Kinetic and Spectroscopic Analyses of Mutants of a Conserved Histidine in the Metallophosphatases Calcineurin and λ Protein Phosphatase*

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Calcineurin, also known as protein phosphatase 2B, consists of a 58-kDa catalytic subunit, calcineurin A, and a 19-kDa regulatory subunit, calcineurin B. It is a serine/threonine protein phosphatase whose activity is regulated by Ca2+/calmodulin. Calcineurin is the target of the immunosuppressant drugs cyclosporin A and FK506 (1, 2). These drugs bind to intracellular proteins, termed immunophilins; cyclophilin is the binding protein for cyclosporin A, and FK506 binds to the FK506-binding proteins. The complex of immunosuppressant drug and immunophilin in turn binds to and inhibits the phosphatase activity of calcineurin. Calcineurin inhibition prevents the transcriptional activation of the interleukin 2 gene in helper T cells, leading to suppression of the immune response.

Calcineurin is a member of the class of serine/threonine protein phosphatases, whose members include protein phosphatases 1 (PP1)1 and 2A (PP2A), phosphatases essential for a number of signal transduction pathways in eukaryotic cells (3, 4). Another protein phosphatase from bacterioophage λ, APP, also belongs to this family (5). In addition, a number of less characterized enzymes containing the “phosphoesterase” consensus motif of this family, DXH(X)nGDXXD(X)nGNHD/E, have been identified via protein sequence comparisons (6, 7). It has been hypothesized that this motif provides a scaffold for an active site dinuclear metal center (7, 8), similar to the dinuclear metal centers in PP1 (9, 10) and calcineurin (11–13). A variety of experimental evidence indicates that this cluster in calcineurin is an Fe3+-Zn2+ center.

Although little is known about the catalytic mechanism of the serine/threonine protein phosphatases, several pieces of experimental data indicate that the dinuclear metal center is a key component of the active site. First, x-ray crystallographic data of calcineurin and PP1 indicate that the dinuclear metal center has a ligand environment nearly identical to that of mammalian and plant purple acid phosphatases, enzymes that contain either dinuclear Fe–Fe or Fe–Zn centers that have been demonstrated to be essential for catalytic activity (14, 15). Second, these crystallographic studies indicate that the product of the reaction, phosphate, and the product analog, tungstate, directly coordinate both metal ions (10, 11, 16). Third, redox titrations of either the Fe3+-Zn2+ (12) or Fe2+-Fe2+ forms2 of calcineurin indicate a correlation between enzyme activity and the oxidation state of the bound metal ions.

In addition to the dinuclear metal center, there are several conserved amino acids within the active site which are likely to contribute to catalysis. One of these residues in calcineurin is histidine 151 (numbering based on the rat calcineurin A sequence (18)). His-151 is not a ligand to either metal but is within 5 Å of both metal ions and is conserved in other metallophosphoesterases such as PP1 (histidine 125) and APP (histidine 76) (7). In one crystal structure, His-151 was modeled to participate in a hydrogen bond to a metal-coordinated solvent molecule (13). The importance of this residue has been demonstrated by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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strated by site-directed mutagenesis of both γPP and PP1 which found substantial effects on catalytic activity and/or protein stability.

In this study the conserved histidine residue in calcineurin A, His-151, was changed to glutamine by site-directed mutagenesis. After recombination of calcineurin B, the calcineurin H151Q heterodimer was purified to homogeneity. EPR spectroscopy was used to assess whether mutagenesis affected the ligand environment of the dinuclear metal center. Protein concentrations were determined by UV-visible spectrophotometry (27) assay using the Pierce Coomassie Plus Protein Assay Reagent with known extinction coefficient agreed within 10% with concentrations of 22,000 M−1 cm−1.

Calmodulin was prepared from bovine brain (19, 20) and coupled to myristoyl transferase. The construction of pT7-7 plasmids containing myristoylated calcineurin B, and purification of the calcineurin heterodimer were performed as described previously (21, 22).

