclusively that calcineurin-B influences calmodulin-dependent activity of calcineurin. The isolated calmodulin binding domain peptide from skMLCK has been shown to affect the affinity of calmodulin for Ca^{2+} to a greater degree than the skMLCK itself (*34*). The difference between the isolated peptide and the intact enzyme is presumably due to energetically unfavorable conformational changes in the skMLCK induced by calmodulin binding (*34*). This work is another example of distant sites regulating the affinity of calmodulin binding domains for calmodulin. It also demonstrates a clear interaction between the two regulators of calcineurin activity, calmodulin and calcineurin-B.

One of the most important and defining features that distinguishes calcineurin from other Ser/Thr phosphatases is the fact that it is positively regulated. This has traditionally been examined from the perspective that the most important regulation of the enzyme is how it is turned on. However, the most important attribute of calcineurin as a regulatory enzyme may be that it can be turned off. When considered from this perspective, the absolute need for calcineurin to be regulated by both calcineurin-B and calmodulin begins to make sense. The role of calcineurin-B may be to suppress enzyme activity when intracellular Ca^{2+} is at resting levels. Activation of calcineurin is only possible when Ca^{2+} is bound to the activating sites of calcineurin-B. The role of the amino half of calcineurin-B is not limited to this enabling, however, as Ca^{2+} binding to site 2 increases the affinity of the enzyme for calmodulin. This can be considered a type of disinhibition as the affinity of isolated calmodulin binding domain peptides for calmodulin is higher than the affinity of the whole protein. Therefore, Ca^{2+} binding to site 2 in calcineurin-B diminishes a constraint on the calmodulin binding domain of calcineurin-A, resulting in an increased affinity of that site for calmodulin.

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