Evidence That Calcineurin Accommodates an Active Site Binuclear Metal Center

Lian Yu, Alice Haddy,† and Frank Rusnak*

Section of Hematology Research and the Department of Biochemistry and Molecular Biology Mayo Clinic and Foundation, Rochester, Minnesota 55905

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Calcineurin is a phosphatase found predominantly in neural tissues.[1−3] Physiologically, it is involved in diverse cellular functions such as the regulation of nitric oxide synthase,[4] gene transcription,[5,6] and neutrophil chemotaxis.[7] Calcineurin has also been shown to have a role in T-cell receptor signal transduction by use of the immunosuppressant drugs cyclosporin A (CsA) and FK506,[8] which inhibit calcineurin when complexed to their respective cytoplasmic receptors, cyclophilin and FK506-binding protein (FKBP).[9,10] The discovery of inhibition by CsA/cyclophilin and FK506/FKBP has spurred interest in calcineurin particularly regarding aspects related to drug-mediated inhibition and its mechanism of catalysis.

Calcineurin is composed of a 58 kDa catalytic subunit, calcineurin A, and a 19 kDa calcium-binding subunit, calcineurin B. Its activity is regulated by Ca^{2+}/calmodulin and is also stimulated by divalent metal ions, particularly Mn^{2+} and Ni^{2+}.[11−14] Little is known regarding the catalytic mechanism of calcineurin or related members of the family of serine/threonine phosphoprotein phosphatases including protein phosphatases 1 (PP1) and 2A (PP2A).[15] Early reports indicated that calcineurin contained approximately 1 equiv each of iron and zinc,[14] while a comparison of the sequences of calcineurin, PP1, and PP2A with purple acid phosphatases (PAPases) led Vincent et al. to hypothesize that these phosphatases contain active site metal clusters isostructural with the binuclear metal centers in eukaryotic PAPases.[16] In this report we provide data indicating that the iron and zinc ions of native bovine brain calcineurin are present as a binuclear metal cluster, albeit in a ligand environment different from that in PAPase. Furthermore, we show that dithionite reduction of the bound ferric ion leads to loss of activity which can be quantitatively recovered upon reoxidation, indicating that the oxidation state of bound iron is important for enzymatic function.

Calcineurin was purified from bovine brain as described.[17] Metal ion analysis indicated 0.88 ± 0.05 equiv of iron and 0.77 ± 0.03 equiv of zinc per mole of protein. Low-temperature electron paramagnetic resonance (EPR) spectroscopy of native calcineurin indicated two signals. One signal with g-values of 8.38 and 5.69, representing the majority of the Fe^{3+} in the sample, corresponds to a high-spin Fe^{3+} center with a modest rhombic splitting (Figure 1A). Comparable signals are observed in the Fe^{3+}/Zn^{2+} forms of kidney bean PAPase[18] and uteroferrin.[19] In addition, a minor component with a g-value of 4.27 arising from a high-spin Fe^{3+} center with a large rhombicity is observed and is most likely due to adventitious iron.

When calcineurin was incubated anaerobically in the presence of β-mercaptoethanol and $Fe(NH_4)_2(SO_4)_2$,[20] it was found that approximately half of the zinc was removed and the number of equivalents of iron increased accordingly. Thus, Fe-substituted calcineurin contained 1.59 ± 0.12 and 0.38 ± 0.01 equiv of iron and zinc, respectively. EPR spectra of the Fe-substituted calcineurin showed a signal with resonances at $g = 1.94$, 1.78, and 1.65 and the loss of the signal with $g = 8.4$ and 5.7 (Figure 1B). The new resonance with $g_{av} < 2.0$ is characteristic of a spin-coupled binuclear $Fe^{3+}-Fe^{2+}$ center, similar to the oxo-bridged binuclear iron centers found in a variety of metalloproteins which have ground states $S = {}^1/_2$ due to antiferromagnetic coupling between high-spin Fe^{3+} ($S_1 = {}^5/_2$) and high-spin Fe^{2+} ($S_2 = 2$) ions.[21,22] Resonances from binuclear iron centers exhibit a strong temperature dependence due to the population of excited state multiplets. This was also the case for the new resonance, which deviated from Curie law behavior and disappeared at $T \geq 20$ K. Spin quantitation of the $g_{av} < 2.0$ resonance against a EDTA·Cu(II) standard yielded 0.65 spin/mol protein,[23] consistent with the metal analysis.