Expression and Purification of Wild Type γPP—Expression of wild type γPP was performed as described (5). All purification steps were performed at 4 °C. After growth and induction with isopropyl β-D-thiogalactopyranoside, the cells were harvested by centrifugation at 3,000 x g for 30 min, washed twice with 250 mM Tris-Cl, pH 7.5, and resuspended at 4,200 x g for 20 min. The cells were resuspended in 20 mM Tris-CI, pH 8.0, 20% glycerol, 1 mM EGTA (TGE buffer) and lysed by three passages through a French pressure cell operating at 16,000 p.s.i. The cell lysate was subsequently centrifuged at 39,000 x g for 3 h. The supernatant (40 ml) was batch adsorbed onto 150 ml of DEAE-Sepharose column with TGE buffer. The sample was washed in a fritted funnel with 300 ml of TGE and eluted with TGE buffer containing 0.1 M NaCl. Fractions containing γPP were pooled and precipitated by the addition of ammonium sulfate to 50% saturation. After centrifugation at 34,800 x g, the protein pellet was resuspended in TGE buffer + 0.5 M NaCl and applied to a phenyl-Sepharose column (20 x 1 cm diameter) previously equilibrated with TGE buffer + 0.5 M NaCl. The column was washed with 200–300 ml of the same buffer and then with 250 ml of 20 mM Tris-CI, pH 7.5. The enzyme was eluted with 250 ml of 50 mM Tris-CI in 50% glycerol, pH 7.5. Fractions were assayed using γPP as a substrate, pooled, and stored at −70 °C in 50 mM Tris-CI and 50% glycerol, pH 7.5.

Expression of γPP(H76N)—The γPPT77(H76N) plasmid (25) was transformed into BL21(DE3) cells and single colonies were inoculated into 10 ml of Luria Bertani medium/ampicillin (0.1 mg/ml) for overnight culture at 37 °C. Overnight cultures were then used to inoculate 5 liters of 2YT/ampicillin medium (10 g/liter yeast extract, 20 g/liter tryptone, 10 g/liter NaCl, 0.05 g/liter ampicillin) in a New Brunswick Bioflo 3000 fermentor. Cells were grown overnight at 22 °C maintaining aeration at 30% of air saturation to a cell density that gave an absorbance of 550 nm of 0.9. Glucose was added to a final concentration of 0.4%, and the cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside. Another aliquot of glucose was added to a final concentration of 0.4% when the cell density corresponded to an absorbance at 595 nm of 16. The cells were harvested after 20 h postinduction by centrifugation at 3,400 x g for 20 min. The cell pellet was resuspended in −2 ml of 50 mM Tris-CI, pH 7.5, 0.5 g/liter of cells, wet weight. To this suspension, 0.4 mg/ml lysozyme, 25 mM EDTA, and 0.05% Triton X-100 were added sequentially with stirring on ice for 30 min followed by a freeze/thaw process to lyse the cells. To reduce the viscosity, MgCl2 (20 mM), Dnase I (0.1 unit/ml final concentration), and 2% proteamine sulfate (1/6 total volume) were added sequentially with stirring on ice. After centrifugation at 10,000 x g for 1 h, the protein was purified as described above for wild type γPP.

Circular Dichroism Measurements—Circular dichroism spectra were recorded at 25 °C on a Jasco J-710 circular dichroism spectrometer. A quartz cell of 0.0202 cm path length was used for all measurements. Mean residue ellipticities were calculated from the relationship [θ]m = 100 n [θ]u/[λc] for the observed ellipticity in millidegrees, [θ]u is the mean residue ellipticity, and [λc] is the mean residue molar concentration, and the molar residue ellipticity, [θ]m, is expressed in deg cm2/mole. Samples of calcineurin (18 µM) and CN(151Q) (24 µM) were examined in 10 mM Heps, pH 7.5, 1 mM MgCl2, 0.1 mM EGTA, 0.2 mM DTT.

pNP Assays—Calcineurin-dependent phosphatase activity of calcineurin and CN(151Q) was measured using pNP as a substrate at 30 °C in 25 mM MOPS, pH 7.0, 1 mM MnCl2, 0.1 mM CaCl2, 1 mM MnCl2, and 21–23 mM wild type calcineurin or 710 nM CN(151Q). Wild type γPP and γPP(H76N) activities were measured at 30 °C in 100 mM Tris-CI, pH 7.8, 10 mM DTT, 1 mM MnCl2, and 0.64 mM wild type γPP or 860 nM γPP(H76N). After incubation for 5 min at 30 °C, reactions were started by the addition of pNP. Specific activity was measured by following the increase in absorbance at 410 nm with time using ε520 = 7,180 M−1 cm−1 at pH 7.0 and 14,400 M−1 cm−1 at pH 7.8 based on a θk of 7.17 and ε520 = 17,800 M−1 cm−1 for the p-nitrophenolate anion. The concentration of pNP was varied from 2 to 100 mM, and the kinetic parameters kcat and Km were determined by fitting the data to the Michaelis-Menten equation using a nonlinear least squares analysis method.

[γP] γP γPP—Peptide Assays—[γP] γPP was phosphorylated with γPP to a specific activity of 333 µCl/µmol using the catalytic subunit of bovine cardiac cyclic-AMP dependent protein kinase and purified as described (28). Assays were done as described (28) in 100 mM MOPS, pH 7.0, 1 mM MnCl2, 0.1 mM CaCl2, 0.5 mM DTT, 0.1–28 µM calcineurin, and 1.0 mg/ml bovine serum albumin. [γP] γPP-γPP was diluted with [γP] γPP peptide and substrate added in final concentrations from 9 µM to 1 mM. Calcineurin concentrations were 10 µM for wild...
type and 7.7–15 μM for CN(H151Q). Data were obtained in duplicate and fitted to the Michaelis-Menten equation by a nonlinear least squares analysis method.

Phosphoryl Phosphate Assays—Assays were performed by determining the amount of inorganic phosphate released during hydrolysis of phosphoryl phosphate as described (29). Assays were done in 100 mM Tris-Cl, pH 7.8, containing 1 mM MnCl₂ and 10 mM DTT. Phosphoryl phosphate concentrations were varied from 1 to 70 mM for APP and 1 to 90 mM for αPP(H76N). Wild type protein concentrations ranged from 7.0 to 630 nM, whereas αPP(H76N) concentrations ranged from 2.2 to 12 μM.

Enzyme was incubated 30 °C, 5 min, and reactions started by the addition of phosphoryl phosphate. At various times from 0.5 to 7.0 min, 50 μl of the reaction was taken and added to 800 μl of a solution containing a 3:1 ratio of 0.045% malachite green hydrochloride to 4.2% ammonium molybdate in 4 N HCl. After 1 min, 100 μl of 34% sodium citrate was added and the absorbance at 660 nm measured. Free phosphate was determined from a standard curve prepared using solutions of KH₂PO₄.

Addition of Orthophosphate to CN(H151Q)—To the iron-reconstituted EPR sample of CN(H151Q), a solution of 0.5 M potassium phosphate as described (29). Assays were done in 100 mM Tris-Cl, pH 7.5, containing 1 mM MnCl₂ and 10 mM DTT. Phenyl phosphate concentration was determined from a standard curve prepared using solutions of KH₂PO₄. Kinetic parameters for APP and αPP(H76N) were determined as described above.

Reconstitution of CN(H151Q) and αPP(H76N) with Iron—About 0.4 mg/ml CN(H151Q) in 20 mM Tris-Cl, pH 7.5, 100 mM KCl, 1.0 mM magnesium acetate, 1.0 mM DTT, 0.1 mM EGTA, or 0.6 mg/ml αPP(H76N) in 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10% glycerol was added and the absorbance at 660 nm measured. Free phosphate was determined from a standard curve prepared using solutions of KH₂PO₄. Kinetic parameters for APP and αPP(H76N) were determined as described above.

Metal analysis was performed by the Mayo Clinic Metals Laboratory using inductively coupled plasma emission spectrometer operating at 9 GHz (X-band) microwave frequency equipped with an Oxford Instruments ESR 900 continuous flow cryostat operating at 9 GHz (X-band) microwave frequency equipped with an Oxford Instruments ESR 900 continuous flow cryostat for temperature regulation. Background cavity resonances were subtracted from all spectra.

Metal Analysis—Metal analysis was performed by the Mayo Clinic Metals Laboratory using inductively coupled plasma emission spectrometry.

RESULTS

Preparation of Recombinant CN(H151Q) and λPP(H76N) Proteins—The relationship of His-151 in calcineurin relative to the dinuclear metal center can be seen in Fig. 1. His-151 was mutated to a glutamine by site-directed mutagenesis to investigate the effect on enzyme activity and assembly of the dinuclear metal cofactor. The mutant calcineurin A subunit was expressed in E. coli in a fashion identical to that of the wild type calcineurin A subunit and reconstituted with myristoylated calcineurin B to generate the mutant protein CN(H151Q).