The optical spectra of both the native Fe^{3+}/Zn^{2+} and the Fe-substituted forms of calcineurin (≤25 μM) were devoid of detectable electronic absorbance for wavelengths ≥300 nm. PAPases, on the other hand, give rise to an intense visible absorbance band at ~550 nm due to a ligand-to-metal charge transfer band resulting from a tyrosine ligand to the ferric center.[24] Both the Fe^{3+}/Fe^{2+} and the Fe^{3+}/Zn^{2+} forms of the PAPases exhibit this absorbance.[19,25]

The activity of Fe-substituted calcineurin was indistinguishable from untreated calcineurin. Using p-nitrophenyl phosphate, the activity of Fe-substituted calcineurin followed Michaelis−Menten kinetics with a K_m of 13.8 mM and V_{max} of 1.17 μmol·min^{-1}·mg^{-1}, similar to native enzyme (Table 1A).

* To whom correspondence should be addressed. Telephone: (507) 284-2289. FAX: (507) 284-8286.

† Present address: Department of Chemistry, The University of North Carolina at Greensboro, Greensboro, NC 27412-5001.

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(20) Calcineurin was added to a final concentration of 0.1 mg/mL to a septum-sealed vial containing 50 mM MOPS (pH 7.0) and 0.15 M β-mercaptoethanol. After 5 min, an anaerobic solution of 30 mM $Fe(NH_4)_2(SO_4)_2$ was added slowly to a final concentration of 0.38 mM and the reaction incubated at room temperature for 24 h. Fe-substituted enzyme was buffer exchanged into 50 mM MOPS, pH 7.0, 1 mM β-mercaptoethanol, by passage through a Sephadex G-25 gel filtration column and concentrated in a Amicon centricon concentrator.

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(23) Spin quantitation of the $g_{av} < 2$ EPR resonance was performed using EDTA·Cu^{2+} as a standard. The EPR spectrum of the EDTA·Cu^{2+} standard was measured at 20 K while the EPR spectra of Fe-substituted calcineurin were recorded at several temperatures to assess relaxation behavior. The 3.6 K spectrum was used for integration. The EPR signal intensity of Fe-substituted calcineurin at 20 K was determined via extrapolation from low temperatures from a graph of signal intensity vs $1/T$.

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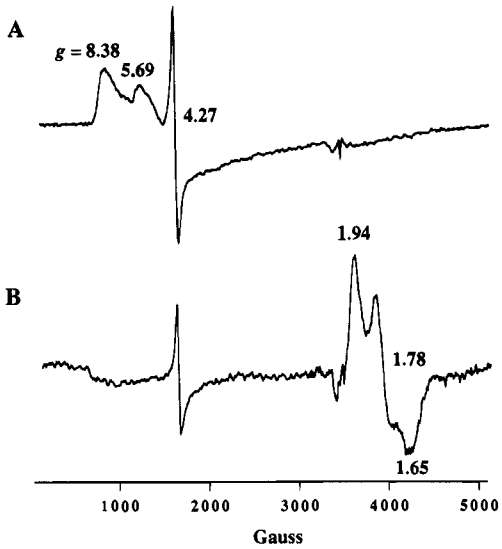

Figure 1. EPR spectra of native and Fe-substituted calcineurin. (A) EPR spectrum of $Fe^{3+}-Zn^{2+}$ form of native calcineurin at 3.6 K. The sample contained 40 mg·mL^{-1} calcineurin in 20 mM Tris, 0.1 mM EDTA, 1.0 mM magnesium acetate, 1 mM DTT, 0.15 M KCl, pH 7.5. Spectrometer conditions: microwave power, 2.0 mW; microwave frequency, 9.443 GHz; modulation amplitude, 10 G at 100 kHz. (B) EPR spectrum of Fe-substituted calcineurin at 3.6 K. Spectrometer conditions were the same as in A except that the microwave power was 20 mW. The sample contained 14 mg·mL^{-1} Fe-substituted calcineurin in 50 mM MOPS, pH 7.0. Fe-substituted calcineurin was prepared as described[20] from the same enzyme preparation as the sample in part A.

Table 1. Kinetic Parameters for the Hydrolysis of Substrates by Native and Iron-Substituted Bovine Calcineurin[a]

A. *pNPP as Substrate*

enzyme	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (mM)
native	1.35	13.5
iron substituted	1.17	13.7

B. *[^{32}P]-R_{II} Peptide as Substrate*

	sp act. (nmol·min^{-1}·mg^{-1})	
enzyme	+Mn	−Mn
native	12 ± 0.08 (100)[b]	3.12 (26)
iron substituted	10 ± 0.013 (82)	2.92 (24.3)

[a] Assays were performed at 30 °C in a solution containing 25 mM MOPS, pH 7.0, 0.1 mM $CaCl_2$, 0.2 μM calmodulin, and except where indicated, 1.0 mM $MnCl_2$. [b] Number in parentheses represents the percent activity relative to native calcineurin in the presence of $MnCl_2$.