The presence of the proper codon as well as the lack of inadvertently introduced mutations in the entire calcineurin A gene were confirmed by DNA sequence analysis. Although the yield of CN(H151Q) was less than that obtained for the wild type reconstituted protein, enough material could be obtained and purified for homogeneity for biochemical and spectroscopic (EPR) analyses. Two typical purification yielded approximately 1 mg of CN(H151Q) protein/liter of culture cell.

The analogous residue in λPP, identified by primary sequence comparisons as His-76 (7), was mutated to an asparagine residue (25). In this study, the APP(H76N) protein was purified to homogeneity as described under “Methods” to yield approximately 17 mg/liter of culture.

Circular dichroism analysis of wild type calcineurin and CN(H151Q) provided evidence for a native-like conformation of CN(H151Q); CD spectra from 200 to 250 nm of both recombinant wild type calcineurin and CN(H151Q) are comparable to spectra of bovine calcineurin (30) (data not shown).

Phosphatase Activities of Wild Type Calcineurin and CN(H151Q)—Kinetic parameters using pNPP and [P]-R II peptide as substrates for wild type calcineurin and CN(H151Q) in the presence of 1 mM MnCl₂ are compared in Table I. Using either substrate, the values of kcat for the mutant enzyme were significantly lower than the kcat values for wild type enzyme. Thus, the kcat for CN(H151Q) using pNPP as a substrate, 5.6 × 10⁻² s⁻¹, is 460-fold lower than recombinant wild type calcineurin prepared in an identical fashion. Using [P]-R II peptide, the kcat values for wild type calcineurin and CN(H151Q) were 1.2 × 10⁻³ s⁻¹ and 9.0 × 10⁻³ s⁻¹, respectively, a difference of 1,300-fold. Using [P]-R II peptide, Kₘ values for both forms of calcineurin were the same within the error of the measurement. However, a 10-fold decrease in Kₘ for CN(H151Q) was observed compared with wild type calcineurin using pNPP as a substrate.

Phosphatase Activities of Wild Type λPP and αPP(H76N)—Kinetic parameters using pNPP and phenyl phosphate as substrates for wild type λPP and APP(H76N) are compared in Table II. These parameters were also determined by inclusion of 1.0 mM MnCl₂ in assay buffers. Using pNPP as substrate, the kcat values for wild type λPP and APP(H76N) were 8.1 × 10⁻¹ s⁻¹ and 6.0 × 10⁻¹ s⁻¹, respectively. This represents a 590-fold difference, which is comparable to the decrease in kcat observed for calcineurin for the analogous substitution. The difference in kcat for λPP is less than the ~10⁶-fold difference found previously (25) mostly because of a 30-fold higher activity measured for the APP(H76N) protein isolated in the present study. Using phenyl phosphate as substrate, the kcat value for APP was 1.7 × 10⁻² s⁻¹, compared with 3.2 × 10⁻² s⁻¹ for APP(H76N), a difference of 530-fold. The Kₘ values were very similar for both mutant and wild type proteins using either substrate.

EPR Analysis of Iron-reconstituted Wild Type Calcineurin
Active Site Mutants of Calcineurin and \( \lambda \) Protein Phosphatase

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and CN(H151Q)—Although not a metal ligand, H151Q in calcineurin is close enough to either metal ion such that mutagenesis might perturb the surrounding environment of the metal cluster. To investigate the effect of mutagenesis on the dinuclear center, wild type calcineurin and CN(H151Q) were reconstituted with iron to generate a mixed valence Fe\(^{3+}\)-Fe\(^{2+}\) cluster as a spectroscopic probe of the active site and analyzed by EPR spectrometry. The EPR spectra of wild type calcineurin, CN(H151Q), and CN(H151Q) in the presence of 20 mM potassium phosphate are compared in Fig. 2. Several features are evident in the EPR spectra including a minor high spin Fe\(^{3+}\) species with \( g \) values of 9.2 and 4.3 (about 700–2,000 Gauss region, not shown for clarity), a minor radical species centered at \( g_{av} = 2.0 \), and a component with \( g_{av} < 2.0 \) representing the major paramagnetic species.

Simulations of the EPR signal in Fig. 2A to an \( S = 1/2 \) species yielded \( g \) values of 1.93, 1.77, and 1.64. This signal is identical to the signal observed previously in bovine brain calcineurin reconstituted with iron and arises from a dinuclear iron center in the mixed valence (Fe\(^{3+}\)-Fe\(^{2+}\)) oxidation state (12). The spin Hamiltonian \( H \), that describes the magnetic properties of the dinuclear iron center is

\[
H = D_1 (S_1^2 - S_0 (S_1 + 1/3)) + E_D (S_1^2 - S_0^2) + \beta S_1 \cdot g_1 \cdot H + D_2 (S_2^2 - S_0 (S_2 + 1/3)) + E_D (S_2^2 - S_0^2) + \beta S_2 \cdot g_2 \cdot H + \sum g_i \cdot S_i \cdot H
\]

where \( H \) is the external magnetic field, \( \beta \) is the Bohr magneton, \( J \) is the exchange-coupling constant, and \( D_1, E_D \), and \( g_i \) (\( i = 1, 2 \)) represent the zero field splitting terms and \( g \) tensors for each metal ion, respectively. Antiferromagnetic coupling (\( J > 0 \)) between the high spin ferric (\( S_1 = 5/2 \)) and high spin ferrous (\( S_2 = 3/2 \)) ions of the cluster yields a ground state with \( S = 1/2 \) which gives rise to this EPR signal.

Fig. 2B shows the EPR spectrum of iron-reconstituted CN(H151Q). An EPR signal representative of a dinuclear iron center in the mixed valence state is evident and indicates that a dinuclear metal center can be assembled in the mutant enzyme. This signal, however, is broader than the signal observed from the mixed valence center in wild type enzyme. Metal analyses of the EPR sample found 1.5 mol each of iron and zinc/mol of protein, consistent with the formation of a dinuclear iron center but also indicating the possible presence of a mixed metal Fe-Zn center or adventitious zinc, either of which would contribute to the EPR spectrum in the \( g_{av} < 2.0 \) region.

Further proof that the signal in Fig. 2B results from an active site metal center was demonstrated by adding 20 mM potassium phosphate to the sample. The addition of phosphate led to a noticeable sharpening of the EPR signal (Fig. 2C), whereas none of the other species was affected, suggesting that phosphate coordinates to one or both of the metal ions of the dinuclear iron center.

**EPR Analysis of Iron-reconstituted \( \lambda PP \) and \( \lambda PP(H76N) \)—In a fashion similar to that for calcineurin, \( \lambda PP \) and \( \lambda PP(H76N) \) were reconstituted with iron to generate a spectroscopic probe of the active site metal cluster. \( \lambda PP \) as purified contains very little iron, zinc, or manganese as determined by metal analysis using inductively coupled plasma emission spectrometry (≤ 0.05 mol of iron, 0.09 mol of zinc, and 0.01 mol of manganese/mol of protein). Likewise, \( \lambda PP(H76N) \) also contained low amounts of these metals (≤ 0.3 mol of iron, 0.09 mol of zinc, and 0.01 mol of manganese/mol of protein). Reconstitution of wild type \( \lambda PP \) and \( \lambda PP(H76N) \) with iron yielded samples that exhibited low temperature EPR spectra with \( g_{av} < 2.0 \) (Fig. 3, A and B). Metal analysis of both EPR spectra samples found 1.74 mol of protein and 0.20 zinc/mol of protein for wild type \( \lambda PP \), and 1.7 iron/mol of protein and 0.05 zinc/mol of protein for \( \lambda PP(H76N) \). Thus both \( \lambda PP \) and \( \lambda PP(H76N) \) can accommodate dinuclear metal clusters. As in the case of calcineurin, the EPR spectrum of the \( \lambda PP(H76N) \) mutant is different from the spectrum of wild type \( \lambda PP \).