Almost identical activities for both were obtained in the presence or absence of Mn^{2+} using a synthetic phosphopeptide substrate, [^{32}P]-R_{II} peptide. With that substrate, Fe-substituted calcineruin gave a specific activity of 10 ± 0.013 nmol·min^{-1}·mg^{-1} in the presence of Ca^{2+} and calmodulin, while native enzyme gave 12 ± 0.08 nmol·min^{-1}·mg^{-1} (Table 1B). Both native and Fe-substituted enzyme showed a significant (~3-fold) stimulation by exogenous Mn^{2+} [11-14] over the activity in the presence of Ca^{2+} and calmodulin alone.

To determine whether the binuclear metal cluster was required for enzymatic activity, the Fe^{3+} ion of native calcineurin was reduced anaerobically with dithionite.[26] Dithionite reduction of calcineurin completely abolished both the EPR signals and the *p*-nitrophenylphosphatase activity of calcineurin (Table 2). Inactivation was reversible in that complete activity was recovered after aerobic removal of the reducing agents. Treatment with a weaker reducing agent, ascorbic acid, had no effect on either the EPR spectrum or the enzyme activity. These results suggest that the oxidation state of the bound iron is important for catalytic activity and indicate that the binuclear Fe^{3+}/Zn^{2+} cluster is an essential active site cofactor.

Table 2. Bovine Calcineurin *p*-Nitrophenylphosphatase Activity in the Presence of Different Reducing Agents

conditions	% act.
control[a]	100
reduced enzyme	
2.0 mM $Na_2S_4O_2$ + 50 μM methyl viologen[a]	0
after desalting into 50 mM MOPS (pH 7.0)[a]	98.7
50 mM ascorbic acid[b]	100
after desalting into 50 mM MOPS (pH 7.0)[b]	104

[a] Assays were performed as in Table 1 using 10 mM *p*-nitrophenyl phosphate. [b] Assays contained 100 mM Tris (pH 7.5) in place of 25 mM MOPS. The percentage of the activity was obtained by comparison with native bovine calcineurin.

These data support a model in which calcineurin contains a binuclear iron/zinc metal cluster at the active site. Although the EPR data have established that the iron atom of native calcineurin is present in the Fe^{3+} oxidation state, it is not possible to determine, on the basis of that data alone, whether the binding site for Zn^{2+} resides close to the ferric ion. Replacement of the diamagnetic Zn^{2+} ion with the paramagnetic Fe^{2+} ion, however, perturbs the magnetic properties of the bound ferric ion, yielding a spin-coupled binuclear iron cluster that is readily identified by an EPR signal with $g_{av} < 2.0$ that exhibits a strong temperature dependence. Analogous EPR signals arising from oxo- and hydroxo-bridged dinuclear iron clusters have now been observed in PAPases,[18,19,27,28] hemerythrin,[29,30] methane monooxygenase,[31] and ribonucleotide reductase.[32]

While this manuscript was being reviewed, the three-dimensional structure of calcineurin complexed with FK506–FKBP was determined at 2.5 Å resolution.[33] Our data is in agreement with the crystal structure, which observes two bound metals separated by 3.0 Å. The spectroscopic and biochemical data presented herein confirm the presence of a dinuclear (Fe^{3+}/Zn^{2+}) metal center and demonstrate its role as an active site cofactor.

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(26) Calcineurin was added to a final concentration of 1 mg/mL to an anaerobic solution of 50 mM MOPS (pH 7.0) containing 2 mM $Na_2S_2O_4$ and 50 μM methyl viologen. Anaerobic conditions were achieved by degassing and purging with oxygen-free argon. Methyl viologen was included to monitor anaerobic conditions and to serve as a mediator for electron transfer. An aliquot of reduced calcineurin was then transferred anaerobically into a 500 μL oxygen-free cuvette and assayed using 10 mM *p*-nitrophenyl phosphate. The enzyme was subsequently reoxidized by removing the reducing reagent aerobically using a Sephadex G-25 column equilibrated in 50 mM MOPS, pH 7.0.

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