**DISCUSSION**

Recent crystallographic models of calcineurin (11, 13), PP1 (9, 10), and purple acid phosphatase (16, 31) have identified a conserved histidine in each active site which, although not coordinated to either metal of the dinuclear metal center, is within ~5 Å of both metal ions. In this study we have mutated the corresponding residue of calcineurin (His-151) to glutamine to explore its significance in catalysis and effect, if any, on the active site dinuclear metal center. An analogous histidine in the bacteriophage \( \lambda \) protein phosphatase has been previously

| Enzyme | \( k_{cat} \) | \( K_m \) | Relative \( k_{cat} \) |
|---|---|---|---|
| pNPP hydrolysis Wild type calcineurin | 2.6 \( \times 10^4 \pm 3 \) | 23 \( \pm 7 \) mM | 1 |
| CN(H151Q) | 5.6 \( \times 10^{-2} \pm 1 \times 10^{-3} \) | 2.2 \( \pm 0.20 \) mM | 460 |
| PP-H76N | 1.2 \( \times 10^{-1} \pm 1 \times 10^{-1} \) | 110 \( \pm 40 \) μM | 1 |
| [P]-RiI | 9.0 \( \times 10^{-3} \pm 1 \times 10^{-3} \) | 130 \( \pm 60 \) μM | 1,300 |

* Relative \( k_{cat} \) compared with wild type calcineurin for each substrate listed.

**TABLE II**

Kinetic parameters for recombinant \( \lambda PP \) and histidine mutant using pNPP and phenyl phosphate as substrates

| Enzyme | \( k_{cat} \) | \( K_m \) | Relative \( k_{cat} \) |
|---|---|---|---|
| pNPP hydrolysis Wild type \( \lambda PP \) | 3.9 \( \times 10^2 \pm 1 \times 10^1 \) | 11 \( \pm 1 \) | 1 |
| 2.0 \( \times 10^0 \) | 10 |
| APP(H76N) | 6.6 \( \times 10^{-1} \pm 4 \times 10^{-2} \) | 16 \( \pm 3 \) | 590 |
| 2.2 \( \times 10^{-2} \) | 4.1 |
| Phenyl phosphate hydrolysis Wild type \( \lambda PP \) | 1.7 \( \times 10^{-2} \pm 2 \) | 14 \( \pm 5 \) | 1 |
| 3.2 \( \times 10^{-2} \pm 3 \times 10^{-3} \) | 22 \( \pm 6 \) | 530 |

* Relative \( k_{cat} \) compared with wild type \( \lambda PP \) for each substrate listed.
modified to asparagine (αPP(H76N)) (25). The H76N mutation resulted in a 10⁶-fold reduction in \( k_{\text{cat}} \) toward pNPP, a 40-fold increase in the \( K_m \) for Mn²⁺, the divalent metal ion activator used in assay buffers, yet little change in \( K_m \) for substrate. In study by Lee and colleagues (32), the comparable residue in PP1 (His-125) was mutated to a number of residues. In that study it was found that most of the substitutions resulted in the production of insoluble protein except for two mutations, H125A and H125S, where a fraction of the protein was soluble and could be purified by affinity chromatography. Although neither PP1 mutant exhibited any detectable phosphatase activity, the upper limit for activity and/or fold reduction relative to wild type PP1 was not reported.

Similar to the results noted in the PP1 study, the level of expression of H151Q soluble protein was also lower than that found for wild type calcineurin A. Hence, the yield of the reconstituted H151Q heterodimer was lower (4-fold) than wild type calcineurin A. Hence, the yield of the expression of H151Q soluble protein was also lower than that obtained for wild type PP1, yet little change in \( K_m \) for both enzymes (5, 33, 34). Manganese is known to incorporate into one or both metal sites to obtain the maximum activity for both enzymes (5, 33, 34). Manganese in the catalytic site and a decrease in \( k_{\text{cat}} \) of 590-fold relative to wild type αPP. This difference is more than 100-fold lower than the 10⁵ noted previously (25) primarily because of a difference in substrate.

Using pNPP as substrate, αPP(H76N) also exhibited little difference in substrate \( K_m \) and a decrease in \( k_{\text{cat}} \) of 590-fold relative to wild type αPP. This difference is more than 100-fold lower than the 10⁵ noted previously (25) primarily because of a difference in substrate.

Although pNPP is a substrate for calcineurin (33), the \( K_m \) of 10–20 mM is significantly greater than the \( K_m \) of 0.1 mM for \([\text{P}]\)–RII peptide. Furthermore, calcineurin activity using \([\text{P}]\)–RII peptide is progressively inhibited in the presence of increasing concentrations of pNPP with an IC₅₀ equivalent to the \( K_m \) for pNPP (data not shown). These results indicate that both pNPP and \([\text{P}]\)–RII peptide utilize the same active site, and hence a comparison of their kinetic parameters is valid.

5 L. Yu and F. Rusnak, unpublished results.

3 P. Mertz, L. Yu, R. Sikkink, and F. Rusnak, unpublished results.
the pK_a of a coordinated water molecule, the putative nucleophile in the reaction. His-151/His-76 could be functioning in concert with this solvent molecule to either position a lone pair on the oxygen atom for optimum in-line attack on the phosphorus atom of the substrate or to serve as a general base to take up a proton concomitant with solvent nucleophilic attack. At least in one crystal structure model of calcinurin, the Ne atom of His-151 was H-bonded to one of two solvent molecules coordinated to the iron atom (13). In the crystal structure of PP1 with microcystin bound, the Ne atom of the analogous histidine, His-125, was also within H-bonding distance of a water molecule, but that water was modeled \( \geq 3.2 \) Å away from the metal ions (9). Further evidence for this model is provided by mutagenesis studies of PP1 examining the influence of a conserved aspartic acid residue, Asp-95, on catalytic efficiency. This conserved aspartate residue is part of the phosphoesterase consensus motif (6, 7). PP1 residue Asp-95 is within H-bonding distance of the conserved histidine, and mutagenesis to asparagine resulted in a 71-fold decrease in activity compared with wild type using phosphorylase \( \alpha \) as substrate (32). The analogous mutant in APP, D52N, resulted in a 36-fold reduction in activity using pNPP as substrate (25). The corresponding residue in calcineurin, Asp-121, is also within H-bonding distance of His-151 (Fig. 1). Thus, the interaction of this conserved histidine/aspartate pair with a solvent molecule is analogous to the catalytic aspartate/histidine/serine motif of serine proteases and could be thought of as a “catalytic tetrad” with the metal ion serving as a Lewis acid to lower the pK_a of the nucleophile.

If His-151 participates in a hydrogen bond with a metal-coordinated solvent, mutagenesis will disrupt this interaction and is likely to affect the spectroscopic properties of the dinuclear metal center. We have reconstituted calcinurin and APP with iron to generate an active site dinuclear iron center for use as a spectroscopic probe of the active site. The Fe^{3+}-Fe^{2+} oxidation state of this cluster gives rise to a signature EPR resonance with \( g_{av} < 2.0 \) (14, 15) which is sensitive to changes in the metal environment via perturbation of zero-field splitting (\( D_{ij}, E_{ij} \)) and spin coupling (\( J \)) constants in Equation 1 (39). The EPR spectrum of iron-reconstituted CN(H151Q) exhibited \( g \) values consistent with the formation of a \( \text{Fe}^{3+}-\text{Fe}^{2+} \) center, indicating that the H151Q mutant enzyme is still able to support a dinuclear metal center. However, the overall shape of this spectrum was quite different from that of wild type calcinurin. In comparison, the EPR spectrum of the iron-reconstituted APP(H76N) also exhibited the characteristic \( g_{av} < 2.0 \) signal with a shape distinct from the corresponding spectrum of wild type APP.

The fact that phosphate addition to iron-reconstituted CN(H151Q) caused a change in the shape of the EPR resonance indicates that it arises from an active site metal center. Interestingly, in both wild type calcinurin and purple acid phosphatase, phosphate binding to the mixed valence cluster led to a broadening of the corresponding EPR signal, a result of a decrease in the spin coupling constant, \( J \), caused by the phosphate ion bridging the two metal ions of the cluster (39).2 With CN(H151Q), on the other hand, phosphate caused a sharpening of the EPR resonance. Further spectroscopic analysis is required to understand the structural basis for these differences. These results demonstrate that the conserved histidine in the metallophosphatases calcinurin and APP is an essential component of the active site since disruption led to significant decreases in activity. Loss of activity may have resulted from removal of an active site base and the disruption of an essential H bond to a metal-coordinated solvent molecule. Future experiments to confirm this are in progress.

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6 The rate constant for nonenzymatic hydrolysis of pNPP, \( k_{uncat} = 7 \times 10^{-6} \text{s}^{-1} \) at pH 7.4, 39 °C (41). Hydrolysis of serine phosphate occurs with a rate constant \( k_{uncat} = 6.7 \times 10^{-6} \text{s}^{-1} \) at 25 °C (17).
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